Cellular & Molecular ONCOLOGY

A CONCISE PRIMER

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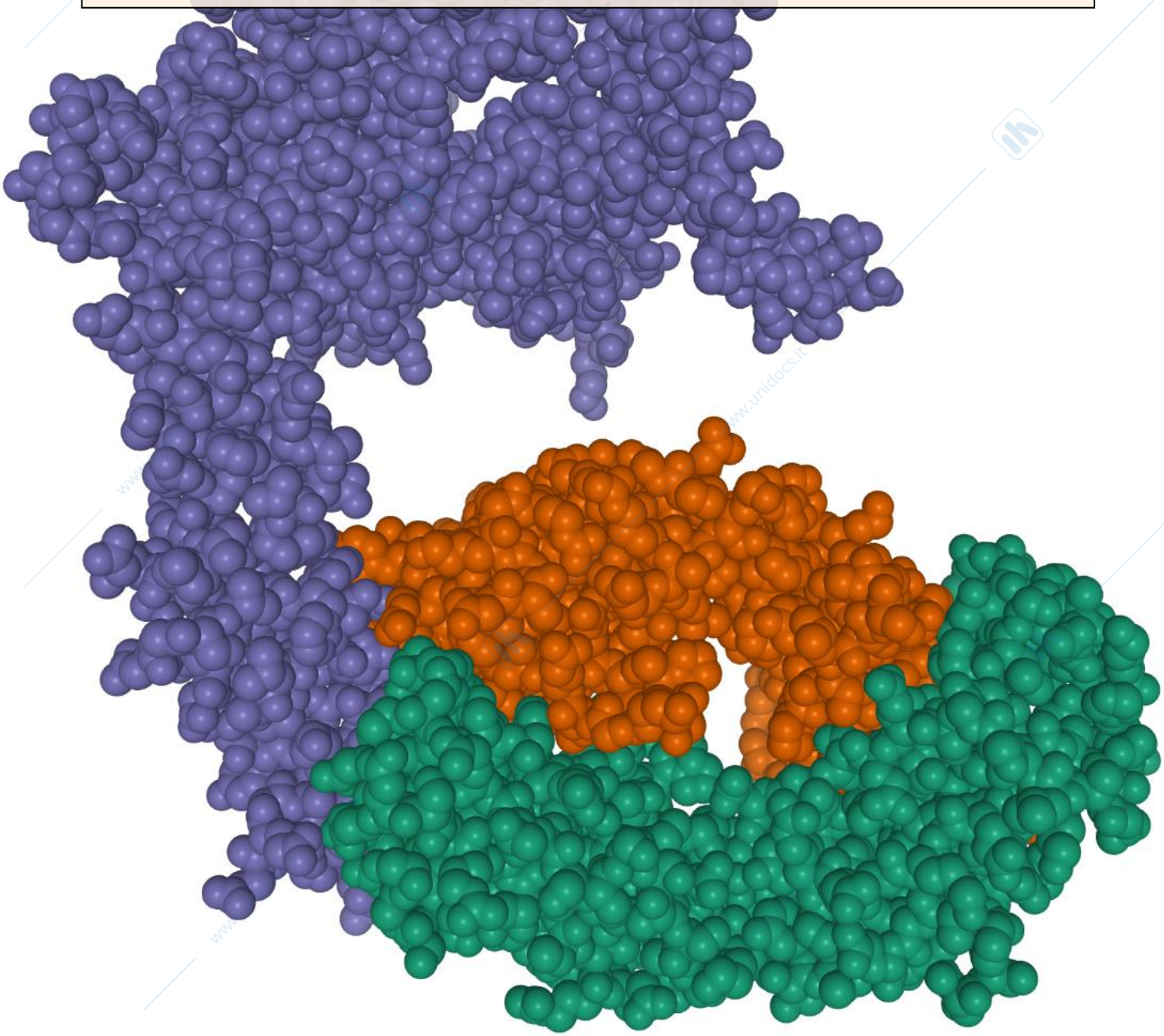

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Chapter 1. What Is Cancer? An Overview

What is cancer?

Cancer is a group of diseases involving an unrestricted increase in cell number.

The number of normal cells is tightly regulated in some tissues and organs, for example the epidermis, by the balance between continuous cell proliferation and continuous cell loss (labile tissues, according to the definitions of the Italian histologist Bizzozero); in others, like the brain, by a lack of cell proliferation (perennial tissues); in some cases (e.g. hepatocytes) cells are normally quiescent, but can restart proliferation if cell loss occurs (stable tissues).

The control of cell number in normal tissues is operated through four major mechanisms, cell proliferation, cell death, cell differentiation and cell migration. Cancer is the result of alterations in such homeostatic mechanisms, leading to a continuing increase in the number of cells of a given tissue.

Benign and malignant tumors

Some tumors, in addition to growth deregulation, invade the surrounding tissues and give rise to distant replicas called metastases (Figure 1.1). Invasion and metastasis are the hallmarks of malignant tumors. In contrast, benign tumors only grow at the anatomical site of origin.

Metastasis is the major cause of death of cancer patients. Most local tumors are easily removed by surgery, but the systemic nature of metastases preempts local therapies, such as surgery or radiotherapy, moreover the simultaneous growth of multiple metastases cannot be controlled by pharmacological therapies.

Glossary

"Tumor" and "neoplasm" are synonymous, referring to both benign and malignant types; "cancer" implies malignancy. "Oncology" is the science that deals with

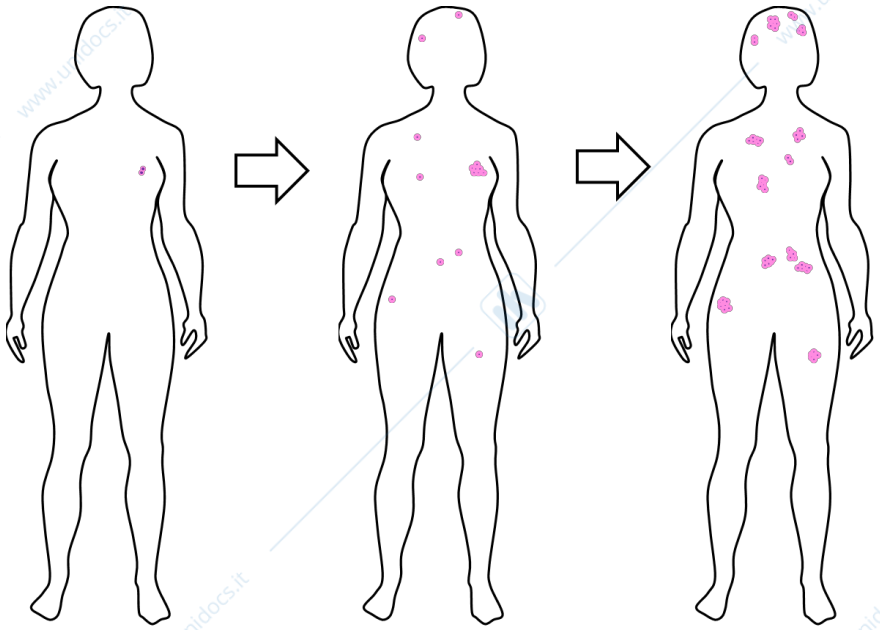


Figure 1.1. Tumor growth and metastatic spread. In this example, a local tumor arising in the breast (left *panel*) grows locally and spreads systemically (*central panel*); the growth of metastases continues after the surgical removal of the primary tumor (*right panel*).

tumors; "cancerology" is a less used synonym.

The generic names of the various cancer types indicate the cells and tissue of origin:

- carcinomas are of epithelial origin;
- sarcomas derive from connective tissues;
- leukemias and lymphomas derive from blood cells;
- melanomas from melanocytes.

Impact of cancer on human health

Cancer is a frequent disease worldwide. The Global Cancer Observatory (https://gco.iarc.fr) estimated that in 2018 there were 18 million new cancer cases in the world, and more than 9 million deaths. Cancer affects both sexes, with a slightly higher incidence and mortality in males (partly due to tobacco smoke). In Italy, there are more than 350.000 new cancer cases each year, and 180.000 deaths; the total number of cancer patients and survivors is about 3.5 million.

In Italy, as in most Western countries, cancer is the second cause of death after cardiovascular diseases (Table 1.1), accounting for one-fourth to one-third of all deaths.

Table 1.1. Causes of death in Italy*

Cause of death	Deceased	%
Circulatory system diseases	232 992	35.8%
Tumors	180 085	27.7%
Respiratory diseases	53 372	8.2%
Digestive diseases	30 692	4.7%
Diabetes	29 519	4.5%
Nervous system diseases	23 261	3.6%
External causes	25 411	3.9%
Other causes	75 302	11.6%
Total	650 614	100.0%

*Year 2017, data from ISTAT 2019.

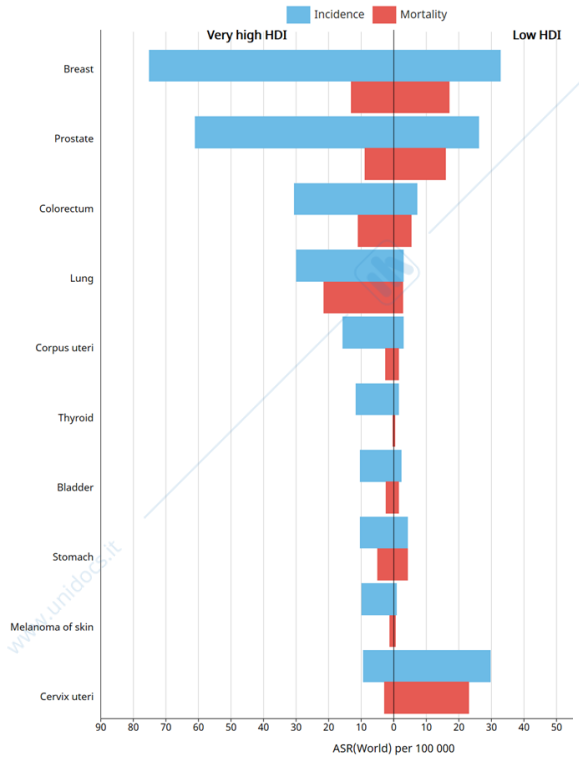


Figure 1.2. Cancer and human development. Estimated age-standardized incidence and mortality rates in 2018. Data source: GLOBOCAN 2018, http://gco.iarc.fr.

The geographical distribution of cancer cases in the world is not homogeneous. Overall, cancer incidence is higher in countries with a high level of human development, as measured by the human development index, HDI, a composite index developed by the United Nations, combining life expectancy, years of schooling and gross national income (Figure 1.2). However, when individual cancer types are analyzed, considerable variations emerge. For example, the geographical distributions of breast and uterine cervix cancer are completely different. Some differences are attributable to different exposures to known carcinogens, for example, higher levels of carcinogenic viruses and bacteria in low-HDI countries, higher

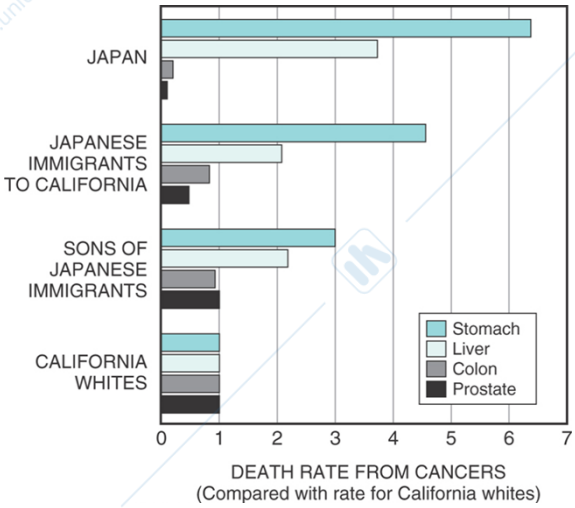


Figure 1.3. Variations in cancer mortality after migration from Japan to California. From Kumar et al: Robbins & Cotran Pathologic Bases of Disease, 8th Edition, Copyright 2009 by Saunders, an imprint of Elsevier.

incidence of tobacco addiction in medium- to high-HDI countries.

A better understanding of the underlying causes of geographical differences can be obtained by the study of cancer in migrant populations. In general, it is assumed that immigrants are soon exposed to the exogenous risk factors of the adopted country, whereas endogenous risk factors, such as genes or microbiome, change more slowly (Figure 1.3). It is important to note that such epidemiological studies provide important clues and hypotheses regarding the underlying causes of various cancer types, that molecular studies can subsequently elucidate.

Cancer in history

During the 20th century, cancer went from relatively rare to very frequent. This led to some speculation that cancer was an entirely new disease, such as AIDS or CoViD-19. However, this is ostensibly not the case. In addition to fossil evidence that cancer affected dinosaurs, human paleopathologists found bone cancer in Egyptian mummies dating back to 3000 before Christ. Furthermore, written documents suggest that surgery of tumors was practiced in ancient Egypt and Greece. Modern medical terms, such as "carcinoma" and "oncos", the root of "oncology", were already used by Hippocrates and Galen.

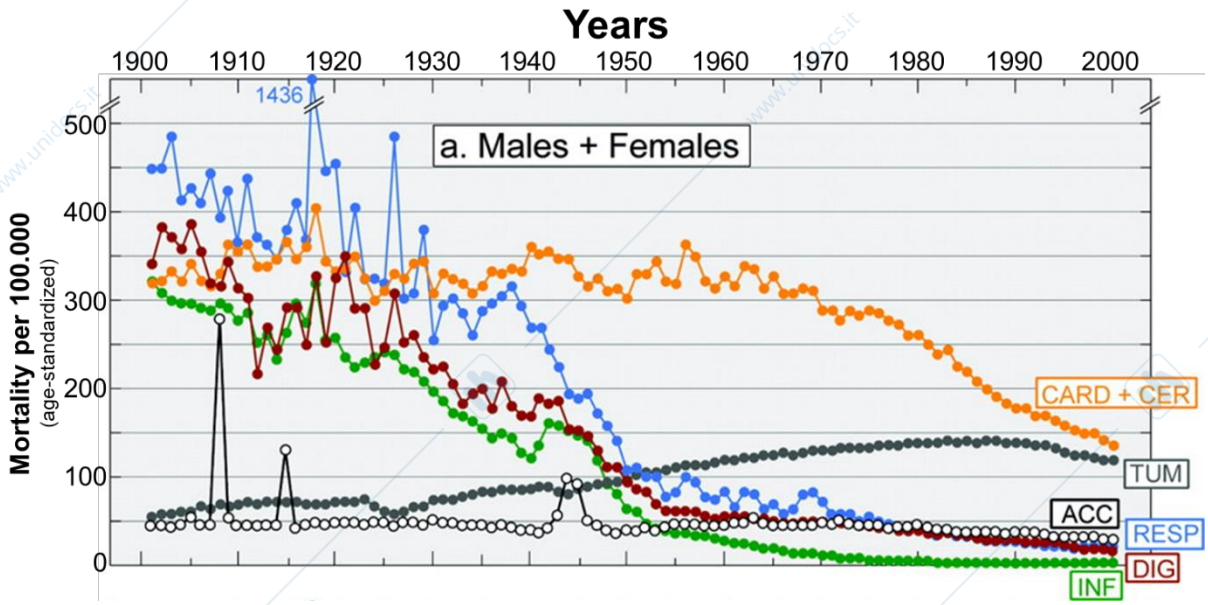


Figure 1.4. Evolution of mortality during the 20th century in Italy.
CARD+CER: cardiovascular + cerebrovascular; TUM: tumors; ACC: accidental; RESP respiratory; DIG: digestive; INF: infectious. From De Flora et al., FASEB J., 19: 892.

It is true that during the last century the magnitude of cancer greatly increased, both in absolute terms and relative to other diseases. In fact, the whole landscape of human diseases underwent profound changes in the 20th century. The analysis of Italian mortality data (Figure 1.4) shows a strong and continuing decrease in infectious diseases (which also include most diseases labeled as respiratory or digestive), brought about initially by improvements in public hygiene, then by the advent of antibiotics. Starting in the 1970s, also cardiovascular mortality began a steady descent. Thus, cancer related mortality became more prominent because other diseases gradually became less deadly. However, there was also an important increase in the absolute magnitude of cancer mortality, which almost tripled from 1900 to 1990. A significant contribution to this increase came from the diffusion of tobacco smoke.

Aging and cancer

The progressive reduction in mortality by infectious and cardiovascular diseases translated into a continuing increase in longevity (Figure 1.5). When Italy was unified, in 1870, life expectancy was about 30 years, almost the same of ancient Romans. Now Italy is among the countries

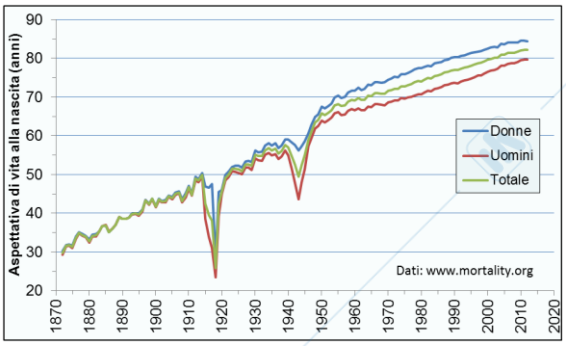


Figure 1.5. Evolution of life expectancy in Italy since 1870.

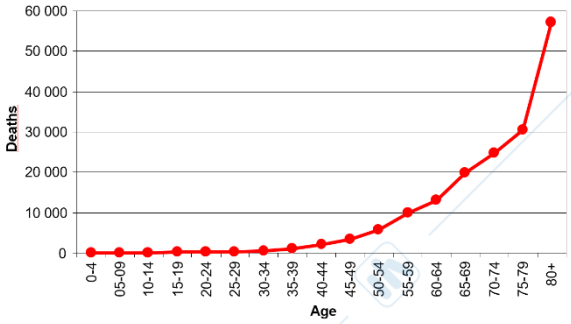


Figure 1.6. Cancer deaths in Italy by age. Data: ISTAT 2006

with the highest life expectancy in the world, with values well above 80 years.

The risk of cancer increases exponentially with age (Figure 1.6), thus the increasing longevity of the world population also entails an absolute increase in the number of new cases of cancer. Compounded with the prolongation of patient's survival brought about by modern cancer therapies, this explains the rising prevalence of cancer patients and cancer survivors worldwide.

Main tumor types

Tumors arise in all tissues and organs of the human body, but incidence and mortality are widely different (Figure 1.7). The four most common anatomical sites are the breasts, the prostate, the lungs, and the colorectal tract, accounting for 40% to 50% of all tumors in Western countries. Even within this limited set of tumors types, mortalities are widely different: low for breast and prostate, around 50% for colorectal, very high for lung cancer.

As it happened for other diseases, also the incidence and mortality of the various cancer types underwent huge variations in the last century (Figure 1.8). The most prominent change was the huge increase of lung cancer, first in males, and later also in females, until the 1990s, followed by a sharp drop in males, while in females the

Estimated New Cases

	Males			Females	
Prostate	180,890	21%		Breast	246,660	29%
Lung & bronchus	117,920	14%		Lung & bronchus	106,470	13%
Colon & rectum	70,820	8%		Colon & rectum	63,670	8%
Urinary bladder	58,950	7%		Uterine corpus	60,050	7%
Melanoma of the skin	46,870	6%		Thyroid	49,350	6%
Non-Hodgkin lymphoma	40,170	5%		Non-Hodgkin lymphoma	32,410	4%
Kidney & renal pelvis	39,650	5%		Melanoma of the skin	29,510	3%
Oral cavity & pharynx	34,780	4%		Leukemia	26,050	3%
Leukemia	34,090	4%		Pancreas	25,400	3%
Liver & intrahepatic bile duct	28,410	3%		Kidney & renal pelvis	23,050	3%
All Sites	**841,390**	**100%**		**All Sites**	**843,820**	**100%**

Estimated Deaths

	Males			Females	
Lung & bronchus	85,920	27%		Lung & bronchus	72,160	26%
Prostate	26,120	8%		Breast	40,450	14%
Colon & rectum	26,020	8%		Colon & rectum	23,170	8%
Pancreas	21,450	7%		Pancreas	20,330	7%
Liver & intrahepatic bile duct	18,280	6%		Ovary	14,240	5%
Leukemia	14,130	4%		Uterine corpus	10,470	4%
Esophagus	12,720	4%		Leukemia	10,270	4%
Urinary bladder	11,820	4%		Liver & intrahepatic bile duct	8,890	3%
Non-Hodgkin lymphoma	11,520	4%		Non-Hodgkin lymphoma	8,630	3%
Brain & other nervous system	9,440	3%		Brain & other nervous system	6,610	2%
All Sites	**314,290**	**100%**		**All Sites**	**281,400**	**100%**

Figure 1.7. Ten leading cancer types in the USA.
From Siegel *et al.*, CA Cancer J. Clin., 66: 7, 2016

decrease started later and was less pronounced. Another notable change was the decrease of stomach cancer, which was the leading cause of cancer mortality at the beginning of the 20th century, but nowadays ranks much lower. As hinted before in this chapter, the underlying causes are the secular trends in tobacco smoke for lung cancer and the advent of modern food hygiene for stomach cancer, which is caused by a bacterial infection.

What can we do?

Given the high impact of cancer on human health, a reduction in cancer incidence and mortality would significantly improve human welfare and further increase life expectancy.

The four main strategies for cancer control are:

- Prevention: avoidance of exogenous carcinogens
- Chemoprevention: Development of drugs to reduce the risk of cancer
- Early diagnosis: To catch early tumors, which are smaller and less malignant
- Therapies: To cure established tumors and metastases

Cancer prevention

About one-half of all tumors are caused by modifiable, exogenous carcinogens, which include physical, chemical and

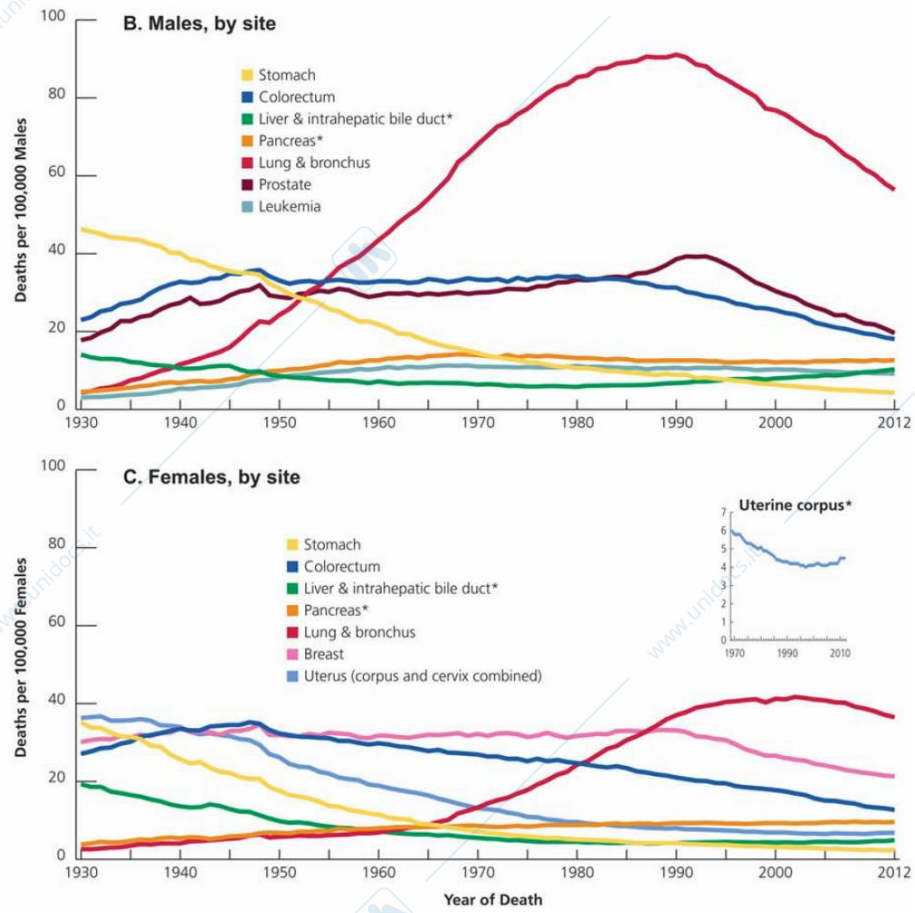


Figure 1.8. Evolution of cancer mortality in the US since 1930, by cancer type.
From Siegel *et al.*, CA Cancer J. Clin., 66: 7, 2016.

biological agents. In principle, human exposure to these carcinogens can be completely avoided. In high-HDI countries, occupational exposure to carcinogens is now tightly regulated and strongly reduced in comparison to the last century. Much less controllable are the exposures related to the lifestyle, such as tobacco smoke, alcoholic beverages and diet, which are currently the major sources of exogenous carcinogenesis.

Cancer is also caused by a plethora of endogenous factors, including hormones, growth factors and chronic inflammatory processes. A promising strategy to counteract these factors is chemoprevention, i.e. the development of drugs that, with various mechanisms, limit the exposure to endogenous carcinogens. Similar approaches are being used with great success against cardiovascular diseases, for example drugs that lower blood pressure or reduce cholesterolemia, but the field of oncological chemoprevention is much less advanced: some drugs do indeed prevent breast or colorectal cancers but their unfavorable toxicological profiles allow the use only in persons at high risk of cancer. The only treatment successfully applied to the general population is the vaccination against cancer-causing viruses, such as the hepatitis B virus (HBV) or papilloma viruses (HPV).

Early diagnosis and therapy

Tumors take several years, or decades, to develop from normal cells to full-fledged metastatic cancers. During this period, tumor cells accumulate mutations and pass through various stages of increasing

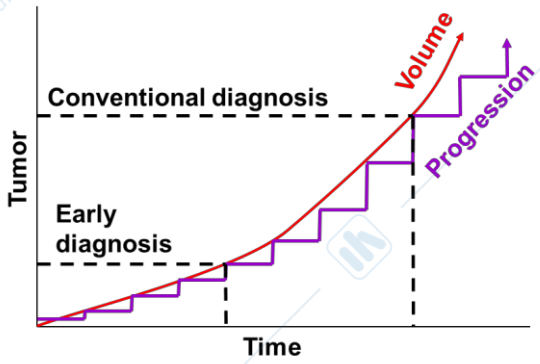


Figure 1.9. Early diagnosis can discover tumors that are smaller and less malignant.

malignancy, a process called tumor progression. The knowledge of tumor progression leads to the concept that an early diagnosis can discover tumors that are not only smaller, but also less malignant and more easily curable, than symptomatic tumors. Population screenings with appropriate technologies, such as mammography or occult fecal blood assays, can indeed lead to the diagnosis and treatment of very early tumors. However, it should be noted that early diagnosis can also discover innocuous tumors, for example those that would have spontaneously regressed in the future, leading to an undesired phenomenon called overdiagnosis.

Cancer therapies

The three pillars of cancer therapy are surgery, radiotherapy and pharmacological therapies. Surgery can cure most primary tumors (the exception being those tumors that cannot be radically removed, such as invasive brain tumors), but the metastatic spread of malignant tumors requires the systemic administration of drugs that can reach metastatic deposits throughout the body.

The combination of multiple therapeutic approaches is a common theme in oncology: most malignant primary tumors are surgically excised, then the patient receives radiotherapy and/or pharmacological treatments with multiple drugs.

Some continuing trends in cancer therapy are:

- Surgery
  - Conservative surgery
  - Robotic surgery
- Radiotherapy
  - Machines and computer algorithms to focus on tumor lesions, sparing normal tissues and organs
- Drug therapy
  - Molecularly targeted agents ("target therapy")
  - Precision medicine, i.e. personalized treatments on the basis of the molecular features of individual tumors
  - Therapeutic regimes without cytotoxic drugs ("chemotherapy-free"), to avoid the systemic toxicity of those drugs that indiscriminately kill proliferating cells
  - Drugs against normal cells that support tumor growth, such as endothelial cells forming new blood vessels which supply oxygen and nutrients to tumors
  - Immunotherapies that exploit the anti-tumor mechanisms of bodily defenses.

Chapter 2. Cancer Classifications

The pathological classification of individual cancer cases is a pillar of cancer diagnosis, and the basis of unbiased communication among the various cancer experts that will interact in the treatment of the patient.

Neoplasia *vs.* hyperplasia

Cancer is a neoplastic disease, clearly distinct from hyperplastic conditions. As defined in the previous chapter, neoplasia results from uncontrolled alterations in tissue homeostasis caused by cell-inheritable defects in proliferation, migration, differentiation, and death.

Hyperplasia is a controlled, non-progressive and reversible increase in cell number. Physiological hyperplasia is an adaptive response to stimuli, e.g. increased erythropoiesis in people living at high altitudes (low oxygen). Pathological hyperplasia is a reactive response to persistent insults, e.g. mechanical friction induces callus formation.

Origin and classification of tumors

Tumors retain some distinctive traits of the cell and tissue of origin. Notably, morphological features allow the pathological classification of tumors upon microscopical examination. When morphology is not sufficiently distinctive, immunohistochemistry with antibodies against specific molecules is used to identify the tumor histotype.

All human tumors are sampled and studied by experienced pathologists to classify their histological features, which contribute to the definition of prognosis and to decisions concerning therapeutic treatments. It can be said that, traditionally, cancer therapy is mainly determined by the tumor histotype.

Modern diagnosis and classification are also based on the study of molecular features, which can be assessed either by immunohistochemistry or by molecular technologies, such as gene sequencing.

Table 2.1. Features of benign and malignant tumors

Feature	Benign tumors	Malignant tumors
Mitotic index*	Low	High
Mitoses	Normal	Abnormal
Nucleoli	Normal size	Large
Nucleus/cytoplasm ratio	Normal	High
Chromosome number	Euploid	Aneuploid
Differentiation	Almost normal	Poor
Functionality°	Functional	Altered functionality
Surrounding capsule	Present	Absent

*Number of mitoses per microscopic field
°*e.g.* glandular secretion

Thus, molecularly targeted therapeutic agents are only administered to those patients whose tumor expresses the target molecule, an approach referred to as precision oncology.

Currently, therapeutic decisions are guided by a combination of morphological and molecular information. In the late 2010s, it was demonstrated that, in some cases, therapy can be effectively decided purely on molecular features. For example, all tumors harboring mutations in NTRK genes can be treated with TRK inhibitors. Such treatments, which are independent of the tumor histotype, are called "tissue-agnostic".

Classification of benign and malignant tumors

The classification of a tumor as benign or malignant is of paramount importance for prognosis and therapy, especially for what concerns the risk of occult distant metastases after the surgical removal of the primary mass. The pathological classification of tumors is traditionally based on cytological and histological features that correlate with tumor behavior (Table 2.1).

Naming of tumors

Pathologists recognize more than 500 tumor types; hence, a precise nomenclature is essential for tumor identification. We have seen that some terms, such as carcinoma, date back to ancient Greece, thus it is no wonder that the naming of cancer types does not follow a fully rational, unifying scheme.

Tumors are classified and named on the basis of the tissue/cell of origin. Macroscopic and stromal features also contribute to classification and nomenclature, e.g. some tumors are classified as inflammatory if they contain a peculiar leukocyte infiltrate and display other features of inflamed tissues.

A prefix usually indicates the tissue/cell of origin, e.g. *adeno-* indicates a glandular origin, *fibro-* from stromal fibroblasts, *chondro-* from chondrocytes, *rhabdomyo-* from striated muscle.

The names of benign tumors usually include the suffix *–oma*, e.g. adenoma, fibroma, chondroma. Confusingly, the names of various malignant tumors follow the same scheme: melanoma, lymphoma, myeloma.

In malignant tumors, the suffix *carcinoma* indicates epithelial tumors, e.g. adenocarcinoma. The suffix *-sarcoma* indicates mesenchymal tumors, *e.g.* fibrosarcoma, osteosarcoma, rhabdomyosarcoma. The suffix *-blastoma* indicates tumors of precursor, poorly differentiated cells: chondroblastoma, glioblastoma, retinoblastoma. Hematopoietic neoplasms are leukemias, originating in the bone marrow, and lymphomas, originating in lymph nodes.

Incidence of malignant tumors

Adult neoplastic pathology is dominated by epithelial tumors (Table 2.2), whereas all other histotypes are rarer. The likely

Table 2.2. Proportions of human tumor histotypes

Tumor type	Percentage
Carcinoma	85%-90%
Sarcoma	1%-2%
Leukemia	3%
Lymphoma	4%
Other	5%

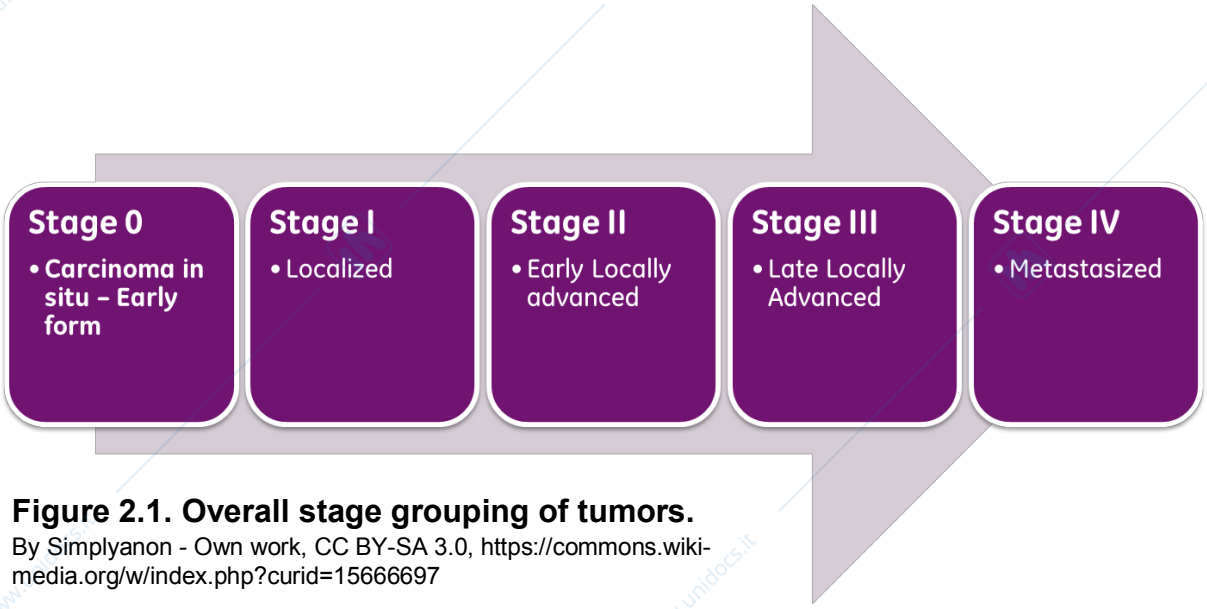


Figure 2.1. Overall stage grouping of tumors.
By Simplyanon - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=15666697

explanation of these differences is the preferential neoplastic transformation of labile tissues, which in adults mostly include epithelial tissues. On the contrary, non-epithelial tumors are common in pediatric pathology.

Staging of malignant tumors

Staging is a standardized synthesis of tumor extension. Staging is important to assign therapies and to predict prognosis. An attempt to define a common staging system for all tumors is the TNM (Tumor, lymphNode Metastasis) classification, which, however, is not applied to hematological, CNS and gynecological tumors, and is frequently replaced by specific classifications in other tumor types.

In the TNM system, T followed by a number quantifies primary tumor size, from T0 (no evidence of primary tumor) to T4 (large). N identifies regional lymph node metastases, from N0 (no metastases) to N3 (numerous). M signals whether distant metastases are absent (M0) or present (M1). Pathological, *i.e.* microscopical, evaluations are prefixed with a lower case "p", *e.g.* pN0 is used when the absence of lymph node metastases was assessed microscopically.

All staging systems are periodically revised to include diagnostic advancements and refinements. A notable evolution of modern staging systems, including TNM, is the gradual inclusion of molecular parameters.

Overall stage grouping synthesizes in a single roman numeral, from 0 to IV, the progression of a tumor. Definitions of the various stages are dependent on the specific type of cancer. Figure 2.1. shows a common system ranging carcinomas from *in situ* to metastatic.

Grading of tumors

Grading is a pathological evaluation of tumor anaplasia and differentiation.

Low-grade tumors resemble well-differentiated normal tissues, whereas high-grade tumors are poorly differentiated and anaplastic. In general, low-grade tumors have a better prognosis than high-grade ones.

An example of a commonly used grading system is the Gleason score of prostate carcinomas (Figure 2.2).

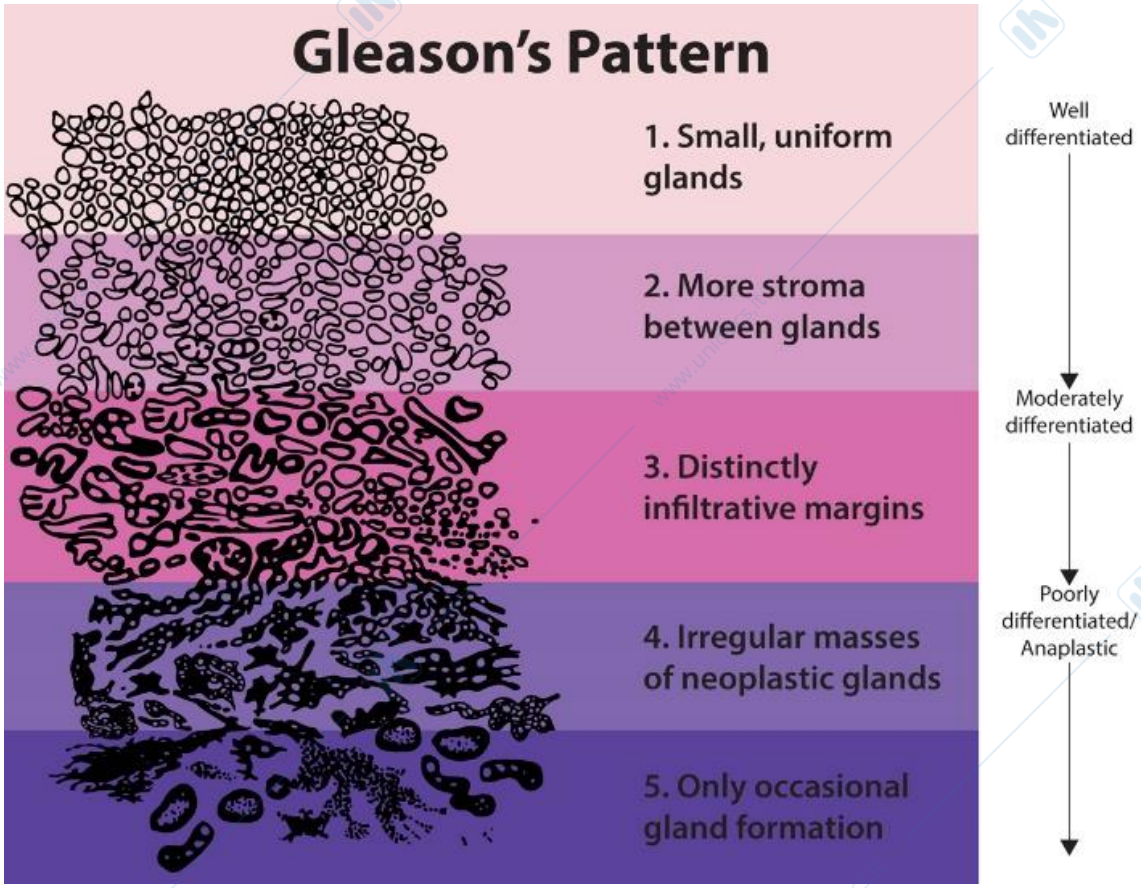


Figure 2.2. Gleason score of prostate cancer. Public Domain, https://commons.wikimedia.org/w/index.php?curid=440437

Chapter 3. Cancer Genes

Cancer is a disease of genes

Cancer is caused by mutations and other alterations causing structural or expression variation in specific genes. Only somatic cells are affected in most cases (sporadic tumors). In some cases (about 5% of all tumors), germline mutations predispose to hereditary cancer syndromes.

The genic nature of cancer was first hypothesized by Theodor Boveri in the 1910s, but gained traction only in the late 1960s, with the development of cancer cytogenetics and mutagenesis.

Gene alterations leading to cancer (Figure 3.1) are caused both by exogenous agents (carcinogens) and by endogenous events, which include several sources of spontaneous mutation (Figure 3.1).

Mutation is unavoidable

Evolution is fueled by germline mutations that increase the fitness of the individual. In a multicellular organism, the same process at the cellular level, *i.e.* somatic mutations that increase the fitness of an individual cell, leading to its unrestricted proliferation, result in the development of a tumor (Figure 3.2). Just as evolution is unstoppable, a baseline level of carcinogenic mutations is unavoidable.

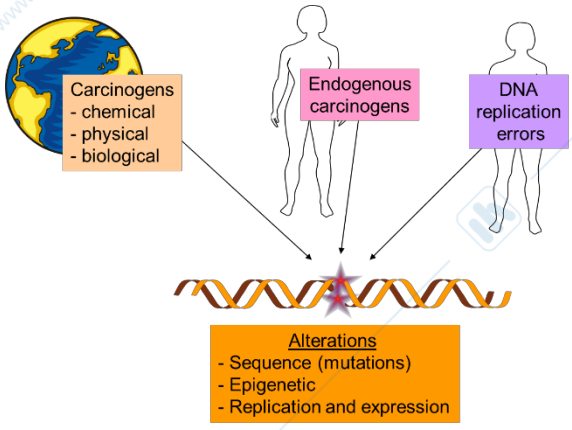


Figure 3.1. The causes of cancer.

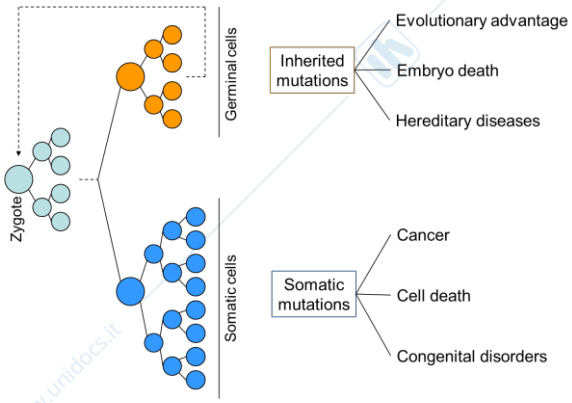


Figure 3.2. The consequences of inherited and somatic mutations.

A major source of spontaneous mutations is linked to the insufficient fidelity of DNA polymerases. Actually, the fidelity of DNA polymerases is quite high, they introduce one error every 1-100 billion bases, but they have a daunting task, as every human needs to incorporate in their DNA about 10^{25} bases. Thus, all humans accumulate trillions of DNA mutations throughout their life.

Water itself is an endogenous mutagen, as DNA in aqueous solutions undergoes spontaneous hydrolysis. Other endogenous mutagens include alkylating agents, such as S-adenosylmethionine, and reactive oxygen species (ROS) produced by some enzymatic reactions and by phagocytes.

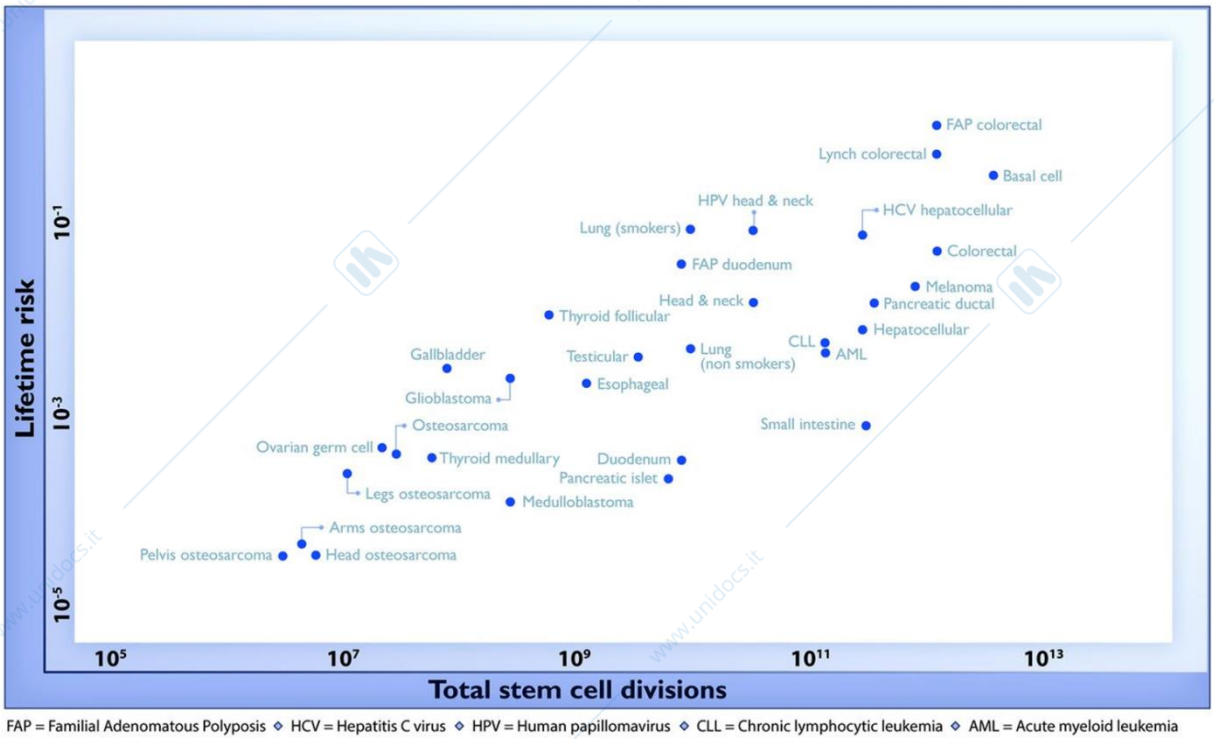


Figure 3.3. The lifetime risk of cancer in each tissue or organ is proportional to the number of stem cell divisions. From Tomasetti & Vogelstein, Science, 347: 79

All DNA-modifying processes are intrinsically error-prone and can cause mutations. Some examples are DNA damage repair, DNA recombination, VDJ recombination and somatic hypermutation of lymphocyte antigen receptors, anti-viral cytidine deaminases and transposable elements.

Fortunately, only some mutations have consequences at the protein level, because a large part of the human genome has no coding or regulatory functions. Furthermore, the genetic code is degenerate, i.e. a mutant codon can encode the same amino acid as the normal one, and even when the amino acid is different, it can be functionally similar to the original one (conservative mutations).

DNA mutation and cell proliferation

Most spontaneous mutations are introduced in the genome when the cell replicates. Thus, there is a strong correlation between cell proliferation in various tissues and their probability of tumor onset. This fact was recently brought to the attention of cancer researcher by Bert Vogelstein and co-workers, who showed the correlation between the number of stem cell divisions in the lifetime of a given tissue and the lifetime risk of cancer in that tissue (Figure 3.3).

This explains why the incidence of carcinomas is much higher than that of sarcomas, (see previous chapter), because epithelial tissues proliferate throughout adult life, unlike mesenchymal ones. Vogelstein and co-workers proposed a tripartite classification of human carcinogenesis, which can be attributed to hereditary, replicative, or environmental causes (Figure 3.4). In each tissue and organ the proportion of tumors attributable to the different causes is different, for example in the brain all tumors are attributed to

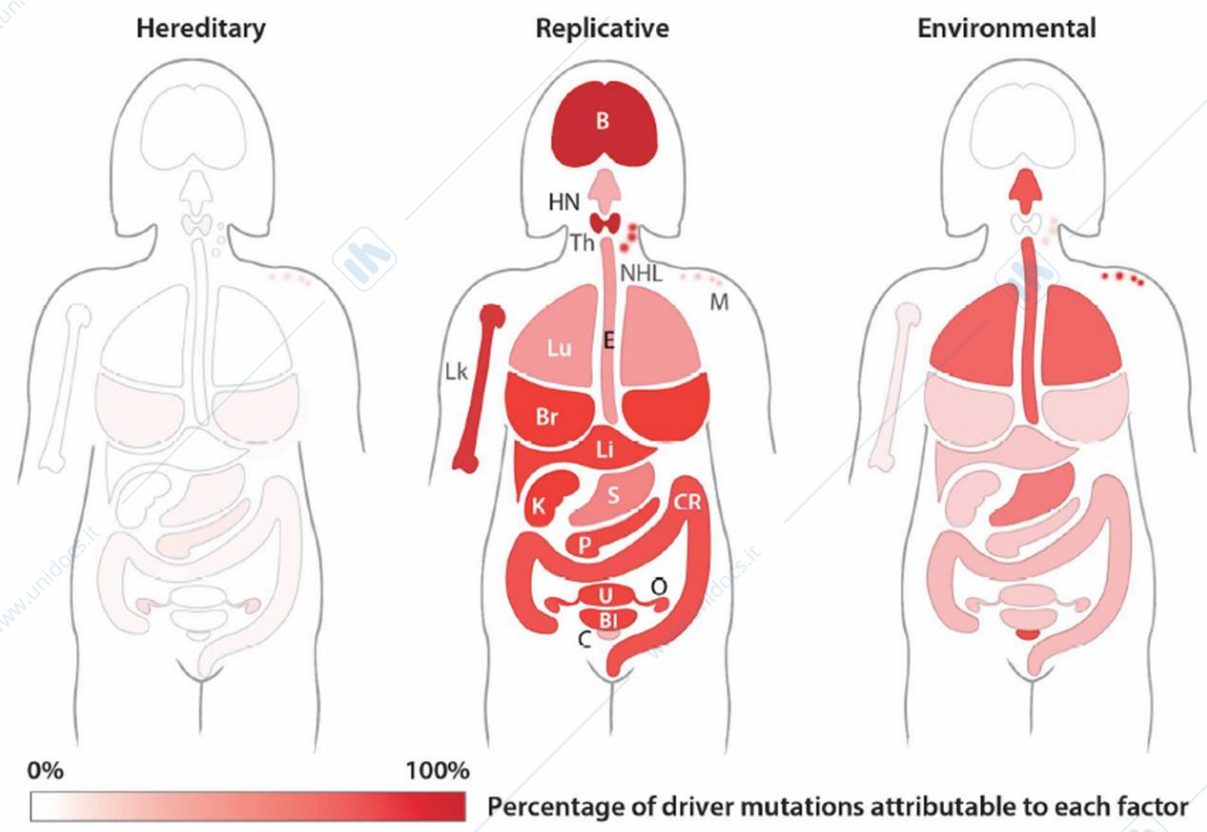


Figure 3.4. Proportions of tumors attributable to hereditary, replicative, or environmental causes. B, brain; HN, head & neck ;Th, thyroid; NHL, non-Hodgkin lymphoma; M, melanoma; Lk, leukemia; Lu, lung; E, esophagus; Br, breast; Li, liver; K, kidney, S, stomach; P, pancreas; CR, colon-rectum; U, uterus; Bl, urinary bladder; O, ovary; C, cervical. From Tomasetti & Vogelstein, Science, 355: 1330.

replicative errors, while a fraction of breast cancers is also caused by environmental and hereditary factors, whereas in the lung carcinogenesis is predominantly environmental.

These considerations fueled a heated debate, because they seem to attribute a stronger importance to unavoidable causes of cancer, and to detract from the importance of cancer prevention, but it must be considered that the estimates proposed by Vogelstein have wide margins of error, and that not all causes of replicative error are unavoidable, for example we already have drugs that can reduce the risk of breast cancer due to estrogen-fueled proliferation. In any case, the great importance of this work is the reminder that spontaneous mutation and cell proliferation are major causes of human cancer.

The mutational landscape of human cancer

DNA sequencing of human cancer revealed wide variations in the number of mutations of individual tumors (Figure 3.5). On the average, each tumor type has a characteristic number of mutations, which appears to be directly related to environmental mutagens; for example, the highest average numbers of mutations are found in melanoma (UV radiation), lung and bladder cancers (tobacco).

Within each tumor type there is a broad variation – more than two orders of magnitude – among individual patients. Mutations in the coding region of genes could

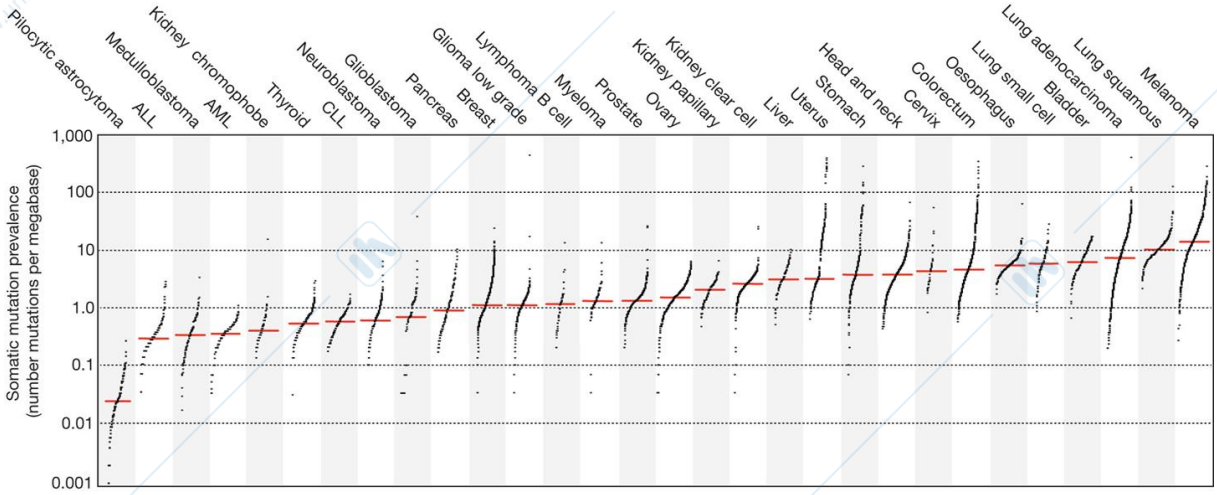


Figure 3.5. The mutational landscape of human tumors.
LB Alexandv *et al. Nature* **500**, 415-421.

result in the appearance of new proteins variants that are recognized by the immune system of the host (neoantigens). Thus it was found that a high number of mutations in a tumor (tumor mutation burden, TMB) can be used in the clinics to select patients for immunotherapeutic treatments.

Drivers and passengers

Within each tumor, only a few mutations actually play a pathogenetic role, whereas a large fraction of the total mutation burden randomly affects non-coding regions, or genes unrelated to the neoplastic phenotype. Using a bus metaphor, those mutations that control tumorigenicity and malignancy (e.g. proliferation, apoptosis, invasiveness, genomic stability, etc.) are called driver mutations, all other mutations are called passengers.

Oncologists are usually more interested in driver mutations, because they are tumor-specific targets against which novel drugs can be directed. However, also passenger mutations can be of clinical use, for example as a source of neoantigens for cancer immunotherapy.

Oncogenes and tumor suppressor genes

The two major types of cancer genes are oncogenes and tumor suppressor genes. Oncogenes are positive regulators of cell growth, affected by activating, dominant gain-of-function mutations. Tumor suppressor genes include negative regulators of cell growth and controllers of genome stability, affected by inactivating, recessive loss-of-function mutations.

In addition to canonical cancer genes, many other genes involved in cell adhesion, non-coding RNAs, metabolism, etc. play functional roles in carcinogenesis, even though they are not usually called oncogenes or tumor suppressor genes.

The Catalogue of Somatic Mutations in Cancer (COSMIC, https://cancer.sanger.ac.uk/cosmic) includes the Cancer Gene Census (CGC), an expert-curated description of the genes driving human cancer. In 2019 GCC listed >500 genes with strong evidence of being cancer genes, equally distributed between oncogenes and tumor suppressor genes (some genes were classified as both oncogene and tumor suppressor, depending on the tumor type).

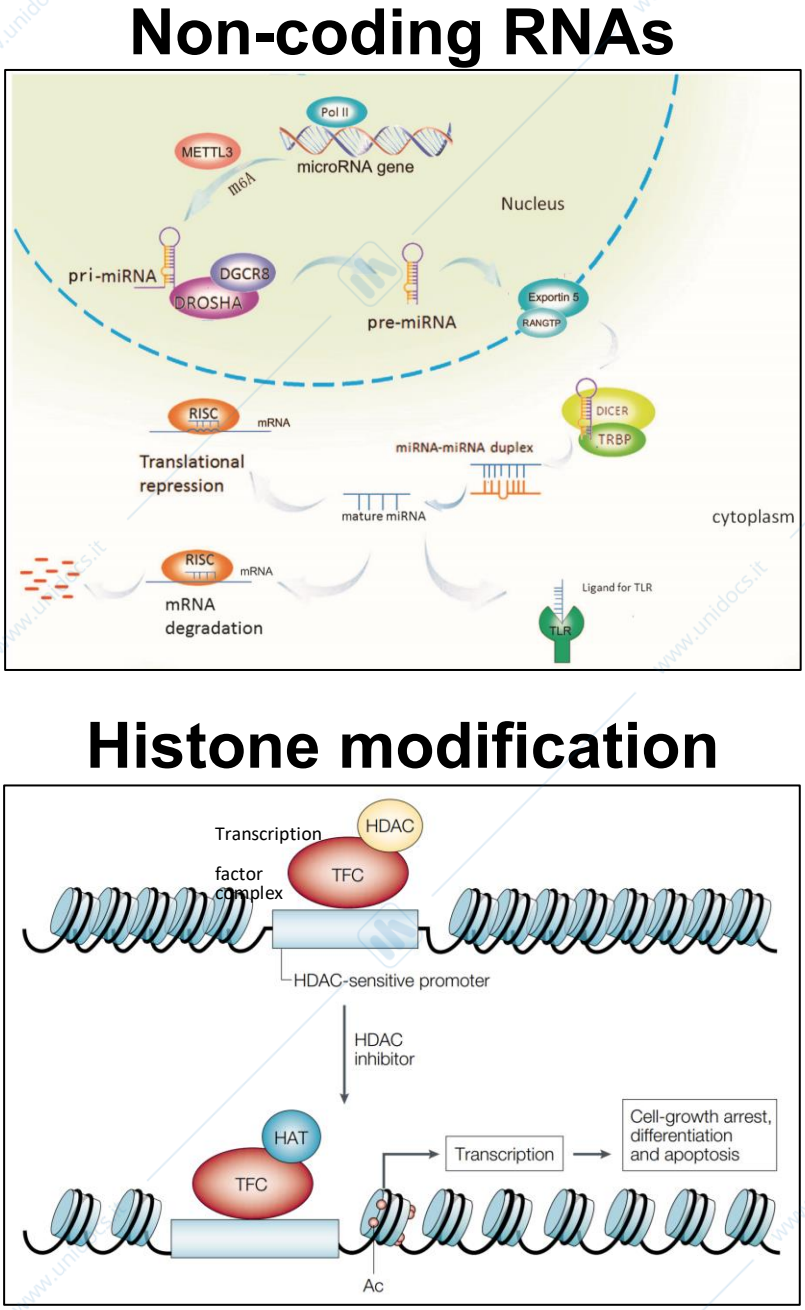


Figure 3.6. Epigenetic controls of gene expression. DNA methylation By Mariuswalter - Own work, CC BY-SA 4.0, https:// commons.wikimedia .org/w/index.php?curid=54319988. Non-coding RNAs: Peng & Croce, Signal Transduction and Targeted Therapy 1: 15004. Histone modification: Marks, Nat Rev Cancer 1, 194.

Epigenetics of cancer

Epigenetic, i.e. non-mutational, mechanisms contribute to the regulation of cancer genes. (Figure 3.6).

Methylation of CpG islands by DNA methyltransferases (DNMT) silences gene expression. Hypermethylation of tumor suppressor genes in cancer (amid a landscape of hypomethylation) can be countered by clinically active DNMT inhibitors, e.g. 5-azacytidine. The methylome, i.e. the methylation pattern of whole human genome, is being studied as a possible prognostic/predictive tool.

Networks of non-coding RNAs, both small (e.g. miRNA) and long (lncRNA) regulate the expression of cancer genes. Functionally behaving both as oncogenes (oncomiR) or tumor suppressor genes, non-coding RNAs are being developed as drugs and prognostic/predictive biomarkers.

Enzymatic histone modification alters chromatin structure and gene expression. Histone acetyltransferases (HAT) and deacetylases (HDAC) are recruited by some cancer genes (RB, PML-RARα), and also modify non-histone substrates (p53, E2F). HDAC inhibitors are under development as drugs.

Chapter 4. Oncogenes

Oncogenes are genes that, when upregulated / deregulated, increase cell growth. The definition of human oncogenes is based on three lines of evidence: neoplastic transformation of gene-transduced cells *in vitro*, carcinogenicity in genetically modified animal models, and consistent alterations in human tumors, which can be inhibited by specific drugs. Note that several genes functionally fulfilling the definition are not commonly called oncogenes.

The discovery of oncogenes began in the 1970s, with the definition of oncogenic sequences (v-onc) of retroviruses and the discovery that v-onc are cell genes (c-onc) transduced, i.e. transported, by retroviruses from one cell genome to another. Transfer to normal cells of the transformed phenotype from (non-virus related) tumor cells by means of transfection then led to the cloning of murine and human oncogenes that are not transduced by retroviruses.

In this chapter we will first examine the general features of oncogenes, then we will analyze in detail some notable human oncogenes.

Oncogene functions

Oncogenes have various functions related to cell proliferation. A useful way to visualize those functions is to follow a mitogenic signal from the outside of a cell to the nucleus (Figure 4.1).

- Soluble signaling molecules: growth factors, cytokines, hormones, e.g. WNT, PDGFB

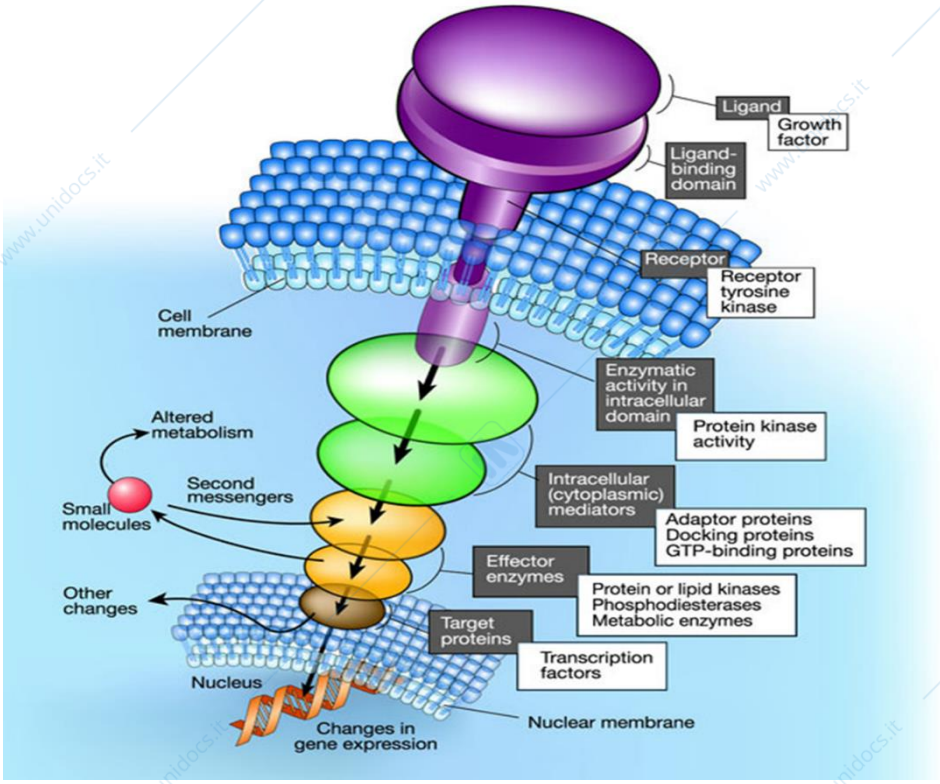


Figure 4.1. Oncogene functions. From J. Downward, Nature, 411: 759.

- Growth factor receptors, e.g. EGFR, HER-2, KIT, MET, RET, TRK
- Intracellular signal transducers, e.g. RAF, RAF, ABL
- Cell cycle regulators, e.g. CCND1, CDK4 and 6
- Transcription factors, e.g. MYC, JUN, FOS
- Apoptosis inhibitors, e.g. BCL-2

Oncogene activation

Normal genes involved in cell growth undergo pathological alterations that activate their oncogenic potential. The term "proto-oncogene" refers to the normal gene, "oncogene" to the activated one (proto-oncogene is mostly used when dealing with both forms, otherwise oncogene can also indicate the normal gene).

There are several mechanisms that can activate proto-oncogenes:

- Point mutations that lock the protein in an activated configuration, e.g. RAS, BRAF
- Truncation of protein domains that control protein activation, e.g. EGFR, HER-2
- Gene amplification, e.g. HER-2, NMYC
- Chromosomal translocations that either generate fusion proteins, e.g. BCR-ABL or move an oncogene to a transcriptionally active region, e.g. MYC
- Insertional mutagenesis, i.e. insertion of viral promoters and/or enhancers near a cellular oncogene, e.g. hepatitis B virus integration near cyclin E1 or telomerase genes. Insertional mutagenesis also occurred in an infamous gene therapy clinical trial in which the retroviral vector activated the LMO2 oncogene.
- Epigenetic alterations can cause the biallelic expression of genes normally expressed by a single allele, e.g. loss of imprinting (LOI) at the insulin-like growth factor 2 (IGF2) gene occurs in hereditary Beckwith-Wiedemann syndrome (risk of rhabdomyosarcoma and Wilms tumor) and in about one-half of sporadic colorectal carcinomas

HER/ERBB family

The HER family comprises four genes, the epidermal growth factor receptor (EGFR, also called HER-1), HER-2, also called ERBB2, HER3 and HER-4, all expressed on the cell membrane (Figure 4.2).

EGFR binds EGF, transforming growth factor α (TGF- α) and other ligands. Its main physiological role is in the development and adult homeostasis of the epidermis and other epithelial tissues. EGFR behaves as a canonical tyrosine kinase receptor. Upon ligand binding, two receptor molecules dimerize and cross-phosphorylate, creating docking phosphotyrosines recognized by adaptor molecules, such as GRB2, that relay the mitogenic signal downstream.

HER-3 is a receptor, but during evolution its tyrosine kinase domain was inactivated, thus HER-3 can bind ligands, but cannot send signals downstream. HER-2 comes to the rescue, having a specular condition: it lacks natural ligands (for which it is called an orphan receptor), but has a strong kinase activity. Hence ligand-bound HER-3 can heterodimerize with HER-2 to relay mitogenic signals within the cell. HER-2 can heterodimerize also with other members of the family, and most importantly can homodimerize. In normal cells HER-2 molecules are relatively rare, hence the level of uncontrolled activity of HER2-2 dimers is low. The major physiological role of HER-2 is in the embryogenesis of the heart; HER-2 knockout mice die *in utero* with a heart lacking

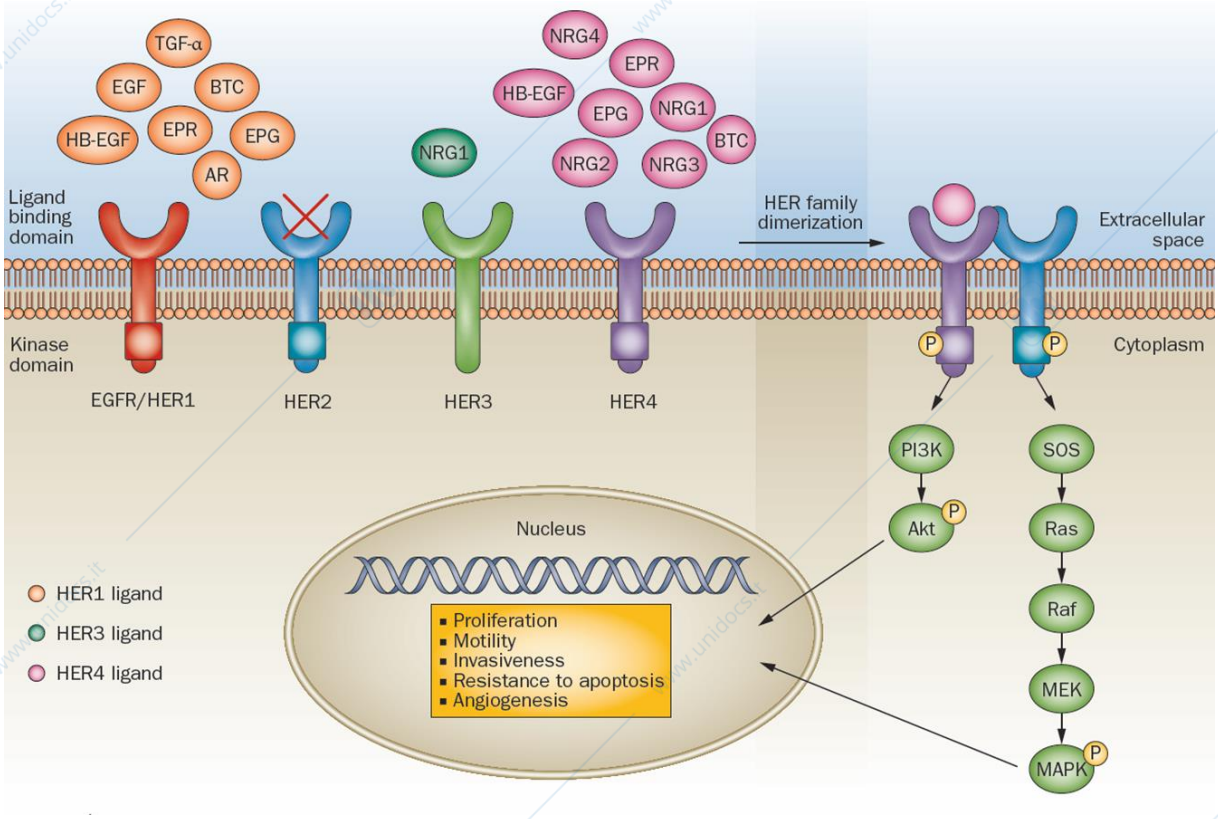


Figure 4.2. The HER/ErbB oncogene family. Arteaga et al., Nat. Rev. Clin. Oncol. 9:16.

internal chambers. In adults HER-2 maintains a minor role in the heart.

EGFR in human cancer

EGFR can be activated by point mutations, small deletions, or gene amplification. It is altered in more than 20% of all human cancers: 40% of lung adenocarcinomas, most head and neck tumors and one-half of glioblastomas (with a typical EGFRvIII deletion).

Therapeutic agents targeting EGFR include monoclonal antibodies that bind to the extracellular portion and small kinase inhibitors. As tumors can develop mutations resistant to kinase inhibitors, second-generation inhibitors were in turn developed to target resistant mutations. The therapeutic activity of these drugs requires a normal downstream flow of signals, for example activating RAS mutations render anti-EGFR agents useless.

HER-2

HER-2 is frequently activated by gene amplification; the presence of an overwhelming number of HER-2 homodimers results in an unrelenting mitogenic signaling. HER-2 can be also activated by point mutations. Post-translational, activating protein truncations were also reported.

The highest prevalence of HER-2 alterations is found in breast (20% of cases) and bladder cancers (10%), low percentages (1%-2%) are found in various other tumor types.

Target therapy is mainly based on monoclonal antibodies, but small kinase inhibitors are also approved. Therapeutic agents were first developed for breast cancer, but positive results against rare cases with HER-2 alteration in other tumor histotypes illustrate the opportunities of tissue-agnostic therapy.

Demonstration of gene amplification is required for the administration of anti-HER-2 therapy. Strong staining (so-called "+++", or "3+" cases) of tumor sections by anti-HER-2 monoclonal antibodies provides first-level evidence of gene amplification. Lower levels of staining (++) require confirmation of gene amplification by fluorescent *in situ* hybridization (FISH). Patients expressing low levels of wild-type HER-2 (+) are not treated with targeted agents. Point mutations can only be diagnosed at the DNA level.

RAS family

The three members of the RAS family, KRAS, HRAS and NRAS, are monomeric guanine nucleotide-binding proteins (G proteins) anchored to the inner membrane by a fatty acid tail. Physiologically they are activated (bound to GTP) by adaptors like SOS, downstream of tyrosine kinase receptors; they are inactivated (bound to GDP) by their intrinsic GTPase activity and by GTPase accelerating (GAP) proteins (Figure 4.3).

Oncogenic activation is by point mutations (hot-spots in codons 12, 13 and 61) that inactivate intrinsic GTPase function. About 20% of all human tumors harbor oncogenic RAS mutations. Mutant KRAS is found in two-thirds of pancreatic, one-third of colorectal and small intestine, one-fifth of lung and endometrial carcinomas. NRAS mutations are found in 25% of acute non-lymphocytic leukemias.

RAS is a coveted therapeutic target, also because RAS mutations mediate resistance to drugs against upstream therapeutic targets, such as EGFR. However, the first generation of specific drugs,

Figure 4.3. RAS signaling activity.

From Vinay et al.: Robbins basic pathology, Copyright Elsevier 2005.

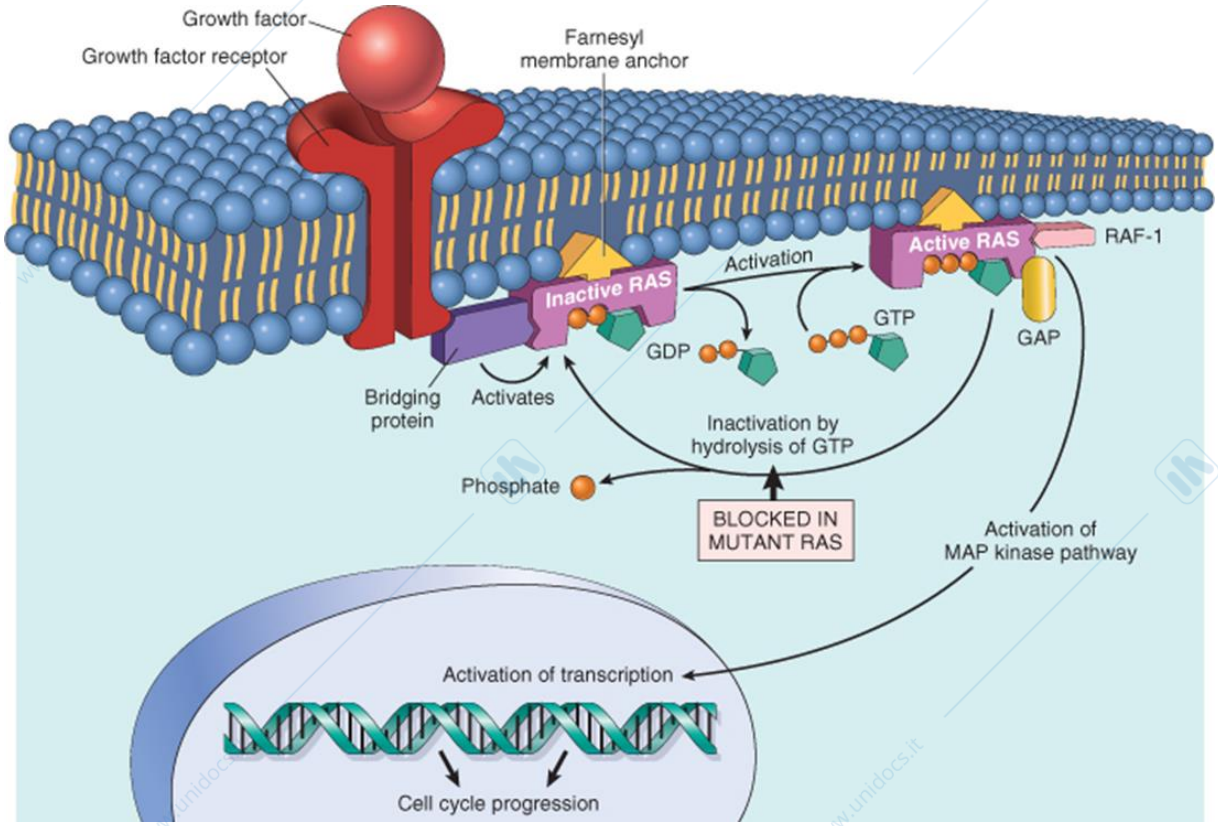
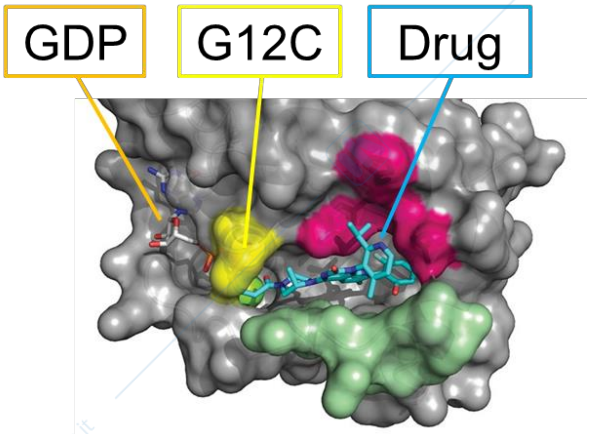


Figure 4.4. Structure of a mutation-specific RAS inhibitor. Credit: Amgen



based on the inhibition of farnesyltransferases that link the protein to the inner membrane, was unsuccessful. Mutant RAS is antigenic, and can be recognized by cytotoxic T cells, but only one case report of successful immunotherapy is found in the literature. A problem encountered in the rational design of drugs is that RAS presents a smooth external surface, offering no anchorage for inhibitors. Structural analyses showed that a G to C mutation in codon 12 indeed offers a link, which was exploited to produce a novel inhibitor endowed with clinical activity (Figure 4.4). It remains to be determined whether this approach will allow the synthesis of inhibitors specific for other mutations.

RAF family

GTP-bound RAS activates a cascade of cytoplasmic kinases that relay the mitogenic signal to the nucleus. The first members of this cascade are RAF serine-threonine kinases, a family that includes RAF, also referred to as c-RAF, A-RAF and B-RAF.

B-RAF mutations, in particular the V600E, are as common as RAS mutations, affecting about 20% of all human tumors. A series of specific inhibitors, with names including the "rafenib" suffix, is in clinical use.

MYC family

A family of three transcriptional activators, MYC, LMYC and NMYC, which bind specific DNA sequences (E-box), activating the transcription of proliferation-related genes. They are regulated through the formation of dimers with two other proteins, MAX and MAD. MYC-MAX heterodimers are transcriptionally active, whereas MAX-MAX homodimers are inactive, and MAD-MAX dimers are inhibitory.

Oncogenic activation is through gene amplification (NMYC) or chromosomal translocation (MYC). Commonly affected human neoplasms include small cell lung cancer (30% of cases), advanced neuroblastoma (hence the N of NMYC), glioblastoma, Burkitt lymphoma and acute T cell leukemia.

In Burkitt lymphoma, a B cell neoplasm, an intact MYC is translocated from chromosome 8 to chromosomes 14 (most frequently), 2 or 22 (Figure 4.5), under the

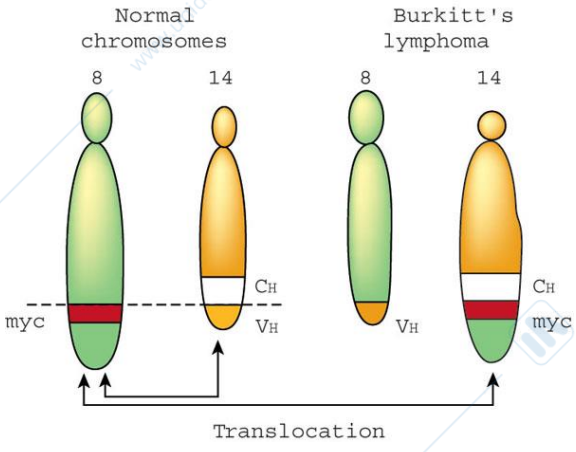


Figure 4.5. The chromosomal translocation of Burkitt lymphoma. *From GJV Nossal, Nature 2003, 440-444*

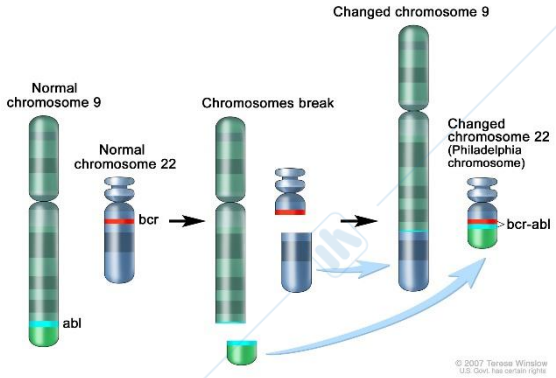


Figure 4.6. BCR-ABL chromosomal translocation. *Copyright 2007 Terese Winslow. U.S. Government has certain rights.*

control of immunoglobulin heavy or light chain genes, which are obviously very active in B cells, thus leading to hyperexpression of MYC. Burkitt lymphoma is endemic in African regions affected by malaria, which constantly stimulates B cell immunity. The VDJ rearrangement of immunoglobulin genes can be viewed as an intra-chromosomal rearrangement, thus it is no wonder that, with malaria parasites eliciting billions of rearrangements, sometimes the B cell makes a mistake and performs an inter-chromosomal rearrangement involving MYC instead. In the chapter on viral carcinogenesis we shall see that these processes require a B cell immortalized by the Epstein-Barr virus.

BCR-ABL translocation

A different type of chromosomal translocation affects the ABL oncogene, which encodes a cytoplasmic/nuclear non-receptor tyrosine kinase controlling normal leukocyte proliferation, differentiation, and apoptosis.

In chronic myeloid leukemia (all cases) and some acute lymphoblastic leukemias (20% of cases), part of chromosome 9 undergoes a reciprocal translocation with chromosome 22, giving rise to a typical 22 derivative called Philadelphia chromosome (Figure 4.6). The translocation encodes chimeric BCR-ABL oncoproteins (p210 in CML, p185 in ALL) which are unique of neoplastic cells.

The specific ABL inhibitor imatinib was one of the first instances of successful targeted therapy. It also showed that, on the one hand, tumor cells can develop resistance to tyrosine kinase inhibitors through specific mutations that prevent drug access to the ATP binding pocket, but on the other hand pharmacologists can design second generation inhibitors targeting resistant mutations (and so on…).

BCL-2 family

The BCL-2 family includes more than 20 genes that regulate apoptosis (Figures 4.7 and 4.8), either positively or negatively, through mitochondrial outer membrane permeability. BCL-2 is anti-apoptotic, and it is in turn inhibited by proteins containing the BH3 domain (Figure 4.7).

BCL-2 is activated by a t(14;18) translocation in follicular B cell lymphoma and is hyper-expressed in chronic lymphocytic leukemia. Several therapeutic approaches were developed over the years to target

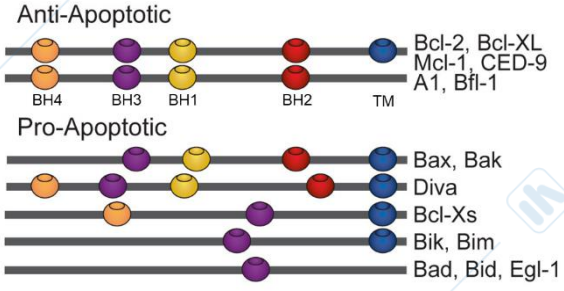


Figure 4.7. BCL family members and BH domains. *By en:User:Kosigrim - designed and donated to Wikipedia by en:Abgent, graphic execution by en:User:Kosigrim, https://commons.wikimedia.org/w/index.php?curid=2747390, modified. Public Domain.*

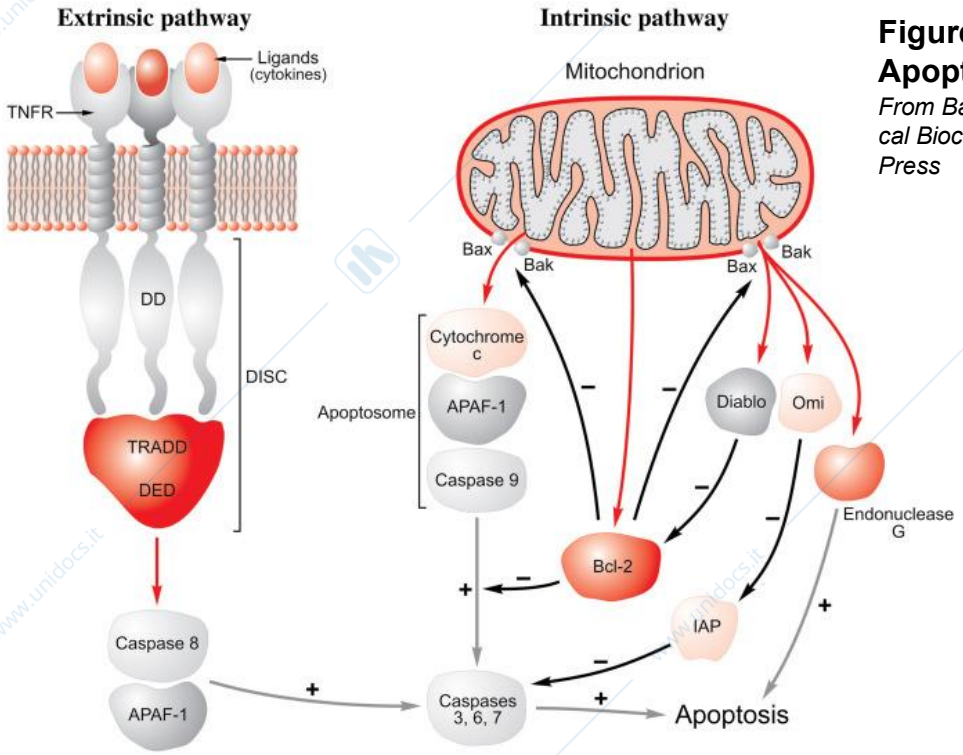


Figure 4.8. Apoptotic pathways.
From Banco & Blanco, Medical Biochemistry, Academic Press

BCL-2, including antisense oligonucleotides. Clinical success was eventually obtained with BH3 mimetics, such as venetoclax.

Oncogenes: clinical implications

The discovery of oncogenes and the endeavors to design truly tumor-specific therapeutic approaches revolutionized oncology and introduced novel lasting notions.

Precision oncology is based on the determination of individual molecular features of tumors, leading to targeted therapies that avoid the one-size-fits-all approach of standard chemotherapy and strive (not always successfully) to avoid undesired toxicities.

No matter how specific or intelligent the therapeutic approach is, tumors can develop resistances based on a variety of molecular mechanisms. In the case of small kinase inhibitors, resistance is mediated by mutations which leave intact the oncogenic potential while preventing inhibitory drug activity, but these resistant mutations can in turn be targeted by novel, specifically designed inhibitors.

Therapeutic agents directed against a given molecular alteration can be used to treat all patients harboring the same alteration, regardless of the tumor type, thus transcending the conventional histogenetic approach to cancer therapy.

Chapter 5. Tumor Suppressor Genes

Tumor suppressors are genes that predispose to cancer development when inactivated. **Gatekeeper** tumor suppressor genes are negative regulators of cell growth; their inactivation directly enhances cell growth. **Caretaker** tumor suppressor genes are involved in genome maintenance and DNA repair. Their inactivation results in genomic instability and facilitates mutations in other cancer genes, which are responsible for the enhancement of cell growth.

The discovery of tumor suppressor genes, between the 1970s and the 1980s, begun with studies by Alfred G. Knudson of hereditary and sporadic retinoblastomas, which led to the formulation of his two-hit hypothesis. The development of techniques to fuse cells *in vitro* showed that hybrids between normal and neoplastic cells can be normal, leading to the idea of a tumor-suppressor phenotype. Eventually, molecular studies allowed the cloning of the retinoblastoma gene and the discovery of p53, which however was initially thought to be an oncogene.

Functions of tumor suppressor genes

Gatekeeper tumor suppressors genes are involved in the regulation of cell proliferation and apoptosis:

- Growth inhibitory factors and downstream inhibitory signaling, e.g. SMAD4
- Inhibitors of mitogenic signaling, e.g. APC, PTEN
- Cell cycle checkpoints, e.g. RB
- Controllers of cell fate and differentiation, e.g. CDH1, VHL
- Apoptosis inducers, e.g. CASP8

Caretaker tumor suppressor genes control genome stability:

- DNA damage response, e.g. p53, ATM
- DNA repair machinery, e.g. BRCA, XPs, MLH1

Inactivation of tumor suppressor genes

- Biallelic mutations, for example deletions, leading to a complete absence of functional protein, e.g. RB, APC
- Monoallelic mutations that reduce the amount of functional protein by 50%, e.g. PTEN (in genetics this is referred to as haploinsufficiency)
- Dominant-negative mutations leading to the functional inactivation of the product of the normal allele by that of the mutant allele. This usually affects multimeric proteins, e.g. p53
- Methylation of the promoter, e.g. mismatch repair genes
- Protein degradation by viral gene products, e.g. p53, RB

Retinoblastoma gene RB

Actually, a family of three genes, RB1, RBL1 and RBL2. RB1 is usually called RB, RB2 refers to RBL2. In normal proliferating cells RB is cyclically phosphorylated and dephosphorylated. Hypophosphorylated RB binds to the E2F/DP complex during the G1 phase of the cell cycle, thus preventing progression to the S phase (mitotic checkpoint). Phosphorylation by cyclin-dependent kinases (CDK) releases E2F/DP, thus allowing the transcription of genes required for cell proliferation and progression through the S phase of the cell

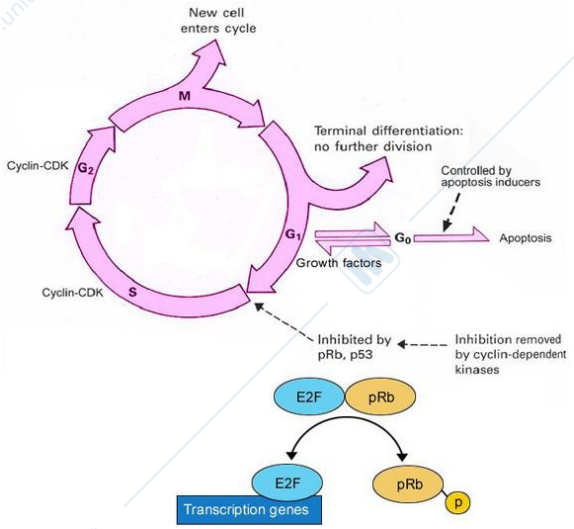


Figure 5.1. The cell cycle and RB.
Ch'ng S. Tan S.T. Frontiers in Bioscience 2009, 14: 918-928

cycle, at the end of which RB is dephosphorylated by a phosphatase (Figure 5.1).

RB is the prototypic tumor suppressor genes inactivated by biallelic mutation, both in hereditary and in sporadic tumors. Mutations are found in most retinoblastomas, osteosarcomas, and small cell lung carcinomas; RB can be also altered in breast, prostate and bladder carcinomas. In tumors caused by DNA viruses, such as cervical carcinoma, the RB gene is normal, but the protein is degraded by the activity of viral oncogenes, like E7 of human papilloma virus.

A therapeutic approach to (indirectly) modify RB is through CDK4/6 inhibitors, which, in combination with anti-estrogenic drugs, were found to be effective in hormone receptor-positive breast cancer.

APC gene

APC (adenomatous polyposis coli, not to be confused with the identical acronym for antigen presenting cells) is a key element of the WNT/β-catenin pathway. In normal quiescent cells, APC form a cytoplasmic complex that binds β-catenin, leading to its degradation. When growth factor WNT binds to its receptor Frizzled, the APC complex disassembles, and β-catenin can migrate to the cell nucleus where, in association with TCF, it stimulates the transcription of proliferation genes; the APC protein is also associated with the kinetochores, the chromosomal structures where microtubules attach

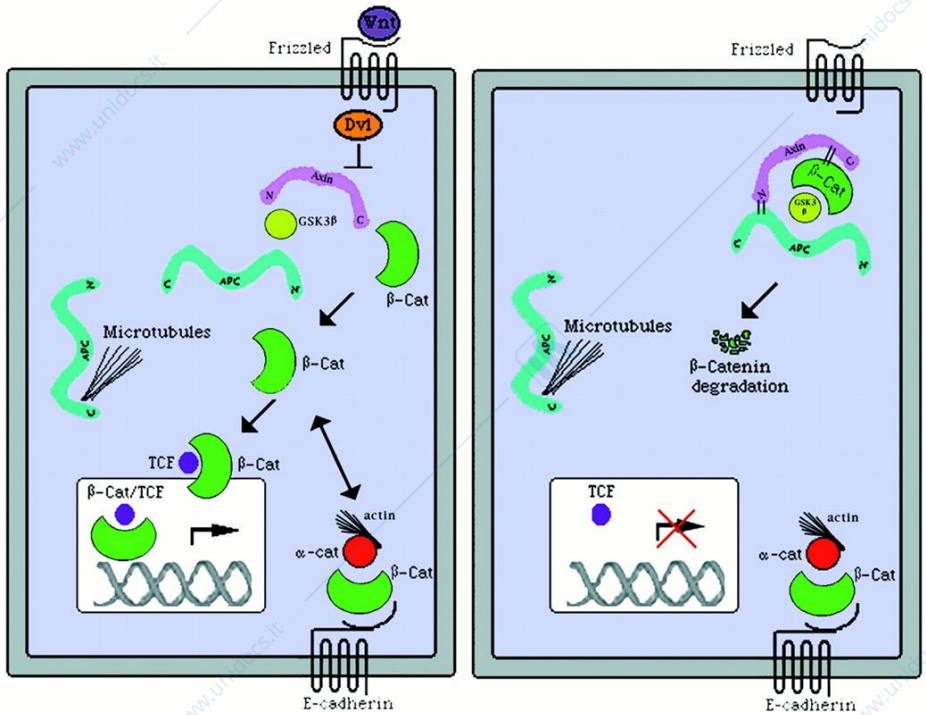


Figure 5.2. APC. *Right panel*: without the growth factor Wnt, APC forms a complex causing the cytoplasmic degradation of β-catenin. *Left panel*: binding of Wnt to its receptor Frizzled causes the dissociation of the APC complex, allowing β-catenin to reach the nucleus, From Fearnhead et al, Human Molecular Genetics, 10: 721

during the formation of the mitotic spindle (Figure 5.3).

In human tumors APC is mainly affected by biallelic inactivating mutations. Monoallelic mutations (i.e. haploinsufficiency) or methylation are found in some cases. In liver cancer the WNT/APC/β-catenin pathway is deregulated by viral proteins HBx and *core*.

Tumor suppressor genes were sometimes referred to as anti-oncogenes, but in most instances, they do not act directly on oncogene products. APC instead is a true anti-oncogene, because it directly causes the degradation of β-catenin, which is an oncogene.

APC inactivation directly enhances cell proliferation, hence APC is a gatekeeper tumor suppressor gene. APC is also a caretaker, because cells lacking APC display chromosomal instability and aneuploidy, resulting from kinetochore alterations that cause abnormal chromosomal segregation.

PTEN

PTEN (phosphatase and tensin homolog on chromosome 10) is a negative regulator of the PI3K / AKT / mTOR pathway. PTEN dephosphorylates phosphatidyl-inositol triphosphate (PIP_3) to PIP_2, antagonizing the activity of phosphatidyl-inositol-3 kinases (PI3K).

PTEN is a gatekeeper tumor suppressor gene. It is inactivated by haploinsufficiency or epigenetic mechanisms. As biallelic deletion activates cell senescence, in tumors PTEN is necessarily monoallelic (obligate haploinsufficiency).

Alterations are very frequent, low expression levels are found in most sporadic human tumors and in rare hereditary cancer syndromes. Drugs that inhibit PI3K

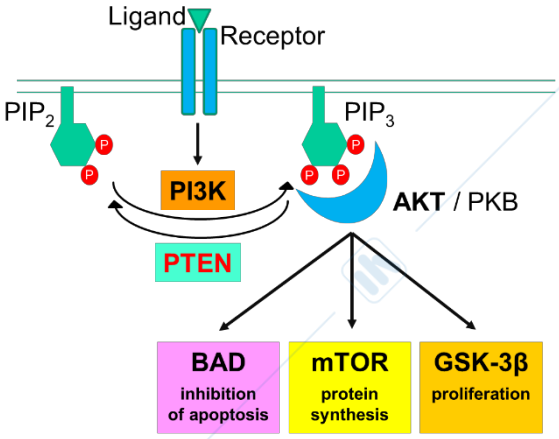


Figure 5.3. PTEN and PI3K.

can be considered as PTEN mimetics, as they take on the function of PTEN.

p53

The p53 family also includes p63 and p73. p53, also referred to as TP53, is a homotetrameric transcription factor activated by noxious stimuli, including DNA damage, oxidative stress, abnormal oncogene expression and viral DNA. Phosphorylation by DNA damage kinases frees p53 from its negative controller, the ubiquitin ligase MDM2, allowing the transcription of a plethora of target genes that mediate the inhibition of cell proliferation, the activation of DNA repair and the induction of apoptosis (Figure 5.4).

In tumor cells, p53 can be inactivated by several mechanisms, in addition to biallelic mutations that cause the lack of the protein.

Some missense mutations encode a dominant-negative protein that inactivates the product of the normal allele (Figure 5.5). In practice, dominant negative p53 behaves as an oncogene (e.g. presents a monoallelic, dominant, gain-of-function mutation), which is why p53 was originally thought to be an oncogene. The half-life of dominant-negative p53 is increased from 20 minutes to 3-7 hours, thus causing

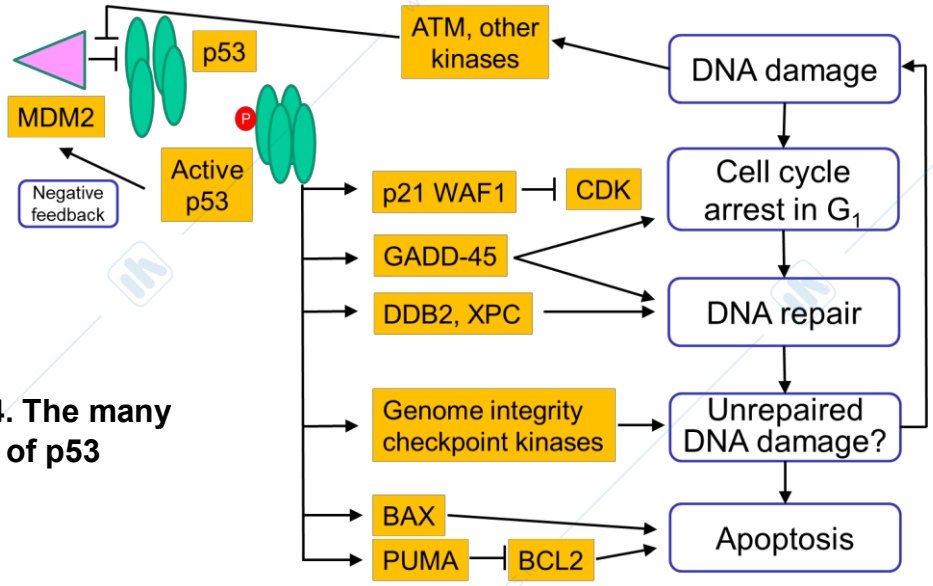
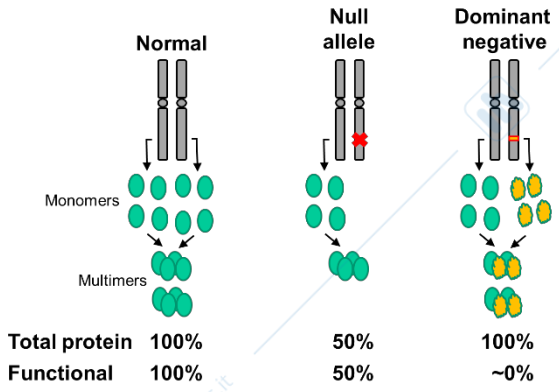


Figure 5.4. The many functions of p53

a strong increase in the level of nuclear p53. This is exploited by pathologists, because mutant p53 is visible using conventional immunohistochemistry, unlike normal p53. Hence a positive staining is diagnostic of p53 mutation. Obviously, negative staining does not allow a distinction between normal and deleted p53, requiring further molecular analysis.

The oncogenes of various DNA tumor viruses, such as E6 of human papilloma virus, induce proteolytic degradation of p53, leading to tumor cells that have intact p53 genes, but lack the protein.

Figure 5.5. Dominant-negative p53.



Inactivation of this caretaker gene is extremely frequent in all tumor types, about 60% of human tumors harbor p53 alterations. In the absence of a functional p53, unrepaired DNA mutations can hit oncogenes and other tumor suppressor genes. Furthermore, mutations randomly affect the two copies of the genome, resulting in asymmetric daughter cells, which is one of the causes of intra-tumor heterogeneity. Finally, both radiotherapy and chemotherapy exploit death mechanisms downstream of DNA damage to kill tumor cells, but p53-negative tumors are poorly sensitive to cytotoxic DNA-damaging agents.

Experimental data show that restoration of normal p53 expression can block the proliferation of tumor cells. Various attempts were made to translate these results into clinically active therapeutic strategies, so far without success. Some proof-of-principle results were obtained with gene therapy, using viral vectors to transduce a normal copy of p53, with oncolytic viruses modified to selectively replicate in p53-negative cells, and with pharmacological inhibitors of the MDM2-p53 interaction.

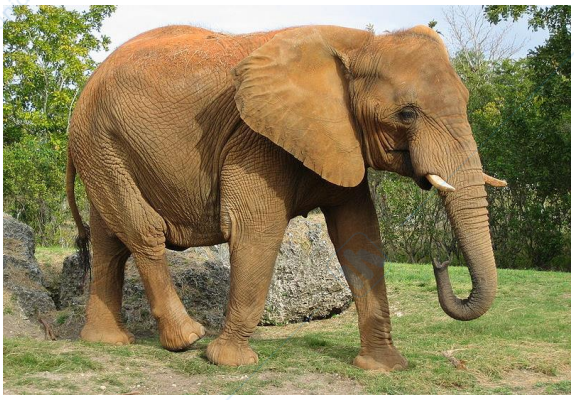


Figure 5.6. Eugenia & Julian, https://www.flickr.com/photos/eugeniayjulian/4881142/

Why elephants do not get cancer?

Cell proliferation and age are major cancer risk factors. Hence, very large, long-lived animals should be exceedingly cancer-prone, but this is not the case. It is called the Peto paradox, from the name of the researcher who first highlighted this contradiction.

We have fragmentary information about cancer incidence in whales, but we know much more about elephants, which are carefully monitored in many zoos, and are also exploited as working animals in India. Available evidence indeed shows that cancer is much rarer in elephants than in humans, despite a comparable longevity and a much larger number of cells in elephants.

Genome sequencing provided a first clue to the mechanism that protects elephants from cancer: in the elephant genome there are 20 copies of p53, thus a loss of p53 function is much less likely than in humans, who have a single copy.

Tumor suppressor genes: Clinical implications

Deletion of tumor suppressor genes presents a formidable problem to targeted therapy, i.e. the absence of a target. Furthermore, restoration of those caretakers that, unlike p53, have no direct activity on cell proliferation could have only a preventive, rather than therapeutic impact. Nonetheless, several approaches were set forth, and some were also rewarded by clinical success.

Replacement gene therapy with viral vectors carrying a functional copy of the missing gene can indeed restore tumor suppressor gene expression, but current gene therapy lacks vectorial systems that can reach multiple metastases throughout the body.

Drugs promoting read-through of premature stop codons are being tested in some hereditary, non-neoplastic diseases, and might find applications in oncology.

The most realistic approach is to find upstream or downstream targets that can be attacked by targeted drugs. We have seen some working examples for RB (CDK4/6) and PTEN (PI3K).

Synthetic lethality is based on the inhibition of pathways that become critical for the survival of tumor cells once the pathway mediated by the tumor suppressor gene is blocked. A prime example was found in hereditary breast cancer (see next chapter).

Finally, tumors lacking caretaker genes can accumulate coding mutations that generate variant proteins recognized by the immune system (neoantigens), opening the way to immunotherapy.

Chapter 6. Hereditary Cancer

Hereditary cancer is relatively rare, representing about 5% of all human tumors (Figure 6.1). It is caused by hereditary alterations of cancer genes. Even though the number of oncogenes and tumor suppressor genes in the human genome is similar, hereditary cancer is mostly caused by tumor suppressor genes, because gain-of-function, dominant mutations in most oncogenes are not compatible with normal embryo development, whereas tumor suppressor gene mutations in a single allele are mostly harmless, allowing normal development.

Features of hereditary tumors

Hereditary tumors have peculiar oncological features that can be used to pinpoint families that should be further investigated using molecular analyses to demonstrate the hereditary nature of their diseases.

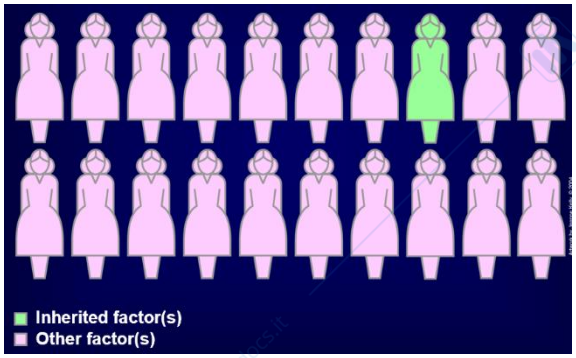
A familial cluster of tumors can be indicative of a hereditary cancer syndrome only if the tumor is rare in the general population, e.g. retinoblastoma. However, if the tumor is frequent in the general population, e.g. breast or colorectal carcinomas, a number of sporadic cases can occur by chance in a large family, hence further elements are required.

Hereditary tumors arise at a younger age than the corresponding sporadic tumors. It takes time to accumulate the mutations needed for cancer development. If one mutation is already present in the genome, then the time to tumor development is shortened. Anticipation varies by tumor type: sporadic retinoblastoma is a rare pediatric eye tumor with an average age of onset at 5 years, whereas the hereditary form can arise in the first semester of life; for colorectal cancer the anticipation is from about 65 years to about 45; sporadic breast cancer is mostly post-menopausal, but the hereditary form can be premenopausal.

Most cancer patients are affected by one tumor throughout their life, whereas bilateral or multiple primary tumors of the same histotype occur frequently in hereditary cancer. The most striking instance is colonic polyposis, whose patients can be affected by hundreds or thousands of tumors.

Many neoplastic hereditary conditions are syndromic, i.e. patients are affected by multiple tumor types. If the mutant gene plays important roles in different tissues or organs, then multiple primary tumors of different histotypes can develop in the same patient, either simultaneously or consecutively. Hereditary retinoblastoma survivors can develop osteosarcomas in

Figure 6.1. About 5% of all human tumors are hereditary. Artwork by Jeanne Kelly, ©2004. https://commons.wikimedia.org/w/index.php?curid=15666697



adolescence. Hereditary breast cancer can be accompanied by ovarian cancer; note that different tumors can occur in different individuals, but a family tree displaying an excess of tumors known to be associated in the same hereditary syndrome should be further investigated, in particular for alterations in the BRCA1 gene, which is known for this association.

Tumors can also arise in the non-affected sex, e.g. male breast cancer. Again, a family tree with multiple female and male cases of breast cancer should be analyzed, in particular for what concerns BRCA2, known to increase the risk of male breast cancer.

Oncogenes and hereditary cancer

Only a few types of hereditary tumor are caused by oncogenes. Hereditary mutations were reported for RET, MET, and cyclin-dependent kinase 4 (CDK4). Inheritance is usually autosomal dominant.

Type 2 multiple endocrine neoplasia (MEN2) is caused by mutations of the RET oncogene, the glial-derived neurotrophic factor (GDNF) receptor. MEN2 patients are affected by thyroid, parathyroid and adrenal tumors; unselected series of medullary thyroid carcinomas can include up to 15% of hereditary cases.

Gatekeeper tumor suppressor genes

We will use the study of hereditary cancer syndromes to broaden the notion of what is a tumor suppressor gene, as we will encounter various examples caused by genes that are not usually thought as tumor suppressors.

In most instances, inheritance follows the classical "double hit" model, set forth

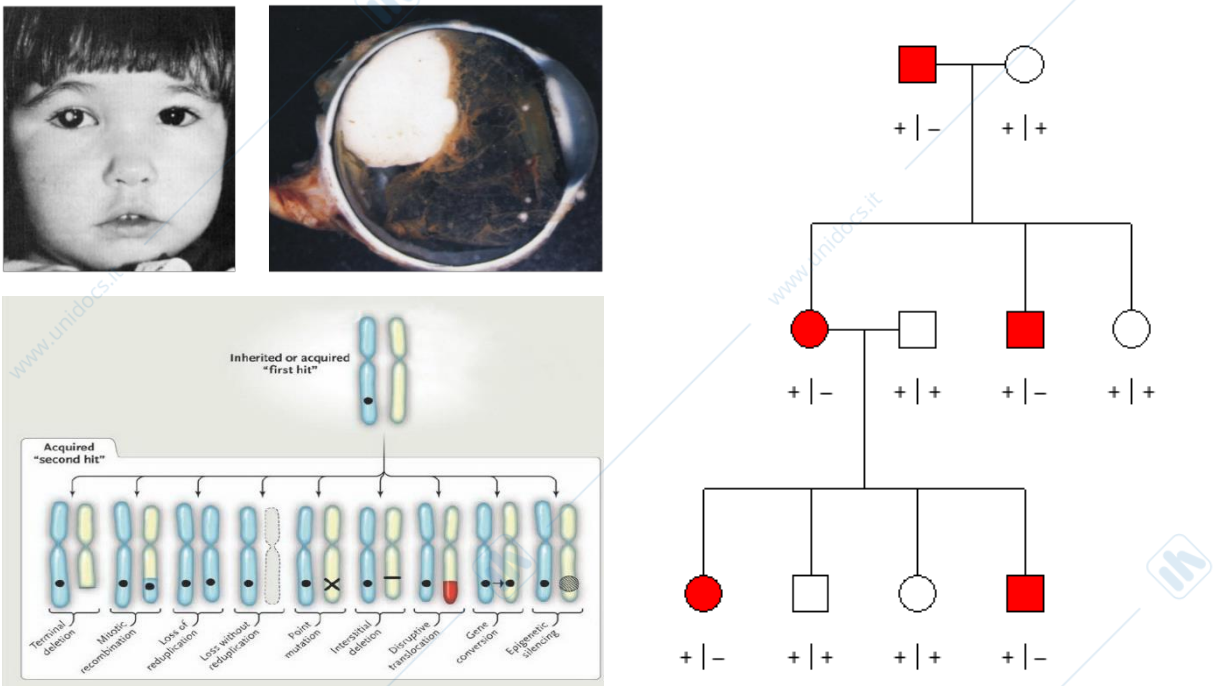


Figure 6.2. Hereditary retinoblastoma. *Upper left:* A child affected by retinoblastoma and the enucleated eye showing a large mass and various smaller neoplastic lesions (pictures *from Nussbaum et al, Thompson & Thompson Genetics in Medicine, WB Saunders Co.*). *Bottom left*: The double hit model, with one chromosome bearing the hereditary mutation and the other chromosome various alternative somatic mutations. *Right*: a typical hereditary tree, with affected individuals in all generations.

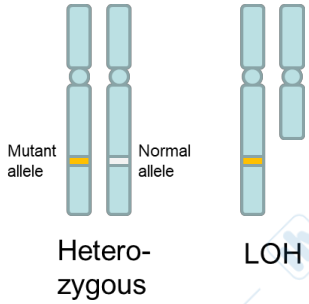


Figure 6.3. Loss of heterozygosity (LOH). In a cell bearing one normal and one pathological allele, i.e. heterozygous, loss of the normal allele is referred to as loss of heterozygosity. In cancer genetics, loss of heterozygosity is usually seen in the context of tumor suppressor genes. Note that, in general genetics, "loss of heterozygosity" has broader meanings.

in the 1970s by Alfred G. Knudson for retinoblastoma (Figure 6.2), a rare ocular tumor, well before the actual cloning of the RB gene. The first "hit", i.e. mutation, is hereditary, thus all the cells of the affected individual have one normal and one mutant allele (i.e. are heterozygous) and display a normal phenotype. The second hit is somatic and causes tumor onset, thus in tumor cells both alleles are mutant (loss of heterozygosity, Figure 6.3).

In genetics, the terms dominant and recessive should be applied to phenotypes, not to genotypes. In hereditary cancers we could consider two phenotypes, one that we could call "cancer predisposition" is dominant, i.e. heterozygous individuals are predisposed to cancer, the other phenotype, "neoplastic transformation", is recessive at the cellular level, i.e. only cells with mutations in both alleles become neoplastic.

Some patients have heterozygous tumors, with one normal and one mutant allele, indicating that a 50% decrease in the level of the protein product can also predispose to tumor onset (haploinsufficiency), in combination with somatic alterations of other cancer genes.

Familial adenomatous polyposis (FAP)

Patients are affected by multiple (up to thousands) colorectal adenomas (polyps) (Figure 6.4). Polyps are benign tumors, but they can have severe consequences if they obstruct the colon (without surgery this can cause the death of the patient), or if they bleed copiously, causing a severe anemia. Furthermore, as each adenoma carries a small risk of progression to adenocarcinoma, the sum of all risks can approach certainty.

Many other neoplasms can arise in addition to colorectal polyps: duodenal polyps, thyroid carcinomas and pancreatic adenocarcinomas. Gardner syndrome (polyps, osteomas, and fibromas) and Turcot syndrome (polyps and central nervous system tumors) are now

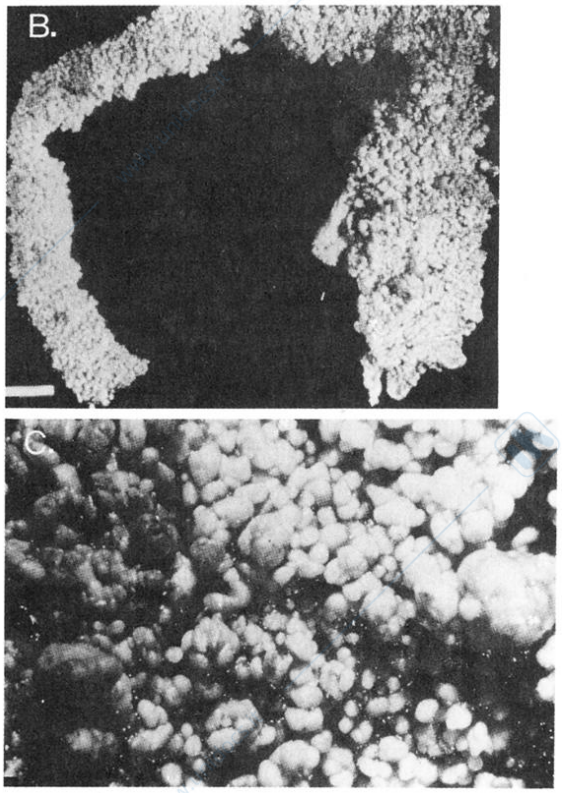


Figure 6.4. The colon of a FAP patient. From Gelehrter et al, Principles of medical genetics, Williams & Wilkins.

considered as part of the FAP spectrum of diseases.

The responsible gene is APC (adenomatous polyposis coli), FAP inheritance usually follows the classical two-hit model, but some cases of haploinsufficiency were also reported. APC is also altered in sporadic colon cancer, but mutational spectra of inherited and somatic mutations are partially different.

Familial diffuse gastric cancer

Patients develop low-grade multifocal tumors in the stomach, unrelated to *Helicobacter pylori* infection or to gastritis. The mutant gene is E-cadherin (CDH1), inheritance follows the classical two-hit model, in some cases the normal allele is present, but is epigenetically silenced (promoter methylation).

This type of hereditary cancer demonstrates that E-cadherin actually is a tumor suppressor gene. In normal epithelia, cell-cell adhesion regulates cell polarity and cell proliferation. Without E-cadherin, the proliferative control exerted by neighboring cells is missing, and the loss of cell polarity alters the orientation of mitotic spindles and the position of daughter cells.

von Hippel-Lindau (VHL) syndrome

A rare (1:30.000-40.000 live births) hereditary cancer syndrome with a spectrum of different tumors: hemangioblastomas (Figure 6.5), renal carcinomas, adrenal tumors (pheochromocytoma), in addition to pancreatic and renal cysts.

The VHL gene was discovered in this syndrome and was found to play a novel tumor suppressor role in the control of hypoxia.

Normal cells are always prepared for hypoxic emergencies through the continuing synthesis of the α subunit of hypoxia-inducible factor (HIF). Under normoxic conditions a ubiquitin ligase complex comprising the product of VHL, pVHL, constantly ubiquitinates HIF-α, which is then degraded by the proteasome, whereas under hypoxic conditions HIF-α is no longer degraded and can form a complex with HIF-β, leading to the transcription of hypoxic response genes, which includes several growth factors that stimulate erythropoiesis and angiogenesis.

Loss of pVHL triggers a chronic hypoxic response in the presence of normal oxygen levels. The resulting growth factors cause tumors through a constant stimulation of target cells. It is interesting to note that VHL hemangioblastomas can be considered as paracrine tumors. Unlike retinoblastomas, in which the cells that lose both RB alleles give rise to the tumor, in VHL hemangioblastomas stromal cells, lacking both VHL alleles, release growth factors acting on endothelial cells, with

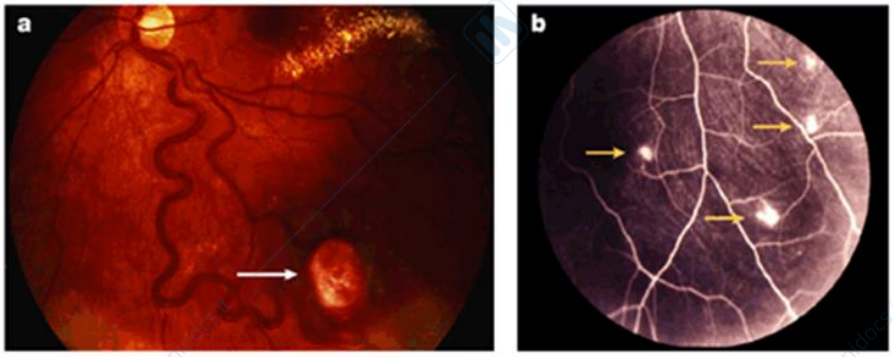


Figure 6.5. VHL retinal hemangioblastomas.
Modified from From Kaelin, Nature Reviews Cancer 2, 673.

one normal allele, which make up the bulk of the tumor.

In addition to the control of HIF-α, pVHL has multiple functions, including the degradation of other proteins, the control of fibronectin assembly in the extracellular matrix and the stabilization of microtubules. Variable expressivity of the tumor phenotype in the VHL syndrome is linked to mutations that selectively alter the various functions of pVHL.

VHL is mutated or deleted in most sporadic renal cell carcinomas.

Caretaker tumor suppressor genes

With the exception of p53, which is directly involved in the control of cell proliferation, the deletion of a caretaker tumor suppressor gene does not directly cause alterations in cell growth, but it opens the doors to widespread mutations affecting oncogenes and gatekeeper tumor suppressor genes, which are the ultimate cause of tumor onset.

Some caretaker tumor suppressors are linked to hereditary cancer syndromes, notable examples are p53, mismatch repair genes and BRCA genes. Inheritance follows the pattern seen before for gatekeeper genes, i.e. two-hit in most cases, and sometimes haploinsufficiency.

Other caretakers have broader effects, which result in syndromes that include non-neoplastic pathologies along with tumors. Some examples are xeroderma pigmentosum (skin lesions and skin tumors), Fanconi anemia (developmental abnormalities, anemia and leukemias) and ataxia-telangiectasia (movement difficulties, dilated blood vessels, lymphomas and leukemias). These syndromes, which display prominent cytogenetic abnormalities, are collectively referred to as chromosomal instability syndromes; inheritance is autosomal recessive.

The DNA damage response

Any kind of cell DNA damage, caused by radiation, chemical mutagens or viruses, elicits a sequence of events aimed at the neutralization of potentially harmful consequences (Figure 6.6.). First, the cell cycle is arrested, to avoid the replication of damaged DNA, then specific repair systems are activated; if the damage is satisfactorily repaired, then the cell can re-enter the cell cycle, otherwise apoptosis is triggered.

As we have seen in the previous chapter for p53, alterations of DNA repair mechanisms have multiple oncological consequences. Exposure to exogenous mutagens (e.g. tobacco smoke) causes an increased number of unrepaired mutations. Replication of cells harboring DNA alterations results in a heterogenous population that allows the selection of more aggressive clones. Those cells that survive despite extensive DNA damage are poorly sensitive to DNA-damaging agents, such as cytotoxic chemotherapy and radiotherapy. On the positive side,

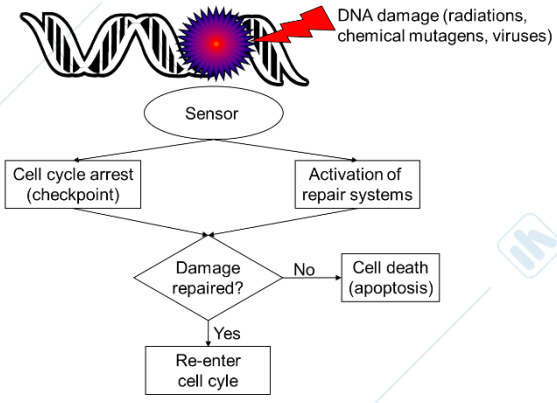


Figure 6.6.
The DNA damage response

mutations can encode neoantigens recognized by the immune system, either spontaneously or after immunotherapy.

DNA repair mechanisms

A variety of DNA repair mechanisms is active in all living organisms (Table 6.1.). Most human repair systems are similar to bacterial systems, some proteins are even interchangeable, but in general human systems are more complex and involve a larger number of repair genes. We will focus on those repair systems that have oncological implications.

Direct repair of O^6 alkylguanines, which would pair with T instead of C, is operated by alkyl transferases (MGMT, methyl guanine methyl transferase) which remove the alkyl group. Killing of tumor cells by DNA alkylation is a mechanism exploited by various cytotoxic drugs; tumor cells with overly active direct repair become resistant to these therapeutic agents.

Nucleotide excision repair (NER) removes pyrimidine (C or T) dimers caused by UV ray exposure of the skin, which would be read by DNA polymerases as different bases, causing dinucleotide mutations. As UV rays only damage superficial cells, inherited deficiencies of the ER systems cause xeroderma pigmentosum (XP), a rare autosomal recessive syndrome with a variety of cutaneous and ocular lesions caused by UV exposure, including cutaneous tumors in about one-half of the patients. It is interesting to note that, as long as XP children are protected from UV exposure, lesions do not develop, thus providing a practical demonstration that the lack of caretaker gene products *per se* is not the cause of cancer.

Mismatch repair (MMR) corrects erroneous base pairings during DNA replication. Inherited MMR alterations cause Lynch syndrome, a type of hereditary colorectal cancer.

DNA strand breaks, which can be caused by ionizing radiation, are repaired by different systems, including non-homologous end joining (NHEJ), which joins

Table 6.1. DNA repair mechanisms

Mechanism	DNA lesion	Enzymatic systems
Direct repair	O^6 alkylguanine (pairs with T instead of C)	Alkyltransferase (MGMT)
Base excision repair (BER)	1-13 damaged bases (e.g. by alkylation)	Glycosidases, polymerases, ligases
Nucleotide excision repair (NER)	Thymidine dimers, "bulky" adducts	XP, other enzymes
Mismatch repair (MMR)	Incorrect base pairings	MSH1-6, MLH/PMS1-16
Double strand break repair (DSBR)	Double strand breaks	Homologous recombination (HR), non-homologous end-joining (NHEJ), BLM, BRCA1-2, DNA recombination systems
Cross-link repair	Cross-links between the DNA filaments	FANC A-D, BRCA2

two free-hanging DNA filaments, and homologous recombination (HR), which uses as templates the sister chromatids of the other chromosome. Hereditary breast cancer is caused by mutations of homologous recombination genes.

Covalent interstrand cross links between the two DNA filaments, which impede the separation of the two strands, are produced by a variety of chemical mutagens, including aldehydes and bifunctional alkylating agents. Cross link repair makes use of other repair systems, including NER and HR, in addition to specific genes (FANC) that were discovered through the study of Fanconi anemia.

Li-Fraumeni syndrome

Hereditary mutations of p53 cause the Li-Fraumeni syndrome (~1:10.000 live births), a testament to the widespread importance of p53. Other hereditary cancer syndromes include a limited spectrum of tumors, frequently sharing histological origin, e.g. carcinomas, whereas Li-Fraumeni patients can be affected by carcinomas, especially breast cancer, soft tissue sarcomas, such as rhabdomyosarcomas, and leukemias.

Hereditary breast cancer

Hereditary breast cancer is one of the most frequent hereditary tumors, amounting to ~5% of all breast cancer cases. Hereditary cases are usually triple-negative (i.e. lacking HER-2 and both estrogen and progesterone receptors), thus among triple-negative patients the percentage of hereditary cases is higher, up to 14%.

The genes responsible for most cases are BRCA1 (50% of affected families) and BRCA2 (35% of families), both involved in homologous recombination (Figure 6.7); inheritance follows the two-hit model. The spectrum of tumors includes ovarian cancer in BRCA1 families; ovarian (less frequent than in BRCA1 families), prostate, pancreatic and lung cancers along with male breast cancer in BRCA2 families.

About 15% of families with inherited breast cancer lack BRCA1 or 2 mutations, but there is not a third high-risk breast cancer gene, which was in the past sometimes referred to as "BRCA3" or

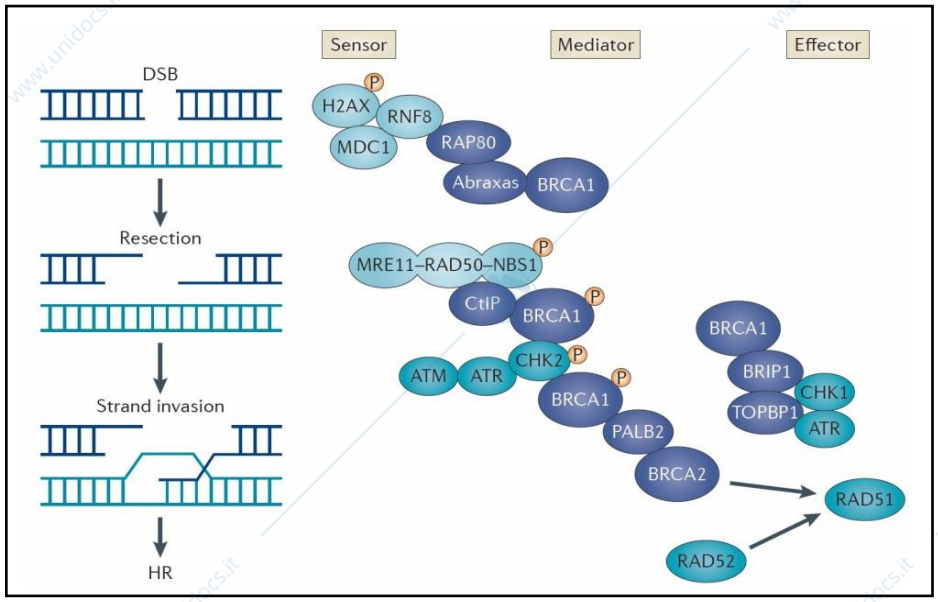


Figure 6.7. BRCA genes are involved in double stand break repair. From Roy et al., Nat. Rev. Cancer 12: 68.

"BRCAX". The remaining 15% can be attributed to other known high/moderate-risk tumor suppressor genes, such as p53, PTEN, ATM, etc., and to several common alleles associated with low increases in breast cancer risk.

Penetrance increases with age, with a sizeable proportion of pre-menopausal cases, and is highly variable. In some families, penetrance at 70 years of age is very high (above 80%), in others it is much lower, around 40%-60%. Such differences can be attributed both to allelic heterogeneity (i.e. different alleles cause different cancer risks) and, in families carrying the same allele, to modifier genes in the background.

The most effective way to prevent hereditary tumor onset is to surgically remove the tissue at risk with a bilateral mastectomy at young age, possibly followed by the removal of ovaries if there is a sizeable risk of ovarian cancer.

In normal cells, the mechanisms that repair DNA strand breaks are redundant, but BRCA-negative tumor cells cannot use homologous recombination and are critically dependent on other mechanisms. It was found that inhibitors of PARP (poly ADP-ribose polymerase), an enzyme that repairs single-strand DNA breaks, are selectively toxic for homologous recombination-deficient tumor cells (an instance of synthetic lethality) and various drugs ("parib" suffix in the common name) were approved for the therapy of homologous recombination-deficient.

Lynch syndrome – Hereditary, non-polyposis colorectal carcinoma (HNPCC)

HNPPC accounts for about 3% of all colorectal cancer cases, on a par with FAP. The main difference is that poorly differentiated adenocarcinomas start appearing around 45 years of age, without a clinical history of polyps. Patients also have a high risk of endometrial, ovarian, renal, pancreatic, gastric and bladder carcinomas.

Various genes involved in mismatch repair (Figure 6.8) are responsible for

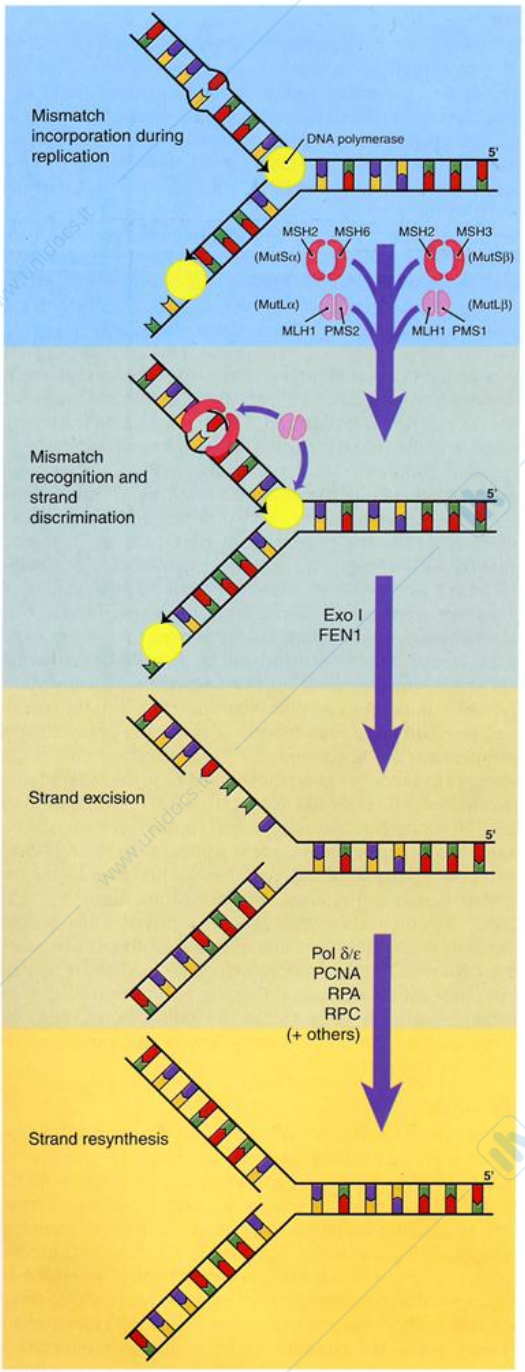


Figure 6.8 Mismatch repair. From the R&D Systems 2003 catalog.

HNPCC: MLH1 (50% of cases), MSH2 (35%), MSH6 (10%), and others. Inheritance follows the two-hit model, penetrance is almost complete.

A defective mismatch repair causes widespread mutations throughout the genome, a so-called "mutator" phenotype, also referred to as RER+ (replication error-prone). This is exploited for diagnostic purposes, as sequence length variations can be easily detected in microsatellite (short tandem repeats) DNA sequences, thus defining a patient/tumor phenotype called microsatellite instability (MSI).

Microsatellite instability, caused by the methylation of the promoters of mismatch repair genes, was also found in ~12% of sporadic colon cancers and in ~4% of all human tumors. The genomic instability of microsatellite-unstable tumors leads to the accumulation of mutations coding for immunogenic protein variants that can be recognized and targeted by the immune system of the host. MSI tumors, independently of histotype, can now be treated with immunotherapy, an instance of histology-agnostic therapy.

Chapter 7. Tumor Progression

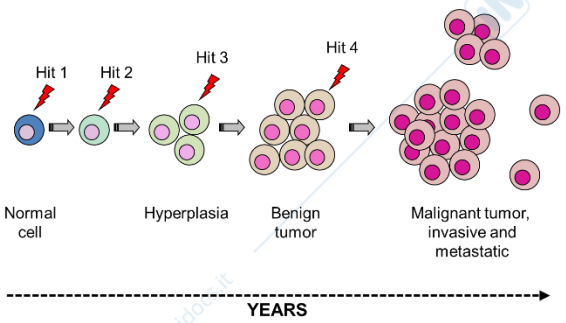
Tumors are dynamic entities, because deregulated cell proliferation, coupled with genomic instability, causes a continuous flow of gene alterations. Tumor progression is the process whereby cells accumulate incremental genetic damage, leading first to cancer development, then to increasing malignancy. The expression "natural history" refers to the timeline of events leading to progression, from normal cells to full-fledged tumors.

Tumor progression is better known in carcinomas and some hematopoietic neoplasms, which develop over several decades with distinct cellular changes, allowing detailed studies of their natural history. On the contrary, sarcomas mostly arise *ex abrupto* (suddenly), hence their natural history is largely unknown.

How many hits to make a tumor?

Multiple genetic hits are required for the development of malignant tumors (Figure 7.1). Accumulation of successive hits takes time, hence the long natural history of most human tumors. A minimal number of hits is required for pediatric tumors, as attested by their relatively short latency, for example it is generally assumed that 2 mutations (affecting both RB alleles) are enough to cause the onset of retinoblastomas. Adult tumors, that develop over several decades, are the result of many more hits: estimates range from 7 or more for colorectal carcinomas to 10-20 for lung cancer.

Figure 7.1. Tumor progression is driven by multiple genetic hits.



Monoclonal or polyclonal?

Is an individual tumor mass derived from the neoplastic transformation of a single cells, or from many different cells? In other words, are tumors monoclonal or polyclonal in origin?

Earlier studies of tumor clonality examined the expression of phenotypes that are heterogeneous in normal cells. For example, heterozygous X-linked genes are inactivated randomly, thus some female cells express one allele and others the second allele (mosaicism). Another example, present in both sexes, is the clonal antigenic specificity of T and B cells. If the origin of a tumor is polyclonal, then it should contain a mixture of cells expressing the various alleles, otherwise all tumor cells will express the same allele. Most tumors examined with these techniques resulted monoclonal in origin (Figure 7.2).

Some exceptions were found among tumors caused by very powerful carcinogenic stimuli, such as hereditary conditions or potent chemical and viral carcinogens. Examples are hereditary retinoblastomas, intestinal polyposis, or tumors caused by chronic exposure to tobacco smoke. In such cases, multiple micro-

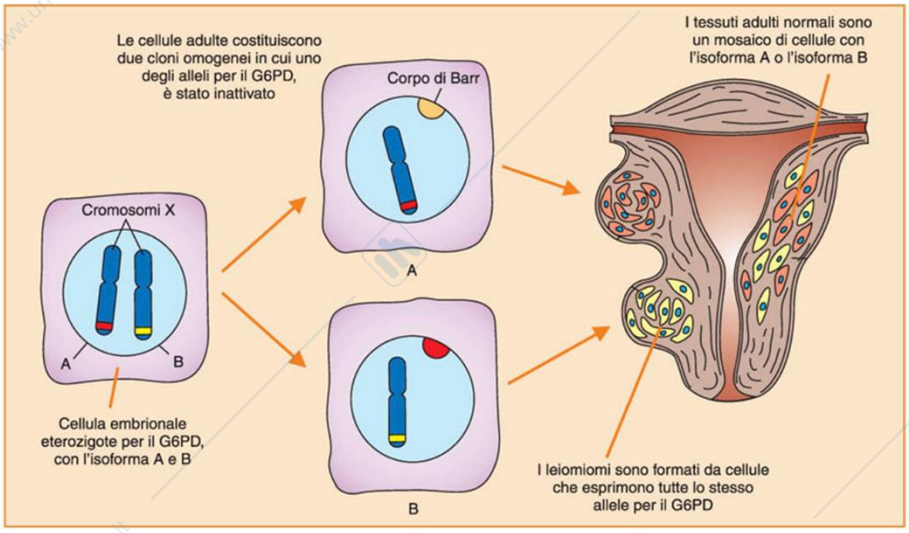


Figure 7.2. Monoclonal origin of uterine leiomyomas, as assessed by means of X-linked G6PD isoforms.
From Pontieri, Russo, Frati. Patologia Generale, Piccin.

scopic monoclonal tumors arise, then coalesce in a single polyclonal mass. This phenomenon is called field carcinogenesis because tumors grow from a field of mutant cells.

Heterogeneity and clonal evolution of tumors

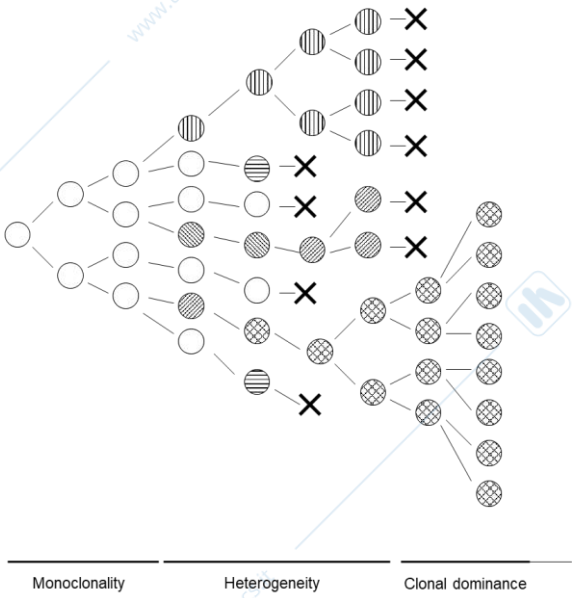
Cancerous tissues, unlike their normal counterparts, frequently display heterogeneous phenotypes encompassing any conceivable cells property, including cell morphology and differentiation, protein expression, metabolism, chromosomal abnormalities, drug sensitivity, antigenicity, invasiveness and metastatic ability.

Even if a tumor is originally monoclonal, genomic instability soon generates heterogeneous clonal variants, giving rise to a situation akin to Darwinian evolution. The various clones compete for available resources (e.g. blood supply, nutrients, oxygen), and the environmental selection (including biological conditions and therapeutic treatments) drives tumor evolution (Figure 7.3). Advanced tumors can eventually return to a quasi-monoclonal condition if one aggressive clone overcomes all other clones, a condition called clonal dominance.

Cancer stem cells (CSC)

The notion of stem cells in normal tissues has spawned the idea that also cancerous tissues contain cancer stem cells. According to the cancer stem cell model, a tumor mass contains varying proportions of stem cells, which can initiate tumor development, and of dispensable non-stem cells. Therapies that kill non-stem cells, but spare stem cells, are eventually

Figure 7.3. Evolution of clonality during tumor progression.



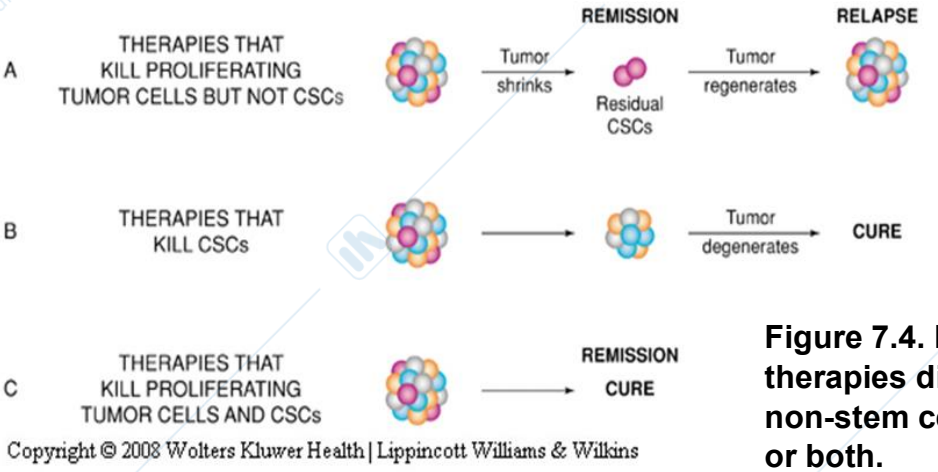


Figure 7.4. Efficacy of therapies directed against non-stem cells, stem cells, or both.

ineffective, because stem cells would then regenerate the tumor (Figure 7.4). The study of stem cells in a variety of tumors has defined a common set of properties that differentiate CSC from the bulk tumor population:

- Self-renewal
- Asymmetric cells division
- Slow proliferation
- Expression of molecular pumps that extrude cytotoxic drugs
- Can be rare, representing less than 1% of all cells in a tumor
- Are highly tumorigenic, even a few cells can generate a tumor
- Defined by combinations of present / absent molecules, rather than by single specific markers, e.g. $CD24^{-/low};CD44^{+}$ in breast cancer.

The cancer stem cell model is an attractive conceptualization, but it contains several critical issues that limit its clinical usefulness. Firstly, CSC were documented in several human tumor types, such as leukemias; breast, colorectal, prostate, head-and-neck cancers; brain tumors and melanoma, but it is not clear whether the paradigm is indeed applicable to all human tumors. Secondly, CSC are bad therapeutic targets, because we lack specific target molecules, and they are intrinsically resistant to cytotoxic drugs. Finally, differentiation is not unidirectional, i.e. in addition to the forward differentiation of CSC into non-stem cancer cells, there can be a reverse differentiation of non-stem cells into CSC, thus preempting the efficacy of any CSC-specific therapy.

Tumor progression: From bad to worse

Growth autonomy and insensitivity to homeostatic controls are positively selected during tumor cell progression. Early neoplastic lesions, slowly growing locally, evolve into invasive cancers which then diffuse systemically, giving rise to distant metastases (Figure 7.1). Even before drug treatment, genomic instability can generate cell variants with drug-resistant phenotypes; therapeutic treatments will then kill only drug-sensitive cells, but drug-resistant variants will regenerate a neoplastic mass that is now fully resistant to therapy. Furthermore, many anti-tumor drugs are mutagenic, and can generate novel mutant variants that were not present before treatment.

Tumor regression: A rare event

Some tumors can evolve in a favorable (for the patient) direction, either giving

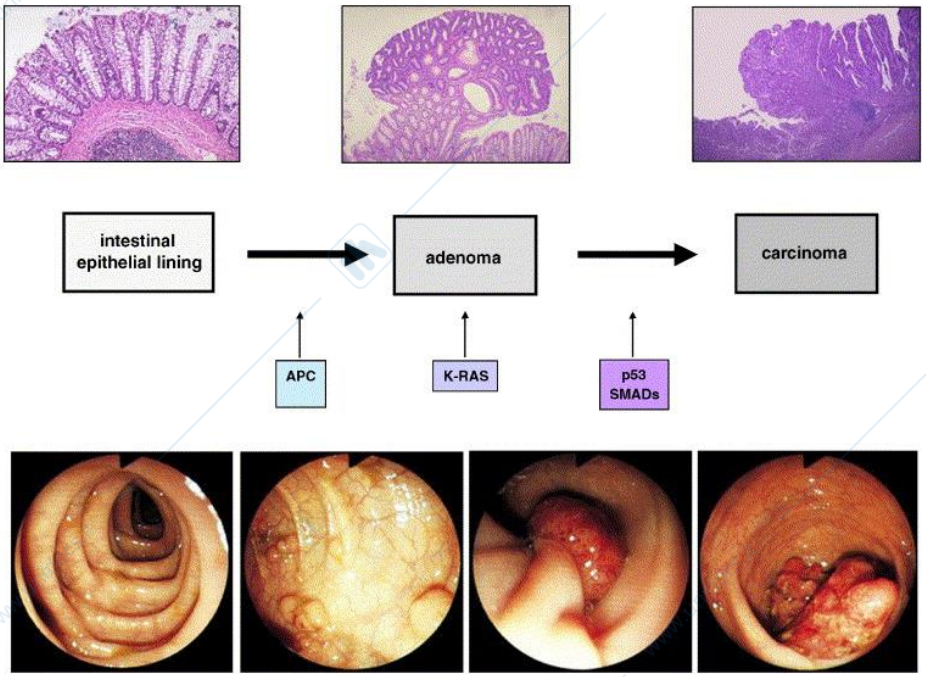


Figure 7.5. Progression of colon cancer.
From BBA Reviews on Cancer 1175: 103-137, 2007.

rise to less malignant tumor variants or regressing completely. There are several well-documented cases, but our understanding of the underlying molecular mechanisms is still lacking.

Almost all infantile hemangiomas (benign vascular tumors) go from a rapid early growth to a slow involution in childhood. Neuroblastoma, a malignant pediatric peripheral nerve tumor, can differentiate to benign ganglioneuroma; the TRK oncogene is thought to be involved. Rare malignant melanomas and renal cell carcinomas heal spontaneously, possibly because they are recognized and killed by the immune system of the host.

Molecular events in the progression of human tumors

The application of molecular analyses to successive stages of human tumor progression led to the discovery of the genes involved in the successive stages of various cancer histotypes.

Bert Vogelstein and Kenneth Kinzler, through the analysis of hereditary and sporadic tumors, first succeeded in elucidating the sequence of molecular events that drives the progression of colon tumors, from normal cells to carcinoma, passing through successive adenoma stages (Figure 7.5). The genes involved are first APC, then KRAS, finally p53, with intervening contributions from alterations in mismatch repair genes, E-cadherin, SMAD4 (TGF-β pathway) and INK4A (inhibitor of cyclin-dependent kinases).

Analogous studies led to the discovery of genes involved in the progression of carcinomas of the breast, lung, pancreas, and others (Figure 7.6). The sequence of molecular events in these tumors is less straightforward than in the Vogelstein-Kinzler original model, probably because the differentiation of various cell types contributes to the architecture of the normal tissues, and carcinogenesis can follow multiple overlapping pathways.

From progression to prevention

The predictable nature of tumor progression is the basis of various approaches to

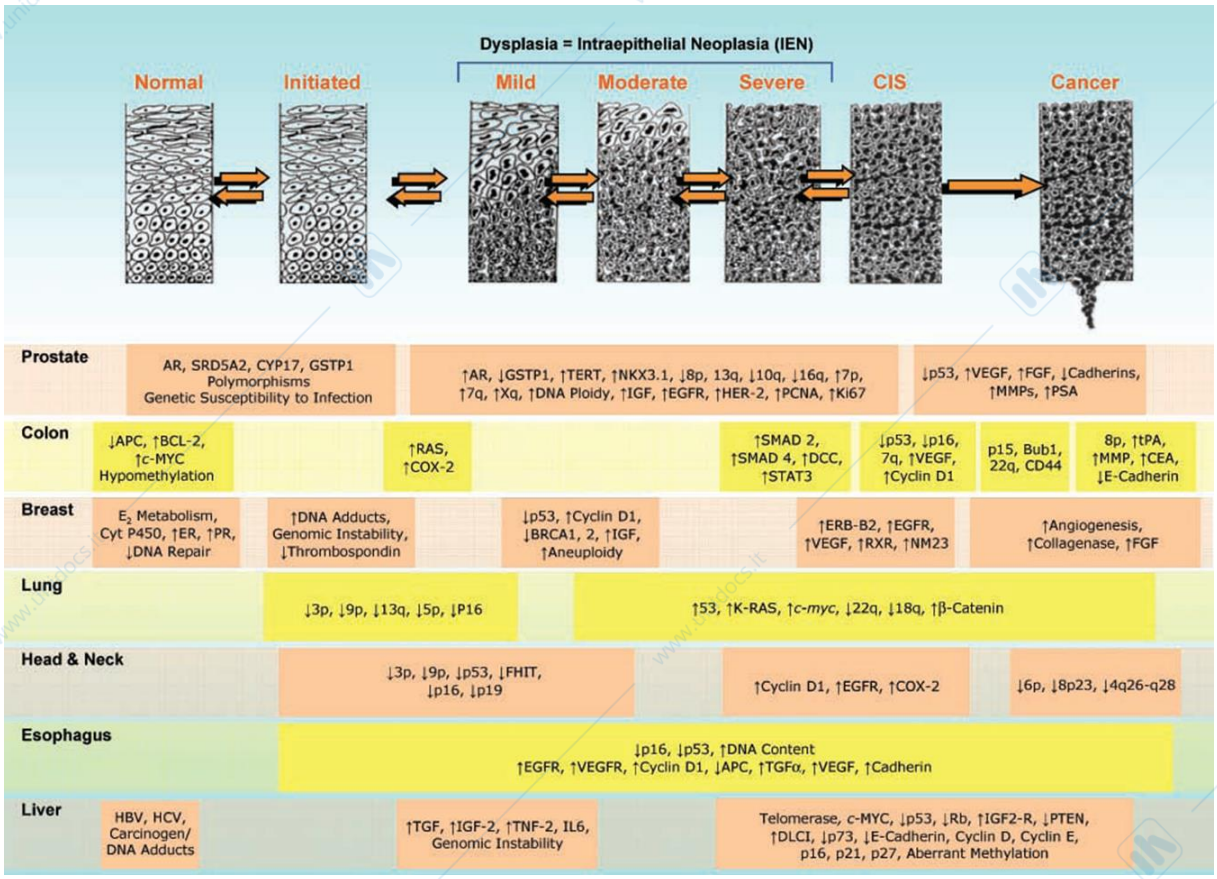


Figure 7.6. Molecular events in the progression of various tumor types.
From Kelloff et al., Clin. Cancer Res. 12:3661.

tumor prevention, e.g. early diagnosis. Unfortunately, progression is a random process that we can mostly predict on a statistical basis, not individually for each patient. For example, in a cohort of early tumors which we know as having a 50% probability of progression to malignancy, we are not yet able to discriminate those tumors that will undergo progression from those that will remain harmless. This is at the root of overdiagnosis, an undesirable consequence of mass screening that will be discussed in the chapter on cancer prevention.

Chapter 8. Cancer Cells

The hallmarks of cancer

Douglas Hanahan and Robert Weinberg, first in 2001, then in 2011, published a review entitled "The hallmarks of cancer", which summarizes the features of tumors and became an instant classic in oncological literature. Figure 8.1 overlays the iconic figure of Hanahan and Weinberg hallmarks with a distinction between those that are intrinsic cell properties and those that depend on tumor-host interactions. In this chapter we will focus on the intrinsic features of cancer cells, using Hanahan and Weinberg nomenclature as a guide; note that several hallmarks are interconnected, e.g. cell death and immortality, thus the classification of a given mechanism as part of either hallmark is somewhat arbitrary.

Cancer cells: The transformed phenotype

The alterations of oncogenes and of tumor suppressor genes trigger a cascade of modifications in gene expression that transform the cell phenotype from normal to neoplastic. Our understanding of the transformed phenotype is at the root of

Figure 8.1. The hallmarks of cancer.
Modified from Hanahan & Weinberg, Cell 144: 646-674, 2011

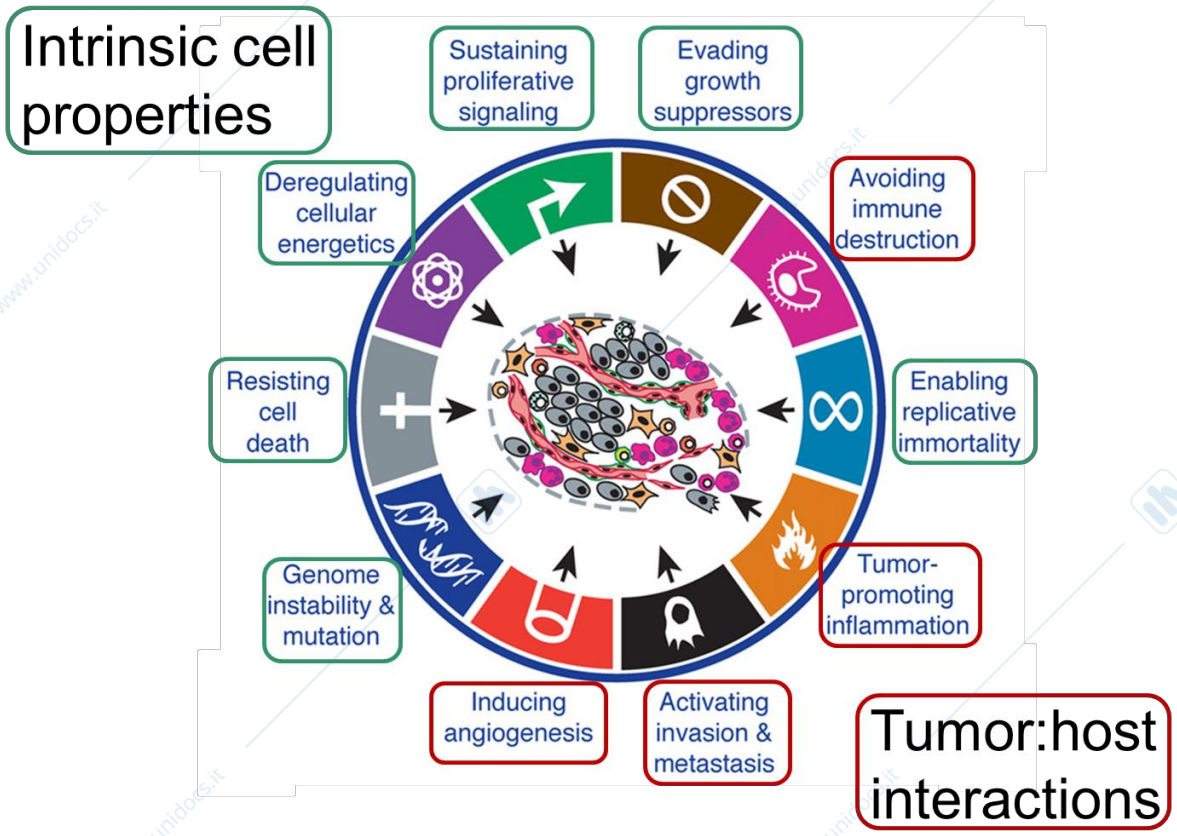


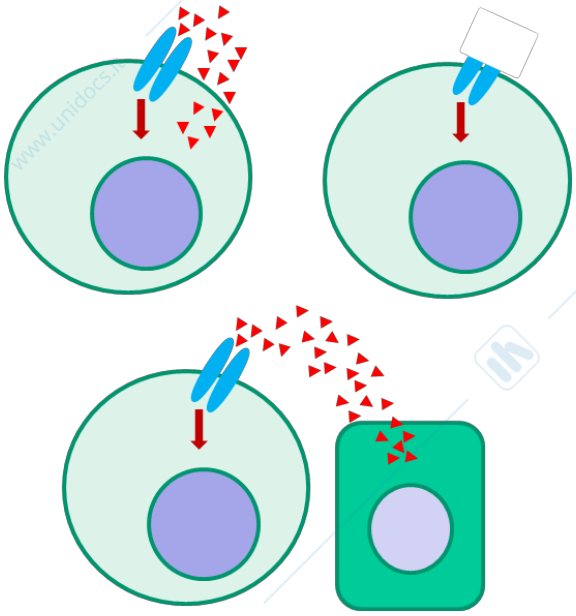
Table 8.1. Classical* *in vitro* features of cancer cells

Normal cells	Neoplastic cells (transformed cells)
Vigorous cell growth requires high-serum supplementation	Growth at low serum percentages
Adherent cells die if not attached to a substrate (e.g. glass or treated plastics)	Adherent cells also grow in semisolid medium (e.g. agar)
Lymphocytes undergo rapid apoptosis *in vitro*	Immortal
Long-term fibroblast cultures become senescent after 40-60 cell generations (Hayflick limit)	Cultures propagate indefinitely
Confluent monolayers stop proliferating (contact inhibition)	No contact inhibition, overlapping cell growth

*Defined in the 1960s-1980s using specific cell types and culture conditions, not necessarily applicable to all cell types or modern culture technologies.

tumor cell identification (diagnosis) in carcinogenesis, malignancy and response to therapy. Many phenotypes, which were defined studying cell cultures, are better understood in terms of cells cultured *in vitro* (Table 8.1)

Figure 8.2. Growth autonomy of tumor cells. Autocrine signaling (*upper left*); constitutively active growth factor receptor (*upper right*); paracrine signaling (*bottom*).



Proliferative signaling

The proliferation of cancer cells is independent, or at least less dependent than that of normal cells, from control signals coming from the environment. Growth autonomy of tumor cells can be mediated by different mechanisms (Figure 8.2):

- Autocrine loops: tumor cells produce a growth factor and express its functional receptors, thus stimulating their own proliferation. Note that autocrine loops are also active in normal cells, for example interleukin 2 in T cells, but are tightly regulated by additional signals, e.g. antigen stimulation through the TCR, and/or balanced by death mechanisms, e.g. apoptosis.
- Hyper-expressed or constitutively active growth factor receptors, or activated downstream mitogenic signaling. Several examples were encountered in the chapter on oncogenes, e.g. EGFR, RAS.
- Paracrine growth signals by normal neighbor cells. A prime example are vascular tumors, such as von Hippel-

Lindau hemangioblastomas, or Kaposi sarcomas.

Evading growth suppressors

The proliferation of tumor cells is also sustained by defects in negative growth signals, typically controlled by gatekeeper tumor suppressor genes.

- Inactivation of growth-inhibitory signaling, e.g. transforming growth factor β (TGF-β) or interferons. SMAD4, which is involved in the progression of colorectal cancer (see previous chapter) is a transcription factor of the TGF-β pathway.
- Alterations of growth-inhibitory cell-cell or cell-matrix contacts, for example E-cadherin. We will further analyze these mechanisms in the following chapters.
- Evasion of mitotic checkpoints, such as RB.
- Inability to attain a post-mitotic state, e.g. by terminal differentiation. In many normal tissues, cell number is regulated by the continuous exit of cells from the proliferative compartment through terminal differentiation; defects in the control of terminal differentiation can rapidly cause the accumulation of proliferating cells, i.e. a tumor.

Resisting cell death

Normal proliferation processes may be terminated by physiological cell death, e.g. amplification of T and B cell clones during immune responses, or skin keratinization. Tumor cells do not die when they should, for a variety of reasons:

- Increase in survival signals, e.g. through the activation of the insulin-like growth factor axis (IGF) axis
- Lack of apoptotic signals, or alterations in the control of apoptosis, e.g. p53, BCL-2
- Resistance to anoikis, that is death by lack of attachment to a substrate (the Greek term means homelessness). This is particularly important for circulating tumor cells that give rise to distant metastases.

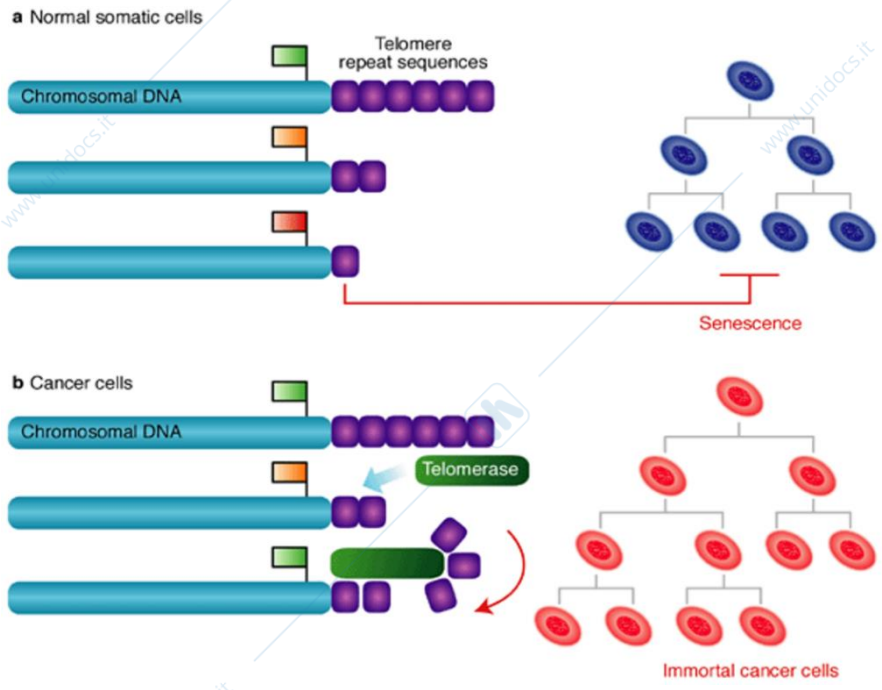


Figure 8.3. Telomerase. From Expert Reviews in Molecular Medicine, Copyright 2002 Cambridge University Press.

Enabling replicative immortality

The extremities of a linear DNA sequence are not duplicated by the normal replication machinery, hence linear chromosomes become shorter after each replication cycle (Figure 8.3). Telomeres are non-coding DNA sequences that protect chromosome extremities and prevent the erosion of coding sequences.

Excessive telomere shortening causes chromosomal abnormalities and cell death, thus preventing infinite cell replication in most cell types. Normal cells with unlimited proliferative potential, e.g. stem cells or lymphocytes, activate telomerase, a ribonucleoprotein that maintains telomere length (Figure 8.3). Its catalytic subunit is encoded by the TERT gene.

All tumor cells must activate telomere maintenance to avoid senescence and to proliferate indefinitely. Most (85%-90%) tumors use telomerase (TERT), others use alternative (ALT) systems. Given their ubiquitous nature, telomere maintenance mechanisms in tumors make tantalizing therapeutic targets, against which we do not have effective drugs.

In addition to telomere maintenance, replicative immortality can be conferred by viral gene products, e.g. the Epstein-Barr virus causes the immortalization of B cells, a phenomenon that was also exploited in the laboratory to obtain the so-called lymphoblastoid cell lines, also used to establish biobanks of living samples from patients affected by a variety of hereditary diseases.

Genome instability

Genome instability is a feature of neoplastic cells that generates cellular heterogeneity, contributing to tumor progression.

A major cause of genome instability is the inactivation of caretaker tumor suppressor genes by mutations or epigenetic mechanisms (e.g. methylation).

Chromosome alterations, in particular aneuploidy, may also result from alterations of mitotic spindles, caused for example by APC gene deletion, or by alterations in cell polarity. Foreign bodies within replicating cells, e.g. asbestos fibers, can also interfere with the organization of mitotic spindles.

In tumors, telomere maintenance is activated after progressive telomere shortening. Very short telomeres, interpreted as double strand breaks by DNA repair, are stitched together, generating dicentric chromosomes, which contribute to genomic instability.

Hypoxic conditions, which are frequent within growing tumors as a consequence of abnormal angiogenesis, should activate caretaker systems, such as p53, which may be in turn defective, resulting in increased mutation rates and genomic instability.

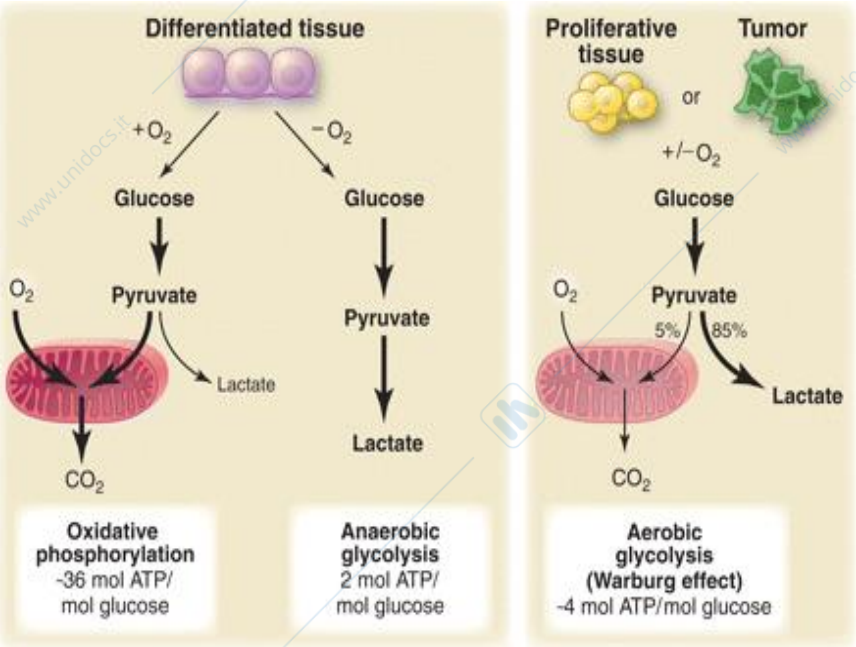


Figure 8.4. The Warburg effect (aerobic glycolysis).
From MG Vander Heiden, LC Cantley, CB Thompson. Science 2009.

Deregulating cellular energetics: tumor metabolism

Neoplastic cells display major metabolic deviations from quiescent normal cells. Some of these changes are tumor-specific, others are also present in proliferating normal cells

A common metabolic feature of solid tumors is the use of aerobic glycolysis (Warburg effect) instead of oxidative phosphorylation (Figure 8.4). Mechanisms include activation of the fetal form of pyruvate kinase (PKM2) and down-regulation of glucose transporters by p53.

In medicine, the high consumption of glucose by tumor cells is exploited to image metabolically active neoplastic masses by positron emission tomography (PET) using (^{18}F) fluorodeoxyglucose (FDG) as a tracer. Note that FDG is not tumor-specific, hence metabolically active tissues and organs, such as the brain, are also imaged. Functional imaging is used to discriminate between active metastases and quiescent or fibrotic lesions, which may be indistinguishable by radiography or CT scans.

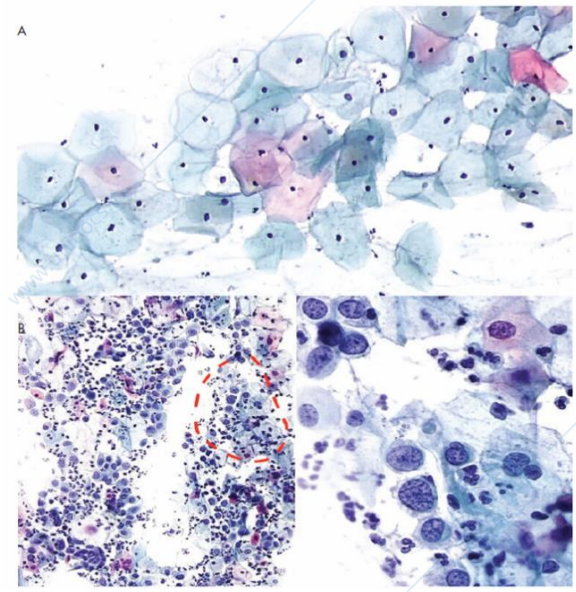


Figure 8.5. Tumor cell morphology.
Upper panel: normal uterine cervix cell; *lower panels*: high-grade squamous intraepithelial lesion (HSIL). From Boyle & Levin (eds), World Cancer Report 2008, IARC.

Cell and tissue morphology

Genetic and metabolic alterations cause major alterations in the morphology of neoplastic cells and tissues. Such recognizable changes have a fundamental role in the pathological diagnosis of tumors (Figure 8.5).

Each tumor histotype undergoes specific morphological changes, but some general features of cancer morphology are present in most cases:

- Morphological heterogeneity: within a normal tissue, cells sharing the same level of differentiation are similar to one another, whereas tumor cells are markedly heterogeneous
- Especially in carcinomas, the shape of tumor cells is different than that of normal cells due to cytoskeletal abnormalities and to cell motility (epithelial-to-mesenchymal transition, see next chapter)
- Tumor cells have a high nucleus to cytoplasm ratio, and chromatin has a coarse aspect
- Nucleoli are multiple and irregular
- The number of chromosomes is not diploid (aneuploidy), and heterogeneous among tumor cells
- The proliferative index is high. Proliferation can be evaluated morphologically, by counting the number of mitoses per microscopic field in conventional slides, but for diagnostic purposes this was largely superseded by immunohistochemistry with antibodies against mitotic proteins, such as Ki-67

Cell differentiation

Differentiated cells of many organs and tissues withdraw from the cell cycle and do not proliferate. Some cell types retain the ability to restart proliferation (e.g. hepatocytes), others reach a post-mitotic, irreversible, terminally differentiated state (e.g. striated myofibers).

The differentiation of tumor cells is usually incomplete and anomalous, at most resembling the proliferative stages of normal tissue. The development of experimental technologies to stimulate the differentiation of normal cells, first in hematology, then in non-hematopoietic cells, led to the idea that differentiation-inducing drugs could be used to restore the differentiation of tumors, causing the extinction of the proliferative compartment through non-cytotoxic mechanisms.

So far, differentiation therapy of human tumors has found limited clinical success, but an effective example is the use of retinoids against acute promyelocytic leukemia (PML), which is mainly caused by the PML-RARα (retinoic acid receptor) translocation. The chimeric PML-RARα protein occupies retinoic acid responsive elements (RARE) in the genome, inhibiting terminal differentiation of granulocytes. Leukemic cells are thus stuck in a proliferative differentiation stage resembling promyelocytes. Treatment of patients with a combination of all-trans retinoic acid (ATRA) which binds RARα and arsenic trioxide, which binds PML, restores terminal differentiation (and exerts other therapeutic activities as well), and represents an effective therapy of promyelocytic leukemia.

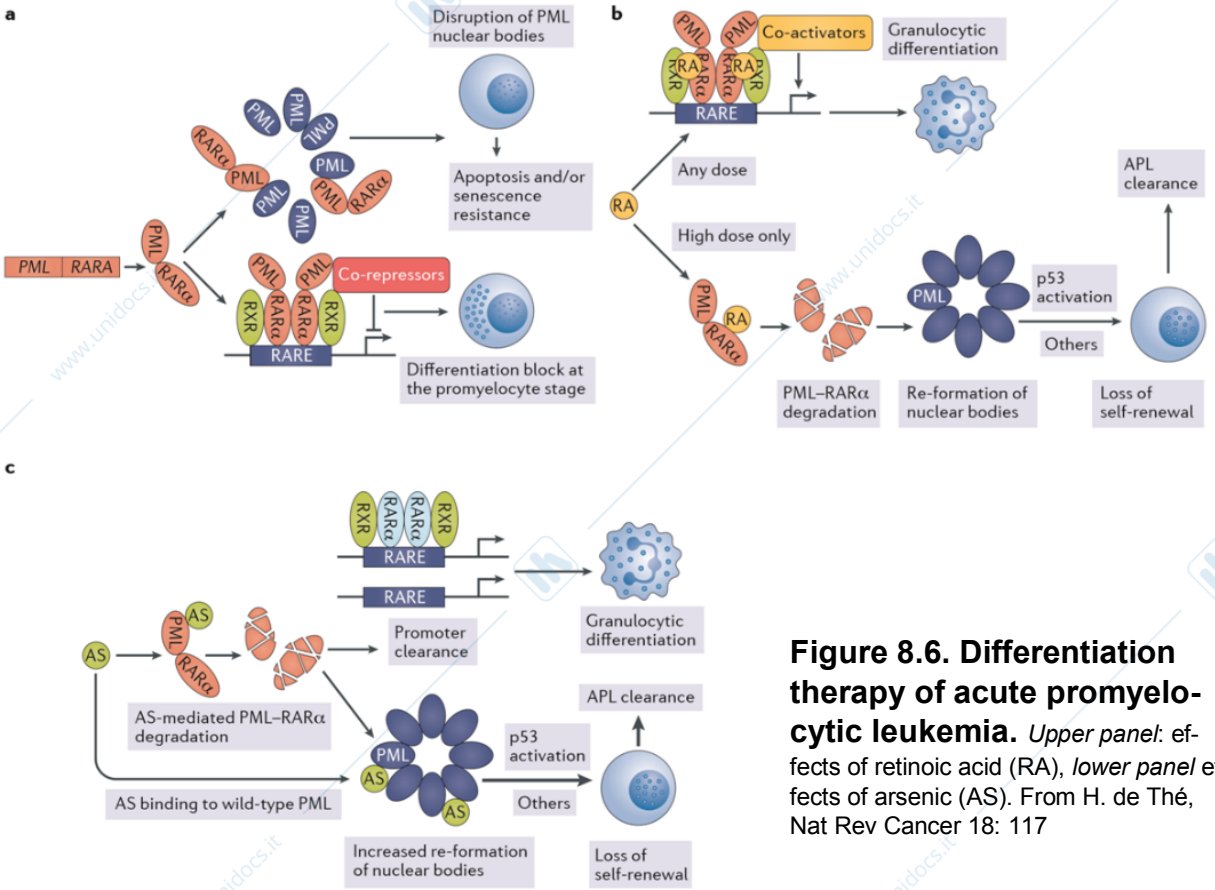


Figure 8.6. Differentiation therapy of acute promyelocytic leukemia. *Upper panel*: effects of retinoic acid (RA), *lower panel* effects of arsenic (AS). From H. de Thé, Nat Rev Cancer 18: 117

Chapter 9. Introduction to Tumor-Host Interactions

Notwithstanding their isolationist propensity, tumor cells interact with a myriad of normal host components. Some solid tumors actually contain more normal cells and molecules, by number or by weight, than tumor cells, thus tumor cells are immersed in a sea of host components. Host interactions play fundamental roles, both positive and negative, in carcinogenesis, progression and therapy (Table 9.1).

In this chapter we will introduce the general elements of tumor-host interactions, the most important relationships will be examined in detail in the following chapters.

Table 9.1. Processes controlled by tumor-host interactions

Interaction	Processes
Microenvironment	• Tumor onset • Invasion
Angiogenesis	• Tumor growth • Invasion and metastasis • Therapy
Inflammation	• Tumor onset • Progression • Immune response
Immune system	• Tumor onset • Therapy
Tumor metabolism and secretions	• Paraneoplastic syndromes • Cachexia

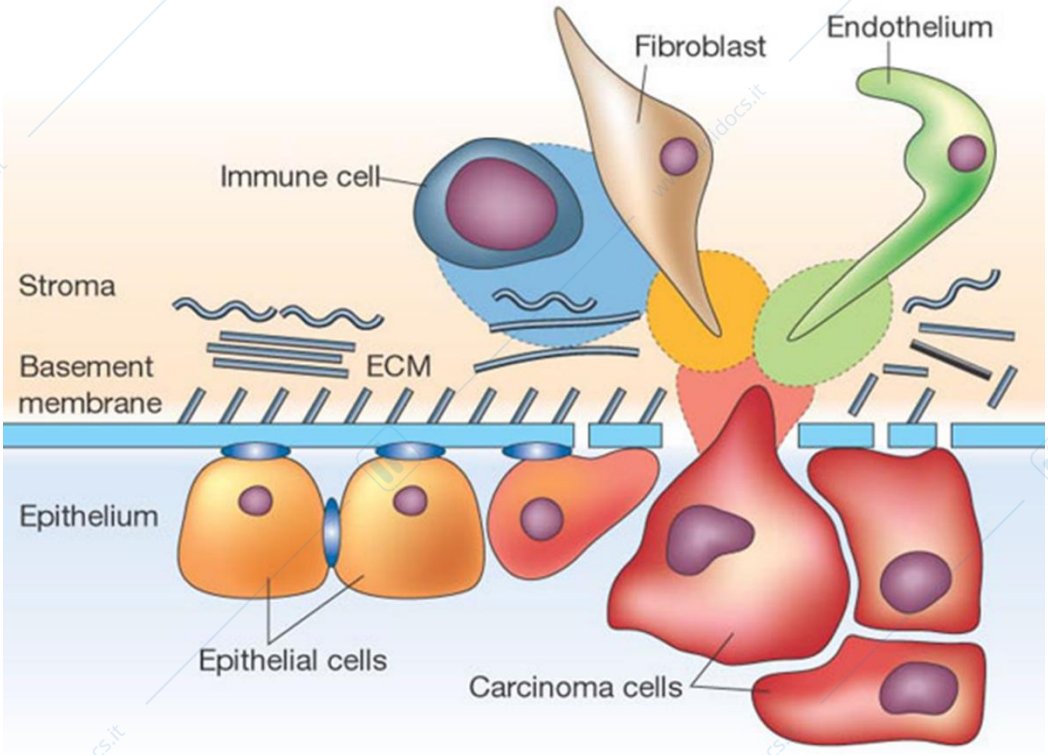


Figure 9.1. Tumor-microenvironment interactions.
Lance A. Liotta and Elise C. Kohn. Nature 411: 375-379, 2001.

The major host elements with which tumor cells interact are (Figure 9.1):

- Normal cells of the tissue of origin
- Extracellular matrix
- Stromal cells
- Tissue stroma
- Inflammatory cells
- Blood and lymphatic vessels
- Immune system
- Coagulation and other blood components
- Hormones, cytokines and growth factors

Epithelial cell interactions

From an oncological point of view, epithelial cell-cell and cell-matrix interactions are tumor suppressors, as attested by E-cadherin mutations in familial diffuse gastric cancer (see chapter on hereditary cancer).

Carcinoma cell typically show (Figure 9.2):

- Reduced intercellular junctions (claudins, cadherins)
- Reduced cell-cell communications (connexins)
- Reduced/altered matrix adhesion (integrins)
- Increased matrix remodeling (metalloproteases)
- Loss of polarity

Epithelial-mesenchymal transition

Epithelial cells spend most of their time attached to one another, but several processes require detachment and possibly movement, such processes include proliferation, embryogenesis and wound healing.

Detachment from epithelial tissues can trigger a complex program that converts cells from epithelial to fibroblast-like, the epithelial to mesenchymal transition, EMT (Figure 9.3).

EMT can be reversed, giving rise to mesenchymal to epithelial transition,

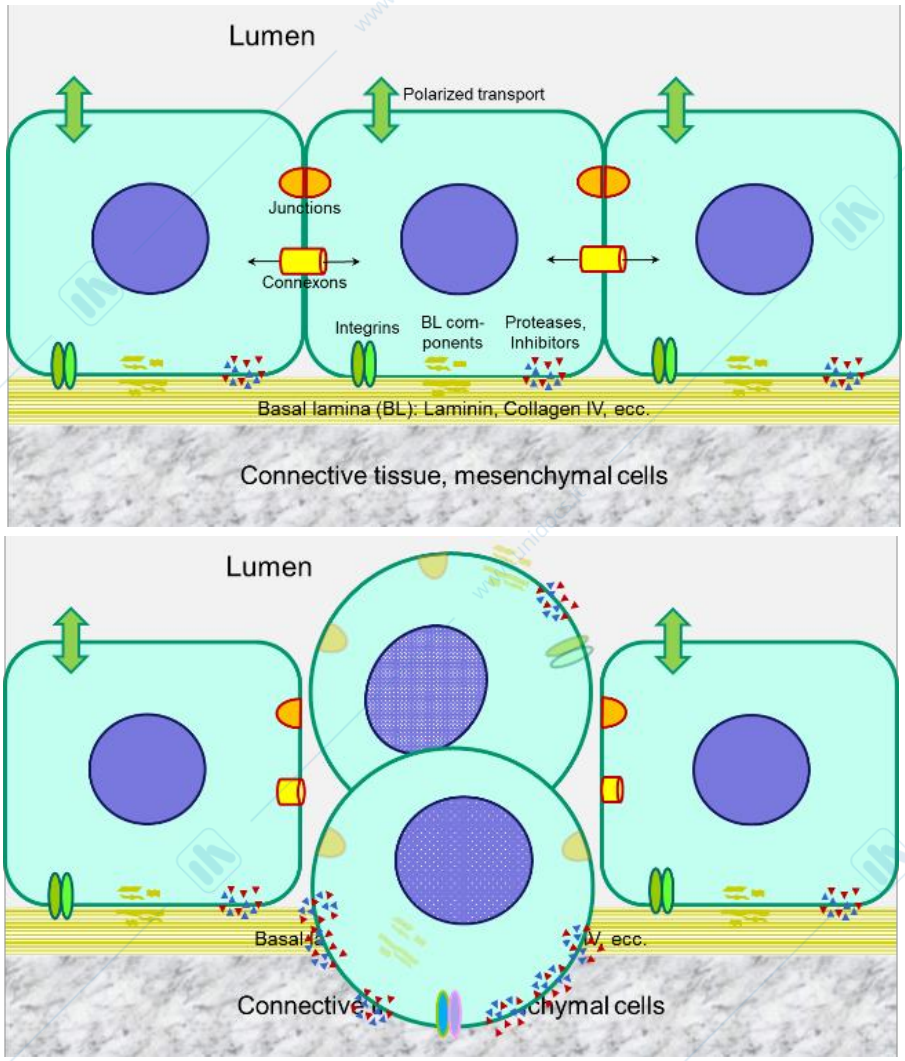


Figure 9.2. Neoplastic alterations of cell-cell and cell-matrix relations. *Upper panel*: normal epithelium, *lower panel*: neoplastic.

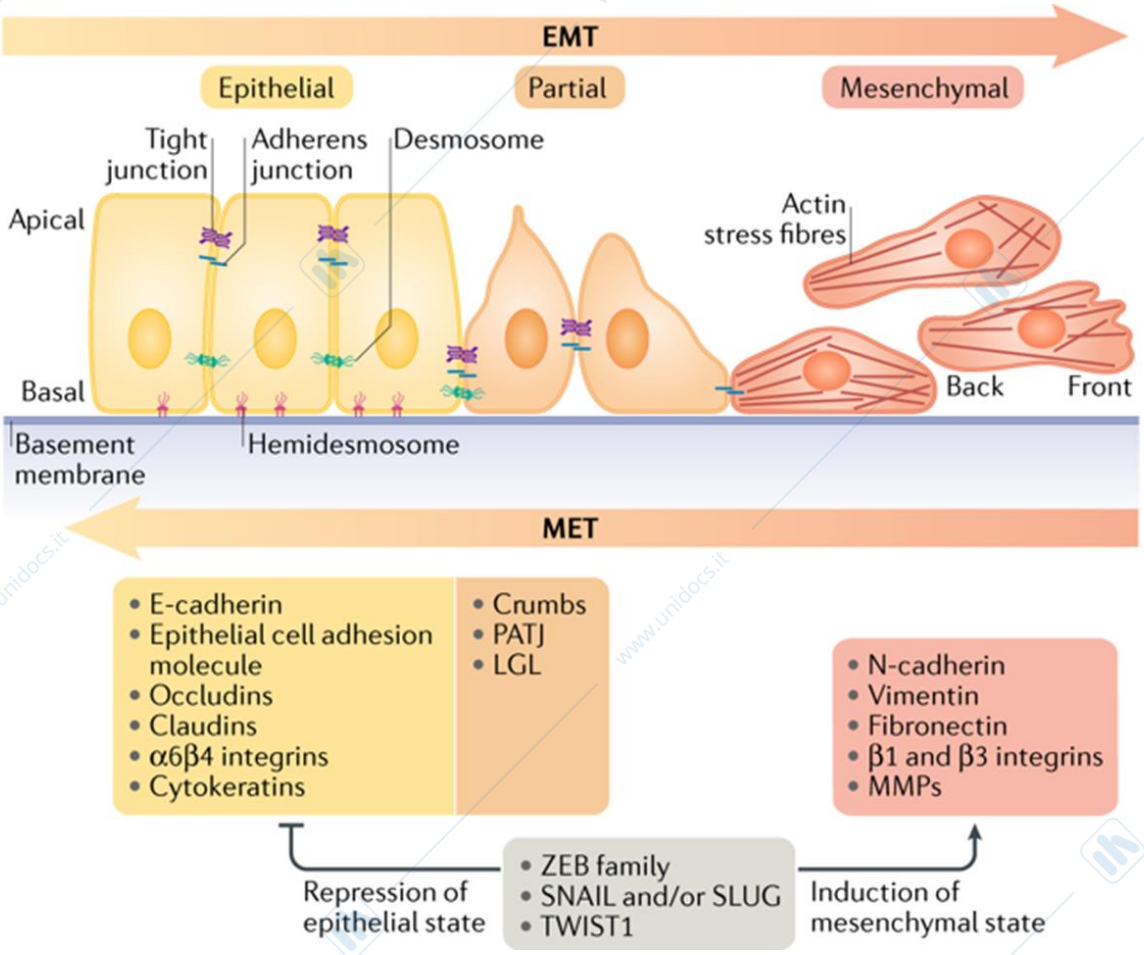


Figure 9.3. Epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET). From Dongre & Weinberg, Nat. Rev. Mol. Cell. Biol. 20:69.

MET (not to be confused with the MET oncogene)

In epithelial tumors, EMT and MET are involved in invasion and metastasis.

Circadian rhythms as tumor suppressors

Many cancer-related phenomena undergo circadian variations:

- Cell cycle and cell proliferation
- Metabolism
- DNA repair, p53 activity
- Inflammation and immunity
- Drug efficacy and toxicity

The expression of rhythm genes (Per1-3, Clock, Cry1-2, Bmal1) is altered in most tumor types. Mice with rhythm alterations are cancer-prone, more sensitive to carcinogens and more permissive to transplantable tumors. Human night shift workers have increased risk of breast, prostate and colorectal cancer; night shift work was classified as a probable human carcinogen.

Altogether these data indicate that circadian rhythms act as tumor suppressors. Furthermore, the fact that the efficacy and toxicity of some cancer drugs are also subject to circadian variations, suggests that treatments administered at specific times of the day might maximize efficacy while minimizing toxic side effects (chronotherapy, see chapter on cancer therapies).

Chapter 10. Tumor Angiogenesis

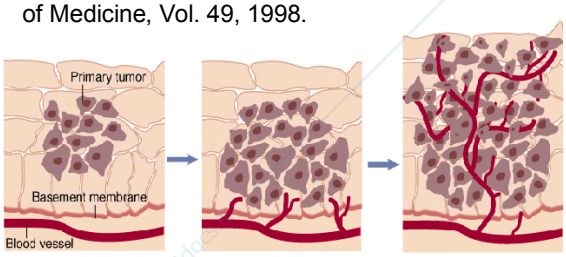
The macroscopic growth of solid tumors depends on blood supply. Tumor cells can thrive on diffusion until the mass reaches a diameter of ~1 mm, then growth stops, unless newly formed vessels (neoangiogenesis) provide fresh blood supply (Figure 10.1). Thus, neoangiogenesis is a general property of all macroscopic, i.e. clinically relevant, tumors, which makes angiogenesis an outstanding target for cancer therapy. Note that avascular microscopic tumors and metastases can remain vital for decades (tumor dormancy).

Vasculogenesis and angiogenesis

The first blood vessels are generated from the mesoderm during embryogenesis by the process of vasculogenesis. Subsequently, new vessels derive from pre-existing ones, the process is called angiogenesis (Figure 10.2). Vasculogenesis and angiogenesis are indispensable for embryogenesis and growth to adulthood.

In adults, angiogenesis is relatively quiescent. It can be triggered by oxygen demand caused by a physiological or pathological increase in tissue mass. Adult angiogenesis is required for reproduction (estrous cycle, pregnancy), tissue healing (wounds, vascular occlusion) and to increase tissue mass (physical exercise).

Figure 10.1. Tumor angiogenesis. Avascular tumor remains small (*left panel*) until endothelial cell growth, stimulated by factors released by the tumor (*central panel*) creates a vascular network that allows unlimited tumor growth (*right panel*). B.R. Zetter, Annual Review of Medicine, Vol. 49, 1998.



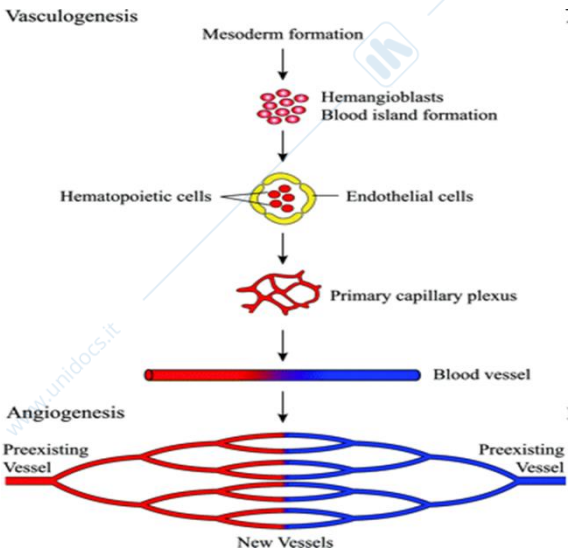


Figure 10.2. Vasculogenesis and angiogenesis. L. Lamalice, F. Le Boeuf, J. Huot. Circulation Research.100:782.

The angiogenic process

Angiogenesis is a complex multiphasic process (Figure 10.3) triggered by angiogenic stimuli, mainly hypoxia, which cause the release of various angiogenic growth factors and mediators acting on pre-existing endothelial cells.

Activated endothelial cells proliferate and migrate toward the source of angiogenic stimuli. Growing endothelial cells degrade the sub-endothelial matrix and invade the surrounding tissues (a physiological type of invasiveness).

New endothelial cells form tubular structures that connect to the existing vascular network (loop formation), allowing

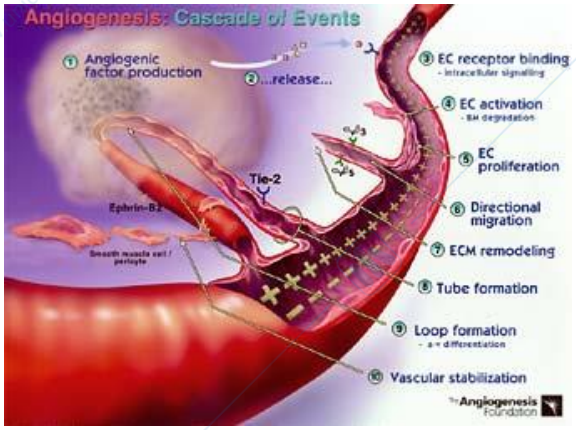


Figure 10.3. The angiogenic process. ©2000, The Angiogenesis Foundation.

the circulation of blood. Remodeling of the surrounding extracellular matrix and the recruitment of pericytes and smooth muscle cells conclude the process.

Angiogenic inducers and inhibitors

Physiological angiogenesis is coordinated by multiple inducers and inhibitors, produced by stromal cells and/or by the endothelial cells themselves, which regulate endothelial proliferation, invasiveness, and migration.

Angiogenic factors (inducers):

- Vascular endothelial growth factor (VEGF, Figure 10.4)
- Basic fibroblast growth factor (bFGF)
- Platelet-derived growth factor (PDGF)
- Angiopoietins
- Other growth factors (EGF, TGF-α, HGF)

Inhibitors

- Thrombospondin 1 (TSP-1)
- Platelet factor 4 (PF-4)
- Angiostatin (cleavage of elastase by plasminogen)
- Endostatin (cleavage of collagen XVIII)

Lymphangiogenesis

Like blood vessels, lymphatic vessels are mostly produced during embryogenesis and early life. In adults, lymphangiogenesis is mainly seen during tissue repair and pathological processes. Lymphangiogenesis is mediated by factors and receptors partially overlapping those involved in angiogenesis (Figure 10.4).

Tumor lymphangiogenesis contributes to fluid dynamics, invasiveness, metastatic dissemination to lymph nodes and distant organs, lymphocyte traffic.

Pathological angiogenesis

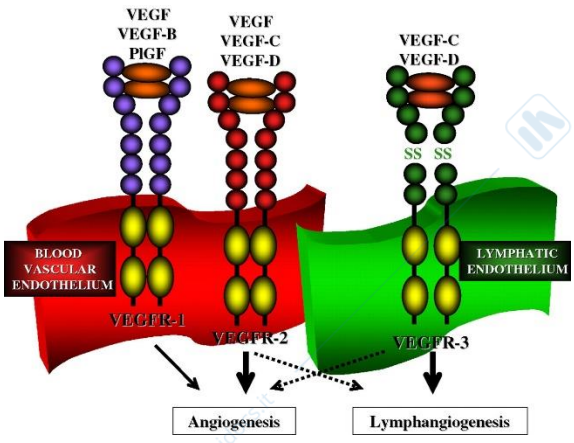
Inappropriate or deregulated activation of angiogenesis is a major component of various human pathologies:

- Proliferative diabetic retinopathy
- Age-related macular degeneration of the retina
- Rheumatoid arthritis
- Tumor angiogenesis
- Neoplastic transformation of angiogenic cells, giving rise to angiomas and angiosarcomas, also called hemangiomas and hemangiosarcomas

Tumor vessels are abnormal

The vascular network inside tumor masses is highly irregular because tumors

Figure 10.4. Vascular endothelial growth factors (VEGF) and their receptors. Tammela T et al. Cardiovasc Res 2005; 65: 550-563.



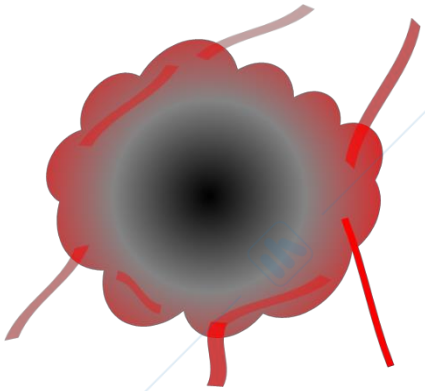


Figure 10.5. Hypoxic and necrotic zones in the tumor center are the consequence of an irregular blood supply.

produce an unbalanced cocktail of angiogenic factors. Tumor vessels are poorly organized, immature, tortuous, dilated, fenestrated, lacunar, with scant basement membrane and pericytes.

In large tumors, only some zones (e.g. the periphery) receive an optimal blood supply, others (e.g. the center) are hypovascular, hypoxic and necrotic (Figure 10.5.

The abnormalities of tumor vessels can also favor tumor growth and malignancy, because metastatic tumor cells find an easy access to the vascular network, while the distribution of therapeutic agents within the tumor mass is irregular, allowing the formation of pockets in which therapeutic concentrations cannot be reached. Furthermore, hypoxic zones are poorly sensitive to radiotherapy, whose therapeutic effect is dependent on oxidative damage.

Angiogenesis-independent tumor blood supply

There are several ways by which a tumor can get sufficient blood supply without the induction of neoangiogenesis.

- Tumor cells can give rise to tubular structures providing blood supply through anastomoses with regular vessels ("vasculogenic mimicry", "vascular mimicry"). The relevance of vascular mimicry in human cancer is debated.
- Tumors can grow around existing normal vases ("vessel co-option").
- Hematogenous metastases were found to grow within vessels. It is generally assumed that circulating tumor cells first extravasate, then proliferate, giving rise to a metastatic mass that will need angiogenesis for macroscopic growth, but the existence of intravascular proliferating tumor cells indicates that alternative sequences of events are also possible.

Angiogenesis as a therapeutic target

Angiogenesis is a coveted therapeutic target in cancer and other pathologies. In cancer it is a potentially universal target, as all progressive tumors need angiogenesis. Unlike therapeutic treatments directed at genetically unstable tumor cells, angiogenesis is a normal process, devoid of the neoplastic mechanisms of drug resistance.

The major foreseeable critical issues of anti-angiogenic cancer therapies are the redundancy and heterogeneity of angiogenic mediators active in different tissues, which may frustrate attempts directed at a single target, and the risks of severe toxicities caused by the inhibition of infarction repair, which is required after a stroke or a heart attack; furthermore, in women systemic anti-angiogenic treatments interfere with the estrus cycle and with pregnancy.

Before considering specific anti-angiogenic therapies, it should be kept in mind that several cytotoxic drugs also inhibit

endothelial cell proliferation and neoangiogenesis. Some examples are doxorubicin, bleomycin, taxanes, thalidomide and tamoxifen; it can be said that these anti-tumor chemotherapies are also anti-angiogenic therapies.

Many diverse strategies were set forth to specifically inhibit tumor angiogenesis, but only a few were successfully applied to patients. We will first examine a series of interesting approaches that are not currently in clinical use, then we will move to approved treatments.

Potential anti-angiogenic strategies and agents

- Natural inhibitors from turmeric, green tea, soy, ginseng, licorice, etc. Coming from vegetables which are normally consumed by humans, these substances might find application in preventive treatments, think for example diets for people at risk of cancer development, or cancer survivors at risk of metastasis.
- Direct physiological inhibitors of angiogenesis, such as angiostatin or endostatin, however early clinical trials failed to provide promising results.
- Cytokines that (also) inhibit angiogenesis, for example the IL-12 → IFNs → CXCL10/IP-10 axis.
- Inhibitors of cell invasiveness: tissue inhibitors of metalloproteinases (TIMP) -1 and -2.
- Agents targeting integrins (such as $\alpha_v\beta_3$) expressed by endothelial cells during angiogenesis
- Vessel normalization, instead of angiogenesis inhibition, could be a therapeutic strategy to enhance drug penetration and tumor sensitivity to radiotherapy.
- Metronomic chemotherapy is based on the administration of cytotoxic drugs at low dose on a continuous or frequent schedule.

Targeted anti-angiogenic strategies in clinical use

The main targets are VEGF and its VEGFR receptors (Figure 10.4), which are tyrosine kinases.

VEGF can be intercepted using monoclonal antibodies, such as bevacizumab, or recombinant chimeric proteins of a VEGFR fused with an immunoglobulin (aflibercept).

VEGFR, in particular VEGFR-2 (also named KDR), can be inhibited by monoclonal antibodies (ramucirumab) or by small tyrosine kinase inhibitors (TKI). Note that TKIs in clinical use inhibit VEGFRs and other tyrosine kinases, such as PDFGR, KIT, RAF (sunitinib, sorafenib, pazopanib, cediranib, axitanib, ecc.), which are also expressed by tumor cells, hence their therapeutic activity is only partially attributable to the inhibition of angiogenesis.

Finally, it should be noted that the promise of a universal cancer therapy has not yet materialized. A case in point is bevacizumab, initially approved for the treatment of both colorectal and breast cancer, but no longer approved for breast cancer because of insufficient benefit/risk ratio. Anti-angiogenic drugs are effective in specific tumor types, just as any other cancer therapy.

Chapter 11. Inflammation and Cancer

Inflammation is a response to tissue damage involving the coordinated activation of vascular elements, leukocytes, and tissue repair. Acute inflammation can eliminate the stimulus and rapidly switch down, restoring tissue integrity. If inflammatory stimuli persist, chronic inflammation becomes a major cause of long-term tissue damage.

Tumor onset, growth and progression are frequently associated with varying levels of chronic inflammation, caused by infectious agents or other causes of chronic tissue damage, including therapeutic treatments like radiotherapy and chemotherapy. The carcinogenicity of chronic inflammation is due to the release of mutagenic reactive oxygen species (ROS) by phagocytes and to the continuous stimulation of cell proliferation to repair the damage inflicted to the tissue. A testament to the carcinogenic role of chronic inflammation is the preventive effects of non-steroidal anti-inflammatory drugs (NSAIDS) in humans. Further details can be found in the chapters on carcinogenesis and on cancer prevention.

Established tumors are infiltrated by inflammatory leukocytes and fibroblasts, which can have both pro- and anti-tumor activities (Figure 11.1).

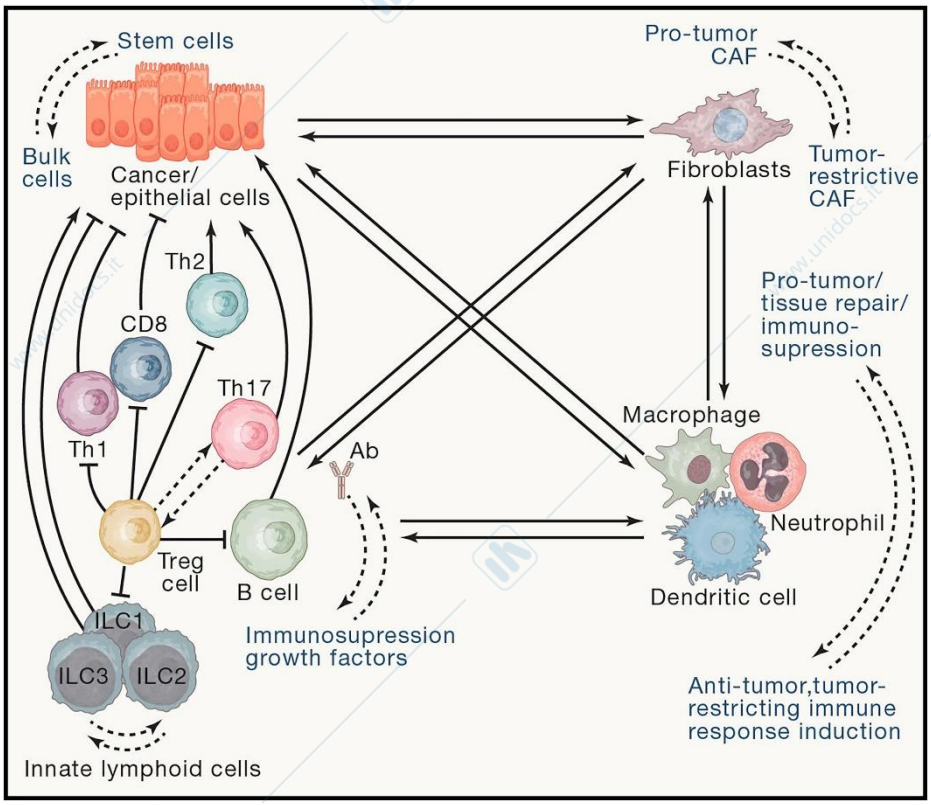
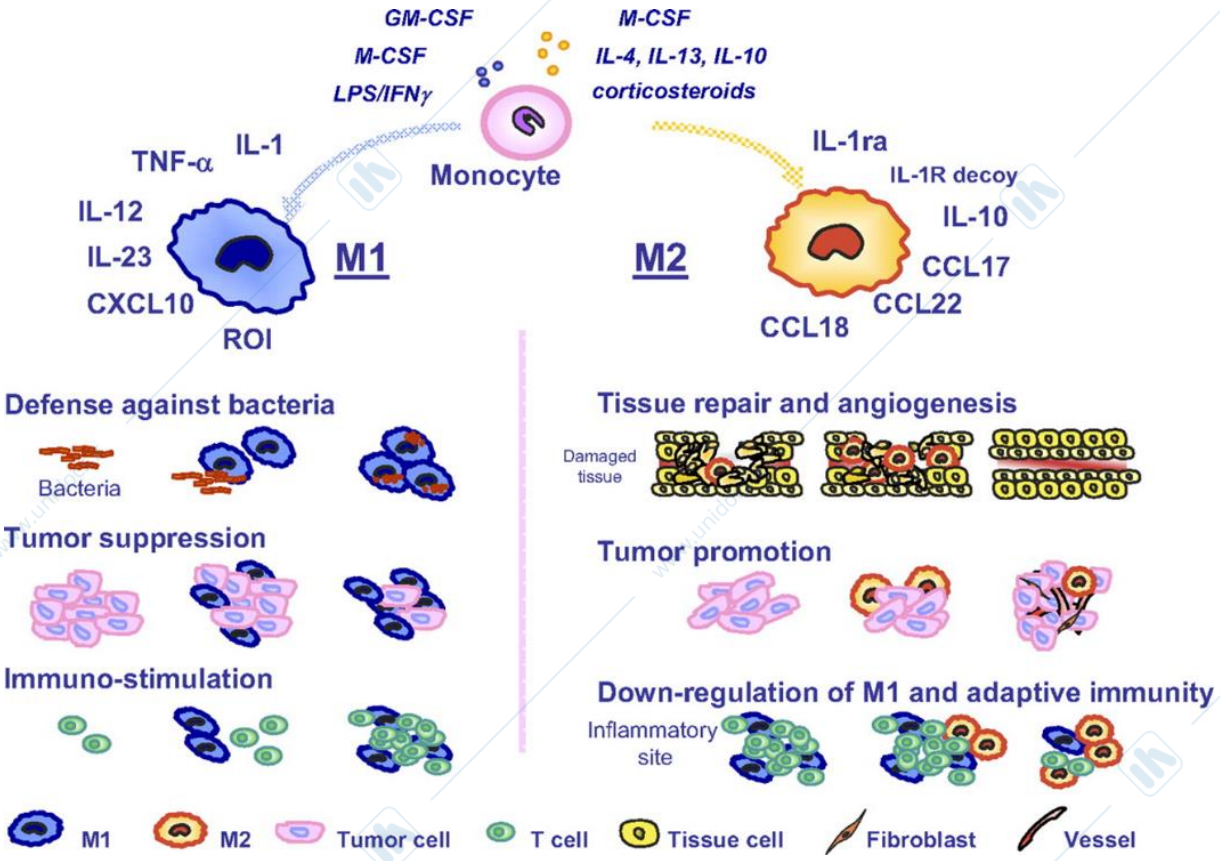


Figure 11.1. Pro- and anti-tumor interactions among immune and inflammatory cells.
FR Greten, Immunity 51: 27.

Figure 11.2. Macrophage polarization. Solinas G. et al., J. Leukoc. Biol., 86: 1065-73.



Tumor-associated macrophages (TAM)

Macrophages are attracted ("recruited") to growing tumors by growth factors, cytokines and chemokines produced by tumor cells or released by stromal cells in the tumor microenvironment, some examples are CSF-1 (M-CSF), CCL2 (MCP-1), CCL3 (MIP1α), CCL4 (MIP1β), IL-1β, IL-6. Further macrophage attractants are molecules released by tumor and stromal cells dying by necrosis and hypoxia.

Macrophages play two different roles in inflammation (Figure 11.2): immune defense (microbe phagocytosis, activation of adaptive immunity) and tissue repair (induction of cell growth, angiogenesis). In analogy with the polarization of helper T cells (Th1/Th2), macrophages were classified as M1 (immune defense) or M2 (tissue repair); different classifications are also used to define the same fundamental functions.

In human cancer, tumor-associated macrophages (TAM) typically favor tumor growth (Figure 11.3) through the release of mediators that in a normal context would contribute to tissue repair:

- Growth factors, e.g. EGF
- Angiogenic and lymphangiogenic factors, e.g. VEGF, PDGF, TGF-beta, FGF, COX2
- Metalloproteinases (MMP2, 7, 9, 12)
- Inhibitors of adaptive immune responses, such as IL-10, TGF-β and arginase

In experimental tumors, macrophages can efficiently kill tumor cells and enhance

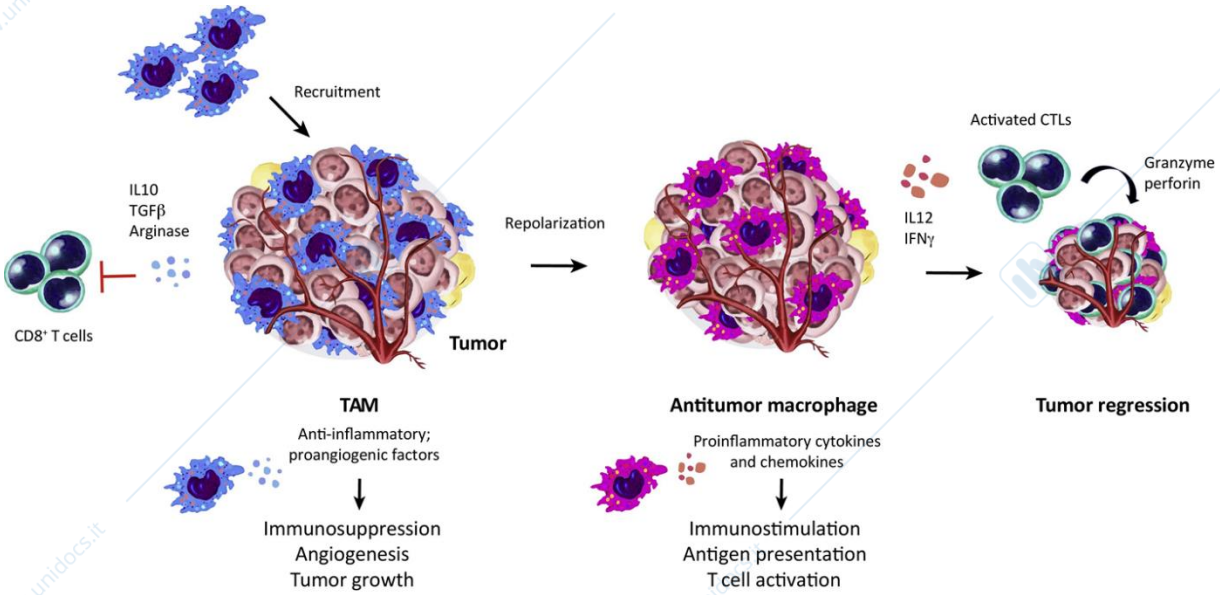


Figure 11.3. Pro- and anti-tumor activities of macrophages.
P Pathria, Trends Immunol 40: 310.

immune responses through a variety of mechanisms (Figure 11.3):

- Direct phagocytosis of tumor cells
- Killing of tumor cells by complement-dependent cell-mediated cytotoxicity (CMC) and antibody-dependent cell-mediated cytotoxicity (ADCC)
- Antigen presentation and activation of T and NK cell cytotoxicity

Conversion of TAMs from a pro-tumor to anti-tumor (TAM repolarization) is a potential therapeutic strategy. Candidate therapeutic agents include macrophage activators, such as cytokines or bacterial products, which however have had limited success so far, and a novel series of drugs aimed at the inhibition of CCR2 (the receptor of CCL2 / MCP-1), CSF1R, PI3Kγ, IL1Ra.

Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSC) are immature myeloid precursors elicited by tumor-derived factors such as CSFs and VEGF. MDSC are present within tumors and in the bloodstream. Large numbers of circulating MDSC can skew the leukocyte formula towards granulocytes; granulocytosis is frequently detected in cancer patients. Advanced tumors can induce such huge numbers of MDSC as to simulate leukemia ("leukemoid reaction").

Cancer-associated fibroblasts

Fibroblasts and myofibroblasts are involved in inflammatory tissue repair and fibrosis. Fibrosis associated with long-term, advanced chronic inflammation is a known cancer risk factor, as seen for example in lung asbestosis and liver cirrhosis (see chapters on chemical and biological carcinogens).

Laboratory evidence shows that, like macrophages, cancer-associated fibroblasts can play a dual role towards tumors (Figure 11.1). During tumor onset, interactions of epithelial cells with fibroblasts inhibit early carcinogenesis. In established tumors, cancer-associated fibroblasts (CAF) favor tumor growth and invasion through the release of growth factors,

such as IGFs, the induction of angiogenesis, the remodeling of extracellular matrix (metalloproteinases) and the production of type I collagen (fibrogenesis).

Pharmacologic inhibition of CAFs could be a therapeutic approach, but there is no clinical evidence so far.

Chapter 12. Tumor Immunology

The existence of anti-tumor immune responses was first proved mid-20th century using inbred mice (see Box 22.1) and their tumors in immunization-challenge experiments (Figure 12.1). Original experiments used transplantable tumors, routinely passed from one inbred mouse to another through the transplantation of tumor fragments; nowadays we can obtain the same results using tumor cell lines (see chapter on cancer modeling). Immunization was carried out with irradiated tumor cells (or other equivalent systems), after some time, immunized mice were challenged with live tumor cells. The lack of tumor growth in immunized mice showed that vaccination could induce tumor rejection. On the contrary, mice immunized with a given tumor, but challenged with a different one, did not reject the challenge. Altogether these experiments, which were repeated in a great variety of mouse models, demonstrate two main features of adaptive immune responses: specificity and memory.

Which elements of the immune system are involved in tumor immunity?

Some clues can be obtained by the analysis of the immune responses elicited in immunization-challenge experiments; however, powerful tumor vaccines can induce practically all types of immune response.

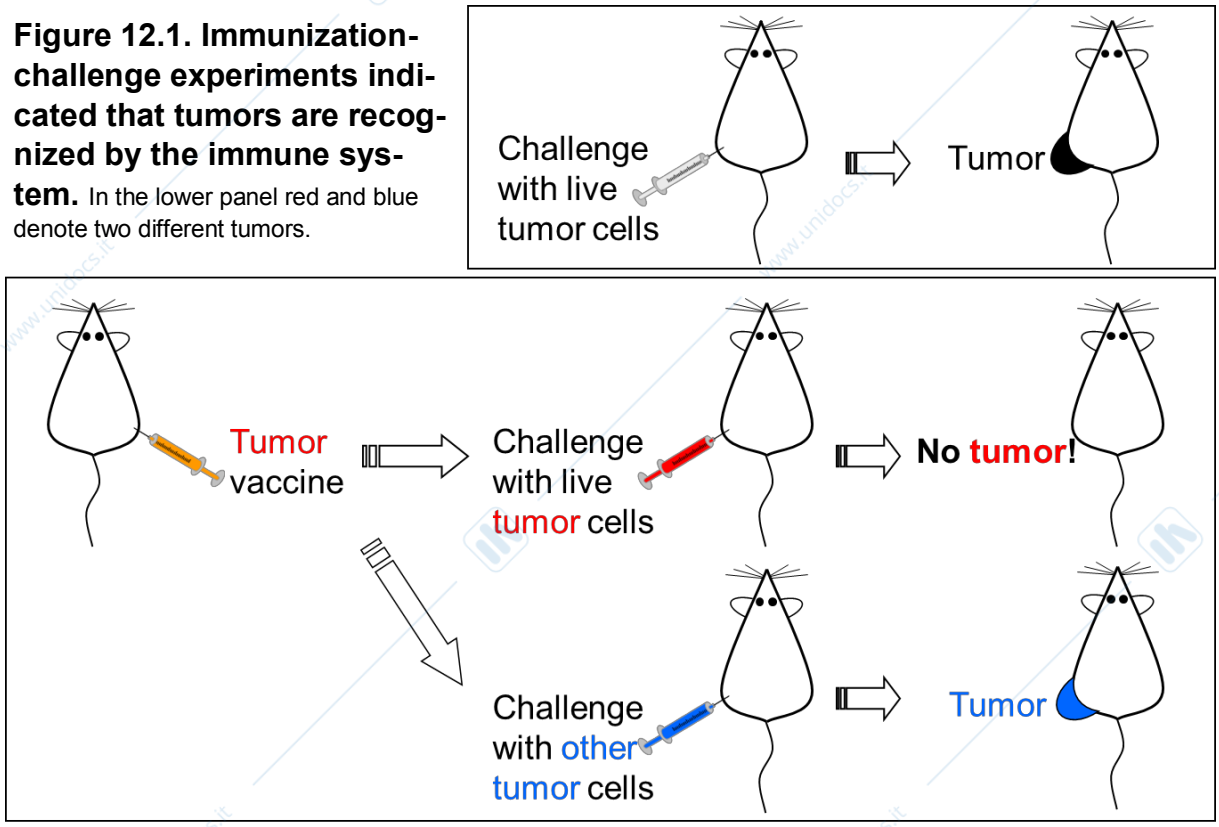


Figure 12.1. Immunization-challenge experiments indicated that tumors are recognized by the immune system. In the lower panel red and blue denote two different tumors.

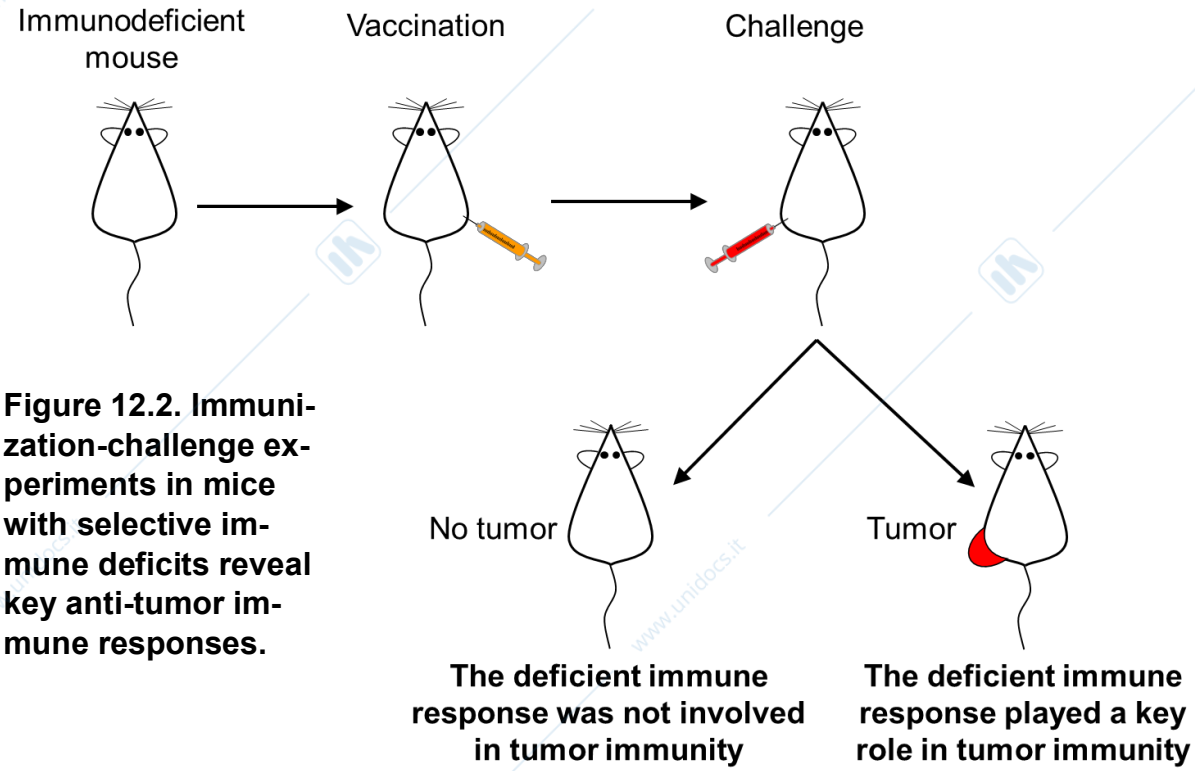


Figure 12.2. Immunization-challenge experiments in mice with selective immune deficits reveal key anti-tumor immune responses.

Mice with selective immune deficiencies, either congenital of induced by exogenous treatments can be used to formally dissect the responses involved in tumor rejection: if vaccination fails to protect the immunodeficient mouse, then the missing immune response plays a causal role, otherwise it is not involved in tumor rejection (Figure 12.2).

The roles played by phagocytes were examined in the previous chapter.

Dendritic cells (DC) are the professional antigen-presenting cells (the acronym is APC, no relation with the APC tumor suppressor gene) at the root of adaptive immune responses. Tumor cells usually lack the co-stimulatory signals that are needed to present their antigens to T cells, hence anti-tumor T cell response require cross-presentation of tumor antigens by DC. Like phagocytes, DC can play both positive and negative roles in the orchestration of anti-tumor immune responses.

Natural killer (NK) cells are active both in tissues and in the bloodstream, where they can kill circulating tumor cells, thus blocking metastatic spread. In mice, depletion of NK enhances hematogenous metastasis by two orders of magnitude.

The depletion of B cells in immunization-challenge experiments, is mostly uneventful, in some cases it could even improve the effect of vaccines. These results downplayed the importance of B cells and antibodies in anti-tumor immune responses, in part because most solid tumors express complement-inhibitory molecules (e.g. CD55 and CD59) that make them impervious to antibody-mediated cytotoxicity. The development of therapeutic monoclonal antibodies, the best application of tumor immunology to cancer therapy, is a testament to the ingenuity of

tumor immunologists, who used biotechnology to overcome the limits of nature.

The study of tumor immunology is dominated by T cells, to the point that some authors lamented a "T cell chauvinism". Cytotoxic T cells (CTL) are the final effectors that kill tumor cells, but their activity is subject to fundamental activating and suppressive actions of helper (Th) and regulatory (Treg) cells. After decades of intensive studies, only recently T cell-based immunotherapies had a significant clinical impact, with the advent of (antibody!) treatments that release cytotoxic responses from suppressive mechanisms, and of genetically engineered chimeric T cell receptors (CAR-T).

The knowledge of the various leukocyte subpopulations involved in cancer immunity is also being exploited by pathologists, in fact the composition of the leukocyte infiltrate within a primary tumor, as assessed using various indexes (e.g. immunoscore) can help in the prediction of both prognosis and response to immunotherapy.

Immune surveillance theory

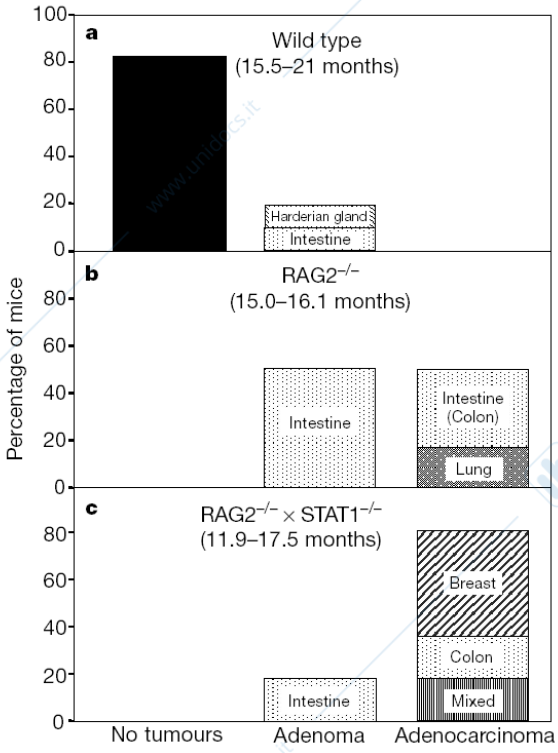
Between the 1960s and 1970s, the immune surveillance theory set forth the idea that the physiological function of the immune system is to defend the host not only from microbes, but also from tumors. The organism is continuously exposed to the onset of microscopic tumors, which are mostly eliminated by immune responses; tumors that become clinically evident must have escaped immune defenses.

This theoretical framework leads to two verifiable predictions, *a*) as the immune system protects the host from tumor onset, immunodeficient individuals should develop more tumors than immunocompetent ones; *b*) as the immune system kills easily recognized (i.e. antigenic) tumor cells, those tumors that grow despite the immune system should be poorly immunogenic.

Tumor development in immunodeficient hosts

Early studies of tumor development in immunodeficient hosts used *nude* mice, a spontaneous mutant with epithelial alterations (hence the lack of hair) which also affect the thymic epithelium, hampering T cell maturation. Spontaneous tumor incidence in *nude* mice was found to be similar to that of immunocompetent mice, leading to the conclusion that the immune surveillance theory was false.

Figure 12.3. The lack of T and B cell immunity (RAG2$^{-/-}$ mice) strongly increases the risk of tumor development, which is further enhanced by the lack of interferon responses (STAT1$^{-/-}$). Shankaran et al., Nature 410: 1107, 2001.

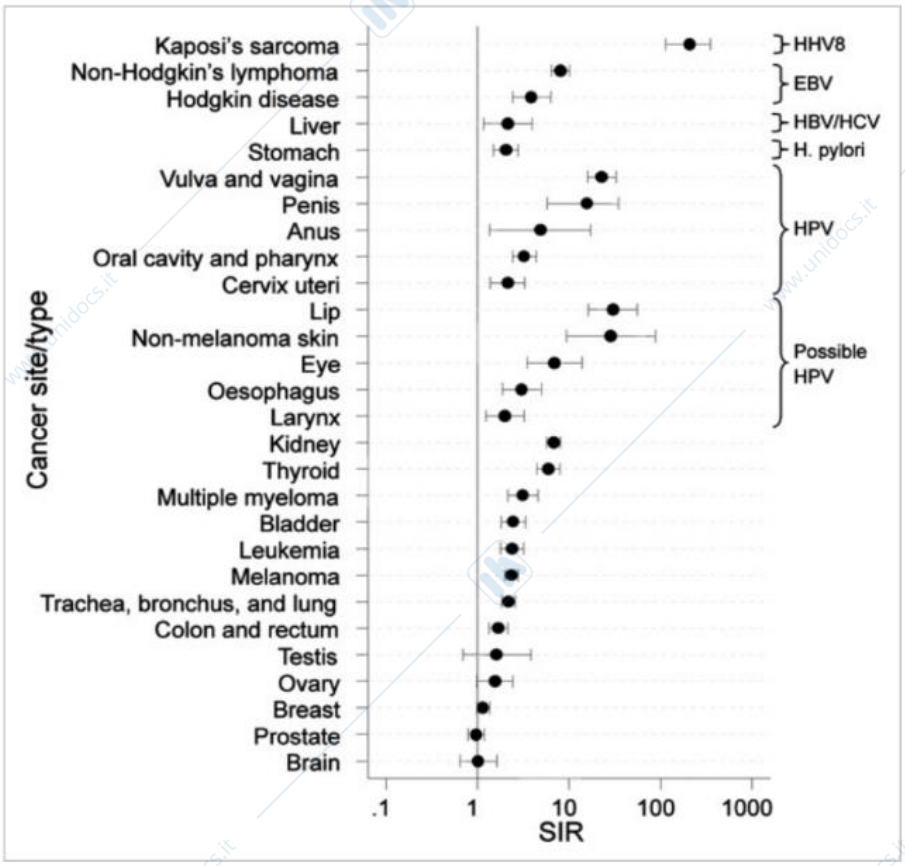


However spontaneous tumor development in the *nude* mouse, as in all hosts, is associated to old age, and aging *nude* mice are not truly immunodeficient, because over time T cells can develop extrathymically, moreover *nude* mice have a partial B cell immunity and strong NK activity. In summary, the demise of the immune surveillance theory based on *nude* mice data was premature.

Only the development of genetically-modified mice, bearing permanent, specific immune defects allowed the study of tumor onset in fully immunodeficient hosts (Figure 12.3). The analysis of spontaneous and carcinogen-induced tumors in knockout mice demonstrated that indeed the lack of adaptive immunity (i.e. T and B cells) leads to an enormous increase in the incidence of benign and malignant tumors. The additional knockout of genes of the innate immune responses further increased the proportion of malignant tumors. Thirty years after the misleading studies in *nude* mice, the immune surveillance theory was finally vindicated.

In the meantime, human immunologists performed studies of tumor incidence in immunodeficient patients. Such studies are not feasible in congenital severe combined immune deficiencies, because life expectancy is too short, while the bulk of tumors appear with aging. The clinical series in which studies of tumor incidence are feasible are transplant recipients, who receive chronic immunosuppressive drug treatments, and AIDS patients, in which HIV progressively kills helper T cells, thus compromising the entire immune response. Both clinical series showed huge increases in the incidence of specific tumor types, such as Kaposi sarcoma or anogenital carcinomas (Figure 12.4). Infectious origin was the common element of tumors with an increased incidence in these immunodeficient patients. It should be noted that the incidence of some common tumor types, such as breast or prostate

Figure 12.4. Cancer risk in organ transplant recipients.
Modified from Vajdic & van Leeuwen, Int. J. Cancer 125: 1747.



carcinomas, was identical to that of immunocompetent individuals.

Combining the results of murine and human studies, we can finally reach firm conclusions on the immune control of tumor onset. In the presence of a complete and long-lasting deficiency of the immune response, the incidence of all tumors increases. If the immunodeficiency is partial or transient, only some tumor types increase, e.g. viral tumors or lymphoid neoplasms.

Studies in genetically engineered mice also allowed to verify the second prediction of the immune surveillance theory, concerning the influence of the immune system on tumor antigenicity. It was found that tumors arising in immunodeficient hosts are immunogenic, whereas those arising in immunocompetent hosts are poorly immunogenic. This phenomenon, which is now called "immunoediting", shows that the immune system negatively selects immunogenic tumor cells, and only those cells that escape immune defenses, thanks to a low immunogenicity, can give rise to macroscopic tumors. Obviously, this is bad news for cancer immunotherapists, because it means that clinically relevant cancers are pre-selected to be poor targets. These conclusions do not apply to tumors caused by powerful mutagens, such as tobacco smoke or UV rays, which develop aggressively despite immune defenses, retaining a strong antigenicity (see chapter on cancer genes).

Tumor antigens

The identification of antigens recognized by the immune system on tumor cells also took a long time and was dependent on the development of suitable technologies. Early studies, starting in the 1950s, injected human tumor cells in rabbits and mice to induce antibodies that were then used to characterize the corresponding antigens. Such studies defined a plethora of molecules which are still called tumor antigens, but in most instances are not recognized as such by the human immune system. A clear distinction must be made with "true" tumor antigens, which can be the target of immunotherapy in cancer patients. The immunologically savvy practitioner must be aware of this ambiguity, which is still common in current medical parlance.

Circulating tumor antigens

Some molecules are released by human tumors in such large quantities as to be easily identifiable with simple blood analyses. The oldest examples are the carcinoembryonic antigen (CEA), a surface molecule of epithelial cells which is copiously shed by many carcinomas, especially colorectal cancers, and α-fetoprotein, an embryonic equivalent of albumin, which is produced by adult liver cancers. In both cases it should be stressed that these molecules are not tumor-specific: sizeable blood levels are associated with tissue damage, and various non-neoplastic chronic conditions yield positive laboratory tests, for example smokers (bronchitis), liver diseases, ulcerative colitis.

The advent of monoclonal antibodies brought a new generation of circulating tumor antigens, such as CA125 (ovarian cancer and other carcinomas), CA15.3 (breast cancer and others), CA19.9 (pancreatic cancer), and many others. However, also these markers are not truly tumor-specific, even though the fraction of positive non-neoplastic samples is lower than with the previous generation.

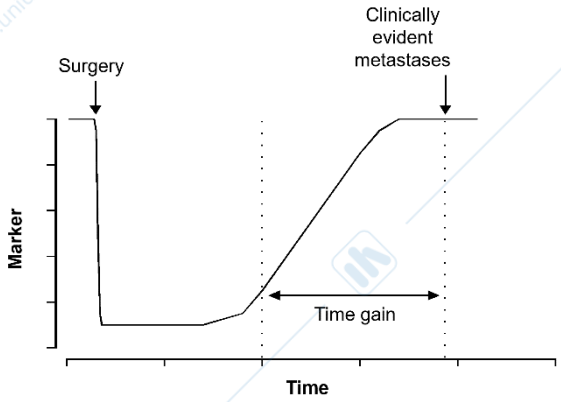


Figure 12.5. The levels of circulating tumor antigens can raise the suspicion of relapse before metastases become clinically evident.

The only tumor-specific marker in this whole bunch is chrorionic gonadotropin, but only because it is expressed by some testicular tumors, while high non-neoplastic blood levels are only found in pregnancy.

The worst case was prostate specific antigen (PSA, a prostatic protease), whose widespread use led to an explosion of prostate cancer diagnoses in the 1990s, most of which are now classified as overdiagnosis (see chapter on cancer prevention).

What is the use of circulating tumor antigens? Despite the sensitivity of modern analytical methods, they cannot be used for early cancer diagnosis, because the variety of positive non-neoplastic conditions would lead to huge numbers of false positives. Blood test are usually performed for all suspect cancer cases, but their contribution to the final diagnosis is relatively minor in comparison with imaging and clinical parameters. The best use of tumor markers is for the follow-up of patients at risk of metastasis development (Figure 12.5). In this case the blood level of the antigen is taken to be proportional to the tumor burden; patients start with a very high level, caused by the primary tumor mass, which rapidly fall after surgical removal. Periodical follow-up blood tests could then reveal the growth of metastatic lesions well before the appearance of symptoms, allowing early therapeutic interventions.

Tumor antigens for cancer immunotherapy

Starting in the 1990s, cloning technologies based on T cell recognition allowed the discovery of tumor antigens that are effectively recognized by the immune system of the host and can be targeted by immunotherapeutic endeavors. Hundreds of antigenic molecules are now known, which can be broadly grouped according to biological and oncological properties.

Cancer-testis antigens are molecules normally involved in male gametogenesis which were originally cloned from human tumors, the first one was called MAGE (Melanoma Antigen GEne). Nowadays MAGE is a family comprising several members, and several other melanoma-testis antigens were also discovered (BAGE, GAGE, NY-ESO-1). Gene sequencing showed that cancer-testis antigens of tumors are not mutated; the relative specificity of immune responses is attributed to the fact that the testes are immunologically-privileged sites, normally not subject to immune surveillance, hence ectopic expression in tumors can elicit immune responses that do not cause autoimmunity.

Differentiation antigens are expressed within the differentiation lineage of the tumor. Notable examples are CD19 and CD20 (B cells and lymphomas), tyrosinase and Melan-A/MART-1 (melanocytes and melanoma). Effective immunotherapies

killing tumor cells also cause the death of normal cells expressing the antigen, e.g. B cell depletion in lymphomas and vitiligo in melanoma patients; such toxicities can be also taken as clinical evidence that the immunotherapy is working.

Shared tumor antigens are molecules expressed by different tumor types, some example are HER-2, which is both an oncogene and an antigen, expressed by breast, ovarian, gastric and other carcinomas: the catalytic subunit of telomerase, TERT (90% of human tumors) and MUC-1, a mucin expressed by colorectal and other carcinomas; CEA can be also considered a shared tumor antigen. In general, these tumor antigens are identical to the molecules expressed by normal cells, but some tumor-specific molecule also exist, for example tumor MUC-1 glycosylation is abnormal, and HER-2 is subject to post-translational modifications.

Tumor-specific antigens are the products of mutational events that include both driver and passenger mutations. It has been shown that mutant oncogenes (RAS), tumor-suppressor genes (dominant-negative p53) and chromosomal translocations (BCR-ABL) contain peptides that can be taken by the antigen-processing machinery of the cells, exposed on the surface in the groove of MHC molecules and finally recognized by T cell receptors that trigger the lytic activity of CTLs. Unfortunately, so far, such immune responses had very little clinical impact. Tumor antigens resulting from passenger mutations were poorly characterized before next-generation genome sequencing, because such random mutations generate individual antigens, different from one tumor to another, also according to the HLA of the patient. Now individual tumors can be sequenced in search of mutations that encode potential antigens, against which personalized vaccines are rapidly synthesized and administered to the patient. The feasibility of this approach has already been demonstrated in clinical trials; its therapeutic efficacy remains to be determined.

A further class of tumor-specific antigens is encoded by viral genes expressed in tumors caused by papilloma (HPV) and other oncogenic viruses (see chapters on biological carcinogenesis and cancer prevention). In the case of HPV, vaccines against structural viral proteins (L1) are effective in the prevention of virus infection but have no therapeutic activity. Experimental therapeutic vaccines made with viral oncogene products (E6 and E7) as antigens have shown clinical activity against early cancer lesions.

The antigen is necessary, but not sufficient

To elicit an effective anti-tumor immune response, the presence of an antigen is just the first step (Figure 12.6). The antigen must be taken up by the antigen processing machinery of the tumor cells and exposed on the cell membrane by MHC molecules; cell-cell contact with T cells must be stabilized by adhesion molecules; T cell activation is conditioned by the expression of stimulatory and inhibitory surface molecules and soluble cytokines.

Tumor cells are not passive spectators of their own destruction by cells of the immune system. As it happens with pathogenic microbes, there is a competition between tumor cells and the immune system, and tumor cells will try to evade immune responses, or even to counter-attack the cells of the immune system.

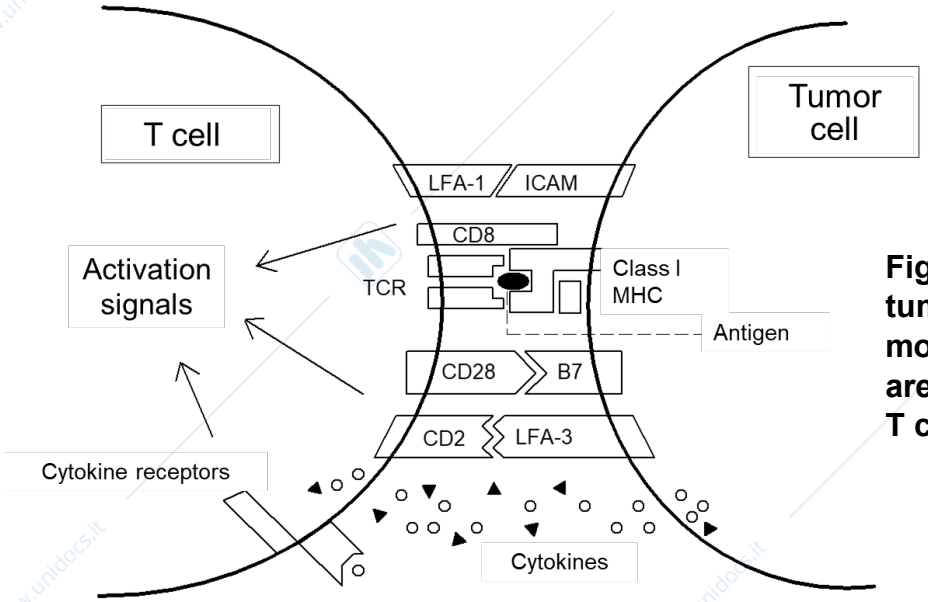


Figure 12.6. Besides tumor antigens, many molecular interactions are required to activate T cells.

The first requisite for T cell lysis is the recognition of a peptide-MHC complex on the surface of tumor cells, however this is rarely the case. The study of antigen expression in melanoma metastases has demonstrated a frequent loss of antigenic specificities expressed by primary tumors, thus any therapy designed on the basis of the antigenic profile of the primary tumor would be ineffective against antigen-loss variants. The situation of class I MHC expression is even worse: comparisons with surrounding normal tissues revealed a loss of expression in most human tumors (Table 12.1), making MHC loss one of the most frequent molecular alterations found in human tumors.

Tumor cells can actively inhibit immune responses using a variety of mechanisms, including complement-inactivating surface molecules (CD55 and CD59), anti-phagocytic "don't eat me" receptors (CD47), inhibitory cytokines (TGF-β), pro-apoptotic surface molecules of the TNF superfamily and T cell-inhibitory receptors (PD-L1). All these molecules are potential targets of immunotherapies aimed at removing their inhibitory activity.

Regulatory T cells are a physiological safety mechanism to prevent autoimmunity, but tumors can stimulate both Treg and MDSC to inhibit anti-tumor immune responses. After decades of poorly effective immunotherapeutic attempts aimed at positively stimulating anti-tumor responses, the advent of monoclonal antibodies directed against inhibitory molecules, such as CTLA4, PD1 and its ligand PD-L1 ('immune checkpoint inhibitors') finally produced significant clinical results in melanoma, lung cancer and other tumors.

Table 12.1. Prevalence of HLA loss (all phenotypes) in human tumors
Garrido & Algarra, Adv. Cancer Res. 83: 117.

Tumor type	Percentage
Bladder	84%
Breast	96%
Cervix	96%
Colorectal	87%
Head & neck	70%
Melanoma	63%
Prostate	87%

Impact of tumor immunology on human health

Various preventive and therapeutic strategies derived from tumor immunology are being applied to human patients. Some will be further discussed in the chapters on cancer prevention and cancer therapy.

- Prophylactic vaccines are highly effective in the prevention of viral tumors, such as hepatocellular carcinoma (HBV) and cervical carcinoma (HPV).
- Therapeutic vaccines against tumor antigens so far had a poor clinical impact.
- Monoclonal antibodies have a strong impact on cancer therapy through different strategies:
  - To directly inhibit tumor cells.
  - To inhibit tumor angiogenesis.
  - To unlock the immune response.
- Genetically modified T cells with chimeric antigen receptors (CAR-T) were approved in the 2020s for the therapy of $CD19^+$ B cell lymphomas, other applications are in the pipeline.

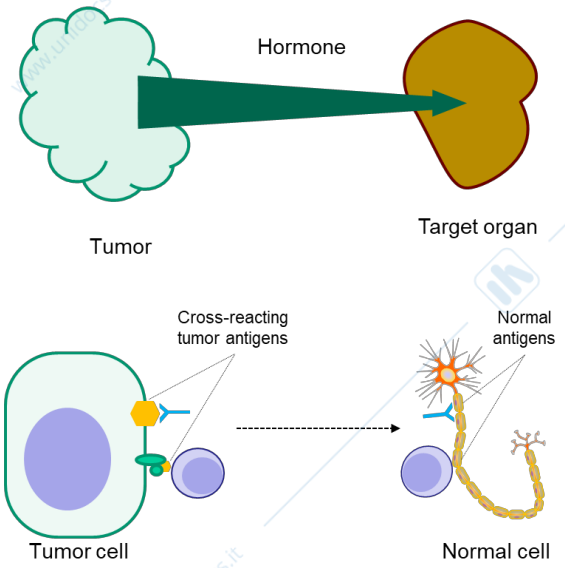
Chapter 13. Paraneoplastic Syndromes and Cachexia

Paraneoplastic syndromes

Paraneoplastic syndromes (PNS) are pathological conditions not directly caused by local tumor growth or metastasis. Two major causes are recognized, the hyper-activation of physiological functions by hormones and cytokines released by tumors, and autoimmune responses elicited by cross-reacting antigens expressed by tumor cells (Figure 13.1).

Paraneoplastic syndromes affect ~10% of all cancer patients, especially advanced patients with a large tumor burden. Their morbidity is widely variable, from asymptomatic to lethal. Therapies leading tumor to regression may resolve endocrine PNS, while autoimmune PNS may persist in the absence of tumor.

Figure 13.1. Hormones released by tumor cells and cross-reacting immune responses cause paraneoplastic syndromes.



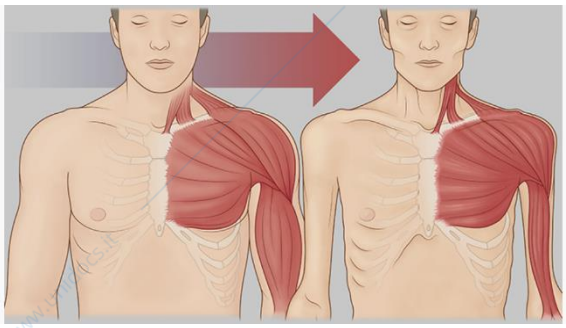


Figure 13.2. Cancer cachexia. From Ohio State University Comprehensive Cancer Center https://cancer.osu.edu/research-and-education/clinical-research/cancer-cachexia

The main types of PNS are endocrine, hematologic, gastrointestinal, neurologic, dermatologic and nephrological. Some notable examples are described in Table 13.1.

Cancer cachexia

Cachexia is a multiorgan, multifactorial syndrome characterized by ongoing loss of muscle tissue (Figure 13.2). Cachexia affects more than one-third of all cancer patients, causing 10%-20% of all cancer deaths.

Reduced food intake (anorexia) is one cause of cachexia, however cachexia cannot be fully reversed by optimal nutritional measures, hence other mechanisms must be at work. Molecular studies (Figure 13.3) highlighted the involvement of:

- Degradation and/or reduced synthesis of muscle proteins, mediated by:
  - Impairment of the IGF axis

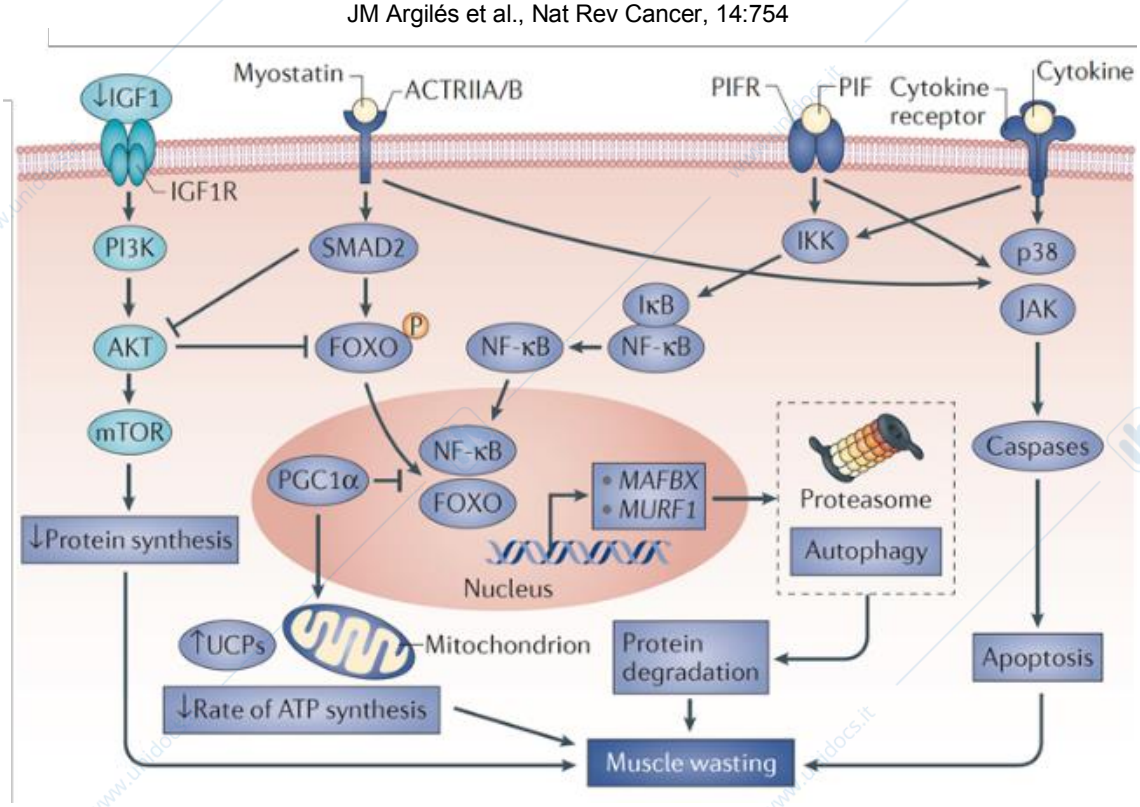
Table 13.1. Some paraneoplastic syndromes

Syndrome*	Mechanisms	Associated to	Notes
Granulocytosis	Colony-stimulating factors (CSF), IL-3, IL-5	Many tumor types	15% of all cancer patients. Induction of myeloid-derived suppressor cells (MDSC)
Hypercalcemia	Parathyroid hormone-related protein (PTHrP)	Breast, lung, kidney carcinomas, lymphomas, myeloma	10% of all cancer patients. Poor prognosis
Hypercortisolism (Cushing syndrome)	Adrenocorticotropic hormone (ACTH) or corticotropin releasing factor	Neuroendocrine lung tumors	
Thrombocytosis	IL-6	Lung carcinoma	Asymptomatic
Cerebellar ataxia	Onconeural antibodies	Small cell lung carcinoma, myeloma, lymphomas	
Myasthenia gravis	Anti-acetylcholine receptor autoantibodies	Thymoma	
Pure red cell aplasia	Autoreactive T cells	Thymoma, hematological neoplasms	

**Paraneoplastic and non-paraneoplastic forms exist for each syndrome.*

Figure 13.3. Molecular mechanisms in cancer cachexia.

JM Argilés et al., Nat Rev Cancer, 14:754



  - Myostatin, a cytokine of the TGF-β family
  - TWEAK, a cytokine of the TNF family
  - PIF, proteolysis inducing factor (documented in mouse models, but the existence of a human counterpart is disputed)
- Inflammatory cytokines, in particular IL-1β, IL-6, TNF-α
- Energy wasting, through uncoupling proteins (UCP).

There is no single approved agent or therapeutic protocol for cancer cachexia. It is usually approached with a combination of nutritional support; anabolic and orexigenic (i.e. appetite stimulant) drugs, including synthetic progestins, androgens, ghrelin receptor agonists and cannabinoids; anti-inflammatory drugs, such as NSAIDS, corticosteroids, biological cytokine inhibitors and thalidomide.

Chapter 14. Metastasis

Metastatic spread is the process whereby a localized primary tumor gives rise to distant secondary tumors, called metastases (sometimes "mets" in medical jargon). It can be considered the terminal part of tumor progression towards malignancy.

Metastases cause about 90% of all cancer deaths. At the moment of diagnosis, one-third of cancer patients are metastasis-free, one-third have occult micrometastases and one-third have overt metastases. Metastasis-free patients are cured by the surgical removal of their primary tumor. Patients with overt metastases have the worst prognosis, even after treatment with the most effective combinations of surgery, radiotherapy and pharmacological therapies. The survival of patients with occult micrometastases, after the removal of the primary tumor, will ultimately depend on the efficacy of treatments (adjuvant therapy, see chapter on cancer therapies) aimed at preventing the outgrowth of metastases, which can occur months to years later.

Metastatic spread is a sequential process that starts in the localized primary tumor and ends with the growth of secondary nodules in distant organs (Figure 14.1). It is intrinsically more complex than tumor growth, because metastatic cells exploit and subvert a variety of biological systems, in addition to cell proliferation.

Metastasis begins in the local tumor

Patients bearing the same type of malignant primary tumor may have widely different risks of metastasis development, therefore a personalized prediction of metastasis risk, based on primary tumor

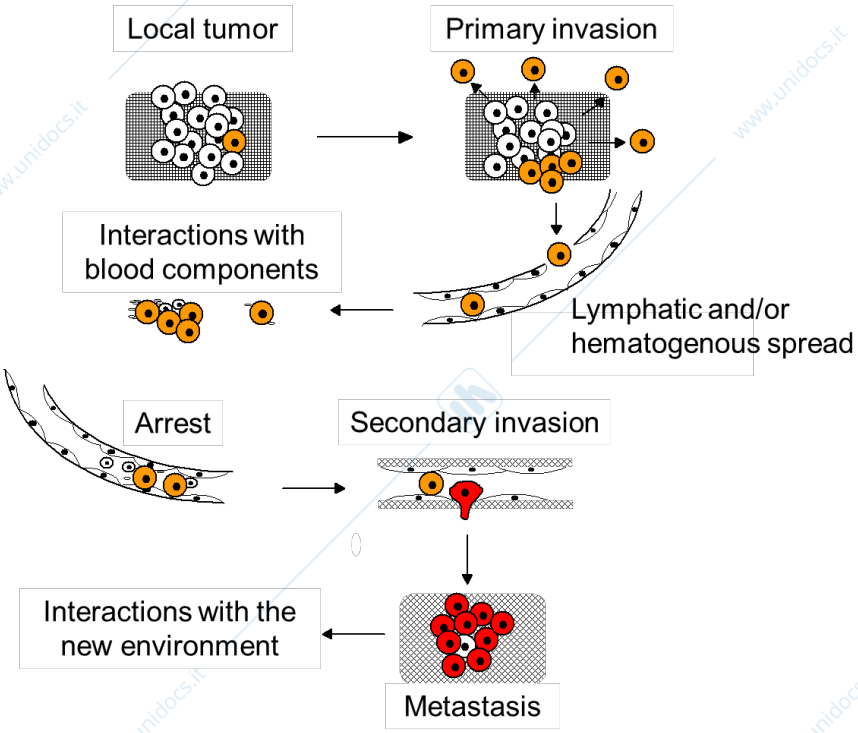


Figure 14.1. The metastatic cascade.

features would be of paramount clinical import.

Time (tumor age) and size (=cell proliferation) roughly correlate with metastasis risk. The relationships between tumor cell proliferation and metastatic spread are complex. Some cells physiologically express features of the metastatic process, e.g. invasiveness and motility: leukocytes, osteoblasts, endothelial cells, fibroblasts, melanocytes, embryonic tissues and trophoblast. Uncontrolled proliferation of these cells is sufficient to unleash a malignant tumor. Furthermore, some proliferation-related cancer genes also control various phenotypes related to the metastatic process, e.g. APC / β-catenin / E-cadherin; p53; MET; SRC; etc. Tumors with alterations in these genes have an intrinsic propensity to metastasize.

Different conceptual models were proposed over the years to reconstruct the natural history and the dynamics of metastatic cell subpopulations, however human tumors do not seem to follow a single unified model. Some tumors have gene profiles predisposing to early metastatic spread, others need to randomly accumulate additional hits, leading to late spread by selected cells.

With the advent of gene expression profiling and next-generation DNA sequencing, a considerable attention was paid to the comparison of primary tumors differing in metastatic propensity. Many genes were found to be differentially expressed between metastatic and non-metastatic cells; prominent pathways include cell proliferation/cell growth and invasiveness. Gene expression signatures (i.e. sets of expression patterns) of primary tumors can predict the risk of metastasis development and are already in clinical use (e.g. in breast cancer) to evaluate prognosis and to make therapeutic decisions.

Invasion

Malignant tumor cells leave the primary tumor mass and invade the surrounding tissues. Local invasiveness correlates with metastatic propensity; thus it is one of the

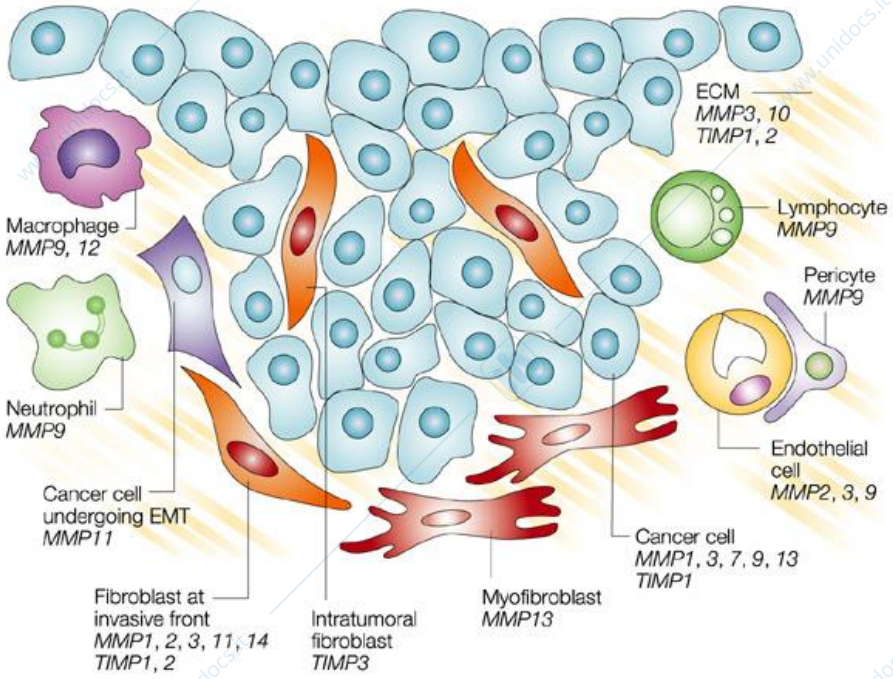


Figure 14.2. Normal cell infiltrate contributes to the balance of matrix metalloproteinases and their inhibitors within tumors. M Egeblad & Z Werb, Nature Reviews Cancer 2, 161.

properties of primary tumors that contribute to the evaluation of prognosis. Note that invasiveness *per se* can be lethal, as demonstrated by human glioblastomas, which only grow within the brain, and recidivating head & neck tumors.

The main cellular and molecular determinants of the invasive phenotype (especially in carcinomas) are:

- Loss of polarity, epithelial-mesenchymal transition
- Loss of homotypic adhesion molecules (e.g. cadherins)
- Loss of adhesion to the basement membrane (integrins)
- Basement membrane remodeling and degradation (metalloproteinases and their inhibitors)
- Interactions with stromal components (integrins, degradation enzymes)
- Cell motility (growth factors, chemokines, matrix)
- Presence of naturally invasive normal cells (e.g. macrophages, fibroblasts, endothelial cells) that can contribute to tumor cell invasiveness

Matrix metalloproteinases

Metalloproteinases are a class of more than 20 zinc-dependent proteolytic enzymes involved in the physiological turnover of the extracellular matrix. Some matrix metalloproteinases (MMP) involved in invasion and metastasis (MMP2, MMP9) degrade basement membrane components, such as type IV collagen ("type IV collagenase"). Within a tumor, the net degrading activity results from the balance between MMPs and their physiological inhibitors (TIMPs), produced both by tumor and by normal cells (Figure 14.2). MMPs are also involved in the activation of growth stimulatory (IGF) and inhibitory (TGF-β) pathways. Other lytic enzymes, released by neoplastic or normal cells, also contribute to invasiveness, e.g. cathepsins, plasminogen activators, endoglycosidases, etc.

Motility of invasive tumor cells

To leave the local tumor, motile invading cells follow different stimuli (Figure 14.3):

- Chemotaxis, i.e. chemical gradients of growth factors (PDGF, IGFs, insulin), or soluble matrix components (laminin, fibronectin).
- Haptotaxis, i.e. movement on solid guides, e.g. fibers: laminin, fibronectin, type IV collagen.
- Chemokinesis, i.e. random brownian motion induced by motogenic factors, such as the scatter factor (also known as hepatocyte growth factor, HGF) or the autocrine motility factor (AMF).

Extracellular matrix degradation contributes to cell motility through the release of soluble matrix components and of matrix-bound growth factors, and the remodeling of the fibrous meshwork.

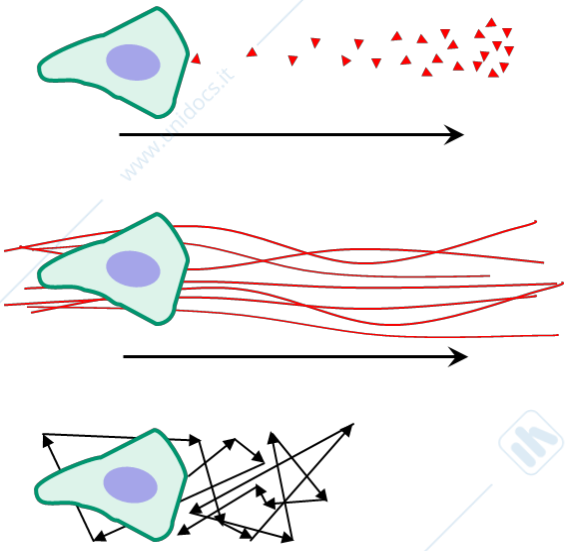
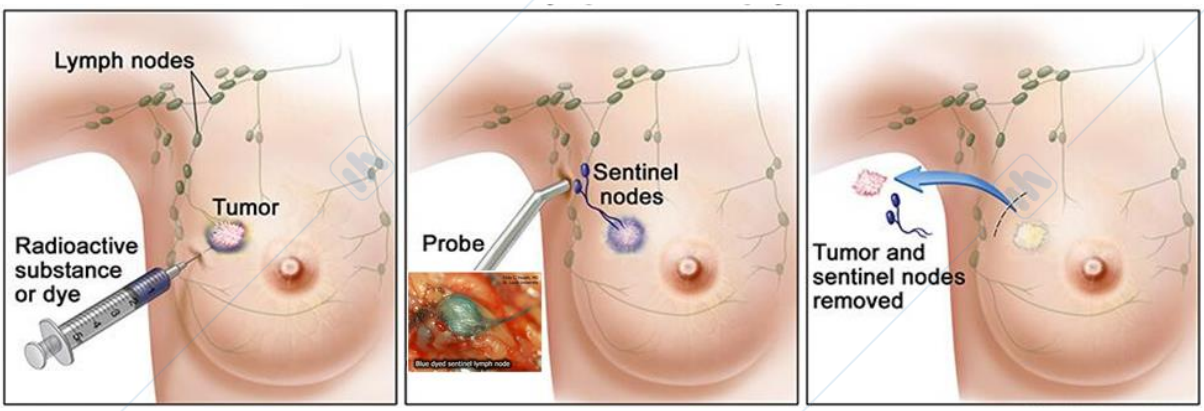


Figure 14.3. Cell motility: chemotaxis (*top*), haptotaxis (*middle*), chemokinesis (*bottom*).

Figure 14.4. Sentinel lymph node biopsy. Drawings are ©2010 Terese Winslow. U.S. Govt. has certain rights. Lymph node image: Eddy C. Hsueh, MD, St. Louis University.



Invasiveness: Clinical implications

Tumor invasiveness has important clinical implications. The future risk of distant metastases must be estimated from local parameters of the primary tumor, hence the pathological evaluation of invasiveness is an important risk factor. Furthermore, the use of conservative surgery for invasive tumors increases the risk of local relapse, because tumor cells that have infiltrated the surrounding tissues can avoid surgical removal, and would subsequently find an ideal environment in wound repair. To reduce the risk of local relapse, conservative surgery is combined with prophylactic irradiation of the surgical field.

Routes of metastatic dissemination

Metastatic spread can take different routes:

- Lymphatic – Many types of carcinoma first colonize draining lymph nodes, then disseminate systemically
- Hematogenous – Sarcomas and some carcinomas tend to spread directly via the blood vessels
- Cavity – Ovarian tumors produce metastatic nodules on peritoneal surfaces
- Contact – Direct surface contact, e.g. lower lip to upper lip

Lymph node metastases

The presence of lymph node metastases at diagnosis ("positive lymph nodes", "N+") provides a strong indication that the tumor has metastatic potential. Positive lymph nodes are a fundamental prognostic indicator that guides therapeutic choices.

In breast cancer, the removal of axillary lymph nodes, which causes long-term arm lymphedema, has been replaced by the sentinel lymph node biopsy (Figure 14.4), i.e. the pathological examination of only the tumor-draining lymph node, pinpointed through the injection of tracers within the tumor prior to surgery. The success of sentinel lymph node in breast cancer fostered the use of similar systems in other tumors, for example in melanoma.

Circulating tumor cells

Tumor cells that gain access to the bloodstream and to systemic circulation are exposed to a variety of interactions that determine their survival. On the whole, the metastatic process is extremely inefficient: billions of disseminating cells die to produce a few metastatic nodules. Even in highly selected experimental models, ratios are >1000:1. A major bottleneck is

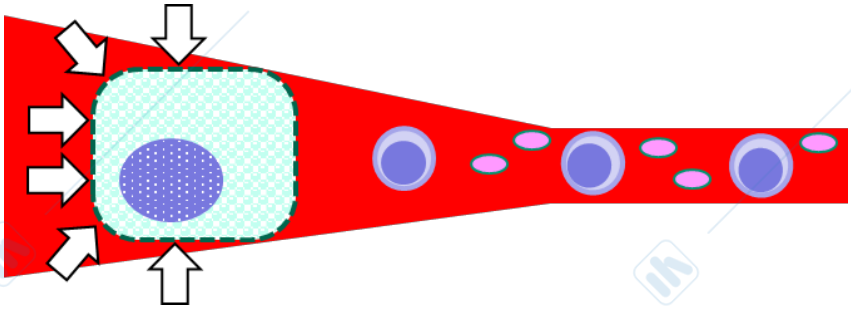


Figure 14.5. Cells from solid tumors can get stuck within capillaries and burst under the hydrostatic pressure of blood.

blood circulation itself, because cells from solid tumors are not adapted to survive within blood vessels for more than a few hours. Circulating tumor cells also find interactions that facilitate their dissemination.

Physical factors are a major determinant of the destruction of circulating tumor cells: capillaries are tailored for the passage of leukocytes, but cells coming from solid tumors have diameters that are twice that of leukocytes, furthermore the stiffness of tumor cell membranes is even higher than that of normal cells; tumor cells get stuck within capillaries, and burst under the hydrostatic pressure of blood (Figure 14.5). Furthermore, circulating tumor cells have a limited capacity to survive without substrate anchorage (anoikis), and are actively killed by NK cells.

Single cells are fragile, but the formation of cell aggregates (emboli), also with leukocytes, or blood clots (thrombi) protects circulating tumor cells from the dangers outlined above and increase their metastatic efficiency (Figure 14.6). The fact that neoplastic emboli and thrombi mainly provide mechanical protection is demonstrated by the fact that even aggregates consisting mainly of dead cells are more metastatic than individual tumor cells.

After surgical removal of the primary tumor, circulating tumor cells (CTC) become rare, but can be purified from the blood of patients using tissue specific (e.g. epithelial) markers. CTCs are an important source of biomaterials (cells, nucleic acids, etc.) for experimental studies, and attempts are being made to develop CTCs into prognostic and predictive biomarkers, and to monitor the response to anti-metastatic therapies. Analogous attempts are being made to extract information from circulating extra-cellular vesicles, proteins or nucleic acids (liquid biosy, see Box 20.2).

Arrest and extravasation

Tumor cells circulate throughout the body following blood flow. Most cells arrest in the capillary beds of "filter" organs, e.g. lungs, liver, bones; circulating cells can also re-colonize the primary tumor (self-seeding).

To exit from blood vessels, some tumors cells exploit the extravasation mechanisms of leukocytes. In some cases, it was found that cells stuck within a blood vessel can re-start proliferation without even existing the circulation.

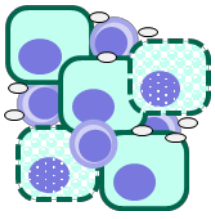


Figure 14.6. Circulating tumor cells in aggregates are protected from mechanical insults.

Metastatic patterns

Some anatomical sites, for example the lungs, are subject to the metastatic colonization by many tumor types, but each tumor type preferentially metastasizes to specific organs, for example colorectal cancer to the liver (Figure 14.7), breast and prostate cancer to the bones, melanoma to the skin. Such organ preferences are not fully accounted for by vessel anatomy or hemodynamics. The *Seed and Soil* theory (Paget, 1889) compared disseminating tumor cells to seeds that sprout only in the right soil, i.e. the right organ microenvironment.

Factors released by the primary tumor (e.g. VEGF) modify the microenvironment of specific distant organs, preparing the soil (pre-metastatic niches) for incoming metastatic cells. Macroscopic metastases will grow from those disseminated cells that find an appropriate soil, i.e. environmental conditions (growth factors, extracellular matrix, angiogenesis, niches, etc.) in the new organ.

Dormancy

The growth of most macroscopic metastases takes from months to a few years, hence the conventional threshold of 5 years to evaluate the survival of patients in most tumor types. In some cases, mainly in breast cancer and melanoma, micrometastases can remain dormant for decades. In these tumors, the awakening of dormant micrometastases is a relevant clinical problem, because it reduces long-term patient survival by 5%-20%.

The sheer duration of tumor dormancy makes it difficult to precisely define the underlying cellular and molecular mechanisms. The main hypotheses concern:

- Replicative cell kinetics, if cell proliferation equals cell death
- Lack of neovascularization, or of other conditions that make the niche fit for metastatic cell growth
- Immunological containment, without complete destruction of the metastatic deposit

Each hypothesis entails different awakening mechanisms. If dormancy is purely based on cell kinetics, then mutations that

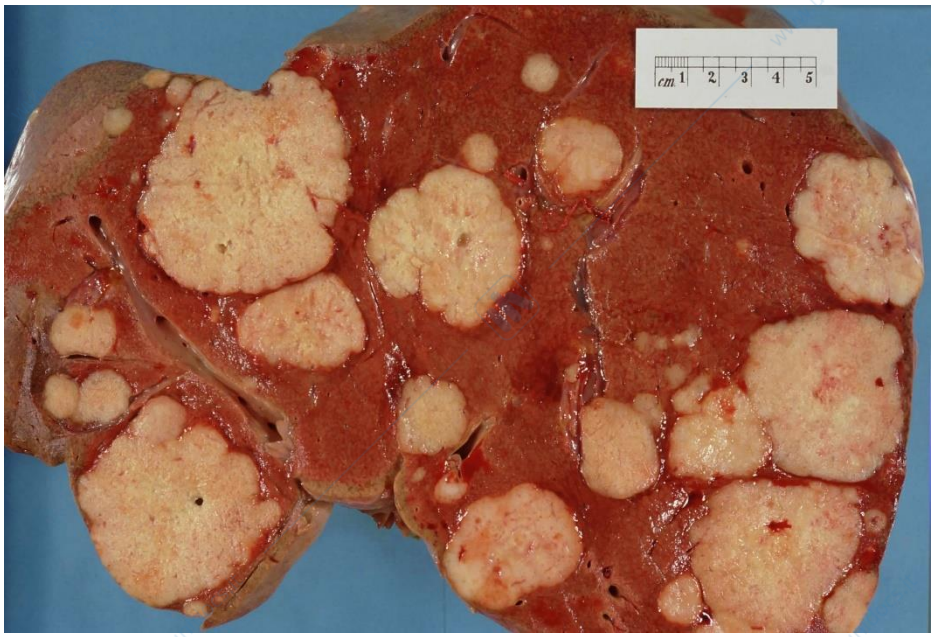


Figure 14.7. Large liver metastases. From Wikipedia, by Hymanj, Public domain.

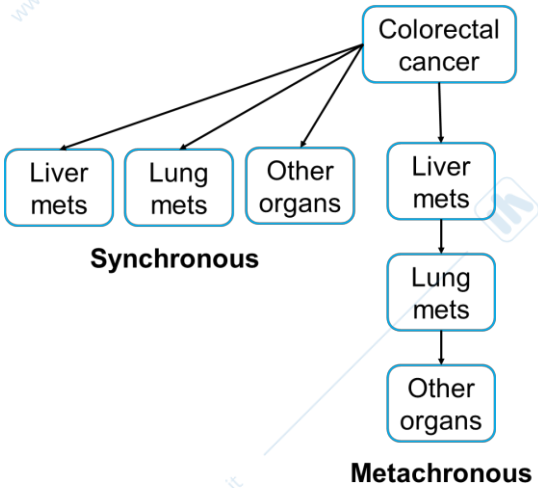
increase cell proliferation, or inhibit apoptosis, can be sufficient to tip the scales in favor of metastasis growth. Similarly, genetically unstable tumor cells can acquire the ability to release paracrine growth factors, such as angiogenic factors, needed to make the niche more hospitable. If the metastasis is contained by the immune response, then the progressive reduction of immune defenses that accompanies human aging, or a temporary immune suppression, can be enough to unleash metastasis growth; alterations in tumor antigen expression or presentations (e.g. MHC loss) can alco be involved.

Metastasis begets metastasis

Secondary metastases, deriving from a primary tumor, can give rise to tertiary metastases in further organs, and so on.

When metastases are found in different organs, an important clinical issue is whether all came in parallel from the primary tumor (synchronous), or in cascade from one another (metachronous) (Figure 14.8). In colorectal cancer, data favor metachronous dissemination, thus supporting the concept that surgical removal of early liver metastases could interrupt the metastatic cascade. Therapeutic excision (metastasectomy) is practiced also for lung nodules.

Figure 14.8. Synchronous *vs.* metachronous metastatic spread.



Development of anti-metastatic therapies

Most pharmacological "cancer therapies" actually target metastases, not local tumors, which are removed surgically. The advice for innovators seeking to invent novel therapies is to think in terms of anti-metastatic therapies, because the need for treatments of primary lesions is mostly limited to brain or head and neck tumors.

The sequential nature of metastatic dissemination indicates that it would be sufficient to block a single step to interrupt the whole process, and the complexity of the process offers a bonanza of potential therapeutic targets beyond cell proliferation and mitogenic signaling, which is still unexploited.

Chapter 15. Fundamentals of Carcinogenesis

The process leading to the onset of a tumor is called carcinogenesis, the causative agents are defined as carcinogens. The discovery of carcinogens and the definition of carcinogenic mechanisms is the basis of cancer prevention. The guiding principle is "if you know them, you can avoid them".

Carcinogens include physical agents, inorganic and organic chemicals, biological agents.

The first study of occupational chemical carcinogenesis was made in 1775 on chimney sweeps by Percival Pott, who pointed to the carcinogenic activity of soot and other combustion by-products.

Extensive studies in the last century discovered strong carcinogens, which now can be banned by law from the workplace or avoided with appropriate lifestyles. Nowadays, open scientific issues are mainly related to weak carcinogens, which can have a significant social impact, if the number of exposed people is high. However, when carcinogenicity is near zero, scientific evidence can be weak or contradictory, which is why deciding whether some substances are indeed carcinogenic can be controversial.

Mechanisms of carcinogenesis

Carcinogenic activity depends on the combination of a limited number of general mechanisms. We will focus here on those mechanisms that are common to all types of carcinogenic agents; the higher complexity of biological carcinogens entails further mechanisms, which will be examined in a subsequent chapter.

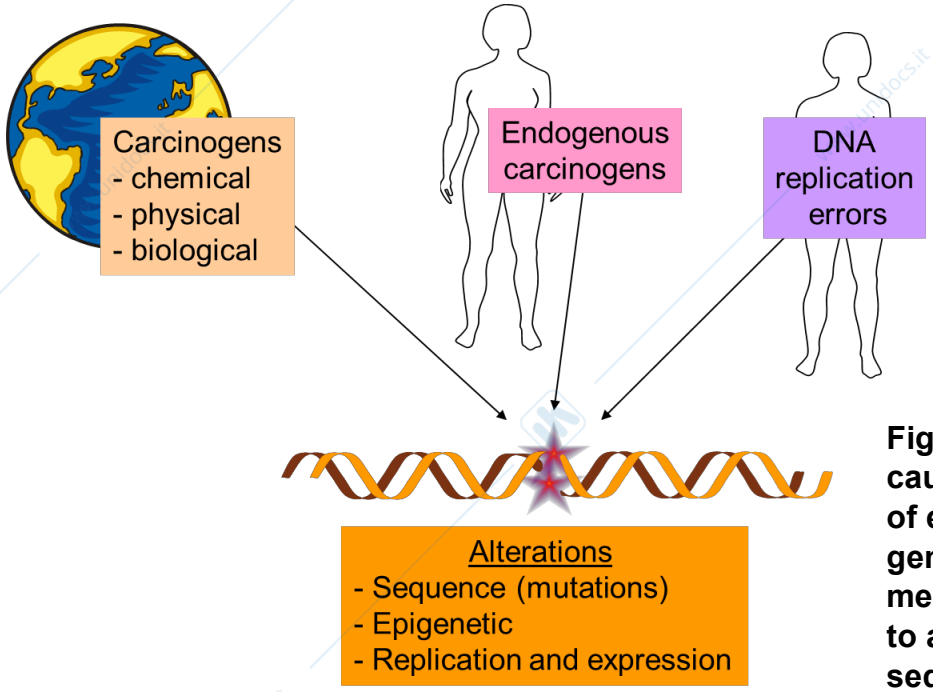


Figure 15.1. Cancer is caused by a combination of exogenous carcinogens and endogenous mechanisms, leading to alterations in DNA sequence and/or expression.

Many physical, chemical and biological agents increase mutation frequency, i.e. are mutagens, causing widespread DNA alterations, which can lead to cancer when oncogenes or tumor suppressor genes are affected. All mutagens are carcinogens, but the opposite is not true, i.e. carcinogens include also non-mutagenic agents, which modify carcinogenic processes such as cell proliferation and inflammation.

We have already considered the risk of neoplastic transformation related to cell proliferation (see chapter on cancer genes). A logical extension is the fact that endogenous or exogenous substances that induce cell proliferation also increase the risk of cancer, i.e. are carcinogens.

Chronic tissue damage is a carcinogenic risk for two reasons, *a*) cell death elicits homeostatic cell proliferation, which we have just examined, and *b*) chronic inflammation cells, associated with tissue damage, release reactive oxygen species (ROS), which can damage DNA, i.e. are mutagenic.

We have seen that immune deficiencies increase cancer risk (see chapter on tumor immunology), it follows that agents that damage the immune system, hampering cancer immune surveillance, are carcinogens.

Biphasic carcinogenesis: initiation and promotion

Studies of experimental carcinogenesis, dating back to the first half of the 20th century, defined two major classes of carcinogens, initiators and promoters.

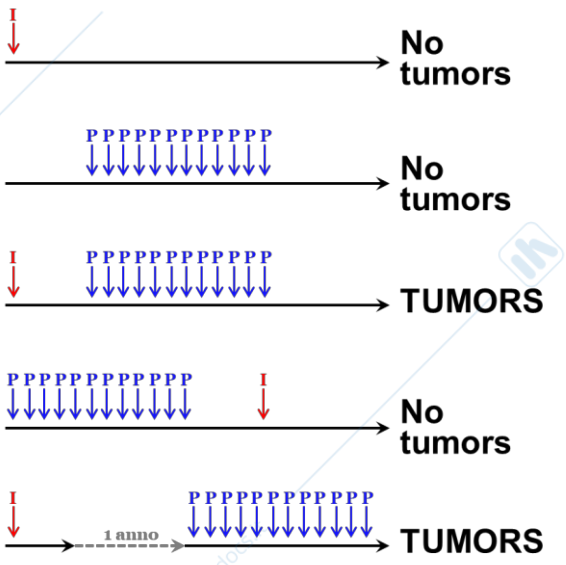
Isaac Berenblum and co-workers established the biphasic model of animal carcinogenesis, in which tumors were only produced by the sequential administration of one chemical, which they called an initiator, followed by repeated administrations of a second substance, called a promoter (Figure 15.2). At the dosages used in biphasic experiments, neither the initiator, nor the promoter alone caused tumors. The reverse treatment sequence, i.e. promoter before initiator also failed to induce tumors, furthermore, the effect of the initiator was permanent, because tumors arose even if treatment with the promoter was delayed by one year (which represents one-third to one-half of the lifespan of experimental animals).

To make a long story short, initiators can be identified with mutagenic carcinogens, and promoters with non-mutagenic carcinogens. The biphasic paradigm is still useful today for the analysis and categorization of carcinogens, and will be used in this book.

Direct and indirect carcinogens

Only a few carcinogens directly alter DNA, i.e. are direct carcinogens. In most cases, chemical carcinogenesis is caused

Figure 15.2. Biphasic carcinogenesis.
I=initiator, P=promoter. From Weinberg, The Biology of Cancer, Garland Science



by metabolites produced by enzymatic systems of the host (metabolic activation of indirect carcinogens).

All living organisms have enzymes that detoxify and excrete foreign compounds (xenobiotics). However, some enzymatic reactions yield toxic or carcinogenic compounds. Most reactions are carried out in the liver; kidneys, lungs and skin are also involved in metabolic activation.

Metabolic activation can be divided in two phases. **Phase I** reactions increase the polarity of water-insoluble xenobiotics through "non-synthetic" reactions, such as oxidoreductions or, cyclization / decyclization, yielding metabolically active products. Main phase I enzymes are P450 cytochromes, alcohol / aldehyde dehydrogenases, monoamine oxidases, esterases, epoxide hydrolases. **Phase II** reactions, using polar groups generated by phase I, conjugate xenobiotics to glutathione, amino acids, glucuronic acid, sulphonates. The end products are usually metabolically inactive and are excreted. Main phase II enzymes are glutathione S-transferase, sulfotransferase, N-acetyltransferase, UDP-glucuronosyltransferase.

The involvement of enzymes introduces various levels of control, which can determine marked individual differences in the sensitivity to carcinogens. Firstly, some enzymatic systems are inducible, determining different levels of activity in individuals subject to continuing vs. intermittent exposure to the carcinogen; secondly, enzyme activity can vary according to sex, age and health; thirdly, in the population there are polymorphisms determining variable levels of enzyme activity.

A prime example is ethanol metabolism, which includes some enzymes that are inducible (cytochromes) and others (dehydrogenases) expressed at different levels in women and men, children and adults; furthermore, alcohol-induced pathologies, such as liver degenerations, alter the amount of healthy tissue involved in the metabolism of alcohol itself.

Many genetic polymorphisms are known to control the metabolism of carcinogens. A classic example was discovered in the pharmacogenetics of the antituberculosis drug isoniazid. Some patients had a good clinical response to the drug, but were frequently subject to toxic side effects (neuropathies), others had a poor response and rare side effects. The difference was found in the activity of N-acetyltransferases (NAT1 and NAT2) that inactivate isoniazid: responders had low enzyme levels (slow acetylators), allowing a longer half-life of the drug, in contrast non responders had high enzyme levels (fast acetylators) that rapidly inactivated the drug. As it frequently happens with polymorphisms, there are strong ethnic differences, for example Asians are mostly (50%-90%) slow acetylators, Caucasians are mostly fast acetylators (80%-95%). The oncological interest of this story is that the same enzymes are also involved in the metabolism of carcinogens, for example some of those contained in tobacco smoke, thus slow acetylators are more susceptible then fast acetylators to chemical carcinogenesis.

Several other enzyme polymorphisms are known to control the activation or deactivation of carcinogens; hence each human being has an individual profile of sensitivity to chemical carcinogenesis. The issue is further complicated by the discovery of polymorphisms influencing carcinogenic lifestyles, such as tobacco smoke. It is well known that some

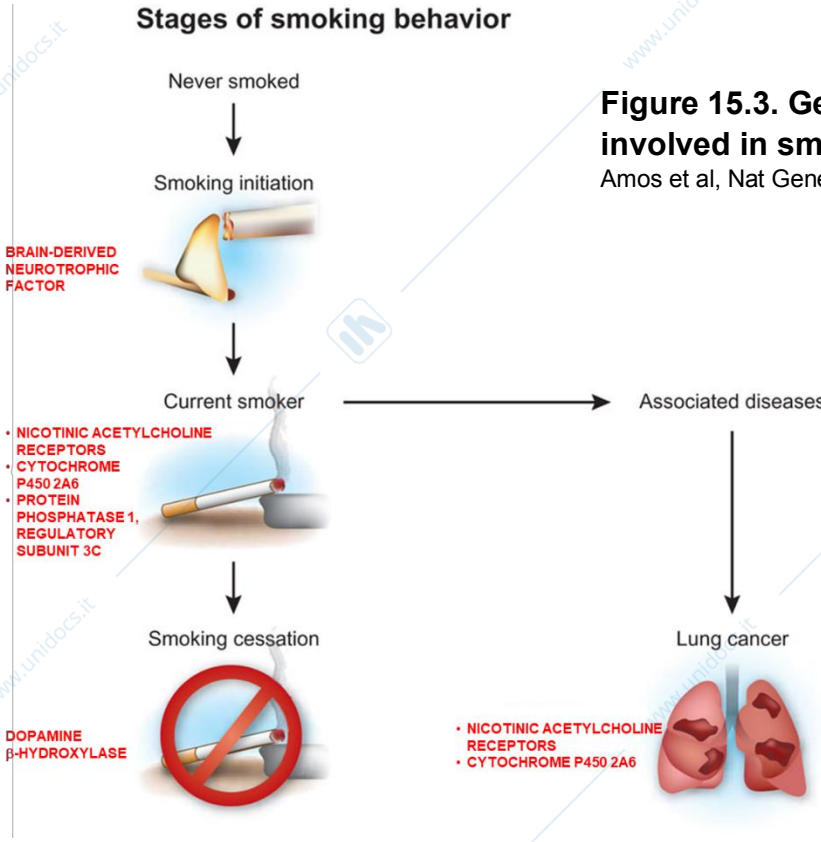


Figure 15.3. Genetic polymorphisms involved in smoking behavior. From Amos et al, Nat Genet 42: 366

smokers can easily quit, whereas others try repeatedly without success. It was found that polymorphic neuromodulators, expressed not in the lungs, but in the brain, affect the individual propensity to take up or give up smoking (Figure 15.3).

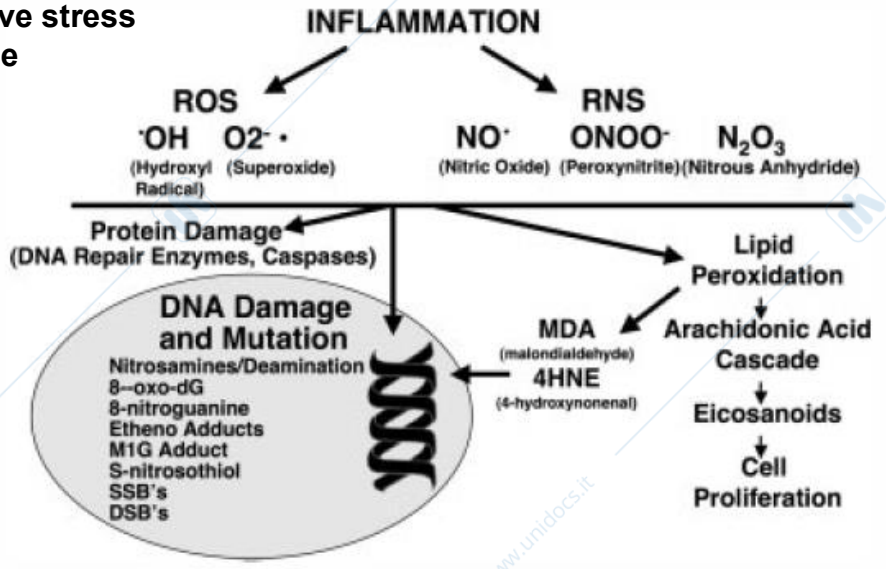
In the future, such complex phenotypes might be used to individually tailor cancer preventative programs, for example through the intensification of psychological counseling and other measures in smokers with unfavorable polymorphisms.

Oxidative stress

Oxidative stress is involved in many types of carcinogenesis because reactive oxygen and nitrogen species (ROS and NOS) are mutagenic, either directly or indirectly.

Inflammation and phagocytes are the major sources of ROS and NOS, which are

Figure 15.4. Oxidative stress causes DNA damage and mutation.
From Perwez Husssain & Harris, Int. J. Cancer, 121: 2373.



harmless as long as they remain within phagocytes, but can damage surrounding tissues if phagocytes die, which is a frequent event in chronic inflammation (Figure 15.4). The importance of inflammation-related oxidative stress should not obscure the fact that the enzymatic reactions that generate ROS are not exclusive of phagocytes, and many others oxidoreductive enzymes are active in all tissues throughout the body.

Definition of carcinogens

The final aim of all the carcinogenesis studies is to unequivocally determine whether a given agent is a carcinogen or not, and to protect the society if it is.

The definition of carcinogens is made by national and international agencies that evaluate all the available scientific evidence, which includes

- Intrinsic properties (molecular structure, etc.)
- *In vitro assays*
  - Bacterial mutagenesis (Ames test in *Salmonella*)
  - Mutagenesis in eukaryotic cells
  - In vitro transformation
- *In vivo* assays
  - Short term
  - Long term / lifetime
- Human exposure (missing for novel agents)
  - Case-control studies
  - Cohort studies

The general properties of carcinogens were summarized in a recent review (MT Smith *et al.*, Environ. Health Persp. 124: 713):

- Electrophilic molecules, directly or after metabolic activation
- Genotoxicity
- Alteration of DNA repair or induction of genomic instability
- Induction of epigenetic alterations
- Induction of oxidative stress
- Induction of chronic inflammation
- Immunosuppressive activity
- Modulation of receptor activity
- Immortalization of normal cells
- Alterations of cell proliferation, cell death or nutrient supply

Some of these properties can be gleaned from structural features of the suspect molecules, others require *in vitro* experiments or *in vivo* studies in animal models.

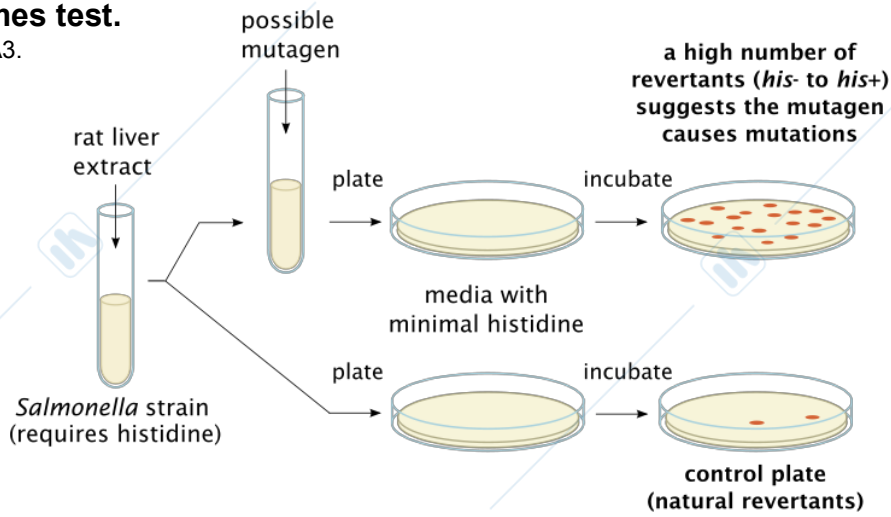
***In vitro* mutagenicity assays**

Exposure of prokaryotic or eukaryotic cells to a suspect carcinogen can measure its mutagenic activity, if the substance is directly active; if it needs metabolic activation, the standard way to provide activating enzymes is through the addition of liver extracts, typically the supernatant obtained after centrifugation at 9000 *g*, called the S9 fraction.

Bruce Ames gave his name to a popular mutagenicity assay using auxotrophic *Salmonella* mutants (Figure 15.5), that can only give rise to colonies in selective media if retro-mutations occur, hence the number of colonies in the presence of the test substance (with or without S9) is proportional to its mutagenicity. For mutagenic carcinogens, the Ames test predicts with good approximation the results of *in vivo* carcinogenicity assays.

A variety of *in vitro* assays can be performed using eukaryotic cells, with or without metabolic activation: mutagenesis assays are conceptually similar to the Ames test, other assays measure nuclear, chromosomal or DNA damages inflicted by the test substance (micronucleus test, sister chromatid exchange, Comet assay); the induction of DNA repair, as a consequence of DNA damage, is evaluated by

Figure 15.5. The Ames test.
From Wikipedia, CC-BY-SA3.



the measure of DNA synthesis in the absence of DNA replication (so-called unscheduled DNA synthesis, UDS); finally, some immortal cell lines (e.g. 3T3) grow in flat, contact-inhibited monolayers, but further oncogenic mutations produce three-dimensional "transformation foci" in which cells lose contact inhibition and grow on top one another.

***In vivo* carcinogenesis assays**

Long-term *in vivo* assays, which can recapitulate the whole process of human carcinogenesis, are the gold standard of carcinogenesis studies. Two different animal species, typically mice and rats, are required to avoid species-specific phenomena. Animal exposure should closely mimic human exposure, which requires some creative and expensive solutions to approximate some human behaviors, think for example cigarette smoke or the use of cell phones. Animal are chronically exposed to the test agent; standard tests last two years, but lifetime studies are also performed (life expectancy of mice and rats is between two and three years). Tumor incidence is carefully recorded throughout the study, and all animals undergo a thorough necropsy, followed by the pathological study of samples taken from all organs and tissues, in search of microscopic tumors. Long-term assays are expensive and use large numbers of animals, for example the test of one compound at 3 doses requires 600 rodents and costs from hundreds of thousands to several millions of Euros, depending on the complexity of the exposure system.

To reduce the duration and costs of *in vivo* assays, several short or medium term assays have been set forth, based on the appearance of lesions that are known to correlate with tumorigenicity (e.g. mutations, chromosomal aberrations, micronuclei, UDS), or using animals with accelerated carcinogenesis (e.g. p53 mutant mice), however none of these tests has effectively replaced the standard long-term assay.

Critical issues in carcinogenesis testing

All experimental systems have shortcomings and critical issues that must be thoroughly understood to avoid mistakes in experimental design and misinterpretations of the results.

All carcinogens are also toxic. As dead cells cannot give rise to tumors, it is obvious that carcinogenicity *in vitro* and *in vivo*

must be investigated using dosages of the test compound that are not (excessively) toxic, otherwise carcinogenicity would be masked by toxicity.

Genotoxic carcinogens are easy, non-genotoxic ones less so. A genotoxic agent would test positive in most, if not all, the *in vitro* and *in vivo* tests described earlier. Non-genotoxic agents are missed by standard *in vitro* assays and can require complex *in vivo* models (e.g. biphasic systems) to reveal their actual carcinogenic potential.

Species differences can be somewhat obviated *in vitro*, for example using human cells or human S9, but *in vivo* assays will be always done in non-human animal species. In-depth knowledge of species-specific peculiarities will at least allow a correct interpretation of the results.

Low-dose extrapolation is an unavoidable pitfall not only of experimental carcinogenesis, but of human studies as well, because the study of high dosages will always be required to detect sizeable effects in a comparatively short time (i.e. years, instead of decades), but general human populations will be exposed to much lower doses over longer time periods. A classic example that we will encounter in the next chapter is the estimate of the carcinogenicity of (very low dose) modern diagnostic radiology from the data of atomic bomb survivors. Diverging curves can be extrapolated from the same set of experimental points, for example a linear model without threshold will predict that any small dose of a carcinogen will cause a correspondingly small, but non-zero, increase in carcinogenicity, whereas a linear model with threshold will suggest that very small doses could be harmless, in contrast a supralinear model would

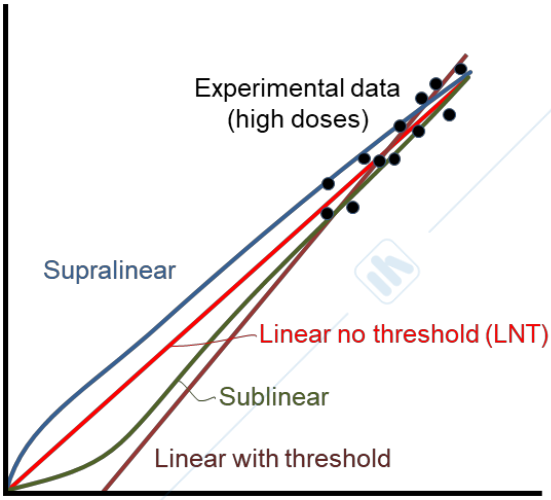


Figure 15.6. Extrapolation from high doses to low doses using different mathematical models.

predict a higher carcinogenicity at low doses than the linear models. There is no single answer, only careful analyses of all available data can provide useful extrapolations at low doses.

Cancer epidemiology

Epidemiology investigates the factors that influence health and disease in human populations. It is the basis of public health interventions and of preventive medicine. **Descriptive epidemiology** describes the distribution of diseases, e.g. by age, geography, history, etc.; many results of descriptive cancer epidemiology are scattered throughout the book, starting with the first chapter. Human carcinogens are the subject of **analytical epidemiology**, which investigates the relationships between risk factors and disease, e.g. diet and diabetes, smoking and cancer, etc., including molecular and genetic factors (molecular epidemiology). **Experimental epidemiology** designs and implements interventions to prevent or modify diseases through the modification of known risk factors.

Table 15.1. Measures of association

	Disease	Health
Exposed	a	b
Non-exposed	c	d

- **Incidence of disease**
  Exposed (IE) = Disease/Exposed = a/(a+b)
  Non-esposed (IN) = Disease/Non-esposed = c/(c+d)
  Population (IP) = (a+c)/(a+b+c+d)
- **Relative risk**, RR (cohort studies)
  Ratio of disease incidence between exposed and non-exposed = IE/IN
- **Population attributable risk** (PAR)
  Reduction in incidence in the absence of exposure = IP-IN
  Relative to exposeds only (AR) = IE-IN

- **Odds Ratio**, OR (case-control studies)
  Ratio of exposure odds in disease (a/c) and health (b/d) = ad/bc
  If the disease is rare, a+b→b, c+d→d, hence OR approximates RR

Odds
If the likelihood than an event will take place is *p* and the likelihood that it will not is *q* (with *p*+*q*=1), then the odds in favor of the event are *p*/*q*

The most straightforward way to study a suspect carcinogen is to compare cancer incidence (see Table 15.1 for definitions) between groups (cohorts, in epidemiological parlance) of exposed and non-exposed individuals, for example smokers and non-smokers. Prospective **cohort studies**, in which healthy individuals exposed or non-exposed to the risk factor are observed until the disease appears, ideally for the entire duration of their life, yield solid results, but take a very long time; in the next chapter we will discuss the prospective study of atomic bomb survivors, which started after World War II and is still ongoing, after more than 70 years.

A faster alternative is to reverse the order: while cohort studies start from exposure **case-control studies** start from the disease. A group of patients affected by a specific disease, along with a group of matched controls, are studied to obtain information about their past exposures to risk factors, for example by means of questionnaires. Case-control studies are faster and cheaper than cohort studies, but more subject to bias.

In most instances, even large epidemiological studies, comprising thousands of subjects, have insufficient statistical power to determine whether the observed results are significant or not; even worse, when the suspect carcinogen is very weak, different studies could yield contradicting results. **Meta-analyses** (Figure 15.7) are statistical combinations of multiple independent studies of the same subject, which can boost the statistical power and provide a solid ground for evidence-based medicine.

Epidemiological studies are affected by **random variability**, **confounding factors,** and **bias**. The standard way to minimize random errors is through the increase in the number of subjects; meta-analysis is just one way of doing that. Confounding is an interpretation error that occurs when some factor related to both exposure and disease is ignored. For example, chronic tobacco smoke is involved in a large

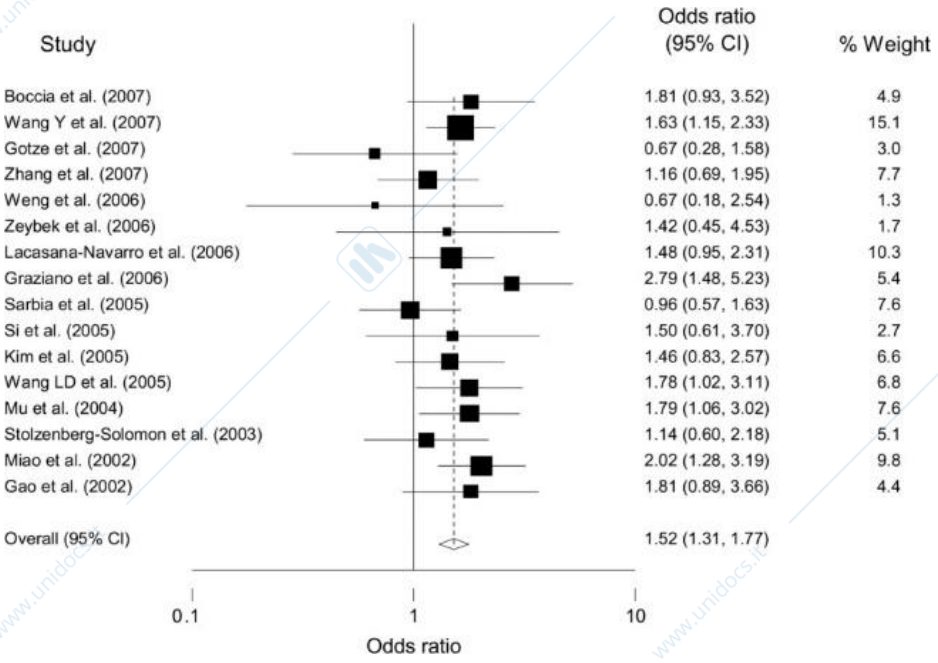


Figure 15.7. A *forest plot* showing the results of a meta-analysis. Each line represents one study, the size of each square is proportional to the number of patients in the study. The lozenge in the last line shows the combined result of the meta-analysis. From Boccia et al., Am. J. Epidemiol. 167: 505.

spectrum of diseases; even if the disease under study is thought to be unrelated to tobacco, it is unwise to pool together smokers and non-smokers. Modern epidemiological studies analyze dozens of potential confounding factors, including age, sex, income, body weight, tobacco smoke, alcohol consumption, blood pressure, diet, etc. Bias is a systematic error, which cannot be reduced through an increase in the number of subjects. Bias is a complex subject, to which epidemiology textbooks dedicate entire chapters, thus a comprehensive treatment is beyond the scope of this short paragraph. Classification bias can occur in all types of epidemiological study when systematic errors affect either the diagnosis or the assessment of exposure. Case-control studies can be affected by selection bias, if either cases or controls are not representative of the respective population, or by recall bias, because the onset of disease can alter in patients the perception and memory of exposure. The long duration of cohort studies entails the risk of attrition bias, that is a selective loss of subjects in the course of the study.

Classification of carcinogens

Once enough data has been collected, using many different assays, the overall evaluation of the carcinogenicity of a given agent is made by committees of experts. A pioneering organization is the International Agency for Research on Cancer (IARC), based in Lyon, France, now part of the World Health Organization (WHO), which has been carrying out and publishing (www.iarc.fr) its evaluations for more than 50 years. IARC evaluations have no normative value but are highly influential and are taken into consideration by all normative bodies. In many countries, governmental agencies provide evaluations that are used for legislative purposes. In general, the classification systems are comparable (Table 15.2), and the evaluations are homogeneous worldwide for what concerns strong carcinogens. Controversies can arise when weak

carcinogens are evaluated, because the margins of error of scientific data allow contradicting interpretations as to whether carcinogenicity is significantly different from zero. The presence of multiple agencies within large confederations, such as the European Union or the United States of America, sometimes leads to conflicting evaluations, with confusing consequences for public awareness and medico-legal decisions. For example, in the USA some substances are considered carcinogenic in California, but not in other States.

The IARC classifies the carcinogens in four groups, based on the strength of the available scientific evidence (Table 15.2); IARC classifications do not take into account carcinogenic potency: both strong and weak carcinogens can be grouped together, as long as the level of certainty is the same. **Group 1** includes agents classified as carcinogenic to humans based on unequivocal scientific evidence. **Group 2**, which includes agents with a lower degree of certainty, because of missing or contradictory scientific evidence, is

Table 15.2. Classification of carcinogens by different agencies.
NOTA BENE: The equivalence of different classifications is approximate.

IARC	EU	USA (NTP)*	ONU (GHS)
Group 1 – Carcinogenic to humans	Category 1A – Substances known to have carcinogenic potential for humans	Known to be human carcinogens	Category 1A – Known to have carcinogenic potential for humans (the placing of a substance in this category is largely based on human evidence)
Group 2A – Probably carcinogenic to humans	Category 1B – Substances presumed to have carcinogenic potential for humans	Reasonably anticipated to be human carcinogens	Category 1B – Presumed to have carcinogenic potential for humans (largely based on animal evidence)
Group 2B – Possibly carcinogenic to humans	Category 2 – Suspected human carcinogens		Category 2 – Suspected human carcinogen
Group 3 – Not classifiable as to its carcinogenicity to humans**			

*In the USA other agencies (EPA, NIOSH, California state, etc.) use different classifications.
**Group 4, "Probably not carcinogenic to humans", was merged with group 3 in 2019; past evaluations will remain in effect.

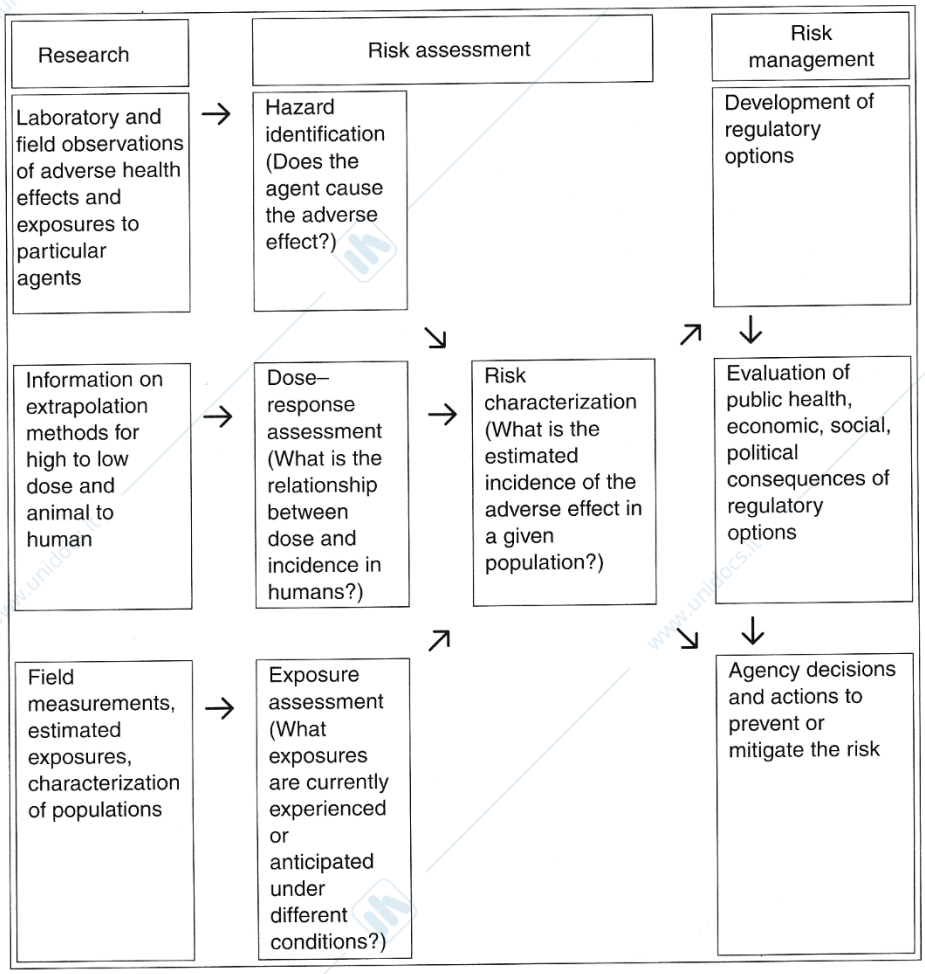


Table 15.3. Risk management and risk assessment.
From Warhawsky & Langdon, Molecular Carcinogenesis and the Molecular Biology of Human Cancer, Taylor & Francis.

further subdivided into **groups 2A** (probably carcinogenic to humans) and **2B** (possibly carcinogenic to humans), with a decreasing level of certainty. Agents not classifiable as to their carcinogenicity to humans are included in **group 3**; since 2019, group 3 includes those agents previously classified in group 4 as probably not carcinogenic to humans. Note that the IARC only takes into consideration suspected carcinogens, thus very few agents end up in group 3, as most evaluations lead to a group 1 or 2 classification.

Risk assessment and risk management

In a wider perspective, transcending the scope of this book, the classification of carcinogens is the first step (hazard identification) in the practice of carcinogenic risk assessment and management. In risk assessment (Table 15.3), hazard is defined as any source of potential damage, risk as the probability that a person will be harmed if exposed to a hazard. Quantitative carcinogenic risk assessment is the foundation of rational, evidence-based decisions and actions, as opposed to acritical applications of the so-called precautionary principle, which may lead to the abandonment of potentially useful products if the risks are misunderstood.

Chapter 16. Physical Carcinogenesis

The electromagnetic spectrum can be divided in three parts, from the point of view of interactions with living matter and carcinogenesis: ionizing radiation, which includes X-rays, gamma-rays and subatomic particles; solar and ultraviolet (UV) radiation; and so-called electromagnetic fields, which include the emissions of mains electricity and cell phones (Figure 16.1).

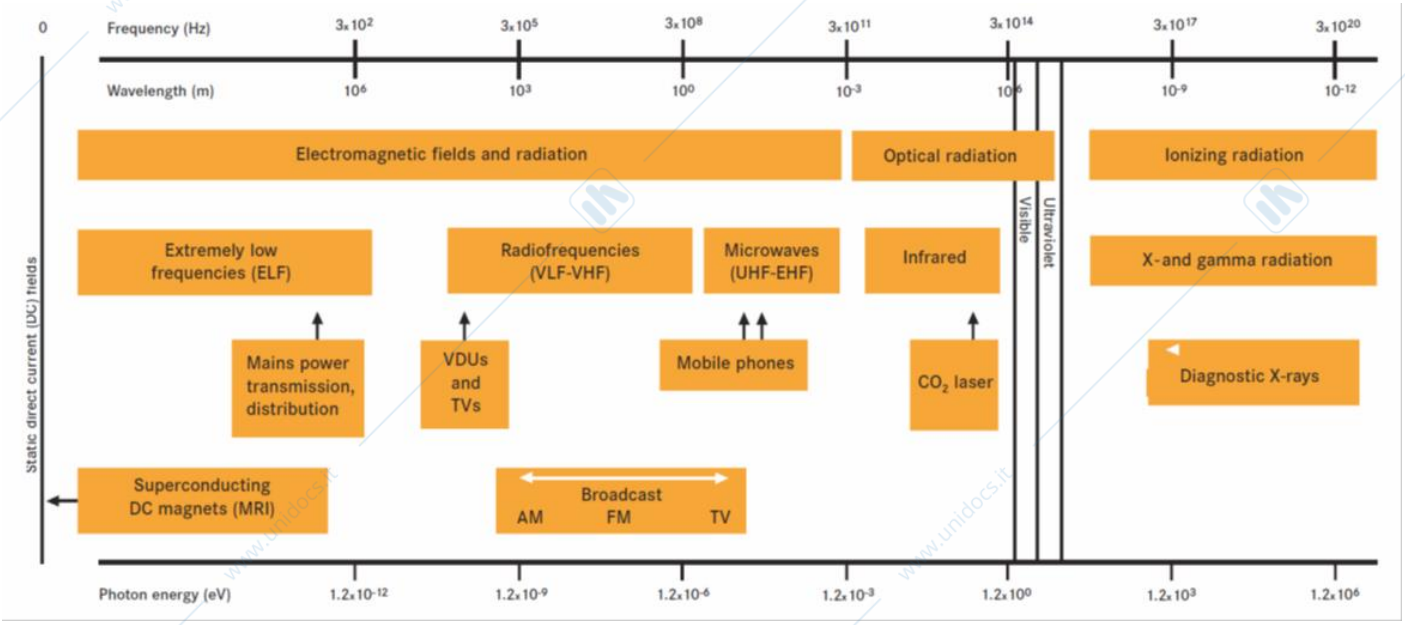
Ionizing radiation

Ionizing radiation, which is the most energetic part of the electromagnetic spectrum, can cause severe damage to DNA molecules, including single and double strand breaks, abasic sites and base modifications. Such damages can be clustered in a short stretch of DNA, further complicating the work of repair enzymes. Direct hits to DNA molecules, however, are comparatively rare, because on a molar basis DNA is a minor cell component. The most frequent damage is water ionization, which generates ROS and oxidative damage.

Radioactivity is measured in Becquerel (Bq), the number of nuclear decays per second of a given material. Linear energy transfer (LET) is the energy transferred per unit of length traversed. Low-LET radiation, which includes X and gamma rays, photons and electrons, passes through the cell rapidly, causing few ionization events, whereas high-LET particles (neutrons, protons, alpha particles) cause many ionizations events, resulting in a much higher level of damage.

To take into account LET and differences in the radiosensitivity of biological targets (e.g. different tissues, organs or animal species), in addition to the absorbed dose of radiation (measured in Grays, Gy), radiobiologists also use the Sievert, Sv, to measure the effective dose, i.e. the

Figure 16.1. The electromagnetic spectrum.
From Boyle & Levin (eds), World Cancer Report 2008, IARC



damage actually inflicted to the tissue. Sv = Gy x constant. For low-LET radiation, Sieverts are equivalent to Gray, whereas for high-LET radiation Sievert are higher than Grays.

In this chapter we are considering the damages inflicted by ionizing radiation to normal cells, but the flip side of the coin is the therapeutic efficacy of radiotherapy. The considerations made in the previous paragraphs explain why modern radiotherapy is adding high-LET radiation to its armamentarium, to better kill tumor cells, and why neoplastic radiosensitivity is dependent on intratumoral levels of oxygen, because hypoxia hampers the generation of effective oxidative damage. As oxygenation in turn depends on vascularization, this establishes a direct relation between angiogenesis and the effectiveness of radiotherapy, explaining why some therapeutic endeavors aim at vessel normalization, rather than inhibition.

The mutagenicity of ionizing radiation can be demonstrated by *in vitro* irradiation of cells, and its carcinogenicity is evident in animal models. The bulk of human data come from the study of individuals exposed to atomic explosions, in particular those of Hiroshima and Nagasaki (Japan) at the end of World War II (1945). The epidemiological study of survivors, begun in the 1950s, includes hundreds of thousands of exposed and control individuals and is still ongoing. Nuclear explosions released a mixture of high- and low-LET radiation, a major problem was to precisely determine individual exposure (dosimetry), which was still being refined as late as 2002, to correlate tumor incidence with radiation dose. Over the years, survivors developed an excess of all types of neoplasm. Leukemias appeared within a few years, whereas the excess of solid tumors was distributed over the following decades, peaking more than 60 years after the explosions (Figure 16.2). In a biphasic perspective, ionizing radiation is an initiating agent; in the course of their life, survivors were exposed to a normal load of promoters, which caused an excess of tumors because they carried in their DNA the initiating events (i.e. mutations) caused by

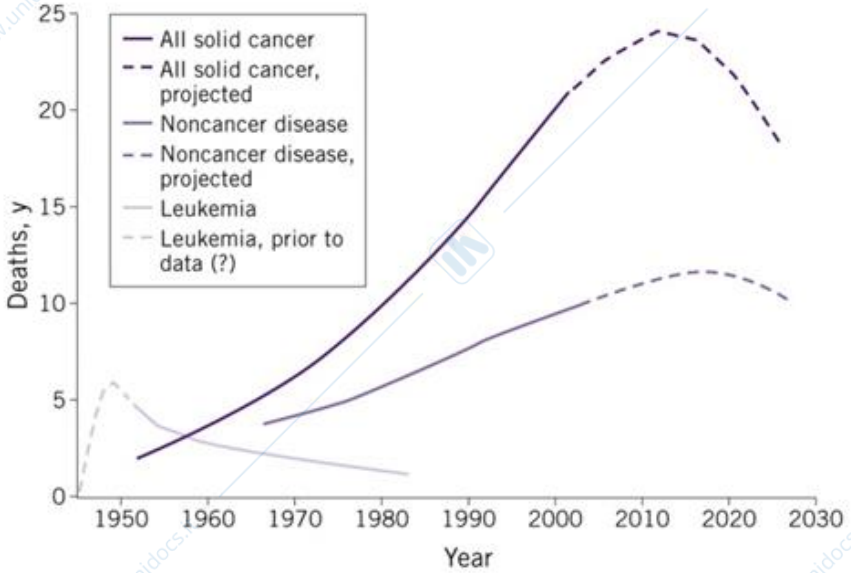


Figure 16.2. Causes of death in atomic bomb survivors, by year.
From Douple et al., Disaster Med Public Health Prep., 5 Suppl 1:S122-33, 2011.VII, Phase 2. National Academies Press.

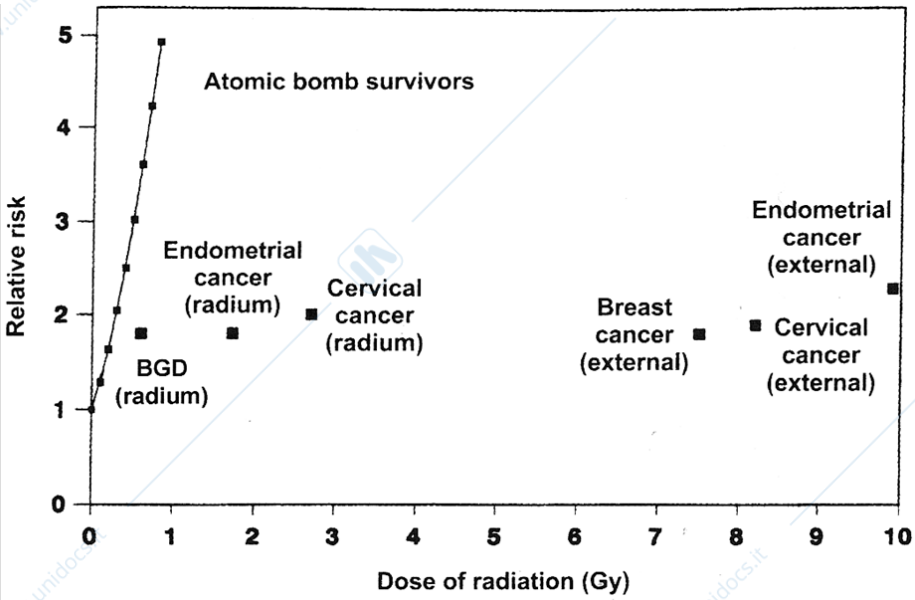


Figure 16.3. Risk of leukemia in atomic bomb survivors vs. radiotherapy patients. BGD=Benign gynecologic disease. From Boice *in* Schottenfield & Fraumeni, Cancer Epidemiology and Prevention, Oxford University Press

atomic explosions. An important consequence of this epidemiological study was the setup of risk models for low-dose exposures, e.g. diagnostic X-rays; a supralinear model was found to best fit leukemia incidence; linear no-threshold (LNT) is the favored extrapolation model for the risk of solid tumors after low-dose radiation exposure (see Figure 15.6).

Returning for a moment to radiotherapy, it should be noted that the risk of carcinogenesis is not proportional to the dose administered (Figure 16.3), because the therapeutic beam is focused on the tumor mass, and the few normal cells that are hit receive a very toxic dose of radiations, which causes cell death rather than neoplastic transformation. Nonetheless, radiotherapy can be estimated to double the risk of tumor development in the irradiated tissues (Figure 16.3).

Biological damage from ionizing radiation also depends on the chemical nature of the radioactive material, because living organisms can metabolize radioactive elements, for example iodine is concentrated by the thyroid and strontium can replace calcium in the bones. A prime example is the Chernobyl disaster. A nuclear reactor exploded on April 26, 1986 in Chernobyl (now Ukraine), releasing a radioactive cloud containing ^{131}I, ^{137}Cs, ^{90}Sr and Pu. After more than 30 years, the most evident increase in tumor incidence was thyroid cancer, attributable to ^{131}I, especially in younger individuals (odds ratio = 5.5-8.4 at 1 Gy dose). The involvement of metabolism also offers some opportunities to reduce the absorption of radioactive elements through the administration of high doses of "cold", i.e. non-radioactive elements, e.g. iodine pills. The Chernobyl disaster raised a considerable alarm throughout Europe, but it is now estimated that in Western Europe the excess of tumors related to Chernobyl will be so low as to be epidemiologically undetectable.

A ubiquitous source of exposure is background radiation (Figure 16.4), which is averaged at 2.4 milliSievert/year (mSv/yr) worldwide. Background radiation includes natural sources, such as cosmic rays and radioactive elements in the ground, along with man-made sources, which by definition include also medical

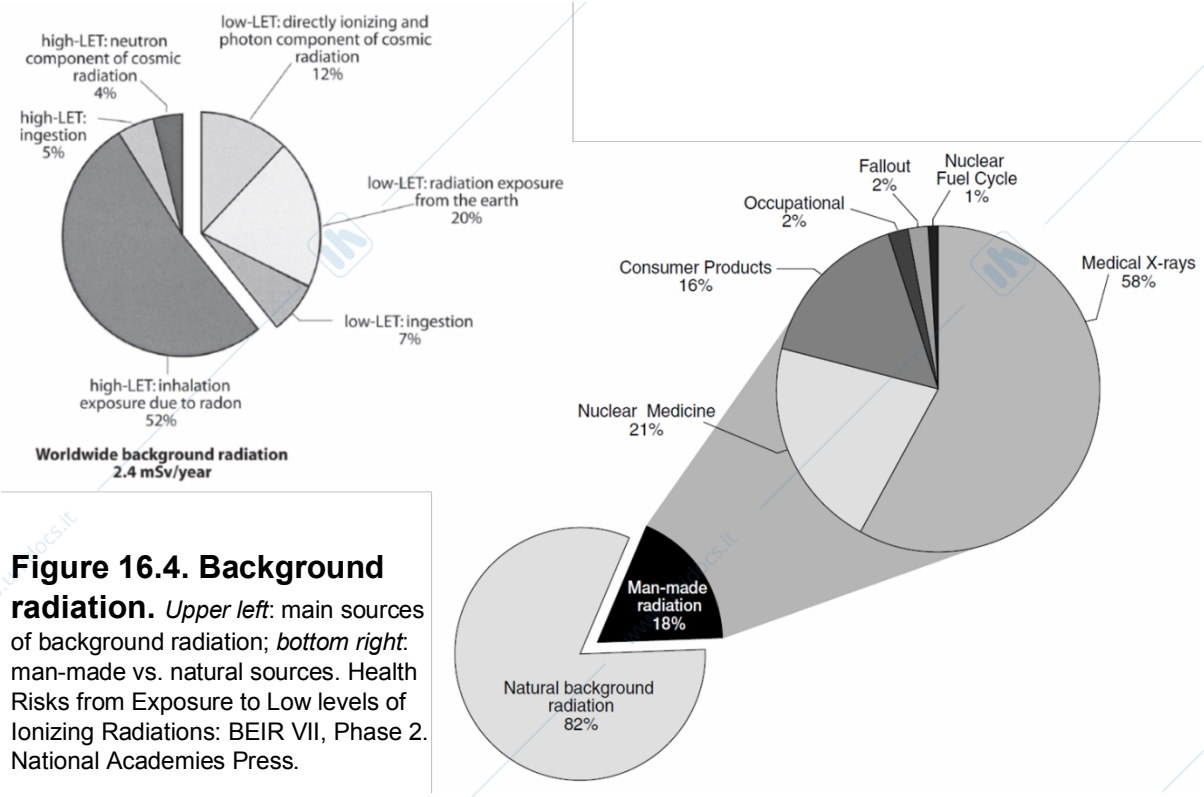
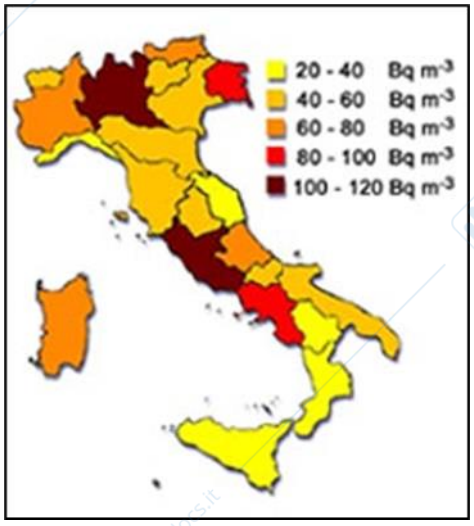


Figure 16.4. Background radiation. *Upper left*: main sources of background radiation; *bottom right*: man-made vs. natural sources. Health Risks from Exposure to Low levels of Ionizing Radiations: BEIR VII, Phase 2. National Academies Press.

uses of ionizing radiation, thus diagnostic radiology is considered as part of the background. While natural sources are mostly constant, the use of radiodiagnostics is constantly increasing, and modern tomographic technologies, such as CT or PET administer higher doses of radiation (up to 20 mSv) to obtain 3D images. In the US, medical uses of radiation caused an increase in total background radiation from 3.6 mSv/yr in 1980 to 6.2 mSv/yr in 2006.

Among natural sources, the major contribution comes from radon (Figure 16.4), which accounts for one-half of environmental background radiation. Radon (Rn) is an inert gas produced by the decay of uranium; the most common isotope, ^{222}Rn, is a high-LET emitter with a half-life of about 4 days. Radon inhalation is carcinogenic for the lung (IARC group 1), as first demonstrated in uranium miners. In the general population radon is one of the causes of lung cancer in non-smokers and contributes to the risk of smokers, it is estimated to be responsible for 3-4% of all lung cancers. Exposure of the population (low levels for long periods) occurs

Figure 16.5. Indoor radon levels in Italy. Data from Bocchicchio et al., Health Phys., 71: 741



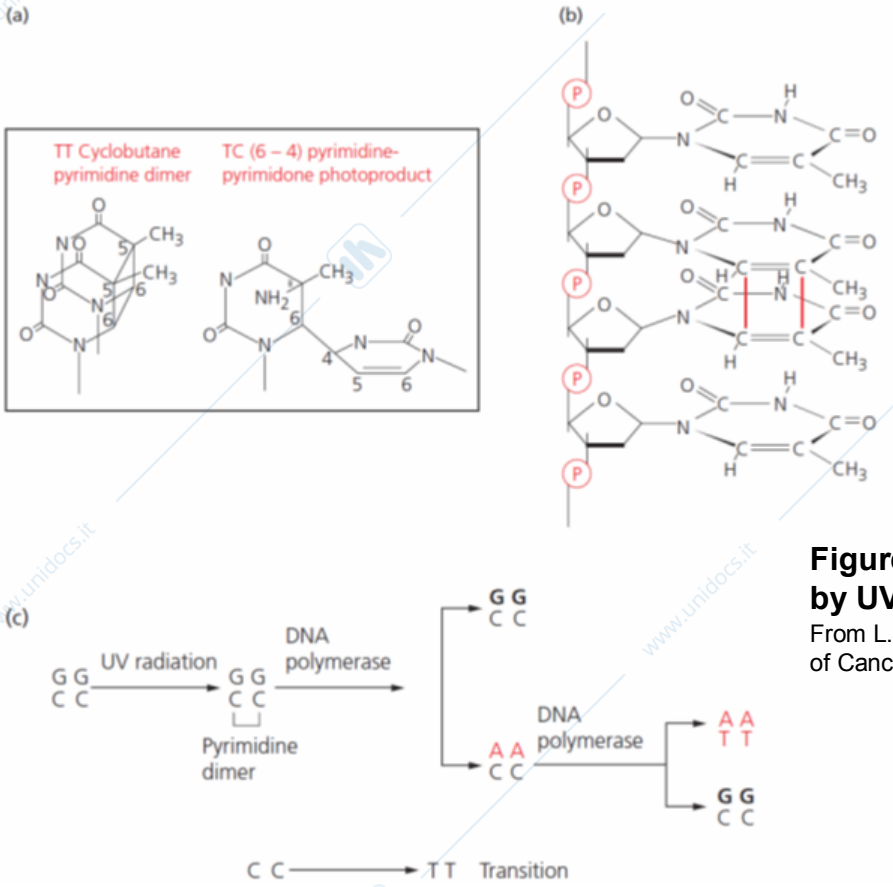


Figure 16.6. DNA damage by UV radiation.
From L. Pecorino, Molecular Biology of Cancer, Oxford University Press.

indoor, by radon released from the ground (especially in volcanic and seismic regions) or by building materials. Indoor radon concentrations are highly variable, because the gas is easily dispersed by ventilation, and simple modifications, such as wallpaper, can block emissions from building materials, therefore radon levels must be measured house-by-house, over long periods (months). Indoor recommended limits range from 200 to 500 Bq/m³. In Italy, indoor levels are within limits, ranging from 20 to 120 Bq/m³ (Figure 16.5).

Ultraviolet and solar radiation

UV radiation (400-100 nm), both natural (sun) and artificial (UV lamps) is mutagenic. UV directly damages DNA in the skin and eyes (UV does not penetrate beyond the epidermis), producing pyrimidine (T/C) dimers (Figure 16.6), which are mostly repaired by the NER system. Exposure to UV, or a deficiency in NER system (xeroderma pigmentosum, see chapter on hereditary tumors), increases the risk of skin and eye tumors.

The sun is the main UV source. The UV spectrum is conventionally divided in UVA (400-315 nm), UVB (315-280 nm) and UVC (280-100 nm). UVC rays are the most mutagenic, as they are the most energetic, and their wavelength corresponds to the peak of DNA absorbance, however solar UVC radiation is completely blocked by the ozone layer, whereas UVB is only partially (70-90%) absorbed by ozone, thus representing the major mutagenic danger of solar exposure.

The level of human UV exposure depends on three orders of factors:

- Astronomical, geographical, and atmospheric factors

Table 16.1. Human phototypes
Modified from Astner & Anderson, J. Invest. Dermatol., 122: 30.

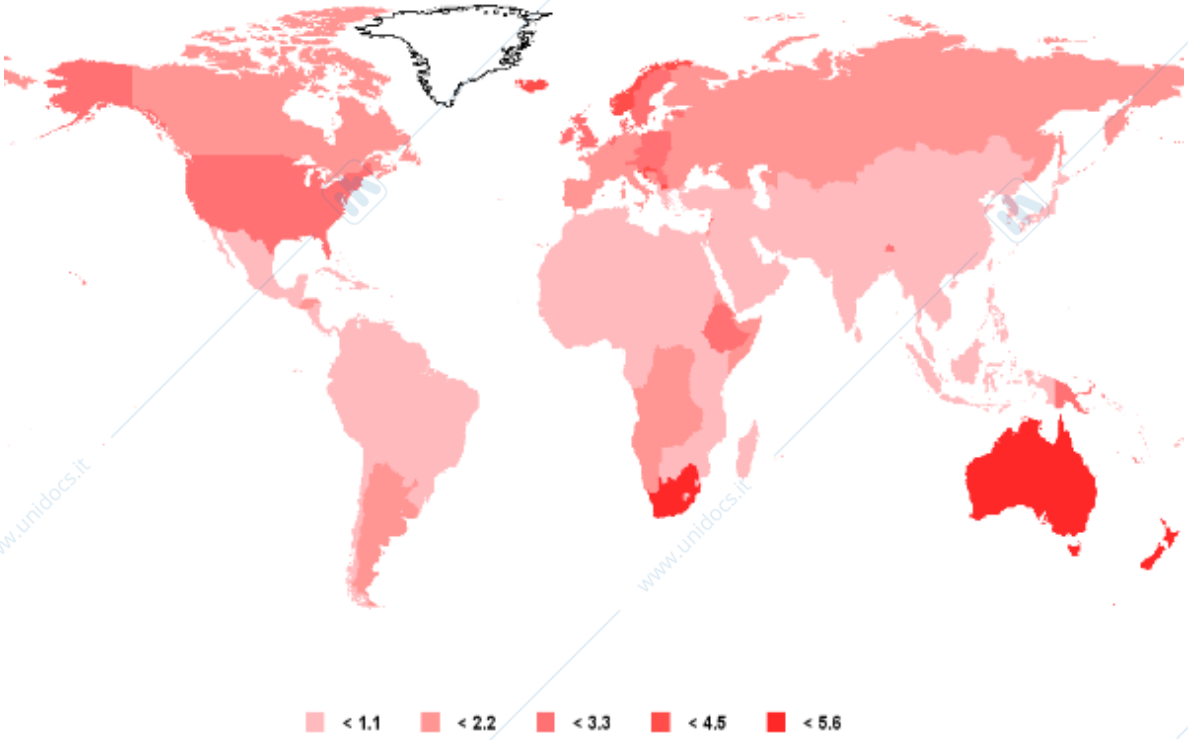
Phototype	Sunburn & Tanning history (defines the phototype)	Immediate pigment darkening	Delayed tanning	Constitutitve color (unexposed buttock skin)
I	Burns easily, never tans	None (–)	None (–)	Ivory White
II	Burns easily, tans minimally with difficulty	Weak ± (± to +)	Minimal to weak (± to +)	White
III	Burns moderately, tans moderately and uniformly	Definite +	Low +	White
IV	Burns minimally, tans moderately and easily	Moderate + +	Moderate + +	Beige-Olive, Lightly tanned
V	Rarely burns, tans profusely	Intense (brown) + + +	Strong, intense brown + + +	Moderate brown or tanned
VI	Never burns, tans profusely	Intense (dark brown) + + +	Strong, intense brown + + +	Dark brown or black

  - Latitude (Equator>Poles)
  - Altitude (mountains>sea level)
  - Season (summer>winter)
  - Time of the day (noon>morning/afternoon)
  - Atmospheric conditions (clear sky> cloudy/polluted)
- Phototype – As melanin protects from UV rays, UV susceptibility is determined by the skin phenotype (phototype, Table 16.1) and by the distribution of melanin, e.g. palm vs. back of the hand. The phototype of human populations is normally adapted to the level of local solar radiation, but mass migrations created some notable imbalances, for example in South Africa and Australia (Figure 16.7), where people of European origin are exposed to tropical UV levels, leading to a disproportionately high levels of UV-related tumors.
- Intentional vs. non-intentional exposure – In the early 20th century, chronic sunlight exposure was mainly associated with specific jobs, e.g. farmers and sailors, but progressive improvements in working conditions have greatly reduced this type of exposure. Meanwhile, suntanning was initially promoted for medical reasons (rickets prevention) but is now widely practiced mainly for esthetical purposes.

UV (A, B, C) and solar radiations are IARC Group 1 carcinogens, causing cutaneous melanoma and skin cancers (squamous cell and basal cell carcinomas). Melanoma is correlated to intermittent, intentional exposure, squamous cell carcinoma to non-intentional exposure. The risk of carcinomas is directly proportional to the absorbed doses, there is no threshold. The risk of melanoma is proportional to the number of sunburns, which add tissue damage and inflammation to the mutagenicity of UV. Risks are higher for fair phototypes and for juvenile exposures.

In principle, prevention of UV-related tumors is easy. The use of high (>5 at least) protection factor creams reduces the absorbed dose and the risk of tumors.

Figure 16.7. Melanoma mortality rates in males. From GLOBOCAN 2008 (IARC).



However, some people compensate by increasing the duration of exposure, thus voiding the protective effect of creams.

UV lamps and tanning beds are also carcinogenic, they are now classified by the IARC in group 1. Tanning beds are illegal in Australia and Brazil and are forbidden to minors in many countries worldwide. To disincentivize tanning centers and the use of tanning equipment, the US instituted a specific taxation, which goes under the name of tanning tax.

Electromagnetic fields

Many modern technologies generate electromagnetic fields, notable examples are cell phones, radio and television broadcasts, and mains electricity. Alternate current at 50 (Europe) or 60 (US) Hz is commonly referred to low-frequency, whereas cellular telephony (300 to 3000 MHz) and other sources in the microwave range are collectively called high-frequency electromagnetic fields (Figure 16.1). By analogy with ionizing and UV radiation, the carcinogenic potential of these electromagnetic fields is being actively investigated, even though the associated energies are much lower. As billions of people worldwide are exposed to these sources, even a tiny carcinogenic hazard would translate into a sizeable risk.

Neither high-frequency, nor low-frequency fields have direct mutagenic activity. In addition to some effects on gene expression, the major biological consequence of cell exposure to high-frequency fields is the heating of intracellular water, similar to what happens in microwave ovens, which could have a pro-inflammatory activity, especially within the skull. Some animal studies of older generation cell phones have shown slight increases in tumor incidence. It must be considered that technologies move faster that both *in vivo* and epidemiological studies, hence the results will always become available when

the technology under investigation is already obsolete.

Epidemiological studies are also affected by various biases. The dosimetry of retrospective studies is affected by large uncertainties, as nobody will precisely remember the use of cell phones in the past; the most reliable data come from professionals, who track their use for economical purposes. Furthermore, case-control studies are affected by the recall bias, a known effect by which illness influences the memory of past events, a trivial example is that of people using the right ear for phone calls, who after the development of some illness in the left side of the head might erroneously report the habitual use of the left ear.

For low-frequency electromagnetic fields, the consensus is that there is no risk for the general population exposed to household electrical supply. Some small clusters of childhood leukemia occurring near power plants (Sellafield in the UK), or high-voltage pylons were thoroughly investigated, but the evidence was insufficient to draw firm conclusions as to carcinogenicity.

The situation is more complex for what concerns cell phones. Large studies did not show an increased risk of cancer for the general population, but some animal studies and some human case-control studies in professional users evidenced an increase in gliomas, acoustic neuromas and parotid tumors. Consequently, the IARC classified radiofrequency electromagnetic fields as Group 2B, possibly carcinogenic to humans. For those interested in avoiding exposure to these fields, the technological solution is simply a wired headset, which removes the source of microwaves, i.e. the phone body, from the immediate vicinity of sensitive tissues (obviously a wireless headset does not solve the problem, it just replaces the phone with a different electromagnetic source).

Chapter 17. Chemical Carcinogenesis

The common feature of chemical carcinogens is the ability to damage the DNA, either directly or indirectly. Carcinogenic compounds have many different chemical structures, there is no single class of chemical carcinogens. Here we will follow what is (hopefully) a logical path, from inorganic to organic molecules, to natural carcinogens, produced by living organisms.

In this chapter we will deal with single molecules, focusing on their mechanisms of action, in the next chapter we will analyze complex mixtures.

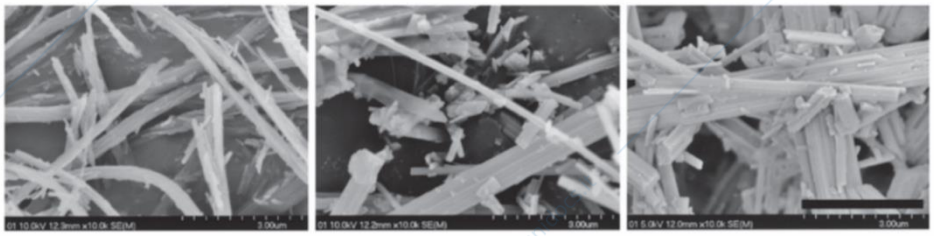
Asbestos and other inorganic carcinogens

Here we will focus on asbestos. Other inorganic carcinogens include arsenic and its compounds, which can pollute drinking water (see next chapter), and heavy metals and their compounds (e.g. chromium, nickel, cadmium and beryllium), which are mainly of concern in occupational carcinogenesis.

Asbestos (Figure 17.1), in Italian also known as *amianto*, is a group of fibrous minerals widely used for the low cost and unique properties: resistance to fire, electrical and thermal insulation, mechanical and chemical resistance. The main uses were in buildings (asbestos-cement, trade name Eternit) and transport industry (ships, cars, railways), also as textile fibers for fire-resistant clothes.

Because of carcinogenicity, the extraction, production and use of asbestos were abandoned in the second half of the 20th century in most developed countries, whereas less rich countries still produce and use asbestos in huge quantities. In Italy, as in most countries that banned asbestos, the main issue is the

Name	Composition	Source		Morphology
Chrysotile	$Mg_6Si_4O_{10}(OH)_8$	U.S. and Canada	White	Curly, pliable
Crocidolite	$Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$	South Africa Western Australia	Blue	Rodlike, durable
Amosite	$(Fe, Mg)_7Si_8O_{22}(OH)_2$	South Africa	Brown	Rodlike, durable
Anthophyllite	$(Mg, Fe)_7Si_8O_{22}(OH)_2$	Finland		Rodlike, durable
Tremolite	$Ca_2Mg_5Si_8O_{22}(OH)_2$	Exists in some deposits of Canadian chrysotile		Rodlike, durable
Actinolite	$Ca_2(Mg, Fe)_5Si_8O_{22}(OH)_2$	Not mined		Rodlike, durable



Chrysotile Crocidolite Amosite

Figure 17.1. Types of asbestos. From Toyokuni, Nagoya J Med Sci, 71: 1.

disposal of residual materials containing asbestos, still frequently found in old buildings and industrial sites.

Asbestos fibers are inhaled, causing long-term chronic inflammation and fibrotic lesions (asbestosis) in the lungs. Early studies demonstrating the carcinogenicity of asbestos date back to the 1950s. Mesothelioma is a rare, highly malignant pleural (90% of cases) or peritoneal tumor, almost always associated with asbestos exposure. Asbestos is strongly carcinogenic also for the lung epithelium, synergizing with tobacco smoke, and for other organs (larynx, ovary, etc.). The latency of tumors caused by asbestos is 30 or more years, hence excess tumors appear long after the ban of asbestos. Note that there is no treatment that can reduce the risk of cancer in people previously exposed to asbestos, we can only offer a continuing medical surveillance to diagnose tumors as soon as possible.

How can such an inert substance cause cancer? There are several possible mechanisms, which operate simultaneously (Figure 17.2). Asbestos is not mutagenic, but small fibers, with subcellular dimensions, when inside the cell can interfere with mitosis, directly causing chromosomal aberrations (substances causing chromosomal aberrations are called clastogens). Furthermore, asbestos contributes to oxidative stress both through the redox activity of iron (Fenton reactions) and the release of ROS in the environment by inflammatory macrophages that, having phagocytized asbestos fibers, are unable to digest them (frustrated phagocytosis). Finally, asbestos fibers and the surrounding inflamed

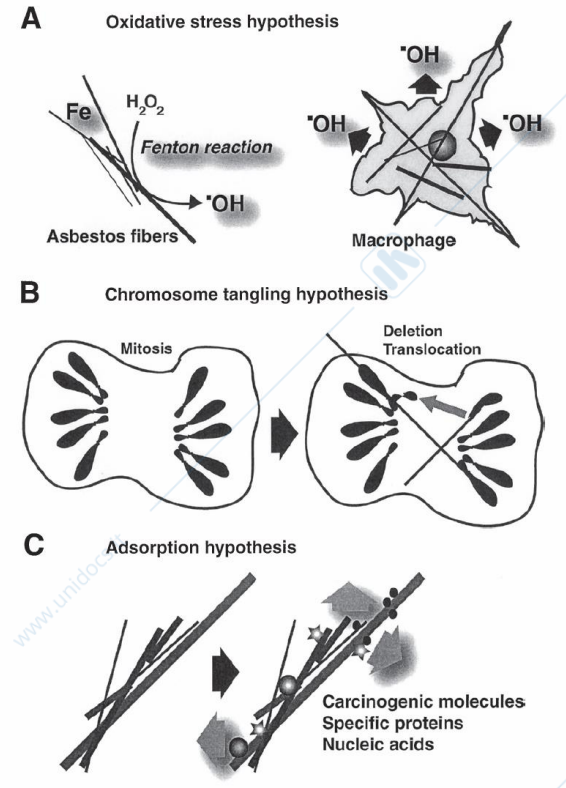


Figure 17.2. Mechanisms of asbestos carcinogenicity.
From Toyokuni, Nagoya J Med Sci, 71: 1.

tissues act as sponges for other lung carcinogens (e.g. air pollutants and tobacco smoke), which are found at higher concentrations than in the remaining lung tissue.

Foreign body carcinogenesis

Implantation within the human body of other "inert" foreign bodies can also cause tumors. A recent iatrogenic example is the appearance of lymphomas associated to breast implants. The physicochemical properties of the materials are relevant, e.g. lymphomas are associated with textured, but not smooth breast prostheses.

In general, the mechanisms of foreign body carcinogenesis can include:

- Inflammation, oxidative stress

- Fibrosis, formation of zones not accessible to immune surveillance
- Chronic local infections
- Direct effects, e.g. release of carcinogenic plastics monomers

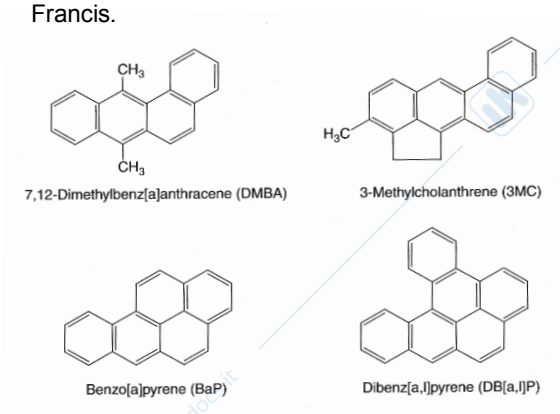
Polycyclic aromatic hydrocarbons

Carcinogenic polycyclic aromatic hydrocarbons (PAH) are made of a few condensed aromatic rings (peak carcinogenicity at 4-5 rings) surrounding a gulf-shaped "bay region" (Figure 17.3). Any kind of incomplete combustion of organic matter releases a mixture of PAHs: tobacco smoke, use of fossil fuels, barbecued meat, etc. Benzo[a]pyrene (BaP) is used as the reference compound; the carcinogenicity of mixtures can be compared in terms of "benzo[a]pyrene equivalents".

PAH are indirect carcinogens, activated by phase I enzymes in the lungs and other organs to reactive diol epoxide and quinone derivatives, which can form DNA adducts (see Box 17.1) with guanine and adenine.

The carcinogenicity of PAH, already implicit in the findings of Percival Pott

Figure 17.3. Some polycyclic aromatic hydrocarbons. From Warhawsky & Langdon, Molecular Carcinogenesis and the Molecular Biology of Human Cancer, Taylor & Francis.



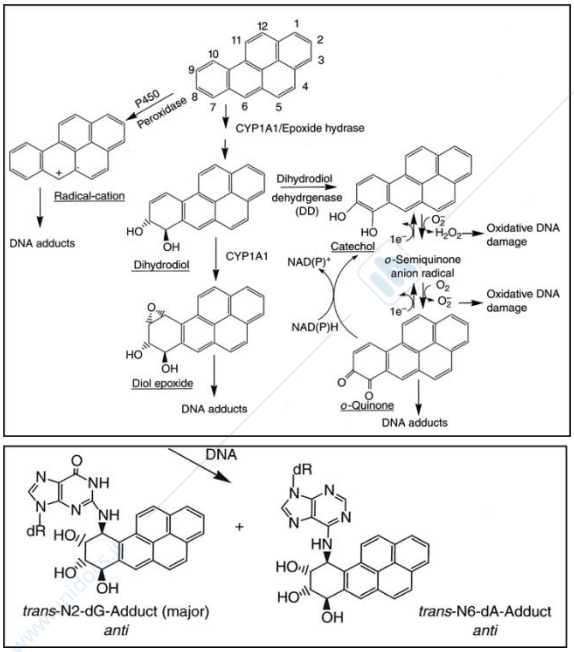


Figure 17.4. BaP metabolic activation and DNA adducts. Modified from Warhawsky & Langdon, Molecular Carcinogenesis and the Molecular Biology of Human Cancer, Taylor & Francis.

in London chimney sweeps, is known since 1918, when Yamagiwa and Ichikawa obtained tumors by painting rabbit ears with tar. PAH are powerful carcinogens in all animal species, causing both epithelial and mesenchymal tumors, depending on the route of administrations. Human exposure is mainly by inhalation or ingestion of PAH mixtures, which are among the main substances responsible for lung cancer in smokers.

Benzene and other carcinogenic hydrocarbons

Benzene (Figure 17.5) is present in fuels, has various industrial uses and is also produced by combustion reactions. In the organism it gives rise to cyclic (phenols) and linear (muconaldehyde) metabolites. Occupational exposure (e.g. gas station attendants) is related to the risk of myeloid leukemia; benzene is a group I IARC carcinogen.

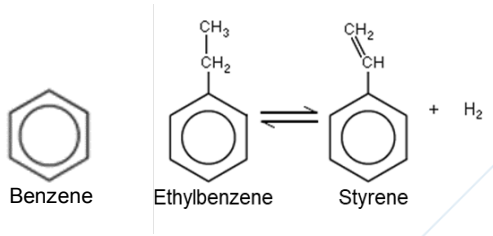
Box 17.1. Adducts.

The term adduct is frequently encountered when dealing with chemical carcinogens, but what is an adduct? Formally (IUPAC) an adduct is "*A new chemical species AB, each molecular entity of which is formed by direct combination of two separate molecular entities A and B in such a way that there is change in connectivity, but no loss, of atoms within the moieties A and B*."

Chemical carcinogenesis is focused on adducts to DNA and to other biomolecules, resulting from the binding of active carcinogenic compounds. Adducts are also found when dealing with physical carcinogens, for example the DNA alterations caused by UV radiation (see previous chapter) are photoadducts.

Note that adducts are not mutations: they are unstable and can be eliminated by DNA repair, for example alkyl groups bound to guanine can be removed by alkyl transferases, but adducts can cause mutations.

Figure 17.5. Benzene and styrene.



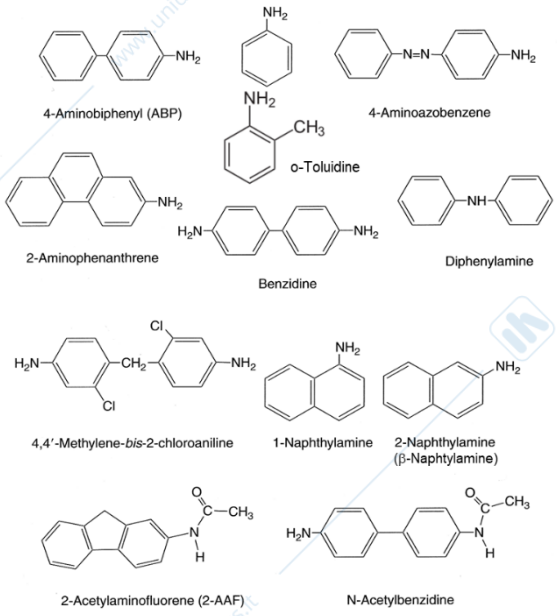
Styrene (vinylbenzene, Figure 17.5), i.e. the monomer of polystyrene, and ethylbenzene are potential carcinogens (IARC 2B). In general, plastics polymers are poorly reactive and non-carcinogenic, whereas their monomers are highly reactive and can be carcinogenic. Polymers can be hazardous if they release monomers under normal use. For this reason, plastics approved for the storage of foods must not release monomers in the presence of weak acids (e.g. lemon juice, vinegar), nor when in contact with hot foods.

Aromatic amines

Aromatic amines (Figure 17.6) are widely used as colorants for paper, wood, leather and plastics. They are also produced by incomplete combustion of organic matter in the presence of nitrogen, under conditions that also generate PAH. Unlike PAH, aromatic amines are activated by phase II enzymes in the kidney and accumulate in the urinary bladder (Box 17.2).

The carcinogenicity of aromatic amines was already noted in the XIX century in textile workers. Aromatic amines are powerful carcinogens in various animal species. Human exposure is

Figure 17.6. Some aromatic amines.
Modified from Warhawsky & Langdon, Molecular Carcinogenesis and the Molecular Biology of Human Cancer, Taylor & Francis.



Box 17.2. IPCA, the cancer factory

The *Industria Piemontese dei Colori di Anilina*, IPCA, produced aniline-based dyestuff in Cirié, near Turin in Piedmont, since 1922. In the 1950 the working environment at IPCA was outdated and highly unsafe, workers were constantly exposed to benzidine and naphthylamines.

"*Color mills workers urinate in the same colors they work with (blue, yellow, violet, etc.), until they begin to urinate blood*" (La fabbrica del cancro, Einaudi 1977).

More than 150 IPCA workers developed bladder cancer. In a cohort of 394 workers, 56 died of bladder cancer, as compared to 3.4 expected cases (JNCI 102: 1096). In 1977 the owners, some executives and the factory doctor were sentenced to 4-6 years in prison. IPCA closed in 1982.

mostly by cutaneous absorption (occupational) and by inhalation (smokers). The main tumor type caused by aromatic amines is bladder carcinoma; bladder carcinogenesis in smokers is mainly attributed to aromatic amines.

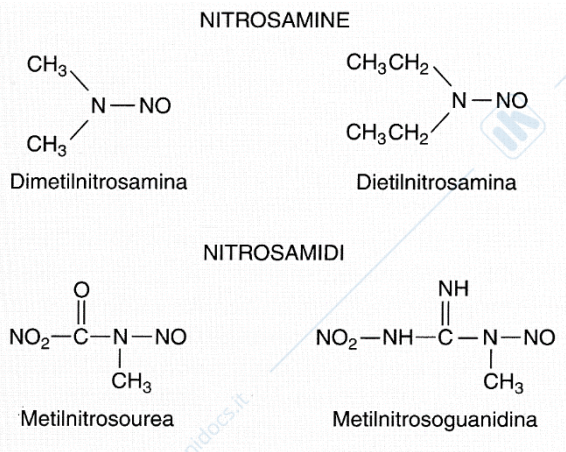
Nitrosamines and nitrosamides

Nitrosamines and nitrosamides (Figure 17.7) are N-nitroso (N–N=O) compounds used in the industry. They are also present in cured tobacco and in smoke (see next chapter). Moreover, dietary nitrites (NO_2^-) and nitrates (NO_3^-), also widely used as food preservatives (E249, E250, E251, E252), are converted to N-nitroso compounds within the organism, for example by reaction with digestive juices. Nitrosamines are activated by phase I enzymes, yielding alkylating derivatives.

Nitrosamides are used as anti-cancer drugs. They are unstable and decompose spontaneously, also giving rise to alkylating agents.

Animal studies demonstrated the carcinogenicity of nitrosamines and nitrosamides. Human exposure to nitrosamines is mainly caused by tobacco (inhalation) and diet (ingestion), contributing to carcinogenesis in the lung and in the digestive tract.

Figure 17.7. Some nitrosamines and nitrosamides. From Pontieri *et al.* Patologia Generale, Piccin, Padova.

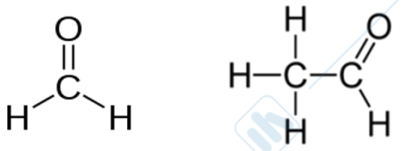


Aldehydes

Two aldehydes are important carcinogens, acetaldehyde, a metabolic derivative of ethanol, which we will analyze in the next chapter, and formaldehyde (Figure 17.8).

Formaldehyde is used in the plastic industry and for the fixation of biological materials in histology and embalming. Its fixative properties are due to the cross-linking of proteins, but formaldehyde also cross-links proteins and DNA,

Figure 17.8. Formaldehyde (left) and acetaldehyde (right).



causing mutations. Human exposure, mainly through inhalation, is linked to nasopharyngeal carcinomas and leukemias.

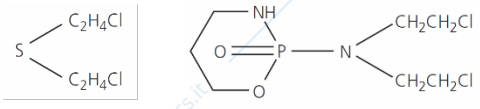
Alkylating agents

Various compounds can alkylate DNA molecules. In addition to the binding of alkyl groups, alkylating agents with two reactive groups (bifunctional) can cross-link DNA chains.

Alkylating agents include various industrial compounds, such as beta-propiolactone and benzyl chloride. Ethylene oxide is used in hospitals for sterilization.

The major medical interest for alkylating compounds is their activity as anticancer drugs. They derive from chemical weapons (poison gases) of World War I, when it was noted that soldiers exposed to gases had a strong decrease in the number of leukocytes, leading to the idea that some derivatives could be used to cure pathologies with too many leukocytes, i.e. leukemias and lymphomas. In the period between the two world wars all the research on these molecules was a military secret, but immediately after the end of World War II publications began to appear that described the therapeutic potential of early alkylating drugs. Modern derivatives, such as cyclophosphamide (Figure 17.9) and its analogues, are still widely used in cancer therapy.

Figure 17.9. A chemical warfare agent (*left*) and a cancer drug, cyclophosphamide (*right*).



Halocarbons

Halocarbons (organo-halogens), especially chlorocarbons, are widely used in agriculture and in various industries. Halocarbons are highly stable and persist in the environment. Some are electrophiles that interact directly with DNA, others require metabolic activation.

Halocarbons are a highly heterogeneous group of molecules, their carcinogenicity is highly variable, in some cases acute toxicity is prevalent. Demonstrations of carcinogenicity, mainly based on animal studies, led to the ban of various pesticides and to a tight regulation of the industrial uses of vinyl chloride (the monomer of PVC), trichloroethylene (TCE) and polychlorinated biphenyls (PCB). Chlorofluorocarbons (CFC), used in spray cans and air conditioners, are sometimes considered as carcinogens by proxy, because they contribute to UV carcinogenesis through ozone layer depletion.

Naturally occurring carcinogens

Many living organisms produce toxic substances for self-defense, for example plants contain pesticides to avoid being eaten by parasites. Bruce Ames once calculated that 99% of dietary pesticides are natural plant products, rather than

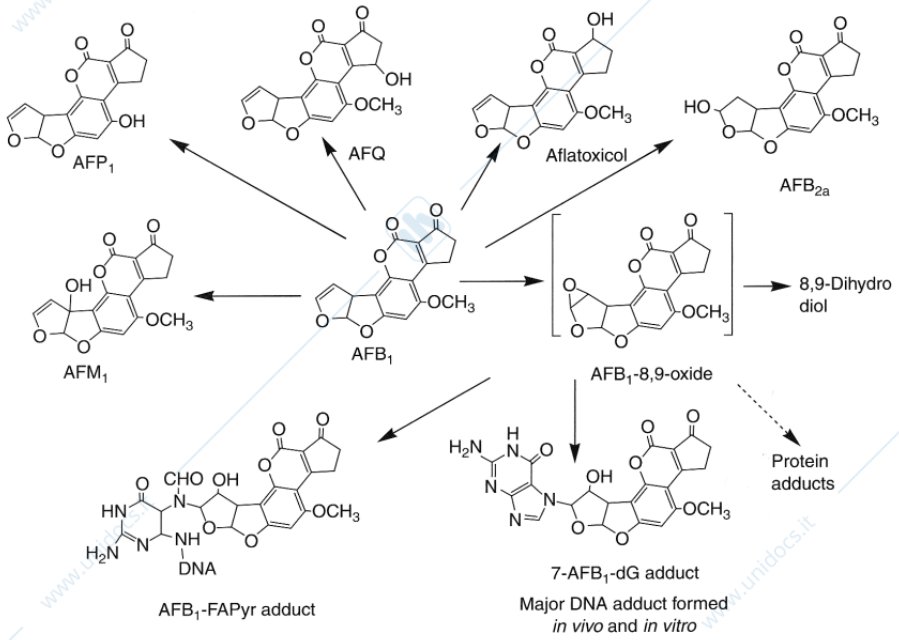


Figure 17.10. Metabolic activation of aflatoxin B1 (AFB_1) by hepatic mixed-function oxygenase. From Warhawsky & Langdon, Molecular Carcinogenesis and the Molecular Biology of Human Cancer, Taylor & Francis.

synthetic man-made compounds. Some of these molecules are also carcinogenic. Note that natural carcinogens are distinct from carcinogenic micro-organisms (i.e. viruses, bacteria and parasites), which are classified as biological carcinogens (see next chapter).

One of the most potent carcinogens overall is a natural molecule, aflatoxin B_1 (Figure 17.10). It is produced by *Aspergillus flavus*, a fungus contaminating seeds (wheat, rice) and nuts (cereals, peanuts, pistachios) stored under warm and damp conditions. Aflatoxin contaminates mainly African and Asian products, but food globalization led to an increase in contaminated imports in the EU and USA.

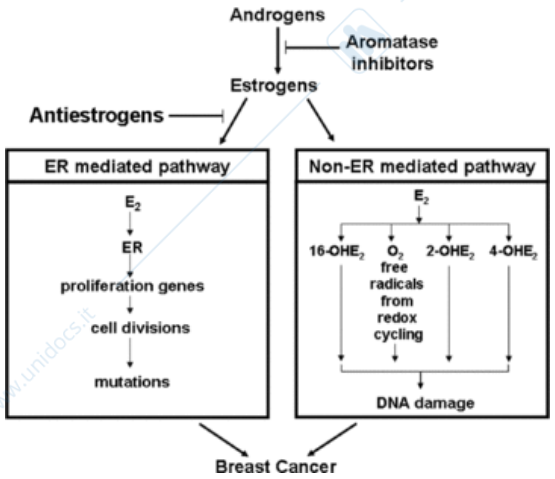
Dietary aflatoxin B_1 is activated in the liver, causing mainly liver cancer. Most other carcinogens induce DNA mutations that are indistinguishable from one another, or from spontaneous mutations, but aflatoxin causes a peculiar p53 mutation, G249T, which is considered as a signature of aflatoxin exposure.

Carcinogenic drugs and hormones

Some therapeutic drugs are carcinogenic, in particular the genotoxic inhibitors of cell proliferation. Their medical use must be based on accurate risk-benefit analyses; carcinogenic drugs are only used for the treatment of severe or life-threatening conditions, e.g. cancer or the prevention of transplantation rejection. Patients treated with carcinogenic drugs have a lifetime increase in the risk of cancer, which is the highest for leukemia patients who received the treatment in childhood.

Synthetic and natural sex steroids (estrogens and progestins) have various pharmacological uses, most prominently as oral contraceptives for fertile women and for hormone replacement in perimenopause. Sex steroids are mainly carcinogenic promoters, because they induce the proliferation of many cell

Figure 17.11. Promoting (*left*) and initiating (*right*) carcinogenic activities of estrogens. From Santen *et al.*, Ann NY Acad Sci, 1155: 132.



types (Figure 17.11), which is a known risk factor of neoplastic transformation; some steroid metabolites damage DNA, either directly or through oxidative stress (Figure 10.11), thus steroids can also be considered as initiating carcinogens. Depending on tissue-specific control of cell proliferation, sex steroids can enhance the risk of cancer in one organ while reducing the risk in another.

Oral contraceptives increase by 20% the risk of breast cancer in all women, and strongly increase the risk of cervical carcinomas in women infected by carcinogenic papilloma viruses; on the other hand, oral contraceptives significantly reduce the risk of endometrial, ovarian and colorectal carcinomas (Table 17.1). On the whole, it can be said that they reduce the risk of cancer in papilloma-negative women, but an evaluation must be done on a case-by-case basis, for example in the presence of a familial risk of breast cancer.

Hormone replacement therapy (HRT), to alleviate the side effects of menopause, made use of purely estrogenic formulations ('unopposed estrogen'), or of combinations of estrogens and progestins. In both cases, the prolonged use of these drugs strongly increased the risk of breast and endometrial cancers (Table 17.1). Especially in the US, HRT became extremely popular in the last part of the 20th century, and the number of prescriptions soared. In the 2000s, after large epidemiological studies (Women Health Initiative and HERS), based on millions of women, clearly demonstrated the carcinogenicity of HRT, the number of prescriptions plummeted. A few years later, the incidence of breast cancer in the US showed an unexpected decrease, which some epidemiologists attributed to the reduction in the use of HRT. In biphasic carcinogenesis, the effect of initiating agents takes many years, as we have seen for

Table 17.1. Relative risk of site-specific cancer in women exposed to exogenous steroids. *Green*: decrease, red: *increase*. Modified from Lacey et al., in Schottenfield & Fraumeni, Cancer Epidemiology and Prevention, Oxford University Press.

Cancer site	Oral contraceptive	Postmenopausal hormone therapy	
		Unopposed estrogen	Estrogen+ Progestin
Breast	1.2	1.3-1.5	1.9-2.2
Uterus	0.2-0.7	2.8-9.5	1.4-3.5[a] 1.0[b]
HPV+ cervical	2.2-4.0	i.d.[c]	i.d.[c]
Ovarian	0.5-0.6	2.0-3.0	i.d.[c]
Colorectal	0.8	0.9	0.6-0.8

[a]Sequential E+P; [b]Continuous; [c]i.d.=insufficient data

ionizing radiation in atomic bomb survivors, whereas promoters can enhance the growth of existing lesions in much shorter time spans. Given that sex steroids are mainly promoters, the correlation between the abandonment of HRT and the decrease in breast cancer incidence is not implausible.

Box 17.3. Breast cancer: Endogenous and exogenous hormonal risk factors

Most breast cancers express estrogen receptors (ER) and grow in response to estrogens. Major risk factors for these tumors are related to the length of the reproductive life of women: an early menarche and a late menopause increase the risk, whereas pregnancies and breastfeeding are protective factors. All these factors can be included in mathematical models used to estimate the individual future risk of breast cancer development.

In addition to endogenous hormones, we have seen that steroidal drugs can significantly increase the risk. A logical extension is the idea that anti-estrogenic drugs, such as ER antagonists or inhibitors of estrogen synthesis, could reduce the risk of breast cancer. This is indeed the case, even though they have important side effects, as we will see in the chapter on cancer prevention.

Risk	Endogenous factors	Exogenous factors
Increase	Early menarche Late menopause Nulliparity	Estrogenic drugs: - Contraceptives - Hormone replacement therapy
Decrease	Duration of breastfeeding Number of pregnancies Early first pregnancy	Inhibitors of estrogen synthesis (aromatase inhibitors) Estrogen receptor antagonists

Chapter 18. Tobacco, Alcohol, Diet and Other Complex Carcinogenic Exposures

Exposure to pure chemical carcinogens is mostly limited to occupational conditions, instead the general population is exposed to complex mixtures of carcinogens. If each carcinogen in a mixture acts independently, the total effect is presumed to be additive, but interactions can determine synergistic or antagonistic effects. The evaluation of complex mixtures, especially when carcinogenic potency is low, relies mainly on large epidemiological studies.

Note that, in the context of mixtures, "co-carcinogen" refers to a non-carcinogenic agent that modifies the activity of a carcinogen, e.g. by modifying exposure or metabolism.

Major population exposures to complex carcinogen mixtures are dependent on individual lifestyles, including tobacco smoke, alcohol consumption and diet. Some of these exposures are voluntary (tobacco, alcohol), which implies that exposure can be reduced to zero, whereas non-voluntary exposures (like natural radiation or environmental pollution,) can only be modulated as to quality and quantity. Others comprise both voluntary and non-voluntary exposures, for example solar radiation (day-to-day exposure vs. intentional tanning) and diet, which in Western countries includes an excess of food, consumed for reasons independent of the maintenance of bodily functions. From an investigational point of view, voluntary exposures allow ideal epidemiological studies, that compare exposed and non-exposed individuals, yielding clear-cut results.

Tobacco

Tobacco smoke is a complex mixture of more than 4000 chemical compounds, of which 60 are carcinogens and 10 strong carcinogens (Figure 18.1).

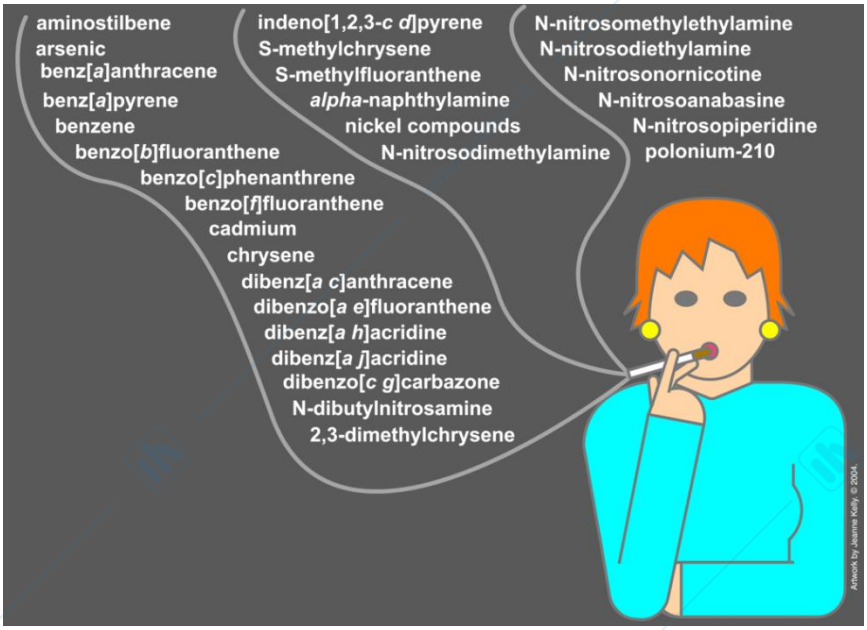
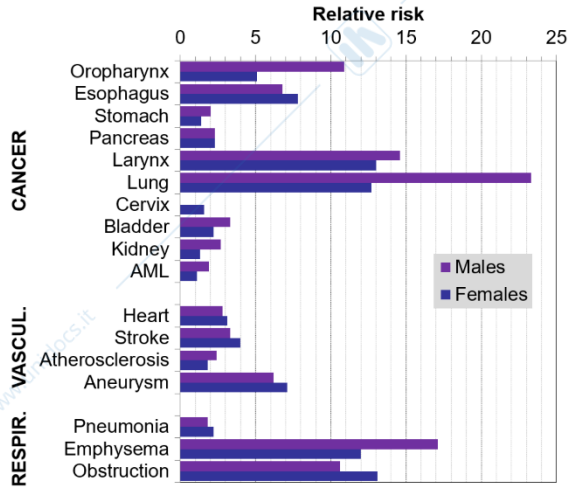


Figure 18.1. Some cancer-causing chemicals in tobacco smoke. From Kleinsmith et al., Understanding cancer, http://www.cancer.gov/cancertopics/understandingcancer/cancer

Figure 18.2. Relative risk of death in smokers, by disease. USA 1995-1999, data from Thun & Henley, *in* Schottenfield & Fraumeni, Cancer Epidemiology and Prevention, Oxford University Press.



Main tobacco carcinogens are polycyclic aromatic hydrocarbons, e.g. benzo[a]-pyrene, and amines, e.g. β-naphthylamine, nitrosonornicotine (NNN) and nitrosaminoketone (NNK). About 50% of NNN is already present in cured tobacco, hence it is also present in smokeless tobacco products. Nicotine is not classified as a carcinogen, but experimental results demonstrate weak initiating and promoting activities. Other carcinogens in tobacco smoke include benzene, formaldehyde, heavy metals and polonium-210. Tobacco smoke also contains co-carcinogens and promoters, moreover it damages respiratory tissues, causing chronic inflammation and cell proliferation. Synergistic or sub-synergistic carcinogenic effects are caused by exposure to alcohol (oropharynx), asbestos and radon.

About one-third of all male tumors and one-fourth of female tumors are attributed to tobacco use, which causes many tumor types in addition to lung cancer, not only in tissues directly exposed to smoke (upper aerodigestive tract), but also in the urogenital system, in particular kidneys and bladder (aromatic amines) and in the hematopoietic system (acute myeloid leukemias, mainly attributed to benzene). Relative risks for smokers are very high, more than 10 for oropharyngeal and laryngeal tumors, more than 20 for lung cancer (Figure 18.2). Here, we are only dealing with cancer, but strong risk increases are registered also for cardiovascular diseases, such as aneurysm, heart attack and stroke, and for obstructive respiratory diseases and emphysema (Figure 18.2).

At the beginning of 20th century, cigarettes were not popular, and lung cancer was rare. Male cigarette smoking increased during World War I (WWI), when cigarettes and alcohol were freely distributed to soldiers in the trenches, and further increased between the wars and during WWII (Figure 18.3). In the 1950s about two-thirds of European and American males were cigarette smokers, then they gradually began to quit smoking. In the 2010s, the percentage of male smokers in most Western countries is around 20%. The incidence of lung cancer trailed the trends of cigarette smoking, with a delay of 20-40 years, i.e. the average latency of lung cancer in smokers. The female population took up cigarette smoking much later than males, mainly after WWII, but the downward trend began much later, after year 2000; currently, the proportion of female smokers is similar to that of males. Having about 20% of smokers in the population is clearly better than what happened in the past, but it still a very high proportion, leading to a dramatic number of cancer cases. The trajectories of cigarette smoking and lung cancer in many countries with a low human development

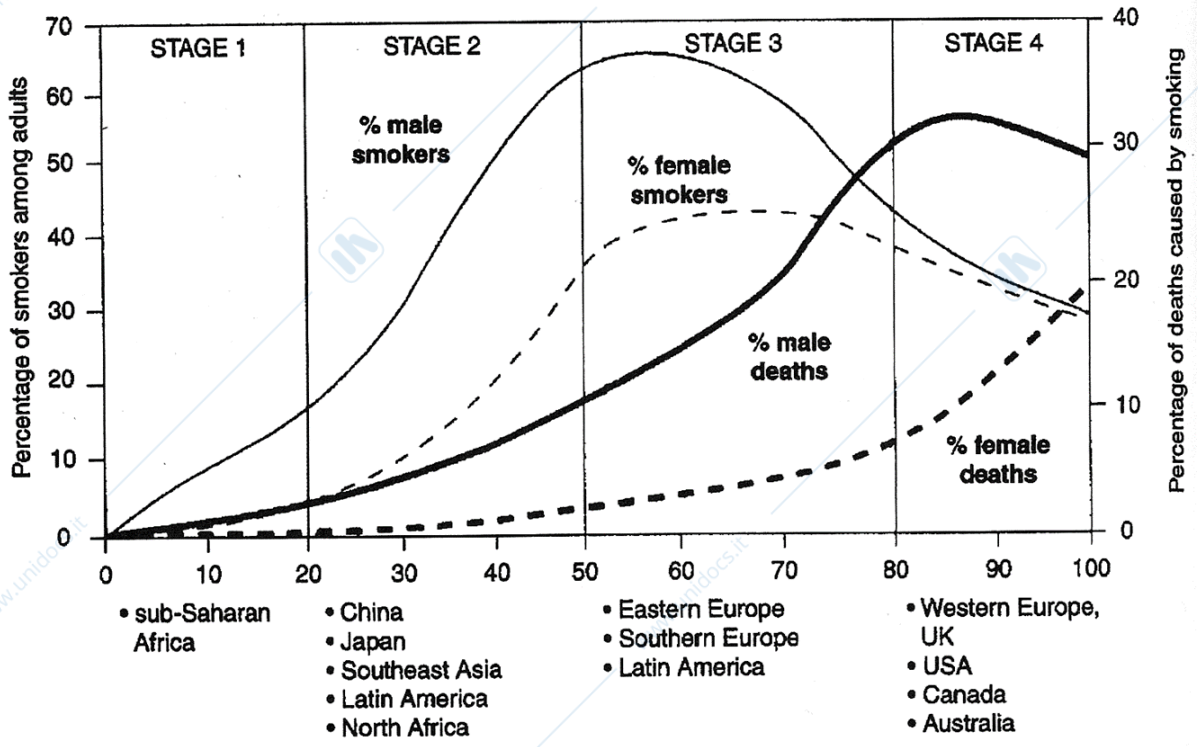


Figure 18.3. Smoking trends and mortality. From Thun & Henley, *in* Schottenfield & Fraumeni, Cancer Epidemiology and Prevention, Oxford University Press.

index resemble those of Western countries, with a delay measurable in years or decades. Some countries, especially in Africa, are still at the beginning of these deadly curves (Figure 18.3) and have the possibility to pursue a different destiny.

The damage inflicted to the body by smoking is not permanent (Figure 18.4). In ex-smokers, the cancer-promoting effects cease, and even the mutation burden decreases, because cells bearing mutations can be replaced by newer cells with an

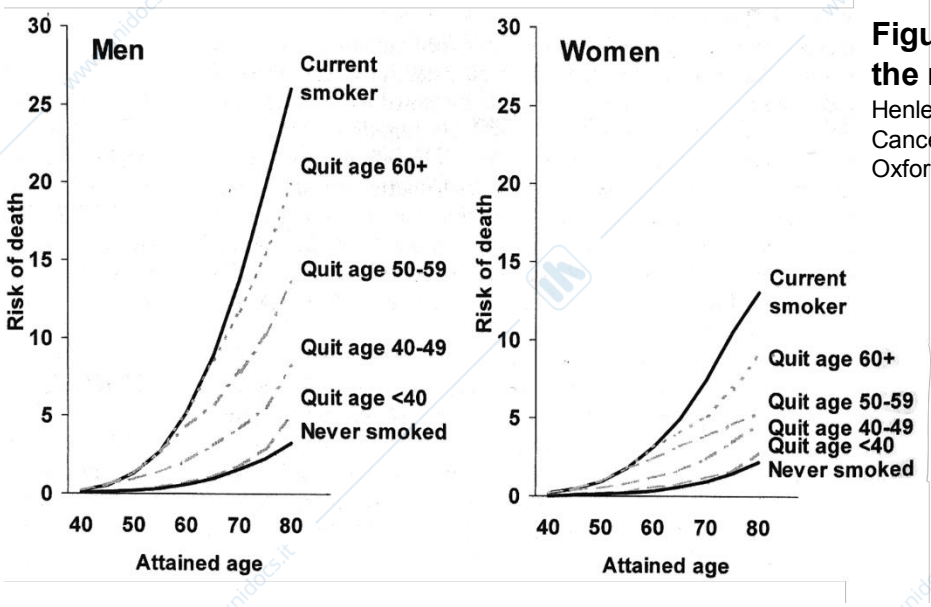


Figure 18.4. Quitting reduces the risk of death. From Thun & Henley, *in* Schottenfield & Fraumeni, Cancer Epidemiology and Prevention, Oxford University Press.

intact genome. Thus, quitting strongly reduces cancer risk: the advantage is inversely proportional to the total number of cigarettes, or years of smoking. For those who quit at a very young age, the risk of smoking-related death becomes very low.

In addition to cigarette smokers, many others are exposed to tobacco. First, indirect cigarette smoke is harmful, increasing the risk of cancer by about 20% in people who spend many hours in the company of smokers. These data led to the ban of smoking in working places and public venues. Pipe and cigar smoke, which is not usually inhaled, increase the risk of cancer in the upper aerodigestive tract. Smokeless tobacco uses, such as chewing or "snuffing", increase the risk of cancer in the exposed anatomical locations, such as the nose or the mouth, because carcinogenic nicotine derivatives, such as NNN, are present in all tobacco products. All over the world, combinations of tobacco and other substances (e.g. betel or areca in India) used for recreational purposes were invariably found to be carcinogenic. In summary, all tobacco exposures are carcinogenic.

Tobacco substitutes

Various substitutes are now available to administer nicotine:

- Nicotine chewing-gums
- Nicotine patches
- Electronic nicotine delivery systems, i.e. electronic cigarettes, "e-cigs"
- Electronic cigarettes containing tobacco

In principle (as long-term data, especially for electronic cigarettes, are not yet available), tobacco-free substitutes strongly reduce the risk of cancer and respiratory diseases, but not the cardiovascular risk and the psychotropic effects of nicotine. The studies on the safety of electronic cigarettes are still in progress. Basically, electronic cigarettes heat and dispense an aqueous solution of pure nicotine with additives to make smoke and optionally some flavoring. Apart from the effects of nicotine, the components should be relatively harmless, but some studies reported the formation of carcinogenic nicotine derivatives, such as NNN, also in the smoke of some electronic cigarettes, as a result of heating and chemical reactions with additives.

Are electronic cigarettes good or bad? Early studies show that e-cigarettes can indeed help smokers to overcome addiction and quit completely, however many become "dual users", still smoking conventional cigarettes at home, resorting to electronic cigarettes where smoking is forbidden, or not socially acceptable. Even dual use is an improvement because the number of conventional cigarettes is significantly reduced. On the other hand, especially in the US, a rising proportion of teenagers use electronic cigarettes, which is worrying, because they can become nicotine addicts, and easily switch to conventional cigarettes in the future. As younger generations seem to be attracted by flavored e-cigarettes, the FDA is trying remove this incentive by banning flavored products.

Alcoholic beverages and cancer

Alcoholic beverages are carcinogenic, they are classified as group 1 by the IARC.

The highest risk of tumor development is for anatomical sites in direct contact with alcohol, such as the oropharynx and the esophagus (Table 18.1). Smaller increases are found for cancers of the liver, the intestine and the breast, however the

Table 18.1. Alcohol and cancer

Cancer§	RR*	Deaths°
Oropharynx	5.1	2347
Larynx	2.7	745
Esophagus	5.0	2716
Liver	2.1	2699
Intestine	1.4	5467
Breast	1.6	7310

§Data from LoConte et al., J. Clin. Oncol., 35: 2017 (relative risks) and from Nelson et al., Am. J. Public Health, 103:641 (deaths).
*Relative risk
°Attributable yearly deaths in the USA.

frequency of these tumors is very high, thus even small risk increments generate large number of cases, for example the highest number of attributable cases is for breast cancer. Breast cancer is thus the most sensitive index of the carcinogenicity of alcoholic beverages; large epidemiological studies have shown that the risk of cancer is directly proportional to ethanol consumption, even one or two glasses of wine per day are sufficient to significantly increase the risk of breast cancer.

Ethanol is directly responsible for most effects of alcoholic beverages on human health, including carcinogenesis. Ethanol is converted by dehydrogenases to acetaldehyde (Figure 17.8), which can directly bind DNA and cause mutations; in addition to this initiating activity, ethanol is also a promoter, because it kills cells, causing epithelial proliferation. Alcoholic beverages contain variable proportions of many other molecules, some toxic, some beneficial (e.g. resveratrol in red wine), however their effect is negligible in comparison to ethanol, which is also responsible for the reduced risk (about 20%) of cardiovascular diseases. Considering all the effects of alcoholic beverages, the standard medical advice is to drink with moderation, limiting alcohol consumption to 15 (women) – 30 (men) grams of ethanol per day, the equivalent of one-two drinks per day.

Table 18.2. Increased risk of cancer per 5 Kg/m^2 increase in BMI. Red: statistically significant increase. From Kandekar et al., Nat. Rev. Cancer, 11:886, 2011

Cancer type	Men (95% CI)	Women (95% CI)
Breast		1.12 (1.08–1.16)
Colon	1.24 (1.20–1.28)	1.09 (1.05–1.13)
Endometrial		1.59 (1.50–1.68)
Oesophageal	1.52 (1.33–1.74)	1.51 (1.31–1.74)
Kidney	1.24 (1.15–1.34)	1.34 (1.25–1.43)
Leukaemia	1.08 (1.02–1.14)	1.17 (1.04–1.32)
Melanoma	1.17 (1.05–1.30)	0.96 (0.92–1.01)
Myeloma	1.11 (1.05–1.18)	1.11 (1.07–1.15)
Non-Hodgkin's lymphoma	1.06 (1.03–1.09)	1.07 (1.00–1.14)
Pancreatic	1.07 (0.93–1.23)	1.12 (1.02–1.22)
Prostate	1.03 (1.00–1.07)	
Rectal	1.09 (1.06–1.12)	1.02 (1.00–1.05)
Thyroid	1.33 (1.04–1.70)	1.14 (1.06–1.23)

Diet and physical activity

The complex and individual nature of human diet makes it difficult to precisely define and measure its pro- and anti-carcinogenic activities. Various estimates attribute to the diet a large proportion (15-35%) of all human tumors.

Body mass index (BMI), i.e. the ratio between weight and the square of height (Kg/m^2), of human populations is increasing worldwide. In Italy, 10% of the population is obese (BMI higher than 30) and 30% is overweight (BMI 25-30), similar values are recorded in the rest of Europe, whereas in America the percentages of obese + overweight individuals are much higher, around 70%. An increase in BMI by 5 Kg/m^2, i.e. a transition from normal weight to overweight, or from overweight to obesity, correlates with a significantly higher risk of cancer

(Table 18.2). Two major phenomena emerge from the studies on BMI and cancer, *a*) obesity is a multiorgan carcinogen, which affects not only the digestive systems, but most organs throughout the body, in fact excess tumors include breast cancer, melanoma, lymphomas and leukemias, kidney and thyroid cancers; *b*) in comparison to the risks of smoke (>10) and alcohol (~5), the risks are much lower, mostly below 1.5. Even if the risks are low, the widespread increase in BMI values, which in most countries have been on the rise since the end of World War II, forecasts a continuing increase in the number of diet-related cancer cases worldwide.

Physical activity is obviously correlated with BMI, but many epidemiological studies have shown that physical activity *per se*, independently of BMI, reduces the risk of several tumors, including colorectal and breast cancers. In addition to the control of body weight, the cancer-preventive effect of physical activity is attributed to its antioxidant and anti-inflammatory properties, and to the general wellbeing of the individual.

Which foods are associated with the risk of cancer? A first line of evidence comes from the study of dietary habits. Note that such studies standardize the results by BMI, to avoid the confounding effects of overweight and obesity. Vegetarians and people eating seafood, but not meat (so called pescetarians) have lower cancer mortalities than carnivores (Figure 18.5).

Studies on the consumption of specific foods confirm the protective activity of non-starchy vegetables and fruits, which have a low-calorie density and contain antioxidants and vitamins. Dietary fiber is associated with a modest decrease in

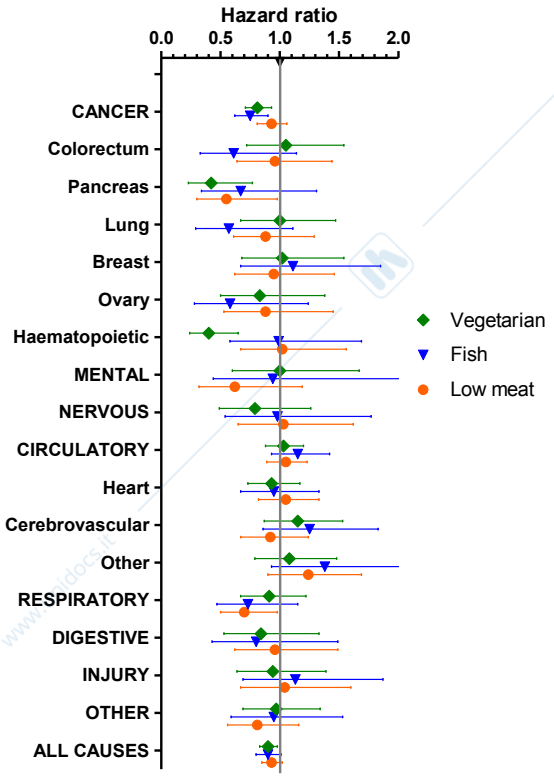


Figure 18.5. Mortality and diet. Risk of death by diet type in Oxford Vegetarian and EPIC-Oxford studies. BMI-standardized data of individuals who did not change the type of diet during the study. Reference group: meat 5 or more times weekly. Data from Appleby at al., Am. J. Clin. Nutr., 103: 218.

intestinal carcinogenesis, mediated by a faster intestinal transit of digested food and by the absorption of dietary carcinogens. Popular beverages, such as coffee, tea and mate, are also a source of plant anti-oxidants, and their use is mostly correlated with a reduction in cancer incidence; some compounds extracted from green tea (catechins) are being tested as cancer-preventive drugs. As these beverages are mostly consumed at temperatures ranging from warm to hot, it should be noted that the ingestion of hot liquids damages the esophagus (like alcohol) and increases the risk of cancer.

The pro-carcinogenic activity of meat is mostly associated with red meat (IARC

group 2A) and processed meat (IARC group 1); in addition to high-calorie density, also due to the associated animal fat, red meat has a higher iron content than white meat, which can contribute to oxidative stress (see previous chapter) and is mostly cooked using methods that produce PAH and other carcinogens (e.g. barbecue). Most processed meats contain preservatives (nitrates, nitrites) that can give rise to carcinogens in the digestive system. Note that 'processed' is an umbrella term that includes meats that are only cured with air drying and/or sodium chloride (e.g. Parma ham), which in principle should be less carcinogenic.

Milk and cheese share with meat the presence of animal fats, but are also important sources of calcium, which has some protective activity, possibly mediated by the precipitation of biliary acids; low-fat and no-fat dairies provide the best calcium/fat ratio.

Can we synthesize in a pill all the beneficial component of a healthy diet? Most studies of dietary supplementation with exogenous vitamins, antioxidants and minerals failed to show significant benefits in terms of cancer prevention. An infamous Swedish study aimed at preventing cancer in ex-smokers with beta-carotene actually increased the incidence of lung cancer; it was later shown that beta-carotene can promote carcinogenesis by acting on cell proliferation and cell differentiation. Some pro-tumor effects are also attributed to vitamin E and omega-3 fatty acids. To sum up, anti-cancer dietary supplements are not yet ready for prime time.

Table 18.3. Diet and cancer: General recommendations by the World Cancer Fund.

Be a healthy weight	Keep your weight within the healthy range and avoid weight gain in adult life
Be physically active	Be physically active as part of everyday life – walk more and sit less
Eat a diet rich in wholegrains, vegetables, fruit and beans	Make wholegrains, vegetables, fruit, and pulses (legumes) such as beans and lentils a major part of your usual daily diet
Limit consumption of 'fast foods' and other processed foods high in fat, starches or sugars	Limiting these foods helps control calorie intake and maintain a healthy weight
Limit consumption of red and processed meat	Eat no more than moderate amounts of red meat, such as beef, pork and lamb. Eat little, if any, processed meat
Limit consumption of sugar sweetened drinks	Drink mostly water and unsweetened drinks
Limit alcohol consumption	For cancer prevention, it's best not to drink alcohol
Do not use supplements for cancer prevention	Aim to meet nutritional needs through diet alone
For mothers: breastfeed your baby, if you can	Breastfeeding is good for both mother and baby
After a cancer diagnosis: follow our Recommendations, if you can	Check with your health professional what is right for you

In conclusion, the relationships between diet and cancer are complex, and the mechanisms are complicated, but health professionals need to convey clear messages to the general public, to promote a healthy diet, devoid of carcinogenic components. The World Cancer Fund tried to synthesize current scientific evidence in ten general recommendations (Table 18.3). The first nine are based on evidence discussed in the previous paragraphs, ten tenth deserves a specific comment. Clinical studies show that lean cancer patients who follow a healthy diet have a better survival, not only because they lack obesity-related comorbidities, but also because they respond better to drug therapies and develop less severe side effects. Therefore, the general recommendations on diet are even more important for cancer patients and survivors.

Pollution: air and water

Polluted air, notably urban air, can contain inorganic particles, carrying arsenic, asbestos, chromium, nickel, and gases or particles carrying organic carcinogens, such as benzene or benzo[a]pyrene.

Comparisons between city and country air are affected by confounding factors, e.g. tobacco use, income, diet, health programs, working habits, etc. As always, the most convincing data come from occupational exposures. For example, US truck drivers and railway workers (US trains have diesel engines) have a 50% increase in the risk of cancer [RR=1.47 (1.29-1.67)]. Urban populations exposed to particulate matter (PM_{10}, $PM_{2.5}$) show a 10-30% increase in the risk of lung cancer (especially adenocarcinomas), with an attributable fraction of 3-4%. Overall, the proportion of human cancer attributed to air pollution is 1%.

For what concerns water pollution, drinking water in developed countries is usually microbiologically and chemically pure. Aquifer pollutants, deriving from natural or anthropic sources, include arsenic, nitrates or halocarbons (produced by the reaction of organic pollutants with chloride-based potabilizers).

Arsenic is of particular concern, because high levels of arsenic in drinking water are associated with an increase in lung, bladder, kidney and skin tumors. Arsenic can come not only from anthropic activities, but also from natural sources, if aquifers pass through rocks rich in arsenic compounds. To remove arsenic from drinking water, modern water depurators can be fitted with dearsenification units.

Does studying reduce cancer mortality?

An inverse correlation is found between the years of education and cancer mortality. People with more than 15 years of education (i.e. university level) have overall cancer death rates less than half than people with less than 13 years (Figure 18.6). Clearly it is not education *per se* that reduces cancer mortality, but the causes and consequences of higher education, including census, access to better health care, healthy lifestyles, etc.

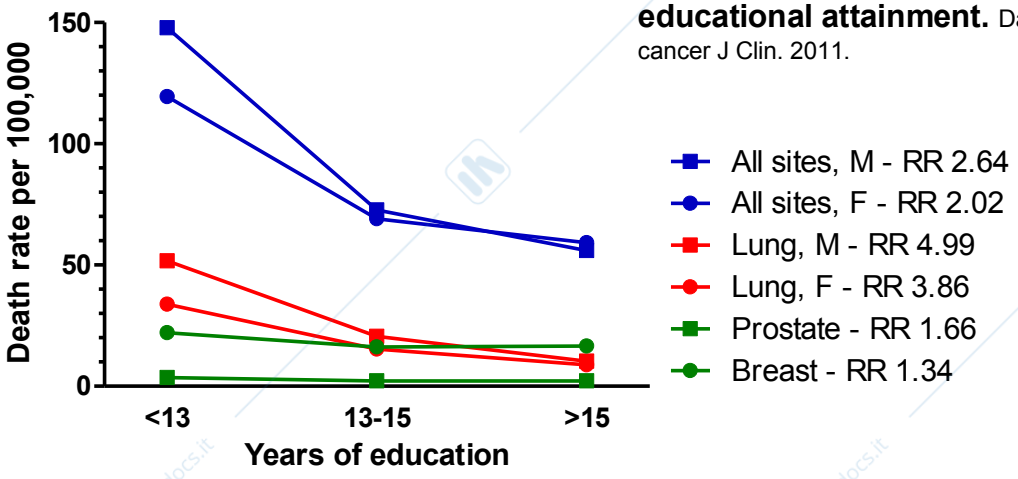


Figure 18.6. Cancer death rates by educational attainment. Data from CA cancer J Clin. 2011.

Chapter 19. Biological Carcinogenesis

About one in six human tumors is attributed to infectious agents. The responsible microorganisms are viruses, bacteria, parasites and cells from organisms of the same species. Just five microbes cause more than 95% of all infectious cancers. Hepatitis B and C viruses (HBV, HCV) cause liver cancer, human papilloma viruses (HPV) a variety of epithelial tumors, notably cervical carcinoma, Epstein-Barr virus (EBV) lymphomas and carcinomas, *Helicobacter pylori* gastric carcinomas and lymphoma (Table 19.1).

Most carcinogenic infectious agents cause a chronic infection, which can be asymptomatic or associated with non-neoplastic pathologies, e.g. inflammation, immune deficiency or autoimmunity. Tumors arise in a minority of infected subjects many years after the original infection. Carcinogenesis can be considered an accidental by-product of the biology of infectious agents, which tamper with the proliferative mechanisms of host cells, because they usually prefer proliferating to quiescent cells. Most carcinogenic microorganisms are necessary, but not sufficient to cause cancer; host cofactors (immune system polymorphisms), chemical carcinogens (smoking, diet), or other microbes are needed for tumor development.

Some specific mechanisms at work in this type of carcinogenesis were not previously encountered when dealing with radiations and chemicals. **Oncogenic gene transduction** occurs when viruses transfer an oncogene from one host to another. Bacteria can insert **oncogenic proteins** inside eukaryotic cells. **Insertional mutagenesis** happens when foreign transcriptional regulators, such as viral enhancer/promoters are inserted in the host genome near some cancer gene, causing its activation. Viral genome **integration** and **de-integration** in the host cell genome also contribute to genome instability and chromosomal aberrations. Microbial products present in the microenvironment or within the cell (e.g. viral nucleic acids) trigger defense mechanisms that may include the DNA damage response and the interferon response, which in turn lead to the arrest of cell proliferation and/or to apoptosis;

Table 19.1. Infection-related human tumors.
Modified from de Martel et al., Lancet Oncol., 2012.

Infectious agent	Cancer	Worldwide cases	
HBV, HCV	Liver	600 000	4.7%
HPV	Cervix, others	610 000	4.8%
Helicobacter pylori	Stomach	660 000	5.2%
EBV	Lymphoma, nasopharingeal ca.	110 000	0.9%
HHV-8	Kaposi	43 000	0.3%
Schistosoma hematobium	Bladder	6 000	<0.1%
HTLV-1	Leukemia	2 100	<0.1%
Liver flukes *Opistorchis, Clonorchis*	Cholangiocarcinoma	2 000	<0.1%
Infection-related		**2 033 100**	**16.1%**
Total cancers (2008)		12 662 500	100.0%

to counteract cellular defenses, some viruses encode proteins that **degrade tumor suppressor gene products**. **Tissue damage** is a general carcinogenic mechanism that in turn elicits cell proliferation and inflammation. Microorganisms can encode products that **modify the immune responses** of the host, thus contributing, either directly or indirectly, to carcinogenesis. The risk of tumor development is further influenced by interactions with **other microorganisms** and by **polymorphisms** of host immune response genes, which modify the severity of infection.

Viral carcinogenesis

Oncogenic viruses can be simply divided (in a microbiologically-incorrect manner) in four groups: retroviruses, hepatitis viruses, small and large DNA viruses.

The contribution of retrovirologists to molecular oncology and to biotechnology has been invaluable. The study of oncogenic retroviruses in the 1970s led to the discovery of many oncogenes, either transduced by the virus itself, or subject to insertional mutagenesis; then retroviruses were developed as vectors for gene transduction, *in vitro* and *in vivo*, and for gene therapy. However, the epidemiological relevance of retroviruses in human carcinogenesis is quite limited. Human immunodeficiency virus (HIV) is not carcinogenic *per se*, but it can be considered a co-carcinogen, because the ensuing immune deficiency (AIDS) favors the onset of tumors caused by other viruses. The only true human carcinogen is human T-lymphotropic virus 1 (HTLV-1), a deltaretrovirus with complex genome, similar to bovine leukemia virus (BLV). It is endemic in South Japan and the Caribbean, where it is transmitted by exchange of infected cells, prevalently through breastfeeding. After

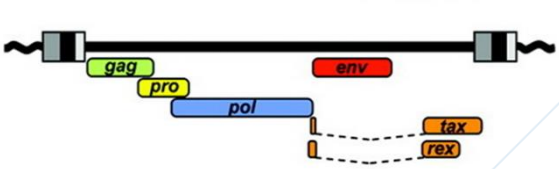


Figure 19.1. HTLV-1 genome. From Voisset et al., Microbiol. Mol Biol. Rev., 72: 157.

decades of asymptomatic infection, 2-7% of those infected may develop adult T-cell leukemia / lymphoma (ATLL) or a progressive myelopathy (tropical spastic paraparesis). Insertional mutagenesis does not seem to be involved in ATLL onset; the main responsible viral product is the trans-activating *tax* protein, homologous to HIV *tat*, which induces cell proliferation, alters the mitotic checkpoints, inhibits apoptosis, interferes with DNA repair and the mitotic spindle.

Hepatitis and cancer

Only two, molecularly unrelated, hepatitis viruses are carcinogenic, HBV and HCV. HBV is a small (3.2 Kb) DNA virus (Figure 19.2) that, like retroviruses,

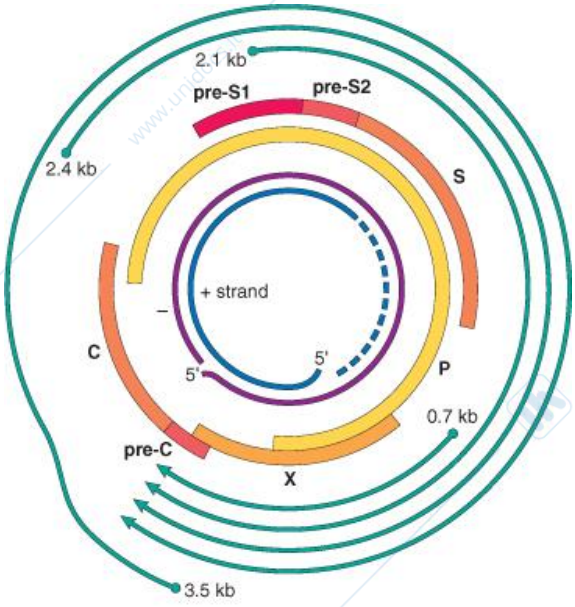


Figure 19.2. The HBV genome. Kumar et al., Le basi patologiche delle malattie, Elsevier.

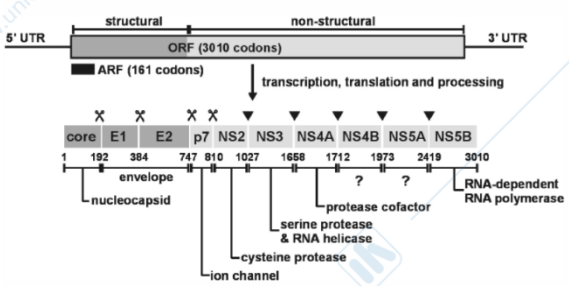


Figure 19.3. HCV genome and proteins. In the lower panel, numbers indicate the positions of the cleavage by cellular (*scissors*) and viral (*inverted triangles*) proteases. From Jin, Front. Biosci, 12: 222.

encodes a reverse transcriptase; it replicates through an RNA intermediate, which after retrotranscription can be integrated in the human genome; in Asia and Africa neonatal/juvenile infection was common, whereas in Western countries infection was prevalently caused by adult exchange of biological materials (e.g. blood transfusion); since the 1980s vaccination prevents infection and all ensuing pathologies. HCV is an RNA flavivirus (Figure 19.3) that does not integrate in the human genome. Infection is mainly by exchange of biological materials; no vaccine is available, but modern (highly expensive) antiviral drugs can eradicate HCV infections.

One common element determining the convergent natural history is the liver response to both viruses (Figure 19.4). Infection by HBV is mostly asymptomatic, only

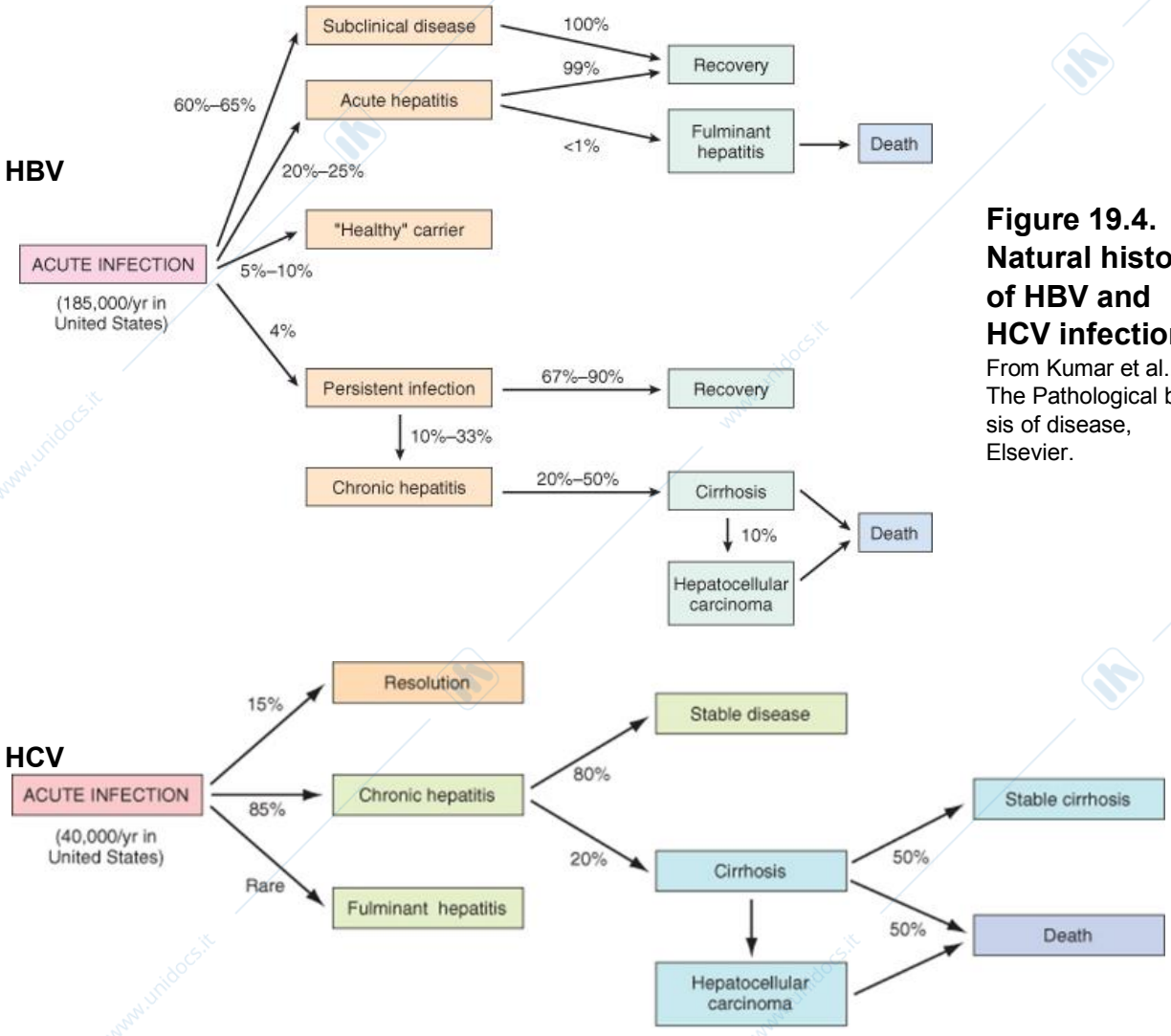


Figure 19.4. Natural history of HBV and HCV infections. From Kumar et al., The Pathological basis of disease, Elsevier.

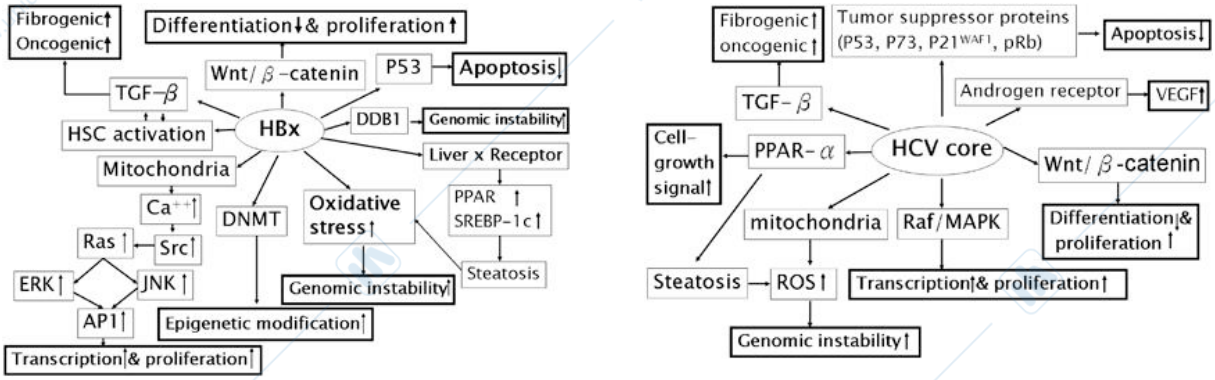


Figure 19.5. The pleiotropic activities of *HBx* and *core*.
From Tsai & Chung, Oncogene, 29: 2309.

detected by laboratory tests, or it determines an acute infection that resolves spontaneously; less than 5% of patients develop a persistent infection that becomes chronic hepatitis, in which chronic inflammation and viral activity can cooperate to induce a severe, sometimes deadly, fibrotic condition called liver cirrhosis, predisposing to the development of hepatocellular carcinoma. The natural history of hepatitis C is similar, but the proportion of patients developing chronic hepatitis is much higher, up to 85%, hence the risk of cirrhosis and carcinoma are correspondingly higher. Of note, HCV infection is also associated with the onset of B cell lymphomas.

Virus-intrinsic mechanism of HBV carcinogenesis include insertional mutagenesis and viral oncogenes. Insertional mutagenicity of Hepadna viruses is well known, a relative of HBV causes liver cancer in woodchucks mainly through the activation of the cellular Myc oncogene, but detailed human studies of HBV insertions are relatively recent. Most HBV^+ cancers have viral genome integrations, but only some integrations occur near cancer genes. TERT (telomerase) and cyclin E1 are among the most frequently affected cellular genes, furthermore, integration contributes to chromosomal instability. HBx is the major viral oncogene, it is a protein with multiple transactivating activities (Figure 19.5) that include the activation of cell proliferation through the interaction with MAPK, JNK, ERK, NFκB, JAK/STAT and transcriptional factors, and the inhibition of p53, DNA repair and apoptosis; HBx also activates the TGF-β pathway, which contributes to the fibrogenesis of cirrhosis. The pre-S region of the viral genome is also involved in the control of hepatocyte proliferation and in the progression to liver cancer. Chronic liver damage and regeneration are further risk factors of cancer development.

HCV has an oncogene called *core* which is unrelated to HBx, but has a similar range of activities (Figure 19.5): it modulates p21, p73, MAPK, JNK and NFκB, interacts with the signaling of various receptors (retinoids, lymphotoxin, PPAR-α), induces oxidative damage and activates TGF-β. Together with NS3 and NS5A gene products it inhibits p53. As for HBV, chronic liver damage and regeneration contribute to liver carcinogenesis by HCV.

If we now change our perspective to that of cancer risk factors, we can appreciate the relative importance of the viruses in the onset of hepatocellular carcinoma.

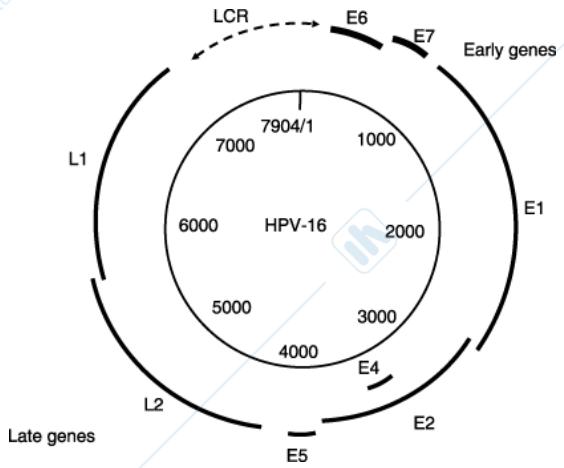


Figure 19.6. HPV.
From Narisawa-Saito & Kiyono, Cancer Sci., 98:1505 (genome) and Doorbar et al., Rev. Med. Virol., 25 (Suppl 1): 1 (electron micrograph).

In fact, about 80% of cases are attributed to either HBV or HCV chronic infection. The balance between acute and chronic infection is determined by the effectiveness of individual immune responses, which are also under genetic control, for example a polymorphism of an interferon-like cytokine, interleukin 28 (IL-28, also called IFN-λ3), determines the efficiency of viral clearance. The other major cancer risk factor is chronic tissue damage; 80%-90% of tumors develop after cirrhosis, which can be also caused by alcohol abuse; liver steatosis, a fatty degeneration caused either by alcohol or by excessive calorie intake, is also a risk factor, much less potent than cirrhosis, but more common in countries with a high incidence of obesity. Aflatoxin (see previous chapter) is a potent liver carcinogen, whereas coffee consumption is associated with a reduced risk. Some liver diseases also increase the risk of tumor development, including autoimmune hepatitis and primary biliary cirrhosis, and hereditary hemochromatosis, which causes iron accumulation.

Papilloma viruses

Human papilloma viruses (HPV) are small (8 Kb) DNA viruses with a circular, double stranded genome (Figure 19.6). Upon entrance in host cells, they first express non-structural, regulatory genes (early genes, denoted by "E"), then structural genes (late "L" genes) encoding capsid proteins. HPV, aided by lesions of epithelial integrity, infects the basal layer of epithelia (Figure 19.7); the expression of early genes interferes with normal cell proliferation and differentiation, and controls the expression of structural viral genes, with the end result that upper epithelial layers become virus factories, which actively release viral particles instead of dying by cornification. Evident cellular alterations are used for diagnostic purposes, e.g. in the PAP test (see chapter on cancer prevention). During early infection phases the virus is episomal, but in

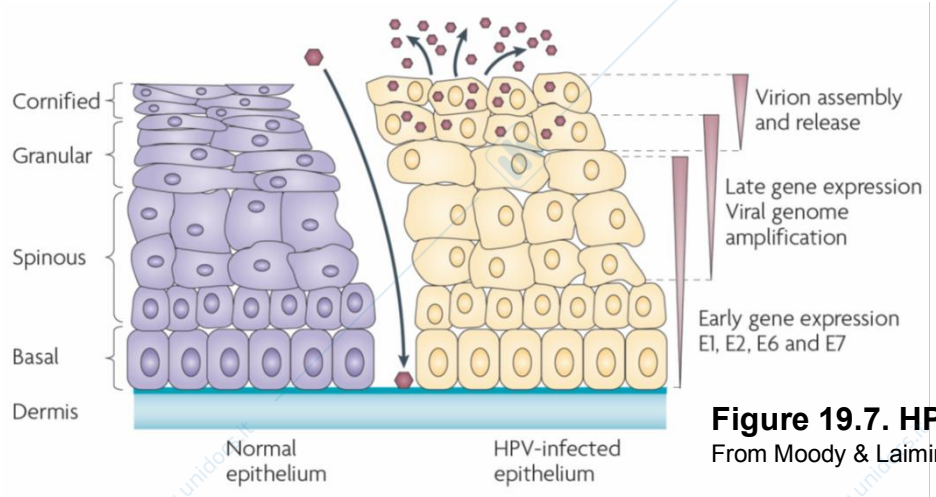
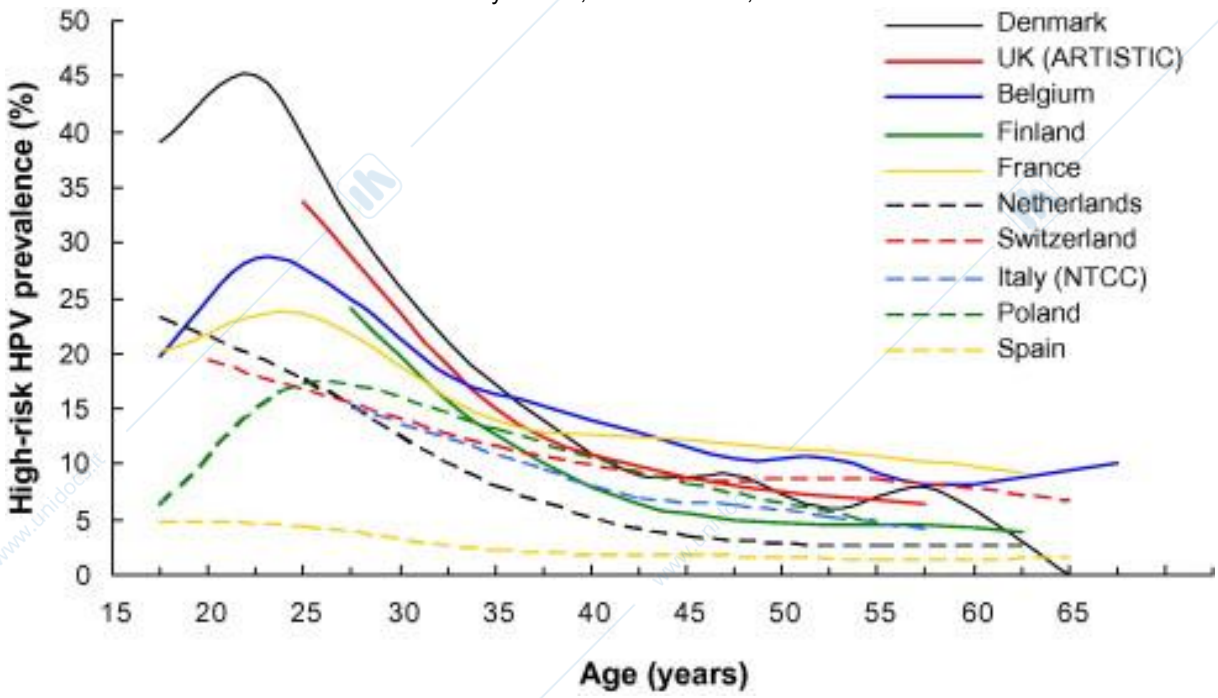


Figure 19.7. HPV infectious cycle.
From Moody & Laimins, Nat. Rev. Cancer, 10: 550.

Figure 19.8. Prevalence of high-risk HPV infections in Europe.
From De Vuyst et al., Eur. J. Cancer, 45: 2632.



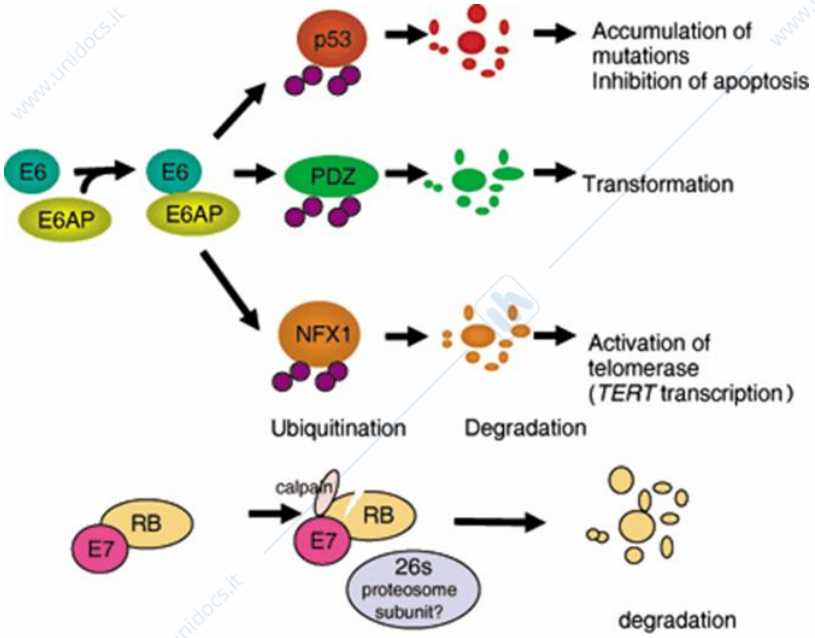
tumors it is also found integrated in the cell genome.

Pathological lesions induced by HPVs are epithelial tumors, ranging from benign (warts) to malignant (carcinomas, affecting especially the anogenital region). Carcinoma of the uterine cervix is the most frequent malignant tumor induced by HPV worldwide.

There are more than 100 HPV genotypes, with variable pathogenetic potency. They are broadly grouped as high-risk (HR) or low-risk (LR), depending on the ability to induce malignant tumors. The IARC classifies genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 as group 1 and genotypes 5, 6, 8,11 as group 2B.

Infection by high-risk HPV is sexually transmitted, prevalently in young age. Large regional / national variations in the prevalence of infection are dependent on several factors, including prevalent

Figure 19.9. HPV oncogenes E6 and E7.
From Narisawa-Saito & Kiyono, Cancer Sci., 98:1505.



sexual behaviors. In the long time, the immune system prevails, and most seropositive cases become negative with age (Figure 19.8). The infection persists indefinitely in ~10% of the population after 30-40 years of age. Vaccines prevent HPV infection (see chapter on cancer prevention), but at present there is no effective therapy of chronic HPV infection.

Three oncogenes are encoded by HPV, E6, E7 and E5 (Figure 19.6). E6 inactivates p53 (Figure 19.9): its protein product activates a cellular ubiquitin ligase (UBE3A, also called E6-AP) forming a ternary complex with p53, which is ubiquitinated and degraded. E6 also has p53-independent transforming activities, because the E6-UBE3A complex can lead to the degradation of other cellular proteins, leading for example to the activation of telomerase.

E7 inactivates RB (Figure 19.9), its product promotes C-terminal degradation of pRB by the cellular protease calpain, then pRB is degraded by the proteasome; most E7 activities are mediated by the inactivation of RB family proteins.

E5 has oncogene-like activities: it stimulates cell growth through the formation of molecular complexes with growth factor receptors (EGFR, PDGFRB, CSF1R); E5 is not necessary for tumor progression, as attested by its frequent deletion in the advanced phases of carcinogenesis, when the HPV genome integrates in the cell genome.

Cofactors contributing to HPV carcinogenesis include indirect factors, such as sexual behavior, which contributes to infection, and molecular factors directly affecting cell transformation. The progression of early neoplastic lesions to full-fledged cervical carcinoma is a multi-step process which requires alterations in **cellular cancer genes**, such as RAS, MYC, HER-2 and PTEN. Involvement of the **immune response** in HPV carcinogenesis is attested by the high incidence of HPV-related tumors in immunodeficient patients (see chapter on tumor immunology); polymorphisms of immune response genes, such as HLA or cytokines, also determine individual variation in the risk of HPV-related cancer. Exogenous **carcinogens** are also involved, tobacco smoke increases the risk of cervical cancer, possibly through the accumulation of carcinogenic metabolites in cervical mucus; oral contraceptives increase the risk of cervical carcinoma in HPV-positive women; sun exposure increases the risk of skin tumors.

The discovery the causal role of HPV has revolutionized the prevention of cervical cancer. In the past we could only detect already existing neoplastic lesions, now we can altogether prevent infection and its neoplastic consequences with vaccines. Furthermore, mass screening, which was based on the discovery of cytological alterations caused by viral interference with cell proliferation and differentiation, now is turning to molecular tests which reveal the presence of viral genomes.

Epstein-Barr virus

Epstein-Barr virus (EBV) is a gammaherpesvirus (HHV-4) with a large, double-helix circular genome of 172 Kb. EBV infection is ubiquitous, affecting 90% of the world population. Infection occurs in young age (earlier in low-development than in high-development countries) and persists for life. EBV causes a variety of diseases worldwide. The virus infects both epithelial cells and B lymphocytes (Figure 19.10). Non-malignant diseases include infectious mononucleosis, a mild,

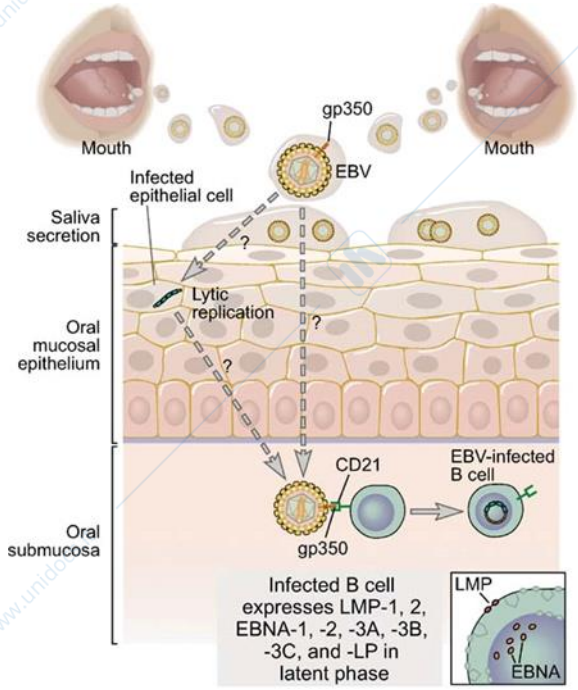


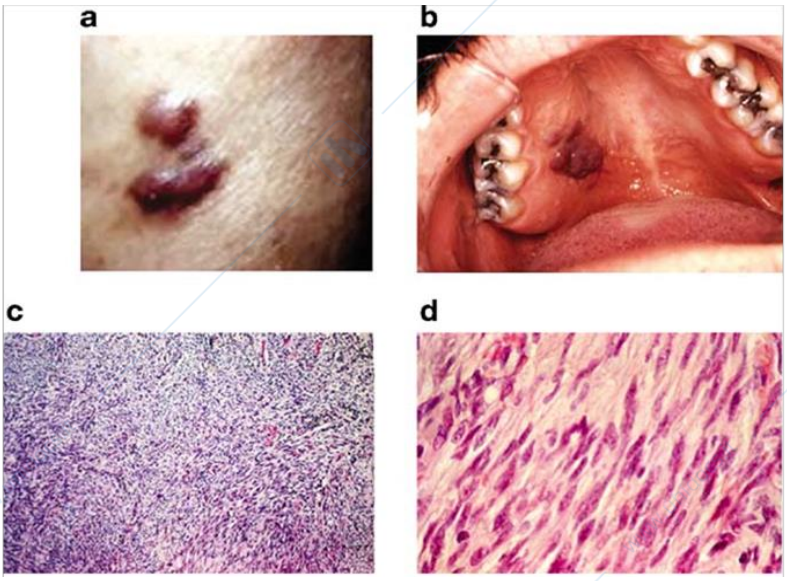
Figure 19.10. EBV infection. From Kutok & Wang, Annu Rev Pathol Mech Dis, 1: 375.98:1505.

flu-like syndrome, and oral leukoplakia, which mainly affects immunodeficient patients. Malignancies include Burkitt lymphoma (the endemic type occurring in Africa is totally attributed to EBV), nasopharyngeal carcinoma (the non-keratinizing type occurring in Asia is also totally attributed to EBV), B cell lymphomas arising worldwide in immunodeficient patients and one-third of Hodgkin lymphoma cases. Altogether these tumors are relatively rare, the discovery of EBV involvement in 5%-10% of gastric cancer cases makes it the most frequent tumor caused by EBV.

The mechanisms of EBV carcinogenesis were mostly investigated in Burkitt lymphoma, other tumors are less thoroughly characterized. Normal B cells cultured *in vitro* in conventional media rapidly undergo apoptotic death, but EBV infection immortalizes B cell. This can be used as a biotechnological tool to produce with high efficiency human lymphoblastoid cell lines (LCL), for example to preserve living samples from patients affected by rare genetic diseases. EBV persists episomally in the host, a phase called latency, expressing a limited set of genes (Figure 19.10); immortalization of host cells is caused by latent phase proteins: latent membrane proteins (LMP) 1 and 2A and Epstein-Barr nuclear antigens (EBNA) 1, 2, 3C and LP. LMP1 mimics T help signaling through CD40-CD40L, while LMP2A promotes antigen-independent B cell survival. EBNAs promote the transcription of LMPs and of cellular oncogenes, and inhibit apoptosis.

EBV infection is ubiquitous, but region-specific carcinogenesis indicates that EBV *per se* is not sufficient to cause cancer, and that environmental and/or host cofactors are needed. For African Burkitt lymphoma, the main cofactor is malaria, as shown by the colocalization of the two diseases in the central part of Africa. The natural history of endemic Burkitt lymphoma begins with childhood infection by EBV, which immortalizes B cells, then malaria and other parasites cause a chronic stimulation of B cell lymphopoiesis, which favors the onset of chromosomal aberrations, in particular the translocation of the MYC oncogene (see chapter on oncogenes) under the transcriptional control of immunoglobulin genes, leading to unrestricted B cell proliferation and lymphoma development. Immunological cofactors are also important, as demonstrated by the frequent onset of B cell lymphomas in immunodeficient patients, and by correlations between lymphoma risk and HLA polymorphisms. The cofactors involved in the genesis of nasopharyngeal carcinoma are less known, possibly including dietary habits, such as the consumption of salt-

Figure 19.11. Kaposi sarcoma. Macroscopic lesions (*a*, *b*) and cellular aspects (*c*, *d*). From Ganem, Annu Rev Pathol Mech Dis, 1: 273.



cured fish and vegetables cooked in pans (*wok*) that release carcinogenic fumes; alcohol and tobacco use also raise the risk of nasopharyngeal carcinoma.

Kaposi sarcoma herpesvirus

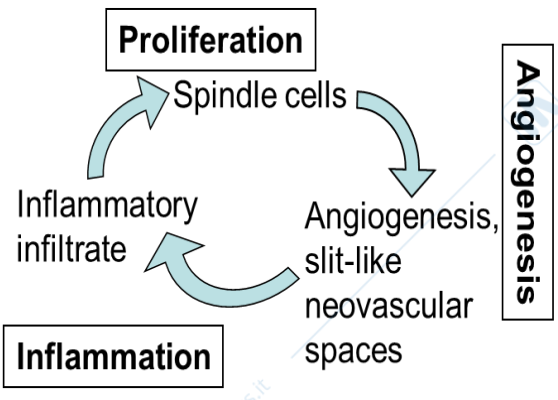
Kaposi sarcoma virus (KSHV / HHV8) is a gammaherpesvirus with a double-helix DNA genome. Kaposi sarcoma (Figure 19.11) is a multifocal vascular tumor frequently arising in immunodeficient patients, in fact one of the early clues that led in the early 1980s to the discovery of a novel immune deficiency syndrome, i.e. AIDS, was an unusual cluster of Kaposi sarcoma cases among homosexual men in California and New York. Kaposi sarcoma is rare in immunocompetent individuals, higher incidence zones are the Mediterranean area, Eastern Europe and Africa.

KSHV carcinogenesis has various features in common with EBV: KSHV is also associated to some B lymphoproliferative disorders (Castleman disease and primary effusion lymphoma); some of its latency proteins induce cell proliferation, block p53 and inhibit apoptosis; co-carcinogenic factors include immunodeficiency and immune system polymorphisms. Specific elements that differentiate KSHV from EBV carcinogenesis are the limited immortalizing activity, because Kaposi sarcoma proliferation is dependent on paracrine circuits (Figure 19.12), and the incomplete latency: in a minority of cells the lytic cycle is switched on, leading to the expression of viral genes that could contribute to neoplastic transformation.

Other human oncogenic retroviruses

Are there further human oncogenic viruses? Many lines of investigation have led to a number of false positive results, and to the coinage of the term "rumor virus", a pun with "tumor virus", applied to viruses indicated as carcinogenic by insufficient or disputable scientific evidence.

Figure 19.12. Paracrine interactions in Kaposi sarcoma.



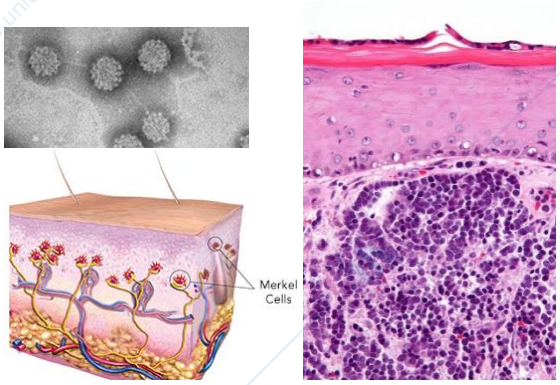


Figure 19.13. Merkel cell virus and Merkel cell carcinoma. Electron micrograph from RM Schowalter, PLoS Pathog. 2013 Aug; epidermis with Merkel cells from the Skin Cancer Foundation, © by Paul Nghiem & Quade Medical Group; histology by Nephron - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=16883126

Modern, highly sensitive molecular techniques allow the discovery of viral sequences either in human tumors or in tumor cells cultured *in vitro*. In most cases, such viruses are not causal agents, but simply freeloaders that exploit the fact that proliferating tumor cells are ideal hosts for opportunistic viral infections. Experimental evidence of neoplastic transformation of non-permissive cells, i.e. cells not allowing viral replication, for example cells of a different animal species, also does not demonstrate carcinogenicity for the natural host, because in replication-permissive cells the virus could be cytotoxic, rather than carcinogenic. In summary, the demonstration of the causal role of a novel virus in human carcinogenesis requires strong mechanistic and clinical evidence that is only rarely obtained.

Some prime examples of rumor viruses can be found among retroviruses, for example XMRV, a true novel retrovirus that originated in a retroviral research laboratory (a common occurrence, which in the past contributed to the discovery of oncogenes) and contaminated human prostate cancer samples, leading to the false claim of an infectious origin of prostate carcinoma. Other unsubstantiated claims concern the existence of human mammary tumor viruses as causes of breast cancer; such viruses do indeed exist in the mouse (MMTV), where they are transmitted from the mother to the newborn with lactation, but no convincing evidence of a human counterpart was ever obtained.

Coming to DNA viruses, an infamous example was the claim that cervical carcinoma is caused by the herpes simplex virus (HSV), antedating the demonstration by Harald zur Hausen that the true cause is HPV, possibly caused by the coexistence of HSV and HPV infections in tumor samples from sexually active women. Simian virus 40 (SV40) is a simian and human polyomavirus encoding an oncogene (large T) which inhibits both p53 and pRB; SV40 transforms rodent cells, but a carcinogenic role in humans is controversial, to say the least. A large accidental human experiment was made in the USA in the 1950s, when SV40 contaminated some batches of polio vaccine administered to the population; long-term follow-up did not reveal any excess cancer incidence in people who received the contaminated vaccine, thus providing a strong evidence against the human carcinogenicity of SV40.

The only *bona fide* new human cancer virus discovered in this century is Merkel cell virus (MCV), a polyomavirus that causes 80% of Merkel cell carcinomas, a rare skin tumor deriving from the eponymous mechanoreceptors (Figure 19.13). Like other polyomaviruses, MCV encodes a large and a small T antigen that are

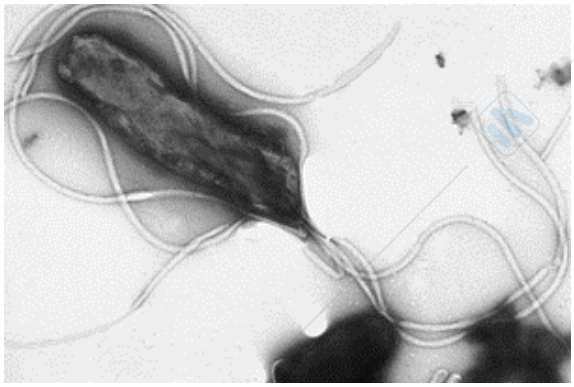
involved in neoplastic transformation. Non-viral cofactors include UV exposure and immune deficiencies.

Helicobacter pylori and other carcinogenic bacteria

Helicobacter pylori (Figure 19.14) is a flagellated, gram-negative epsilonproteobacterium that colonizes the human stomach. Infection is caused by orofecal human transmission, no intermediate host is known. A large proportion of the population is infected worldwide; in countries with low hygiene levels, infection is contracted during childhood, and prevalence is 80%-100%, whereas in countries with higher hygiene levels infection is contracted later in life and prevalence is much lower (50%-70%) and declining. Infection is chronic, unless the bacterium is eradicated by antibiotic treatments.

The optimum pH for *H. pylori* is neutral (6-8), which is obviously not the case in the human stomach, thus the bacterium encodes a urease which produces urea to neutralize the acid environment. Most infections are asymptomatic, actually *H. pylori* is considered a beneficial human symbiont, but in some cases it causes chronic gastritis, which can progress to peptic ulcer, then to gastric carcinoma or lymphoma.

Figure 19.14. *Helicobacter pylori*.
Picture by Tsutsumi, http://info.fujita-hu.ac.jp/~tsutsumi/

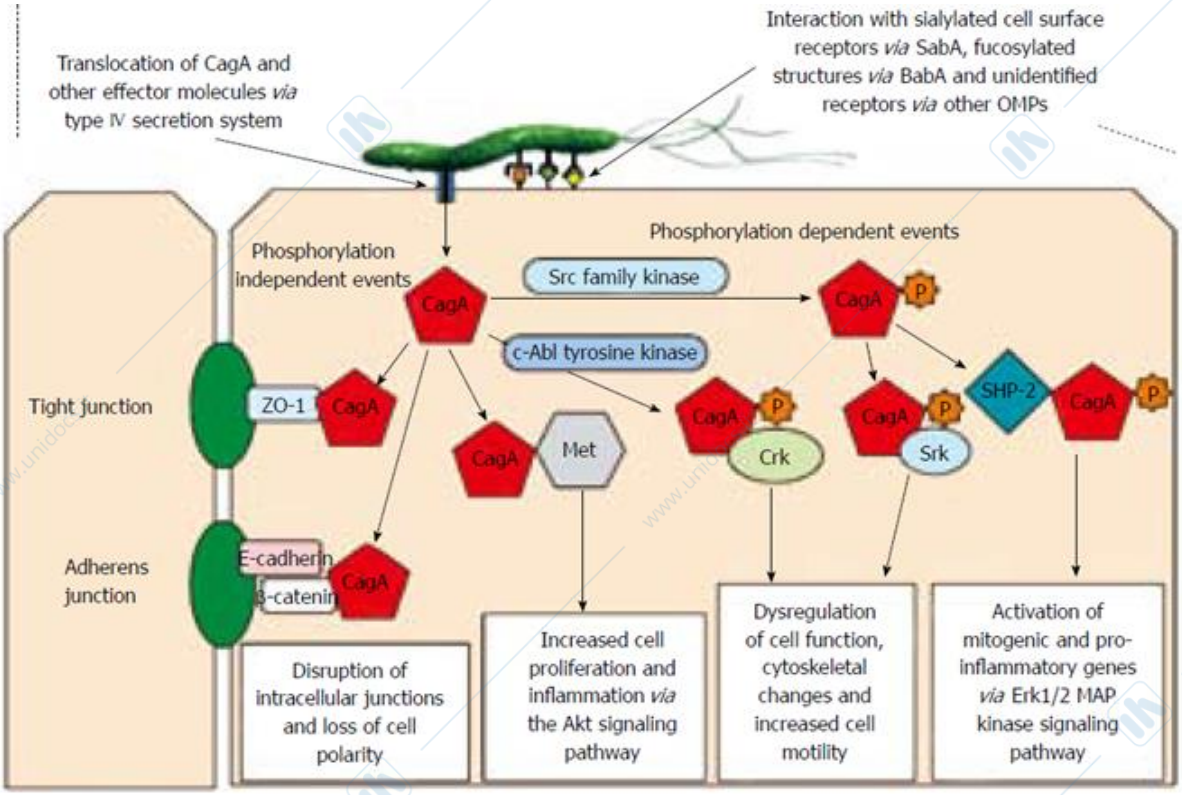


What is the difference between asymptomatic and pathogenetic infections? *H. pylori* genome is highly unstable, because the bacterium lacks mismatch repair, and is transformation-competent, i.e. it is prone to horizontal gene transfer through the uptake of genetic material from the environment. Frequently, multiple genetic variants coexist and compete within the same human host. Some bacterial strains harbor a 40 Kb genomic region called *pathogenicity island*, whose presence correlates with human diseases, which encodes the CagA protein and the molecular machinery to inject CagA into human cells. The percentage of CagA-positive bacterial isolates in different regions of the world is variable, Asian isolates are mostly positive, whereas only 30%-40% of Western isolates are positive. Variants of the VacA and BabA bacterial proteins also correlate with human diseases.

CagA (Figure 19.15) is an oncoprotein. Once inside human cells, CagA stimulates cell proliferation through the activation of the RAS-MAP kinase pathway via SHP2, of the WNT/β-catenin pathway and of the EGF receptor, and alters intercellular junctions, causing loss of epithelial polarity and of cell-cell adhesion. A formal demonstration of its oncogenic potential comes from CagA transgenic mice, which are prone to the development of carcinomas and lymphomas.

The existence of relevant co-carcinogenic factors is highlighted by epidemiological evidence, as only 1-2% of infected individuals develop tumors, and tumor incidence in males is always higher than in females. There are also strong, unexplained geographic differences, for

Figure 19.15. Molecular interactions of the CagA oncoprotein inside human cells.
From Dunne et al., World J Gastroenterol 20: 5610.



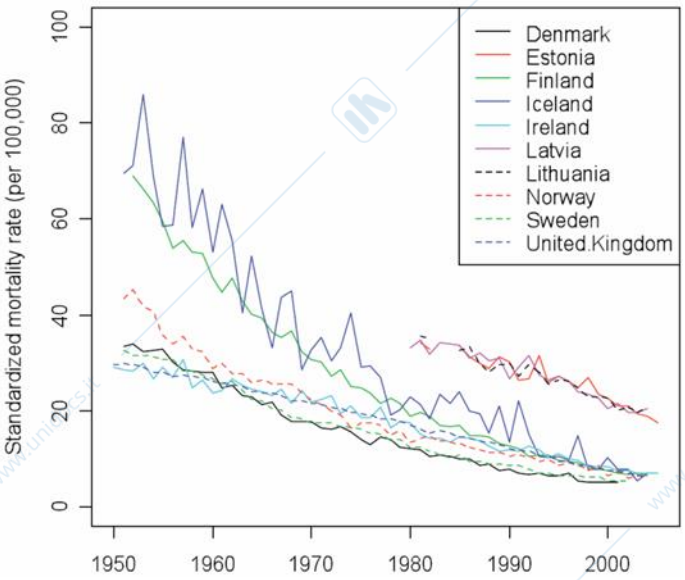
example stomach cancer is relatively rare in Africa, despite a very prevalence of *H. pylori* infection (the so-called "African enigma").

Inflammation and **immunity** are important cofactors. *H. pylori* infection is pro-inflammatory, and polymorphisms in cytokine (IL-1β, IL-8, IL-10, TNF-α) and immune response (HLA, TLR) genes strongly modulate cancer risk, with very high relative risks, up to 27, which is on a par with the risk of strong carcinogens, such as tobacco smoke. Concomitant parasite infestations can induce a type 2 helper (Th2) polarization of immune responses, which down-modulate inflammatory cytokines and reduce cancer risk; as parasites are common in Africa, this is invoked as possible explanation of the African enigma.

Food hygiene reduces the risk of infection. The reduction in gastric cancer incidence registered in Western countries (Figure 19.16) in the last 100 years is attributed mainly to improvements in food storage (refrigeration) and to the corresponding reduction in the use of carcinogenic preservatives (nitrites, nitrates). A vegetarian **diet**, rich in antioxidants, is also associated with a reduced risk of gastric cancer.

Gender differences are poorly explained, but are shared with other infectious neoplasms, such as liver cancer and ATLL. Possible explanations include hormonal factors and immunological differences between the immune systems of males and females. For example, immunity is profoundly modified during pregnancy, which can be considered as an

Figure 19.16. Gastric cancer mortality in Europe since 1950. From Boyle & Levin (eds.), World Cancer Report 2008, IARC.



allogeneic transplant that is not rejected; of note, during pregnancy concomitant inflammatory and autoimmune responses are somewhat quenched.

In addition to gastric cancer, *H. pylori* is also responsible for 90% of gastric lymphomas originating from the mucosa-associated lymphoid tissue (MALT). These are low-grade B cell lymphomas which in most cases depend on the continuing bacterial presence for their growth, in fact 70% of lymphomas regress after *H. pylori* eradication, in practice the only human tumor which can be effectively cured with antibiotics. In the remaining 30% of cases, neoplastic B cells are no longer dependent on antigenic stimulation and require more conventional anti-tumor therapeutic treatments.

Other potentially carcinogenic bacteria, associated with lymphoma onset, are *Chlamidophila psittaci* (MALT lymphomas of ocular annexes) and *Borrelia burgdorferi* (cutaneous lymphomas).

Recent advances in the study of the human microbiome (i.e. the collective genome of commensal bacteria) are beginning to show further correlations between specific bacteria and cancer risk, in particular for what concerns colorectal carcinoma and imbalances in the intestinal microbiome. In the near future, such correlations might lead to significant advancements in early cancer diagnosis and to novel preventive endeavors based on the administration of bacteria to restore an anti-carcinogenic microbiome.

Eukaryotic parasites

A few trematodes (flat worms, "flukes") are associated with a small number of cancer cases occurring in developing countries. *Schistosoma hematobium* infestation is contracted in Africa by contact with freshwater contaminated by snails; it damages the bladder epithelium and causes chronic inflammation, leading to the development of squamous bladder carcinoma. *Chlonorchis sinensis* and *Opistorchis viverrini* infestations in South-Eastern Asia are caused by the consumption of uncooked freshwater fish (raw, salted, marinated or fermented) containing live parasites; they colonize the gallbladder, causing cholangiocarcinoma through mechanisms involving the interaction of nitroso compounds of preserved fish with fluke secretions, tissue damage, and inflammation.

Tumor cell transplantation

Cell transplants, either normal or neoplastic, between MHC-different individuals of the same species are usually rejected by strong immune responses of the host. However, in some cases tumor cell

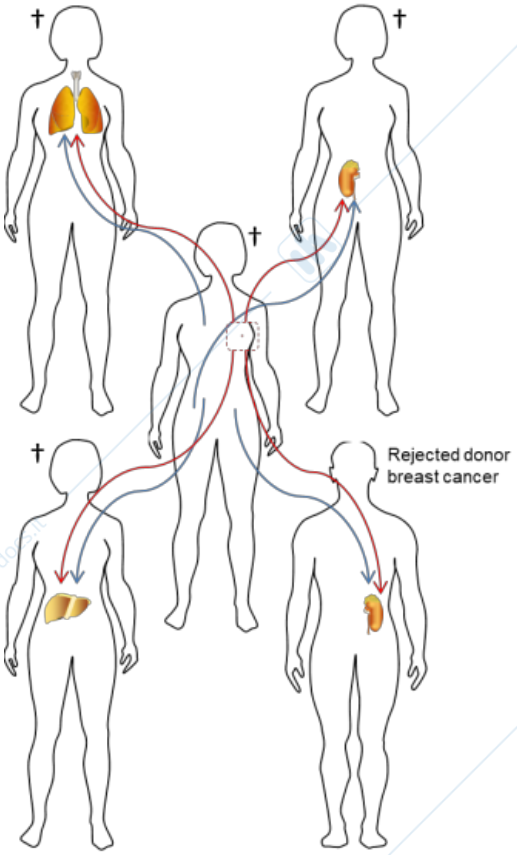


Figure 19.17. A case of human tumor transplantation. Organ transplants from a donor bearing an occult breast carcinoma led to the development of tumors in all recipients. From McCallum & Jones, PLOS Biol., 4: e342.

transplants are not rejected and give rise to neoplastic masses in the host.

Canine transmissible venereal tumor (CTVT) is a tumor that is passed from dog to dog during sexual intercourse. Phylogenetic analyses of CTVT genome indicate that it arose thousands of years ago in a dog ancestor, making it one of the oldest living beings on Earth. A more recent instance of natural transplantable tumor is the facial tumor of the Tasmanian devil, which develops at sites of bites received during fights between males; tumor development is relatively recent (a few decades) in comparison with CTVT, but it could bring to extinction Tasmanian devils, leading to the idea that, in the past, other animals species might have been brought to extinction by transplantable tumors. Other such tumors affect mollusks (clams and mussels) and are currently expanding worldwide.

Humans can be also affected by tumor transplantation as a consequence of organ transplantation (Figure 19.17). Oncologic patients are not eligible as organ donors, but if death occurs for traumatic reasons, such as a road accident, before the tumor is diagnosed in the donors, their organs can be implanted in several hosts, who will be exposed to the risk of tumor development. Nowadays, various organs from a single donor are implanted in different recipients, thus multiplying the risk. In one case, kidneys, lungs and liver from one woman affected by an occult breast cancer were implanted in four different recipients (Figure 19.17), three of which died of cancer, the only survivor was a male in which immunosuppressive therapy was withdrawn, leading to the rejection of both the implanted organ and the tumor.

Why do some tumor transplants grow despite immunological barriers? CTVT has a very low expression of canine MHC antigens, hence it is poorly recognized by alloreactive T cells; facial tumors of Tasmanian devils also have a low MHC expression, coupled with the limited MHC polymorphism of this species, which only lives in Tasmania; tumor cells carried by human organ transplants can grow in the recipient because natural HLA differences are minimized by the selection of donor HLA compatible hosts, and immunosuppressive drug treatments block residual immune responses.

Box 19.1. Carcinogens in the biomedical laboratory

Many readers of this book will work in biomedical laboratories, at the very least for the preparation of their thesis, in some cases for their entire working life. The laboratory is not devoid of carcinogenic risks, which must be thoroughly known, understood and minimized or avoided through the implementation of standard operating procedures (SOP) and the use of protective equipment.

Physical carcinogens include radioisotopes, emitting either beta (e.g. ^{3}H) or gamma (e.g. ^{32}P) radiation, which have been mostly abandoned in favor of less hazardous enzymatic techniques. Exposure to UV rays can result either from the use of UV lamps in specific apparatuses (e.g. spectrophotometers) or from germicidal lamps, used in cell culture laboratories to minimize microbial contaminations. Note that, outside the laboratory, the main risks of natural and artificial UV rays are UVA and UVB, but within the laboratory more hazardous UVC sources are also used, for example because the absorbance maxima of nucleic acids are in the UVC range.

Several **chemical carcinogens** are commonly used in the laboratory. Commercial products must be clearly labeled as such with hazard (H) statements H350 (*may cause cancer*) and H351 (*suspected of causing cancer*); mutagens are labeled H340 (*may cause genetic defects*) or H341 (*suspected of causing genetic defects*). The list includes formaldehyde, widely used for tissue fixation; acrylamide (as with many polymers, the monomer is reactive and dangerous, the polymer less so); various DNA stains, such as ethidium bromide, propidium iodide and trypan blue; some chromogenic substrates like diaminobenzidine (DAB) and Fast red; various organic solvents, like benzene, carbon tetrachloride and trichloroethylene; cytotoxic anti-cancer drugs.

Risks of **biological carcinogenesis** may come from human blood or other human materials harboring carcinogenic microorganisms, or from viral vectors derived from oncogenic viruses, used to transduce oncogenic sequences.

In addition to known carcinogens and mutagens, research laboratories may also use newly synthesized agents, whose carcinogenic risk is totally unknown. It is advisable to handle as potential carcinogens all experimental agents not previously evaluated and/or lacking a material safety data sheet (MSDS).

Chapter 20. Cancer Prevention

The most straightforward way to reduce cancer incidence is to avoid human exposure to all the carcinogens that we have examined in the previous chapters. This is called **primary cancer prevention** and is just one of the strategies that have been conceived to control the impact of cancer on human health. We have seen that malignancy, attained in many cases through tumor progression, is the major cause of cancer death; **secondary cancer prevention** aims at preventing neoplastic progression by means of early diagnosis and therapy. There are also tertiary and quaternary types of prevention, but the reader is advised that these terms are only used by cancer prevention experts, whereas other health professionals, such as medical oncologists, speak of these approaches using different terminologies. **Tertiary prevention** aims at limiting tumor extension through the prevention of relapse and metastasis; two effective approaches, better described in the chapter on therapies, are prophylactic radiotherapy, to reduce the risk of local relapse after conservative surgery, and adjuvant therapy, administered to patients who might have micrometastases, to prevent their development. **Quaternary prevention** is a more recent coinage, to denote the approaches aimed at the reduction of iatrogenic damage, especially in connection with hypermedicalization; we will see in this chapter that some risks derive from the diffusion of secondary prevention, thus it can be said that one approach to quaternary prevention is the prevention of indiscriminate secondary prevention.

Primary cancer prevention

In principle, primary prevention is easy, once you know the carcinogens, you can altogether avoid them. In practice, carcinogenic human exposures have been on the rise for the best part of last century, despite a growing scientific evidence, both in the workplace and in everyday life; a significant decrease in global cancer incidence only began in the 1990s.

Primary prevention in the workplace

Exposure to carcinogens in the workplace is not tolerable by todays' standards, at least in developed countries, which have the resources and the possibility to implement effective (and expensive) measures to protect workers. Primary prevention of occupational carcinogenesis is implemented by mean of laws and regulations. In some cases, the carcinogen can be banned from the workplace (e.g. asbestos); if a total ban is not feasible (e.g. most organic solvents), precise rules define exposure limits and the use of appropriate protective equipment. The definition of exposure limits depends on quantitative scientific evaluations of risks and benefits, without which the "precautionary principle" can be invoked, leading to an indiscriminate ban of potentially useful agents.

The European Union has progressively implemented a very advanced control of chemical risks. REACH is the European regulation concerning the Registration,

Figure 20.1. The logo of ECHA. https://echa.europa.eu

Evaluation and Authorization of CHemical substances; it applies to all chemicals produced or imported in the EU in quantities >1 tonne/year (excluding drugs and foods, which are subject to separate regulations). The European Chemicals Agency, ECHA (Figure 20.1), manages registrations and, for substances of very high concern (SVHC), authorizations and restrictions. SVHC include carcinogens, mutagens and substances toxic for reproduction (CMR), in addition to persistent, bioaccumulable and toxic substances (PBT).

The identification and the management of risks are entrusted to producers and importers, who must compile for each substance a comprehensive file with available data, and must produce relevant data if unavailable. Note that this completely reverses the logic that guided the discovery of hazardous substances in the past, when the evidence was usually obtained by academic researchers using public money, while the producers were actually trying to hide the harmful properties of their products.

Lifestyles and cancer prevention

The bulk of carcinogenic exposures is currently related to lifestyles, a field in which primary prevention is much more difficult than in occupational carcinogenesis. Voluntary carcinogenic exposures can be completely avoided, thus preventing all attributable cancers. This is the case for tobacco, which causes about 30% of all cancer cases, and alcohol, about 4%. Non-voluntary, or mixed exposures cannot be completely avoided, thus only a fraction of the attributable cases can be avoided. For example, only a fraction of the large proportion (up to 35%) of human cancer linked to the diet is effectively preventable; this fraction includes 3% of all tumors, which are attributed to a low consumption of fruit and vegetables, 3% to overweight and obesity and 2% to sedentary life. A further 1% of tumors could be avoided through the control of air pollution.

Altogether, lifestyle modification could prevent more than 40% of all human tumors, but how? The largest social experiment of lifestyle modification was Prohibition, the constitutional ban of alcohol consumption attempted in the USA between 1919 and 1933. As illustrated by countless gangster movies, Prohibition was a failure of historic proportions: alcohol consumption continued, fostering the expansion of the organized crime, which provided illegal beverages, frequently of lower quality and even more harmful than before. The general lesson is that lifestyles are not easily regulated by law.

A major strategy to discourage the production and consumption of carcinogens is through taxes (technically called excises), for example on cigarettes or alcoholic beverages. Note however that excises cannot be increased at will, because excessive taxation, like Prohibition, fosters contraband trafficking and illicit trade, as it happens within the USA, where the price of a packet is above 10$ in New York state, but below 5$ in tobacco-producing states, like Kentucky. Creative uses of taxation can go beyond conventional excises, for example the USA introduced a tanning

tax which is reportedly successful in discouraging the use of artificial tanning, and some attempts are being made at introducing novel taxes on caloric beverages, which contribute to obesity. Laws and regulations that forbid smoking in public venues, conceived for the protection of non-smokers, also represent significant disincentives for smokers.

Educational campaigns convey, to the general public or to selected groups, scientific information on the dangers associated to a given exposure, e.g. cigarette smoking, possibly with advice on how to quit. The impact of these interventions must be assessed quantitatively (e.g., number of smokers before/after) to correctly appraise their efficacy. Most data is available for anti-smoking campaigns, which seem to have some demonstrable efficacy when administered in preventively to young non-smokers, for example in elementary of middle schools, whereas the impact on adult smokers, for example of messages and pictures printed on cigarette packets, is limited.

Less obvious, but very effective messages come from role models, for example pop singers and Hollywood stars, or sports champions. In the past, actors and actresses used to smoke and drink, both in the movies and in real life, also because they were sponsored by tobacco and alcohol industries. Nowadays, most good guys in movies and fictions do not smoke, bad guys can smoke, but they usually die before the end. The impact of smoking in the media can be assessed quantitatively, for example some studies show a good correlation between the number of cigarettes that appear in TV programs in given week and the number of packets sold by tobacconists during the following weeks. Advertising proposes positive role models to sell cigarettes, which some countries, like Italy, counteract with laws specifically forbidding cigarette ads.

We have seen that a sizeable risk reduction is observed in ex-smokers, therefore it is important to provide help to smokers who are trying to quit; psychological help and self-help is more readily available for other types of addictions, for example alcoholism, whereas it could significantly improve the rates of successful cessation also for smokers, possibly in combination with a controlled administration of nicotine. The discovery of gene polymorphisms related to the strength of nicotine addiction suggests that in the future the strength of psychological and pharmacological help might be personalized for each smoker who is trying to quit.

Cancer chemoprevention

Cancer chemoprevention is based on the development of drugs that counteract exogenous or endogenous carcinogens, to reduce the risk of tumor development. We have already encountered one instance of chemoprevention, the administration of high-dose, non-radioactive iodine pills to people exposed to radioactive iodine from nuclear accidents. However, the most intriguing aspect of chemoprevention is the possibility to decrease the risk caused by endogenous carcinogens, which cannot be otherwise modulated.

Chemoprevention is widely and successfully used by cardiologists to reduce the risk of heart attack and other cardiovascular events, for example using drugs that control high blood pressure. What is the status of cancer chemoprevention? Clinical studies have clearly shown that a pharmacological reduction of the risk of cancer is feasible, however all the

available drugs also have important toxicities that severely restrict their use.

The clinical observation that breast cancer patients treated with selective estrogen receptor modulators (SERM), like tamoxifen, had a reduced incidence of second primary tumors fostered prevention trials that demonstrated a 50% reduction of breast cancer risk in healthy women chronically treated with tamoxifen, leading to its approval for breast cancer prevention by the FDA in the US. Analogous results were obtained with other SERM or with inhibitors of estrogen synthesis. However, the inhibition of estrogen activity also has several side effects, some positive, like the reduced risk of osteoporosis, some very dangerous, like the increased incidence of endometrial carcinomas and of thrombo-embolic events. Altogether, the beneficial effect on breast cancer prevention surpasses the negative side effects, but it is clear that drugs with this kind of toxicological profile are not ready for the general population. Contrast this situation, for example, with the toxicological profile of drugs used for the control of blood pressure, which cause no side effects in almost all patients.

A reduction in the risk of breast cancer was also obtained with a completely different type of drug, fenretinide, which is a retinoid. Unfortunately, also in this case side effects were severe, including the risk of blindness caused by interference with the mechanisms of vision.

A further class of effective chemopreventive molecules is that of non-steroidal anti-inflammatory drugs (NSAIDS), which counteract the chronic inflammatory processes associated with various types of cancer. A huge number of individuals worldwide chronically take a small daily dose of acetylsalicylic acid (i.e. Aspirin) for the prevention of cardiovascular accidents; the study of cancer incidence in these people revealed a small (10%-15%), but significant reduction in tumor incidence, especially of colorectal cancer. Here the main toxicity is the risk of severe bleedings, caused by the antiaggregant activity of Aspirin, which would outweigh the benefits in the general population.

In summary, effective cancer preventive drugs already exist, but unfavorable toxicological profiles restrict their use to people at risk, for example to individuals bearing cancer-predisposing hereditary mutations. Scientific research is actively looking for new candidates, especially among natural antioxidants extracted from vegetables associated with a reduced cancer risk, such as green tea, cabbages, chocolate or red wine, however an oncological equivalent of blood pressure medications is not yet on the horizon.

Cancer immunoprevention

Actually, a class of effective cancer-chemopreventive agents is already in general use, if we classify cancer immunoprevention with vaccines as a type of chemoprevention. In the 1980s it was shown that vaccines against hepatitis B reduced by 70% the incidence of hepatocellular carcinoma in Taiwanese children. The same pivotal study also showed the superiority of recombinant versus conventional vaccines and the need of a complete (3 doses) vaccination cycle to obtain optimal protection from cancer onset. It might be objected that the HBV vaccine was primarily made to prevent hepatitis, and that cancer prevention is just a positive side effect. The point is well taken, but it certainly does not apply to the following cancer

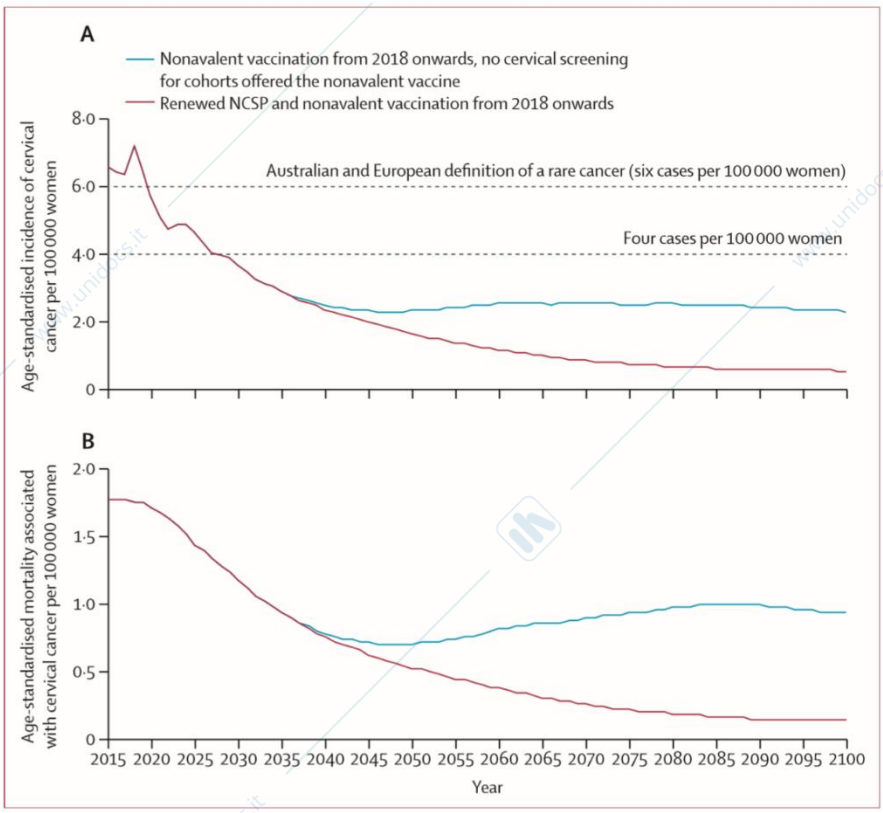
preventive vaccine, against HPV, because papilloma viruses are exquisitely oncogenic.

The general use of HPV vaccines began in 2007, hence population data, especially on long-term cancer incidence, are still premature. Furthermore, three different vaccines were successively used, directed against 2, 4 and 9 HPV genotypes; the current nonavalent (or 9-valent) vaccine has only been used since 2017.

The results of approval prevention trials, which enrolled thousands of healthy women, showed a complete absence of chronic infection by the viral genotypes contained in the vaccine, and a complete absence of cervical carcinomas. Given that HPV is the sole cause of cervical cancer, vaccination offers, for the first time in human history, the opportunity to eradicate a deadly cancer, much as the smallpox vaccine did with smallpox.

However, this appealing theoretical perspective encounters many practical hurdles. In many countries, including Italy, the percentage of vaccinated girls (the vaccine was originally offered only to 11-12 years old females) is far from 100%; even in regions with traditionally high levels of compliance with vaccination programs, like Emilia Romagna, the percentages are below 80%. High cost is also a factor limiting vaccine diffusion in poor countries with high viral prevalence, where it would be most useful. Some ethical concerns also hampered vaccine adoption in some countries, based on the fear that immunity from a sexually transmitted virus could increase the chance of risky sexual behaviors in adolescents, even though clinical studies have shown that this is not the case. Early vaccination programs only included girls, mainly to maximize the benefit/cost ratio, because cervical cancer is the most prevalent HPV-related tumor However, from a medical point of view, the extension to the male population would also have a preventive impact, because HPV induces tumors in males, and would deprive the virus of a huge infectious reservoir, eventually eliciting herd

Figure 20.2. Predicted incidence of cervical cancer (A) and mortality (B) in Australia up to year 2100. NCSP: National Cancer Screening Programme. From Hall *et al.*, Lancet 2018.



immunity. Fortunately, many countries have now approved the vaccine also for the male population.

Early results from countries with high vaccination levels, like Australia, show a constant decrease in the prevalence of HPV genotypes contained in vaccines, and epidemiologists have already produced mathematical models projecting the gradual disappearance of cervical cancer in the next decades (Figure 20.2). Interestingly, the model predicts synergistic effects of vaccination and screening, thus dispelling the fears that secondary prevention of cervical cancer would have been rapidly rendered obsolete by vaccines.

In conclusion, vaccines against oncogenic viruses (HBV, HPV) effectively prevent cancers representing about 10% of all human tumors. Vaccines are not available against HCV or *H. pylori*, but effective drugs can eradicate both infectious agents well before tumor onset, thus raising the percentage of preventable infectious tumors to 15%.

Interestingly, preclinical studies have shown that immunological strategies are also effective in the prevention of non-infectious tumors, thus leading to early clinical trials.

Secondary cancer prevention

The idea that early diagnosis could be an effective means of secondary prevention is based on two fundamental oncological tenets: *a*) small tumors are more curable than large ones, and *b*) over time, tumor progression increases malignancy and reduces curability (Figure 20.3).

At the population level, secondary prevention is implemented through mass screenings, using sensitive and cost-effective diagnostic tests, which are proposed to people with a non-negligible risk of cancer, typically beyond a certain age, for example women older than 40-50 years for breast cancer, or people older than 50 for colorectal cancer.

Secondary prevention was initially proposed as a beneficial medical approach with minimal harmful side effects, but practical implementations revealed that, under certain conditions, mass screening can be useless, or even harmful. If available therapies are totally ineffective, or totally effective, then early diagnosis does not modify mortality and is useless. Early diagnosis can also discover many lesions

Figure 20.3. The accumulation of genetic "hits" leading to tumor growth and progression takes several years (*left panel*), hence tumors discovered through early diagnosis should be smaller and less malignant (*right panel*).

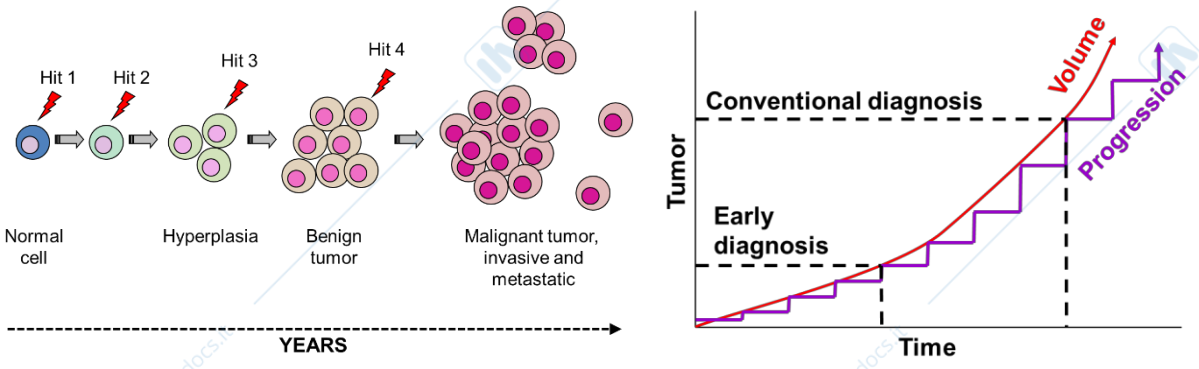
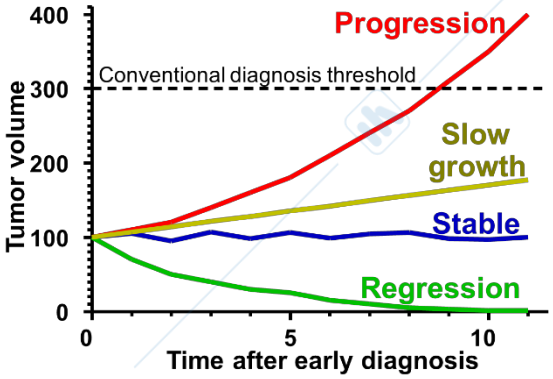


Figure 20.4. Possible fates of a tumor discovered through early diagnosis.



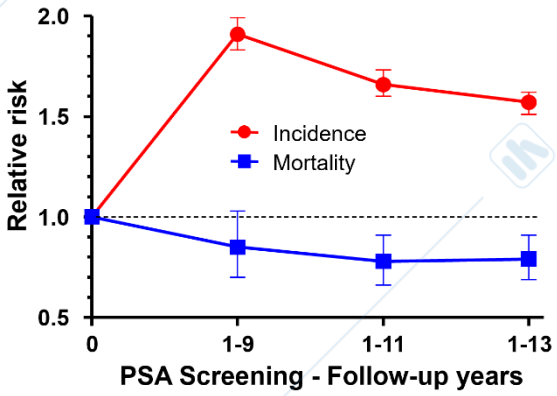
that would have not progressed to full-fledged tumors ("overdiagnosis"), leading to useless or harmful treatments ("overtreatment").

The major problem is overdiagnosis, because current screening technologies are sensitive enough to reveal microscopic *bona fide* early cancers, but are unable to discriminate those that will progressively grow to become clinically relevant from those that will spontaneously regress, or will remain dormant, or will grow so slowly that the patient will succumb earlier to other pathologies (Figure 20.4).

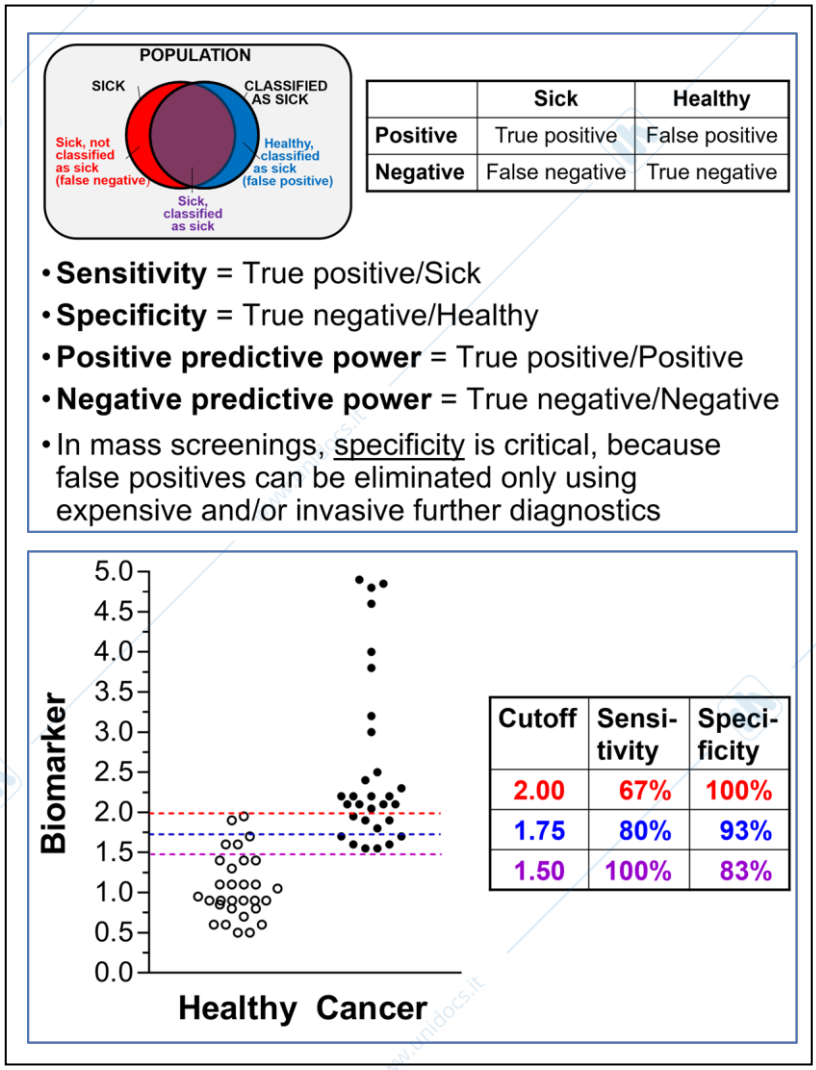
All mass screenings are subject to a certain degree of overdiagnosis. The problem is to obtain a reliable quantitative estimate, to decide whether or not that particular screening is worthwhile (Figure 20.5). The main aim of a mass screening is the reduction of global mortality, but it should be noted that mass screenings by themselves introduce distortions and bias in epidemiological measures. For example, after the start of a mass screening, tumor incidence will increase, because tumors discovered by early diagnosis will add to those of conventional diagnosis; after some years, incidence should return to the original level, otherwise overdiagnosis should be inferred. The apparent survival time of patients can increase, just because tumors are discovered earlier. Take for example a tumor that is diagnosed by conventional means when patients are 60, causing their demise at 63, hence mean survival is 3 years; if early diagnosis discovers the same tumors when patients are 55, but therapies are ineffective and they still die at 63, then effective survival is not modified, however apparent survival time will show a terrific increase from 3 to 8 years; this is called *lead time bias*.

As the effect of mass screening on cancer mortality can only be assessed after many years, some useful proxies are provided by changes in the relative proportions of tumor subtypes known to be correlated with mortality (e.g. decreases in tumor stage or grade, or percentages of non-invasive early neoplastic lesions vs. invasive tumors).

Figure 20.5. PSA screening yielded a modest reduction in prostate cancer mortality *vis-à-vis* a strong increase in incidence.
Data from European Randomized Study of Screening for Prostate Cancer, from AIOM-AIRTUM, I numeri del cancro in Italia 2016.



Box 20.1. Diagnostic tests. *Upper panel*: measures of performance; *lower panel*: influence of cutoff choice on sensitivity and specificity



Mass screenings

Mass screening that have been abandoned because of overdiagnosis include prostate and thyroid cancer. Early diagnosis of **prostate cancer** begins with a simple blood test to detect prostate-specific antigen (PSA, see also chapter on tumor immunology), however, elevated PSA levels, or variations thereof, lead to the discovery of many non-progressive lesions, thus prostate cancer is considered as a prime example of overdiagnosis, even though only a few reliable clinical studies contributed to this evaluation (Figure 20.5). As a result, in most countries, older males, instead of receiving an invitation from the health service to undergo PSA testing, are invited to contact the family doctor, to discuss the pros and cons of actually doing the blood test, and the possible consequences, on the basis of individual and familial conditions. Such changes in the implementation of prostate cancer prevention aim at reducing useless or harmful medical practices, hence are classified as quaternary prevention (see the first paragraph of this chapter).

The gold standard of **breast cancer** screening is mammography, a low-dose radiological technique; breast self-exam, which is still touted as an important way to self-diagnose breast cancer, or even objective breast examination by an experienced physician, have been shown to detect too many false-positive lesions to be considered as effective screeing methods. Epidemiological studies indicate that mammographic screening, which is implemented in many countries for women above 45 years of age, reduces by 50% breast cancer mortality, however recent studies also showed a sizeable proportion of overdiagnosis. Some improvements over conventional mammographic technologies can come from more up-to-date

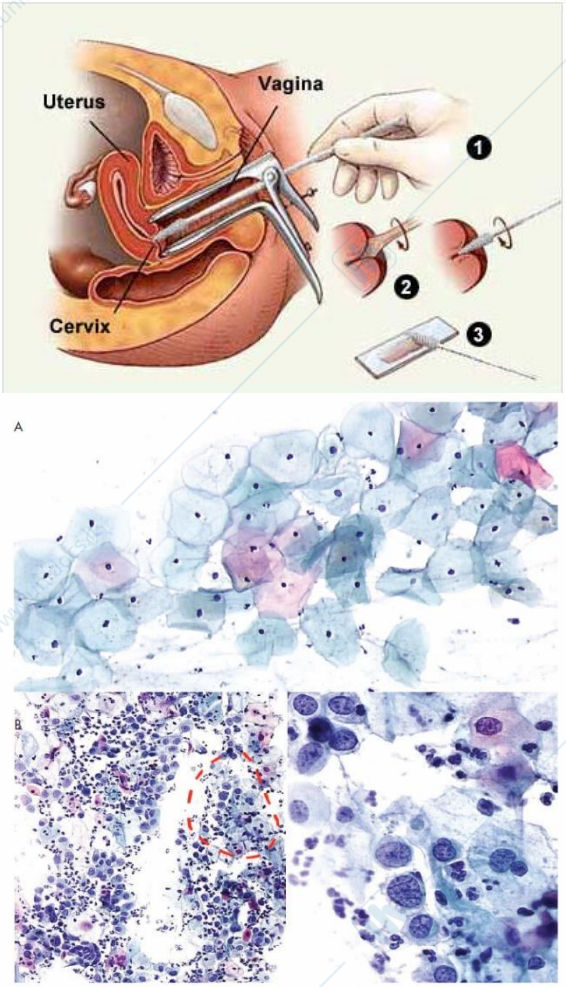


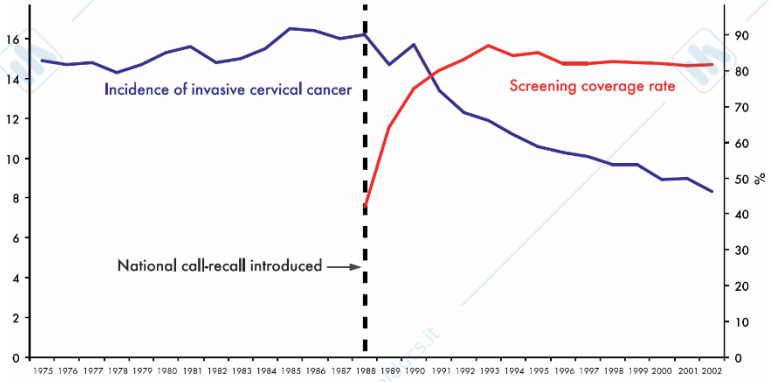
Figure 20.6. The Pap test. *Upper panel* ©Mayo foundation for Medical Education and Research, www.mayo.edu; *micrographs* from Boyle & Levin (eds), World Cancer Report 2008, IARC.

radiological technologies, e.g. digital mammography or tomosynthesis, and from the implementation of personalized screening programs which take into account individual risk factors to schedule mammograms, instead of using a standard interval (typically two years), and combine imaging technologies according to individual tissue features, such as breast density.

Cervical cancer screening was based for many years on the Pap test (Figure 20.6), a cytological analysis of cells scraped from the uterine cervix, devised by George Papanicolaou. Note that the Pap test does not detect papilloma virus, despite the coincidental initials. In the UK, population-wide implementation led to a significant decrease in the diagnosis of cervical carcinomas (Figure 20.7), in favor of less malignant lesions (CIN, cervical intraepithelial neoplasia), which forecast decreases in long-term mortality.

Other technologies are also available: visual inspection of the uterine cervix by experienced health professionals is a low-tech system, frequently used in low-HDI countries, whereas molecular tests detect the presence of HPV DNA sequences. Note that molecular diagnosis can be automated, thus dispensing with the most expensive part of the Pap test, i.e. the experienced human cytologist. Most countries, including Italy, have replaced initial Pap tests with molecular assays. Women older than 30 with positive HPV tests likely have a chronic infection and a risk of cervical carcinoma that can be diagnosed by successive Pap tests, whereas women who are HPV-negative at 30 years of age will only need some subsequent molecular test, to verify that they remain HPV-negative.

Figure 20.7. Pap screening coverage and invasive cancer incidence in England. Data from Cancer Research UK, http://info.cancerresearchuk.org.



Different technologies are also available for **colorectal cancer** screening. Intestinal bleeding is a common feature of these tumors, thus the simplest system is to look for occult blood in the stool by measuring fecal hemoglobin. This cheap and non-invasive system is an effective way to implement mass screening, however it has a low specificity for cancer, because many intestinal pathologies yield false positives. The standard follow-up to a positive occult blood test is optical colonoscopy, using a video camera on the tip of a flexible fiber optics tube inserted into the rectum; colonoscopes also carry instruments to resect small lesions (e.g. polyps) and to collect tissue samples for pathological studies; the use of colonoscopy for screening significantly reduces colorectal cancer mortality. An alternative is virtual colonoscopy, actually an abdominal computed tomography (CT) scan with a computer-aided imaging system that can simulate optical colonoscopy; virtual colonoscopy is obviously less invasive than optical colonoscopy, but it is not totally harmless, as the radiation dose exceeds 10 mSv, and its major drawback is that it does not allow tissue sampling, hence the preference of most gastroenterologists goes to optical colonoscopy. Technological advancements of colorectal cancer screening could come from the replacement of hemoglobin in occult blood assays with more cancer-specific molecular probes, such as mutant cancer genes, which can also be analyzed in circulating DNA.

Screening for **lung cancer** is not yet implemented at the population level, but promising results have been obtained using low-dose CT, sometimes called spiral CT, which has been shown to reduce mortality when applied to smokers and ex-smokers. Also in the case of lung cancer, technological advancements could come from the combination of CT with molecular studies of tumor-specific alterations in circulating DNA or in sputum samples (liquid biopsy, Box 20.2).

Screening has been attempted also for other tumor types, not always with positive, or generally applicable results. Attempts at using the Pap test for the early diagnosis of **endometrial cancer** have been unsuccessful, whereas transvaginal ultrasonography can yield significant results when applied to women at risk, for example after a diagnosis of endometrial hyperplasia. In some high-incidence Asian countries, mass screening for **gastric cancer** have been proposed using imaging approaches analogous to colorectal cancer, i.e. optical gastroscopy or radiological systems. **Oral cancers**, caused by smoking, alcohol or HPV, are frequently preceded by a long history of visible oral lesions; an original idea is the implementation of "opportunistic" screening programs that enroll dentists and dental hygienists, who have periodical access to the oral cavities of large strata of the population.

Box 20.2. Liquid biopsy.

Liquid biopsy is the analysis of tumor materials, such as cells, vesicles, metabolites, nucleic acids, etc., sampled from the blood or other biological fluids. Liquid biopsy is less invasive than conventional tissue biopsy and offers new perspectives for early cancer diagnosis and the follow-up of cancer patients.

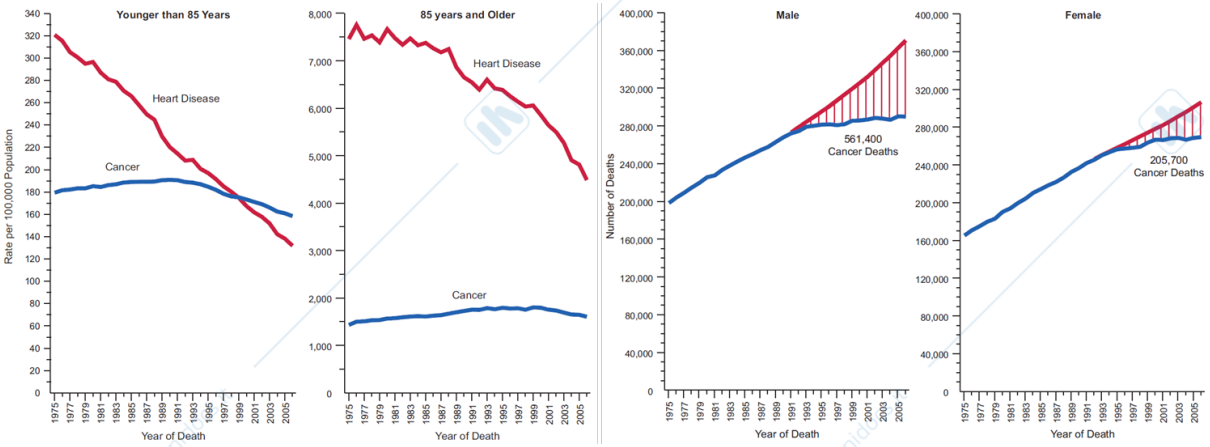
Cancer prevention – How are we doing?

A comparison of the impact of cardiovascular and oncological prevention is unforgiving. In the last half century, the death rates of cardiovascular disease in Americans younger than 85 were reduced by two-thirds, and actually became lower than those of cancer, which in the same period underwent first a small increase, then a small decrease (Figure 20.8). Cardiovascular diseases remain the first cause of death above 85 years of age, but even in this high-risk population death rates underwent a strong decrease, while cancer death rates were stable.

An analysis focusing on cancer deaths in the same population since the 1990s showed that the small (in comparison to cardiovascular) decrease in cancer mortality actually corresponded to a large number of avoided deaths, more than half a million males and 200.000 females (Figure 20.8). The likely causes are past differences in cigarette smoking trends between males and females, which in turn suggests that primary prevention (i.e. smoking cessation) is the major determinant of the observed downwards trend.

These and other patterns indicate that modern disease prevention (and therapy) indeed has a strong impact on public health, as is the case for cardiovascular diseases, but cancer prevention is not (yet) fulfilling its promises.

Figure 20.8. Cancer prevention.
Leftmost panels show US death rates for cancer and heart disease. Rightmost panels show an estimate of the number of cancer deaths avoided. From Jemal et al., CA Cancer J. Clin., 60: 277.



Chapter 21. Cancer Therapy

Cancer is the kingdom of multimodal therapies; many patients first undergo surgical removal of the primary tumor, possibly followed by radiotherapy, then are treated with combinations of cytotoxic and molecularly targeted drugs, to block metastasis growth, angiogenesis and immune suppression. In this chapter we will examine the major therapeutic strategies, mechanisms, and current trends.

Fundamentals of cancer therapy

Any difference between the tumor and normal cells can be a therapeutic target. However, not all targets are actionable, for a variety of reasons, which include the (current) inability to devise pharmacological approaches against entire classes of well-known cancer genes.

Tumors are heterogeneous and genetically unstable; thus, any therapy will exert a selective pressure, leading to the emergence of pre-existent cell variants resistant to therapies; in some cases, the treatment with mutagenic drugs will generate novel resistant variants.

The most effective way to prevent and/or to overcome tumor resistance is through the combination of therapies acting on different targets or with different mechanisms. This is one of the reasons why cancer therapies are frequently based on complex combinations and schedules.

Many molecularly targeted drugs block cell proliferation, but do not immediately kill tumor cells, i.e. are cytostatic rather than cytotoxic. In many instances, cytostatic drugs alone are insufficient to cure cancer, and need to be combined with cytotoxic drugs.

All anti-cancer treatments are toxic. Short-term toxicities are mainly due to acute insults to normal tissues and to the blockade of physiological processes. Long-term toxicities may involve reproductive sterility, neurological syndromes and the onset of novel tumors, if carcinogenic drugs were used.

Molecularly targeted agents produce toxic effects that can depend on the inhibition of the target molecule itself (on-target toxicity), or on the inhibition/activation of other molecules (off-target toxicity). On-target toxicity occurs if the therapeutic target is involved in relevant physiological processes. Off-target toxicities occur if the therapeutic agent is not specific.

Development of novel therapies

The safety and the efficacy of any novel anti-cancer agent must be convincingly demonstrated before it is reliably administered to human patients. Note that the standard testing of novel agents was originally designed for cytotoxic drugs, and may require significant modifications for other therapeutic approaches, such as immunotherapy.

Preclinical studies include *in vitro* studies on tumor cells and *in vivo* studies in which the novel agent is used to treat either immunocompetent mice bearing murine tumors, or immunodeficient mice bearing human tumors. Those agents that yield promising preclinical results will be selected for early human studies. The histotype and molecular features of sensitive

experimental tumors provide some clues as to which types of human tumors should be tested first.

The first problem that needs to be addressed is the dose that can be safely administered to human patients. Some approximate calculations provide mouse-to-human dose conversions, but the only reliable system is the direct *in vivo* testing in human patients. The aim of **phase I clinical trials** is to evaluate toxicity and to determine the dosages to be used in subsequent trials, possibly including the maximum tolerated dose (MTD), that is the maximum dose that can be tolerated by patients without severe toxic effects; first-in-human phase I trials typically recruit patients who no longer respond to available treatments.

The next problem is whether the new agent is effective. **Phase II clinical trials** analyze the responses of groups of patients affected by homogeneous neoplastic pathologies, to evaluate therapeutic efficacy. Phase I and phase II can also be combined in hybrid phase I/II trials that simultaneously aim at determining toxicity and efficacy.

The final problem is whether the novel therapeutic agent provides clinical advantages, in particular if it is more effective, than current therapies. **Phase III clinical trials** compare, in large groups of patients, the efficacy of the novel agent with that of standard therapies. As most treatment regimens include multiple drugs, phase III trials usually compare a standard drug combination with the same combination plus the novel agent, or a combination in which the novel agent replaces a standard one. Patients are randomly assigned to the various treatments; ideally, neither the patient, nor the physician know which treatment is administered, and patients who do not receive the novel agent should receive a placebo instead; such a trial, which would be defined as randomized, double-blinded and placebo-controlled, offers the best guarantee against the biases that could affect the objective evaluation of the novel agent.

Approval by governmental agencies, such as the European Medicinal Agency, EMA, or the US Food and Drug administration, FDA, is the final step of development, certifying that the novel agent is finally ready for clinical use.

Post-marketing clinical studies are labeled as **phase IV**. Drugs are usually approved only for the treatment of those tumor types that were included in previous clinical trials. After approval, further trials against different tumor types can broaden the range of therapeutic indications, or, sometimes, can demonstrate that initial data were too optimistic, leading to the withdrawal of the approval for some tumor types.

Various attempts are being made to innovate and accelerate the whole process, which currently takes many years from conception to regulatory approval, is extremely expensive and is prone to dramatic failures, even in phase III. One such attempt is the design of clinical trials that fuse together two phases, such as the phase I/II trials mentioned before, another is the design of **phase zero clinical trials**, in which sensitive analytical techniques are used to monitor the effects of sub-therapeutic doses, administered to small numbers of patients, for example to select the best candidate for further clinical development among several similar molecules.

Response criteria in oncology

Clinical trials recruit patients with objectively quantifiable lesions, be it the number of leukemic blasts in bone marrow biopsies, or the volume of liver metastases as measured by CT, to allow quantitative statistical analyses of the effect of therapeutic agents.

The best outcome is a **complete response (CR)**, that is the complete disappearance of all lesions. **Partial response (PR)** is a significant regression of lesions, according to pre-defined criteria, e.g. by at least 50% (WHO criteria) or 30% (RECIST criteria). Even a **stable disease (SD)** can indicate a therapeutic response, if the lesions were actively growing before treatment, whereas **progressive disease (PD)**, i.e. lesions increasing by more than 20%, usually means treatment failure.

Survival does not always imply death, rather "survival" in clinical studies means the duration of a given condition. For example, **disease-free survival (DFS)** is the duration of a complete response, whereas **progression-free survival (PFS)** is the duration of a partial response, also called **time to progression (TTP)**. The only instance in which the opposite of survival is death is **overall survival (OS)**, which measures the time from diagnosis to death.

Strategies in cancer therapy

There are several unique approaches in cancer therapy that impact not only the clinical management of patients, but also the type of molecular studies that can be implemented to investigate the responses of tumors and metastases.

It is generally thought that therapies are only administered to patients with disease, but in oncology this is not always the case. What can be done for those patients that, after primary tumor removal, are apparently healthy, but have a high risk of having distant micrometastases? A case in point are breast cancer patients with positive lymph nodes (N+ patients), because we have no way to distinguish the two-thirds of them in which metastases will develop over the years from the one-third that will remain disease-free forever. The solution, devised in the 1970s, is **adjuvant therapy**; all patients who consent will receive the treatment, which will be useless or harmful for those who do not have micrometastases, but for those who do, it could prevent metastasis outgrowth. The fact that adjuvant therapy is administered on a statistical basis is the reason why it is also classified as a type of prevention (tertiary prevention, see previous chapter). Adjuvant therapy has a significant impact on patients' survival and is currently a standard approach in many tumor types for high-risk patients.

Neo-adjuvant therapy shares part of the name with adjuvant therapy, but it is an entirely different approach. Other names, such as **primary therapy** or **pre-operatory therapy** are possibly more descriptive, but "neo-adjuvant" is well entrenched in oncological parlance. Here the problem starts as a surgical issue: the extension of some primary tumors is such that their removal would require a highly invasive intervention, such as the amputation of an arm or a leg in children with limb osteosarcomas. The solution was to reverse the standard sequence (surgery first, chemotherapy later), using chemotherapy to reduce the extension of the tumor, allowing a conservative surgical intervention e.g. limb-sparing in the osteosarcoma example. Neo-adjuvant therapy can also improve survival, and is now

used in various tumor types, such as breast cancer. Neo-adjuvant therapy also has an impact on the implementation of clinical and molecular studies; treatments are typically administered for a few months, and the response of the primary tumor is precisely evaluated, clinically and pathologically, thus neo-adjuvant regimens allow a rapid evaluation of novel therapeutic agents. However, molecular analyses of tumor response are hampered by the fact that responding tumors are highly necrotic, yielding little or no material for laboratory studies.

Other treatment approaches are used for advanced cases, in which the cure is no longer the aim of therapy. **Salvage therapy** defines treatments that are administered to patients who do not respond to various standard treatments. **Palliative care** includes surgical, medical and psychological treatments that can guarantee an acceptable quality of life to patients bearing tumors or metastases that cannot be eradicated.

Oncological surgery

Most primary tumors are completely removed by surgery, only some anatomical locations can limit radical surgery, e.g. head-and-neck, or brain tumors. Malignant tumors that have not yet metastasized are definitively cured by surgery, but the presence of distant metastases (either overt or microscopic) before surgery is the major cause of cancer lethality.

Improvements in surgical techniques fostered a constant trend towards conservative oncological surgery, which can be combined with prophylactic irradiation to reduce the risk of local relapse. Prophylactic radiotherapy, like adjuvant therapy, is given to all patients on a statistical basis, because we cannot distinguish those tumors that will relapse from those that were completely removed, hence it is also classified as a type of tertiary prevention.

Oncological surgery includes many approaches beyond the removal of primary tumors, for example **surgical removal of metastases** can effectively cure patients and interrupt the metastatic cascade or can be used with a palliative intent. **Prophylactic surgery** is used to remove the tissue at risk of neoplastic transformation from individuals carrying high-penetrance hereditary mutations in cancer genes, for example radical bilateral mastectomy for BRCA1/2 carriers. In some instances, surgery is used more for diagnostic purposes (**surgical staging**) than with a curative intent; an example is the removal of sentinel lymph nodes (see chapter on metastases).

Cancer radiotherapy

About 50% of all cancer patients receive radiotherapy, which uses ionizing radiation to kill cancer cells (see chapter on physical carcinogenesis). Standard radiotherapy employs external sources of low-LET gamma-rays (e.g. ^{60}Co); alternative approaches are the implantation of radioactive sources within the body (brachytherapy), the administration of radioactive drugs, such as monoclonal antibodies, and the use of high-LET external sources (adrotherapy) such as protons (proton therapy), which should inflict more damage to the tumor and better spare normal tissues than low-LET radiation.

The efficacy of radiotherapy depends on the characteristics of both tumor cells and microenvironment. Tumor-intrinsic radiosensitivity is determined by the activity of DNA damage responses, including the mechanisms of apoptotic cell

death, all of which are frequently compromised in tumor cells. The extent of oxidative damage inflicted by radiation can be limited by hypoxia, which in turn is dependent on tumor vascularization.

Huge technological advancements have greatly enhanced the efficacy of radiotherapy and reduced the damage to healthy tissues. In addition to the use of more powerful sources of radiation, important advancements came from the implementation of computer-controlled sources that move around the tumor mass while changing the shape and the intensity of the radiation beam (3D-conformal and intensity-modulated radiotherapy).

Modern machines are very expensive but are used to treat thousands of patients over many years, hence radiotherapy is less expensive than either surgery or chemotherapy.

Cytotoxic chemotherapy

Chemotherapy is a generic term that refers to any kind of therapy with chemical drugs, but in oncology it is used, both by physicians and by patients, to indicate cytotoxic chemotherapy.

The main aim of cytotoxic chemotherapy is to kill proliferating cells. The proliferation of tumor cells is uncontrolled, but its velocity is similar to, or frequently slower than that of normal cells. Thus, cytotoxic drugs are toxic (Figure 21.1) because they kill also normal proliferating cells, e.g. in hematopoiesis, mucosae, hair bulbs. The lack of tumor specificity of these drugs is also attested by the fact that some are also used to kill pathogenic non-neoplastic cells in other diseases, for example self-reactive lymphocytes in autoimmune diseases.

Not all tumor cells are killed, some escape because they are not actively proliferating when drugs are administered, others are resistant because they express molecules that inactivate the drugs, or contain such high levels of the target molecules that outcompete intracellular drug concentrations. One outstanding example of the first type is multidrug resistance, a phenomenon by which resistant tumor cells emerging after therapy with one type of drug are no longer sensitive to multiple drug types; the molecular

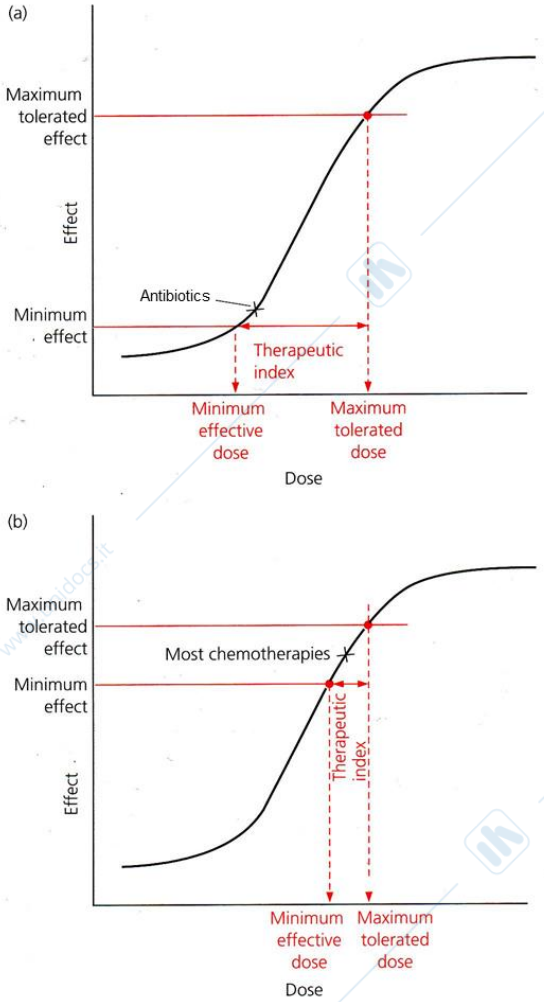


Figure 21.1. The narrow therapeutic index of cytotoxic chemotherapy.
(*a*) Antibiotics exemplify a class of drugs featuring ample margins between effective and toxic doses, whereas (*b*) cancer chemotherapeutics are used at doses approaching the maximum tolerated dose. From Pecorino, Molecular Biology of Cancer, Oxford University Press.

Table 21.1. Cytotoxic antineoplastic drugs.

Class	Mechanism of action	Examples
Antimetabolites	Inibition of nucleic acid synthesis	• Base analogs: 5-fluorouracil, cytarabine, gemcitabine, 6-thiopurine, fludarabine • Methotrexate, pemetrexed
DNA adductors	Formation of adducts that block DNA synthesis and cell proliferation	• Alkylating agents: cyclophosphamide, nitrosoureas, DTIC, temozolomide • Pt compounds: cisplatin, carboplatin • Mitomycin C
Mitotic inhibitors	Interference with tubulin polymerization and assembly of the mitotic spindle	• Vinca alkaloids: vincristine, vinblastine • Taxanes: paclitaxel, docetaxel • Epotilons
Inibitors of DNA unwinding	Inhibition of DNA double-helix unwinding, required for DNA replication and gene expression	• Topoisomerase inhibitors: Topo I: irinotecan, topotecan Topo II: doxorubicin, etoposide
Epigenetic modulators	Re-activation of tumor suppressor genes, induction of cell differentiation and apoptosis	• Inhbitors of DNA methyltransferases: 5-azacitidine, 2'-deoxy-5-azacitidine • Inhibitors of histone deacetylases: valproic acid, belinostat
Others	Various	• Thalidomide • Bleomicin

explanation was found in the hyperexpression of membrane pumps that actively extrude cytotoxic drugs from the cell cytoplasm; the first to be discovered in tumors, originally called MDR1, P-glycoprotein or p170, is a member of the ATP-binding cassette (ABC) family of transporters, now formally designated as ABCB1. One example of the second type is the amplification of the dihydrofolate reductase (DHFR) gene, which is needed by replicating cells to make thymine, and is inhibited by the anti-cancer drug methotrexate; DHFR gene amplification is also exploited as a biotechnological tool, to amplify transfected genes.

A large number of cytotoxic drugs, developed since the end of World War II, exploit a small number of different mechanisms to inhibit cell proliferation (Table 21.1). Actively proliferating cells need purines and pyrimidines for DNA synthesis; **antimetabolites** are base analogs that inhibit DNA synthesis enzymes, or key enzymes of base synthesis, like DHFR. **DNA adductors** exploit another way to damage DNA, through the binding of alkyls or other extraneous chemical groups; we have encountered some of these drugs in the chapter on chemical carcinogenesis. After DNA replication, proliferating cells undergo mitosis, which requires the formation of the mitotic spindle, which is a huge polymer made of tubulin molecules, to drive chromosomal separation; **mitotic inhibitors**, also called spindle poisons, interfere with tubulin polymerization to disrupt spindle assembly; this class of drugs includes several natural molecules, originally extracted from plants (vinca alkaloids, taxanes) or bacteria (epothilones). DNA replication and gene expression

require the continuous coiling and uncoiling of the DNA filaments, which is allowed by specific enzymes, including DNA topoisomerases I and II (familiarly called topo I and topo II); **inhibitors of DNA unwinding** include older drugs, like doxorubicin, and molecules specifically targeted against topoisomerases, which usually include "topo" in the generic name. Finally, **epigenetic modulators** act on the alterations of cancer gene expression that can be mediated by epigenetic controls, such as promoter methylation, which was found to inhibit the expression of various tumor suppressor genes; methylating enzymes (DNA methyltransferases) act on sequences containing cytosine, like CpG islands, hence they can be inhibited by drugs that are cytosine analogs, such as 5-azacytidine.

The individual efficacy and toxicity of chemotherapy can be modulated by various mechanisms, including genetically encoded enzymatic polymorphisms, which could be exploited to avoid the administration of specific drugs to overly sensitive patients, and circadian rhythms, which could be different in tumors than in normal cells, potentially allowing the design of schedules that administer the drug when tumors are maximally sensitive and normal cells relatively resistant (chronotherapy).

Targeted therapy

After several decades of fruitful developments, in the 1980s cytotoxic chemotherapy reached a plateau, and the discovery of novel drugs, let alone novel mechanism of actions, dwindled. Fortunately, at the same time, the findings of cellular and molecular studies, in particular the discovery of oncogenes and the elucidation of their mechanisms of action came to the rescue, with a novel therapeutic framework called molecularly-targeted therapy, or target therapy for short.

The ideal logical sequence leading to the development of a novel targeted therapeutic agent begins with the discovery of molecular differences between normal and tumor cells, and the discrimination of key molecules that drive tumor growth and allow tumor cell survival. The definition of actionable targets then drives the rational design, or the discovery by means of high-throughput systems, of novel drugs that selectively hit cancer cells expressing the target. Molecular studies of tumor samples are finally used to treat only the patients that express significant levels of the target, thus avoiding the one-size-fits-all approach of conventional chemotherapy.

The main approaches and targets of targeted therapies are:

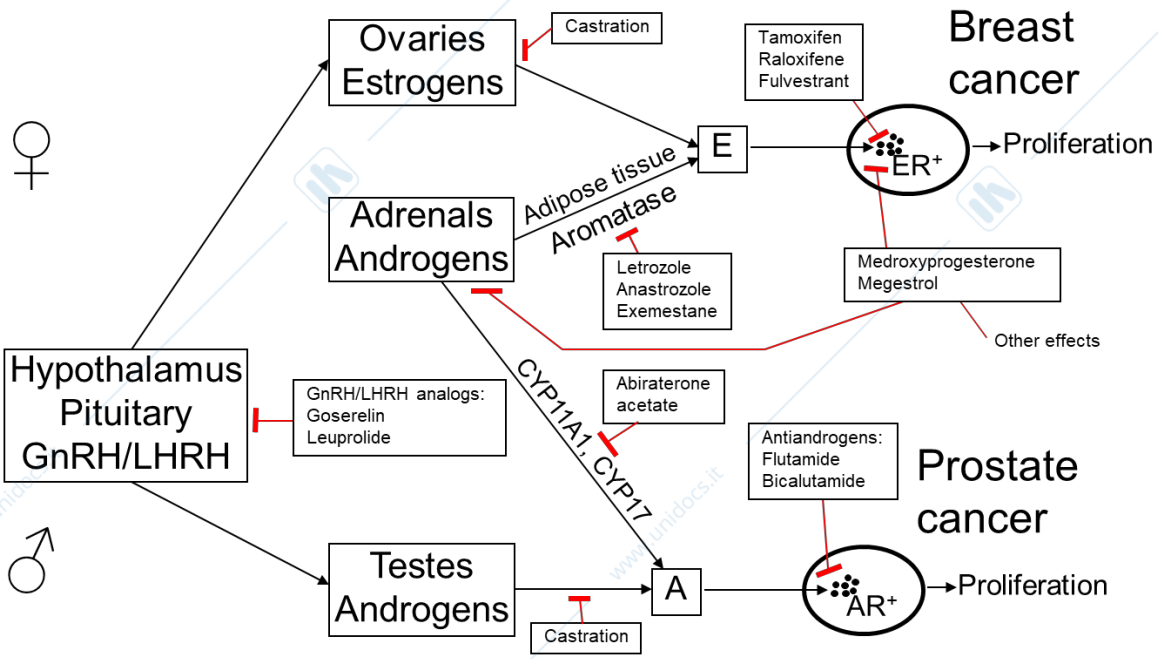
- Hormone therapy
- Growth factors, their receptors and downstream mitogenic signal transduction
- Apoptosis and protein traffic
- Synthetic lethality
- Angiogenesis and stroma
- Immunotherapy

Here we will only deal with hormone therapies and immunotherapy, as all other approaches were detailed in the chapters on oncogenes, tumor suppressor genes and angiogenesis.

Hormone therapies

Hormone therapy has been used to selectively cure cancer patients well before the term target therapy entered oncological lexicon. The growth of high-incidence tumors, such as breast and prostate cancer, is fueled by sex steroids, estrogens and androgens, respectively.

Figure 21.2. Hormone therapy of breast and prostate cancer.



Conceptually, the overall therapeutic strategies are remarkably similar. One class of drugs inhibits hormone binding and activation of the receptors, which are intracellular transcription factors. A second class of drugs inhibits the synthesis of hormones by the adrenal glands. A third approach is the blockade of hormone secretion by the gonads, which was once obtained by surgical removal (castration), but can be effectively obtained with drugs that inhibit the hypothalamic-pituitary axis (chemical castration). Unlike cytotoxic treatments, which are administered for a few months, one year at most, hormone therapies, which are cytostatic, are usually scheduled for five years, and some clinical trials indicate that ten years can yield even better results.

A first major difference is that all prostate cancers are assumed to be initially dependent on androgens, and are treated with hormone therapy, whereas about one-fourth of all breast cancer cases does not express estrogens or progestin receptors (ER or PR), thus is not eligible for hormone therapy. The second major difference is that in women the onset of menopause radically changes estrogen levels. Most cases are post-menopausal, when estrogen levels are low, deriving mainly from the adrenal conversion of androgens by enzymes called aromatases. Aromatase inhibitors and selective estrogen-receptor modulators (SERM) are the mainstays of post-menopausal breast cancer. For pre-menopausal breast cancer, the first priority is the block of gonadal estrogens, which effectively induces an artificial menopause, then patients can be treated as post-menopausal cases.

Immunotherapy

Attempts at inducing therapeutic immune responses against tumors date back to end of the 19th century, when William Coley, having observed the regression of human tumors after severe bacterial infections, tried to elicit "a commotion in the blood" of cancer patients through the

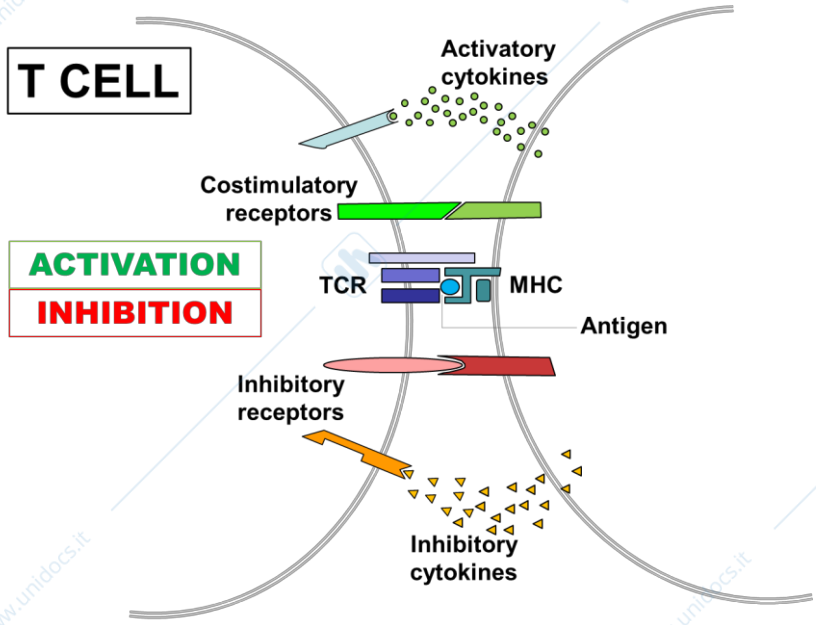


Figure 21.3. Immune activatory and inhibitory signals exploited by cancer immunotherapy.

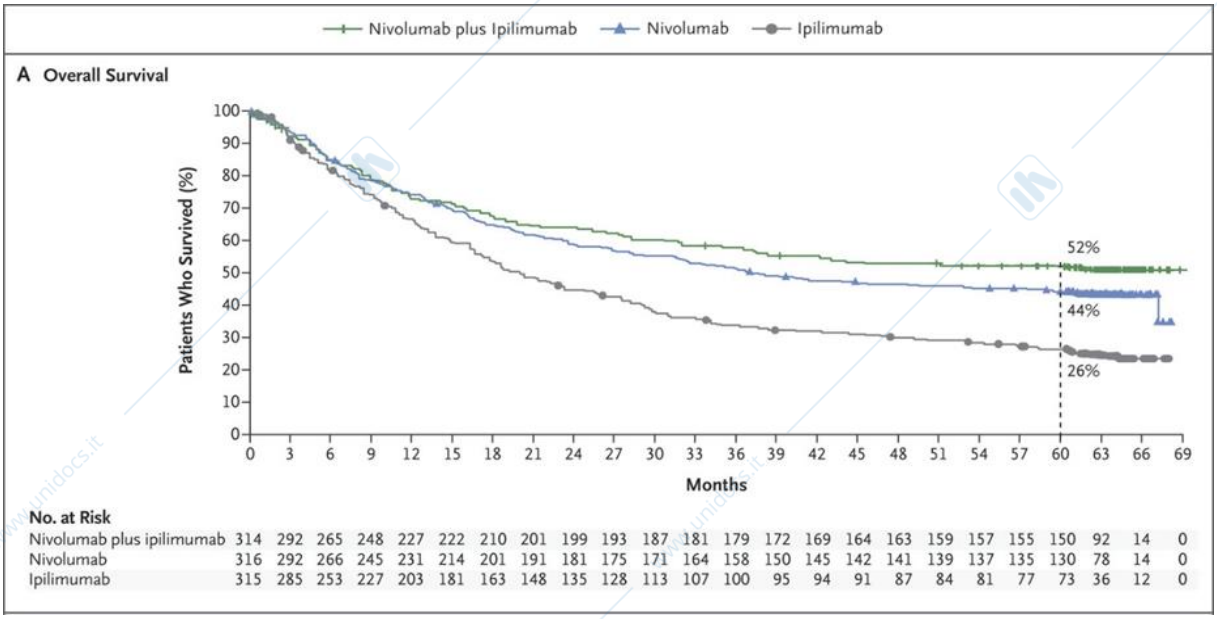
administration of preparations ("Coley toxins") obtained from people with infections.

Major immunotherapeutic strategies can be classified in various ways. The simplest is to distinguish **antigen-specific** from **non-antigen specific** (non-specific for short) approaches, and **active** therapies, which aim at stimulating the immune system of the patient, from **passive** ones, which consist of large amounts of immunological drugs, not requiring (at least in theory) the activation of the immune system of the host; when the "drug" consists of cells, the immunotherapy is called **adoptive**.

Thus, Coley toxins were a first attempt at **non-specific, active immunotherapy**. The following century saw an incredible number of similar attempts, using first bacterial preparations, then cloned cytokines, some of which were even approved by regulatory agencies, for example interferon-α. However, the clinical impact was always quite limited. A huge improvement came from a paradigm change: instead of trying to positively stimulate the immune system, some researchers turned their attention to molecules that inhibit the immune response (Figure 21.3), designated, for analogy with the cell cycle, as "immune checkpoints". Inhibition of CTLA4, PD-1 and its ligand PD-L1 with monoclonal antibodies (immune checkpoint inhibitors, ICI) had a significant clinical impact, not only in melanoma (Figure 21.4), which is known to be sensitive to immunotherapies, but also in non-small cell lung cancer and other tumor types, to the point that for clinical oncologists immunotherapy is now synonymous with ICI. As with all therapeutic treatments, a sizeable percentage of patients do not respond to ICI, prompting for the search of predictive biomarkers, either in the tumor or in the host, in particular in the immune system. Available evidence indicates that ICI are only able to unleash existing immune responses, hence the immunogenicity of the tumor (as predicted, for example, by the tumor mutation burden, see chapter on cancer genes) is an important determinant of therapeutic efficacy.

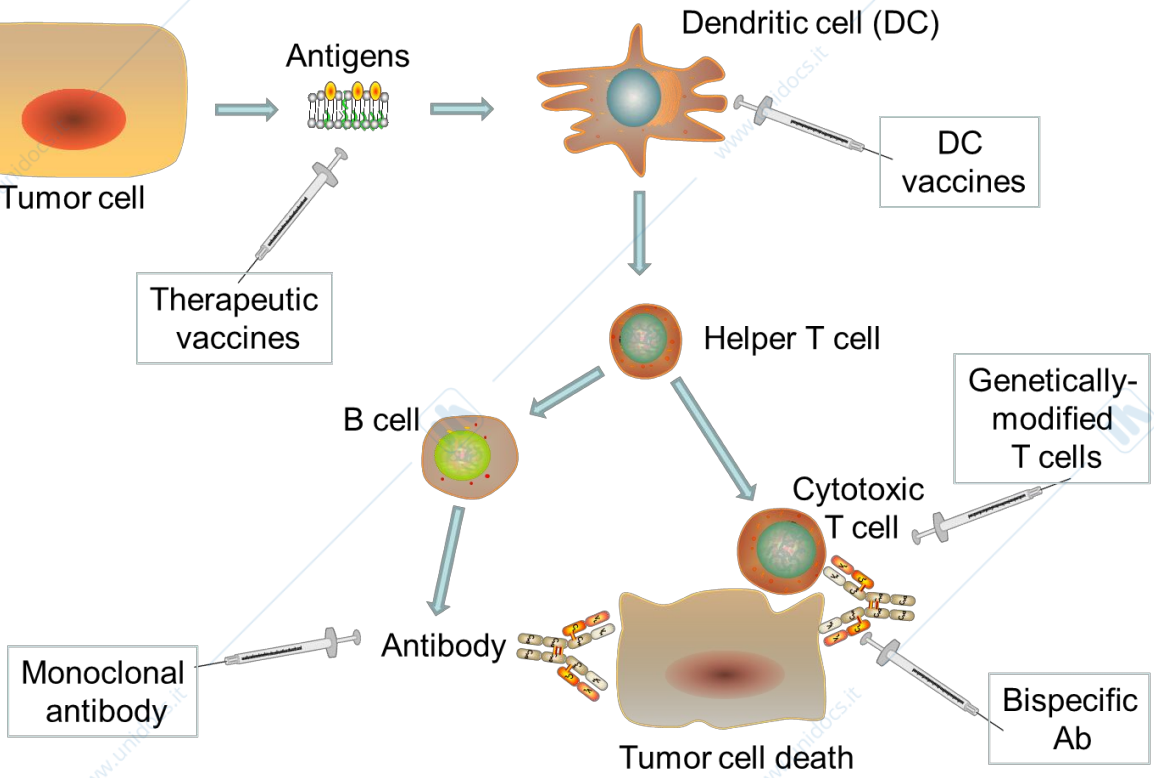
Active, antigen-specific immunotherapy (Figure 21.5) is based on therapeutic vaccines that aim at inducing anti-tumor immune responses through an immunogenic presentation of tumor antigens to the immune system of the host. In

Figure 21.4. Immune checkpoint inhibitors against advanced melanoma.
Monoclonal antibodies anti-PD-1 (nivolumab) and CTLA4 (ipilimumab). From Larkin et al. NEJM 28 Sep 2019.



addition to technologies based on the *in vivo* administration of various antigen preparations, cancer vaccines can also be made by exposing *in vitro* ("pulsing") the dendritic cells DC of the patient to tumor antigens, followed by the *in vivo* infusion of the pulsed DC, which will then present the antigens to the immune system of the patient. A proprietary technology of DC preparation (sipuleucel-T) for the

Figure 21.5. Antigen-specific immunotherapeutic strategies.



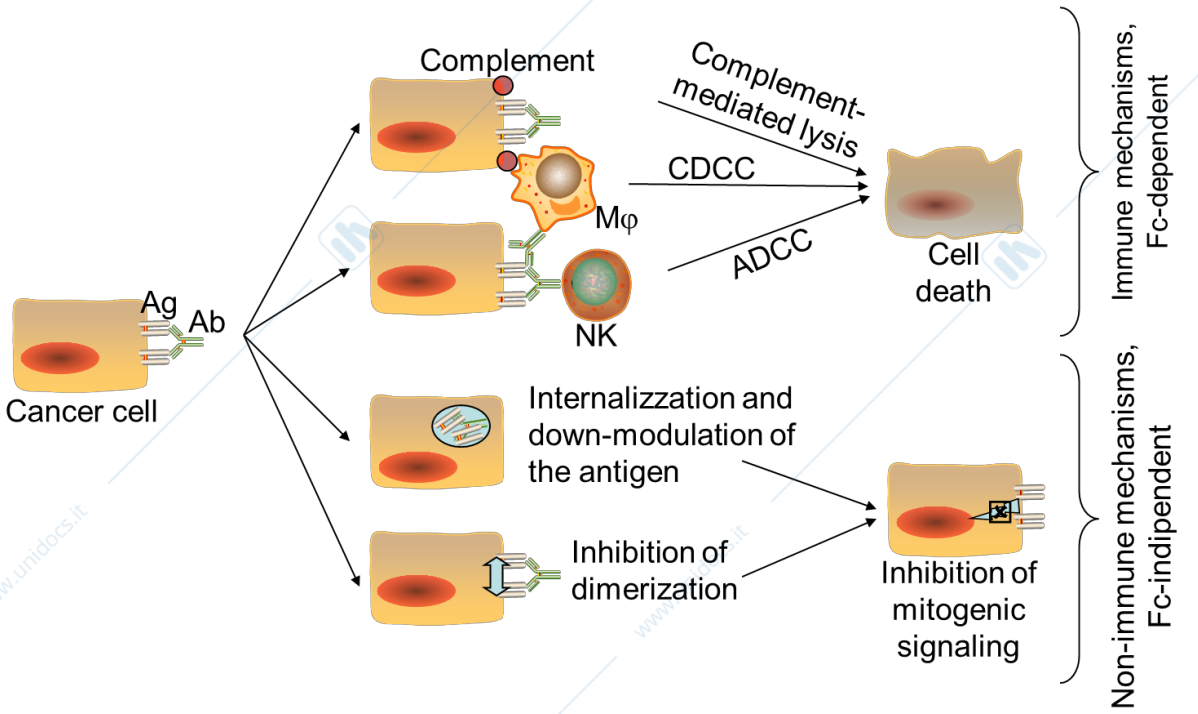


Figure 21.6. Mechanisms of action of monoclonal antibodies directed against tumor cells.

immunotherapy of prostate carcinoma is the only approved therapeutic cancer vaccine. Despite the FDA approval, sipuleucel-T shares with other cancer vaccines a very limited therapeutic efficacy, especially against advanced and metastatic cancer. Some promising results were instead obtained with vaccines directed against early cancer lesions, such as cervical intraepithelial neoplasia (CIN), a precursor of cervical carcinoma.

Monoclonal antibodies (MAb) are the best gift of tumor immunology to cancer therapy. Specific applications were discussed in various parts of this book, here we will only examine some general aspects of MAbs as therapeutic agents.

A major advantage over other targeted agents, which are mainly cytostatic, is that MAb are also cytotoxic (Figure 21.6). Cytotoxicity is mediated by various immune mechanisms that involve the Fc region of the immunoglobulin molecule, including the activation of complement and the binding by Fc receptors expressed by macrophages and natural killer cells, which in turn can directly kill tumor cells. Cytostatic mechanisms are mediated by MAbs activity as receptor antagonists, through the inhibition of receptor dimerization, the induction of receptor recycling and other mechanisms resulting in the inhibition of mitogenic signaling. MAbs are large molecules (the molecular weight of an IgG is around 150,000 Da), which can also be used as vectors of many kinds of therapeutic molecules, including cytotoxic drugs or radioactive isotopes. A further advantage is that immunoglobulins are physiologically endowed with a long half-life in the blood, unlike most other drugs, hence the therapeutic schedules can be more relaxed.

The limits and disadvantages of MAbs include the fact that they can only target extracellular molecules and cannot be administered orally, unlike many modern targeted drugs. The large size of immunoglobulins also hampers their traffic, for example through the blood-brain barrier, resulting in a limited therapeutic efficacy against brain metastases. For many years, the easiest way to make MAbs was through the use of mouse B and myeloma cells, however the resulting mouse immunoglobulins are antigenic in human patients, and repeated administrations elicit neutralizing human anti-mouse antibodies (HAMA). Thus, most therapeutic MAbs are either chimeric or humanized: taking advantage of the modular nature of IgG genes, the constant and framework regions of murine origin were replaced with the human counterparts, leaving the antigenic specificity intact.

T cell responses can effectively kill tumor cells under clinical conditions. This is clearly demonstrated by the therapeutic effect of allogeneic T cells grafts in leukemic patients. In normal transplants (e.g. kidney), the immune system of the host can attack the graft, but in patients receiving transplants of allogeneic bone marrow, which also contain mature T cells, it is the graft that attacks the host, a phenomenon called graft-versus-host (GvH), which can cause a range of severe diseases (GvHD). However, in leukemic patients it was found that GvH also has therapeutic consequences, because allogeneic T cells also kill leukemic cells (graft-versus-leukemia, GvL). The major clinical problem of GvL as a therapeutic strategy is the sword of Damocles of GvHD. Gene therapy has provided a solution through the insertion in the genome of donor T cells of a metabolic suicide gene, which converts an innocuous pro-drug into a cytotoxic agent; genetically-modified allogeneic T cells can thus be infused safely to leukemic patients, because at the first sign

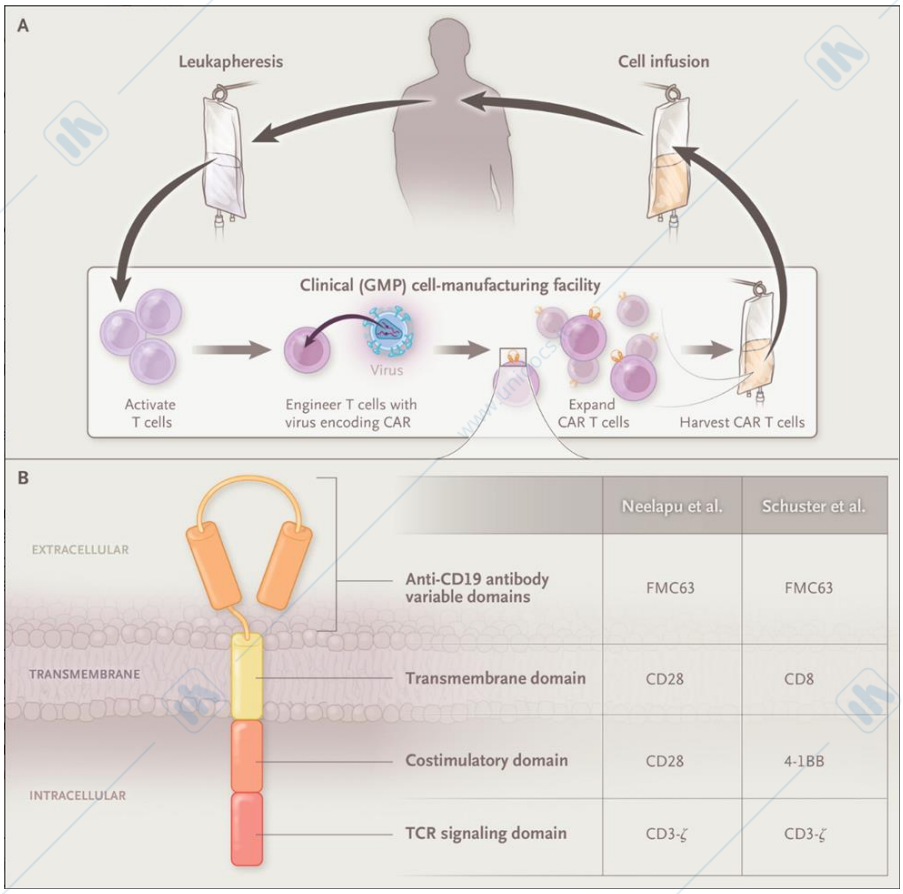


Figure 21.7. CAR-T preparation (*A*) against CD19 using two different molecular assemblies (*B*).
From Tran et al., NEJM 10 Dec. 2017.

of GvH they can be selectively eliminated by the administration of the pro-drug.

The efficacy of GvL depends on the peculiar strength of allogeneic T cell responses. After many decades of intensive research, and many clinical failures, only recently some cancer therapies based on syngeneic T cells effectively entered the clinical arena. In one case the therapeutic agent is actually an antibody, but the mechanism is based on T cell activation. Natural antibodies have two identical antigen-binding sites, but it has long been possible to make biotechnological molecules called bispecific antibodies, with binding sites that recognize two different antigens. In cancer therapy, bispecific antibodies (Figure 21.5) bind tumor cells on the one side and T cells on the other, typically though an anti-CD3 binding site, thus acting as a molecular glue that unites the two cells, forcing the T cell to kill the tumor cell. The first approved bispecific antibody, blinatumomab, is active against CD19-positive acute B cell leukemia.

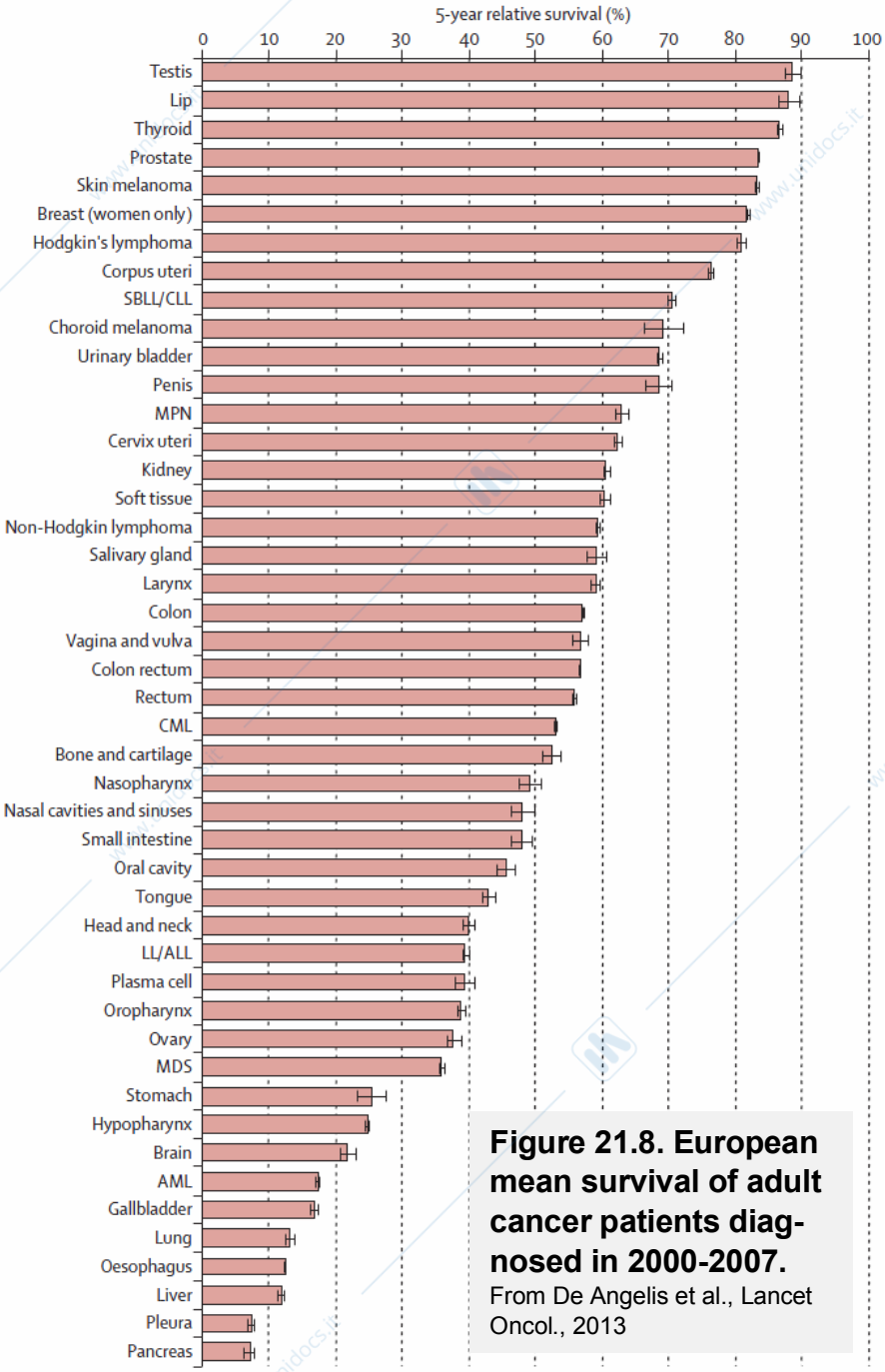


Figure 21.8. European mean survival of adult cancer patients diagnosed in 2000-2007.
From De Angelis et al., Lancet Oncol., 2013

A pure T cell-based cancer therapy was finally obtained through the genetic engineering of the antigenic specificity, after many decades of ineffectual attempts to activate *ex vivo* the cytotoxic T cells from cancer patients. Chimeric antigen receptor T cells (CAR-T, Figure 21.7) are made by transducing patient's T cells of unknown antigenic specificity with a chimeric gene that encodes the antigen-binding sequence of an antibody and various intracellular signaling domains derived from genes involved in T cell activation. The engineered T cells are then re-infused in the patient to kill tumor cells. CAR-T directed against leukemic CD19-

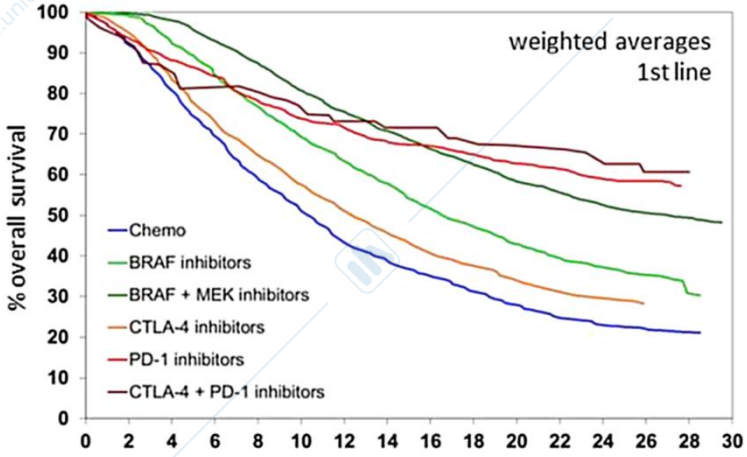


Figure 21.9. New therapies *vs.* chemotherapy against metastatic melanoma
From Ugurel et al., Eur. J. Cancer 83: 247.

positive cells have already been approved for clinical use, and many others are being developed, against hematopoietic and solid neoplasms. As the chimeric receptor is an antibody derivative, CAR-T share with monoclonal antibodies the limitation that only extracellular tumor antigens can be recognized. The engineering of true T cell receptors (TCR) would allow the recognition of antigenic peptides deriving from any kind of tumor antigen, but so far TCR-engineered T cells (TCR-T) have not demonstrated a significant therapeutic activity in the absence of limiting toxicities.

Cancer therapy: how are we doing?

The status of cancer therapies can be gathered from large studies, such as EUROCARE, which analyzed 5-year survival in Europe across the whole spectrum of tumor histotypes (Figure 21.8). Altogether, the mean survival is slightly above 50%, the range practically goes from almost 100% to almost 0%. Some high-incidence tumors, such as breast and prostate cancer or melanoma, have reached very high rates of survival, above 75%, thanks to early diagnosis and the advancements in all treatment modalities. Another big killer, colorectal cancer, ranges near the middle; these visceral tumors too frequently grow unnoticed and are only discovered when metastatic spread has already taken place, which compromises long-term survival. Towards the bottom we find several poorly curable cancers, including lung, pleural, stomach, liver and esophagus tumors, for which we have at least some effective preventive strategies. The

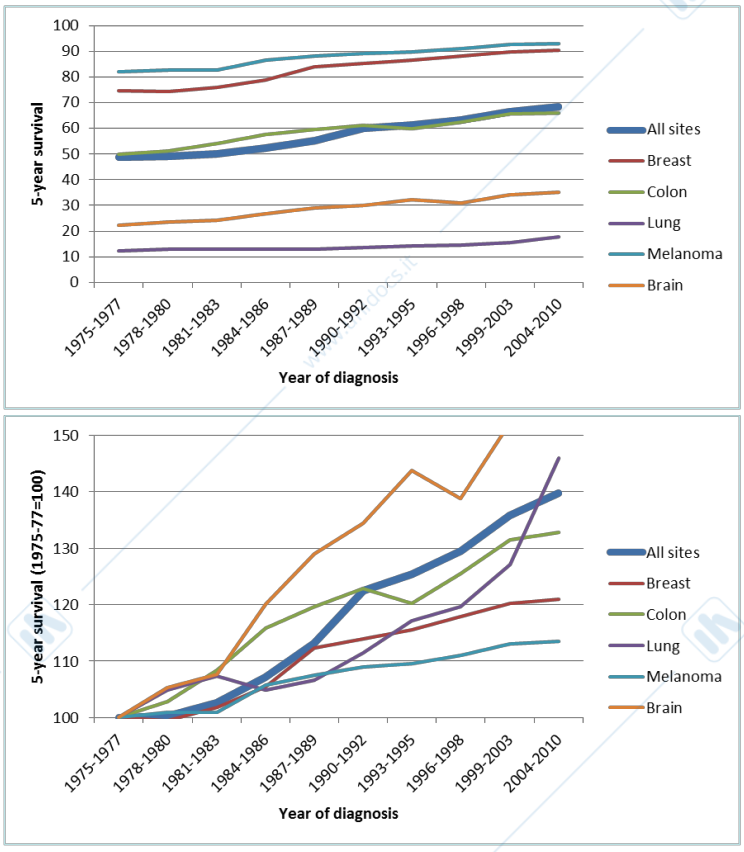


Figure 21.10. Trends of cancer survival.
Data from SEER Cancer Statistics Review 1975-2011.

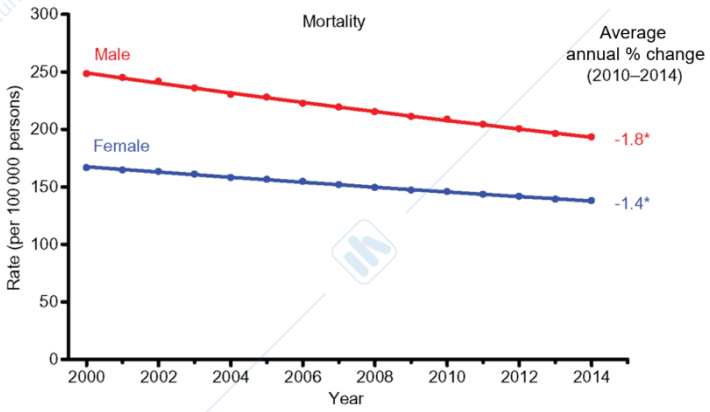


Figure 21.11. Overall cancer mortality trends.
From Jemal et al., JNCI 109 (9) 2017.

worst case at present is pancreatic cancer, for which we have neither prevention, nor effective therapies.

Novel therapeutic strategies, especially targeted therapies and immunotherapy are having a significant impact on patient survival. An excellent example is metastatic melanoma, in which cytotoxic chemotherapy was only able to cure less than 30% of patients. The advent of targeted agents against BRAF and MEK, and of effective immunotherapies against immune checkpoints have progressively increased long-term overall survival well above 50%, up to 70% in some cases (Figure 21.9). Such spectacular increases are found only in a few other tumor types, but the analysis of 5-year survival over the past half-century shows upward trends in all tumor types (Figure 21.10), regardless of whether the starting point was very low or very high; not unsurprisingly, the former showed the highest relative gains.

The analysis of US cancer mortality rate trends in this century shows an unrelenting decrease, with reductions ranging from 1.4% per year in females) to 1.8% per year in males (Figure 21.11).

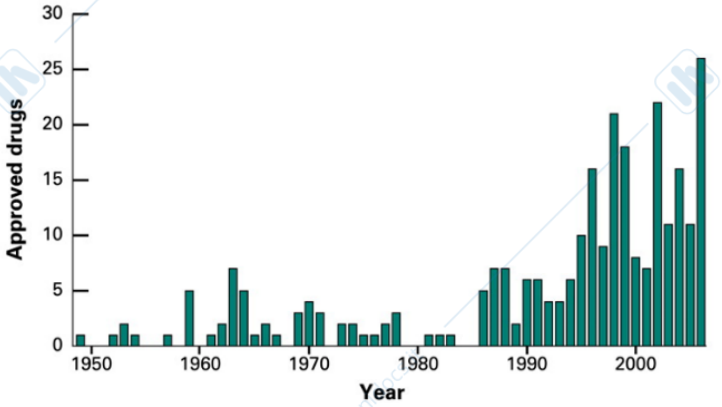
The continuation of these trends, which in future decades could significantly reduce the impact of cancer on human health, will depend on combined efforts at continuously improve primary and secondary cancer prevention, as well as molecular research leading to the discovery of novel therapeutic strategies and agents.

Perspectives of cancer therapy

The discovery of new cytotoxic drugs has been dwindling since the 1980s (Figure 21.12), however some fresh hope comes from newer conceptual approaches, such the application of high-throughput technologies, which mine chemical databases comprising thousands of different molecular structures, and the attempts at using nanotechnologies for the fabrication of novel drugs and drug carriers.

Target therapy is a global philosophy which has already yielded many effective drugs since the 1980s (Figure 21.12). More therapeutic agents are in the pipeline, and several aspects of the biology of tumor cells will lead to the discovery of new targets and novel therapeutic strategies.

Figure 21.12. Number of oncological treatments approved by the FDA, by year.
From J. Natl. Cancer Inst., 99: 344.

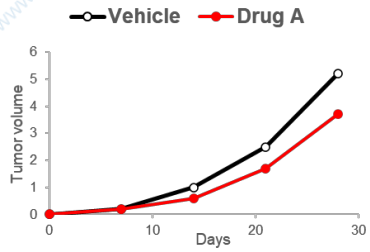


Chapter 22. Modeling Cancer

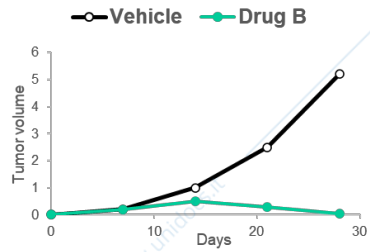
Experimental studies in human cancer patients are severely constrained by ethical and practical reasons, hence model systems are fundamental tools to unravel the complexity of cancer biology and to devise novel therapeutic strategies. The three major modeling domains are *in silico*, *in vitro* and *in vivo*. Note that the three domains are complementary, not mutually exclusive; most experimental frameworks will include all three, in addition to data gathered from humans.

How reliable are cancer models? A considerable debate has been fueled by the undeniable fact that many new drugs, touted as promising after successful testing in preclinical models, fail to demonstrate a therapeutic activity in clinical trials. The expression "lost in translation" (by Robert Frost, before being a movie title) is frequently cited for those treatments that fail the translation from bench to bedside. There is certainly a large space for the improvement of current cancer models, however two important issues must always be kept in mind when working with models: the first is that models will be models; there is no way to convert a map into the corresponding territory, nonetheless a map is extremely useful for those who know how to read it and need to move into an unknown territory. The second problem is wishful thinking; minimal, but statistically significant, proof-of-principle inhibitory effects observed in experimental models are too frequently claimed as evidence that some new treatment should be immediately translated to human patients (Figure 22.1). If a drug kills all tumor cells *in vitro* at sub-nanomolar concentrations and cures all tumor-bearing mice, then there is a faint hope that it could have some effect in patients, any lesser result in preclinical models

Figure 22.1. Critical evaluation of the results of proof-of-principle preclinical studies is mandatory for a successful clinical translation.



p value	Proof-of-principle study	Clinical equivalent
<0.05	Drug A significantly inhibits tumor growth	Tumor progression, failure



p value	Proof-of-principle study	Clinical equivalent
<0.001	Drug B significantly inhibits tumor growth	Complete response, success

$$\frac{\partial}{\partial t} f_i(t,u) = \sum_{j=1}^{n} \eta_{ij} \int_{D_u}\int_{D_u} \mathcal{B}_{ij}(u_*, u^*; u)\, f_i(t,u_*)\, f_j(t,u^*)\, \mathrm{d}u_*\, \mathrm{d}u^*$$
$$- f_i(t,u) \sum_{j=1}^{n} \eta_{ij} \int_{D_u} f_j(t,u^*)\, \mathrm{d}u^*$$
$$+ \sum_{h=1}^{n}\sum_{k=1}^{n} \eta_{hk} \int_{D_u}\int_{D_u} \mu^i_{hk}(u_*, u^*; u)\, f_h(t,u_*)\, f_k(t,u^*)\, \mathrm{d}u_*\, \mathrm{d}u^*$$
$$+ \sum_{j=1}^{n} \eta_{ij} \int_{D_u} \mu_{ij}(u_*, u^*, u)\, f_i(t,u_*)\, f_j(t,u^*)\, \mathrm{d}u_*\, \mathrm{d}u^*.$$

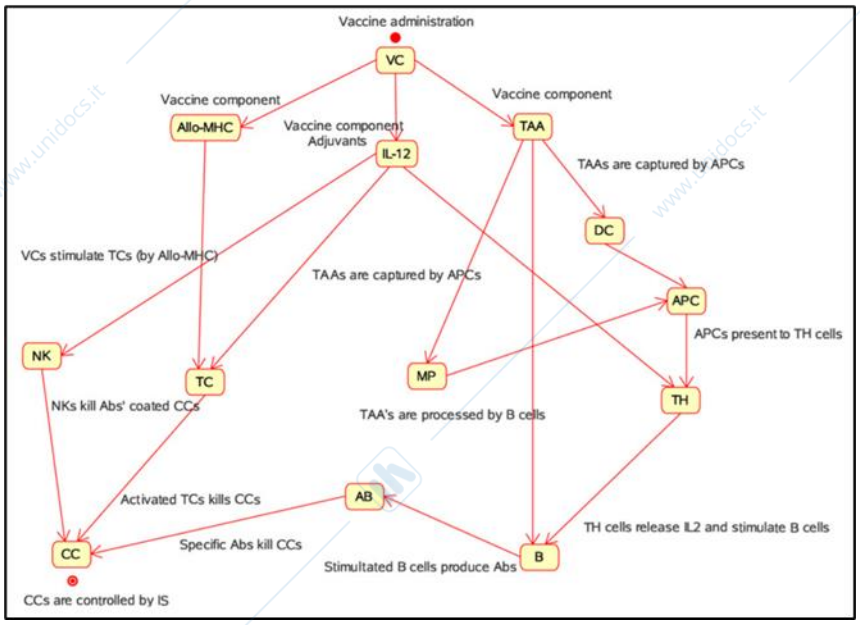


Figure 22.2. Excerpts from mathematical models of tumor immunology, a differential equation (*upper panel*) and an agent-based model (*lower panel*). Upper panel from Bellomo & Forni, Curr. Top. Dev. Biol. 81:485, lower panel from Pappalardo *et al.*, Cell. Immunol. 244: 137.

should discourage clinical translation, prompting instead for further preclinical work to improve and refine a potentially interesting, but still premature therapeutic strategy.

In silico cancer models

Mathematical modeling of oncological phenomena started with simple equations that describe tumor growth (e.g. Gompertz function), then moved to the analysis of the effects of cytotoxic drugs, and is now trying to capture the complexities of tumor-host relationships, including immune responses and neoangiogenesis. The design of useful mathematical models requires a tight cooperation, and mutual understanding, between mathematicians and cancer experts. The willingness to collaborate can be very high, but mutual understanding is frequently limited. The choice of the model system is critical; mathematicians usually favor differential equations, which very few life science researchers are prepared to understand. However, differential equations are not the sole available tool: agent-based models, which in essence resemble board games played with tokens representing cells or molecules, are more easily understood by biomedical researches, allowing more interactive collaborations with mathematicians (Figure 22.2).

In silico models can be easily tailored to reproduce any type of cancer behavior, from growth, to metastasis spread, to the effect of therapeutic interventions, the major difference being that each biological experiment takes months or years, whereas validated mathematical models can perform millions of experiments in a

few seconds. The analysis of the results of *in silico* models can yield new insight into oncological phenomena, leading to the formulation of fresh hypotheses, which can then be tested in biological systems.

***In vitro* studies**

Pioneering experiments of *in vitro* tissue culture date back to the end of the 19th century, but major advancements, leading to the development of modern techniques and culture media, were made by virologists after World War II. The first human cancer cell line, HeLa, was established in 1951 from a cervical carcinoma. Since then, a huge number of normal and neoplastic cell lines was established *in vitro*; more than 120,000 human, mouse and rat cell lines are listed in the July 2020 Cellosaurus database (web.expasy.org/cellosaurus).

There are three main ways to obtain tumor cells for *in vitro* studies: primary cell cultures, directly obtained from tumors, can be used for short time spans (days to weeks), but frequently fail to thrive *in vitro*; only in some instances they give rise to long-term cultures that eventually yield continuous cell lines – conventionally the cell line designation should be attributed only after 20 *in vitro* passages. Neoplastic cell lines can also be obtained from normal cells exposed in vitro to carcinogens or transduced with oncogenes or mutant tumor suppressor genes. Note that the designation of "normal cells" is mistakenly attributed to several continuous cell lines, such as various "3T3" lines, that display some normal phenotypes, such as contact inhibition, but are no longer fully normal, having accumulated various spontaneous mutations *in vitro*; the only source of *bona fide* normal cells are primary cultures from non-neoplastic hosts.

Tumor cell lines are highly useful tools, but several critical issues must be always kept in mind.

- The establishment of a cell line *in vitro* from a tumor entails major bottlenecks, usually most tumor cells die and only a few cells give rise to the cell line, thus the process selects subpopulations of tumor cells endowed with properties that allow them to thrive *in vitro*.
- Tumor cells are genetically unstable, and conventional culture conditions are mutagenic (e.g. high oxygen), thus cell lines must be continuously monitored to avoid phenotypic drift or the emergence of unwanted variants. As the accumulation of mutations is proportional to the time in culture, remember that some popular cell lines have been around for more than 40 years, e.g. HeLa, 1951; B16, 1954; HT-29, 1964; RD, 1969; MCF-7, 1970; 4T1, 1978.
- Specific genotypes or phenotypes are selected positively or negatively *in vitro*, for example high proliferation rate and low differentiation. Some cancer genes are favored *in vitro* in comparison to the original tumor, for example the frequency of p53 mutations among human sarcoma cell lines is much higher than in the corresponding tumors, whereas other genes are necessary for *in vivo* tumor growth but are counterselected *in vitro*, for example cell lines from HER-2 transgenic mammary carcinomas tend to lose HER-2 expression *in vitro*.
- *In vitro* culture conditions are ideal also for many microbes. In most instances the contamination is evident and the culture is immediately discarded, but occult infections by non-cytotoxic microbes, e.g. mycoplasmas, can modify

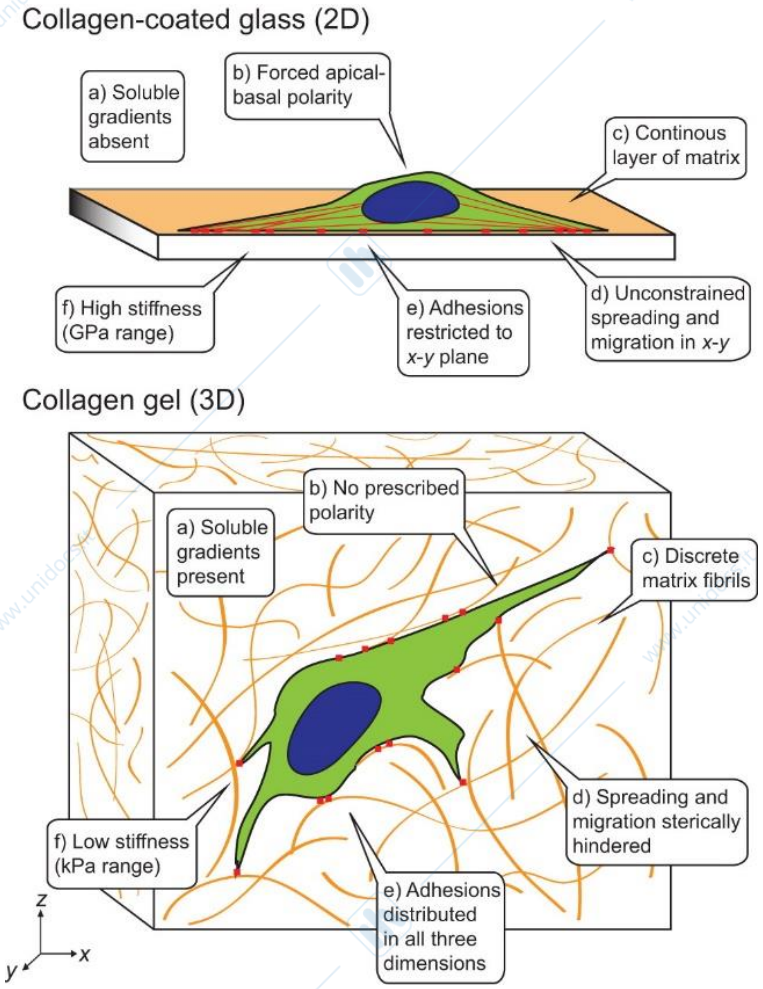


Figure 22.3. 2D *vs.* 3D cell cultures.
From BM Baker & CS Chen, J Cell Sci 125: 3015.

multiple phenotypes of eukaryotic cells, such as metabolism, interferon response, etc., without any visible sign.

- Most laboratories simultaneously maintain many different cell lines and primary cultures, thus misidentification of the starting material and cross-contamination by other cell lines are constant risks. Some notorious cell lines, such as HeLa or RD, cross-contaminated dozens of others tumor cell lines, which were inadvertently used for many years before the contamination was discovered.

To avoid these and other problems, some standard pieces of advice are:

- Preferentially obtain cell lines from reputable biobanks, such as the American Type Culture Collection, ATCC. Quarantine new arrivals from external sources and carefully check for possible microbial contaminations.
- Authenticate all cell lines using genetic polymorphisms and known phenotypes.
- For a set of experiments, use a few *in vitro* passages, as close as possible to the original source.
- Implement procedures to avoid cross-contaminations and periodically monitor cell genotype/phenotype and microbiological contaminations.

From 2D to 3D

In vivo, both normal and neoplastic cells form three-dimensional (3D) structures, like acini, villi, polyps, emboli, metastases, etc. Conventional culture conditions constrain all cell types to grow in plastic flasks under conditions that favor the bi-dimensional (2D) growth of adherent cells, but many matrixes, each with specific features, are now available to allow 3D cell growth *in vitro*:

- Plastic surfaces not treated for cell adhesion
- Semisolid media, e.g. agar
- Extracellular matrix gels
- Decellularized scaffolds, e.g. bone
- 3D-printed scaffolds

Culture of 3D tumor organoids, though more cumbersome, allows the expression

of phenotypes missing or altered in 2D (Figure 22.3), for example:

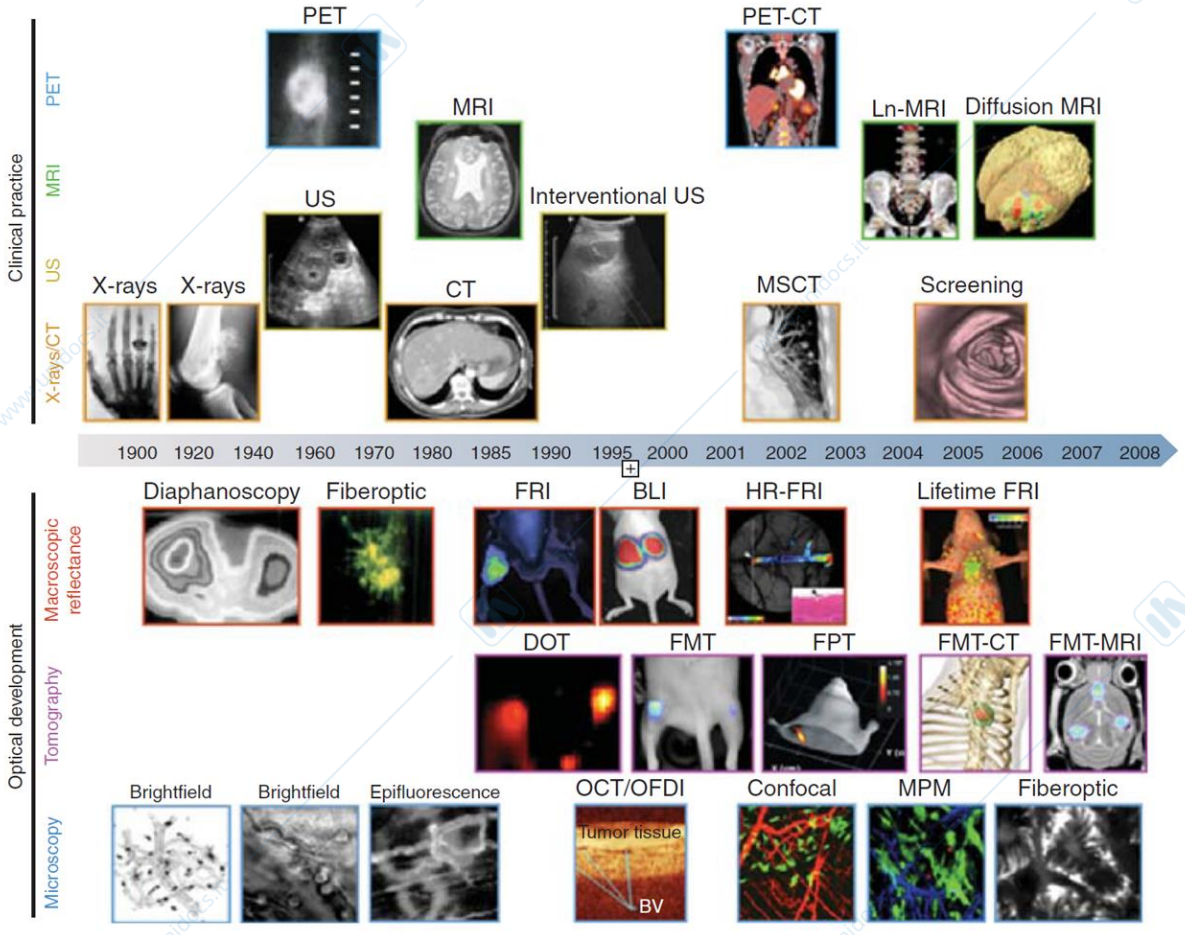
- Tissue organization and cell polarization
- Cell differentiation, release of mediators
- Hypoxia, cell death
- Invasiveness
- Drug sensitivity or resistance

Most attempts at establishing tumor cell cultures entail a constant struggle to get rid of normal elements, such as fibroblasts, that are thought as "contaminants" of pure tumor cell lines. However, real tumors are organ-like structures made of tumor cells that constantly interact with microenvironmental host-derived elements, like angiogenesis, fibroblasts, inflammatory cells, immune infiltrate, etc. The addition of host components to 2D and 3D tumor cell cultures aims at the reconstitution of a faithful tumor microenvironment in vitro, allowing studies previously restricted to *in vivo* models. However, even the most complex tumor organoids are still incomplete approximations of *in vivo* interactions, especially for what concerns systemic phenomena, such as metastatic spread or anti-tumor immune responses, hence *in vivo* studies will be always required to fully appraise tumor behavior.

In vivo models

Animal research is regulated worldwide by laws and guidelines. The EU reference

Figure 22.4. A timeline of imaging techniques.
From Condeelis & Weissleder, Cold Spring Harb Perspect Biol 2010;2:a003848.



is Directive 2010/63. Italian law (DL 26/2014) implements additional restrictions. In Italy, all research projects using vertebrates or cephalopods must be approved by an institutional committee, e.g. the *Comitato per il benessere animale*, CoBA, at Bologna University, and by the Ministry of Health. The general guiding principles of all animal research are summarized in the "three Rs" (3R):

- Replacement: Use alternative methods whenever possible
- Reduction: Implement experimental designs that minimize the number of animals
- Refinement: Improve experimental methods to enhance animal welfare

A general way to implement reduction and refinement is through the use of imaging technologies (Figure 22.4), which for example allow the study of sequential time points in the same experimental animal. Furthermore, imaging technologies enhance and improve *in vivo* studies. Specialized "micro" versions of human imaging technologies are available for animal studies, including ultrasound, radiology, TC, NMR, PET and multi-modal machines, such as PET-TC. Special techniques have been developed for *in vivo* visualization of tumor cells labeled with imaging tracers, either permanently by gene transduction, or after *in vitro* treatment prior to *in vivo* injection; bioluminescence, for example through transduction with a firefly luciferase gene and imaging by luciferase substrate injection in tumor-bearing mice, is a popular system to sequentially monitor tumor growth and metastasis.

Meaningful and fruitful oncological research can be performed in all whole-organism biological models, from *E. coli* to the yeast, from *Drosophila* to the zebrafish. However, the royalty of cancer models is represented by rodents, and the mouse is the absolute king. Many varieties of mouse models are available for specific types of scientific research:

- Mice captured in the wild (wild mice), which are mostly used in zoological and genetic research
- Inbred mice (Box 22.1), to obtain an unlimited supply of genetically identical individuals
- Outbred mice, obtained by random mating, which are generally less expensive than inbred mice and in principle are more genetically diverse, even though many years of closed breeding in specialized establishments brings about a very limited polymorphism
- F_1 hybrids of inbred strains, which are used for genetic and pharmacological studies

Box 22.1. Inbred mice

Inbred mice are obtained through repeated brother x sister mating; after 20 or more generations, all mice are operationally identical, like human monozygotic twins. Hundreds of inbred mouse strains are currently available, each with specific phenotypes.

Within a given inbred strain, transplants of normal or neoplastic tissues from one individual to another are not rejected, whereas transplants between inbred strains differing at major histocompatibility complex (MHC) genes are rejected by strong immune responses. These experiments led to the discovery of the major histocompatibility complex (MHC) and to the definition of basic transplantation 'laws'; George D. Snell was awarded the Nobel Prize for this work.

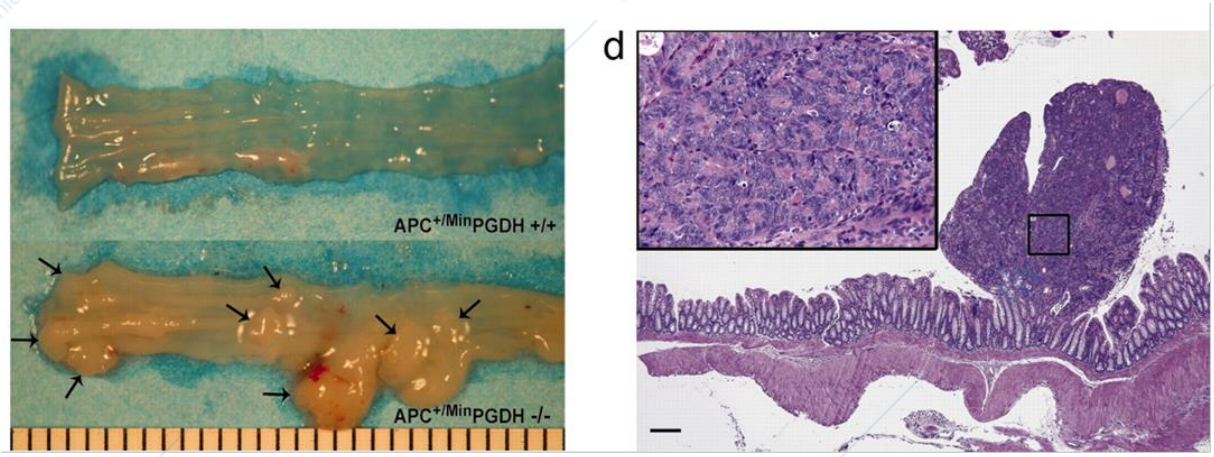


Figure 22.5. Intestinal polyps in Apc^{Min} mice.
From Y. Yamada *et al.*, PNAS 102: 13580.

- Spontaneous mutants, among which the most used in oncological research is the athymic *nude* mouse
- Genetically modified mice, with "designer" deletions (knock-out) or insertions (transgenic, knock-in) of specific genes

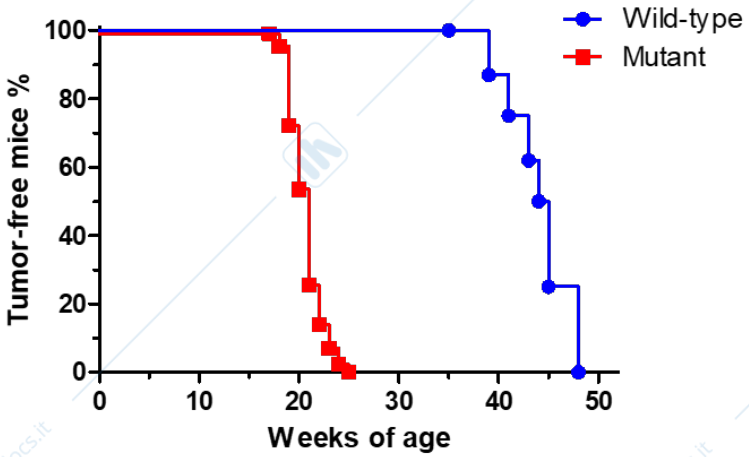
Modeling tumor onset

Animal studies of tumor onset allow the analysis of carcinogenic steps and processes that cannot be studied in humans. Spontaneous tumors, in mice as in humans, are relatively unpredictable and mainly affect old age; the study of some strains with unusual high incidence of tumors affecting young mice allowed the discovery of several oncogenic viruses and of many oncogenes. As we have seen in the chapters on carcinogenesis, *in vivo* treatments mimicking human exposure are used to study suspect carcinogens; moreover, the inhibition of standardized carcinogenic treatments can be used to study the activity of potential cancer preventive agents. Mice harboring spontaneous and induced mutations of oncogenes and tumor suppressor genes, which are prone to highly reproducible carcinogenic processes, allow a precise analysis of the molecular mechanisms that drive tumor progression downstream of specific cancer gene alterations, and are also used to investigate tumor prevention strategies. Genetic engineering techniques allow the choice of constitutive or inducible promoters and the selection of target organs and tissues. Frequently used models include Apc mutants. HER-2 transgenics and p53 knockouts.

Apc^{Min} (mutation induced by chemical carcinogenesis) heterozygous (homozygosis is lethal *in utero*) mice are prone to intestinal adenoma development (Figure 22.5) and are used as a model of human polyposis (see chapter on hereditary cancer) and for the study of intestinal carcinogenesis. Major differences with human polyposis are the prevalent development of lesions in the small intestine rather than in the colon, and the infrequent spontaneous progression to carcinoma. Many genetically engineered Apc mouse lines were developed to improve the faithfulness of the model, including different mutations, e.g. Apc^{1309}, and combinations with other cancer genes, such as p53.

HER-2 transgenic mice are prone to mammary carcinoma development. A

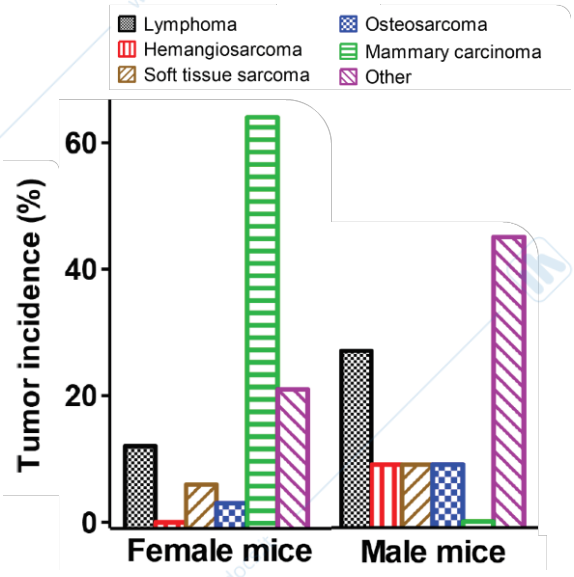
Figure 22.6. Mammary carcinoma onset in mice carrying a wild-type or a mutant HER-2 transgene



variety of mouse lines are available, differing in molecular features, tumor incidence, progression, and malignancy. The oncogene can be of rat or human origin, with a wild-type sequence or with activating point mutations that accelerate mammary carcinogenesis (Figure 22.6). A number of different promoters are also available; early transgenic mouse lines used mouse mammary tumor virus (MMTV) long terminal repeats (LTR) or the pregnancy-dependent promoter of the whey acidic protein (WAP), subsequently, transgenic lines were also made using the natural promoter or conditional promoters that allow to switch on and off HER-2 expression to study oncogene control of different phases of the mammary carcinogenesis. Notably, the carcinogenic potency of HER-2 transgenes was widely different in different hosts (e.g. FVB, BALB/c, C57BL/6, F_1 hybrids), thus illustrating the importance of the genetic background, which can contain various modifier genes.

Many engineered mutations of p53 are available, including deletions and dominant-negatives. As in human Li-Fraumeni syndrome, mice develop carcinomas, lymphomas and sarcomas (Figure 22.7), the relative proportions depend also on the genetic background. In heterozygous ($p53^{+/-}$) mice, tumor development typically begins after the occurrence of a somatic mutation in the remaining p53 allele, i.e. the tumors are $p53^{-/-}$, according to the classical Knudson two-hit model. Homozygous mice are viable and show a highly accelerated carcinogenesis. In addition to the study of p53-mediated carcinogenesis, p53 mutant mice can be used as a platform for many other oncological studies, for example to accelerate the *in vivo* analysis of suspect carcinogens (see chapter on the fundamentals of carcinogenesis), or to combine polygenic modifications that mimic human tumor development, for example p53 + Apc mutation yields

Figure 22.7. Development of multiple tumor types in p53 heterozygous ($p53^{+/-}$) mice. From Landuzzi et al., Oncotarget. 5: 11924.



intestinal tumors, p53 + Ras yields lung carcinomas, p53 + HER-2 or Fos yields rhabdomyosarcomas.

Human-in-mouse systems

Human tumor xenografts are rejected by immunocompetent hosts, but some can grow in immunodeficient rodents. The starting material can be either human neoplastic cells cultured *in vitro*, or human tumor specimens (Figure 22.8); models obtained with tumor specimens were given a variety of designations: patient-derived xenografts (PDX), xenopatients and avatars. Wide variations were found in PDX take and growth, depending on tumor histotype, implantation site, type of host immunodeficiency, etc.

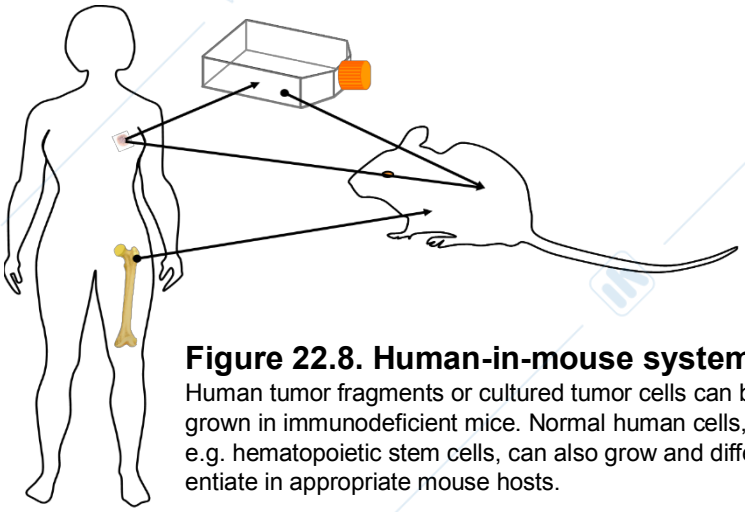


Figure 22.8. Human-in-mouse systems.
Human tumor fragments or cultured tumor cells can be grown in immunodeficient mice. Normal human cells, e.g. hematopoietic stem cells, can also grow and differentiate in appropriate mouse hosts.

Early studies used *nude* mice, which harbor a spontaneous Foxn1 mutation causing epithelial defects. A partial T cell immune deficit is an indirect consequence of thymic epithelium atrophy. However, the *nude* mouse is quite inefficient, as many highly malignant human tumors fail to grow. Early alternatives (SCID, bg/nu/xid, *nude* +anti-NK treatments) provided marginal benefits. Knockout of the common gamma subunit of interleukin receptors (Il2rg) was the key to a novel generation of T, B and NK deficient mice ($Rag1^{-/-}$/$Il2rg^{-/-}$, $Rag2^{-/-}$/$Il2rg^{-/-}$, NOD/SCID/$Il2rg^{-/-}$).

All immunodeficient mice have residual immune responses that interfere with the processes under study. *Nude* mice have a T cell maturation defect and a secondary B cell impairment due to the lack of T cell help, but retain a strong NK response, which can impair the local growth and hematogenous metastatic spread of tumor cells. Mice with Il2rg + recombination gene (Rag) knockouts are more permissive than *nude* mice to the growth and the multi-organ dissemination of human metastatic tumors.

The major shortcoming of human tumor implantation in these mice is the lack of immune responses, which rules out most immunological studies, and introduces significant bias in the study of therapeutic agents, as the immune system is known to be involved in the mechanisms of action of cytotoxic and targeted treatments. However, it was found that highly immunodeficient mice can be engrafted with some normal human cells (Figure 22.8), leading to the development of mice harboring a (partially) functional human immune system after the implantation of human hematopoietic stem cells, thus allowing the analysis of some aspects of human tumor immunology without the human body.

Modeling metastatic spread

Even though the major unsolved clinical problem is the control of metastatic spread, most experimental studies use tumors growing locally in mice. Apart from technical hurdles, this is due to the fact

Box 22.2. Heterotopic *vs.* orthotopic tumor implantation

The easiest routes for tumor cell implantation in mice are the subcutaneous (s.c.) and the intramuscular (i.m.), however most tumor histotypes would find an alien microenvironment in these sites.

Orthotopic injection places the tumor in an anatomical site similar to the site of origin (e.g. brain, intestine, mammary fat pad, prostate).

Various functional studies suggest that tumors implanted orthotopically mirror the features of the original tumor better than heterotopic implants, including metastatic spread.

Orthotopic injection is generally more cumbersome than heterotopic, in some instances requiring surgical procedures, which explains why heterotopic implantation is still used in many studies.

that experimental models of metastatic spread are inefficient, as demonstrated by the fact that several cell lines and PDX derived from metastatic human tumors fail to metastasize in mice. This is the reason why a small set of proven human and mouse metastatic cell lines are used in most experiments, e.g. mouse B16, 4T1, TS/A; human MDA-MB-231, SK-OV-3, HT1080. It can be argued that this introduces a kind of "experimental model selection bias", which possibly contributes to the translational problems that we have examined at the beginning of this chapter. Some improvements were obtained through the use of hosts lacking NK cells and of orthotopic tumor cell injections (Box 22.2), however the metastatic efficiency of experimental models remains suboptimal.

A commonly used modification to improve metastatic efficiency is the intravenous (i.v.) injection of tumor cells. Direct injection in the bloodstream only reproduces the post-intravasation events of the metastatic process, whereas spread from tumors includes local phases. Despite its intrinsic incompleteness, injection in the bloodstream has distinct advantages: higher efficiency, fast and reproducible development of metastatic nodules, testing of short-lived in vitro pretreatments, target organ specificity. I.v. injection in a tail vein is commonly used in mice,

Figure 22.9. Features of major cancer model systems.

Tuveson & Clevers, Science 364: 592

GEMM, genetically engineered mouse model; MDO, murine-derived organoid; MDOX, murine-derived organoid transplantation; CLs, cell lines; PDX, patient-derived xenograft; iPS, inducible pluripotential stem cell; PDO, patient-derived organoid; PDOX, patient-derived organoid transplantation.

	GEMM	MDO	MDOX	CLs	PDX	iPS	PDO	PDOX
Wild-type cell culture	+	+	+	–	–	+	+	–
Preinvasive cancer models	+	+	+	–	–	+	+	+
Invasive cancer models	+	+	+	+	+	+	+	+
Metastatic cancer models	+	+	+	+	+	+	+	+
Cost	$$$$	$$	$$$	$	$$	$$	$$	$$$
Time	++++	+	++	+	++++	+++	++	+++
Success rate	high	med	med	med	med	low	med	med
Throughput therapies	low	med	low	high	low	high	med	low

+ denotes 1 month or less; ++, 1–2 months; +++, 1–6 months; ++++, oftentimes more than several months.

typically resulting in lung metastases. Jugular vein or intracardiac injections are used to obtain brain metastases, intrasplenic injection for liver metastases.

Conclusions

We have examined the advantages and the shortcomings of many cancer models. Even though no single model can recapitulate the complexity of human oncology, an extensive understanding of the possibilities offered by experimental models (Figure 22.9), and the combined use of different model systems, have been, and will always be, the key to major advancements in our understanding of human tumors and in the development of effective cures.

Further Reading

- Vincent T. DeVita Jr., Steven A. Rosenberg, Theodore S. Lawrence (eds.). Cancer: Principles and Practice of Oncology. 11th edition Lippincott Williams & Wilkins, 2018.
- Lauren Pecorino. Molecular Biology of Cancer: Mechanisms, Targets, and Therapeutics. 4th edition, Oxford University Press, 2016).
- Robert A. Weinberg. The Biology of Cancer. 2nd edition, Garland Science, 2013.

Cellular & Molecular Oncology – A Concise Primer
By Pier-Luigi Lollini

Preliminary edition, November 2020

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Front Cover
The therapeutic monoclonal antibody trastuzumab (orange and green) bound to the extracellular domain of the oncogene HER-2 (blue).

About the book
This book is set in 11.5 pt. (text) and 22 pt. (titles) Palatino Linotype.
Figure legends and tables are set in Arial.
To ease printing and e-reading, all the customary white pages were omitted. Bibliophiles will excuse the unsightly consequences, such as chapters that begin on a left page.