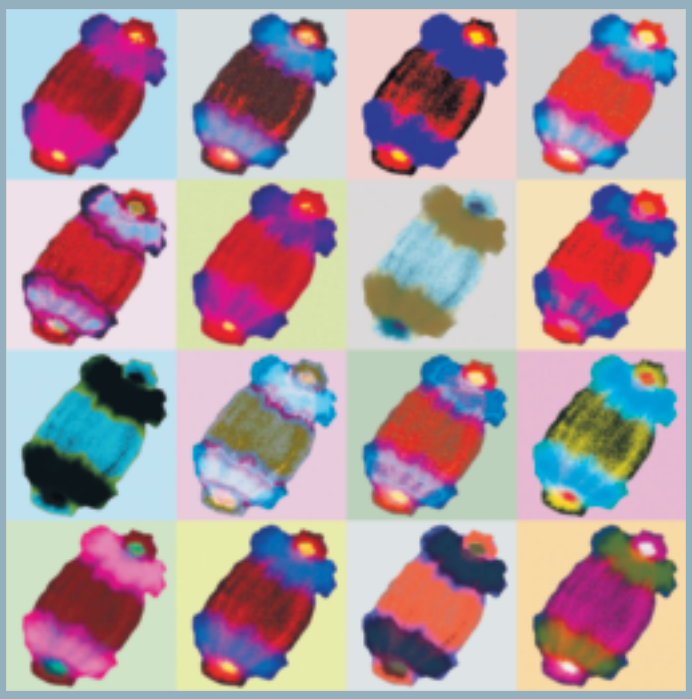
Mechanisms of tumour development

The phenotypic changes which a cell undergoes in the process of malignant transformation is a reflection of the sequential acquisition of genetic alterations. This multi-step process is not an abrupt transition from normal to malignant growth, but may take place over 20 years or more. The mutation of critical genes, including suppressor genes, oncogenes and genes involved in DNA repair, leads to genetic instability and progressive loss of differentiation. Tumours enlarge because cancer cells lack the ability to balance cell division by cell death (apoptosis) and by forming their own vascular system (angiogenesis). The transformed cells lose their ability to interact with each other and exhibit uncontrolled growth, invade neighbouring tissues and eventually spread through the blood stream or the lymphatic system to distant organs.



MULTISTAGE CARCINOGENESIS

SUMMARY

> Tumours consist of cells whose growth and morphological characteristics are markedly different from those of normal cells. Criteria for malignancy include increased cell proliferation, loss of differentiation, infiltrative growth and metastasis to other organs.

> Malignant transformation is a multistage process, typically a progression from benign lesions (e.g. adenoma) to malignant tumours (e.g. carcinoma). This evolution of malignant cells is caused by the sequential accumulation of alterations in genes responsible for the control of cellular proliferation, cell death and the maintenance of genetic integrity.

> The development of cancer may be initiated by environmental agents (chemical carcinogens, radiation, viruses) and inherited genetic factors (germline mutations).

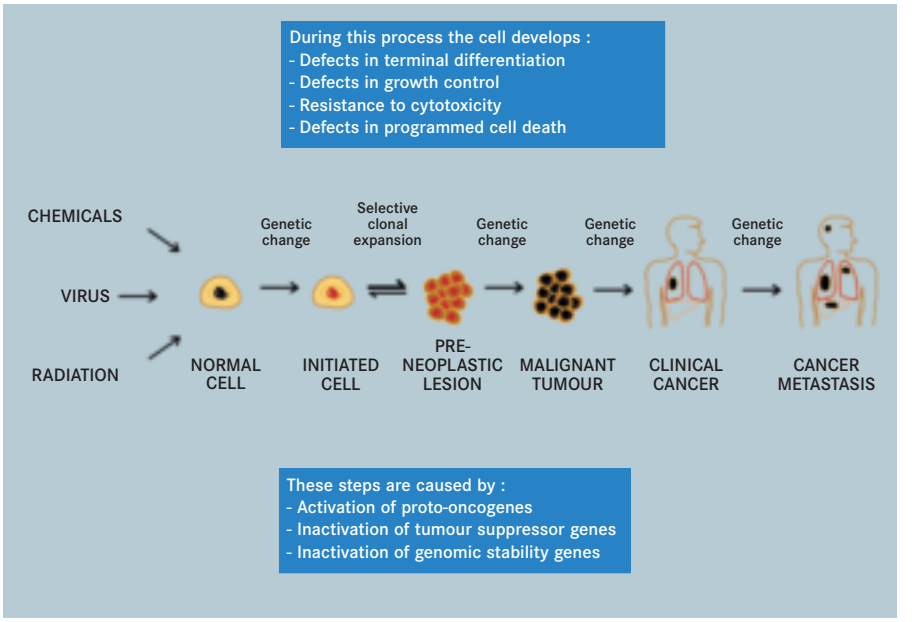


Fig. 3.1 Carcinogenesis is a multistage process involving multiple genetic and epigenetic events in proto-oncogenes, tumour suppressor genes and anti-metastasis genes.

Cancer arises from a single cell

Malignant tumours (or "cancers") are described as monoclonal, meaning that each tumour arises from a single cell. The development of a malignant tumour from a normal cell usually occurs over a considerable fraction of our lifetime. Such a long period is reflected, for example, by the difference between the age at which people start smoking and the age at which diagnosis of lung cancer most often occurs. The long "latent period" in lung cancer and almost all other malignancies is not explicable on the basis of a single-step transition from a normal cell to malignant one. Rather, the tumour is the outcome of an evolutionary process involving successive generations of cells, which are progressively further advanced towards cancerous growth [1].

Human histopathological observations support this scenario, and a range of pre-malignant lesions have been identified [2]. Likewise, in experimental animals, specific cell populations may be identified as marking a commitment towards malignancy, and these may be exploited as an early indicator in the context of carcinogen testing [3]. Thus, wholly on morphological grounds, cancer may be perceived as the outcome of a complex biological process.

Multiple steps are required for a cancer to arise

Animal "models" of cancer development, most commonly involving treatment of rodents with carcinogenic chemicals or other cancer-inducing agents, have provided clear evidence that specific stages in malignant transformation can occur discretely [4]. Chemicals which cause cancer in animals without the need for other treatment are sometimes called "complete carcinogens" (although "carcinogens" would be appropriate). Most such carcinogens cause damage to DNA of cells or tissues exposed to them. DNA-damaging activity may be identified on the basis of defined protocols (sometimes called "short-term tests", to emphasize their difference from chronic lifetime bioassay in rodents). Chemicals which exhibit mutagenic activity in short-term tests, which typically involve sensitive bacterial strains and cell-free extracts to catalyse metabolism of the test compound, are characterized as "genotoxic" [5]. Genotoxic agents may be complete carcinogens, but can also act as "initiating agents". After a single treatment with an initiating agent, tumour growth may be facilitated by chemicals (or treatments) which stimulate cell proliferation, sometimes by inducing mild toxic damage in exposed tissue. These agents are termed "promoters" (Table 3.1). As well as these genotoxic chemicals, a range of non-genotoxic agents can cause cancer in humans and/or experimental animals [6].

The stages in tumorigenesis have been designated "initiation", which encompasses damage to, and then division of exposed cells such that their growth potential is changed irreversibly, and "progression", denoting multiple rounds of cell replication mediating the gradual transition of an initiated cell towards autonomous, cancerous, growth. Ultimate spread of malignant cells resulting in multiple tumour sites has been termed "metastasis". The unequivocal identification by the mid-1970s of these various phases was one indication that carcinogenesis is a multistage process. Arguably, the greatest achievement of cancer research during the last decades of the 20th century has been the elucidation of multistage carcinogenesis at the molecular genetic level.

The molecular basis of tumour pathology

In a seminal publication, Vogelstein and colleagues [7] provided evidence that the different stages in the cellular evolution of colon cancer in humans, histologically identified as hyperplasia, early-stage adenoma, late-stage adenoma etc., could be identified with specific successive genetic changes (Fig. 3.2). The genetic changes included oncogene activation by mutation at specific sites and loss of chromosomal regions (necessarily involving multiple genes) which were subsequently shown to be the location of tumour suppressor genes. Since that initial description, knowledge of the molecular genetic basis for human colon cancer has been massively extended (*Colorectal cancer*, p198). For most tumours, the genetic changes are not inherited from our parents but arise in a previously normal cell. The progeny of this cell after cell division carry the same genetic change but the surrounding cells remain normal. Because these genetic changes affect only the cancer cells, they are not passed on to the children of cancer patients. However, in a minority of cases some critical changes are inherited, giving a familial predisposition to colon or other cancers.

Factor		Cancer site/cancer
Hormones	Estrogens, progesterone	Uterus, mammary gland
	Gonadotrophins	Ovary, testis, pituitary
	Testosterone	Prostate gland
Pharmaceutical products	Oral contraceptives	Liver
	Anabolic steroids	Liver
	Analgesics	Renal pelvis
Miscellaneous substances	Bile acids	Small intestine
	Saturated fatty acids	Colon
	Salt	Stomach
	Tobacco	Oral cavity, lung, bladder etc.
	Saccharin, uracil, melamine, tetraphthalic acid and other xenobiotics causing urinary stones	Urinary bladder
	Dichlorobenzene, trimethylpentane (lead-free gasoline), perchloroethylene	Kidney
	Butylated hydroxyanisole, propionic acid	Stomach
	Nitrilotriacetate	Kidney

Table 3.1 Promoting agents: non-genotoxic agents that facilitate carcinogenesis by stimulating cell division. Tobacco smoke also contains genotoxic carcinogens.

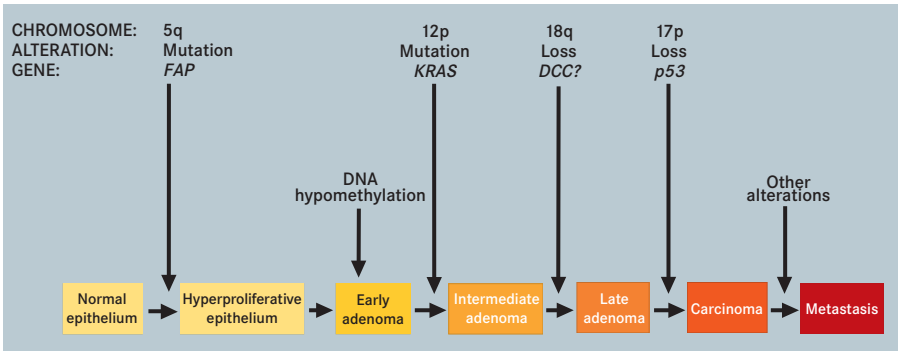


Fig. 3.2 The original Vogelstein model for the genetic and histological evolution of colon cancer. (*Colorectal cancer*, p198).

Commonality and heterogeneity

The molecular biological basis of multistage carcinogenesis initially described for colon cancer appears to have application to all tumour types, although there is marked variation in the extent to which genes relevant to particular tumours have been identified [8]. Some genes, and the corresponding change associated with tumorigenesis (mutation, overexpression, deletion and/or amplification) are common to a number of tumour types. However, each tumour type is associated with a distinctive set of gene alterations. The genes in question are discussed under the subheading *Pathology and genetics* for each of the tumour types included in Chapter 5. Such enumeration of relevant genes necessitates a degree of simplification. There is clear heterogeneity between individual tumours of the same type. In

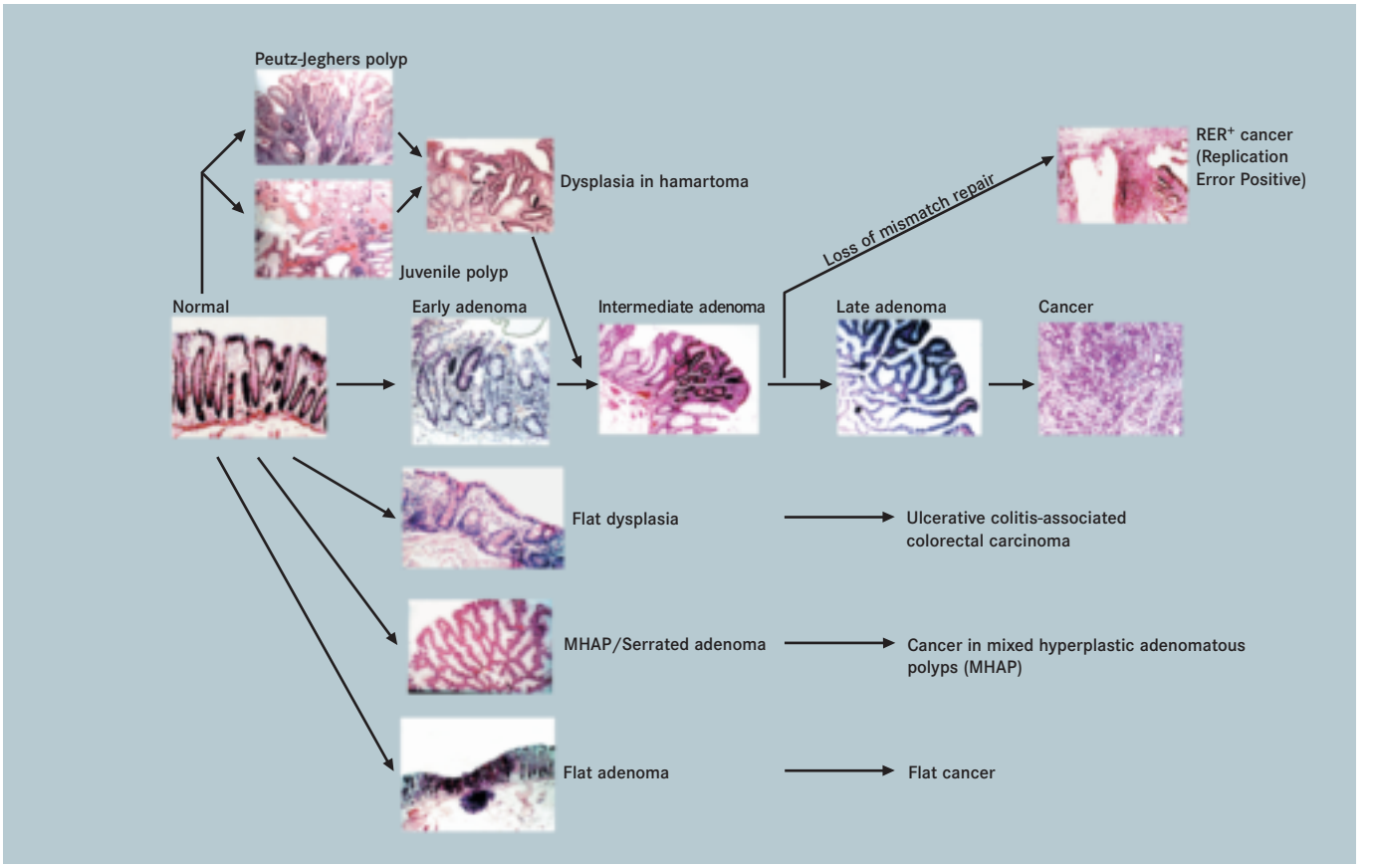


Fig. 3.3 Histological representation of the pathogenesis of colorectal cancer. Phenotypic changes in the morphology of the colonic mucosa reflect the sequential acquisition of genetic alterations.

other words, not every tumour will necessarily exhibit all the genetic changes established for the tumour type in question. Moreover, there is often marked heterogeneity within an individual tumour: adjacent cells differ. Mapping and identification of genes involved in malignant transformation has been a major component of the study of the molecular mechanisms of carcinogenesis.

Multiple genetic changes required

The emergence of a malignant cell population is understood to be the cumulative effect of multiple (perhaps five, ten or more) genetic changes, such changes being accumulated in the course of the evolution of the cell from normal to malignant. The genes designated as oncogenes and tumour suppressor genes *(Oncogenes and tumour suppressor genes,* p96*)* have been identified in terms of their biological function [9]. Such genes are among those that facilitate transmission of growth control signals from the cell membrane to the nucleus (that is, signal transduction), that mediate cell division, differentiation or cell death and, perhaps most critical of all, that maintain the integrity of genetic information by DNA repair and similar processes (*Carcinogen activation and DNA repair*, p89). Since mutations are normally infrequent events, it seems unlikely that in the course of a human lifetime a cell would acquire all the mutations necessary for cancer to develop, unless at some point the developing cell lost its ability to protect itself against mutation and gained what is called a "mutator" phenotype [10]. Thus, alterations in gene structure and expression which bring about carcinogenesis are being progressively identified [11]. As noted earlier, members of some cancer-susceptible families inherit mutations in particular genes that contribute to cancer development, and hence to their individual risk of disease. However, with most cancers, the genetic change critical to carcinogenesis results from damage to DNA by chemicals, radiation and viruses (Fig. 3.1). This damage is not entirely and perhaps not predominantly produced by exogenous agents but by natural processes, such as the production of reactive oxygen species or the spontaneous deamination of the 5-methylcytosine naturally present in DNA [13]. Furthermore, as shown as the second step in Fig. 3.2, biological change that is heritable may result from non-genetic processes including the modulation of gene expression by hypermethylation [12].

PRECURSOR LESIONS IN CHEMOPREVENTION TRIALS

Trials of agents for chemopreventive activity which are based on assessment of malignant disease are almost unmanageable because of the long period of time (perhaps decades) potentially involved. Attention has therefore been focused on lesions, either cellular or molecular, demonstrated to be valid indicators of the subsequent development of malignancy. A trial may then evaluate the effect of the putative chemopreventive agent on such precursor lesions.

The best-validated precursor lesions are benign tumours, such as colorectal adenomas. It is established that adenoma number, size, and severity of dysplasia are predictive factors for colorectal cancer incidence. It has been estimated that 2-5% of all colorectal adenomas progress to adenocarcinomas if not removed or treated. The risk is greater for large and severely dysplastic polyps. Cancer risk is decreased by polyp removal, and a strong correlation exists between the relative prevalence of adenomas and cancers across populations (Winawer SJ et al., *N Engl J Med,* 328: 901-906, 1993). Several epidemiological studies have shown that regular use of aspirin or related drugs is associated with a reduced adenoma incidence (*IARC Handbooks of Cancer Prevention. Vol. 1,* Lyon, 1997). This provides further confirmation that adenomas are precursor lesions for colon cancer, since aspirin is known to reduce the incidence of malignant colon cancer.

Potential precursor lesions of carcinogenesis include both phenotypic and genotypic markers (Miller AB et al. *Biomarkers in Cancer Chemoprevention, IARC Scientific Publications 154*, Lyon, 2001). Thus oral leukoplakia is a recognized precursor for cancer of the oral cavity. Histological modulation of a precancer (often called intraepithelial neoplasia) has been used as a precursor lesion in prevention trials (Kelloff GJ et al., *Cancer Epidemiol Biomarkers Prev, 9*: 127-137, 2000). Additionally, genetic lesions such as progressive genomic instability as measured by loss of heterozygosity or amplification at specific microsatellite loci, have been considered (Califano J et al. *Cancer Res*, 56: 2488-2492, 1996). Other potential precursor endpoints include proliferation and differentiation markers, specific gene and general chromosomal damage, cell growth regulatory molecules, and biochemical activities (e.g. enzyme inhibition). Serum proteins are of special interest because of their availability. Thus prostate-specific antigen (PSA) is being used as a "surrogate" marker for prostate cancer. It is expected that the number and variety of biomarkers for precursor lesions will continue to expand In parallel with the advances in understanding of the genetic and cellular basis of carcinogenesis.

Fig. 3.6 Tubular adenoma of the colon is a precursor lesion for colorectal cancer.

Ageing

Apart from multistage development, certain other processes are fundamental to malignant disease. Principal amongst these is ageing, which can be considered both in relation to the whole individual, and also at the cellular level. In humans, as well as in other mammals, the incidence of cancer rises dramatically with age. An exponential increase occurs from mid-life [14]. Passage of time is also critical to cell biology. Normal cells do not divide indefinitely due to senescence (Box: *Telomeres and Telomerase*, p108). Senescent cells cannot be stimulated to divide further, become resistant to apoptotic cell death and acquire differentiated functions. Senescence may be an anti-cancer mechanism that limits accumulation of mutations. However, when maintained in culture, cells treated with carcinogenic chemicals or infected with oncogenic viruses may avoid senescence and proliferate indefinitely. Such cell populations are described as being "transformed" and when further maintained in culture, once-normal cells acquire the same characteristics as cells cultured from malignant tumours. These and various other alterations in growth characteristics are recognized as the experimental counterpart of multistage carcinogenesis through which tumours develop in intact animals or humans. The genetic basis for senescence, and its relationship to malignancy, is a subject of intense investigation [15].

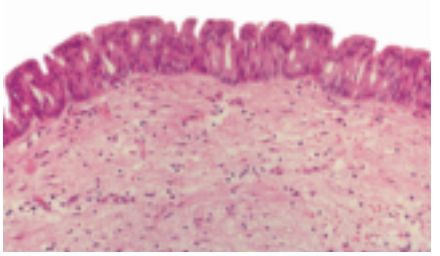


Fig. 3.4 Severe intraepithelial neoplasia (dysplasia) in the epithelium of an intrahepatic large bile duct, a condition caused by hepatolithiasis.

Preventing cancer

The significance of multistage carcinogenesis extends beyond facilitating understanding of how a transition from normal to malignant cell growth occurs. The fundamental cellular studies outlined earlier provide a basis for preventing can-

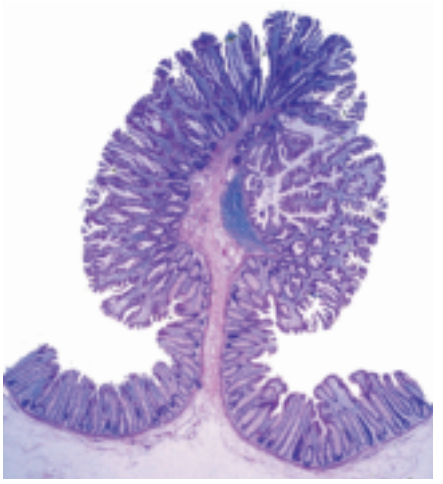


Fig. 3.5 Pedunculated hyperplastic polyp of the colon.

cer (see chapter 4). The fact that particular patterns of cell morphology and growth precede emergence of an unequivocally malignant cell population is the basis of secondary prevention of cancer.

Examples include detection of polyps in the large bowel (Fig. 3.5) and of morphological change which is the basis of the Papanicolaou smear test for early detection of cervical cancer. Moreover, dietary or pharmaceutical interventions calculated to prevent or reverse such lesions are the basis of chemoprevention [16]. Most importantly, knowledge of the genetic basis underlying tumour growth should provide new criteria for individual determination of diagnosis and prognosis. The mechanisms now known to operate in the proliferation of cancer cells provide a basis for the development of new, more efficient therapies without the side-effects that currently often afflict cancer patients [17].

REFERENCES

1. Foulds L, ed. (1969) *Neoplastic Development,* Vol. 1, London, Academic Press.

2. Correa P (1996) Morphology and natural history of cancer precursors. In: Schottenfeld D, Fraumeni JF, eds, *Cancer Epidemiology and Prevention*, New York, Oxford University Press, 45-64.

3. Ito N, Imaida K, Asamoto M, Shirai T (2000) Early detection of carcinogenic substances and modifiers in rats. *Mutat Res*, 462 : 209-217.

4. Weinstein IB (1982) Carcinogenesis as a multistage process–experimental evidence. In: Bartsch H, Armstong B, eds, *Host Factors in Human Carcinogenesis* (*IARC Scientific Publications No. 39*) Lyon, IARCPress, 9-25.

5. Vainio H, Magee PN, McGregor DB, McMichael AJ, eds (1992) *Mechanisms of Carcinogenesis in Risk Identification* (*IARC Scientific Publications No. 116*), Lyon, IARCPress.

6. Yamasaki H, Ashby J, Bignami M, Jongen W, Linnainmaa K, Newbold RF, Nguyen-Ba G, Parodi S, Rivedal E, Schiffmann D, Simons JW, Vasseur P (1996) Nongenotoxic carcinogens: development of detection methods based on mechanisms: a European project. *Mutat Res*, 353: 47-63.

7. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL (1988) Genetic alterations during colorectal-tumor development. *N Engl J Med*, 319: 525-532.

8. Balmain A, Harris CC (2000) Carcinogenesis in mouse and human cells: parallels and paradoxes. *Carcinogenesis*, 21: 371-377.

9. Evan GI, Vousden KH (2001) Proliferation, cell cycle and apoptosis in cancer. *Nature*, 411: 342-348.

10. Loeb LA (2001) A mutator phenotype in cancer. *Cancer Res*, 61: 3230-3239.

11. Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, Weinberg RA (1999) Creation of human tumour cells with defined genetic elements. *Nature*, 400: 464-468.

12. Esteller M, Corn PG, Baylin SB, Herman JG (2001) A gene hypermethylation profile of human cancer. *Cancer Res*, 61: 3225-3229.

13. Marnett LJ, Plastaras JP (2001) Endogenous DNA damage and mutation. *Trends Genet*, 17: 214-221.

14. Armitage P, Doll R (1954) The age distribution of cancer and a multistage theory of carcinogenesis. *Br J Cancer*, 8: 1-12.

15. Wynford-Thomas D (1999) Cellular senescence and cancer. *J Pathol*, 187: 100-111.

16. Bartsch H (2000) Studies on biomarkers in cancer etiology and prevention: a summary and challenge of 20 years of interdisciplinary research. *Mutat Res*, 462: 255-279.

17. Kallioniemi OP, Wagner U, Kononen J, Sauter G (2001) Tissue microarray technology for high-throughput molecular profiling of cancer. *Hum Mol Genet*, 10: 657-662.

CARCINOGEN ACTIVATION AND DNA REPAIR

SUMMARY

>Many chemical carcinogens require spontaneous or enzymatic activation to produce reactive intermediates which bind to DNA. The resulting carcinogen-DNA adducts may be eliminated from DNA by various enzyme-mediated repair processes.

>In cells and tissues with deficient DNA repair, replication of carcinogen-damaged DNA may result in the mutation of genes that regulate cell growth and differentiation in target cell populations. Such genetic alterations typically lead to progressive genetic instability resulting in uncontrolled growth, loss of differentiation, invasion and metastasis.

Experimental studies in rodents and in cultured cells have led to the classification of chemical carcinogens into two broad classes: genotoxic and non-genotoxic. Genotoxic carcinogens alter the structure of DNA, mostly by covalent binding to nucleophilic sites. These lesions, that is, the chemical entity of carcinogen bound to DNA, are called DNA "adducts". The replication of DNA containing unrepaired adducts may result either in the generation of sequence changes (mutations) in the newly synthesized daughter strands of DNA or in DNA rearrangements evident as chromosome aberrations.

This critical, irreversible genetic event can thus result in fixation of the original structural change in DNA as permanent, transmissible, genetic damage, or in the loss of genetic information through alterations in chromosomes. Such heritable change has the potential to perturb growth control in the affected cell, and is sometimes referred to as the "initiation" step of the tumorigenic process (Fig. 3.7).

Carcinogen activation

The first indication that certain cancers were associated with exposure to chemicals arose from observations by clinicians in the 18th and 19th centuries. The field of experimental chemical carcinogenesis started in 1915 with the experiments of Yamagiwa and Ichikawa, who showed that application of tar to the ears of rabbits induced skin tumours. In the 1940s, experiments on mouse skin demonstrated the stepwise evolution of cancer and allowed the characterization of two classes of agents, initiators and promoters [1]. Most chemical carcinogens are subject to metabolism that results in their elimination, but in the course of which reactive intermediates are generated. Such metabolic activation results in the modification of cellular macromolecules (nucleic acids and proteins) [2]. Accordingly, mutagenicity tests using bacteria and mammalian cells in culture were developed and are extensively used to identify potential carcinogens. Not all chemicals known to cause cancer, however, can be demonstrated to bind to DNA and hence be classified as "genotoxic".

Activation of chemical carcinogens in mammalian tissue mostly occurs through oxidation by microsomal mono-oxygenases (cytochromes P450, phase I enzymes). Cytochromes P450 are located in the endoplasmic reticulum (internal membranes of the cell) and constitute a superfamily of proteins; about 50 are now known in humans. The oxidation products are substrates for other families of enzymes (transferases, phase II enzymes) which link the carcinogen residues to a glutathione, acetyl, glucuronide or sulfate group; the resulting conjugates are hydrophilic and thus can be easily excreted. Carcinogenic electrophilic metabolites arise as by-products of these metabolic reactions. The metabolic pathways are well characterized for the major classes of chemical carcinogens (Fig. 3.8), including polycyclic aromatic hydrocarbons, aromatic amines, *N*-nitrosamines, aflatoxins and vinyl halides, which yield electrophilic species through phase I activation [3]. Other metabolic pathways are known. For example, dihaloalkanes are activated to carcinogenic metabolites by glutathione transferases.

Understanding of carcinogen-DNA interactions (Fig. 3.9) has resulted largely from the development of sensitive and specific methods for determining DNA adducts [4]. The most frequently used methods include immunoassays using adduct-specific anti-sera or antibodies, ^{32}P-postlabelling, fluorescence spectroscopy, electrochemical detection and mass spectrometry. Measurement of carcinogen-DNA adducts in rodents has revealed correlations between the concentration of the carcinogen in the environment, DNA adduct levels in tissues where tumours may arise and cancer incidence. It is therefore accepted that DNA adducts may be used as indicators

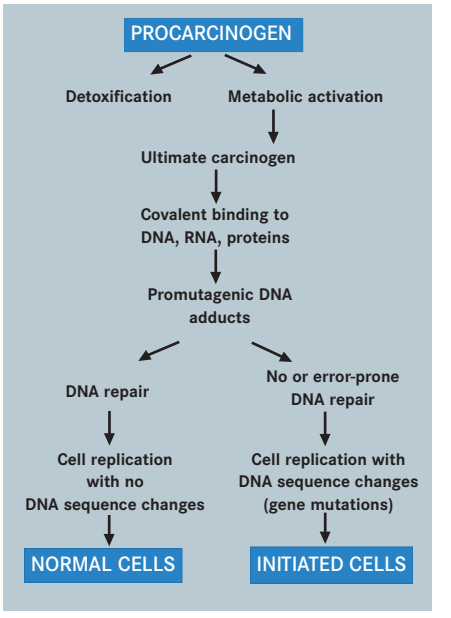


Fig. 3.7 Critical stages in the process of initiation by genotoxic chemicals.

of the effective biological exposure, and hence of carcinogenic risk in humans [5]. However, analysis of DNA adducts in human cells and tissues remains difficult, due to the very low levels of adducts present in DNA (typically, one adduct per 10^7–10^8 parent nucleotides).

Activities of the enzymes involved in carcinogen metabolism vary greatly between individuals due to induction and inhibition processes or to gene polymorphisms that can affect activity. These variations can affect the formation of carcinogen-DNA adducts, together with other genetic determinants that regulate DNA repair or cell cycle control, for example, and thus affect the outcome of exposure to DNA-damaging agents and influence cancer risk in different individuals [6]. Many studies have sought to correlate genetic polymorphisms, adduct levels and cancer risk in human populations (*Genetic susceptibility*, p71). These studies have hitherto provided some correlations for risk prediction at the population level. However, due to the great number of enzymes and polymorphisms involved, large-scale studies and high throughput assays (based on DNA microchips, for example) will be required to fully elucidate the complex nature of such gene-environment interactions.

Mutational spectra

Adducts of DNA and proteins can be used as early markers of exposure to carcinogens as indicated. However, because adducts only persist for a short time (typically, for a few hours or days for DNA

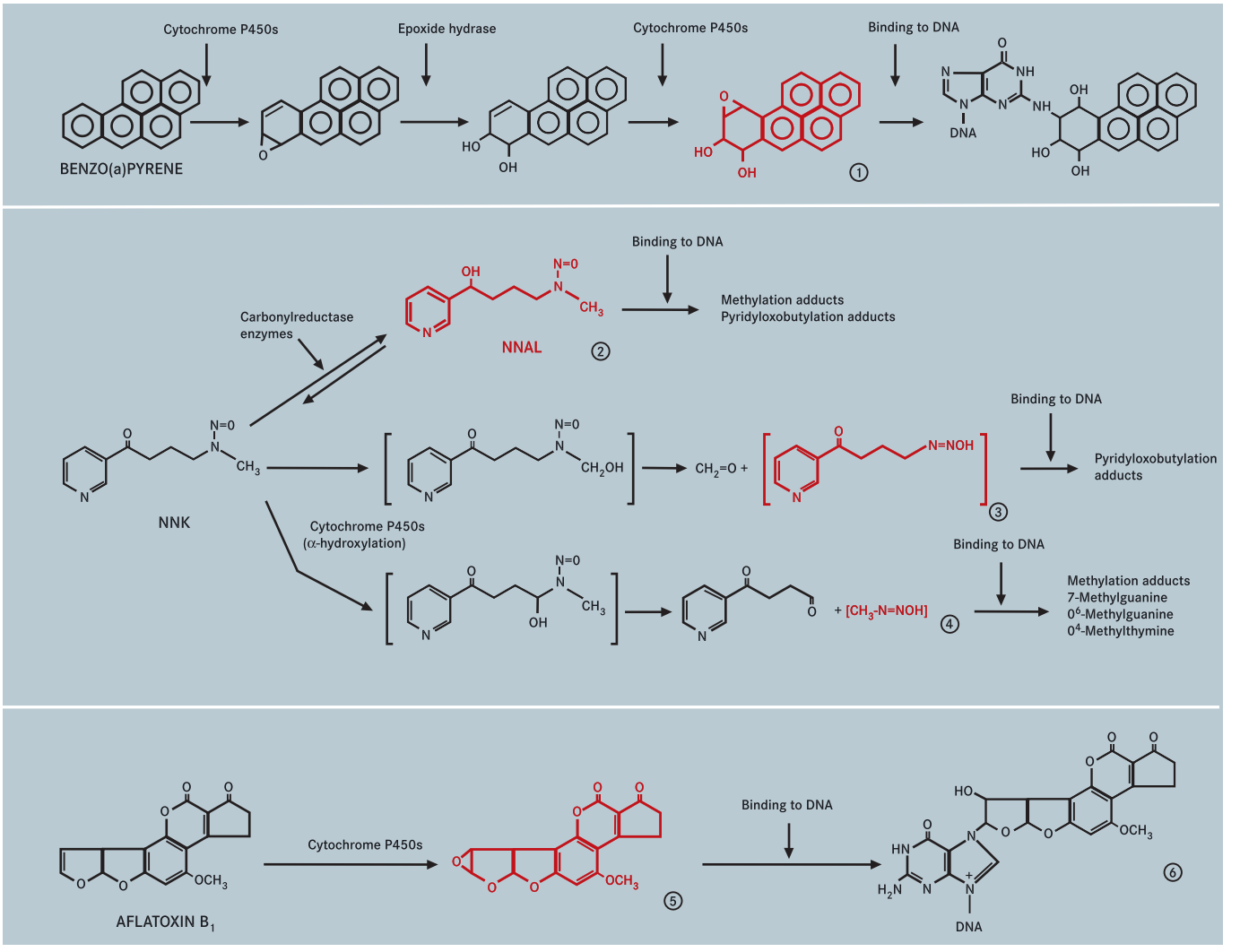


Fig. 3.8 Carcinogen activation by mammalian enzymes: reactions catalysed during metabolism of benzo[a]pyrene and NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), both contained in tobacco, and of aflatoxin B_1, produce reactive intermediates (ultimate carcinogens, in box), which bind to DNA. Other reaction pathways leading to the formation of glucuronides and other esters, which are excreted, are not shown. **1.** Benzo(a)pyrene-7, 8-diol-9, 10-epoxide; **2.** 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; **3.** Diazohydroxide; **4.** Diazohydroxide; **5.** Aflatoxin B_1-8,9-oxide; **6.** 2,3-Dihydro-2-(N^7- guanyl)-3-hydroxyaflatoxin B_1.

adducts, a few weeks or months for albumin or haemoglobin adducts), their usefulness as exposure markers is limited. Mutations in specific genes can be used as longer-term "biomarkers" of early biological effects or of disease [7]. Indeed, gene mutation patterns are probably the only biological marker that can be characteristic of a past exposure to a carcinogenic agent or mixture. Study of such mutations will increasingly assist in the identification of etiologic agents, in risk prediction and in cancer prevention studies. Mutation spectra can be analysed either in normal tissues (including blood cells) or in tumour tissues. Analysis of mutations in normal tissues remains difficult, because the mutant cell or DNA must be identified against a background of a very large excess of non-mutant cells or DNA, and a selection or an enrichment step is required. In contrast, mutations in tumour cells often favour growth and are amplified due to clonal expansion of the tumour cell population.

A few genes are suitable markers ("reporters") of mutation induction in experimental animals and in humans. Thus the hypoxanthine-guanine phosphoribosyl-transferase gene *HPRT*, when inactivated by mutation, renders cells resistant to growth inhibition by 6-thioguanine; such mutant cells can therefore be isolated by culture in the presence of this agent. Studies in humans have associated increases in the frequency of *HPRT* mutations (measured in circulating lymphocytes) with exposure to environmental genotoxic agents. However, in contrast to observations made in rodents, in which mutation profiles often reflect the relatively extreme DNA damage that induced them, characteristic *HPRT* mutation spectra (i.e. the types and positions of the base changes within the DNA sequence of the *HPRT* gene) are more difficult to observe in humans.

The identification of oncogenes and tumour suppressor genes (*Oncogenes and tumour suppressor genes*, p96) has led to the characterization of gene mutations which are more directly associated with carcinogenesis. The *RAS* family of oncogenes was among the first that was recognized as being mutated in a wide variety of human cancers. *p53* is the most commonly altered tumour suppressor gene in human cancer, being mutated in over 50% of almost all tumour types. A large database of *p53* mutations has been generated. Mutational spectra have been identified that provide evidence for the direct action of environmental carcinogens in the development of certain cancers (i.e. in these cases, cancer can be linked causally to past exposure to a defined carcinogenic agent). These mutations, which could in principle be used to identify exposure to particular agents, have been termed "signature" mutations. They result from the formation of specific DNA adducts. For example, *p53* mutations characteristic of the known or suspected etiological agent occur in lung cancer (attributable to benzo[a]pyrene in tobacco smoke) and hepatocellular carcinomas (due to aflatoxin B_1 in contaminated food) (Box: *Geographic variation in mutation patterns*, p102). In general, however, it is often not practical to obtain DNA from healthy tissue to analyse for potentially tumorigenic mutations, as invasive methods of sampling are required. Fortunately, the protein products of the mutated genes and, even the mutated DNA itself, can be detected and measured in body fluids or secretions, such as blood plasma, that have been in contact with the malignant tissue.

Presumed signature mutations have also been identified in "normal" tissues (non-pathological but probably containing initiated cells) from exposed individuals. For example, the *p53* mutation associated with exposure to aflatoxin B_1 has been found in liver tissue and in plasma DNA from healthy subjects (without cancer) who have consumed food contaminated with aflatoxins. Therefore, mutations in cancer genes could be used, in certain cases, as early indicators of risk before disease diagnosis.

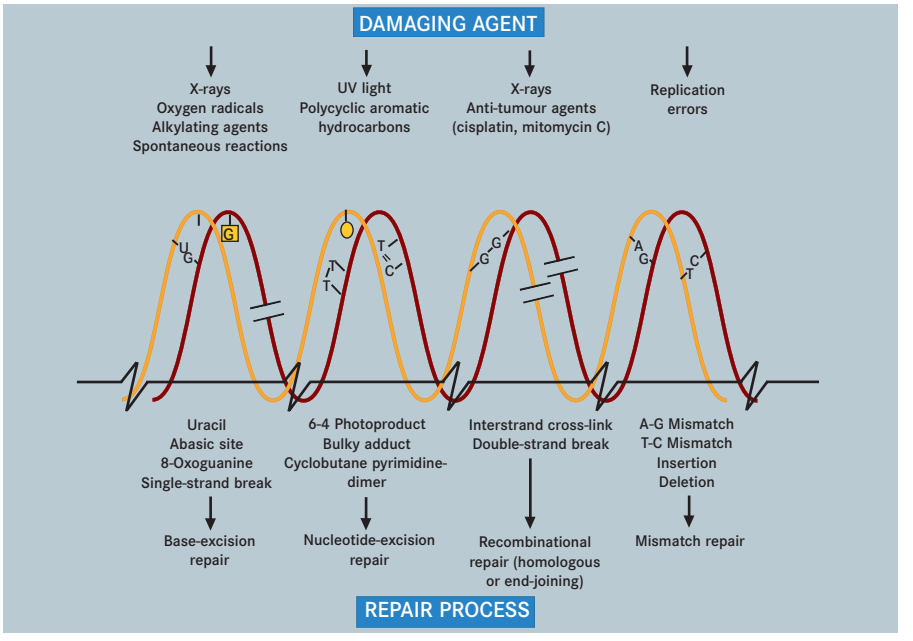


Fig. 3.9 Common DNA damaging agents, examples of DNA lesions induced by these agents and the most important DNA repair mechanism responsible for the removal of these lesions.

DNA repair

The 3 x 10^9 nucleotides of the DNA within each human cell are constantly exposed to an array of damaging agents of both environmental origin, exemplified by sunlight and tobacco smoke, and of endogenous origin, including water and oxygen [8] (Table 3.2). This scenario necessitates constant surveillance so that damaged

nucleotides may be removed and replaced before their presence in a DNA strand at the time of replication leads to the generation of mutations [9]. Restoration of normal DNA structure is achieved in human cells by one of several DNA repair enzymes that cut out the damaged or inappropriate bases and replace them with the normal nucleotide sequence. This type of cellular response is referred to as "excision repair" and there are two major repair pathways which function in this manner: "base excision repair" which works mainly on modifications caused by endogenous agents and "nucleotide excision repair" which removes lesions caused by environmental mutagens. UV light is probably the most common exogenous mutagen to which human cells are exposed and the importance of the nucleotide excision repair pathway in protecting against UV-induced carcinogenesis is clearly demonstrated in the inherited disorder xeroderma pigmentosum. Individuals who have this disease lack one of the enzymes involved in nucleotide excision repair and have a 1,000 times greater risk of developing skin cancer following exposure to sunlight than normal individuals. The genes in question have been named *XPA*, *XPB*, etc. [10].

One of the great achievements of the last two decades has been the isolation and characterization of the genes, and their protein products, involved in base excision repair and nucleotide excision repair. It has become apparent that certain proteins so identified are not exclusively involved in DNA repair but play an integral part in other cellular processes such as DNA replication and recombination.

Excision repair

The first step in both base excision repair and nucleotide excision repair is the recognition of a modification in DNA by enzymes that detect either specific forms of damage or a distortion in the DNA helix. Recognition of damage is followed by an excision step in which DNA containing the modified nucleotide is removed. Gap-filling DNA synthesis and ligation of the free ends complete the repair process.

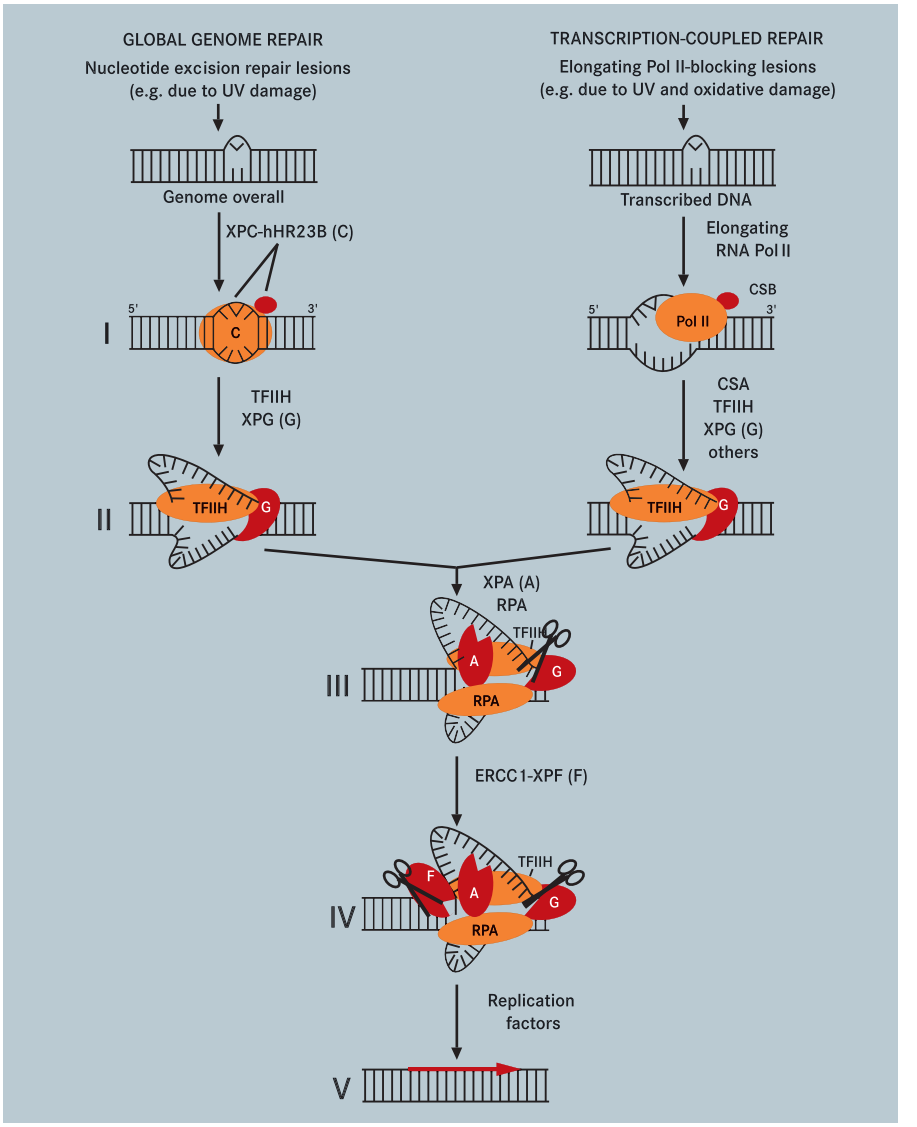


Fig. 3.10 Nucleotide excision repair (NER). Two NER pathways are predominant for removal of UV light- and carcinogen-damaged DNA. In global genome NER, the lesion is recognized by the proteins XPC and hHR23B while in transcription-coupled NER of protein-coding genes, the lesion is recognized when it stalls RNA polymerase II. Following recognition, both pathways are similar. The XPB and XPD helicases of the multi-subunit transcription factor TFIIH unwind DNA around the lesion (II). Single-stranded binding protein RPA stabilizes the intermediate structure (III). XPG and ERCC1-XPF cleave the borders of the damaged strand, generating a 24-32 base oligonucleotide containing the lesion (IV). The DNA replication machinery then fills in the gap (V).

Nucleotide excision repair may occur in the non-transcribed (non-protein-coding) regions of DNA (Fig. 3.10, steps I to V). A distortion in DNA is recognized, probably by the XPC-hHR23B protein (I). An open bubble structure is then formed around the lesion in a reaction that uses the ATP-dependent helicase activities of XPB and XPD (two of the subunits of TFIIH) and also involves XPA and RPA (II-III). The XPG and ERCC1-XPF nucleases excise and release a 24- to 32-residue oligonu-

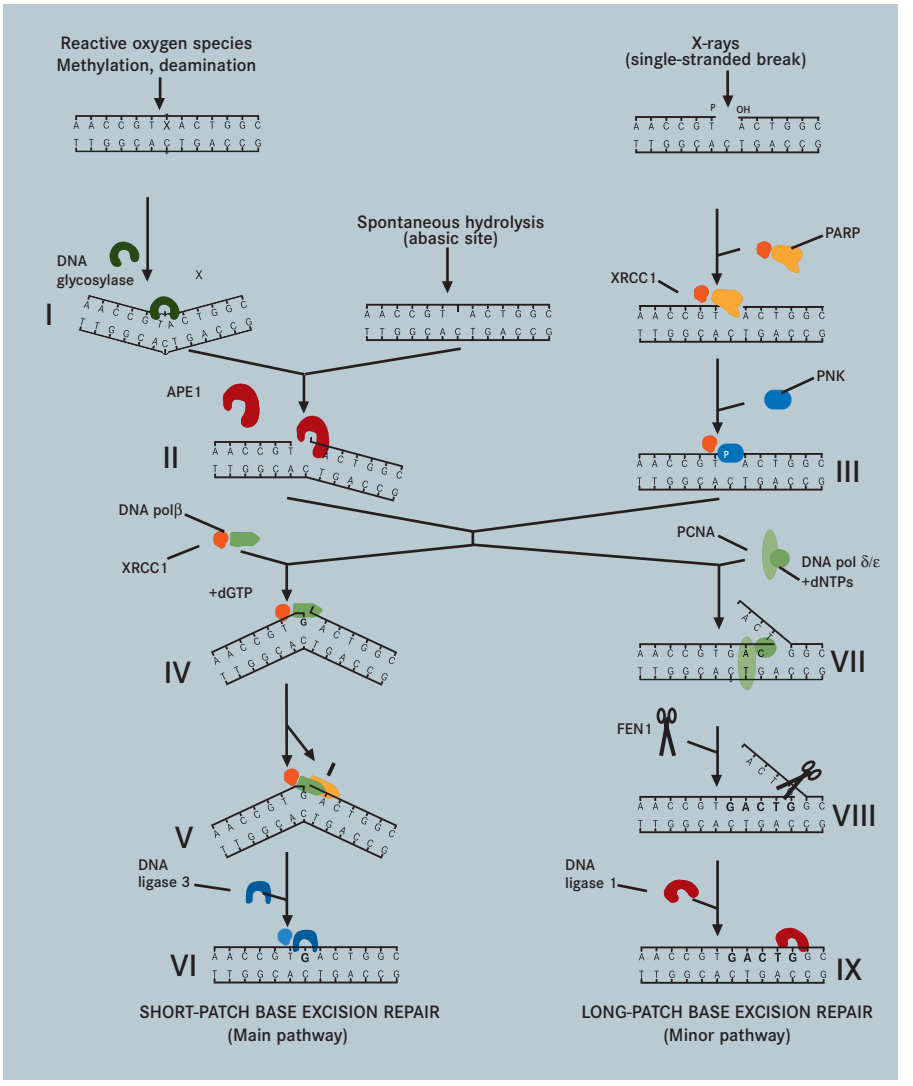


Fig. 3.11 Stages of base excision repair. Many glycosylases, each of which deals with a relatively narrow spectrum of lesions, are involved. The glycosylase compresses the DNA backbone to flip the suspect base out of the DNA helix. Inside the glycosylase, the damaged base is cleaved, producing an "abasic" site (I). APE1 endonuclease cleaves the DNA strand at the abasic site (II). In the repair of single-stranded breaks, poly(ADP-ribose)polymerase (PARP) and polynucleotide kinase (PNK) may be involved. In the "short-patch" pathway, DNA polymerase β fills the single nucleotide gap and the remaining nick is sealed by DNA ligase 3. The "long-patch" pathway requires the proliferating cell nuclear antigen (PCNA) and polymerases β, ε and δ fill the gap of 2-10 nucleotides. Flap endonuclease (FEN-1) is required to remove the flap of DNA containing the damage and the strand is sealed by DNA ligase 3.

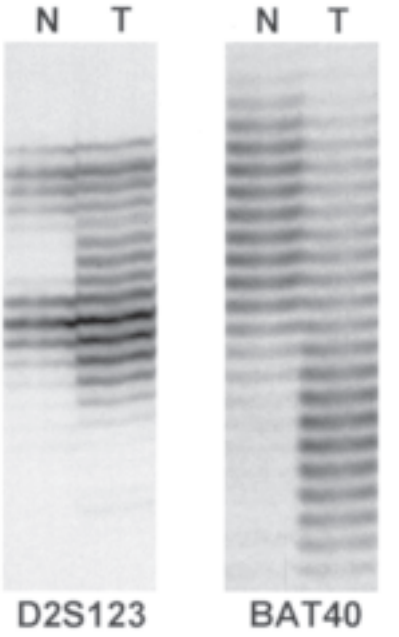


Fig. 3.12 In the human genome there are numerous places where short sequences of DNA are repeated many times. These are called microsatellites. In DNA from a patient with hereditary nonpolyposis colorectal cancer, there are changes in the number of repeats in the microsatellites. Note the difference in the microsatellite pattern between normal (N) and tumour tissue (T) from the same patient. This microsatellite instability is caused by errors in post-replicative DNA mismatch repair.

cleotide (IV) and the gap is filled in by PCNA-dependent polymerases (POL) epsilon and delta and sealed by a DNA ligase, presumed to be LIG1 (V). Nucleotide excision repair in regions which are transcribed (and hence code for proteins) requires the action of TFIIH [11].

DNA base excision repair (Fig. 3.11, steps I to VI or steps III to IX) involves the removal of a single base by cleavage of the sugar-base bond by a damage-specific DNA glycosylase (e.g. hNth1 or uracil DNA glycosylase) and incision by an apurinic/apyrimidinic nuclease (human AP1) [12]. Gap-filling may proceed by replacement of a single base or by resynthesis of several bases in the damaged strand (depending on the pathway employed).

More complex and unusual forms of damage to DNA, such as double strand breaks, clustered sites of base damage and non-coding lesions that block the normal replication machinery are dealt with by alternative mechanisms. Inherited human diseases in which the patient shows extreme sensitivity to ionizing radiation and altered processing of strand breaks, such as ataxia telangiectasia and Nijmegen breakage syndrome, constitute useful models to study the repair enzymes involved in these processes. Indeed, if elucidation of base excision repair and nucleotide excision repair was the great achievement of the late 1990s, then understanding strand

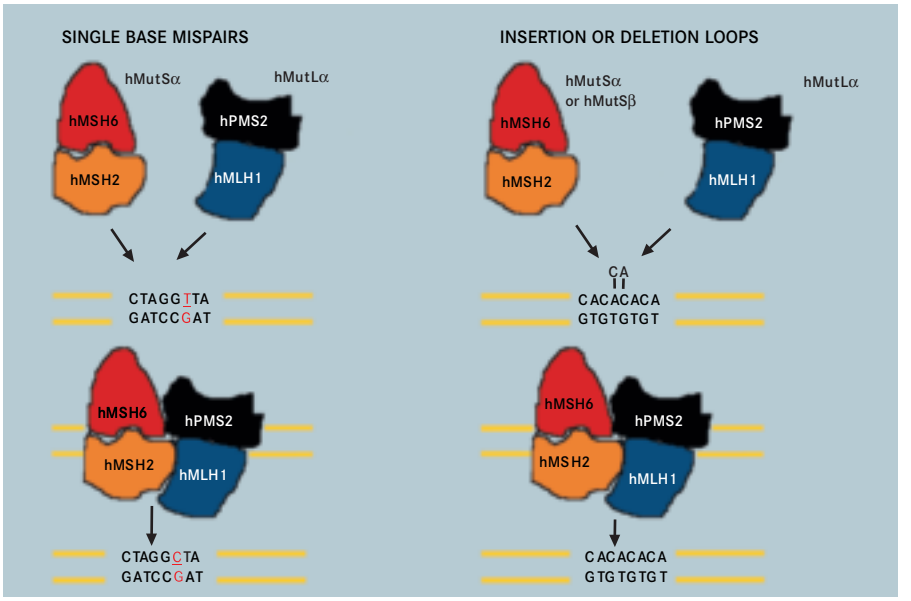


Fig. 3.13 Mismatch repair pathways: after DNA synthesis, base pairing mistakes that have escaped the editing function of DNA polymerase are recognized by mismatch repair proteins.

break repair will probably be the great achievement of the next decade. This will have important consequences. Certain cancers are often treated with radiotherapy (*Radiotherapy*, p277) and a small percentage of patients show considerable sensitivity to their treatment, with the result that treatment schedules are reduced to try to avoid adverse reactions. A better understanding of the possible causes of this radiosensitivity, including characterization of the enzymes involved in the repair of DNA damage produced by ionizing radiation, may lead to better tailoring of radiotherapy doses to individual patients.

Other repair pathways

Human cells, in common with other eukaryotic and prokaryotic cells, can also perform one very specific form of damage reversal, the conversion of the methylated adduct, O^6-methylguanine, in DNA back to the normal base (Fig. 3.14). O^6-Methylguanine is a miscoding lesion: both RNA and DNA polymerases "read" it incorrectly when they transcribe or replicate a DNA template containing it. As this modified base can pair with both the base cytosine (its correct partner) and the base thymine (an incorrect partner), its presence in DNA can give rise to transition mutations by mispairing of relevant bases. A specific protein, O^6-alkylguanine-DNA-alkyltransferase, catalyses transfer of the methyl group from the guanine base to a cysteine amino acid residue located at the active site of the protein [13]. This error-free process restores the DNA to its original state but results in the inactivation of the repair protein. Consequently, repair can be saturated when cells are exposed to high doses of alkylating agents and synthesis of the transferase protein is required before repair can continue.

Mismatched bases in DNA arising from errors in DNA replication, for instance guanine paired with thymine rather than cytosine, are repaired by several pathways involving either specific glycosylases,

Agent	Mutation hotspot	Type of mutation (> = changes to)	Tumours associated
Benzo[a]pyrene (tobacco smoke)	Codons 157, 158, 248, 273	G>T transversions	Lung, larynx
4-Aminobiphenyl (aromatic dyes, tobacco smoke)	Codons 280, 285	G>C transversions G>A transitions	Bladder
Aflatoxin B_1	Codon 249	AGG>AGT (arginine > serine)	Hepatocellular carcinoma
Ultraviolet (UV)	Codons 177-179, 278	C>T transitions CC>TT transitions	Skin cancer (not melanoma)
Vinyl chloride	Several codons	A>T transversions	Angiosarcoma of the liver
Endogenous mechanism (enhanced by nitric oxide)	Codons 175, 248, 273, 282	C>T transitions at CpG dinucleotides	Colon, stomach Brain cancers

Table 3.2 Spectra of *p53* mutations caused by environmental carcinogens or endogenous mechanisms.

which remove the mismatched bases, or long-patch mismatch repair involving homologues of the bacterial genes *MUTS* and *MUTL* (Fig. 3.13). Insertion or deletion loops at microsatellite sequences can be recognized by hMutSα (a heterodimer of hMSH2 and hMSH6) or hMutSβ (a heterodimer of hMSH2 and hMSH3). Subsequent recruitment of hMutLα (a heterodimer of hMLH1 and hPMS2) to the altered DNA targets the area for repair, which requires excision, resynthesis, and ligation. Single nucleotide mispairing events require hMutSα function for recognition. One important requirement of such repair processes is that they are able to distinguish the correct base from the incorrect one in the mispair. Since both bases are normal constituents of DNA, this cannot be achieved by an enzyme that scans the DNA for a lesion or structure that is not a normal constituent of the DNA. Defects in at least four of the genes whose products are involved in mismatch repair, namely hMSH2, hMLH1, hPMS1 and hPMS2, have been associated with hereditary nonpolyposis colorectal cancer. This is one of the most common genetic diseases and affects as many as 1 in 200 individuals and may account for 4-13% of all colorectal cancers (*Colorectal cancer*, p198). Affected individuals also develop tumours of the endometrium, ovary and other organs. The DNA of hereditary nonpolyposis colorectal cancer tumours is characterized by instabilities in simple mono-, di- and trinucleotide repeats which are common in the human genome (Fig. 3.12). This instability is also seen in certain sporadic colorectal tumour cells and arises directly from alterations in the proteins involved in mismatch repair [14]. Generally speaking, genomic instability is considered an indicator of, and fundamental to the nature of, malignant cell growth.

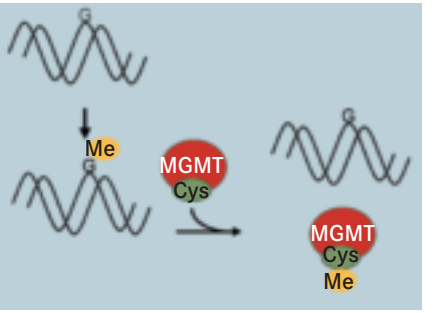


Fig. 3.14 The repair of O^6-methylguanine by O^6-alkylguanine-DNA-alkyltransferase.

REFERENCES

1. Miller EC, Miller JA (1979) Milestones in chemical carcinogenesis. *Semin Oncol*, 6: 445-460.

2. Miller JA, Miller EC (1977) Ultimate chemical carcinogens as reactive mutagenic electrophiles. In: Hiatt HH, Watson, JD, Winsten, JA eds, *Origins of Human Cancer* (Book B), Cold Spring Harbor, Cold Spring Harbor Laboratory, 605-627.

3. Guengerich FP (2000) Metabolism of chemical carcinogens. *Carcinogenesis*, 21: 345-351.

4. Hemminki K, Dipple A, Shuker DEG, Kadlubar FF, Segerbäck D, Bartsch H, eds (1994) *DNA Adducts. Identification and Biological Significance* (*IARC Scientific Publications No. 125*), Lyon, IARCPress.

5. Toniolo P, Boffetta P, Shuker DEG, Rothman N, Hulka B, Pearce N, eds (1997) *Application of Biomarkers in Cancer Epidemiology* (*IARC Scientific Publications No. 142*), Lyon, IARCPress.

6. Vineis P, Malats N, Lang M, d'Errico A, Caporaso N, Cuzick J, Boffetta P, eds (1999) *Metabolic Polymorphisms and Susceptibility to Cancer* (*IARC Scientific Publications No. 148*), Lyon, IARCPress.

7. McGregor DB, Rice JM, Venitt S, eds (1999) *The Use of Short- and Medium-Term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation* (*IARC Scientific Publications No. 146*), Lyon, IARCPress.

8. Friedberg EC, Walker GC, Siede W, eds (1995) *DNA Repair and Mutagenesis*, Washington DC, ASM Press.

9. Lindahl T (2000) Suppression of spontaneous mutagenesis in human cells by DNA base excision-repair. *Mutat Res*, 462: 129-135.

10. de Boer J, Hoeijmakers JH (2000) Nucleotide excision repair and human syndromes. *Carcinogenesis*, 21: 453-460.

11. Benhamou S, Sarasin A (2000) Variability in nucleotide excision repair and cancer risk: a review. *Mutat Res*, 462: 149-158.

12. Cadet J, Bourdat AG, D'Ham C, Duarte V, Gasparutto D, Romieu A, Ravanat JL (2000) Oxidative base damage to DNA: specificity of base excision repair enzymes. *Mutat Res*, 462: 121-128.

13. Pegg AE (2000) Repair of O^6-alkylguanine by alkyltransferases. *Mutat Res*, 462: 83-100.

14. Pedroni M, Sala E, Scarselli A, Borghi F , Menigatti M, Benatti P, Percesepe A, Rossi G, Foroni M, Losi L, Di Gregorio C, De Pol A, Nascimbeni R, Di Betta E, Salerni B, de Leon MP, Roncucci L (2001) Microsatellite instability and mismatch-repair protein expression in hereditary and sporadic colorectal carcinogenesis. *Cancer Res*, 61: 896-899.

WEBSITES

A comprehensive listing of human DNA repair genes:
http://www.sciencemag.org/cgi/content/abstract/291/5507/1284

DNA Repair Interest Group (NCI):
http://www.nih.gov:80/sigs/dna-rep/

ONCOGENES AND TUMOUR SUPPRESSOR GENES

SUMMARY

> Human cells become malignant through the activation of oncogenes and inactivation of tumour suppressor genes. The pattern of genes involved varies markedly at different organ sites.

> Oncogenes stimulate cell proliferation and may be overexpressed by gene amplification *(*e.g. *MYC)*. In addition, oncogenes may be activated by mutations (e.g. the *RAS* gene family).

> Tumour suppressor genes are typically inactivated by gene mutations in one allele (gene copy), followed by loss of the intact allele during cell replication (two-hit mechanism). This leads to loss of expression and abolition of the suppressor function, which is particularly important in cell cycle control.

> Mutational inactivation of suppressor genes in germ cells is the underlying cause of most inherited tumour syndromes. The same type of mutation may arise through mutations occurring during an individual's lifetime.

Definitions

The multi-step nature of carcinogenesis has long been recognized (*Multistage carcinogenesis*, p84). Over the past 20 years, experimental studies in animals and molecular pathological studies have converged to establish the notion that each step in malignant transformation is determined by a limited number of alterations in a small subset of the several thousands of cellular genes [1]. The terms "oncogene" and "tumour suppressor gene" are commonly used to identify the sets of genes involved in such sequences of events [2]. Both groups of genes are extremely diverse in terms of nature and function. An oncogene is a gene whose function is activated in cancer. This can be achieved by a number of simple molecular mechanisms, including point mutations that constitutively activate an enzyme, deletions that remove negative regulatory regions from proteins, or increased expression resulting from promoter deregulation or from multiplication of the number of copies of the gene (a phenomenon called "amplification" [3]). Activation of an oncogene is a dominant mechanism, since alteration of a single allele is sufficient to confer a gain of function for cancer onset or progression. The non-activated counterpart of an oncogene is sometimes called a "proto-oncogene". A proto-oncogene is in fact a "normal" gene in all respects, often with important functions in the control of the signalling of cell proliferation, differentiation, motility or survival.

A tumour suppressor gene is a gene whose alteration during carcinogenesis results in the loss of a functional property essential for the maintenance of normal cell proliferation. Loss of function of a tumour suppressor gene is typically a recessive mechanism. Indeed, in many instances both copies of the gene need to be inactivated in order to switch off the corresponding function. Inactivation of tumour suppressor genes proceeds by loss of alleles (most often through the loss of entire chromosomal sections encompassing several dozen genes), small deletions or insertions that scramble the reading frame of the gene, transcriptional silencing by alteration of the promoter region, or point mutations that change the nature of residues that are crucial for the activity of the corresponding protein. Recently, it has emerged that tumour suppressor genes can be conveniently subclassified into two major groups. The genes of the first group are nicknamed "gatekeepers". Their products control the gates on the pathways of cell proliferation. Typically, gatekeeper genes are negative regulators of the cell cycle, acting as "brakes" to control cell division. The genes of the second group are called "caretakers", as their primary function is not to control the speed or timing of cell division but rather its accuracy. Caretaker genes are usually involved in DNA repair and in the control of genomic stability. Their inactivation does not enhance cell proliferation *per se* but primes the cell for rapid acquisition of further genetic changes [4]. The combined activation of oncogenes and inactivation of tumour suppressor genes drive the progression of cancer. The most evident biological consequences of these alterations are autonomous cell proliferation, increased ability to acquire genetic alterations due to deregulated DNA repair, ability to grow in adverse conditions due to decreased apoptosis, (*Apoptosis*, p113) capacity to invade tissues locally and to form distant metastases, and ability to activate the formation of new blood vessels (a process called angiogenesis). Together, these five biological phenomena may be caricatured as pieces of the "cancer jigsaw" [5] (Fig. 3.15). None alone is sufficient in itself, but cancer arises when they interact together into a chain of coordinated events that profoundly modifies the normal cellular pattern of growth and development.

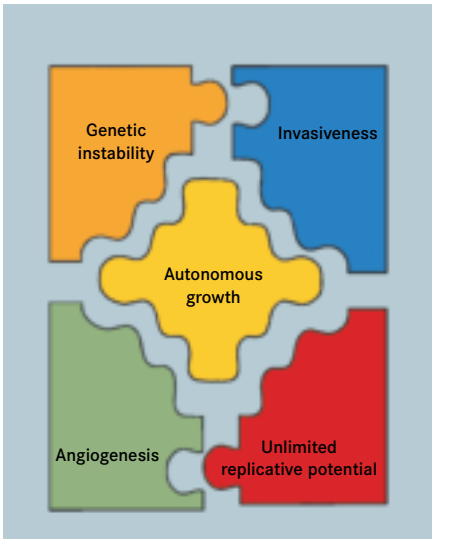


Fig. 3.15 The cancer jigsaw: multiple functions must be altered for tumorigenesis to occur.

Common human oncogenes

Many common proto-oncogenes encode components of the molecular cascades that regulate the cellular response to mitogenic signals [6]. They include growth factors (e.g. *TGFA*), growth factor receptors (e.g. the receptors for epidermal growth factor, *EGF* and its close homologue, *ERBB2*), receptor-coupled signal transduction molecules (in particular, several small guanosine triphosphate (GTP)-binding proteins located on the inner face of the cell membrane, such as the various members of the *RAS* family), kinases (*SRC*, *ABL*, *RAF1*), regulatory subunits of cell cycle kinases (*CCND1* and *CCNA*), phosphatases (*CDC25B*), anti-apoptotic molecules (*BCL2*) and transcription factors (*MYC*, *MYB*, *FOS*, *JUN*). The cumbersome nomenclature of these genes (Box: *Naming genes and proteins*, p101) owes much to the way they were discovered and identified. The *SRC* gene, for example, was the first oncogene identified, in 1976, as a modified version of a cellular gene incorporated in the genome of a highly transformant chicken retrovirus, the Rous sarcoma virus. The *MYC* gene was also originally identified in the genome of an avian retrovirus inducing promyelocytic leukaemia. The *RAS* genes were first identified as activated genes capable of inducing the formation of rat sarcomas, and various members of the family were found in different murine retroviruses, such as the Harvey sarcoma virus (*HRAS*) and the Kirsten sarcoma virus (*KRAS*).

The most commonly activated oncogenes in human cancers are *ERBB2* (in breast and ovarian cancers), members of the *RAS* family (in particular *KRAS* in lung, colorectal and pancreatic cancers, and *MYC* (in a large variety of tumours such as cancers of the breast and oesophagus and in some forms of acute and chronic leukaemia). These three examples give an excellent illustration of the diversity of the mechanisms of oncogene activation and of their consequences for cell growth and division.

ERBB2

In the case of *ERBB2*, oncogenic activation is almost always the result of amplification of the normal gene [7] (Fig. 3.16). This gene is located within a region of the genome which is amplified in about 27% of advanced breast cancers, leading to a spectacular increase in the density of the molecule at the cell surface. *ERBB2* encodes a transmembrane protein with the structure of a cell-surface receptor, the intracellular portion of which carries a tyrosine kinase activity. Overexpression of *ERBB2* leads to constitutive activation of the growth-promoting tyrosine phosphorylation signal. The elucidation of this mechanism has led to the development of neutralizing antibodies and specific chemical inhibitors of tyrosine kinase activity as therapeutic approaches to the blocking of *ERBB2* action.

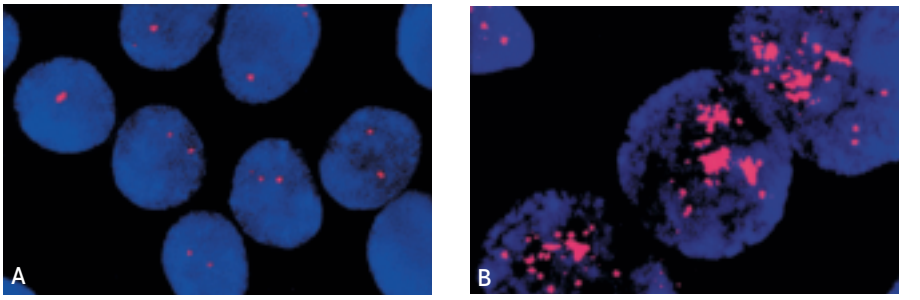


Fig. 3.16 Analysis of the status of the *ERBB2* oncogene by fluorescent in situ hybridization (FISH) with a rhodamine-labelled *ERBB2* probe (pink). In breast tumour cells without amplification of the gene, each nucleus possesses two copies of *ERBB2* **(A)**. In tumour cells with high-level amplification of the gene, numerous signals are evident in each nucleus **(B)**.

RAS

The *RAS* genes are located one step downstream of *ERBB2* in growth signalling cascades. The protein products of the *RAS* genes are small proteins anchored at the cytoplasmic side of the plasma membrane by a lipidic moiety. They indirectly interact with activated tyrosine kinases and act as "amplifiers" to increase the strength of the signal generated by the activation of cell-surface receptors [8]. In their active form, ras proteins bind guanosine triphosphate (GTP) and catalyse its hydrolysis into guanosine diphosphate (GDP) returning to their inactive form. Oncogenic forms of activated *RAS* genes often carry missense mutations at a limited number of codons within the GTP-binding site of the enzyme, making it unable to hydrolyse GTP and thus trapping it in the active form. Activation of *RAS* genes thus induces the cell to behave as if the upstream, Ras-coupled receptors were being constantly stimulated.

MYC

The *MYC* oncogene may be seen as a prototype of the family of molecules which lies at the receiving end of the signal transduction cascades. *MYC* encodes a transcription factor which is rapidly activated after growth stimulation and which is required for the cell to enter into cycle [9].

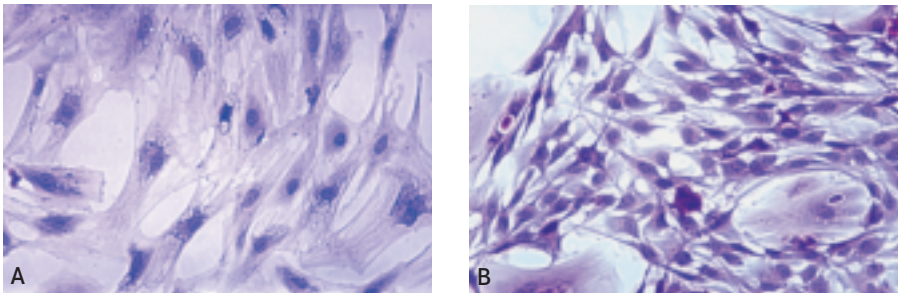


Fig. 3.17 In cell cultures, activation of a single oncogene may result in a changed morphology from "normal" **(A)** to "transformed" **(B)** and this often corresponds to a change in growth properties. Malignant transformation appears to require the co-operation of at least three genes.

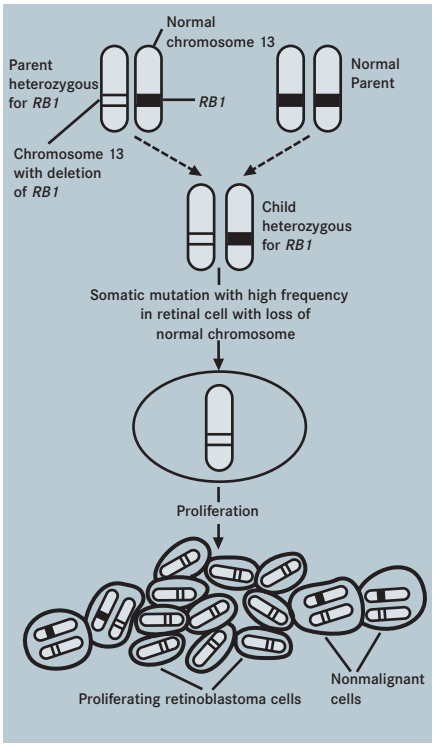


Fig. 3.18 The retinoblastoma gene is a paradigm for tumour suppressor genes: if a child inherits a mutation or deletion of one copy ("allele") of the retinoblastoma gene, the remaining normal copy tends to be lost at a high frequency in cells of the retina, resulting in loss of function and in the formation of tumours. The diagram shows loss of the whole normal chromosome but the normal allele can also be lost by mutation, deletion, gene conversion or mitotic recombination.

Myc transactivates a number of other cellular genes and has a wide spectrum of molecular effects (a phenomenon that may explain why Myc is activated in many different types of cancer cells). Activation of Myc often proceeds through amplification of the region containing the gene on chromosome 8, but Myc is also commonly activated by chromosomal translocation in some forms of B-cell leukaemia (*Leukaemia*, p242).

BCL2

The *BCL2* gene (activated in B cell lymphomas) exemplifies another kind of oncogene. Initially identified as a gene located within a chromosomal breakpoint in some forms of leukaemia, *BCL2* was found to encode a protein capable of extending the life span of a cell by preventing the onset of programmed cell death, or apoptosis [10] (*Apoptosis*, p113). Biochemical studies have revealed that *BCL2* encodes a regulator of the permeability of the mitochondrial membrane. Mitochondrial damage and cytoplasmic leakage of mitochondrial components is one of the important signals that lead a cell to apoptosis. By helping to keep the mitochondrial permeability pores closed, Bcl-2 protein prevents this leakage and thus allows the survival of cells that would otherwise have been eliminated by a physiological process.

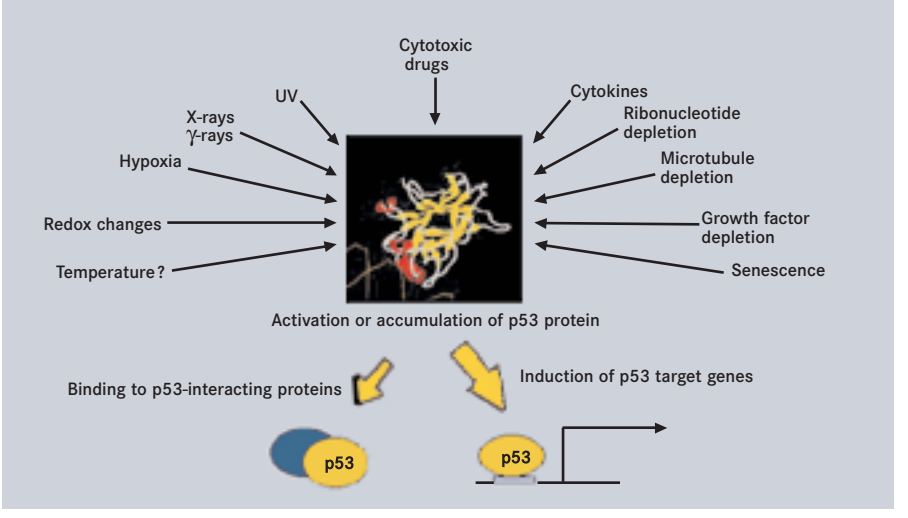


Fig. 3.19 Many types of biological stress lead to a p53-mediated response.

Tumour suppressor genes: history of a concept

Whereas the study of retroviruses and gene transfection experiments were the keys to the discovery of oncogenes, tumour suppressor genes were identified through the study of large DNA viruses and the analysis of familial tumour syndromes.

Retinoblastoma

In 1971, Knudsen proposed the now popular "two hits" hypothesis to explain the inheritance of retinoblastoma, a rare childhood tumour type [11,12] (*Genetic susceptibility*, p71). He postulated that, in a familial setting, individuals may inherit only one normal copy of the gene (localized by linkage studies to chromosome 13q14), the other being either lost, partially deleted or otherwise inactivated. Consequently, these individuals would just need one additional mutagenic step to switch off the remaining copy of the gene, thus totally losing the corresponding function (Fig. 3.18). The very same type of cancer may also occur in a sporadic manner, but in this case it would require two consecutive "hits" (mutagenic events) to inactivate the two copies of the gene in the same cell. This theory paved the way for the modern concept of recessive tumour suppressor genes. In 1988, the gene responsible for familial retinoblastoma was identified [13]. The *RB1* gene encodes a protein that binds and inactivates transcription factors that are essential for the progression of the cell cycle, thus fulfilling the functions of a molecular "brake" on cell division.

Large DNA viruses

In parallel with events previously outlined, it became evident that many DNA viruses associated with cancer encode complex viral proteins that are capable of sequestering and inactivating cellular proteins [14]. This is the case of a tumorigenic simian virus, SV40, of several adenoma and polyoma viruses and of oncogenic forms of human papillomaviruses. In the case of SV40, the virus encodes a large protein (called LT for Large Tumour antigen) which binds two cellular proteins, the

product of the *RB1* gene (pRb) and an ubiquitous protein that was conservatively called p53. In the case of oncogenic human papillomaviruses, the viruses encode two distinct proteins, E7 (which neutralizes pRb) and E6 (which neutralizes p53). Thus it was suggested that pRb and p53 might have similar, complementary functions, operating jointly in the control of cell division.

The "missing link" in this conceptual edifice was the discovery of alterations in the gene encoding p53. This was achieved in 1989, when it emerged that the *p53* gene was often mutated and/or deleted in many forms of cancers [15]. In 1991, inherited loss of *p53* was found to be associated with a rare familial syndrome of multiple cancers, the Li-Fraumeni syndrome, in which afflicted family members suffer vastly increased incidence of many tumour types [16]. Today, about 215 families worldwide affected by this syndrome have been described and the *p53* mutations they exhibit are compiled in a database maintained at IARC.

Tumour suppressor genes and familial cancer syndromes

Most familial cancer syndromes are inherited as a recessive trait, and correspond to the constitutive inactivation of an important tumour suppressor gene, as described above in the case of familial retinoblastoma. Over the past 15 years, many loci containing tumour suppressor genes have been identified by linkage studies in cancer-prone families.

Colorectal cancer

In colorectal cancers, two different familial cancer syndromes have been found to be associated with the constitutive alteration of two distinct sets of tumour suppressor genes (*Colorectal cancer*, p198). Patients with familial adenomatous polyposis, a disease that predisposes to the early occurrence of colon cancer, often carry alterations in one copy of the adenomatous polyposis coli (*APC*) gene [17]. This gene plays a central role in a signalling cascade that couples cell-surface receptors, calcium-dependent adhesion molecules and transcription factors that regulate cell proliferation. Loss of APC function sets these transcription factors free, an event that favours not only the formation of polyps but also their transformation into adenomas and carcinomas.

Breast cancer

Two genes have been identified as involved in familial breast cancer risk, *BRCA1* and *BRCA2* [18]. These genes encode large proteins with complex functions in many aspects of cell regulation, such as cell cycle control and DNA repair. However, how their inactivation contributes to the onset or development of breast cancer is still largely unknown.

Others

In the case of hereditary Wilms tumours, a rare type of kidney cancer, the gene identified encodes a protein essential for the correct differentiation of the nephron. This very specific role may explain why the hereditary loss of this gene does not seem to be associated with cancers at any other site.

This short overview gives only a few examples of the diversity of tumour suppressor genes, and there is little doubt that many still remain to be identified. Given the breadth of the concept of "tumour suppressors", many genes encoding components of stress response pathways have the potential to behave in this fashion (as their alteration may prevent cells from mounting an adequate response to genotoxic, potentially

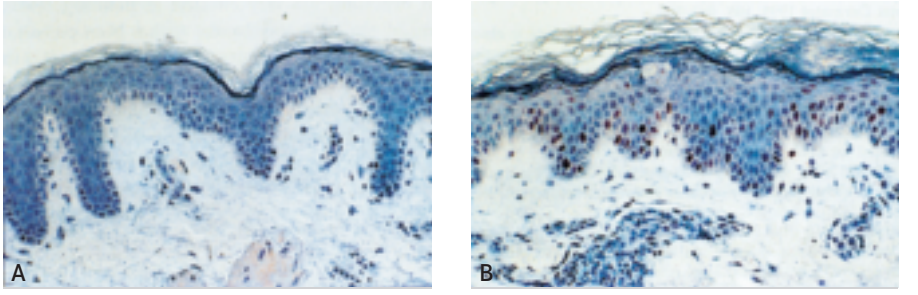


Fig. 3.20 Accumulation of p53 in human epidermis after exposure to sunlight. Unexposed skin shows no immunostaining against p53 protein (A). Exposed skin (B) shows a dense dark nuclear coloration of epidermal cells due to positive immunostaining for p53 protein.

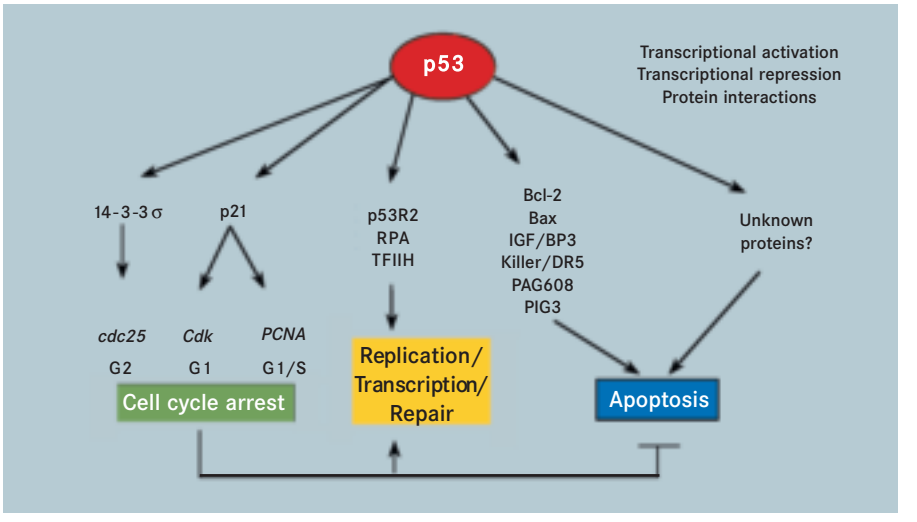


Fig. 3.21 Multiple response pathways are triggered by the accumulation of p53 in the cell nucleus.

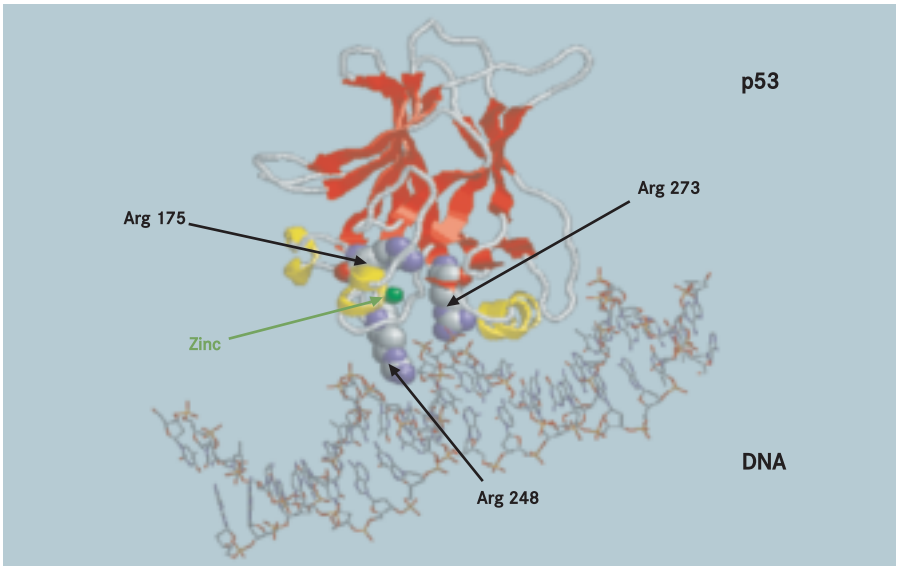


Fig. 3.22 Molecular modelling of part of the p53 protein (DNA-binding domain), showing its interaction with DNA. The amino acids labelled (arginine 175, 248, 273) are important for maintaining biological activity and are among the "hotspots" for mutations in cancer. The zinc atom is required for stabilizing the complex three-dimensional structure of the p53 oligomer.

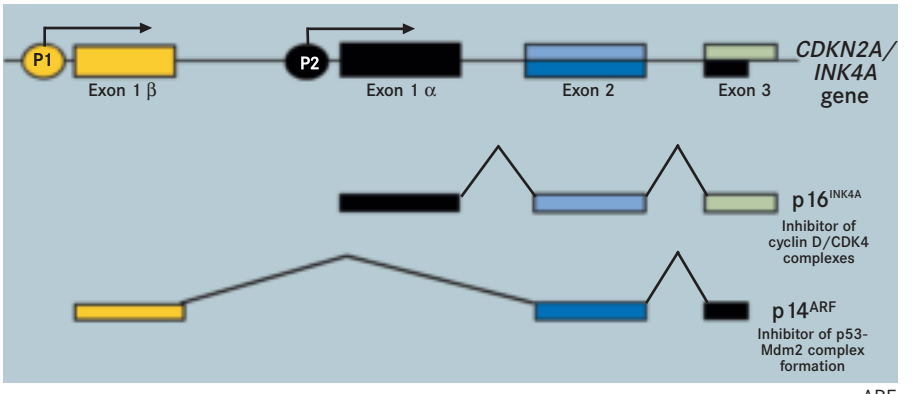


Fig. 3.23 Generally, a single segment of DNA codes for a single protein. However, the p16 and $p14^{ARF}$ proteins are both encoded by a single region of DNA. P = promoter.

oncogenic forms of stress). The genes responsible for complex inherited diseases such as ataxia telangiectasia or xeroderma pigmentosum (*Carcinogen activation and DNA repair*, p89) belong to this category [19]. Alteration of such genes results in many defects, including hypersensitivity to radiation and therefore to the development of cancers such as skin tumours.

Tumour suppressor genes and sporadic cancers

Many of the tumour suppressor genes associated with familial cancer syndromes are also mutated at variable rates in many forms of sporadic cancer. However, two of them, *p53* and *CDKN2A*, are very commonly altered in almost every kind of human cancer.

p53, the guardian of the genome

The *p53* gene encodes a phosphoprotein of molecular weight 53,000 daltons, which accumulates in the nucleus in response to various forms of stress, in particular, DNA damage (Fig. 3.20). In this context, p53 acts as a transcriptional regulator, increasing or decreasing the expression of several dozen genes involved in cell cycle control, in the induction of apoptosis, in DNA repair and in differentiation control. Together these genes exert complex, anti-proliferative effects (Fig. 3.21). Essentially, when cells are subjected to tolerable levels of DNA-damaging agents, activation of p53 will result in cell cycle arrest, temporarily removing the cells from the proliferative pool or mediating differentiation. However, when faced with highly damaging levels of genotoxic stress, p53 will induce apoptosis, a programmed form of suicide that eliminates cells with potentially oncogenic alterations. This complex role in the protection of the cell from DNA damage has resulted in p53 being described as the "guardian of the genome" [20]. Loss of this function by mutation, as often occurs during carcinogenesis, will allow cells with damaged DNA to remain in the proliferative population, a situation that is essential for the expansion of a clone of cancer cells.

The *p53* gene differs from most other tumour suppressors in its mode of inactivation in human cancers. Whereas most tumour suppressors are altered by loss of alleles or inactivating deletions or insertions, *p53* is commonly the target of point mutations within the portion of the gene that encodes the DNA-binding domain of the protein (Fig. 3.22). These mutations prevent the correct folding of this protein domain, and therefore disrupt the interactions of p53 with its specific DNA targets. However, the mutant proteins are often extremely stable and therefore accumulate to high levels within the nucleus of cancer cells. This accumulated protein can often be detected by immunohistochemistry in primary tumours as well as in distant metastases. Although not all mutations induce accumulation of the protein, p53 accumulation provides a convenient tool for pathologists to assess the possibility of a p53 dysfunction in cancer specimens [21].

Mutation is not the only way to alter p53 protein in cancer. In cervical cancers, *p53* gene mutations are infrequent, but the protein is inactivated by binding of the viral protein E6 which is produced by human papillomavirus. This protein cre-

ates a molecular bridge between p53 and the protein degradation machinery, resulting in the rapid degradation and effective elimination of p53 protein. This interaction plays an important role in cervical cancer (*Cancers of the female reproductive tract,* p215). In normal cells, the degradation of p53 is regulated by the Mdm2 protein. Mdm2 ("murine double minute gene 2") was originally identified in the mouse as the product of a gene amplified in aberrant chromosome fragments called "double minute chromosomes". Amplification of *MDM2* is common in osteosarcomas and is sometimes detected in other cancers, such as carcinomas or brain tumours. *MDM2* thus behaves as an oncogene, since its activation by amplification causes the inactivation of a tumour suppressor gene [22].

The *p53* gene (and its product) is one of the most studied genes in human cancer. In the 20 years since its discovery in 1979, more than 15,000 publications have addressed its structure, function and alteration in cancers. There have been many attempts to exploit this knowledge in the development of new therapies based on the control of p53 activity in cancer cells. Experimental gene therapy has shown that it may be possible to restore p53 function in cells that have lost the gene. More recently, drugs designed to specifically target and restore the function of mutant *p53* have shown promising results in experimental systems. As knowledge of the *p53* pathway improves, it is anticipated that this central molecular event in human cancer will provide a basis for developing new forms of therapy.

CDKN2A: one locus, two genes

CDKN2, or "cyclin dependent kinase inhibitor 2", is known under several names, including *INK4A* (inhibitor of kinase 4A) and *MTS1* (multiple tumour suppressor 1). The *CDKN2A* locus is located at the extremity of the short arm of chromosome 9, the letter "A" serving to distinguish it from the *CDKN2B* gene, which is located just 20 kilobases away.

This gene is unique in that it contains two distinct reading frames, with two different promoters, the same DNA being used to synthesize two proteins that do not have a single amino acid sequence in common [23] (Fig. 3.23). The first reading frame to be discovered encodes p16, an inhibitor of cyclin-dependent kinases 4 and 6, which associates with cyclin D1 in G1 phase of the cell cycle (*The cell cycle*, p104). The p16 protein is thus an archetypal cell cycle "brake", its loss leading to increased cell proliferation and, more specifically, to escape from replicative senescence and extended cellular life span. The other reading frame, named $p14^{ARF}$ for "alternative reading frame" (often called by the same name as its mouse homologue, $p19^{ARF}$), is synthesized from a different portion of the *CDKN2A* locus but shares one exon (exon 2) with p16. However, although the DNA sequence encoding the two products is identical, p16 and $p14^{ARF}$ use different reading frames of exon 2, such that their amino acid sequences are completely different. $p14^{ARF}$ is a protein that controls Mdm2, which in turn regulates p53 protein stability. Activation of $p14^{ARF}$ blocks Mdm2 and therefore results in p53 protein accumulation and activation. Thus the *CDKN2A* locus behaves as two unrelated but interlocked genes. The first gene, encoding p16, directly controls cell cycle progression and senescence. The second, encoding $p14^{ARF}$, controls p53 and all its downstream anti-proliferative functions.

The *CDKN2A* locus is often altered by loss of alleles (which removes both p16 and $p14^{ARF}$), by mutation (most frequently in exon 2, common to both gene products), and by hypermethylation. Increased methylation of specific regions of the DNA within the promoters and some of the coding regions prevents adequate transcription and decreases the levels of protein synthesized. Loss of expression due to hypermethylation may be the most frequent way of altering the *CDKN2A* locus in many forms of cancers, particularly carcinomas.

NAMING GENES AND PROTEINS

Conventionally, a gene (that is, a specific segment of DNA) is identified by a single name, in upper case and italicized (e.g. the oncogene *RAS*) that is indicative of the character or function of the protein encoded, which is designated by the same name, in lower case (ras in the case of the present example). Proteins are also named by reference to their molecular weight, with the corresponding gene in superscript (e.g. $p21^{WAF1}$). The names of genes are often based on acronyms, and are generally the prerogative of the successful investigator. Identification of a novel gene is often followed by discovery of structurally related or "homologous" genes (and corresponding proteins) and these may be given names closely related to the first member of the "family" identified. Such an approach to nomenclature may be inadequate, for example, in those instances in which a single DNA segment encodes multiple proteins through alternative splicing of messenger RNA. Multiple names for the same gene or protein may arise because of independent discovery (and hence, naming) by different investigators. Thus, the cyclin-dependent kinase inhibitor *WAF1* is also known as *CDKN1A*, *CAP20*, *MDA-6*, *PIC-1* and *SDI-1*. In scientific writing, all such names are given in the first instance, after which a single name is used consistently in any one document. The latest estimates from the Human Genome Project suggest that there may be about 30,000 human genes.

Conventions for the naming of genes and proteins are subject to international agreement and are continuously subject to review (HUGO Gene Nomenclature Committee, http://www.gene.ucl.ac.uk/nomenclature/).

GEOGRAPHIC VARIATION IN MUTATION PATTERNS

Mutations in cancer genes are the direct consequence of attack on DNA by exogenous or endogenous agents or of errors in DNA repair systems. By analysing the type and the sequence context of such mutations, it is possible to form hypotheses regarding the nature of the mutagenic mechanism involved. The most interesting genes in this respect are those altered by missense point mutations, such as members of the *RAS* family, *CDKN2A/INK4A*, and, in particular, the *p53* gene.

The *p53* gene is the most frequently mutated gene in human cancer, with over 16,000 mutations reported and compiled in a database maintained at IARC (http://www.iarc.fr/p53). The diversity of these mutations allows the identification of patterns which vary depending on the tumour type, the geographic origin and the risk factors involved. These are often specific for particular agents that have caused these mutations. Thus *p53* gene mutations in cancers may be seen as "fingerprints" left by carcinogens in the human genome, which may help to identify the particular carcinogen involved.

A typical example of such a "fingerprint" is the mutation at codon 249 observed in liver cancers of patients from sub-Saharan Africa and Eastern Asia. In these regions, liver cancer is a consequence of chronic infection by hepatitis viruses and of dietary poisoning with aflatoxins, a class of mycotoxins which contaminates traditional diets (groundnuts) (*Food contaminants*, p43). Experiments in animals and in cell culture have shown that aflatoxins can directly induce the mutation at codon 249. This particular mutation is not found in liver cancers in areas of the world, such as the USA, where exposure to aflatoxins is low.

Specific mutations have also been observed in lung cancers from smokers (due to tobacco carcinogens). In skin cancers, the mutations bear typical chemical signatures of the damage inflicted to DNA by exposure to solar ultraviolet radiation. In other instances, exemplified by patterns of mutation in breast cancer, marked differences have been observed between geographical areas, which may provide information on the nature of risk factors involved.

In many other cancers, mutation patterns also vary from one region of the world to another. This variability may give clues about the genetic heterogeneity of populations, as well as about the diversity of agents involved in causing cancers. For example, in oesophageal cancers, mutation types widely differ between high-incidence and low-incidence regions, suggesting that specific mutagens are at work in causing the excess incidence seen in some parts of the world, such as Northern Iran and Central China.

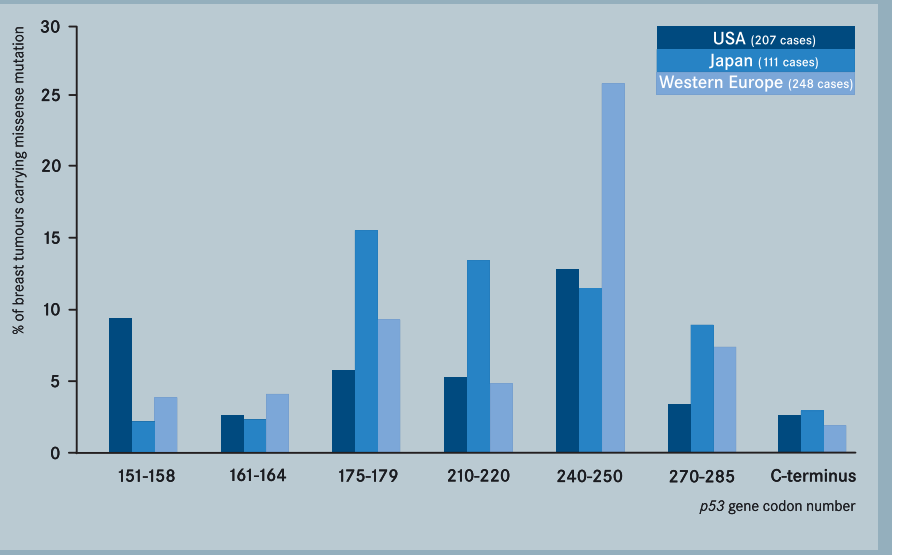


Fig. 3.24 Geographic variations in the prevalence of *p53* gene mutations in breast cancers.

Lesions in the $p16^{INK4A}$–cyclin D, CDK4-pRb and $p14^{ARF}$-Mdm2-p53 pathways occur so frequently in cancer, regardless of patient age or tumour type, that they appear to be fundamental to malignancy [24].

Prospects for the molecular analysis of cancer

More than 200 genes that are altered at variable proportions in different human cancer types have been characterized. Most of these have a powerful impact on tumour growth. However, it is very likely that many critical genes with less penetrant phenotypes remain to be identified. In particular, the genes involved in stress reponses, in the control of oxygen metabolism and in the detoxification of xenobiotics are all candidates for a role as cofactors in the cancer process. Moreover, many biological alterations leading to cancer may not be detectable at the DNA level. Cancer-causing changes may result from modification of RNA levels or processing, and of protein structure and function through a variety of epigenetic phenomena. The systematic profiling of gene expression in cancer cells will probably reveal a whole new set of

potential cancer genes altered through less conspicuous mechanisms. Apart from expression studies, the whole territory of protein structure, dynamics and interactions is still largely unexplored. An understanding of such processes will provide a basis for the development of new means for treatment and diagnosis.

REFERENCES

1. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell*, 61: 759-767.

2. Weinberg RA (1995) The molecular basis of oncogenes and tumor suppressor genes. *Ann N Y Acad Sci*, 758: 331-338.

3. Savelyeva L, Schwab M (2001) Amplification of oncogenes revisited: from expression profiling to clinical application. *Cancer Lett*, 167: 115-123.

4. Kinzler KW, Vogelstein B (1997) Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature*, 386: 761, 763.

5. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell*, 100: 57-70.

6. Hunter T (1991) Cooperation between oncogenes. *Cell*, 64: 249-270.

7. Hung MC, Lau YK (1999) Basic science of HER-2/neu: a review. *Semin Oncol*, 26: 51-59.

8. Wiesmuller L, Wittinghofer F (1994) Signal transduction pathways involving Ras. Mini review. *Cell Signal*, 6: 247-267.

9. Henriksson M, Luscher B (1996) Proteins of the Myc network: essential regulators of cell growth and differentiation. *Adv Cancer Res*, 68: 109-182.

10. Antonsson B, Martinou JC (2000) The Bcl-2 protein family. *Exp Cell Res*, 256: 50-57.

11. Knudson AG (1996) Hereditary cancer: two hits revisited. *J Cancer Res Clin Oncol*, 122: 135-140.

12. Knudson AG, Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A*, 68: 820-823.

13. Bartek J, Bartkova J, Lukas J (1997) The retinoblastoma protein pathway in cell cycle control and cancer. *Exp Cell Res*, 237: 1-6.

14. Lukas J, Muller H, Bartkova J, Spitkovsky D, Kjerulff AA, Jansen-Durr P, Strauss M, Bartek J (1994) DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1. *J Cell Biol*, 125: 625-638.

15. Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, vanTuinen P, Ledbetter DH, Barker DF, Nakamura Y (1989) Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science*, 244: 217-221.

16. Birch JM (1994) Li-Fraumeni syndrome. *Eur J Cancer*, 30A: 1935-1941.

17. Bresalier RS (1997) The gatekeeper has many keys: dissecting the function of the APC gene. *Gastroenterology*, 113: 2009-2010.

18. Zheng L, Li S, Boyer TG, Lee WH (2000) Lessons learned from BRCA1 and BRCA2. *Oncogene*, 19: 6159-6175.

19. Shiloh Y, Rotman G (1996) Ataxia-telangiectasia and the ATM gene: linking neurodegeneration, immunodeficiency, and cancer to cell cycle checkpoints. *J Clin Immunol*, 16: 254-260.

20. Lane DP (1992) Cancer. p53, guardian of the genome. *Nature*, 358: 15-16.

21. Hainaut P, Hollstein M (2000) p53 and human cancer: the first ten thousand mutations. *Adv Cancer Res*, 77: 81-137.

22. Momand J, Jung D, Wilczynski S, Niland J (1998) The MDM2 gene amplification database. *Nucleic Acids Res*, 26: 3453-3459.

23. Chin L, Pomerantz J, DePinho RA (1998) The INK4a/ARF tumor suppressor: one gene—two products—two pathways. *Trends Biochem Sci*, 23: 291-296.

24. Sherr CJ (2000) The Pezcoller lecture: cancer cell cycles revisited. *Cancer Res*, 60: 3689-3695.

WEBSITES

American Tissue Type Collection:
http://www.atcc.org/

Centers for Disease Control and Prevention, Atlanta:
http://www.cdc.gov/

Cancer Genome Anatomy Project:
http://www.ncbi.nlm.nih.gov/ncicgap/

European Bioinformatics Institute:
http://www.ebi.ac.uk/

HotMolecBase (Weizmann Institute, Israel):
http://bioinformatics.weizmann.ac.il/hotmolecbase/

IARC p53 database:
http://www.iarc.fr/p53/

Kyoto encyclopedia of genes and genomes:
http://www.genome.ad.jp/kegg/kegg.html

OMIM (Online Mendelian Inheritance in Man):
http://www3.ncbi.nlm.nih.gov/omim/

Protein Data Bank (a protein structure database):
http://www.rcsb.org/pdb/

THE CELL CYCLE

SUMMARY

> The control of cell division is critical to normal tissue structure and function. It is regulated by a complex interplay of many genes that control the cell cycle, with DNA replication (S phase) and mitosis as major checkpoints.

> The cell cycle is tightly regulated to minimize transmission of genetic damage to subsequent cell generations.

> Progression through the cell cycle is primarily controlled by cyclins, associated kinases and their inhibitors. Retinoblastoma (*RB*) and *p53* are major suppressor genes involved in the G1/S checkpoint control.

> Cancer may be perceived as the consequence of loss of cell cycle control and progressive genetic instability.

Classically, the "cell cycle" refers to the set of ordered molecular and cellular processes during which genetic material is replicated and segregates between two newly generated daughter cells via the process of mitosis. The cell cycle can be divided into two phases of major morphological and biochemical change: M phase ("mitosis"), during which division is evident morphologically and S phase ("synthesis"), during which DNA is replicated. These two phases are separated by so-called G ("gap") phases. G1 precedes S phase and G2 precedes M phase.

During progression through this division cycle, the cell has to resolve a number of critical challenges. These include ensuring that sufficient ribonucleotides are available to complete DNA synthesis, proofreading, editing and correcting the newly-synthesized DNA; that genetic material is not replicated more than once; that the spatial organization of the mitotic spindle apparatus is operational; that the packing and the condensation of chromosomes is optimal; and that there is equal distribution of cellular materials between the daughter cells. Moreover, immediately before or after the cell cycle, various factors interact to determine whether the cell divides again or whether the cell becomes committed to a programme of differentiation or of cell death. Therefore, the term "cell cycle" is often used in a broad sense to refer to, as well as the basic, self-replicating cellular process, a number of connected processes which determine pre- and post-mitotic commitments. These may include the commitment to stop dividing in order to enter a quiescent state, to undergo senescence or differentiation, or to leave the quiescent state to re-enter mitosis.

Molecular architecture of the cell cycle

The molecular ordering of the cell cycle is a complex biological process dependent upon the sequential activation and inactivation of molecular effectors at specific points of the cycle. Most current knowledge of these processes stems from experiments carried out in the oocyte of the frog, *Xenopus laevis,* or in yeast, either *Saccharomyces cerevisiae* (budding yeast) or *Schizosaccharomyces pombe* (fission yeast). The *Xenopus* oocyte is, by many criteria, one of the easiest cells to manipulate in the laboratory. Its large size (over a millimetre in diameter) means that cell cycle progression can be monitored visually in single cells. Microinjections can be performed for the purpose of interfering with specific functions of the biochemical machinery of the cell cycle. The *Xenopus* oocyte has proven to be an invaluable tool in the study of the biochemistry of the cell cycle, allowing, among other findings, the elucidation of the composition and regulation of maturation promoting factor (MPF), a complex enzyme comprising a kinase (p34cdc2) and a regulatory subunit (cyclin B), which drives progression from G2 to M phase [1]. In contrast, the exceptional genetic plasticity of yeast has allowed the identification of scores of mutants with defects in cell cycle progression; in mammalian cells, these mutations would have been lethal and it would therefore have been impossible to characterize them. These mutants were called "cdc", for cell division cycle mutants, and many of them have been accorded wider recognition through the application of their names to the mammalian homologues corresponding to the yeast genes.

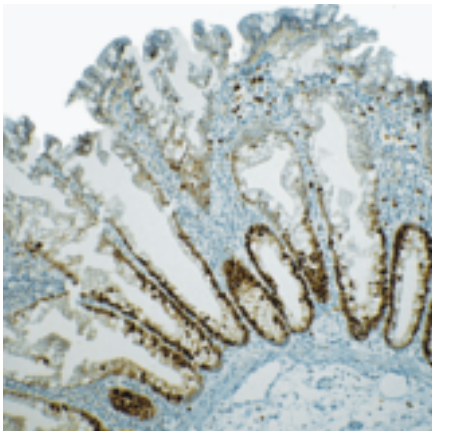


Fig. 3.25 Proliferating cells in the basal parts of the colonic crypts, visualized by immunohistochemistry (stained brown).

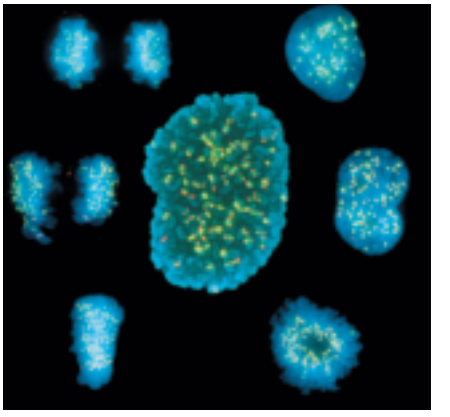


Fig. 3.26 A human osteosarcoma cell nucleus during mitosis. Cell division proceeds clockwise from upper right through interphase, prophase (centre), prometaphase, metaphase, anaphase and telophase. During the cycle, the chromosomes are replicated, segregated and distributed equally between the two daughter cells.

One of the earliest genes to be identified in this way was *cdc2*. Isolated in *S. pombe*, *cdc2* was determined to be able to correct a G2 cell cycle arrest defect. The product of this gene, a serine-threonine kinase of molecular weight 32-34,000 daltons, was subsequently shown to be the yeast homologue of the kinase contained in the *Xenopus* MPF. This enzyme became the paradigm of a class of enzymes now called cyclin-dependent kinases, or CDKs. In their active form, CDKs form heterodimers with cyclins, a class of molecules synthesized in a time-dependent manner during the cell cycle. The progression of the cell cycle depends upon the sequential activation and inactivation of cyclin/CDK complexes [2], a process which requires the synthesis of cyclins, the formation of a complex between a specific cyclin and a CDK and post-translational modification of the CDK to convert the enzyme to an active form (Fig. 3.27).

Progression through the cell cycle as mediated by cyclins is, in turn, determined by factors categorized as having either regulatory (upstream) or effector (downstream) roles. Upstream of cyclin/CDKs are regulatory factors called cyclin-dependent kinase inhibitors (CDKIs), that regulate the assembly and the activity of cyclin/CDK complexes. Downstream of cyclin/CDKs are effector molecules, essentially transcription factors, which control the synthesis of proteins that mediate the molecular and cellular changes occurring during each phase.

CDKIs are small proteins that form complexes with both CDKs and cyclins [3]. Their role is primarily to inhibit the activities of cyclin/CDK complexes and to negatively regulate cell cycle progression. They constitute the receiving end of many of the molecular cascades signalling growth promotion or suppression of growth. Thus CDKIs may be considered as the interface between the cell cycle machinery and the network of molecular pathways which signal proliferation, death or stress responses. However, by virtue of their complexing properties, some CDKIs also play a positive role in cell cycle progression by facilitating the assembly of cyclin/CDK complexes. For example, p21, the product of the *CDKN1A* gene (also known as WAF1/CIP1), promotes the assembly of cyclin D/cdk2 complexes in G1 at a stoichiometric 1:1 ratio, but inhibits the activities of these complexes when expressed at higher levels. There are three main families of CDKIs, each with distinct structural and functional properties: the WAF1/CIP1 family (p21), the KIP family (p27, p57) and the INK4 family (p16, p15, p18) (Fig. 3.27).

Downstream effectors of cyclin/CDKs include proteins mediating three main functional categories: (1) those involved in the control of the enzymes responsible for DNA replication, proof-reading and repair, (2) those involved in chromosome and chromatin remodelling and in the control of genomic integrity and (3) those involved in the mechanics of cell division (including the formation of the centrosome and the mitotic spindle, and in the resorption of the nuclear membrane). These processes require the coordinated synthesis of hundreds of cellular proteins. Transcription factors of the E2F family play a critical role in the control of gene transcription during cell cycle progression (Fig. 3.28). In G1, factors of the E2F family are bound to their DNA targets but are maintained in a transcriptionally inactive state by the binding of proteins of the retinoblastoma (pRb) protein family. At the G1/S transition, the sequential phosphorylation of pRb by several cyclin/CDKs dissociates pRb from the complexes, allowing E2Fs to interact with transcription co-activators and to initiate mRNA synthesis [4].

Gene (chromosome)	Product	Type of alteration	Role in cell cycle	Involvement in cancer
***p53* (17p13)**	p53	Mutations, deletions	Control of p21, 14-3-3σ, etc.	Altered in over 50% of all cancers
***CDKN2A* (9p22)**	p16 and $p19^{ARF}$	Mutations, deletions, hypermethylation	Inhibition of CDK4 and 6	Altered in 30-60% of all cancers
***RB1* (13q14)**	pRb	Deletions	Inhibition of E2Fs	Lost in retinoblastomas, altered in 5-10% of other cancers.
CCND1	Cyclin D1	Amplification	Progression into G1	10-40% of many carcinomas
CDC25A, CDC25B	cdc25	Overexpression	Progression in G1, G2	10-50% of many carcinomas
KIP1	p27	Down-regulation	Progression in G1/S	Breast, colon and prostate cancers

Table 3.3 Cell cycle regulatory genes commonly altered in human cancers.

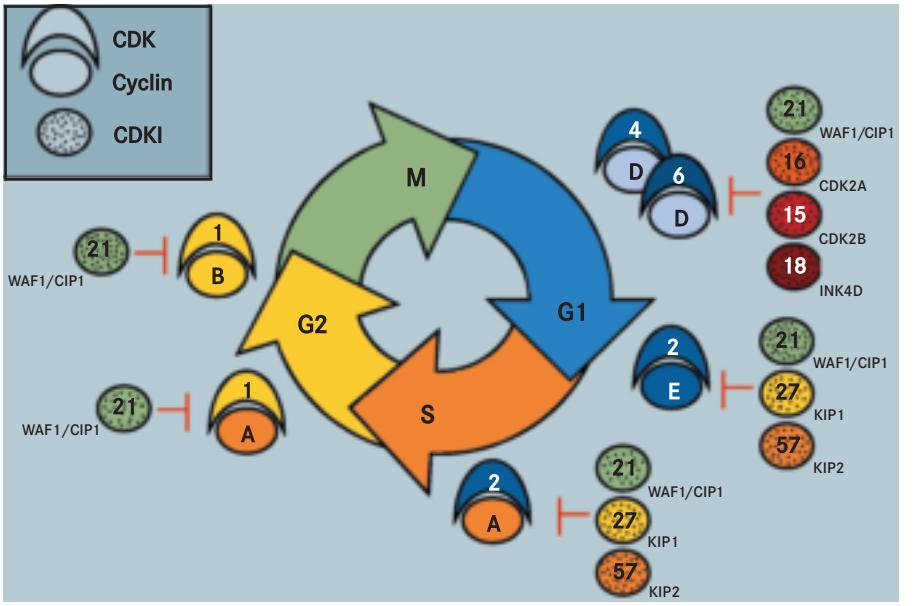


Fig. 3.27 The progression of the cell cycle depends upon the sequential activation and inactivation of cyclin/CDK complexes. This process requires the synthesis of cyclins, the formation of a complex between a specific cyclin and a CDK, and modification of the CDK to convert this enzyme to an active form. The enzyme's activity may be disrupted by a specific inhibitor, a CDKI.

Through this mechanism, E2Fs exert a dual function both as transcriptional repressors in G1, when bound to pRb, and as transcriptional activators in G1/S and in S phase, after dissociation of pRb from the complex. Recent observations suggest that transcriptional repression by E2Fs is essential to prevent the premature activation of cell cycle effectors, which would scramble the temporal sequence of molecular events and preclude cell cycle progression.

Cell cycle checkpoints

The notion of "cell cycle checkpoints" is also derived from early studies in *Xenopus* oocytes and in yeast mutants. In *S. cerevisiae*, commitment to the mitotic cycle requires the crossing of a "restriction point" called the *start* transition. Failure to cross this transition results in cells being blocked in the G1 phase of the cycle. Another control point has been clearly identified after S phase, at the transition between G2 and M phases. Cells unable to cross this checkpoint may remain blocked in a pre-mitotic, tetraploid state. Physiologically, this checkpoint is active in germ cells during the second division of meiosis: cells that have undergone the first, asymmetric division of the meiotic cycle arrest in G2 until completing the second division, which is triggered by fertilization. This concept of "cell cycle checkpoints" was later extended to all mammalian cells [5]. It is now common to envisage the mammalian cell cycle as a succession of checkpoints that have to be negotiated in order for division to be achieved. There is no clear agreement on how many such checkpoints exist in the mammalian cell cycle, or on their exact position.

Control of cdk1 at G2/M transition

The regulation of the complex between cdk1 (also called p34cdc2) and cyclin B exemplifies how different factors co-operate to control the activation of cyclin/CDK complexes at a cell cycle checkpoint. This activation process requires co-operation between three levels of regulation: association between the two partners of the complex, post-translational modifications of the kinase and of the cyclin, and escape from the negative regulation exerted by the CDKIs.

In early G2, cdk1 is in an inactive form. Its activation requires first association with cyclin B, followed by post-translational modification of the kinase itself. This modification includes phosphorylation of a conserved threonine residue (Thr161) by a kinase complex called CAK (CDK-activating kinase), as well as dephosphorylation of two residues localized within the active site of the enzyme, a threonine (Thr14) and a tyrosine (Tyr15). The removal of these phosphate groups is carried out by the dual-specificity phosphatases of the cdc25 group, comprising three isoforms in humans (A, B and C). Activation of these phosphatases is therefore crucial for the activation of cyclin B/cdk1 complexes. The phosphatase is directly controlled by a number of regulators, including plk1 (polo-like kinase), an activating kinase, pp2A, (protein phosphatase 2A), an inhibitory phosphatase and 14-3-3s, a signal transduction molecule which complexes with cdc25, sequesters it in the cytoplasm and thus prevents it from dephosphorylating its nuclear targets. Of course, the action of cdc25 phosphatases is counteracted by kinases that restore the phosphorylation of Thr14 and Tyr15, named wee1 and mik1 [6].

Following the activation process outlined above, the cyclin B/cdk1 complex is potentially able to catalyse transfer of phosphates to substrate proteins. However, in order to achieve this, it has to escape the control exerted by CDKIs, such as p21. The function of this CDKI is itself controlled by several activators, including BRCA1, the product of a breast cancer susceptibility gene (*Oncogenes and tumour suppressor genes,* p96). The p21 protein is removed from the complex by a still poorly understood phosphorylation process, which also drives rapid degradation of the protein by the proteasome. This leaves the cyclin B/cdk1 complex ready to function, after a final step of autophosphorylation, in which cdk1 phosphorylates cyclin B. The complex is now fully active and ready to phosphorylate many different substrates, such as nuclear lamins, during entry into mitosis.

Regulation of the cell cycle and control of genetic stability

During the cell cycle, a number of potential problems may result in damage to the genome. These problems may arise at three distinct stages: (1) during DNA replication, especially if the cell is under conditions of stress that favour the formation of DNA damage (irradiation, exposure to carcinogens etc.), (2) following the termination of DNA replication, when the cell effectively "switches off" its DNA synthesis machinery and (3) during M phase, when the cell has to negotiate the delicate task of segregating chromatids equally. A tight coupling between these processes and cell cycle regulation is therefore crucial to allow the cell to pause during the cell cycle in order to afford the time necessary for the successful completion of all the operations of DNA and chromosome maintenance. Failure to do this may result in both genetic and genomic instabilities, which are hallmarks of cancer. Genetic instability is characterized by an increased rate of gene mutation, deletion or recombination (essentially due to defects in DNA repair). Genomic instability results in chromosome translocations, loss or duplication of large chromosome fragments and aberrant chromosome numbers (aneuploidy).

Tens of molecules have been identified as components of the signalling cascades which couple detection of DNA damage and regulation of the cell cycle. One of these is the product of the tumour suppressor gene *p53* (*Oncogenes and tumour suppressor genes*, p96). *p53* is specifically activated after various forms of direct DNA damage (such as single or double strand breaks in DNA) and regulates the transcription of several inhibitors of cell cycle progression, particularly at the G1/S and G2/M transitions [7]. Other important molecules in this coupling process include the checkpoint kinases chk1 and chk2. Chk1 is activated after replication blockage during S-phase. In turn, chk1 activates wee1 and mik1, two kinases that counteract the action of cdc25 and keep cdk1 in an inactive form. Thus, through activation of chk1, the cell triggers an emergency mechanism that ensures that cells with incompletely replicated DNA cannot enter mitosis.

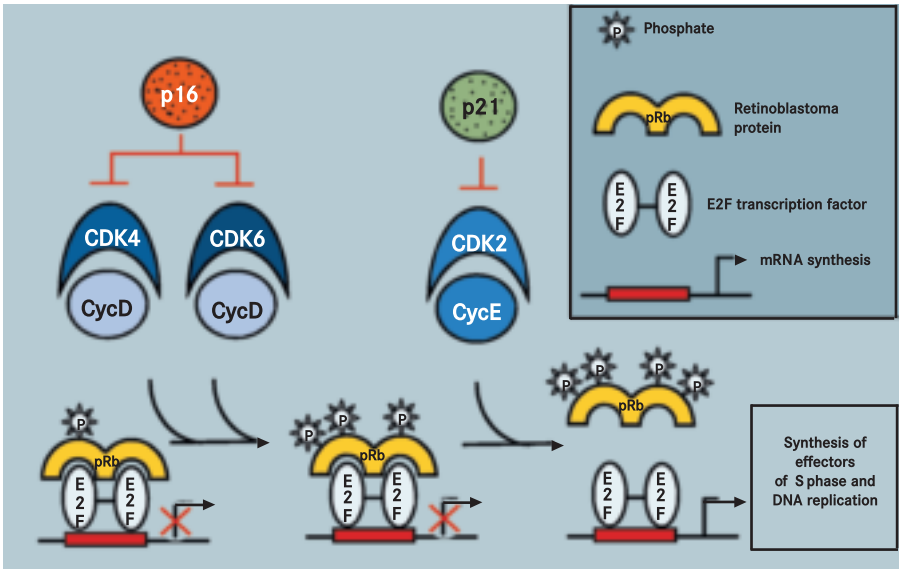


Fig. 3.28 Progression from G1 to S phase is regulated by phosphorylation of the retinoblastoma protein (pRb), in the absence of which DNA replication cannot proceed.

The cell cycle and cancer

Genes involved in cell cycle control are important among those subject to the genetic alterations that give rise to cancer [8]. However, the proliferation of cancer cells requires that the cells retain functional cell cycle processes. The cell cycle alterations seen in cancer are mainly confined to two major sets of regulators: those involved in the negative control of cell cycle progression (inactivation of which leads to accelerated and unchecked cell proliferation) and those involved in coupling the maintenance of genome integrity to the cell cycle (inactivation of which results in cells having gene alterations that progressively accumulate during carcinogenesis) (Table 3.3) [9]. Most of the genes corresponding to these two categories fall within the group of tumour suppressors, and many of them are also direct participants in DNA repair processes.

The gene which encodes p16 (*CDKN2A/INK4A*) has been established as a tumour suppressor gene [10], and mutations and deletions at this site are commonly found in primary human tumours, especially melanoma (although the contribution of another protein encoded by the same locus on chromosome 9p, $p14^{ARF}$, to suppressor activity remains to be determined). Unlike the *CDKN2A/INK4A* gene, the *CDKN1A* gene (encoding p21) is rarely disrupted in cancer. As p21 plays many roles in the negative regulation of almost all phases of the cell cycle, loss of this function might be expected to result in uncontrolled cell division. This is apparently not the case, as mice lacking the *CDKN1A* gene do not show an increased frequency of cancer. This observation illustrates one of the most important characteristics of cell cycle regulatory mechanisms: there is a large degree of redundancy and overlap in the function of any particular effector. Therefore, cancer-causing deregulation of the cell cycle requires a combination of many alterations in genes encoding proteins that, either alone or in concert, are critical for the control of cell division.

Apart from inactivation of negative regulators, a few cell cycle genes may be activated as oncogenes, in that their alteration results in enhanced activity leading to accelerated cell proliferation. The best example of such a cell cycle oncogene is *CCND1*, the gene encoding cyclin D1, a G1-specific cyclin [11]. This gene is located on chromosome 11p13, within a large region that is amplified in up to 20% of

several carcinomas (e.g. breast, head and neck, oesophageal and lung cancers). There is also limited evidence for transcriptional activation of cyclin A (an S-phase cyclin) and for activating mutations of CDK4 (one of the partners of cyclin D1) in some cancers. Indeed, the high complexity of cell cycle effectors provides an extremely diverse range of possibilities for cancer-associated alterations. In this respect, cancer can be seen as, fundamentally, a disease of the cell cycle.

TELOMERES AND TELOMERASE

The ends of eukaryotic chromosomes are referred to as telomeres. These contain many copies of a repetitive DNA sequence, which in vertebrates is the hexanucleotide TTAGGG. The telomeres of normal human somatic cells shorten by 50 to 150 base pairs every time cell division occurs. This appears to act as a cell division counting mechanism: when a cell's telomeres have shortened below a critical length, the cell exits permanently from the cell cycle. Normal cells thus have a limited proliferative capacity, and this acts as a major barrier against carcinogenesis. Cells that have accumulated some carcinogenic changes are unable to form clinically significant cancers unless this proliferation barrier is breached. More than 85% of all cancers achieve this by expressing an enzyme, telomerase, that synthesizes new telomeric DNA to replace the sequences lost during cell division (Shay JW, Bacchetti S, *Eur J Cancer,* 33A: 787-791, 1997).

Telomerase assays have not yet entered routine clinical practice, but there is considerable interest in their possible use for cancer diagnosis and prognosis. For example, telomerase assays of urine sediments may be useful for diagnosis of urinary tract cancer (Kinoshita H et al., *J Natl Cancer Inst,* 89: 724-730, 1997), and telomerase activity levels may be a predictor of outcome in neuroblastoma (Hiyama E et al., *Nature Medicine,* 1: 249-255, 1995).

The catalytic subunit of human telomerase, hTERT, was cloned in 1997 (Lingner J, Cech TR, *Curr Opin Genet Dev* 8: 226-232, 1998). It has subsequently been shown that genetic manipulations of hTERT which result in inhibition of telomerase activity in tumour cells limit their proliferation and often result in cell death. This raises the possibility that telomerase inhibitors may be a very useful form of therapy for many or most types of cancer. However, in tumours with long telomeres, it may take many cell divisions before telomerase inhibitors exert an anti-tumour effect. When such drugs are developed they will therefore need to be carefully integrated with other anticancer treatments.

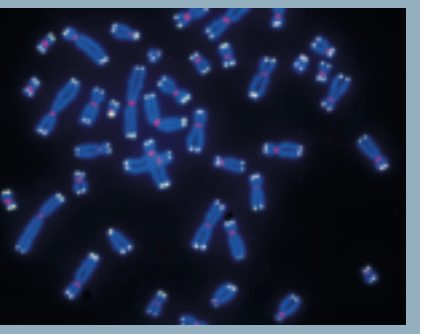


Fig. 3.29 Telomeres contain repetitive DNA sequences that cap the ends of chromosomes. Quantitative fluorescence *in situ* hybridization analysis of human metaphase chromosome spreads is shown, using oligonucleotide probes specific for telomere (white) and centromere (red) DNA sequences, and the DNA dye DAPI (blue). From the laboratory of Drs J.W. Shay and W.E. Wright.

A potential challenge facing telomerase research is the finding that some cancers maintain their telomeres by a mechanism that does not involve telomerase, referred to as alternative lengthening of telomeres, ALT (Bryan TM et al., *Nature Medicine*, 3: 1271-1274, 1997; Reddel RR, *J Clin Invest*, 108: 665-667, 2001).

REFERENCES

1. Hunt T (1989) Maturation promoting factor, cyclin and the control of M-phase. *Curr Opin Cell Biol*, 1: 268-274.

2. Pines J (1995) Cyclins and cyclin-dependent kinases: a biochemical view. *Biochem J*, 308 (Pt 3): 697-711.

3. Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev*, 13: 1501-1512.

4. Weinberg RA (1995) The retinoblastoma protein and cell cycle control. *Cell,* 81: 323-330.

5. Hartwell LH, Weinert TA (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science*, 246: 629-634.

6. Zeng Y, Forbes KC, Wu Z, Moreno S, Piwnica-Worms H, Enoch T (1998) Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. *Nature*, 395: 507-510.

7. Hainaut P, Hollstein M (2000) p53 and human cancer: the first ten thousand mutations. *Adv Cancer Res*, 77: 81-137.

8. Hartwell LH, Kastan MB (1994) Cell cycle control and cancer. *Science*, 266: 1821-1828.

9. Kinzler KW, Vogelstein B (1997) Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature*, 386: 761, 763.

10. Strohmaier H, Spruck CH, Kaiser P, Won KA, Sangfelt O, Reed SI (2001) Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. *Nature*, 413: 316-322.

11. Schuuring E (1995) The involvement of the chromosome 11q13 region in human malignancies: cyclin D1 and EMS1 are two new candidate oncogenes–a review. *Gene*, 159: 83-96.

WEBSITES

Animation of the phases of the cell cycle and of mitosis: http://www.cellsalive.com/

Nature Reviews, "Focus on cell division": http://www.nature.com/ncb/celldivision/

The Forsburg laboratory home pages, a guide to the cell cycle and DNA replication in *S. pombe*: http://pingu.salk.edu/~forsburg/lab.html

CELL-CELL COMMUNICATION

SUMMARY

> Cells communicate by means of secreted molecules which affect neighbouring cells carrying appropriate receptors, and also by direct cell contact, including specifically includes gap junctions.

> Cell contact-mediated communication through gap junctions is controlled by connexin genes and is often disrupted in cancer. This may contribute to uncontrolled and autonomous growth.

> Interventions restoring gap junction communication may provide a basis for therapy.

In complex organisms, neighbouring cells behave and function in harmony for the benefit of the whole organism through the operation of cell-cell communication. During evolution, various types of intercellular communication have developed, which in mammals take two forms: (1) humoral communication and (2) cell contact-mediated communication (Fig. 3.30). Humoral communication is typically mediated by molecules, such as growth factors and hormones, excreted from certain cells and received by receptors of other cells. Intercellular communication based on direct cell-cell contact is mediated by various junctions, including adherence junctions, desmosomes and gap junctions.

During multistage carcinogenesis, genes critically involved in cell growth are altered [1]. Most such genes are known to be directly or indirectly involved in the control of cell replication (*The cell cycle*, p104) or in the death of individual cells [2] (*Apoptosis*, p113). Genes involved in intercellular communication control cellular growth at another level. These genes function to maintain cell growth in harmony with that of the surrounding tissue. Since most cancer cells do not proliferate in harmony with normal neighbouring cells, it is not surprising that the function of genes involved in intercellular communication mechanisms is disrupted in many tumours. Thus, several oncogenes (*Oncogenes and tumour suppressor genes*, p96) encode products involved in humoral intercellular communication: c-erb, c-erbB2 and c-SIS [3]. It has also become clear that cell contact-mediated intercellular communication plays a crucial role in cell growth control [4] and genes involved are often classified as tumour suppressor genes [5]. Cell adhesion molecules are also involved in cell-cell recognition. There are several lines of evidence which suggest that aberrant functions of cell adhesion may be involved in tumour invasion and metastasis [6].

Gap junctional intercellular communication and cancer

Gap junctional intercellular communication is the only means by which cells exchange signals directly from the interior of one cell to the interior of surrounding cells [7]. Since the extent to which tumour cells deviate from cells which exhibit tissue homeostasis is fundamental to the nature of malignancy, it has long been postulated that gap junctional intercellular communication is disturbed in cancer. The first confirmatory evidence was the observation that of a reduced level of gap junctional intercellular communication in one tumour type [8]. This phenomenon has now been observed in almost all tumours [4]. Cell lines established from tumours, as well as cells transformed *in vitro*, usually exhibit impaired function in respect of gap junctional intercellular communication. Gap junctional intercellular communication between transformed cells and neighbouring normal counterparts is selectively defective in murine embryonic BALB/c3T3 cells (Fig. 3.31). A lack of heterologous gap junctional intercellular communication between transformed and normal cells has been observed using rat liver epithelial cell lines and rat liver tumour *in vivo*. It appears that reduced gap junctional intercellular communication is common to many tumour cells. Further studies with multistage models of rat liver and mouse skin carcinogenesis have revealed that there is, in general, a progressive decrease in the level of gap

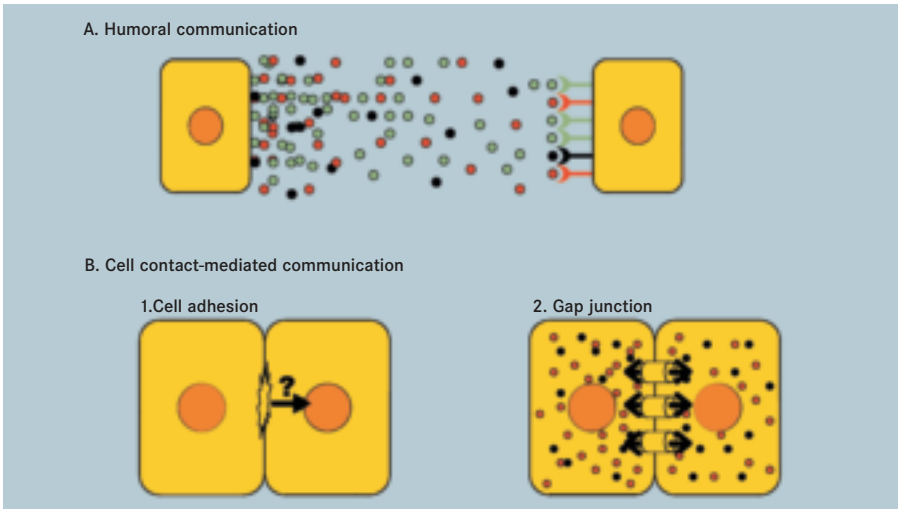


Fig. 3.30 Relationships between cells are maintained by different types of intercellular communication, which may **(B)** or may not **(A)** require cell contact.

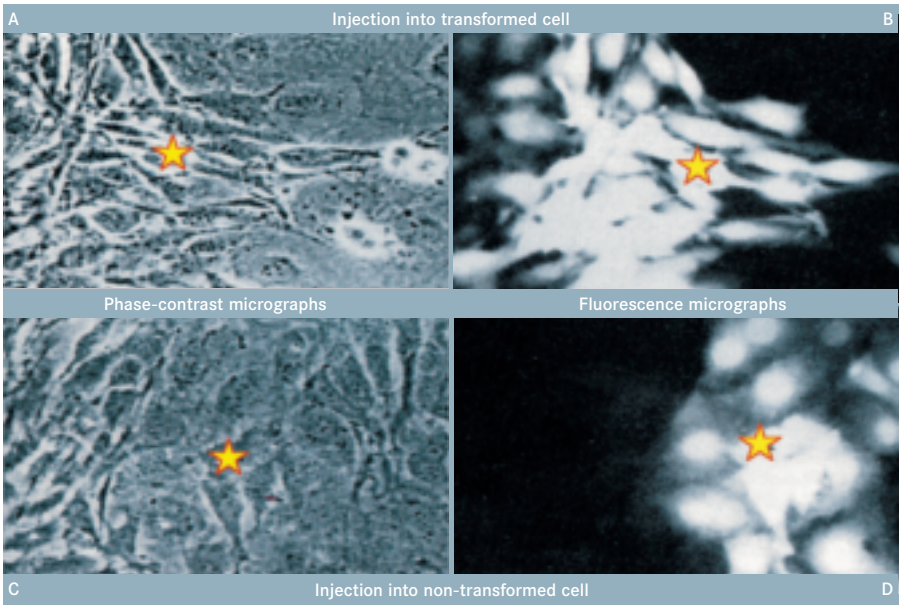


Fig. 3.31 Selective gap junctional communication: cells transformed by a chemical carcinogen (spindle-shaped and criss-crossed) communicate among themselves, but not with their surrounding non-transformed counterparts. When a gap junction-diffusible fluorescent dye is microinjected into a single cell (marked with star) of a transformed focus there is communication between transformed cells but not with the surrounding non-transformed cells **(A, B)**. Injection of the dye into a non-transformed cell which is located near a transformed focus results in communication between non-transformed cells, but not with transformed cells **(C, D)**.

junctional intercellular communication during carcinogenesis and tumour progression.

Another line of evidence, that implies a causal role for blockage of intercellular communication in carcinogenesis, is that agents or genes involved in carcinogenesis have been shown to modulate gap junctional intercellular communication. The mouse skin tumour-promoting agent 12-*O*-tetradecanoylphorbol 13-acetate (TPA) inhibits gap junctional intercellular communication. Many other tumour-promoting agents inhibit gap junctional intercellular communication [9]. In addition to such chemicals, other tumour-promoting stimuli, such as partial hepatectomy and skin wounding, have been demonstrated to inhibit gap junctional intercellular communication. Activation of various oncogenes, including those which encode src, SV-40 T antigen, c-erbB2/neu, raf, fps and ras, also results in inhibition of gap junctional intercellular communication. Conversely, some chemopreventive agents enhance gap junctional intercellular communication [10].

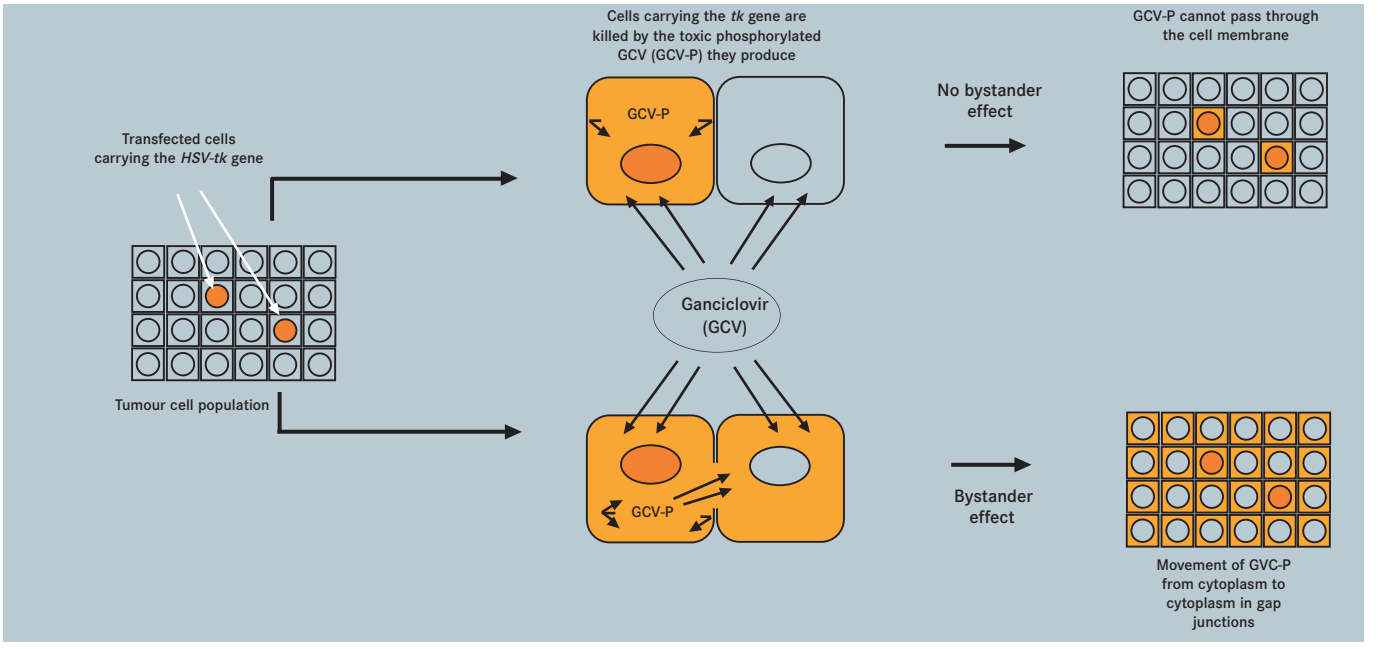


Fig. 3.32 Role of cell-cell interaction in gene therapy. In a tumour cell population, only a few cells can be reached by vectors carrying the *HSV-tk* gene. Expression of the *tk* gene (orange) makes these cells sensitive to ganciclovir: they produce phosphorylated ganciclovir, which is toxic. As phosphorylated ganciclovir cannot pass through the cell membrane, theoretically only cells expressing the *tk* gene will die as a result of ganciclovir treatment. Transmembrane diffusion of phosphorylated ganciclovir from cytoplasm to cytoplasm can induce a bystander effect sufficient to eradicate a tumour cell population, even if only a few cells express the *tk* gene [13].

Connexin genes and tumour suppression

The first indication that normal cells may suppress the growth of malignant cells with which they are in contact came from the work of Stoker and colleagues [11]. More recent evidence for such a direct role of gap junctional intercellular communication in tumour suppression has come from experiments in which connexin genes were transfected into gap junctional intercellular communication-deficient malignant cell lines. In many cases, connexin gene expression reduced or eliminated tumorigenicity of recipient cells [10].

Although tumour suppressor genes, most notably *p53*, are mutated in a high proportion of tumours, few mutations of connexin genes have yet been found in rodent tumours and none has been reported for any human cancer. Although this suggests that connexin gene mutations are rare in carcinogenesis, only a few studies (all from one laboratory) on a limited number of connexin genes (*Cx32*, *Cx37*[α4] and *Cx43*) have been conducted. Several polymorphisms in connexin genes in humans and rats have been described, although there was no apparent correlation between such polymorphisms and the cancer sites examined [10].

Enhancement of cancer therapy by cell-cell communication

A decade ago it was demonstrated that gap junctional intercellular communication could be exploited to distribute therapeutic agents among cancer cells and thereby enhance cancer therapy [12]. One principle of gene therapy is the mediation of selective cytotoxicity by the introduction, into malignant cells, of a gene that activates an otherwise innocuous drug. In practice, only a fraction of the total number of tumour cells sought to be eliminated, are successfully transfected with the gene in question. However, at least in the case of brain tumour therapy based upon the thymidine kinase gene from herpes simplex virus (*HSV-tk*), not only are the cells transfected with the gene affected by treatment with the drug ganciclovir, but neighbouring cells are also killed in the presence of ganciclovir. Several studies have provided strong evidence that this phenomenon, termed "the bystander effect" (Fig. 3.32), is due to connexin-mediated gap junctional intercellular communication; that is, ganciclovir phosphorylated by HSV-tk can diffuse through gap junctions and even those cells without *HSV-tk* gene can be killed. The role of connexin genes in this effect has been confirmed [13].

Signal transduction from intercellular network

The main physiological function of gap junctional intercellular communication is probably to maintain homeostasis by keeping the level of signals mediated by agents of low molecular weight at equilibrium among cells linked by gap junctions. This implies that intercellular communication may control cell growth indirectly. As already noted, such an activity is distinct from that mediated by genes which are directly involved in cell growth and death. One particularly important pathway linking intercellular interaction to signal transduction involves the cell adhesion molecule β-catenin. If the level of β-catenin in the cytoplasm and nucleus rises, it activates transcription factors of the TCF(T-cell factor)/LEF family and increases activity of genes including *C-MYC,* cyclin D1 and connexin-43. Normally the levels of β-catenin in the cytoplasm and nucleus are kept very low because a complex of proteins including the *APC* gene product (adenomatous polyposis coli, *Colorectal cancer*, p198), axin and glycogen synthesis kinase 3β bind the free β-catenin and put a phosphate group onto it which marks it for destruction [14].

In normal cells the level of free β-catenin is regulated by the Wnt ("wingless homologue") signal from outside the cell, which

Gene	Cancer site/cancer	Changes observed
Integrin	Skin, liver, lung, osteosarcoma	Reduced expression
E-cadherin	Stomach, colon, breast, prostate	Mutations: reduced expression
α-catenin	Stomach, colon, breast, prostate, oesophagus, kidney, bladder, etc	Reduced expression
β-catenin	Melanoma, colon	Mutations: reduced expression
γ-catenin	Breast, colon	Loss of expression, translocation into nuclei
Connexins	Liver, skin etc	Reduced expression, aberrant localization

Table 3.4 Examples of cell-cell interaction genes involved in carcinogenesis [10].

increases the level by transiently reducing the activity of the kinase. However, mutations in either the *APC* gene or in the part of the β-catenin (*CTNNB1*) gene which codes for the part of the molecule which accepts the phosphate allow the levels of β-catenin to rise; when this happens the TCF/LEF-controlled genes are permanently activated. The fact that mutations of the *CTNNB1*, *AXIN1*, *AXIN2* and *APC* genes occur in many cancers, including those of the colon, breast and endometrium, shows the importance of this pathway and emphasizes the connection between cell-cell contact and signal transduction.

REFERENCES

1. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell,* 61: 759-767.

2. Kinzler KW, Vogelstein B (1997) Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature*, 386: 761, 763.

3. Heldin CH (1996) Protein tyrosine kinase receptors. *Cancer Surv* , 27: 7-24.

4. Krutovskikh V, Yamasaki H (1997) The role of gap junctional intercellular communication (GJIC) disorders in experimental and human carcinogenesis. *Histol Histopathol*, 12: 761-768.

5. Hirohashi S (1998) Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol*, 153: 333-339.

6. Birchmeier EJ, Behrens J (1994) Cadherin expression in carcinoma: Role in the formation of cell junctions and prevention of invasiveness. *Biochim Biophys Acta*, 1198: 11-26.

7. Bruzzone R, White TW, Paul DL (1996) Connections with connexins: the molecular basis of direct intercellular signaling. *Eur J Biochem*, 238: 1-27.

8. Loewenstein WR, Kanno Y (1966) Intercellular communication and the control of tissue growth: lack of communication between cancer cells. *Nature*, 209: 1248-1249.

9. Trosko JE, Chang CC, Madhukar BV, Klaunig JE (1990) Chemical, oncogene and growth factor inhibition gap junctional intercellular communication: an integrative hypothesis of carcinogenesis. *Pathobiology*, 58: 265-278.

10. Yamasaki H, Omori Y, Zaidan-Dagli ML, Mironov N, Mesnil M, Krutovskikh V (1999) Genetic and epigenetic changes of intercellular communication genes during multistage carcinogenesis. *Cancer Detect Prev*, 23: 273-279.

11. Stoker MG (1967) Transfer of growth inhibition between normal and virus-transformed cells: autoradiographic studies using marked cells. *J Cell Sci,* 2: 293-304.

12. Yamasaki H, Katoh F (1988) Novel method for selective killing of transformed rodent cells through intercellular communication, with possible therapeutic applications. *Cancer Res*, 48: 3203-3207.

13. Mesnil M, Yamasaki H (2000) Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: role of gap-junctional intercellular communication. *Cancer Res*, 60: 3989-3999.

14. Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D, Birchmeier W (1998) Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science*, 280: 596-599.

APOPTOSIS

SUMMARY

> The term apoptosis refers to a type of cell death that occurs both physiologically and in response to external stimuli, including X-rays and anticancer drugs.

> Apoptotic cell death is characterized by distinctive morphological changes different from those occurring during necrosis, which follows ischaemic injury or toxic damage.

> Apoptosis is regulated by several distinct signalling pathways. Dysregulation of apoptosis may result in disordered cell growth and thereby contribute to carcinogenesis.

> Selective induction of apoptosis in tumour cells is among current strategies for the development of novel cancer therapies.

Apoptosis is a mode of cell death that facilitates such fundamental processes as development (for example, by removal of unwanted tissue during embryogenesis) and the immune response (for example, by elimination of self-reactive T cells). This type of cell death is distinguished from necrosis both morphologically (Fig. 3.33) and functionally. Specifically, apoptosis involves single cells rather than areas of tissue and does not provoke inflammation. Tissue homeostasis is dependent on controlled elimination of unwanted cells, often in the context of a continuum in which specialization and maturation is ultimately succeeded by cell death in what may be regarded as the final phase of differentiation. Apart from elimination in a physiological context, cells that have been lethally exposed to cytotoxic drugs or radiation may be subject to apoptosis.

The process of apoptosis can be described by reference to distinct phases, termed "regulation", "effector" and "engulfing" respectively [1]. The regulatory phase includes all the signalling pathways that culminate in commitment to cell death. Some of these pathways regulate only cell death, but many of them have overlapping roles in the control of cell proliferation, differentiation, responses to stress and homeostasis. Critical to apoptosis signalling are the "initiator" caspases (including caspase-8, caspase-9 and caspase-10) whose role is to activate the more abundant "effector" caspases (including caspase-3 and caspase-7) which, in turn, brings about the morphological change indicative of apoptosis. Finally, the engulfing process involves the recognition of cellular "remains" and their elimination by the engulfing activity of surrounding cells.

Identification of genes mediating apoptosis in human cells has been critically dependent on definition of the *ced* genes in the nematode *Caenorhabditis elegans*, members of this gene family being variously homologous to human *BCL2* (which suppresses apoptosis), *APAF-1* (which mediates caspase activation) and the caspases themselves (proteases which mediate cell death). The centrality of apoptosis to cancer biology is indicated by excess tumorigenesis in *BCL2*-transgenic and *p53*-deficient mice. An appreciation of apoptosis provides a basis for the further development of novel and conventional cancer therapy

The role of cell death in tumour growth

Apoptosis, or lack of it, may be critical to tumorigenesis [2]. *BCL2*, a gene mediating resistance to apoptotic stimuli, was discovered at the t(14:18) chromosomal

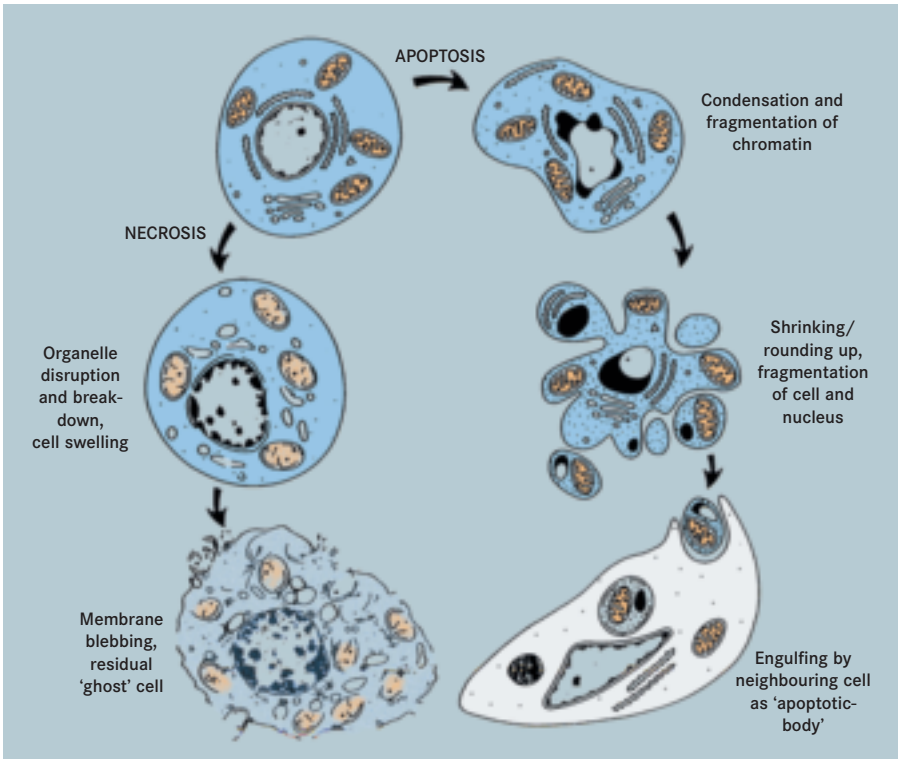


Fig. 3.33 Apoptosis and necrosis are distinguished by characteristic morphological changes.

translocation in low grade B cell non-Hodgkin lymphoma. It thus became apparent that neoplastic cell expansion could be attributable to decreased cell death rather than rapid proliferation. Defects in apoptosis allow neoplastic cells to survive beyond senescence, thereby providing protection from hypoxia and oxidative stress as the tumour mass expands. Growth of tumours, specifically in response to chemical carcinogens, has been correlated with altered rates of apoptosis in affected tissues as cell populations with altered proliferative activity emerge. Paradoxically, growth of some cancers, specifically including breast, has been positively correlated with increasing apoptosis [3].

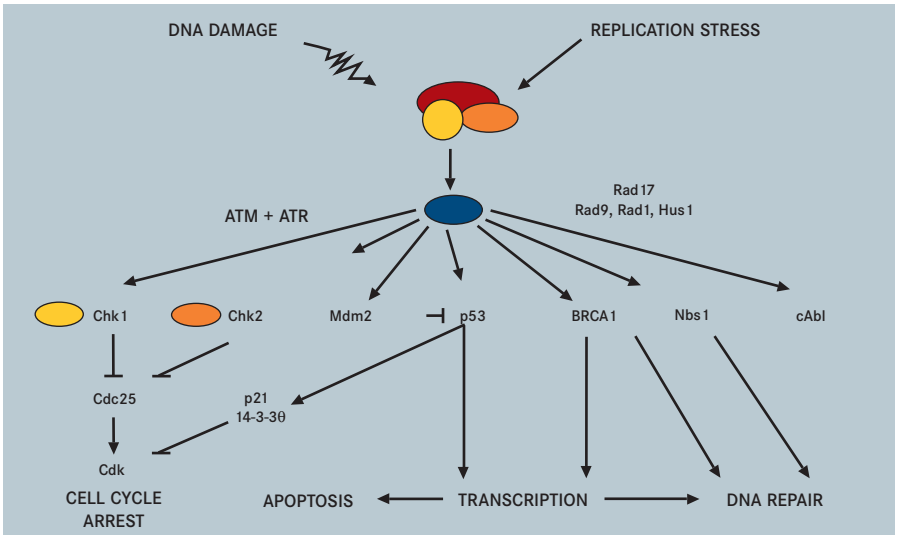


Fig. 3.34 Response to DNA damage is mediated by several signalling pathways and may include apoptosis.

Interrelationships between mitogenic and apoptotic pathways

A dynamic relationship between regulation of growth/mitosis and apoptosis may be demonstrated using a variety of relevant signalling pathways. Many differing promoters of cell proliferation have been found to possess pro-apoptotic activity [4]. Thus, ectopic expression of the *C-MYC* oncogene (normally associated with proliferative activity) causes apoptosis in cultured cells subjected to serum deprivation (which otherwise prevents proliferation). Oncogenes that stimulate mitogenesis can also activate apoptosis. These include oncogenic *RAS*, *MYC* and *E2F*. Mutations in *E2F* that prevent its interaction with the retinoblastoma protein (pRb) accelerate S phase entry and apoptosis. A function of pRb is to suppress apoptosis: pRb-deficient cells seem to be more susceptible to p53-induced apoptosis.

Agents such as radiation or cytotoxic drugs cause cell cycle arrest and/or cell death [5]. The DNA damage caused by radiation or drugs is detected by various means (Fig. 3.34). DNA-dependent protein kinase and the ataxia-telangiectasia mutated gene (*ATM*) (as well as the related ATR protein) bind to damaged DNA and initiate phosphorylation cascades to transmit damage signals. DNA-dependent protein kinase is believed to play a key role in the response to double-stranded DNA breaks. ATM plays an important part in the response to DNA damage caused by ionizing radiation, controlling the initial phosphorylation of proteins such as p53, Mdm2, BRCA1, Chk2 and Nbs1. Other sensors of DNA damage include mammalian homologues of the PCNA-like yeast proteins Rad1, Rad9 and Hus1, as well as the yeast homologue of replication factor C, Rad17. Specific molecules detect nucleotide mismatch or inappropriate methylation. Following exposure of mammalian cells to DNA-damaging agents, p53 is activated and among many "targets" consequently upregulated are the cyclin-dependent kinase inhibitor p21 (which causes G1 arrest) and Bax (which induces apoptosis). Thus, the tumour suppressor gene *p53* mediates two responses to DNA damage by radiation or cytotoxic drugs: cell cycle arrest at the G1 phase of the cell cycle and apoptosis (*Oncogenes and tumour suppressor genes*, p96). The serine/threonine kinase Chk2 is also able to positively interact with p53 and BRCA1. Chk2 and the functionally related Chk1 kinase appear to have a role in the inhibition of entry into mitosis via inhibition of the phosphatase Cdc25 (*The cell cycle*, p104).

The regulatory phase

Two major apoptotic signalling pathways have been identified in mammalian cells (Fig. 3.37). The "extrinsic" pathway

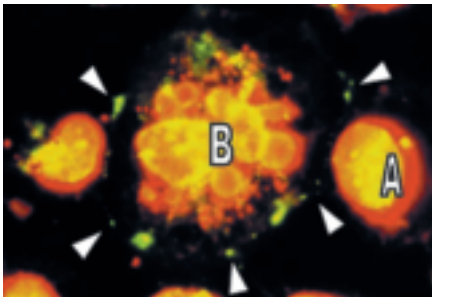


Fig. 3.35 Apoptotic cell death requires gap junctional intercellular communication. Expression and subcellular location of connexin 43 in healthy **(A)** and in apoptotic **(B)** rat bladder carcinoma cells. Arrows indicate location of connexin 43 in areas of intercellular contact between apoptotic **(B)** and non-apoptotic **(A)** cells. Counterstaining of DNA with propidium iodide reveals fragmentation of the nucleus typical of apoptosis **(B)**.

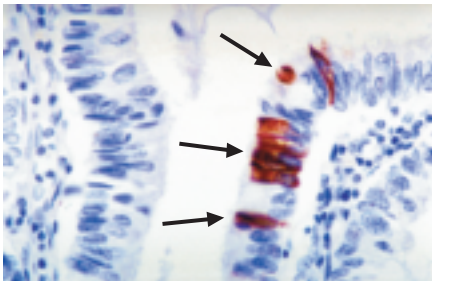


Fig. 3.36 Apoptotic cells in an adenoma, visualized by immunohistochemistry (red). Apoptosis is restricted to single cells, unlike necrosis, which typically involves groups of cells. Apoptosis does not produce an inflammatory response.

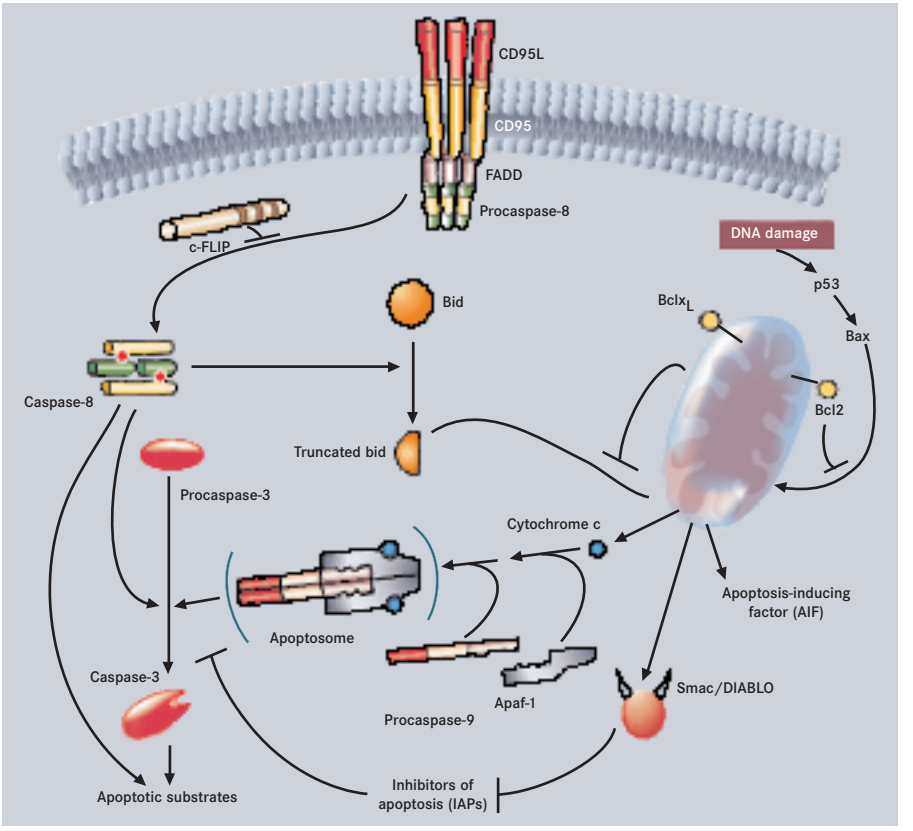


Fig. 3.37 Apoptosis occurs when specific proteases (caspases) digest critical proteins in the cell. The caspases are normally present as inactive procaspases. Two pathways lead to their activation. The death receptor pathway (at the top and left side of the figure) is triggered when ligands bind to death receptors such as CD95/Fas. The mitochondrial pathway is triggered by internal insults such as DNA damage as well as by extracellular signals. In both pathways, procaspases are brought together. They then cleave each other to release active caspase. The binding of ligand (FasL or CD95L) to CD95 brings procaspase 8 molecules together; release of mitochondrial components bring procaspases 9 together. The active caspase 8 and 9 then activate other procaspases such as procaspase 3.

depends upon the conformational change in certain cell surface receptors following the binding of respective ligands. The "intrinsic" pathway involves mitochondrial function and is initiated by growth factor deprivation, corticosteroids or DNA damage induced by radiation or cytotoxic drugs.

Cell surface receptors

Apoptosis may be induced by signalling molecules, usually polypeptides such as growth factors or related molecules, which bind to "death" receptors on the cell surface [6]. Such cell death was initially investigated in relation to the immune response, but has much wider ramifications. The best-characterized receptors belong to the tumour necrosis factor (*TNF*) receptor gene superfamily [7]. In addition to a ligand-binding domain, death receptors contain homologous cytoplasmic sequence termed the "death domain". Members of the family include Fas/APO-1/CD95 and TNF-1 receptor (which binds TNFα). Activation of the Fas (or CD95) receptor by its specific ligand (FasL or CD95L) results in a conformational change such that the "death domain" interacts with the adaptor molecule FADD which then binds procaspase-8. In some cell types, drug-induced apoptosis is associated with Fas activation. Ultraviolet irradiation directly activates the Fas receptor in the absence of ligand. TRAIL (TNF-related apoptosis-inducing ligand, Apo-2L) has 28% amino acid identity to FasL. TRAIL induces cell death only in tumorigenic or transformed cells and not in normal cells [8].

The regulation of apoptosis by BCL2 family genes

While the members of the "death receptor" family and their ligands have structural elements in common, agents and stimuli initiating the mitochondrial pathway to apoptosis are diverse. Common to these stimuli, however, is a change in mitochondrial function, often mediated by members of the *BCL2* family [9]. In humans, at least 16 homologues of *BCL2* have been identified. Several family members (including Bcl-2, Bcl-x_L, Bcl-W) suppress apoptosis, while others induce apoptosis and may be subdivided on the basis of their ability to dimerize with Bcl-2 protein (Bad, Bik, Bid) or not (Bax, Bak). Phosphorylation of Bad protein by a specific (Akt/PKB) and other kinases prevents dimerization with Bcl-2 and promotes cell survival. At least two distinct mechanisms of action are recognized: the binding of Bcl-2 (or other members of the family) with either pro- or anti-apoptotic members of the Bcl-2 family or the formation of pores in mitochondrial membranes. Bcl-x_L is a potent death suppressor that is upregulated in some tumour types. Bax is a death promoter that is inactivated in certain types of colon cancer, stomach cancer and in haematopoietic malignancies. By dint of relevant binding sites, Bax is under the direct transcriptional control of p53.

Involvement of mitochondria

Apoptosis induced by cytotoxic drugs is accompanied by critical changes in mitochondria. Such apoptotic stimuli induce translocation of Bax from cytosol to mitochondria, which induces release of cytochrome c. Loss of transmembrane potential follows cytochrome c release and is dependent on caspase activation (see below), whereas cytochrome c release is not. Bcl-2 and Bcl-x_L reside chiefly in the outer mitochondrial mem-

brane. Bcl-2, Bcl-x_L and Bax can form ion channels when they are added to synthetic membranes, and this may be related to their impact on mitochondrial biology [10]. In the cytosol after release from mitochondria, cytochrome c activates the caspases through formation of a complex (the "apoptosome") with Apaf-1 (apoptotic-protease activating factor-1), procaspase-9 and ATP. It appears that Bcl-2/Bcl-x_L may suppress apoptosis by either preventing release of cytochrome c or interfering with caspase activation by cytochrome c and Apaf-1. Sustained production of nitric oxide (NO) may cause the release of mitochondrial cytochrome c into the cytoplasm and thus contribute to the activation of caspases. However, nitric oxide is involved in several aspects of apoptosis and may act both as a promoter and inhibitor depending on conditions [11].

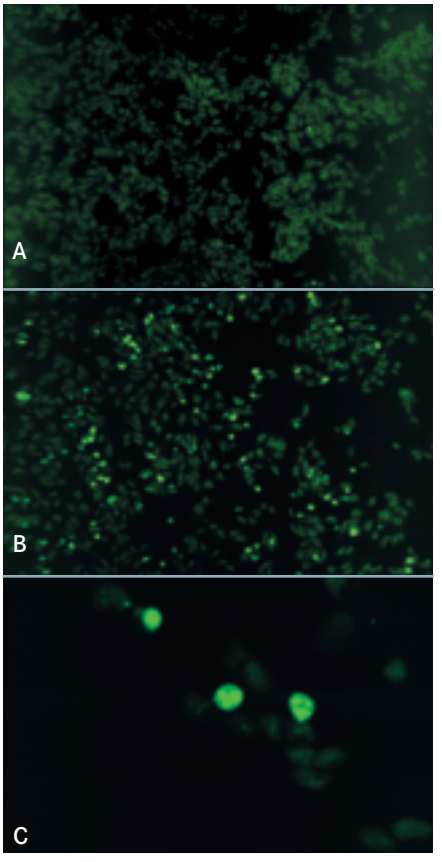


Fig. 3.38 Neuroblastoma cells treated with ionizing radiation undergo apoptosis. The TUNEL assay was used to visualize apoptotic cells (green), before **(A)** and 24 hours after **(B)** treatment with X-rays (5 Gray). Close-up shows that the nuclei of the apoptotic cells are fragmented **(C)**.

The effector and engulfing phases

In mammals at least 13 proteases which mediate the breakdown of cell structure during apoptosis have been identified and are designated caspases-1 through -13 [12]. All possess an active site cysteine and cleave substrates after aspartic acid residues. They exist as inactive zymogens, but are activated by different processes which most often involve cleavage of their pro-forms (designated procaspase-8, etc.) at particular sites, thereby generating subunits which form active proteases consisting of two large and two small subunits. Proteolytic cascades may occur with some caspases operating as upstream initiators (which have large N-terminal prodomains and are activated by protein-protein interaction) and others being downstream effectors (activated by protease cleavage). As noted earlier, at least two pathways of caspase activation can be discerned: one involving FADD or similar protein-protein complexes and the other mediated by release of cytochrome c. In the former, affinity labelling suggests that caspase-8 activates caspases-3 and -7 and that caspase-3 in turn may activate caspase-6. On the other hand, release of cytochrome c into the cytoplasm results in the activation of caspase-9 which in turn activates caspase-3.

Though the intrinsic pathway to caspase-3 activation may be distinguished from the extrinsic pathway (i.e. that activated by Fas, etc.), some interaction is demonstrable. Thus, caspase-9 is able to activate caspase-8. Nonetheless, the pathways are separate to the extent that caspase-8 null animals are resistant to Fas- or TNF-induced apoptosis while still susceptible to chemotherapeutic drugs; cells deficient in caspase-9 are sensitive to killing by Fas/TNF but show resistance to drugs and dexamethasone. Finally, death of some cells may occur independently of caspase-3. Caspases-3, -7 and −9 are inactivated by proteins of the inhibitor of apoptosis family (IAPs) which are suppressors conserved throughout evolution. The IAP protein "survivin" is overexpressed in a large proportion of human cancers. Little is known about the involvement of caspase mutations in cancer.

Caspase substrates and late stages of apoptosis

Apoptosis was initially defined by reference to specific morphological change. In fact, both mitosis and apoptosis are characterized by a loss of substrate attachment, condensation of chromatin and phosphorylation and disassembly of nuclear lamins. These changes are now attributable to caspase activation and its consequences.

Most of the more than 60 known caspase substrates are specifically cleaved by caspase-3 and caspase-3 can process procaspases-2, -6, -7 and -9 [13]. Despite the multiplicity of substrates, protease activity mediated by caspases is specific and seems likely to account for much of the morphological change associated with apoptosis. Caspases cleave key components of the cytoskeleton, including actin as well as nuclear lamins and other structural proteins. Classes of enzymes cleaved by caspases cover proteins involved in DNA metabolism and repair exemplified by poly(ADP-ribose) polymerase and DNA-dependent protein kinase. Other classes of substrates include various kinases, proteins in signal transduction pathways and proteins involved in cell cycle control, exemplified by pRb. Cleavage of some substrates is cell-type specific. Caspase activity accounts for internucleosomal cleavage of DNA, one of the first characterized biochemical indicators of apoptosis. ICAD/DFF-45 is a binding partner and inhibitor of the CAD (caspase-activated DNAase) endonuclease, and cleavage of ICAD by caspase-3 relieves the inhibition and promotes the endonuclease activity of CAD.

Therapeutic implications

In theory, knowledge of critical signalling or effector pathways which bring about apoptosis provides a basis for therapeutic intervention, including the development of novel drugs to activate particular path-

ways. Several options are under investigation [14]. More immediately, attempts are being made to exploit knowledge of apoptotic processes to increase the efficacy or specificity of currently available therapy. Simple answers have not emerged. Thus, for example, relatively increased expression of Bcl-2 (which, under many experimental conditions, inhibits apoptosis) is not necessarily indicative of poor prognosis and the reverse appears true for some tumour types. In experimental systems, cells acquiring apoptosis defects (e.g. *p53* mutations) can more readily survive hypoxic stress and the effects of cytotoxic drugs [15]. However, clinical studies have not consistently established that mutation of *p53* is associated with poor response to chemotherapy [16].

The function of Bcl-2 family members may be subject to interference by small molecules [17]. In preclinical animal models, suppression of Bcl-2 by an antisense oligonucleotide has been shown to retard tumour growth and the approach is currently subject to clinical trial. Likewise, antisense oligonucleotides directed at "survivin" are being evaluated. The possibility of using recombinant TRAIL to induce apoptosis in malignant cells is under investigation. TRAIL is implicated as the basis of *all-trans*-retinoic treatment of promyelocytic leukaemia [18]. Also noteworthy. The drug Gleevec is already in clinical use (*Leukaemia,* p242*)*. It is hoped that in future, a combination of agents targeting parallel pathways, as well as combinations with classical cytotoxic agents will improve the outcome of cancer patients.

DRUGS TARGETING SIGNAL TRANSDUCTION PATHWAYS

In complex multicellular organisms, cell proliferation, differentiation and survival are regulated by a number of extracellular hormones, growth factors and cytokines. These molecules are ligands for cellular receptors and communicate with the nucleus of the cell through a network of intracellular signalling pathways. In cancer cells, key components of these signal transduction pathways may be subverted by proto-oncogenes through over-expression or mutation, leading to unregulated cell signalling and cellular proliferation. Because a number of these components may be preferentially over-expressed or mutated in human cancers, the cell signalling cascade provides a variety of targets for anticancer therapy (Adjei AA, *Current Pharmaceutical Design,* 6: 471-488, 2000).

Different approaches have been used to attack these targets and include classical cytotoxic agents as well as small molecule drug inhibitors. In addition, antisense oligonucleotides, vaccines, antibodies, ribozymes and gene therapy approaches have been utilized.

The diagram illustrates cell signalling pathways that are targeted by anticancer agents currently undergoing clinical testing.

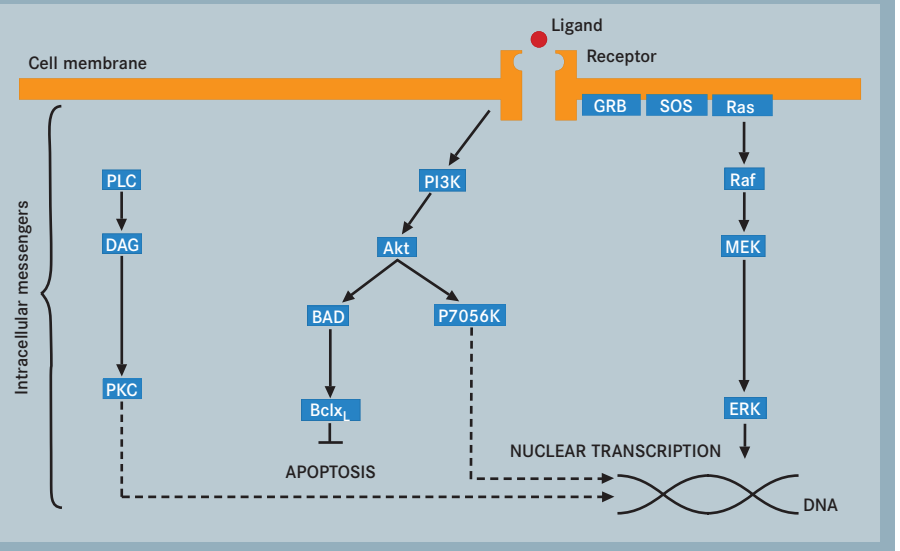


Fig. 3.39 Signaling pathways targeted by anticancer agents. PI3K = phosphoinositide-3-kinase; PLC = phospholipase C; PKC = protein kinase C; MEK = mitogen-activated protein kinase kinase; ERK = extracellular signal-regulated kinase; Akt = protein kinase B (PKB); BAD = Bcl-XL/Bcl-2-associated death protein; VEGF = vascular endothelial growth factor; HER = human epidermal growth factor receptor family; PDGF = platelet derived growth factor; FGF = fibroblast growth factor; SOS = son of sevenless guanine nucleotide exchange protein; GRB = growth factor receptor-bound protein.

Classes of agents and their potential targets include:

- **> *Inhibitors of ligands*, such as recombinant human antibody to VEGF (rHu mAbVEGF)**
- **> *Receptors,* anti-receptor antibodies and tyrosine kinase receptor inhibitors**
- **> *RAS* farnesyltransferase inhibitors**
- **> *RAF* inhibitors**
- **> *MEK inhibitors***
- **> *Rapamycin analogues***
- **> *Protein kinase C (PKC) inhibitors***
- **> *Inhibitors of protein degradation***
- **> *Inhibitors of protein trafficking***

worthy is the development of caspase inhibitors for the treatment of certain degenerative (non-cancerous) diseases characterized by excess apoptosis.

Drugs shown to induce apoptosis specifically include chemopreventive agents (*Chemoprevention*, p151), exemplified by 4-hydroxyphenylretinamide. Butyrate, a short-chain fatty acid produced by bacterial fermentation of dietary fibre, inhibits cell growth *in vitro* and promotes differentiation; it also induces apoptosis. Both roles may contribute to its prevention of colorectal cancer. Moreover, cyclo-oxygenase enzyme (COX-2) expression may modulate intestinal apoptosis via changes in Bcl-2 expression. Aspirin and similar drugs which inhibit COX-2 may promote apoptosis and prevent tumour formation.

REFERENCES

1. Strasser A, O'Connor L, Dixit VM (2000) Apoptosis signaling. *Annu Rev Biochem*, 69: 217-245.

2. Kaufmann SH, Gores GJ (2000) Apoptosis in cancer: cause and cure. *Bioessays*, 22: 1007-1017.

3. Parton M, Dowsett M, Smith I (2001) Studies of apoptosis in breast cancer. *BMJ*, 322: 1528-1532.

4. Choisy-Rossi C, Yonish-Rouach E (1998) Apoptosis and the cell cycle: the p53 connection. *Cell Death Differ*, 5: 129-131.

5. Rich T, Allen RL, Wyllie AH (2000) Defying death after DNA damage. *Nature*, 407: 777-783.

6. Peter ME, Krammer PH (1998) Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. *Curr Opin Immunol*, 10: 545-551.

7. Yeh WC, Hakem R, Woo M, Mak TW (1999) Gene targeting in the analysis of mammalian apoptosis and TNF receptor superfamily signaling. *Immunol Rev*, 169: 283-302.

8. Griffith TS, Lynch DH (1998) TRAIL: a molecule with multiple receptors and control mechanisms. *Curr Opin Immunol*, 10: 559-563.

9. Gross A, McDonnell JM, Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev*, 13: 1899-1911.

10. Matsuyama S, Llopis J, Deveraux QL, Tsien RY, Reed JC (2000) Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat Cell Biol*, 2: 318-325.

11. Chung HT, Pae HO, Choi BM, Billiar TR, Kim YM (2001) Nitric oxide as a bioregulator of apoptosis. *Biochem Biophys Res Commun*, 282: 1075-1079.

12. Kumar S (1999) Regulation of caspase activation in apoptosis: implications in pathogenesis and treatment of disease. *Clin Exp Pharmacol Physiol*, 26: 295-303.

13. Porter AG, Janicke RU (1999) Emerging roles of caspase-3 in apoptosis. *Cell Death Differ*, 6: 99-104.

14. Nicholson DW (2000) From bench to clinic with apoptosis-based therapeutic agents. *Nature*, 407: 810-816.

15. Zhou BB, Elledge SJ (2000) The DNA damage response: putting checkpoints in perspective. *Nature*, 408: 433-439.

16. Brown JM, Wouters BG (1999) Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res*, 59: 1391-1399.

17. Zheng TS (2001) Death by design: the big debut of small molecules. *Nat Cell Biol*, 3: E43-E46.

18. Altucci L, Rossin A, Raffelsberger W, Reitmair A, Chomienne C, Gronemeyer H (2001) Retinoic acid-induced apoptosis in leukemia cells is mediated by paracrine action of tumor-selective death ligand TRAIL. *Nat Med*, 7: 680-686.

WEBSITE

The European Cell Death Organization:
http://www.ecdo.dote.hu/

INVASION AND METASTASIS

SUMMARY

> The ability of tumour cells to invade and colonize distant sites is a major feature distinguishing benign growths from malignant cancer.

> Most human tumours lead to death through widespread metastasis rather than the adverse local effects of the primary neoplasm.

> Often, metastatic spread first involves regional lymph nodes, followed by haematogenous spread throughout the body. Metastases may become clinically manifest several years after surgical resection of the primary tumour.

> Current methods are inadequate for the routine detection of micrometastases and the search for effective, selective therapies directed toward metastatic growth remains a major challenge.

Metastasis (from the Greek meaning "change in location") refers to growth of secondary tumours at sites distant from a primary neoplasm. Metastasis thus distinguishes benign from malignant lesions and is the ultimate step in the multistage process of tumour progression. Metastatic growth is the major cause of treatment failure and the death of cancer patients. Although secondary tumours may arise by shedding of cells within body cavities, the term metastasis is generally reserved for the dissemination of tumour cells via the blood or lymphatics. Spread in the cerebrospinal fluid and transcoelomic passage may also occur. Most (60-70%) cancer patients have overt or occult metastases at diagnosis, and the prognosis of the majority of these patients is poor (Box: *TNM Classification of Malignant Tumours*, p124).

There is a critical need to identify reliable indicators of metastatic potential, since clinical detection of metastatic spread is synonymous with poor prognosis. Current methods of detecting new tumours, including computed tomography (CT) scans or magnetic resonance imaging (MRI), ultrasound, or measurement of circulating markers such as carcinoembryonic antigen (CEA), prostate-specific antigen (PSA) or cancer antigen 125 (CA125) are not sufficiently sensitive to detect micrometastases. A greater understanding of the molecular mechanisms of metastasis is required. It is clear that metastatic growth may reflect both gain and loss of function, and indeed the search for "metastasis suppressor" genes has been more fruitful than identification of genes which specifically and reliably potentiate metastasis [1].

The genetics of metastasis

With the publication of the human genome sequence, and various major initiatives such as the Cancer Genome Project in the UK and the Cancer Genome Anatomy Project in the USA, the search for genes selectively upregulated, mutat-

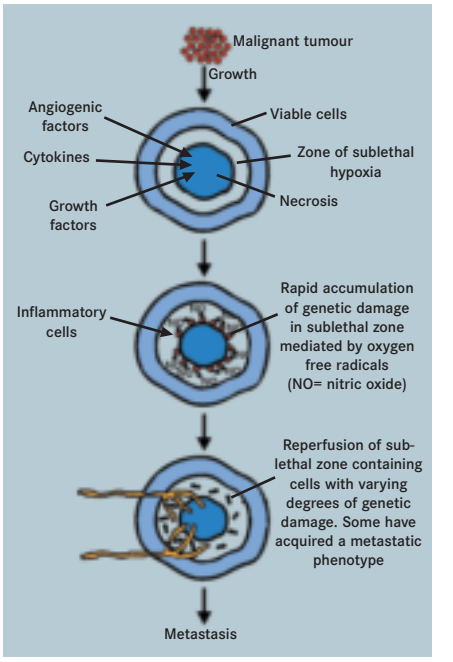


Fig. 3.40 The hypoxia hypothesis suggests that the progression of malignant tumours to a metastatic phenotype is mediated by deficiency of oxygen and resulting tumour necrosis.

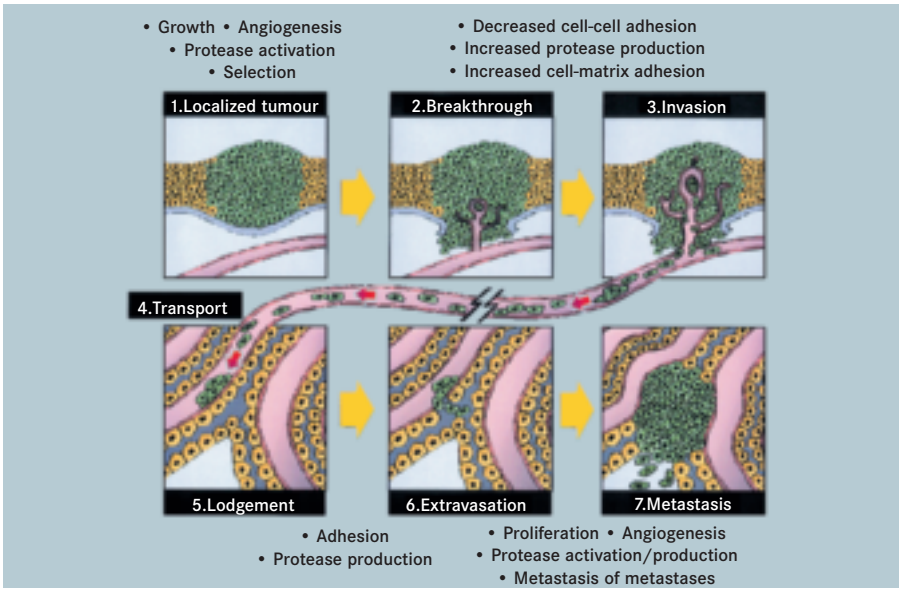


Fig. 3.41 The stages in the metastatic process, illustrated in relation to the spread of a primary tumour from a surface epithelium to the liver.

Gene	Cancer type(s)	Mechanism
nm23 Family (H1-6) of nucleoside diphosphate kinases	Breast (liver, ovary, melanoma)	Cell migration? Signalling via G proteins, microtubule assembly
PTEN/MMAC1	Prostate, glioma, breast	Migration, focal adhesions
KAI1/CD82/C33	Prostate, stomach, colon, breast, pancreas, lung	Cell-cell adhesion, motility
CAD1/E-cadherin	Many adenocarcinomas	Cell-cell adhesion, epithelial organization
MKK4/SEK1	Prostate	Cellular response to stress?
KiSS-1	Melanoma, breast cancer	Signal transduction? Regulation of MMP-9?
BRMS1	Breast	Cell communication, motility
DPC4	Colon, pancreas	?

Table 3.5 Putative metastasis suppressor genes.

ed or lost in metastatic cancers (Table 3.5) has gained momentum. It is now possible, using laser capture microdissection and serial analysis of gene expression (SAGE), to isolate invasive cancer cells and compare their gene or protein expression with non-invasive or normal cells from the same patient [2]. Prior to this, transfection of chromosomes or DNA from metastatic to non-metastatic cells (or *vice versa*), subtractive hybridization/differential display PCR, cDNA array and other strategies resulted in identification of some genes specifically linked to metastasis, although many others so identified are also associated with tumour growth or developmental processes.

The events which lead to cancer metastasis include changes in cell-cell and cell-matrix adhesion, alterations in cell shape, deformability and motility, invasion of surrounding normal tissues, gaining access to lymphatic or vascular channels, dissemination via blood or lymph, survival of host defence mechanisms, extravasation and colonization of secondary sites (Fig. 3.41). There are now many features of cancer cells recognized to potentiate metastasis, and a great deal is known about the cellular and molecular events that underlie the process. However the ability to predict which patient has occult micrometastases, and the discovery of effective, selective therapies for metastatic disease, remain major challenges in oncology.

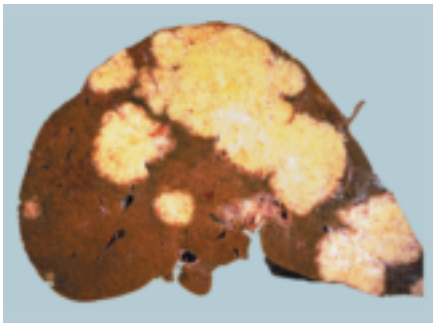


Fig. 3.42 Multiple metastatic growths of an intestinal carcinoma in the liver.

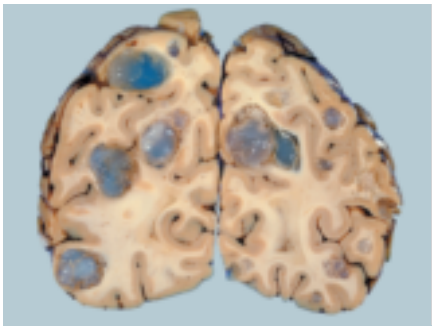


Fig. 3.43 Multiple metastases to the brain from a lung carcinoma.

The biology of metastasis

Growth of tumours beyond a few millimetres in diameter cannot progress without neovascularization, and there is a growing appreciation of how this phenomenon is linked to metastasis [3]. Many genetic changes associated with malignant progression (mutation of *HRAS*, over-expression of *ERBB2* oncogenes, loss of *p53*) induce an angiogenic phenotype (developing blood vessels) via induction of cytokines, such as vascular endothelial growth factor (VEGF-A). VEGF-A is also upregulated by hypoxia in tumours, partly by host cells such as macrophages. The presence of hypoxic areas is a characteristic of solid tumours and has been related to poor response to conventional therapies (Fig. 3.40). In addition, activation of epithelial growth factor receptor (EGFR) and other oncogenic signalling pathways can also upregulate VEGF-C, a known lymphangiogenic cytokine [4]. The receptors for these cytokines (Flk-1 and Flk-4) are expressed on tumour vasculature, and both (in addition to acting as potent mitogens for endothelial cells) also enhance vessel permeability. Thus activation of these signalling pathways may potentiate both vascular and lymphatic invasion and tumour spread. Basic fibroblast growth factor (bFGF) is often upregulated in cancers, particularly at the invasive edge where tumour cells interact with host cells [5].

Epithelial cells are normally bounded by basement membranes which separate them from the underlying stroma and mesenchymal compartments. Breaching this barrier is the first step in the transition from carcinoma *in situ* to invasive and potentially metastatic carcinoma. Basement membrane is composed of a variety of structural proteins including collagen IV (the major component),

laminin, entactin and also heparan sulfate proteoglycans. Interactions of tumour cells with the basement membrane have been considered to comprise three steps, which can readily be demonstrated *in vitro*: adhesion, matrix dissolution/proteolysis and migration [6].

Epithelial cells are normally polarized and firmly attached to each other via desmosomes, tight junctions and intercellular adhesion molecules such as E-cadherin, and also bound to the basement membrane via other adhesion molecules including integrins. Changes in cell-cell and cell-matrix adhesive interactions are common in invasive cancer (*Cell-cell communication*, p109). Indeed, E-cadherin may be designated a tumour suppressor gene, since its loss or functional inactivation is one of the most common characteristics of metastatic cancer, and its reintroduction into cells can reverse the malignant phenotype. The adenomatous polyposis coli gene (*APC*), which is mutated in many inherited and sporadic colon cancers, normally regulates the expression of β-catenin, a protein which interacts with E-cadherin. Mutations in *APC* (or β-catenin) increase cellular levels of the latter and facilitate interactions with transcription factors such as T-cell factor/lymphoid enhancer factor (TCF/LEF) which drive the expression of genes involved in inhibiting apoptosis and stimulating cell proliferation. Other genes commonly lost in cancers (e.g. *DCC*, Deleted in Colon Carcinoma) also encode adhesion molecules.

Integrins

Integrins are heterodimeric proteins that mediate adhesion between cells and the extracellular matrix or other cellular elements. Ligand specificity is determined by the subunit composition; many integrins bind multiple substrates and others are more selective. Far from being an inert "glue", they are capable of transmitting important signals regulating cell survival, differentiation and migration [7]. Many differences in integrin expression between benign and malignant cells have been documented, but the patterns are complex. In addition, their expression and binding affinity can be profoundly influenced by the local microenvironment and soluble factors, enabling the tumour cell to respond to different conditions encountered throughout the metastatic cascade.

Other molecules involved in adhesion

Other adhesion molecules implicated in cancer progression include selectins such as sialyl Le^x and members of the immunoglobulin superfamily, including intercellular adhesion molecules (ICAM-1, ICAM-2, VECAM and PECAM). The latter are upregulated on activated endothelial cells, and can interact with integrins on leukocytes and circulating tumour cells, assisting their arrest and extravasation. CD44 is another adhesion molecule utilized during lymphocyte "homing", and a change from the standard "epithelial" pattern to expression of splice variants associated with haematopoietic cells has been proposed to assist carcinoma cells in haematogenous dissemination. Thrombospondin may mediate adhesion between circulating tumour cells, platelets and endothelial cells, promoting embolization (vessel obstruction) and arrest. Tumour cells then gain access to the subendothelial basement membrane when endothelial cells retract in response to these emboli, and can adhere to exposed proteins. Synthetic peptides containing sequences of amino acids which compete with binding to laminin or fibronectin can inhibit colonization of the lung by intra-

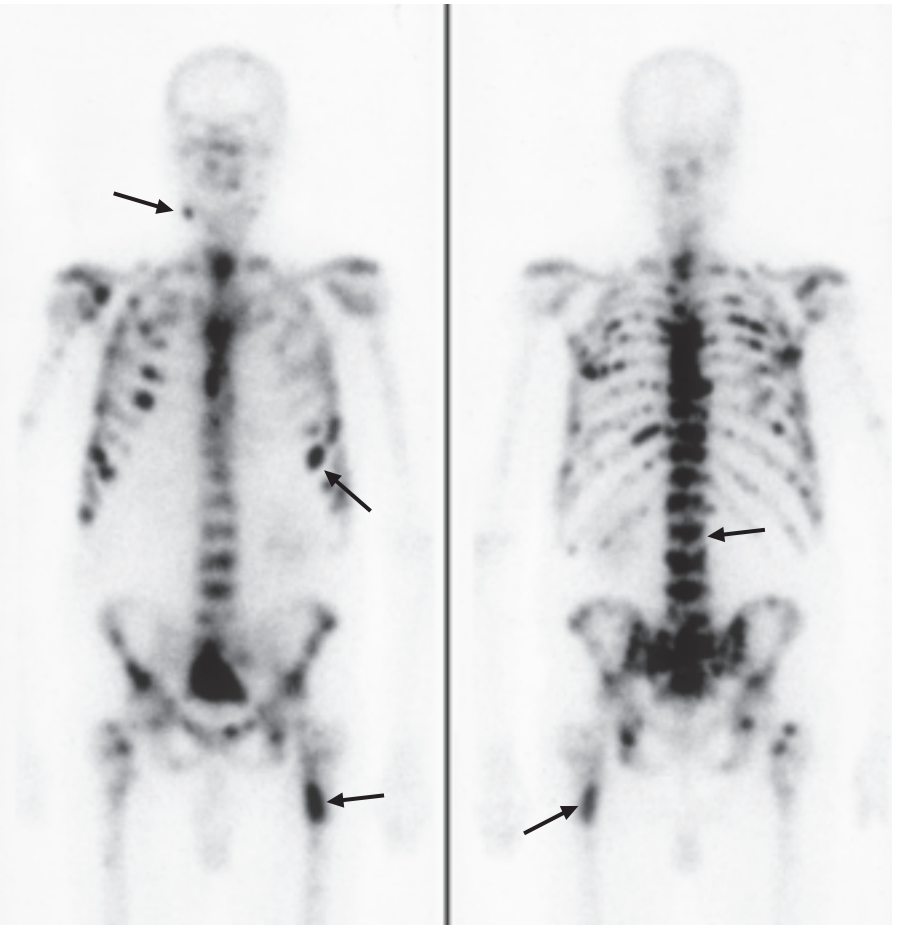


Fig. 3.44 MRI scan showing skeletal metastases in a patient with a primary prostatic carcinoma (front and back views). Some of the larger metastases are marked by arrows. Note the numerous metastases in the ribs and in the spine.

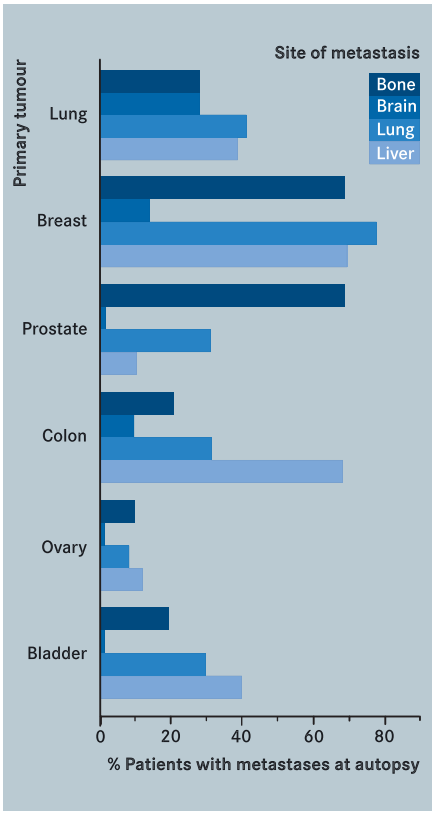


Fig. 3.45 Location of metastases at autopsy for some common cancers, indicating that the site of metastasis is not random.

Primary tumour	Site of metastasis
Bronchial cancer	Adrenal (often bilateral)
Breast ductal carcinoma	Liver
Breast lobular carcinoma	Diffuse peritoneal seeding
Breast	Bone, ovary
Lung	Brain
Ocular melanoma	Liver
Prostate	Bone
Melanoma	Brain

Table 3.6 Some sites of metastasis which are not explicable by circulatory anatomy.

venously injected cells in experimental models.

RHO

The *RHO* gene family of small GTP-hydrolysing proteins contains several members known to be involved in cell migration via regulation of actomyosin-based cytoskeletal filament contraction and the turnover of sites of adhesion. Overexpression of RhoC alone in melanoma cells is sufficent to induce a highly metastatic phenotype [8].

Enzyme functions in invasion and metastasis

Invasive tumour cells show increased expression of many enzymes due to upregulation of genes, enhanced activation of pro-enzymes or reduced expression of inhibitors such as tissue inhibitors of metalloproteinases (TIMPs). In addition, tumour cells may also induce expression of enzymes by neighbouring host cells and "hijack" these to potentiate invasion.

Matrix metalloproteinases

One important group is the matrix metalloproteinases (MMP). Different cancers may show different patterns of expression; for instance squamous carcinomas frequently have high levels of gelatinase B (MMP-9), stromelysins 1-3 (MMP-3, MMP-10 and MMP-11, normally stromal enzymes, but also expressed by these carcinomas) and matrilysin (MMP-7). Adenocarcinomas such as breast may have increased levels of gelatinase A (MMP-2) and colon carcinomas commonly overexpress MMP-7. In addition, MT1-MMP, which activates MMP-2, is often upregulated in tumour and/or neighbouring host tissues. The major substrate of the gelatinases is collagen IV, a major component of the basement membrane, whereas the stromelysins prefer laminin, fibronectin and proteoglycans, and can also activate procollagenase (MMP-1), which in turn degrades the fibrillar collagens of the interstitial tissues. Urokinase plasminogen activator (uPA) is also frequently upregulated in cancer. It controls the synthesis of plasmin, which degrades laminin and also activates gelatinases. Thus, upregulation of these enzymes in cancers leads to proteolytic cascades and potential for invasion of the basement membrane and stroma.

Metalloproteinases also contribute to tumour growth and metastasis by other means [9]. During angiogenesis, "invasion" of capillary sprouts requires local proteolysis (mediated in part by upregulated MMP-2 and MMP-9 together with uPA) and in addition MMP-9 has been implicated in the "angiogenic switch" by releasing VEGF from sequestration in the extracellular matrix [10]. Furthermore, these proteases can contribute to the sustained growth of tumours by the ectodomain cleavage of membrane-bound pro-forms of growth factors, and the release of peptides which are mitogenic and chemotactic for tumour cells.

Heparanase

Apart from the structural proteins cleaved by metalloproteinases in the basement membrane and extracellular matrix, the other major components are glycosaminoglycans, predominantly heparan sulfate proteoglycan (HSPG). Heparanase is an important enzyme which degrades the heparan sulfate side-chains of HSPGs and, like the proteases described above, not only assists in the breakdown of extracellular matrix and basement membrane, but is also involved in the regulation of growth factor and cytokine activity. Basic fibroblast growth factor (bFGF, another potent mitogen and chemotactic factor for endothelial cells) and other heparin-binding growth factors are sequestered by heparan sulfate, providing a localized depot available for release by heparanase. Similarly, uPA and tissue plasminogen activator (tPA) can be released from heparan sulfate by heparanase, further potentiating proteolytic and mitogenic cascades.

Tissue-specific growth factors

Finally, it is possible that release of tissue-specific growth factors may play a role in organ selectivity of metastasis. For example, colorectal carcinoma cells over-expressing EGFR have a predilection for

Target	Example of agent	Comments
Adhesion/attachment	RGD-toxin constructs and RGD-targeted gene therapy	Have not reached clinical trials
	Anti-avfl3 monoclonal antibody (Vitaxin, Medi522)	Cytostasis in patients; anti-tumour and anti-angiogenic in animal models
Proteolysis	Matrix metalloproteinase inhibitors	Cytostatic in patients; rare occurence of tumour partial regressions; stromal fibrosis; activity seen in multiple animal models and in combination with chemotherapy; new agents with varied MMP specificity under development
Motility	No selective agents	
Signal pathways	Squalamine (NHE-3 inhibitor)	Selective for endothelial cells
	PDGFR, KDR and EGFR small molecule inhibitors	Active *in vitro* in animal models; preclinical activity in combinations; phase I trials completed for several agents, some tumour stabilization or regression
	Anti-EGFR monoclonal antibody (C225)	Neutralizing antibody; active *in vitro* in animal models; phase I trials ongoing
	Anti-VEGF antibody	Blocking antibody; active *in vitro* in animal models; preclinical activity alone and in combination; phase I-III trials ongoing
	CAI (non-voltage-gated Ca^{++} uptake inhibitor)	Active *in vitro* in animal models; preclinical activity in combinations; phase I trials of single agents and combinations, some tumour stabilization or regression
Extracellular matrix	Pirfenidone	Suppresses stromal/inflammatory cell Remodelling by stromal expression of TGF-β Phase I trials for pulmonary fibrosis

Table 3.7 Therapeutic agents directed towards stroma-tumour interactions.

growth in the liver where there are high concentrations of its ligands. All of these require proteolytic cleavage for activation. Other enzymes which have been implicated in metastasis include the cysteine proteinases, notably cathepsins B and D. For most of the enzymes described, there are active research programmes seeking selective inhibitors (some of which have reached phase II and III clinical trials) to prevent or treat metastatic disease.

Motility, coupled with proteolysis, is the basis of tumour cell invasion, and is also important during intravasation and extravasation of blood and lymphatic vessels. Many motility factors have been described which may be tumour- or host-derived. Many growth factors, such as transforming growth factor alpha, epidermal growth factor and platelet-derived growth factor, can induce chemotactic responses in tumour cells expressing the cognate receptors. Scatter factor (also known as hepatocyte growth factor, HGF) is a potent host-derived motility factor, and tumour cells themselves secrete a variety of autocrine motility factors including autotaxin and neuroleukin/phosphohexose isomerase.

Organ preference of metastases

The organ distribution of metastases depends on the type and location of the primary tumour, with 50-60% of the secondary sites being dictated by the anatomical route followed by the disseminating cells. Most metastases occur in the first capillary bed or lymph node encountered. The number of involved nodes is a key prognostic factor for many cancers, and this has led to efforts to identify "sentinel" lymph nodes in order to improve predictions of cancer spread. Lymphatic channels present less of a challenge to tumour cell entry than capillaries since they have scanty basement membrane. Once in the lymphatics, tumour cells are carried to the subcapsular sinus of draining nodes, where they may arrest and grow, succumb to host defences, or leave the node via the efferent lymphatics. The propensity for a tumour cell to generate a lymphatic metastasis may depend upon its ability to

TNM CLASSIFICATION OF MALIGNANT TUMOURS

The TNM system for the classification of malignant tumours (http://tnm.uicc.org/) is a form of clinical shorthand used to describe the anatomic extent (staging) of a cancer in terms of:
T - the primary tumour
N - regional lymph nodes
M - distant metastases
These components are given a number that reflects the absence or presence and extent of the disease. For example, a tumour of the colon that is classified as T2N1M0 would have extended into the colon's muscular wall, spread to 1 to 3 regional lymph nodes but without evidence of distant metastasis. Evaluation by the TNM system can therefore help in the planning of treatment by the oncologist and in monitoring the efficacy of this treatment, as well as giving some indication of prognosis. Moreover, the use of a standardized system facilitates the dissemination of information in the clinical community.
The TNM system was developed by Pierre Denoix (President of the UICC, 1973-1978) between 1943 and 1952 (Sobin LH, TNM – principles, history and relation to other prognostic factors. *Cancer*, 91:1589-92, 2001). In 1968, a series of brochures published by UICC describing the classification of cancers at 23 body sites were combined to produce the *Livre de Poche*, which has been subject to regular re-edition, enlargement and revision over subsequent years. In order to prevent unwanted variations in the classification by its users, in 1982 it was agreed that a single international TNM classification should be formulated. This is achieved via meetings of experts that update existing classifications, as well as develop new ones. The present TNM edition (Eds. Sobin LH and Wittekind Ch, *TNM Classification of Malignant Tumours, 6th Edition*, Wiley, 2002) contains guidelines for classification and staging that correspond exactly with those of the 6th edition of the *AJCC Cancer Staging Manual* (2002). TNM, now the most widely used system to classify tumour spread, is published in 12 languages and is accompanied by an illustrated *TNM Atlas* (Eds. Hermanek P et al., 4th Edition, Springer-Verlag, 1997), *a TNM Mobile Edition* (Wiley, 2002) and a TNM supplement (Eds. Wittekind Ch et al., *TNM Supplement 2001. A Commentary on Uniform Use, 2nd Edition*, Wiley 2001) with rules and explanations.
The challenge for the future is the incorporation into TNM of information from new diagnostic and imaging technologies (such as endoscopic ultrasound, magnetic resonance imaging, sentinel node biopsy, immunohistochemistry and polymerase chain reaction). There is an expanding array of known and potential prognostic factors (Eds. Gospodarowicz M et al., *Prognostic Factors in Cancer*, Wiley, 2001) with which TNM could be integrated to form a comprehensive prognostic system. Such integration could potentially be exploited to enhance the prediction of prognosis, and individualize cancer patient treatment.

T = primary tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues
T4	Tumour directly invades other organs or structures and/or perforates visceral peritoneum
N = regional lymph nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1 to 3 regional lymph nodes
N2	Metastasis in 4 or more regional lymph nodes
M = distant metastasis	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 3.8 TNM classification of cancer of the colon and rectum.

adhere to reticular fibres in the subcapsular sinus. These fibres contain laminin, fibronectin and collagen IV, and different integrins expressed by different tumour cells may be responsible for adhesion to these structures and to the lymphatic endothelial cells [11].
Sarcomas tend to metastasize to lungs because the venous drainage returns there; colon carcinoma cells enter the portal circulation which delivers cells to the liver, and so on (Fig. 3.45). However, a non-random element in metastatic patterns has long been recognized (Table 3.6). Stephen Paget developed the "seed and soil" hypothesis in 1889, based on his observations from autopsies of over 700 women with breast cancer. He proposed that specific cancer cells (the seed) had an affinity for certain organs (the soil).
In experimental systems there are many examples showing that primary tumours are heterogeneous, and that cloned cells can vary in their ability to metastasize to different sites. Some of the patterns relate to the ability of malignant cells to adhere to the endothelial cells in target

organs, and to respond to local growth factors once they have extravasated. It used to be thought that escape from the primary tumour and survival in the circulation were the major rate-limiting steps for successful metastasis. However, while there is a good deal of attrition at these stages, both in experimental models and in man, many tumour cells reach distant sites but may remain dormant, either due to lack of appropriate growth factors, or their failure to induce neoangiogenesis. Indeed, using sensitive assays such as immunocytochemistry and polymerase chain reaction (PCR), individual tumour cells (or specific genetic markers) can be found in blood, nodes, bone marrow, body fluids etc, but the significance of "positive" results, and whether they can be used to predict subsequent overt metastases is not yet established.

REFERENCES

1. Yoshida BA, Sokoloff MM, Welch DR, Rinker-Schaeffer CW (2000) Metastasis-suppressor genes: a review and perspective on an emerging field. *J Natl Cancer Inst*, 92: 1717-1730.

2. Simone NL, Paweletz CP, Charboneau L, Petricoin EF, Liotta LA (2000) Laser capture microdissection: Beyond functional genomics to proteomics. *Mol Diagn*, 5: 301-307.

3. Fidler IJ (2000) Angiogenesis and cancer metastasis. *Cancer J Sci Am*, 6 Suppl 2: S134-S141.

4. Eccles SA (2000) Cell biology of lymphatic metastasis. The potential role of c-erbB oncogene signalling. *Recent Results Cancer Res*, 157: 41-54.

5. Fidler IJ (1999) Critical determinants of cancer metastasis: rationale for therapy. *Cancer Chemother Pharmacol*, 43 Suppl: S3-10.

6. Stracke ML, Liotta LA (1992) Multi-step cascade of tumor cell metastasis. *In Vivo*, 6: 309-316.

7. Berman AE, Kozlova NI (2000) Integrins: structure and functions. *Membr Cell Biol*, 13: 207-244.

8. Ridley A (2000) Molecular switches in metastasis. *Nature*, 406: 466-467.

9. McCawley LJ, Matrisian LM (2000) Matrix metalloproteinases: multifunctional contributors to tumor progression. *Mol Med Today*, 6: 149-156.

10. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol*, 2: 737-744.

11. Brodt P (1991) Adhesion mechanisms in lymphatic metastasis. *Cancer Metastasis Rev*, 10: 23-32.

WEBSITE

The Metastasis Research Society (UK):
http://www.metastasis.icr.ac.uk