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Chemical Carcinogenesis

Ainsley Weston, PhD
Curtis C. Harris, MD

Human chemical carcinogenesis is a multistage process that results from exposures, usually in the form of complex chemical mixtures, often encountered in the environment or through our lifestyle and diet (Table 14-1).[1–4] A prime example is tobacco smoke, which can cause cancers at multiple sites including the lung, the bladder, and the head and neck.[5–7] Although most chemical carcinogens do not react directly with intracellular components, they are activated to carcinogenic and mutagenic electrophiles by metabolic processes evolutionarily designed to rid the body of toxins and to modify endogenous compounds. Electrophilic chemical species are naturally attracted to nucleophiles like deoxyribonucleic acid (DNA) and protein, and through covalent bonding to DNA genetic damage results. Once internalized, carcinogens are subject to competing processes of metabolic activation and detoxification, although some chemical species can act directly. There is considerable variation among the human population in these competing metabolic processes, as well as the capacity for repair of DNA damage and cellular growth control. This is the basis for interindividual variation in cancer risk, and is a reflection of gene–environment interactions, which embodies the concept that heritable traits modify the effects of chemical carcinogen exposure.[8] Such variations in constitutive metabolism and DNA repair contribute to the relative susceptibility of individual members of the population to chemical exposures. For example, only 10% of tobacco smokers develop lung cancer, albeit that tobacco use accounts for other fatal conditions, including emphysema, chronic obstructive pulmonary disease, stroke, and heart disease. Within the conceptual framework of multistage carcinogenesis, the primary genetic change that results from a chemical–DNA interaction is termed *tumor initiation*.[9,10] Thus, initiated cells are irreversibly altered and are at a greater risk of malignant conversion than are normal cells. The epigenetic effects of tumor promoters facilitate the clonal expansion of the initiated cells.[10] Selective, clonal growth advantage causes a focus of preneoplastic cells to form. These cells are more vulnerable to tumorigenesis because they now present a larger, more rapidly proliferating, target population for the further action of chemical carcinogens, oncogenic viruses, and other cofactors. Additional genetic changes continue to accumulate. The activation of oncogenes, and the inactivation of tumor suppressor and DNA-repair genes, leads to genomic instability or the so called *mutator phenotype* and an acceleration in the genetic changes taking place.[11,12] This scenario is followed by malignant conversion, tumor progression, and metastasis. The underlying molecular mechanisms that govern chemical carcinogenesis are becoming increasingly understood, and the insights generated are assisting in the development of better methods to investigate human cancer risk and susceptibility.[13] The results of such studies are intended to mold strategies for prevention and intervention. Moreover, insights into the normal operations of so called *gatekeeper* genes,[14] like the tumor suppressor *TP53*, have provided an opportunity to develop new, targeted, therapeutic approaches.[15]

MULTISTAGE CARCINOGENESIS

Carcinogenesis can be divided conceptually into four steps: tumor initiation, tumor promotion, malignant conversion, and tumor progression (Figure 14-1). The distinction between initiation and promotion was recognized through studies involving both viruses and chemical carcinogens.[9,16] This distinction was formally defined in a murine skin carcinogenesis model in which mice were treated topically with a single dose of a polycyclic aromatic hydrocarbon (ie, initiator), followed by repeated topical doses of croton oil (ie, promoter),[9] and this model has been expanded to a range of other rodent tissues, including bladder, colon, esophagus, liver, lung, mammary gland, stomach, and trachea.[17] During the last 50 years, the sequence of events comprising chemical carcinogenesis has been systematically dissected and the paradigm increasingly refined, and both similarities and differences between rodent and human carcinogenesis have been identified.[18,19] Carcinogenesis requires the malignant conversion of benign hyperplastic cells to a malignant state, and invasion and metastasis are manifestations of further genetic and epigenetic changes.[20–22] The study of this process in humans is necessarily indirect and uses information from lifestyle or occupational exposures to chemical carcinogens. Measures of age-dependent cancer incidence have shown, however, that the rate of tumor development is proportional to the sixth power of time, suggesting that at least four to six independent steps are necessary.[23] Partial scheduling of specific genetic events in this process has been possible for some cancers. Exam-

Table 14-1 Selected Examples of Human Chemical Carcinogenesis

[illegible]	[illegible]	[illegible]
Lung	Metals: As, Be, Cd, Cr, Ni	—
	BCME	—
(Small cell and squamous cell)	Tobacco smoke	Asbestos
	Diesel exhaust	—
Pleural mesothelium	Asbestos	—
Oral cavity	Smokeless tobacco	—
	Betel quid	Slaked lime [$Ca(OH)_2$]
Esophagus	Tobacco smoke	Alcohol
Nasal sinuses	Snuff	Powdered glass
	Isopropyl alcohol	—
Skin (scrotum)	Cutting oil	—
	Coal soot*	—
Liver (angiosarcoma)	Aflatoxin B_1	HBV, HCB
	Vinyl chloride	Alcohol
Bladder	Aromatic amines (eg, 4-ABP and benzidine)	—
	Aromatic amines from tobacco smoke†	—
ALL	Benzene	—
Lymphatic and hemapoietic malignancies	Ethylene oxide	—

4-ABP = 4-aminobiphenyl; ALL = acute lymphoblastic leukemia; BCME = bischloromethyl ether; HBV = hepatitis B virus; HCV = hepatitis C virus.

*Early report of occupational chemical carcinogenesis from 225 years ago.[1]

†Strong circumstantial evidence.[150]

A comprehensive treatise on the evaluation of the carcinogenic risk of chemicals to humans can be found in the ongoing International Agency for Research on Cancer monograph program initiated in 1971.[4]

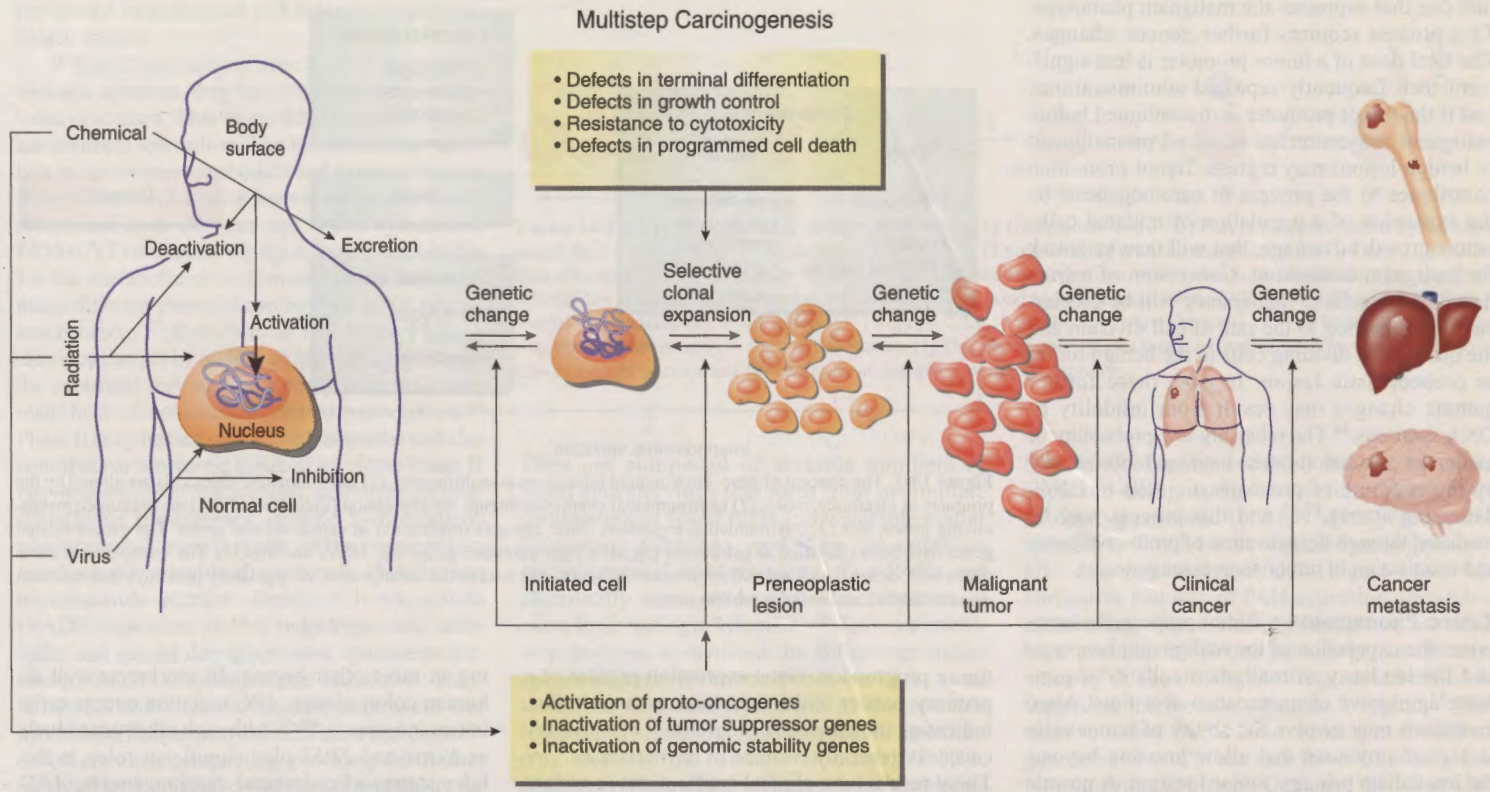

Figure 14-1 Multistage chemical carcinogenesis can be conceptually divided into four stages: tumor initiation, tumor promotion, malignant conversion, and tumor progression. The activation of proto-oncogenes and inactivation of tumor suppressor genes are mutational events that result from covalent damage to DNA caused by chemical exposures. The accumulation of mutations, and not necessarily the order in which they occur, constitutes multistage carcinogenesis.[26,99]

ples of sequential genetic and epigenetic changes that occur with the highest probability are those found in the development of lung cancer[24,25] and colon cancer.[26]

Tumor Initiation The early concept of tumor initiation indicated that the initial changes in chemical carcinogenesis are irreversible genetic damage. However, recent data from molecular studies of preneoplastic human lung and colon tissues implicate epigenetic changes as an early event in carcinogenesis. DNA methylation of promoter regions of genes can transcriptionally silence tumor suppressor genes.[22] For mutations to accumulate, they must arise in cells that proliferate and survive the lifetime of the organism. A chemical carcinogen causes a genetic error by modifying the molecular structure of DNA that can lead to a mutation during DNA synthesis. Most often, this is brought about by forming an adduct between the chemical carcinogen or one of its functional groups and a nucleotide in DNA[17] (the process by which this occurs for the major classes of chemical carcinogens is discussed in detail under "Carcinogen Metabolism"). In general, a positive correlation is found between the amount of carcinogen–DNA adducts that can be detected in animal models and the number of tumors that develop.[27–29] Thus, tumors rarely develop in tissues that do not form carcinogen–DNA adducts. Carcinogen–DNA adduct formation is central to theories of chemical carcinogenesis, and it may be a necessary, but not a sufficient, prerequisite for tumor initiation (the concept of so called nongenotoxic carcinogens is also explored under "Carcinogen Metabolism"). DNA adduct formation that causes either the activation of a proto-oncogene or the inactivation of a tumor suppressor gene can be categorized as a tumor-initiating event (see "Tumor Progression," "Oncogenes and Tumor Suppressor Genes" in this chapter).

Tumor Promotion Tumor promotion comprises the selective clonal expansion of initiated cells. Because the accumulation rate of mutations is proportional to the rate of cell division, or at least the rate at which stem cells are replaced, clonal expansion of initiated cells, produces a larger population of cells that are at risk of further genetic changes and malignant conversion.[20,25,26] Tumor promoters are generally nonmutagenic, are not carcinogenic alone, and often (but not always) are able to mediate their biologic effects without metabolic activation. These agents are characterized by their ability to reduce the latency period for tumor formation after exposure of a tissue to a tumor initiator, or to increase the number of tumors formed in that tissue. In addition, they induce tumor formation in conjunction with a dose of an initiator that is too low to be carcinogenic alone. Croton oil (isolated from *Croton tiglium* seeds) is used widely as a tumor promoter in murine skin carcinogenesis, and the mechanism of action for its most potent constituent, 12-*O*-tetradecanoylphorbol-13-acetate, which occurs via protein kinase C activation, is arguably the best understood among tumor promoters.[30] Chemicals or agents capable of both tumor initiation and promotion are known as complete carcinogens, eg, benzo[*a*]pyrene and 4-aminobiphenyl. Identification of new tumor promoters in animal models has accelerated with the sophisticated development of model systems designed to assay for tumor promotion. Furthermore, ligand binding properties can be determined in recombinant protein kinase C isozymes that are expressed in cell cultures.[31] Chemicals, complex mixtures of chemicals, or other agents that have been shown to have tumor-promoting properties include dioxin, benzoyl peroxide, macrocyclic lactones, bromomethylbenzanthracene, anthralin, phenol, saccharin, tryptophan, dichlorodiphenyltrichloroethane (DDT), phenobarbital, cigarette-smoke condensate, polychlorinated biphenyls (PCBs), teleocidins, cyclamates, estrogens and other hormones, bile acids, ultraviolet light, wounding, abrasion, and other chronic irritation (ie, saline lavage).[17] In addition, protein kinase C is activated and cellular diacylglycerol elevated in laboratory animals maintained on high-fat diets.[32,33]

Malignant Conversion Malignant conversion is the transformation of a preneoplastic cell

into one that expresses the malignant phenotype. This process requires further genetic changes. The total dose of a tumor promoter is less significant than frequently repeated administrations, and if the tumor promoter is discontinued before malignant conversion has occurred, premalignant or benign lesions may regress. Tumor promotion contributes to the process of carcinogenesis by the expansion of a population of initiated cells, with a growth advantage, that will then be at risk for malignant conversion. Conversion of a fraction of these cells to malignancy will be accelerated in proportion to the rate of cell division and the quantity of dividing cells in the benign tumor or preneoplastic lesion. In part, these further genetic changes may result from infidelity of DNA synthesis.[34] The relatively low probability of malignant conversion can be increased substantially by the exposure of preneoplastic cells to DNA-damaging agents,[17,35] and this process may be mediated through the activation of proto-oncogenes and inactivation of tumor suppressor genes.

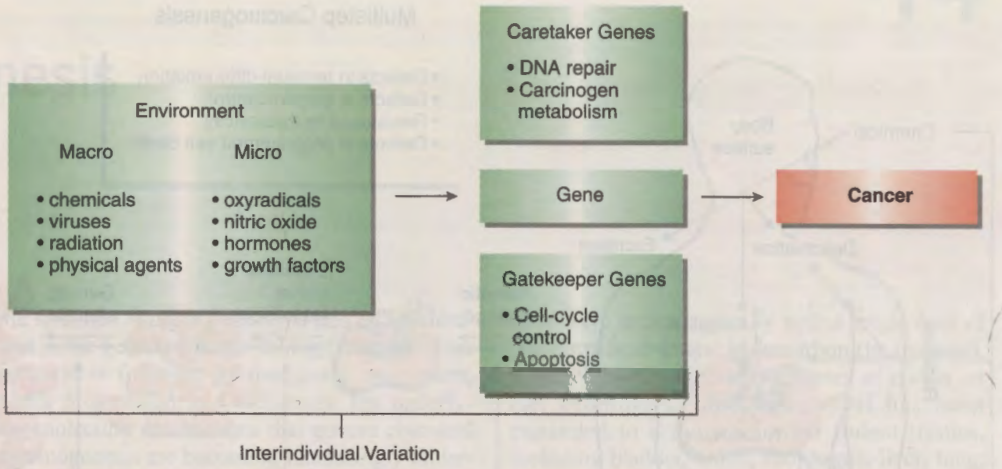

Figure 14-2 The concept of gene–environment interaction is multifaceted: (1) environmental chemicals are altered by the products of metabolic genes; (2) environmental chemicals disrupt the expression (induce or inhibit) of carcinogen metabolizing genes; and (3) environmental exposures cause changes (mutations) in cancer-related genes. The cancer-related genes have been classified as gatekeeper (eg, *APC*) and caretaker genes (eg, *MSH1* and *MLH1*). The interaction of these genes with external and internal environmental agents can lead to the derangement of regulatory pathways that maintain genetic stability and cellular proliferation.

TUMOR PROGRESSION Tumor progression comprises the expression of the malignant phenotype and the tendency of malignant cells to acquire more aggressive characteristics over time. Also, metastasis may involve the ability of tumor cells to secrete proteases that allow invasion beyond the immediate primary tumor location. A prominent characteristic of the malignant phenotype is the propensity for genomic instability and uncontrolled growth.[36] During this process, further genetic and epigenetic changes can occur, again including the activation of proto-oncogenes and the functional loss of tumor suppressor genes. Frequently, proto-oncogenes are activated by two major mechanisms: in the case of the *ras* gene family, point mutations are found in highly specific regions of the gene (ie, the twelfth, thirteenth, fifty-ninth, or sixty-first codons), and members of the *myc*, *raf*, *HER2*, and *jun* multigene families can be overexpressed, sometimes involving amplification of chromosomal segments containing these genes. Some genes are overexpressed if they are translocated and become juxtaposed to a powerful promoter (eg, the relationship of *bcl-2* and immunoglobulin heavy chain gene promoter regions in B-cell malignancies; see also Philadelphia chromosome, under "Clonal Evolution"). Loss of function of tumor suppressor genes usually occurs in a bimodal fashion, and most frequently involves point mutations in one allele and loss of the second allele by a deletion, recombinational event, or chromosomal nondisjunction. These phenomena confer to the cells a growth advantage as well as the capacity for regional invasion, and ultimately, distant metastatic spread. Despite evidence for an apparent scheduling of certain mutational events, it is the accumulation of these mutations, and not the order or the stage of tumorigenesis in which they occur, that appears to be the determining factor.[24–26] Recent evidence from microarray expression analysis of human cancers supports an alternative, and not mutually exclusive, mode of tumor progression. Gene expression profiles of a primary cancer and its metastases are similar, indicating the molecular progression of a primary cancer is generally retained in its metastases.[37,38] These results have clinical implications in molecular diagnosis of primary cancers and therapeutic strategies.

GENE-ENVIRONMENT INTERACTIONS AND INTERINDIVIDUAL VARIATION

A cornerstone of human chemical carcinogenesis is the concept of gene-environment interactions (Figure 14-2).[8] Potential interindividual susceptibility to chemical carcinogenesis may well be defined by genetic variations in the host elements of this compound system. Functional polymorphisms in human proteins that have, or may have, a role in chemical carcinogenesis include enzymes that metabolize (ie, activate and detoxify) xenobiotic substances, enzymes that repair DNA damage, cell surface receptors that activate the phosphorylation cascade and cell cycle control genes (ie, oncogenes and tumor suppressor genes that are elements of the signal transduction cascade).

CARETAKER AND GATEKEEPER GENES Gatekeeper and caretaker genes are characterized by their control of net cellular proliferation or maintenance of genomic integrity, respectively.[15,39] Examples of gatekeeper genes include *APC* and β-*catenin* in colon epithelial cells, *Rb* in retinal epithelial cells, *NF1* in Schwann cells, and *VHL* in kidney cells. The most prominent example of a gatekeeper is the *APC* gene in colorectal cancer. An alteration in *APC* may lead to a derangement of the cellular proliferation pathway, which is necessary for maintaining a constant cell population. However, this function of *APC* has a high specificity for colonic epithelial cells, but is lacking in most other organs. In murine as well as human colon cancer, *APC* mutation occurs early in carcinogenesis.[40–42] Although other genes such as *K-ras* and *TP53* play significant roles in the later stages of colorectal carcinogenesis, *APC* mutation, and the less-common β-*catenin* mutations, are essential events in neoplastic initiation (for a review, see Kinzler KW and Vogelstein B[39]). If this concept of a gatekeeper pathway holds true for the initiation of neoplasia in general, then the identification of other gatekeeper genes can be anticipated.

Unlike gatekeeper genes, caretaker genes generally maintain genomic stability and are not involved directly in neoplastic initiation. Genetic instability caused by mutations in caretaker genes enhances the probability of mutation in other genes, including those in the gatekeeper pathway. Mismatch DNA-repair genes, such as *MSH2* and *MLH1*, are caretaker genes, and abnormalities in these genes enhance genomic instability and increase the risk of human colon cancer. Based on this knowledge of human colon carcinogenesis, animal models have been developed.[43–45] Similar in vivo, as well as in vitro models, should be developed for carcinogenesis in other tissues. Breast cancer-susceptibility genes, *BRCA1* and *BRCA2*, are also included in the list of caretaker genes.[14] The same report suggested that an individual who inherits a mutated caretaker gene is at a lower risk of cancer as compared with an individual who inherits a mutated gatekeeper gene. This difference is attributed to the finding that three or more additional somatic mutations are required to initiate neoplasia in the caretaker pathway, versus only one additional somatic mutation that is required to initiate neoplasia in the gatekeeper pathway. The analysis of potentially preneoplastic lesions for mutations in gatekeeper and caretaker genes could provide short-term and less-expensive pathobiologic end points

for hazard identification and molecular epidemiologic studies.

When chemicals or xenobiotics encounter biologic systems, they become altered by metabolic processes. This is an initial facet of gene–environment interaction. The interindividual variation in carcinogen metabolism and macromolecular adduct formation arising from such processes was recognized 25 to 30 years ago.[46] The cytochrome P450 (CYP) multigene family is largely responsible for the metabolic activation and detoxication of many different chemical carcinogens in the human environment.[47] Cytochrome P450s are Phase I enzymes that act by adding an atom of oxygen onto the substrate; they are induced by polycyclic aromatic hydrocarbons and chlorinated hydrocarbons.[48] Phase II enzymes act on oxidized substrates and also contribute to xenobiotic metabolism. Some Phase II enzymes are methyltransferases; acetyltransferases; glutathione transferases; uridine 5'-diphosphoglucuronosyl transferases; sulfotransferases; nicotinamide adenine dinucleotide (NAD)- and nicotinamide adenine dinucleotide phosphate (NADP)-dependent alcohol dehydrogenases; aldehyde; and steroid dehydrogenases; quinone reductases; NADPH diaphorase; azo reductases; aldoketoreductases; transaminases; esterases; and hydrolases. The pathways of activation and detoxification are often competitive, providing yet further potential for individual differences in propensity for carcinogen metabolism to DNA damaging species.

This scenario is further complicated by a second facet of gene–environment interaction that leads to enzyme induction or inhibition. In this case, environmental exposures alter gene expression, and genes responsible for carcinogen metabolism can be upregulated or repressed by certain chemical exposures.

A third facet of gene–environment interaction occurs when the chemical alters gene structure. Once a procarcinogen is metabolically activated to an ultimate carcinogenic form, it can bind covalently to cellular macromolecules, including DNA. This DNA damage can be repaired by several mechanisms.[49,50] Differences in rates and fidelity of DNA repair potentially influence the extent of carcinogen adduct formation (ie, biologically effective dose) and, consequently, the total amount of genetic damage. The consequences of polymorphisms in genes controlling the cell cycle (serine/threonine kinases, transcription factors, cyclins, cyclin-dependent kinase inhibitors, and cell surface receptors) are much less clear. However, molecular epidemiologic evidence suggests that certain common variants of these types of genes have a role in susceptibility to chemical carcinogenesis.[51,52] The evaluation of polymorphisms as potential biomarkers of susceptibility in the human population is discussed under "Implications for Molecular Epidemiology, Risk Assessment, and Cancer Prevention."

CARCINOGEN METABOLISM

The first chemically identified carcinogens were the polycyclic aromatic hydrocarbons (PAHs).[7,53,54] They are composed of variable numbers of fused benzene rings that form from incomplete combustion of fossil fuels and vegetable matter (including tobacco), and they are common environmental contaminants. The PAHs are chemically inert, and require metabolism to exert their biologic effects.[55,56] This is a multistep process, it involves the following: initial epoxidation (cytochrome P450, CYP1A1 is an inducible isoform), hydration of the epoxide (epoxide hydrolase), and subsequent epoxidation across the olefinic bond (CYP1B1; CYP3A4) (Figure 14-3). The result is the ultimate carcinogenic metabolite, a diolepoxide.[57,58] The biology of CYP1A1 metabolism has been elucidated providing a molecular basis for inducibility and interindividual variation,[46,59,60] and variations in cytochrome levels among humans have been documented.[61]

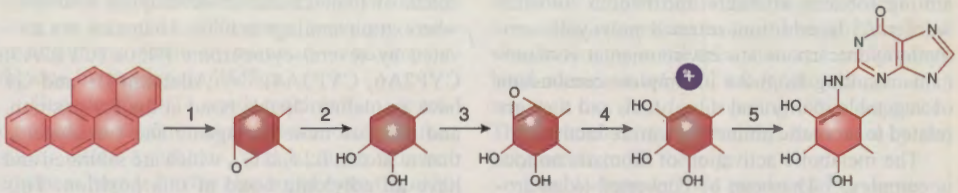

Figure 14-3 Metabolic activation of benzol[*a*]pyrene. (1) Cytochrome P450 (CYP1A1) catalyses initial epoxidation across the 1 - 2, 2 - 3, 4 - 5, 7 - 8 (shown), 9 - 10 and 11 - 12 positions. (2) With the exception of the 1 - 2 and 2 - 3 oxides that convert to phenols, epoxide hydrolase may catalyze the formation of dihydrodiols. (3) Benzo[*a*]pyrene-7, 8-dihydrodiol is further metabolized at the olefinic double bond by cytochrome P450 (CYP1B1 and CYP3A4) to form a vicinal diol-epoxide (r7, t8-dihydroxy-c9, 10 epoxy-7,8,9,10-tetrahydroxybenz[*a*]pyrene). (4) The highly unstable arene ring opens spontaneously to form a carbocation. (5) This electrophic species forms a covalent bond between the 10 position of the hydrocarbon and the exocyclic amino group of deoxyguanosine.

The arene ring of benzo[*a*]pyrene-7,8-diol 9,10-oxide opens spontaneously at the 10 position, giving a highly reactive carbonium ion that can form a covalent addition product (ie, adduct) with cellular macromolecules, including DNA. Several DNA-adducts can be formed, the most abundant being at the exocyclic amino group of deoxyguanosine ([7R]-N^2-[10-{7β,8α,9α-trihydroxy-7,8,9,10-tetrahydro-benz[*a*]pyrene} yl] - deoxyguanosine; BPdG) (Figure 14-4). An alternative pathway of PAH activation, through a mechanism of one electron oxidation, has also been postulated.[62] When benzo[*a*]pyrene is activated by this route, the resulting radical cation is formed at the *meso* position or L-region.[58] The reactive cation forms DNA adducts at the C8 of guanine (BP-6-C8Gua and BP-6-C8dGua), the N7 of guanine (BP-6- N7Gua), and the N7 of adenine (BP-6-N7Ade) (see Figure 14-4). These adducts place strain on the *N*-glycosyl link to the helical backbone and depurination results. Firm evidence for the exfoliation of these adducts in urine was provided recently for exposure scenarios that included coal and tobacco smoke.[63]

Aromatic amines are another class of chemical carcinogens found in cigarette smoke, diesel exhaust, industrial environments and certain cooked foods. The compound, 4-aminobiphenyl, is thought to be responsible for bladder cancer

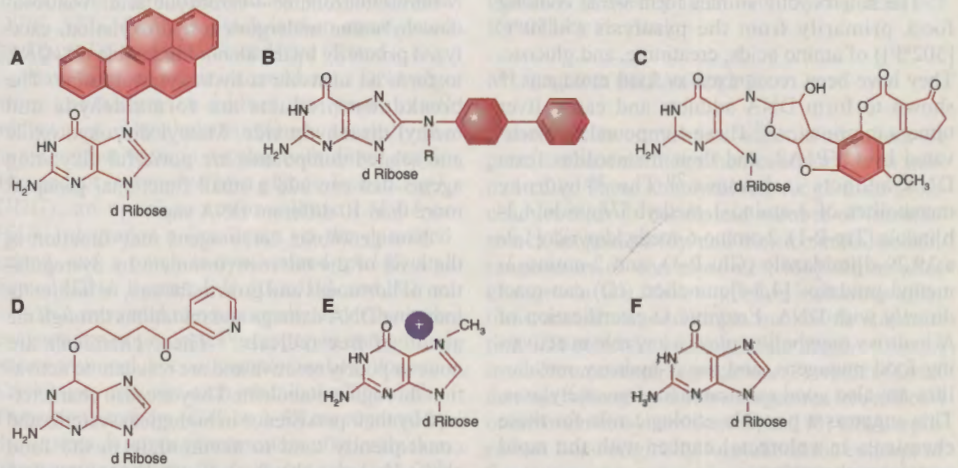

Figure 14-4 Examples of carcinogen–DNA adducts: **A,** N7(benzo[*a*]pyren-6-yl)guanine; **B,** *N*-(deoxyguanosin-8-yl)-{acetyl}aminobiphenyl (when R= H the adduct is not acetylated [R can also be an acetyl group]); **C,** 8,9-dihydro-8-(N5-formyl- 2', 5', 6'-triamino-4'-oxo-N5-pyrimidyl)-9-hydroxy-aflatoxin B1; **D,** O6-[4-Oxo-4(3-pyridyl)butyl]guanine, a mutagenic lesion formed by the metabolism of the tobacco-specific nitrosamine, NNK[73,74]; **E,** N7-methyldeoxyguanosine; and **F,** 3-methyladenosine. Adducts **E,** and **F,** can also result as the small alkyl products of NNK metabolism.[73,74]

among tobacco smokers and rubber industry workers.[64] In addition, nitrated polycyclic aromatic hydrocarbons are environmental contaminants resulting from the incomplete combustion of vegetable matter and diesel fuel, and they are related to aromatic amines by nitroreduction.

The metabolic activation of aromatic amines is complex.[65] They can be converted to an aromatic amide that is catalyzed by an acetyl coenzyme A-dependent acetylation. The acetylation phenotype varies among the population. Persons with the rapid acetylator phenotype are at a higher risk of colon cancer, whereas, those who are slow acetylators are at risk of bladder cancer.[66] This latter association may result from the fact that activation of aromatic amines by *N*-oxidation is a competing pathway for aromatic amine metabolism. Also, the *N*-hydroxylation products when protonated (by acid conditions in the urinary bladder) form reactive electrophiles that bind covalently with DNA or proteins to produce macromolecular damage.

An initial activation step for both aromatic amines and amides is *N*-oxidation by CYP1A2. *CYP1A2* is inducible by phenobarbital, and because it is also responsible for the 3-demethylation of 1,3,7-trimethylxanthine (ie, caffeine), *CYP1A2* phenotype can be determined using this as a probe drug.[67] The reactions of *N*-hydroxyarylamines with DNA appear to be acid catalyzed, but they can be further activated by either an acetyl coenzyme A-dependent O-acetylase or a 3'-phosphoadenosine-5'phosphosulfate-dependent O-sulfotransferase. The *N*-arylhydroxamic acids arise from the acetylation of *N*-hydroxyarylamines or *N*-hydroxylation of aromatic amides; they are not electrophilic and require further activation. The predominant pathway for this occurs through the acetyltransferase-catalyzed rearrangement to a reactive *N*-acetoxyarylamine. Sulfotransferase catalysis forms *N*-sulphonyloxy arylamides. This complex pathway results in two major adduct types, amides (ie, acetylated) and amines (ie, nonacetylated).

The heterocyclic amines form while cooking food, primarily from the pyrolysis (>150°C [302°F]) of amino acids, creatinine, and glucose. They have been recognized as food mutagens,[68] shown to form DNA adducts and cause liver tumors in primates.[69] These compounds are activated by CYP1A2, and their metabolites form DNA adducts in humans.[70] The *N*-hydroxy metabolites of 3-amino-1-methyl-5*H*pyrido[4,3-b]indole (Trp-P-1), 2-amino-6-methyldipyrido[1,2-a:39,29-d]imidazole (Glu-P-1), and 2-amino-3-methyl-imidazo- [4,5-f]quinoline (IQ) can react directly with DNA. Enzymic O-esterification of *N*-hydroxy metabolites plays a key role in activating food mutagens, and the *N*-hydroxy metabolites are also good substrates for transacetylases. This suggests a possible etiologic role for these chemicals in colorectal cancer with the rapid acetylator phenotype.

Aflatoxins (B1, B2, G1, and G2) are metabolites of *Aspergillus flavus* that contaminate cereals, grain, and nuts. A positive correlation exists between dietary aflatoxin exposure and the incidence of liver cancer in developing countries, where grain spoilage is high. Aflatoxins are activated by several cytochrome P450s (CYP2A3; CYP2A6; CYP3A4).[47,71] Aflatoxin B1 and G1 have an olefinic double bond at the 8,9-position, and they are more mutagenic and carcinogenic than aflatoxin B2 and G2, which are saturated and have an ethylenic bond at this position. This implies that the olefinic 8,9-bond is the activation site. Further support for this mechanism comes from studies of DNA-adducts and the prevalence of *TP53* mutations in liver cancer. In people with liver cancer from parts of China and Africa, where food spoilage caused by molds is high, G:C to T:A transversions in codon 249 are frequent.[72] This phenomenon is consistent with metabolic activation of aflatoxin B1 and the formation of depurinating carcinogen–deoxyguanosine adducts.

Carcinogenic *N*-nitrosamines are ubiquitous environmental contaminants and can be found in food, alcoholic beverages, cosmetics, cutting oils, hydraulic fluid, rubber, and tobacco.[73] Tobacco-specific *N*-nitrosamines, such as 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone, are carcinogenic in a wide range of animal species. Unlike exposure to many other carcinogens associated with tobacco use, exposure to tobacco-specific *N*-nitrosamines does not require pyrolysis, therefore they may account for the carcinogenic nature of snuff and chewing tobacco.[74] The tobacco-specific nitrosamines are not symmetric so both small alkyl-adducts and large bulky adducts can be formed; for example, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone metabolism gives rise to either a positively charged pyridyl-oxobutyl ion or a positively charged methyl ion, both of which are able to alkylate DNA.[73,74] Endogenous nitrosation forms nitrosamines when an amine reacts with nitrate alone or nitrite in the presence of acid. Thus, nitrite (used in curing meats) and L-cysteine, in the presence of acetaldehyde (a metabolite of alcohol), form *N*-nitrosothiazolidine-4-carboxylic acid. *N*-nitrosodimethylamine undergoes α-hydroxylation, catalyzed primarily by the alcohol inducible *CYP2E1*, to form an unstable α-hydroxynitrosamine. The breakdown products are formaldehyde and methyl diazohydroxide. Methyl diazohydroxide and related compounds are powerful alkylating agents that can add a small functional group at more than 10 different DNA sites.

Non-genotoxic carcinogens may function at the level of the microenvironment by dysregulation of hormones and growth factors, or indirectly inducing DNA damage and mutations through the action of free radicals.[75] These chemicals are none or poorly reactive and are resistant to activation through metabolism. They are also characterized by their persistence in biological systems and consequently tend to accumulate in the food chain. However, they have the ability to stimulate oxyradical formation. There are at least three mechanisms by which this may occur: in the case of persistent organochlorine species, interaction with the Ah receptor could lead to cytochrome P450 induction, similar to PAHs, and associated oxyradical formation; or interaction with other receptors, like IFN-γ, would stimulate elements of the primary immune response and again generate oxyradicals; agents like asbestos would promote oxyradical formation through interaction with ferrous metal and Fenton chemistry. Oxyradicals generated by any one of these mechanisms can then damage DNA. Some of the so called "nongenotoxic" carcinogens might more appropriately be considered to be "oxyradical triggers." Indeed, chronic inflammatory states, which involve oxyradical formation, can also be cancer risk factors.[76]

DNA DAMAGE AND REPAIR

The chemical structure of DNA can be altered by a carcinogen in several ways: the formation of bulky aromatic-type adducts, alkylation (generally small adducts), oxidation, dimerization, and deamination. In addition, double- and single- strand breaks can occur. Chemical carcinogens can cause epigenetic changes, such as altering the DNA methylation status that leads to the silencing of specific gene expression.[77] A complex pattern of carcinogen–DNA adducts likely results from exposure to tobacco smoke, because of the mixture of different chemical carcinogens present.[78]

r7, t8-dihydroxy-c9, 10 epoxy-7,8,9,10-tetrahydroxybenzo[*a*]pyrene (benzo[*a*]pyrene-7,8-diol 9,10-epoxide, BPDE) reacts with the exocyclic (N2) amino group of deoxyguanosine and resides within the minor groove of the double helix; it is typical of polycyclic aromatic hydrocarbons (see Figures 14-3 and 14-4). This adduct, BPdG, appears to be the most common, persistent adduct of benzo[*a*]pyrene in mammalian systems, but others are possible. Some metabolites bind covalently with deoxyadenosine, and proapurinic adducts form through one electron oxidation (see Figure 14-4). This type of adduct, BPdG and others, is thought to induce *ras* gene mutations, which are common in tobacco-smoking-related lung cancers.[29,63,79,80] Aromatic amine adducts are more complex, because they have both acetylated and nonacetylated metabolic intermediates, and they form covalent bonds at the C8, N2, and sometimes O6 positions of deoxyguanosine as well as deoxyadenosine. The major adducts, however, are C8-deoxyguanosine adducts, which reside predominantly in the major groove of the DNA double helix (see Figure 14-4).[65]

Aflatoxin B1 and G1 activation occurs through hydroxylation of the olefinic 8,9-position and adducts are formed at the N7-position of deoxyguanosine. They are relatively unstable and have a half-life of approximately 50 h at neutral pH; depurination products have been detected in rat and human urine.[81] The aflatoxin B1-N7-deoxyguanosine adduct also can undergo ring opening to yield two pyrimidine adducts; alternately, aflatoxin B1-8,9-dihydrodiol could result. This latter possibility could restore the DNA molecular structure if hydrolysis of the original adduct occurs, but a potentially promutagenic

lesion would result if the 8,9-dihydrodiol results from degradation of open-ring adduct forms.[82]

DNA alkylation can occur at many sites either following the metabolic activation of certain *N*-nitrosamines, or directly by the action of the *N*-alkylureas (*N*-methyl-*N*-nitrosourea) or the *N*-nitrosoguanidines. The protonated alkyl-functional groups that become available to form lesions in DNA generally attack the following nucleophilic centers: adenine (N1, N3, and N7), cytosine (N3), guanine (N2, O6, and N7), and thymine (O2, N3, and O4). Some of these lesions are known to be repaired (O6-methyldeoxyguanosine), while others are not (N7-methyldeoxyguanosine).[73,74] Furthermore, O6-methyldeoxyguanosine is a promutagenic lesion, whereas N7-methyldeoxyguanosine is not.

Oxyradical damage can form thymine glycol or 8-hydroxydeoxyguanosine adducts. Exposure to organic peroxides (catechol, hydroquinone, and 4-nitroquinoline-*N*-oxide) leads to oxyradical damage; however, oxyradicals and hydrogen peroxide can be generated in lipid peroxidation and the catalytic cycling of some enzymes.[83] Tobacco smoke is also a source of oxyradicals, and by inducing an inflammatory response, tobacco smoke can contribute to diseases such as asthma and chronic heart disease, as well as cancer.[76] Also, certain drugs and plasticizers can stimulate cells to produce peroxisomes.[84] In addition, increased oxyradical formation is mediated through protein kinase C when inflammatory cells are exposed to tumor promoters like phorbol esters.[85]

Another potentially mutagenic cause of DNA damage is the deamination of DNA-methylated cytosine residues. 5-Methylcytosine comprises approximately 3% of deoxynucleotides. In this case, deamination at a CpG dinucleotide gives rise to a TpG mismatch. Repair of this lesion most often restores the CpG; however, repair may cause a mutation (TpA).[86] Deamination of cytosine also can generate a C to T transition if uracil glycosylation and G-T mismatch repair are inefficient. Oxyradicals can enhance the deamination rate, so the activity of inducible nitric oxide synthase and production of high concentrations of nitric oxide could contribute to DNA damage by this mechanism.[87]

Maintenance of genome integrity requires abrogation of DNA damage, and diminished DNA-repair capacity is associated with carcinogenesis, birth defects, premature aging, and foreshortened life-span. DNA-repair enzymes act at DNA-damage sites caused by chemical carcinogens, and six major mechanisms are known: direct DNA repair, nucleotide excision repair, base excision repair, nonhomologous end joining (doublestrand break repair), mismatch repair, and homologous recombination (postreplication repair).[50,88]

In the presence of nonlethal DNA damage, cell-cycle progression is postponed for repair mechanisms. This highly coordinated process involves multiple genes. A DNA-damage recognition sensor triggers a signal transduction cascade and downstream factors direct G1 and G2 arrest in concert with the proteins operationally responsible for the repair process. Although there are at least six discrete repair mechanisms, within five of them there are numerous multiprotein complexes comprising all the machinery necessary to accomplish the step-by-step repair function.

Generically, DNA repair requires damage recognition, damage removal or excision, resynthesis or patch synthesis, and ligation. Recent advances have led to the cloning of more than 130 human genes involved in five of these DNA-repair pathways. A list of these genes and their specific functions was published elsewhere.[89] These genes are responsible for the fidelity of DNA repair, and when they are defective the mutation rate increases. This is the mutator phenotype.[34] Mutations in at least 30 DNA-repair-associated genes have been linked to increased cancer susceptibility or premature aging (Table 14-2).[89] Moreover, the role of common polymorphisms in some of these genes are associated with increased susceptibility in a gene–environment interaction scenario (this is discussed under "Implications for Molecular Epidemiology, Risk Assessment, and Cancer Prevention"). Indeed, molecular epidemiologic evidence suggests that tobacco-smoking-related lung cancer is associated with a polymorphism in the nucleotide excision repair gene, *XPC* (*ERCC2*).[90]

Direct DNA repair is effected by DNA alkyltransferases. These enzymes catalyze translocation of the alkyl moiety from an alkylated base (eg, O^6-methyldeoxyguanosine) to a cysteine residue at their active site in the absence of DNA strand scission. Thus, one molecule of the enzyme is capable of repairing one DNA alkyl lesion, in a suicide mechanism. The inactivation of this mechanism by promoter hypermethylation is associated with *K-ras* G to A mutations in colon cancer.[91]

In DNA nucleotide excision repair, lesion recognition, preincision, incision, gap-filling, and ligation are required, and the so-called excinuclease complex comprises 16 or more different proteins. Large distortions caused by bulky DNA adducts (eg, BPDE-dG and 4ABP-dC) are recognized (*XPA*) and removed by endonucleases (*XPF*, *XPG*, *FEN*). A patch is then constructed (*pol* Δ, *pol* ε) and the free ends are ligated.

Base excision repair also removes a DNA segment containing an adduct, however, small adducts (eg, 3-methyladenine) are generally the target so that there is overlap with direct repair. The adduct is removed by a glycosylase (*hOgg1*, *UDG*), an apurinic endonuclease (*APE1* or *HAP1*) degrades a few bases on the damaged strand, and a patch is synthesized (*pol* β) and ligated (DNA ligases: I, II, IIIα, IIIβ, and IV).

DNA mismatches occasionally occur, because excision repair processes incorporate unmodified or conventional, but noncomplementary, Watson–Crick bases opposite each other in the DNA helix. Transition mispairs (G-T or A-C) are repaired by the mismatch repair process more efficiently than transversion mispairs (G-G, A-A, G-A, C-C, C-T, and T-T). The mechanism for correcting mispairings is similar to that for nucleotide excision repair and resynthesis described earlier, but it generally involves the excision of large pieces of the DNA containing mispairings. Because the mismatch recognition protein is required to bind simultaneously to the mismatch and an unmethylated adenine in a GATC recognition sequence, it removes the whole intervening DNA sequence. The parental template strand is then used by the polymerase to fill the gap.

Double-strand DNA breaks can occur from exposure to ionizing radiation and oxidation. Consequences of double-strand DNA breaks are the inhibition of replication and transcription, and loss of heterozygosity. Double-strand DNA break repair occurs through homologous recombination, where the joining of the free ends is mediated by a DNA-protein kinase in a process that also protects the ends from nucleolytic attack. The free ends of the DNA then undergo ligation by DNA ligase IV. Genes known to code for DNA-repair enzymes that participate in this process include *XRCC4, XRCC5, XRCC6, XRCC7, HRAD51B, HRAD52, RPA*, and *ATM*.[87]

Postreplication repair is a damage-tolerance mechanism and it occurs in response to DNA replication on a damaged template. The DNA polymerase stops at the replication fork when DNA damage is detected on the parental strand. Alternately, the polymerase proceeds past the lesion, leaving a gap in the newly synthesized strand. The gap is filled in one of two ways: either by recombination of the homologous parent strand with the daughter strand in a process that is mediated by a helical nucleoprotein (RAD51); or when a single nucleotide gap remains, mammalian DNA polymerases insert an adenine residue. Consequently, this mechanism may lead to recombinational events as well as base-mispairing.

Persistent non-repaired DNA damage blocks the replication machinery. Cells have evolved translesion synthesis (TLS) DNA polymerases to bypass these blocks.[92,93] Most of these TLS polymerases belong to the recently discovered Y-family, have much lower stringency than replicative polymerases and thus are error prone. An increased mutation frequency is an evolutionary trade-off for cellular survival.

MUTATOR PHENOTYPE

Cancer cells contain substantial numbers of genetic abnormalities when compared with normal cells. These abnormalities range from gross changes such as nondiploid number of chromosomes, ie, aneuploidy, and translocations or rearrangements of chromosomes, to much smaller changes in the DNA sequence including deletions, insertions, and single nucleotide substitutions. Therefore, carcinogenesis involves *errors* in (1) chromosomal segregation; (2) repair of DNA damage induced by either endogenous free radicals or environmental carcinogens; and (3) DNA replication. Loeb originally formulated the concept of the mutator phenotype in 1974[94] to account for the high numbers of mutations in cancer cells when compared to the rarity of mutations in normal cells. Recent advances in the molecular analysis of carcinogenesis in human cells and animal

Table 14-2 Examples of Disease Susceptibility and Disease Syndromes Associated with Mutations in DNA-Repair Genes

Cancer Susceptibility		
MMR[a]		
MLH1	Damage recognition	HNPCC2[b], glioma
MLH2	DNA binding	HNPCC1, ovarian cancer
MSH3	—	Endometrial cancer
MSH6	Sliding clamp	Endometrial cancer, HNPCC1
PMS1	Damage recognition	HNPCC3
PMS2	Repair initiation	HNPCC4, glioblastoma
NER		
BRCA-1	Directs *p53* transcription towards DNA-repair pathways	Breast cancer, ovarian cancer
RB1	Cell-cycle restriction	Retinoblastoma, breast cancer, and progression osteosarcoma
DSB		
BRCA-2	Regulation of RAD51	Breast cancer, pancreatic cancer
HR		
RAD54	Helicase	Colon cancer, breast cancer, NHL
Other		
TP53 (DSB, NER, HR)	Cell-cycle control; exonuclease; apoptosis; DNA binding	Colon cancer, common somatic defect in human cancer in general; inherited in Li-Fraumeni syndrome and some breast cancers
hOgg1 (Various)	Glycosylase	Cancer susceptibility
Xeroderma pigmentosum (XP)		
NER		
XPD	DNA Helicase	Skin and neurologic, but later onset than XPA
XPB	DNA Helicase	Skin lesions
XPG	Endonuclease	Acute sun sensitivity, mild symptoms; late skin cancer
XPC (and BER)	Exonuclease	Mental retardation; skin sensitivity; microcephaly
DDB1 and DDB2	Binds specific DNA damage	XPE—Mild skin sensitivity
XPA	Damage sensor	XPA—Skin and neurologic problems: the most severe XP
XPC	Damage sensor	XPC—Skin, tongue, and lip cancer
XPE	Damage sensor	XPE—Neurologically normal
PRR		
POLH	Polymerase	XPV—Mild to severe skin sensitivity; neurologically normal
Other syndromes		
NER		
Cockaynes		
CSB	ATPase	Cutaneous, ocular, neurologic, and somatic abnormalities; short stature, progressive deafness, mental retardation, neurologic degeneration, early death; sometimes presents together with XPB
Juberg-Marsidi		
ATRX	Putative helicase	Thalassemia/mental retardation
SB		
Nijmegen		
NBS1	Nibrin; cell-cycle regulation	Microencephaly; mental retardation; immunodeficiency; growth retardation; radiation sensitivity; predisposition to malignancy
Ataxia-telangiectasia		
ATM	Phosphorylation	Neurologic deficiencies, manifest by inability to coordinate muscle actions; skin and corneal telangiectases. Leukemia, lymphoma, and other malignancies (breast cancer?)
MRE11 (Ataxia-like)	Exonuclease	DNA damage sensitivity; genomic instability; telomere shortening; aberrant meiosis; severe combined immunodeficiency
PRKDC	Ser/Thr kinase	SCID
Bloom's		
BLM	DNA Helicase	High rate of spontaneous lymphatic and other malignancy; high rate SCE[c]
Fanconi anemia		
FANCA-G	Protein control	Multiple congenital malformations; chromosome breaks; pancytopenia. Telomere shortening
Werner		
WRN	DNA Helicase/Exonuclease	Premature senility, short stature, exonuclease rapidly progressing cataracts, loss of connective tissue and muscle, premature arteriosclerosis, increase risk of malignancy
RecQ4	DNA Helicase	Osteosarcoma; premature aging.

[a] Repair mechanisms: BER = base excision; DSB = double strand break; HR = homologous recombination; MMR = mismatch; NER = nucleotide excision; PRR = postreplication; SB = strand break.
[b] Diseases: HNPCC = hereditary nonpolyposis colon cancer; NHL = non-Hodgkin lymphoma.
[c] Other abbreviations: SCE = sister chromatid exchange; SCID = severe combined immunodeficiency.

models have refined the mutator phenotype[12] concept that is also linked to the clonal selection theory proposed by Nowell (Figure 14-5).[95]

The fidelity of DNA replication, and the partitioning of equal numbers of chromosomes to the daughter cells, is remarkably accurate in dividing normal cells. Whereas, the maintenance of the species will tolerate few mutations in germ cells, somatic cells may tolerate many more mutations that cause cancer and other life-shortening diseases. Although rare in the human population, inherited mutations in specific genes that are responsible for rare cancer-prone diseases are consistent with the mutator phenotype concept and indicate examples of functional classes of

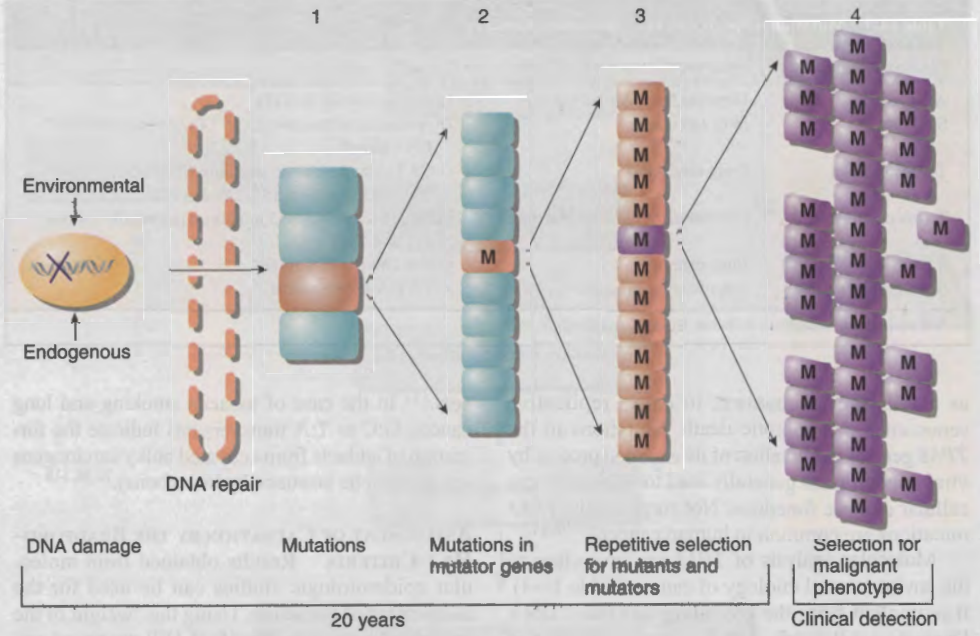

Figure 14-5 Mutation accumulation during tumor progression. (1) Random mutations result when DNA damage exceeds the cell's capacity for error-free DNA repair. (2) These random mutations can result in clonal expansion and mutations in mutator genes (M). (3) Repetitive rounds of selection for mutants yield coselection mutants in mutator genes. (4) From this population of mutant cancer cells, there is selection for cells that escape the host's regulatory mechanisms for the control of cell replication, invasion, and metastasis. (Modified and reproduced with permission from Loeb.[12])

genes that, when mutated, increase the probability of cancer.

Examples of cancer syndromes include those with defective nucleotide excision repair (eg, xeroderma pigmentosum), or mismatch DNA repair (eg, hereditary nonpolyposis colorectal cancer syndrome). Germline mutations in DNA helicases also cause the cancer-prone Bloom and Werner syndromes. Other functional classes of genes required for maintaining genomic stability, that when mutated, may be responsible for the mutator phenotype, include the very genes involved in DNA replication, deoxynucleotide metabolism, cell cycle checkpoints, and mitosis (see Table 14-2). The *TP53* tumor suppressor gene is frequently mutated in human cancer,[96–98] and is involved in DNA repair and recombination, cell cycle checkpoints, and apoptosis.[99] Thus, *TP53* may be an important target gene for a mutator phenotype.

CLONAL EVOLUTION

Clonal evolution in human tumors is complex, because there is evidence that a single progenitor cell gives rise to all the cells in the tumor, as well as evidence for a hierarchical system of stem cells. Both systems likely exist. In tobacco-related cancer of the lung, metachronous bilateral masses have, in some cases, a similar genetic background, whereas in other cases, they are dissimilar.[100,101] In addition, adenosquamous lung carcinomas are most frequently composed of a unique genetic profile, although a small percentage present variability.[102] Together, these findings suggest that multifocal masses may exist where different foci have a common initiated cell as well as multifocal masses where each focus has its own clonal origin.

Cancers of the head and neck are frequently tobacco related and many patients develop metachronous tumors, thus these cancers provide another tumor type in which to approach the question of clonality. Comparative molecular analysis found some paired tumors, arising in the same field, shared a common origin. These findings indicate that a single progenitor cell can replicate, expand, and populate contiguous regions in the process of clonal expansion.[26,103] However, the zonal limits of clonal expansion have not yet been adequately determined.[104,105]

Further evidence for clonality in tumors comes from studies of loss of heterozygosity, where allelic loss invariably affects the same chromosome.[106] Even more convincing is differential methylation in female cancer patients. Inactivation of the X chromosome (by methylation) during embryogenesis is random, so polyclonal female tissues develop with an approximately equal complement of inactivated maternal and paternal X chromosomes. In monoclonal tissues, the same inactive X chromosome should be present in all of the cells. By use of methylation-sensitive restriction enzymes virtually all colorectal tumors in females were found to be monoclonal. [107] Genetic studies have found at least 95% of human tumors to be monoclonal in origin.

In chronic myelogenous leukemia, the early disease phase is characterized by the Philadelphia chromosome, a single reciprocal translocation, t(9;22). The formation of a hybrid gene of *c-abl* with the breakpoint cluster region (*BCR*) activates the proto-oncogene through its in-frame insertion. The resulting gene product has elevated tyrosine kinase activity. The later stages of chronic myeloid leukemia are typified by overgrowth of one or more subclones that have additional karyotypic alterations.[108]

ONCOGENES AND TUMOR SUPPRESSOR GENES

Chronic exposures to carcinogens, accumulation of mutations, development of the mutator phenotype, and clonal selection during several decades result in cancer. Although the phenotypic traits of individual cancers are highly variable, commonly acquired capabilities include limitless replicative potential, self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, tissue invasion, sustained angiogenesis, and metastasis.[109] These phenotypic traits reflect a complex molecular circuitry of biochemical pathways and protein machines within cancer cells.[19]

The genes encoding the proteins within the cancer-associated molecular circuitry are of many functional classes and, historically, have been conceptually divided into oncogenes and tumor suppressor genes. Detailed descriptions of oncogenes and tumor suppressor genes are found in Chapters 4 to 7. The *ras* oncogene and the *TP53* tumor suppressor gene will be used as examples of molecular targets of chemical carcinogens.

Activated *ras* genes predominate as the family of oncogenes to be isolated from solid tumors that are induced by chemicals in laboratory animals. Members of the *ras* gene family code for proteins of molecular weight 21,000 (p21); these proteins are membrane bound, have GTPase activity, and form complexes with other proteins. The *ras* genes code for small G-proteins (guanine nucleotide binding) that exert a powerful proliferative response through the signal transduction cascade. The first direct evidence of proto-oncogene activation by a chemical carcinogen was obtained from in vitro studies.[110] A wild-type recombinant clone of the human *Ha-ras* gene (pEC) was modified with benzo[*a*]pyrene-diolepoxide. The treated plasmid was then used to transfect murine NIH-3T3 cells, with the result that the transformed cell foci contained the same point mutations (in either codon 12 or 61) known to exist in activated *ras* genes isolated from human tumors including the bladder (pEJ). In animal models of chemical carcinogenesis and surveys of different types of human tumors that arise from a variety of environmental exposures, *ras* mutations have been found.[31,99,111] For example, tobacco smoke can mutate *K-ras* during the molecular pathogenesis of human lung adenocarcinoma.[80] In rodents, polycyclic aromatic hydrocarbons (3-methylcholanthrene, 7,12-dimethylbenz[*a*]anthracene and benzo[*a*]pyrene) have been used repeatedly to produce both benign tumors and malignant carcinomas. A large pro-

portion of these premalignant and malignant lesions have mutations in either the twelfth or sixty-first codons. Similarly, treatment of rats with either 7,12-dimethylbenz[*a*]anthracene or *N*-methyl-*N*-nitrosourea resulted in the development of mammary carcinomas containing *ras* codon 12 or 61 mutations. These types of mutations also have been observed in mouse skin after initiation with 7,12-dimethylbenz[*a*]anthracene and tumor promotion with 12-*O*-tetradecanoylphorbol-13-acetate. Mutations in *ras* have been found in mouse liver after treatment with vinyl carbamate, hydroxydehydroestragole, or *N*-hydroxy-2-acetylaminofluorene. The same point mutations have been found in murine thymic lymphomas after treatment with *N*-methyl-*N*-nitrosourea or γ-radiation, and in other rodent skin models after treatment with methylmethanesulfonate, α-propiolactone, dimethylcarbamyl chloride, or *N*-methyl-*N*9-nitro-*N*-nitrosoguanidine.

These data indicate that chemical carcinogens may produce site-specific mutations based, in part, on nucleoside selectivity of the ultimate carcinogen. Persistence of a specific mutation, however, also depends on the amino acid substitution in that the function of the mutant protein is altered to confer on the cell a selective clonal growth advantage. The types of mutations that are found in chemically activated *ras* genes cause conformational changes that alter protein binding (GTPase Activating Protein) in such a way that the *ras*-MAP kinase pathway is permanently activated. Data support the hypothesis that *ras* activation is associated with malignant conversion as well as tumor initiation. Transfection of activated *ras* genes into benign papillomas that did not contain a constitutively activated *ras* gene, caused malignant progression.[111] These and other results implicate *ras* mutations in chemical carcinogenesis. Similarly, malignant transformation occurred when immortalized human bronchial epithelial cells were transfected with an activated *ras* gene.[112,113] *Ki-ras* gene mutations are also one of many changes that can arise either early or late in the development of colorectal carcinoma.[106] These findings indicate that the accumulation of mutations, and not necessarily the order in which they occur, contributes to multistage carcinogenesis. Furthermore, the stage of carcinogenesis in which each mutation occurs is not necessarily fixed. In the model for human colorectal carcinoma, *ras* mutations most often occur during malignant conversion, but can be an early event (ie, tumor initiation), but in the rodent skin models, *ras* mutations appear to be primarily a tumor-initiating event. These differences may reflect the type of exposure, both in terms of chemical class and chronic versus acute exposure, or they may be a function of tissue type.

The *TP53* tumor suppressor gene is central in the response pathway to cellular stress.[106] For example, DNA damage caused by chemical carcinogens activates the p53 tumor suppressor protein by post-translational modification to transduce signals to "guard the genome"[114] by engaging cell cycle checkpoints and enhancing DNA repair, and as a fail-safe mechanism, to cause replicative senescence or apoptotic death. Mutations in the *TP53* gene or inactivation of its encoded protein by viral oncoproteins generally lead to a loss of these cellular defense functions. Not surprisingly, *TP53* mutations are common in human cancer.[96–98,115]

Table 14-3 Mutational Spectra of *TP53* in Human Cancers*

Carcinogen Exposure	Neoplasm	Mutation
Aflatoxin B_1	Hepatocellular carcinoma	Codon 249 (AGG 6 AGT)
Sunlight	Skin carcinoma	Dipyrimidine mutations (CC 6 TT) on nontranscribed DNA strand
Tobacco smoke	Lung carcinoma	G:C 6 T:A mutations on nontranscribed DNA strand (frequently codons: 157, 248 and 273)
Tobacco and alcohol	Carcinoma of the head and neck	Increased frequency p53 mutations (especially codons 157 and 248)
Radon	Lung carcinoma	Codon 249 (AGG 6 ATG)
Vinyl chloride	Hepatic angiosarcoma	A:T 6 T:A transversions

*For reviews see Greenblatt, Hollstein, Hussain, and Harris.[72, 96, 120, 146]

Molecular analysis of *TP53* can give clues to the environmental etiology of cancer (Table 14-3). It is implicit from the preceding text (see "DNA Damage and Repair") that the covalent binding of activated carcinogens to DNA is not random. Therefore, the formation of a particular DNA lesion to some extent may be deduced from the resulting mutation. A dramatic example of this phenomenon is the previously mentioned *TP53* codon 249 mutation, which is detected in almost all aflatoxin-related hepatocellular carcinomas.[97,116] The striking nature of this association could arise by two distinct mechanisms. First, the third base in codon 249 (AGG) may be unusually susceptible to activated aflatoxin B1 mutations. As discussed earlier, aflatoxin B1-8,9-oxide causes a promutagenic lesion by covalently binding to the N7 position of deoxyguanosine. Alternately, cells bearing the codon 249 lesion may have an important selective growth advantage. Evidence that a combination of these factors is responsible has been presented as well.[116] Another prominent example where circumstantial evidence points to specific molecular events is that of *TP53* mutations indicative of pyrimidine dimer formation in ultraviolet light related skin cancers.[117] In the case of tobacco smoking and lung cancer, G:C to T:A transversions indicate the formation of adducts from activated bulky carcinogens (eg, polycyclic aromatic hydrocarbons).[72,96,118]

Assessment of Causation by the Bradford–Hill Criteria Results obtained from molecular epidemiologic studies can be used for the assessment of causation. Using the "weight of the evidence" principle, Bradford-Hill proposed criteria in the assessment of cancer causation, including strength of association (consistency, specificity, and temporality) and biologic plausibility.[119] These criteria can be applied for the analysis of data obtained in molecular epidemiologic studies.[120] Cigarette smoking has been established as a major risk factor for the incidence of lung cancer (Table 14-4). Codons 157, 248, and 273 of *TP53* are designated as mutational hotspots in lung cancer. The majority of mutations found at these codons are G to T transversions. Furthermore, besides lung cancer, codon 157 also constitutes one of the hotspots for G to T transversions in breast, and head and neck cancers. In smoking-associated lung cancer, the occurrence of G to T transversions has been linked to the presence of benzo[*a*]pyrene in cigarette smoke. Interestingly, codon 157 (GTC to TTC) mutations are not found in lung cancer from never smokers.[96–98] A dose-dependent increase in *TP53* G to T transversion mutations

Table 14-4 Assessment of Causation by the Bradford-Hill Criteria*

Hypothesis:	
The chemical carcinogen, benzo[α]pyrene, in tobacco smoke can cause *TP53* hotspot mutations at codons 157, 248, and 273 in human lung carcinogenesis.	
Strength of association	Biologic plausibility
Consistency	Tobacco smoke and benzo[α]pyrene are mutagens.
Cigarette smoking or exposure to coal smoke are associated with a dose-response increase in *TP53* mutations (G to T transversions in human lung cancer).	Benzo[α]pyrene is metabolically activated and forms benzo[α]pyrene diol-epoxide-DNA adducts in human bronchus *in vitro* (75-fold interindividual variation).
Specificity	Benzo[α]pyrene diol-exposide binds to Gs in codons 157, 248, and 273, which are *TP53* mutational hotspots.
Codon 157 (GTC 6 TTC) mutations are uncommon in other types of cancer, including in lung cancer from never smokers.	Benzo[α]pyrene exposure to human cells in vitro produces codon 248 (CGG ≥ CTG) *TP53* mutations.
Temporality	Cigarette smoke condensates or benzo[α]pyrene can neoplastically transform human bronchial epithelial cells in the laboratory.
TP53 mutations can be found in bronchial dysplasia.	

*For reviews see Hill, and DeMarini.[119,151]

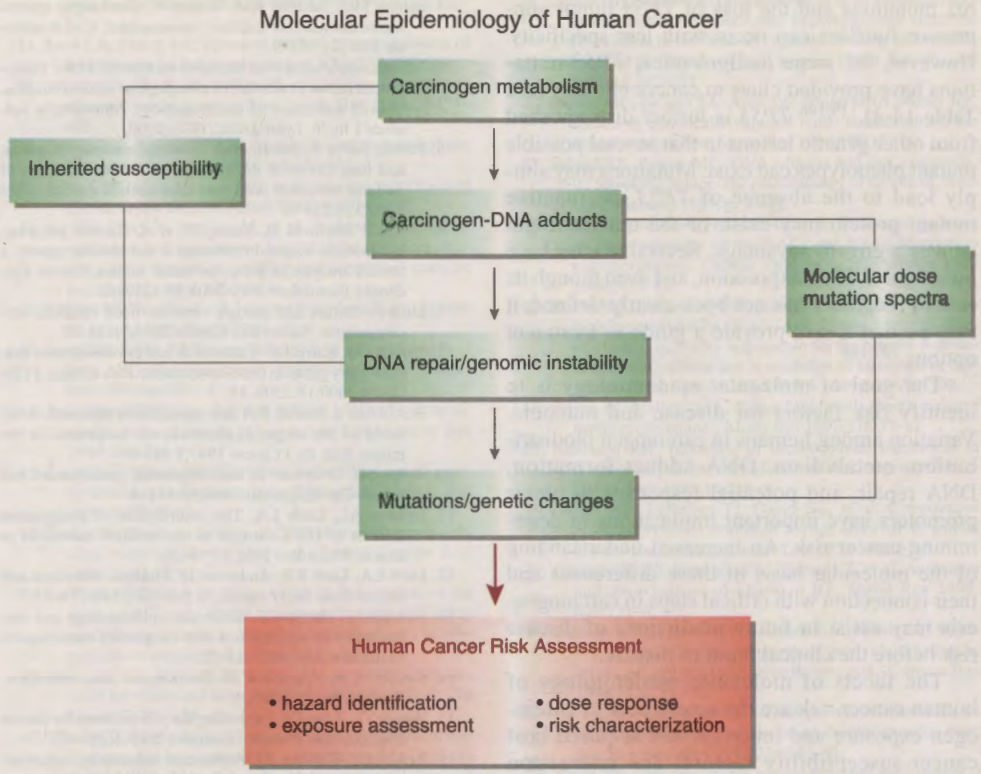

Figure 14-6 Facets of molecular epidemiology that investigate gene–environment interactions. Once internalized, chemical carcinogens are metabolized to reactive species that cause DNA damage (carcinogen DNA adducts). The innate ability to repair DNA damage may reduce or ablate the overall damage burden. Alternately, genetic changes (mutations, clastogenesis) may occur. Carcinogen metabolism and DNA repair are categorizable genetic traits (host factors). DNA adducts (molecular dose) and mutational spectra are measures of exposure. Information from assays designed to investigate host factors and measure exposure can be used for human cancer risk assessment.

with cigarette smoking has been reported in lung cancer.[121] Benzo[α]pyrene diol-epoxide, the metabolically activated form of benzo[*a*]pyrene, has been shown to bind to guanosine residues in codons 157, 248, and 273, which are mutational hotspots in lung cancer.[122] Also, cigarette smoke condensate or benzo[*a*]pyrene neoplastically transforms in vitro human bronchial epithelial cells.[123] In general, molecular and epidemiologic data provide only circumstantial evidence for causation. Bradford-Hill criteria provide a framework for the logical consideration of converging lines of evidence in cancer etiology.

IMPLICATIONS FOR MOLECULAR EPIDEMIOLOGY, RISK ASSESSMENT, AND CANCER PREVENTION

Molecular epidemiology (use of biochemical and molecular biological methods to buttress epidemiological studies) has resulted from the confluence of several disciplines.[124] It encompasses the detection of carcinogen–macromolecular adducts (DNA as a direct genotoxic measure and protein as a surrogate), normal DNA sequence variants (heritable variations), and mutations in target genes (somatic changes). Therefore, these investigations use epidemiologic methods to investigate all aspects of gene–environment interactions and risk assessment in human populations (Figure 14-6).

The biologically effective dose of a chemical carcinogen is governed by the amount that reaches a target tissue in a form that becomes activated to a chemical species capable of causing DNA lesions.[125] Humans are most commonly exposed to complex mixtures of chemicals. Human carcinogen dosimetry at the molecular level requires sensitive and specific methods for carcinogen-macromolecular adduct quantitation. The low levels of adducts that are present in human DNA samples challenge the detection limits of conventional assay systems, and complex mixtures of adducted materials confound simple assay systems.

The most commonly used methods for carcinogen–DNA dosimetry in humans are ^{32}P-nucleotide postlabeling, immunoassays, fluorescence spectroscopy, electrochemical conductance, liquid chromatography/electrospray ionization/tandem mass spectrometry (LC/ESI/MS/MS), and gas chromatography/mass spectroscopy (GC/MS). Each of these techniques currently has its own advantages and limitations, and within the framework of epidemiologic surveys, multiple corroborative end-point analyses seem to provide the most useful information. These methodologies, their application, and their limitations are reviewed extensively elsewhere.[3,29]

For exposure to tobacco smoke, GC/MS has provided a tool to measure aromatic amine protein adducts such as 4-aminobiphenyl hemoglobin. These studies have shown a dose–response relationship between the extent of smoking, type of tobacco used, and the adduct levels.[126] Similarly, tobacco-specific nitrosamine globin adducts have been used to monitor the dose in smokers and snuff dippers. A corroborative approach to the measurement of benzo[*a*]pyrene–DNA adducts has been used in the monitoring of both tobacco and coal smoke exposure. In this study, both GC/MS and fluorescence line-narrowing spectroscopy were used to detect adducts exfoliated in urine.[63,127]

In the case of aflatoxin B1, levels of adducts exfoliated in human urine were measured by GC/MS. 8,9-dihydro-8-(N5-formyl- 2', 5', 6'-triamino-4'-oxo-N5-pyrimidyl)-9-hydroxy-aflatoxin B1 (aflatoxin–N7 guanine) adducts correlated with environmental exposure and disease outcome. Similarly, aflatoxin–albumin adducts provided a corroborative surrogate. Both of these markers were also correlated with 6-hydroxycortisol levels, indicating a role for CYP3A4 in aflatoxin B1 activation. Particularly, the presence of aflatoxin–N7 guanine adducts in urine was associated with liver cancer.[128,129] Based on these findings, a randomized clinical trial of the *interceptor molecule*, chlorophyllin (Derifil), was performed. The test drug or placebo was taken three times daily and urinary AFB1-N7-Gua was monitored by GC/MS. After 12 weeks, adduct levels were > 100% higher among 90 persons taking the placebo than those ($n = 90$) taking chlorophyllin.[81]

Interindividual variation in cancer susceptibility, and, consequently, meaningful human cancer risk assessment, involve determination of inherited host factors as well as exposure assessment. Metabolic polymorphisms have been determined by the use of indicator drugs (eg, caffeine, debrisoquine, dextromethorphan, dapsone, and isoniazid), however, these assays are being replaced by direct genetic assays.[61,67,130] This approach has allowed the investigation of diverse host factors for which indicator drugs were not available, and it has been applied to a wide variety of cancers, including lung, head, and neck.[131,132] Thus, genetic indicators of propensity for carcinogen activation and detoxification, DNA-repair capacity, and cell-cycle control are all features of molecular epidemiologic studies that are complementary to adduct studies because of the implications for a biologically effective dose after exposure.[3]

Cytochrome P450 polymorphisms, involved in carcinogen activation, and glutathione-*S* transferases, uridine diphosphate (UDP) glucuronosyltransferases, sulfotransferases, and *N*-acyltransferases, involved in both carcinogen activation and detoxification, could explain variations in cancer susceptibility among the human population. Evidence that absent protection of a functionally intact *GSTM1* gene correlates with an increased risk of tobacco-related lung cancer.[133,134] Similarly, UDP glucuronosyltransferases (eg, *UGT1A1, UGT1A9, UGT2B7*) have been implicated in cancers of the head and neck. Persons inheriting reduced activity variants of *NAT1* and *NAT2*

genes, resulting in the slow acetylator phenotype, are at a greater risk of aromatic amine-induced bladder cancer. This may include persons exposed through tobacco smoke inhalation.[66] Even though the inducible form of arylhydrocarbon hydroxylase (AHH) (*CYP1A1* and *CYP1A2*) has long been suspected of increasing cancer susceptibility in PAH-exposed persons, molecular epidemiologic studies remain inconclusive. Studies of *CYP2D6* metabolizer status and tobacco smoke-related lung cancer are similarly confusing.[8] However, analysis of multiple traits, for example, *CYP1A1* and *GSTM1*, in the same population may help to resolve these issues. Currently, there is a need for improved epidemiologic study design that integrates DNA adduct measures with indicators of metabolic capacity.[135–137]

Many DNA-repair genes have been described recently, and a growing number of polymorphisms have been identified for which molecular epidemiologic studies have provided evidence that genetic variation in these attributes can be a human cancer risk factor.[90,91,138,139] Typically, these types of molecular epidemiological studies initially focus on high exposure groups such as workers, patients taking therapeutic drugs and tobacco smokers. Several polymorphisms in DNA repair genes have now been implicated in tobacco related neoplasams.[140]

The components of cell-cycle control comprise genes that code for cell membrane receptors, intermediate messengers, and transcription factors. Polymorphisms in a number of these genes have been implicated in various cancers, notably: *TP53, p73, BRCA-1, BRCA-2* and *CCND1*.[141–144] However, there is overlap. This is because many of these genes, like *BRCA-1* and *TP53*, are integral components of the DNA repair system. Indeed they constitute a system of genome surveillance that alerts cells of damage status.

Molecular characterization of tumors, that is, molecular profiling, is an important tool that has both etiologic and clinical application. Molecular profiling is a rapidly advancing area that is being propelled by DNA and protein microarray research.[37,38,145] During chemical carcinogenesis, the genome becomes altered and mutations accumulate. These mutations become evident in genes responsible for growth control and cellular homeostasis (including proto-oncogenes, tumor suppressor genes, and some DNA-repair genes), because corruption of these functions is part of carcinogenesis. In respect to chemical carcinogenesis, the most studied genes are Kirsten *ras (Kras)* and *TP53*. *Kras* is mutated in approximately 30% of lung adenocarcinomas, and may prove to be an indicator of prognosis or a guide to treatment.[80] The *TP53* tumor suppressor gene is mutated in most types of human cancers and it is the most commonly mutated gene yet known (eg, mutations in *TP53* are found in approximately 50% of lung cancers). Unlike *ras* gene mutations that are found in highly specific regions (codons 12, 13, 59, and 61), *TP53* mutations occur more widely. This is presumably because a positive growth advantage is conveyed only with specific *ras* mutations and the loss of *TP53* tumor suppressor function can occur with less specificity. However, for some malignancies, *TP53* mutations have provided clues to cancer etiology (see Table 14-3).[119,146] *TP53* is further distinguished from other genetic lesions in that several possible mutant phenotypes can exist. Mutations may simply lead to the absence of *TP53*, an inactive mutant protein may exist, or the mutant might convey a growth advantage. Several studies have investigated *TP53* expression, and even though its role in prognosis has not been clearly defined, it may be that it will provide a guide to treatment options.[147,148]

The goal of molecular epidemiology is to identify risk factors for disease and outcome. Variation among humans in carcinogen biodistribution, metabolism, DNA adduct formation, DNA repair, and potential responses to tumor promoters have important implications in determining cancer risk. An increased understanding of the molecular basis of these differences and their connection with critical steps in carcinogenesis may assist in future predictions of disease risk before the clinical onset of disease.

The facets of molecular epidemiology of human cancer risk are the assessment of carcinogen exposure and inherited and acquired host cancer-susceptibility factors. The interaction between these facets determines cancer risk. When combined with carcinogen bioassays in laboratory animals and classic epidemiology, molecular epidemiology can contribute to the four critical aspects of cancer risk assessment: (1) hazard identification, (2) dose–response assessment, (3) exposure assessment, and (4) risk characterization. Important bioethical considerations accompany the identification of high-risk individuals; these include autonomy, privacy, justice, and equity. Benefits of the knowledge of risk for an individual may be offset by specific concerns relating to that individual's responsibility to family members and psychosocial anxiety regarding the genetic testing of children. Therefore, the uncertainty of current individual risk assessments and the limited availability of genetic counseling services dictate caution. In addition, it is widely held that genetic testing should be restricted to those situations that are amenable to preventative or therapeutic intervention.[149]

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Front cover: The colon cancer cell line HCT116 was transfected with GFP-Bax, a pro-apoptosis molecule and then treated with a protease inhibitor, MG132. Cells were then stained with the DNA dye Hoeschst 33342. Normal cells have large round nuclei (*blue*) and the GFP-Bax signals (*green*) are diffusive indicating a cytoplasmic distribution. Apoptotic cells have small, condensed or fragmented nuclei (*blue*) and the GFP-Bax signals are punctate indicating the translocation of Bax from the cytoplasm to the mitochondria, where it initiated the apoptotic program. (Images provided by Drs. Xiao-Ming Yin and Wen-Xing Ding, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA.)