Chapter 5
Cancer

Introduction

The 2004 Surgeon General's report, *The Health Consequences of Smoking: A Report of the Surgeon General* (U.S. Department of Health and Human Services [USDHHS] 2004), concluded that the evidence is sufficient to infer a causal relationship between smoking and cancers of the lung, larynx, oral cavity, pharynx, esophagus, pancreas, bladder, kidney, cervix, and stomach, and acute myeloid leukemia. In addition, the report found that evidence suggests a causal relationship between smoking and colorectal and liver cancers. This chapter examines the mechanisms by which cigarette smoking induces cancer. Literature citations for this section's discussion appear in subsequent sections of this chapter, as appropriate. A schematic overview of the pertinent mechanisms discussed in this chapter is presented in Figure 5.1. The figure depicts the major established pathways of cancer causation by cigarette smoking: (1) the exposure to carcinogens (cancer-causing substances), (2) the formation of covalent bonds between the carcinogens and DNA (DNA adduct formation), and (3) the resulting accumulation of permanent somatic mutations in critical genes (genes appear in italics). Somatic mutations lead to clonal outgrowth and, through accumulation of additional mutations, to development of cancer.

Each puff of each cigarette contains a mixture of thousands of compounds, including more than 60 well-established carcinogens. The carcinogens in cigarette smoke belong to multiple chemical classes, including polycyclic aromatic hydrocarbons (PAHs), *N*-nitrosamines, aromatic amines, aldehydes, volatile organic hydrocarbons, and metals. In addition to these well-established carcinogens, others have been less thoroughly investigated. These include alkylated PAHs, oxidants, free radicals, and ethylating agents. Considerable evidence indicates that in human cancers caused by cigarette smoking, PAHs, *N*-nitrosamines, aromatic amines, and certain volatile organic agents play a major role. Extensive data in the literature demonstrate the uptake of these carcinogens by smokers. The data confirm the expected presence of metabolites of these substances in the urine of smokers at higher levels than those in nonsmokers.

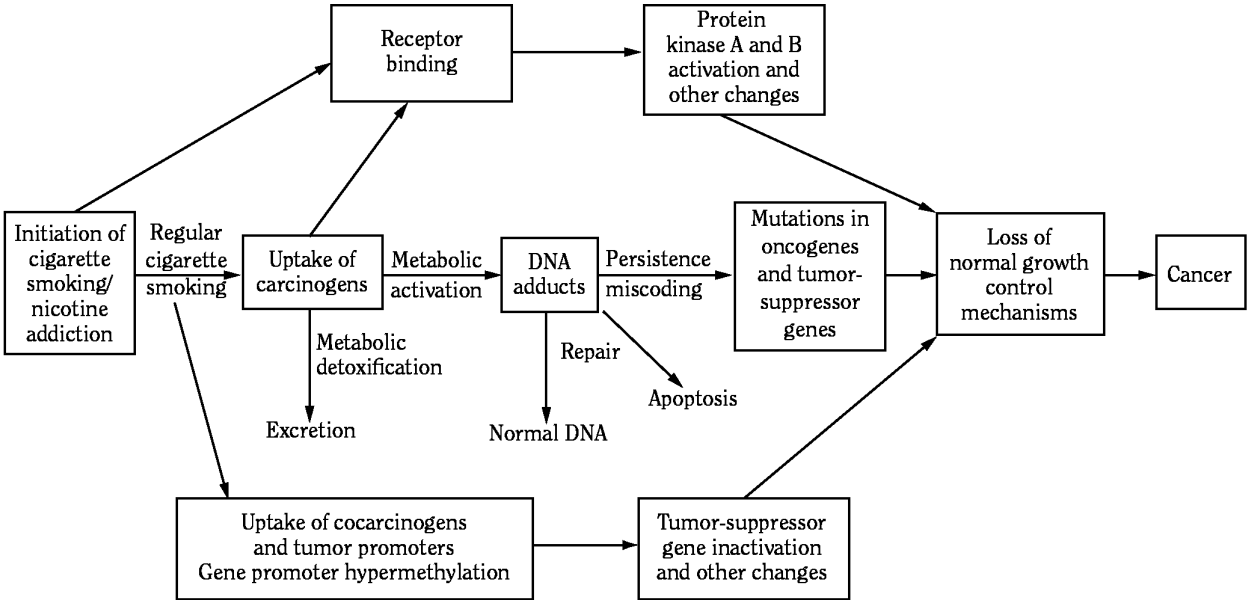
Most carcinogens in cigarette smoke require a metabolic activation process, generally catalyzed by cytochrome P-450 enzymes (P-450s), to convert the carcinogens to forms that can covalently bind to DNA and form DNA adducts. P-450s 1A1 and 1B1, which are inducible by cigarette smoke through interactions with the aryl hydrocarbon receptor, are particularly important in the metabolic activation of PAHs. The inducibility of these P-450s may be a critical aspect of cancer susceptibility in smokers. P-450s 1A2, 2A6, 2A13, and 2E1 are also important in the activation of cigarette smoke carcinogens. Competing with the activation process is metabolic detoxification, which excretes carcinogen metabolites in generally harmless forms and is catalyzed by a variety of enzymes, including glutathione-*S*-transferases (GSTs), uridine-5′-disphosphate-glucuronosyltransferases (UGTs), epoxide hydrolases, and sulfatases. The balance between metabolic activation and detoxification of carcinogens varies among persons and likely affects cancer susceptibility. Persons with a higher activation and lower detoxification capacity are at the highest risk for smoking-related cancers. This finding is supported by considerable evidence from molecular epidemiologic studies of the polymorphisms (variants) in these enzymes.

The metabolic activation of carcinogens results in the formation of DNA adducts, which are absolutely central to the carcinogenic process. However, some carcinogens can directly form DNA adducts without metabolic activation. Since the mid-1980s, extensive studies have examined the presence of DNA adducts in human tissues. Studies that used nonspecific methods, such as ^{32}P-postlabeling and immunoassays, to measure adducts concluded that adduct levels in the lung and in other tissues are higher in smokers than in nonsmokers. Some epidemiologic data link higher adduct levels with a higher probability of developing cancer.

There are ample cellular repair systems that can remove DNA adducts and maintain a normal DNA structure. These systems include direct repair of DNA bases by alkyltransferases, the excision of DNA damage by base and nucleotide excision repair, mismatch repair, and double-strand break repair. If repair enzymes are overwhelmed by DNA damage or for other reasons cannot function efficiently, DNA adducts may persist and increase the likelihood of developing somatic mutations. Inherited polymorphic variants in some DNA repair enzymes are also associated with decreased DNA repair activity and a potentially higher probability of developing cancer.

Persistent DNA adducts can cause miscoding (e.g., insertion of the wrong base) during replication of DNA when DNA polymerase enzymes process the adducts incorrectly. Considerable specificity exists in the relationship between specific DNA adducts caused by carcinogens in cigarette smoke and the types of observed somatic mutations; for example, an O^6-methylguanine adduct causes G→A transitions. These types of mutations are frequently observed in the *KRAS* oncogene in lung

Figure 5.1 Link between cigarette smoking and cancer through carcinogens in tobacco smoke



cancer and in the *TP53* gene in a variety of cancers induced by cigarette smoke. The *KRAS* and *TP53* mutations observed in lung cancer in smokers appear to reflect DNA damage caused by metabolically activated PAHs. However, a number of other carcinogens or toxicants, such as *N*-nitrosamines and aldehydes, as well as oxidative damage, are also likely to be involved. Animal studies have firmly established the cancer-causing role of mutations in these genes.

Gene mutations can cause the loss of normal functions in control of cellular growth, ultimately resulting in cellular proliferation and cancer. Studies have strongly linked chromosome damage in cells throughout the aerodigestive tract to exposure to cigarette smoke. The protective process of programmed cell death (apoptosis) can counterbalance these mutational events by removing cells with DNA damage. The balance between mechanisms leading to apoptosis and those suppressing apoptosis has a major impact on tumor growth. In addition, researchers have observed numerous cytogenetic changes in lung cancer.

The central track of Figure 5.1 that proceeds through genetic damage is clearly established as a major pathway by which carcinogens in cigarette smoke can cause cancer. However, the top and bottom tracks of Figure 5.1 indicate that other pathways also contribute to carcinogenesis. Nicotine and tobacco-specific nitrosamines bind to nicotinic receptors and other cellular receptors. This binding then leads to the activation of protein kinase B (AKT, also known as PKB), protein kinase A (PKA), and other key biologic pathways for cytogenetic changes. Cigarette smoke activates EGFR and COX-2, both known to be important in cell proliferation and transformation. Furthermore, the occurrence of cocarcinogens and tumor promoters in cigarette smoke is well established. Although these compounds are not carcinogenic, they clearly enhance the carcinogenicity of cigarette smoke carcinogens through mechanisms that usually lead to stimulation of cell proliferation. The reversibility of cancer risk after smoking cessation supports the role of tumor promoters and other epigenetic factors in tobacco carcinogenesis. However, the specifics of these effects have not been fully elucidated. An important epigenetic pathway is the enzymatic hypermethylation of promoter regions of genes, which can result in gene silencing. If this occurs in tumor-suppressor genes, the result can be unregulated cellular proliferation.

Carcinogen Exposure, Metabolism, and DNA Adducts

Carcinogens in Cigarette Smoke

Carcinogens in cigarette smoke that were evaluated by the International Agency for Research on Cancer (IARC 2004) are listed in Table 5.1. All are carcinogenic in laboratory animals, and 15 are rated as carcinogenic in humans (group 1 carcinogens). Similar evaluations have been published by the USDHHS (2005). The total exposure of smokers to these compounds is approximately 1.4 to 2.2 milligrams (mg) per cigarette (Table 5.1). This estimate is based on machine measurements and may underestimate actual exposure. Some of the strongest of these carcinogens are PAHs, *N*-nitrosamines, and aromatic amines, which occur in the lowest amounts, and some of the weaker carcinogens, such as acetaldehyde and isoprene, occur in the highest amounts. Thus, a simple addition of the amounts of carcinogenic agents could be misleading. For other carcinogens in cigarette smoke that IARC has not evaluated (e.g., broad spectra of PAHs and aromatic amines), data on frequency of occurrence, levels, and carcinogenic activities are incomplete (IARC 1986).

PAHs are incomplete combustion products first identified as carcinogenic constituents of coal tar (Phillips 1983). These products occur as mixtures in tar, soot, broiled foods, automobile engine exhaust, and other materials generated by incomplete combustion (IARC 1983). Generally, PAHs are carcinogens that act locally. Some PAHs, such as benzo[*a*]pyrene (B[*a*]P), have powerful carcinogenic activity. Studies have typically evaluated PAH carcinogenicity by application to mouse skin, but PAHs also induce tumors of the lung, trachea, and mammary gland, depending on the route of administration and the animal model used (Dipple et al. 1984).

Heterocyclic compounds include analogs of PAHs containing nitrogen, as well as simpler compounds such as furan, which is a liver carcinogen. *N*-nitrosamines are a large class of carcinogens with demonstrated activity in at least 30 animal species (Preussmann and Stewart 1984). They are potent and systemic carcinogens that affect different tissues depending on their structure. Two of the most important *N*-nitrosamines in cigarette smoke are the tobacco-specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N′*-nitrosonornicotine (NNN) (Hecht and Hoffmann 1988). NNK caused lung tumors in all species tested, and activity in rats was particularly high. Studies using animal models have demonstrated that NNK also induces tumors of the pancreas, nasal cavity, and liver. In addition, NNN produces esophageal and nasal tumors in rats and respiratory tract tumors in mice and hamsters (Hecht 1998).

Aromatic amines in cigarette smoke are combustion products that include the well-known human bladder carcinogens 2-naphthylamine and 4-aminobiphenyl (4-ABP), which were first characterized as human carcinogens attributable to industrial exposures in the dye industry (Luch 2005). Heterocyclic aromatic amines are also combustion products and are best known for their occurrence in broiled foods (Sugimura 1995), but they also occur in cigarette smoke.

Aldehydes such as formaldehyde and acetaldehyde occur widely in the human environment and are endogenous metabolites found in human blood (IARC 1995c, 1999; Gao et al. 2002). The phenolic compounds catechol and caffeic acid are common dietary constituents. High doses of catechol cause glandular stomach tumors when administered in the diet. Catechol can also act as a cocarcinogen, enhancing the activity of carcinogens such as B[*a*]P (IARC 1999). Dietary caffeic acid caused renal cell tumors in female mice (IARC 1993). The volatile hydrocarbons include 1,3-butadiene, a powerful multiorgan carcinogen in mice that was shown to have weaker activity in rats, and benzene, a known human leukemogen (IARC 1982, 1999). 1,3-butadiene and benzene are arguably the two most prevalent potent carcinogens in cigarette smoke, on the basis of toxicologic criteria (Fowles and Dybing 2003).

Other carcinogenic organic compounds in cigarette smoke include the human carcinogens vinyl chloride in low amounts and ethylene oxide in substantial quantities (IARC 1979). Ethylene oxide is associated with malignancies of the lymphatic and hematopoietic systems in both humans and laboratory animals (IARC 1994). Diverse metals such as the human carcinogen cadmium are also present in cigarette smoke, as is the radioisotope polonium 210, which is carcinogenic to humans.

Cigarette smoke also contains oxidants such as nitric oxide (about 600 micrograms [µg] per cigarette) and related species (Hecht 1999). Free radicals have been detected by electron spin resonance and spin trapping (Hecht 1999). Researchers postulate that the major species of free radicals are a quinone-hydroquinone complex. Other compounds may also be involved in the oxidative damage produced by cigarette smoke. In addition, several studies demonstrate the presence in cigarette smoke of an uncharacterized ethylating agent, which ethylates both DNA and hemoglobin (Hb) (Carmella et al. 2002a; Singh et al. 2005).

Table 5.1 IARC evaluations of carcinogens in mainstream cigarette smoke

Carcinogen[a]	Quantity (per cigarette)	IARC evaluations of evidence of carcinogenicity in humans: In animals	In humans	IARC group[b]	*IARC Monograph*[c] (volume, year)
Polycyclic aromatic hydrocarbons					
Benz[*a*]anthracene	20–70 ng	Sufficient		2A	*32*, 1983; *S7*, 1987
Benzo[*b*]fluoranthene	4–22 ng	Sufficient		2B	*32*, 1983; *S7*, 1987
Benzo[*j*]fluoranthene	6–21 ng	Sufficient		2B	*32*, 1983; *S7*, 1987
Benzo[*k*]fluoranthene	6–12 ng	Sufficient		2B	*32*, 1983; *S7*, 1987
Benzo[*a*]pyrene	8.5–17.6 ng	Sufficient	Limited	1	*32*, 1983; *S7*, 1987; *92*, in press
Dibenz[*a,h*]anthracene	4 ng	Sufficient		2A	*32*, 1983; *S7*, 1987
Dibenzo[*a,i*]pyrene	1.7–3.2 ng	Sufficient		2B	*32*, 1983; *S7*, 1987
Dibenzo[*a,e*]pyrene	Present	Sufficient		2B	*32*, 1983; *S7*, 1987
Indeno[*1,2,3-cd*]pyrene	4–20 ng	Sufficient		2B	*32*, 1983; *S7*, 1987
5-methylchrysene	ND–0.6 ng	Sufficient		2B	*32*, 1983; *S7*, 1987
Heterocyclic compounds					
Furan	20–40 µg	Sufficient		2B	*63*, 1995a
Dibenz[*a,h*]acridine	ND–0.1 ng	Sufficient		2B	*32*, 1983; *S7*, 1987
Dibenz[*a,j*]acridine	ND–10 ng	Sufficient		2B	*32*, 1983; *S7*, 1987
Dibenzo[*c,g*]carbazole	ND–0.7 ng	Sufficient		2B	*32*, 1983; *S7*, 1987
Benzo[*b*]furan	Present	Sufficient		2B	*63*, 1995a
***N*-nitrosamines**					
N-nitrosodimethylamine	0.1–180 ng	Sufficient		2A	*17*, 1978; *S7*, 1987
N-nitrosoethylmethylamine	ND–13 ng	Sufficient		2B	*17*, 1978; *S7*, 1987
N-nitrosodiethylamine	ND–25 ng	Sufficient		2A	*17*, 1978; *S7*, 1987
N-nitrosopyrrolidine	1.5–110 ng	Sufficient		2B	*17*, 1978; *S7*, 1987
N-nitrosopiperidine	ND–9 ng	Sufficient		2B	*17*, 1978; *S7*, 1987
N-nitrosodiethanolamine	ND–36 ng	Sufficient		2B	*17*, 1978; *77*, 2000
N′-nitrosonornicotine	154–196 ng	Sufficient	Limited	1	*37*, 1985; *S7*, 1987; *89*, in press
4-(methylnitrosamino)-1-(3-pyridyl) 1-butanone	110–133 ng	Sufficient	Limited	1	*37*, 1985; *S7*, 1987; *89*, in press
Aromatic amines					
2-toluidine	30–200 ng	Sufficient	Limited	2A	*S7*, 1987; *77*, 2000
2,6-dimethylaniline	4–50 ng	Sufficient		2B	*57*, 1993
2-naphthylamine	1–22 ng	Sufficient	Sufficient	1	*4*, 1974; *S7*, 1987
4-aminobiphenyl	2–5 ng	Sufficient	Sufficient	1	*1*, 1972; *S7*, 1987
Heterocyclic aromatic amines					
2-amino-9*H*-pyrido[2,3-*b*]indole	25–260 ng	Sufficient		2B	*40*, 1986; *S7*, 1987
2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole	2–37 ng	Sufficient		2B	*40*, 1986; *S7*, 1987
2-amino-3-methylimidazo[4,5-*f*]quinoline	0.3 ng	Sufficient		2A	*S7*, 1987; *56*, 1993
3-amino-1,4-dimethyl-5*H*-pyrido [4,3-*b*]indole	0.3–0.5 ng	Sufficient		2B	*31*, 1983; *S7*, 1987
3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole	0.8–1.1 ng	Sufficient		2B	*31*, 1983; *S7*, 1987
2-amino-6-methylpyrido[1,2-*a*:3′, 2′-*d*]imidazole	0.37–0.89 ng	Sufficient		2B	*40*, 1986; *S7*, 1987
2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole	0.25–0.88 ng	Sufficient		2B	*40*, 1986; *S7*, 1987
2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine	11–23 ng	Sufficient		2B	*56*, 1993

Table 5.1 Continued

Carcinogen[a]	Quantity (per cigarette)	IARC evaluations of evidence of carcinogenicity in humans: In animals	In humans	IARC group[b]	*IARC Monograph*[c] (volume, year)
Aldehydes					
Formaldehyde	10.3–25 µg	Sufficient	Sufficient	1	*S7*, 1987; *62*, 1995b
Acetaldehyde	770–864 µg	Sufficient		2B	*S7*, 1987; *71*, 1999
Phenolic compounds					
Catechol	59–81 µg	Sufficient		2B	*S7*, 1987; *71*, 1999
Caffeic acid	<3 µg	Sufficient		2B	*56*, 1993
Volatile hydrocarbons					
1,3-butadiene	20–40 µg	Sufficient	Limited	2A	*S7*, 1987; *71*, 1999
Isoprene	450–1,000 µg	Sufficient		2B	*60*, 1994; *71*, 1999
Benzene	12–50 µg	Sufficient	Sufficient	1	*29*, 1982; *S7*, 1987
Nitrohydrocarbons					
Nitromethane	0.5–0.6 µg	Sufficient		2B	*77*, 2000
2-nitropropane	0.7–1.2 ng	Sufficient		2B	*S7*, 1987; *71*, 1999
Nitrobenzene	25 µg	Sufficient		2B	*65*, 1996
Miscellaneous organic compounds					
Acetamide	38–56 µg	Sufficient		2B	*S7*, 1987; *71*, 1999
Acrylamide	Present	Sufficient		2A	*S7*, 1987; *60*, 1994
Acrylonitrile	3–15 µg	Sufficient		2B	*S7*, 1987; *71*, 1999
Vinyl chloride	11–15 ng	Sufficient	Sufficient	1	*19*, 1979; *S7*, 1987
1,1-dimethylhydrazine	Present	Sufficient		2B	*4*, 1974; *71*, 1999
Ethylene oxide	7 µg	Sufficient	Limited	1	*60*, 1994; *S7*, 1987
Propylene oxide	0–100 ng	Sufficient		2B	*60*, 1994; *S7*, 1987
Urethane	20–38 ng	Sufficient		2B	*7*, 1974; *S7*, 1987
Metals and inorganic compounds					
Arsenic	40–120 ng	Sufficient	Sufficient	1	*84*, 2004
Beryllium	0.5 ng	Sufficient	Sufficient	1	*S7*, 1987; *58*, 1993
Nickel	ND–600 ng	Sufficient	Sufficient	1	*S7*, 1987; *49*, 1990
Chromium (hexavalent)	4–70 ng	Sufficient	Sufficient	1	*S7*, 1987; *49*, 1990
Cadmium	41–62 ng	Sufficient	Sufficient	1	*S7*, 1987; *58*, 1993
Cobalt	0.13–0.20 ng	Sufficient		2B	*52*, 1991
Lead (inorganic)	34–85 ng	Sufficient	Limited	2A	*23*, 1980; *S7*, 1987; *87*, in press
Hydrazine	24–43 ng	Sufficient		2B	*S7*, 1987; *71*, 1999
Radioisotope polonium-210	0.03–1.0 picocurie	Sufficient		1	*78*, 2001

Source: Adapted from Hoffmann et al. 2001 and International Agency for Research on Cancer 2004 with permission from American Chemical Society, © 2001 and International Agency for Research on Cancer, © 2004.
Note: **IARC** = International Agency for Research on Cancer; **ND** = not detected; **ng** = nanograms; ***S7*** = Supplement 7; **µg** = micrograms.
[a]Virtually all these compounds are known carcinogens in experimental animals, and IARC found sufficient evidence for carcinogenicity in animals for all the compounds.
[b]Using data on cancer in humans and, in some cases, other data, IARC established classifications for compounds as group 1 (carcinogenic to humans), group 2A (probably carcinogenic to humans), and group 2B (possibly carcinogenic to humans).
[c]If more than two IARC evaluations were performed, only the two most recent monographs are listed.

In summary, cigarette smoke contains diverse carcinogens. PAH, *N*-nitrosamines, aromatic amines, 1,3-butadiene, benzene, aldehydes, and ethylene oxide are probably the most important carcinogens because of their carcinogenic potency and levels in cigarette smoke.

Biomarkers of Carcinogens in Smokers

Measurements of carcinogens or their metabolites in urine, blood, and breath can provide convenient and reliable quantitative information on human exposure to carcinogens. The information provided by these measurements, which are biomarkers of exposure, is critical to objective evaluation of carcinogen doses in smokers.

Urinary Biomarkers

Urinary biomarkers are the most widely applied biomarkers of carcinogen exposure in smokers (Hecht 2002b). Urine is relatively simple to obtain in large quantities, and obtaining study participants' consent and specimens for testing is almost never a difficulty. Carcinogens in cigarette smoke and/or their metabolites are frequently present in substantial quantities in urine. Therefore, reliable quantitation is generally feasible. This section provides an overview of some of the urinary biomarkers most commonly used to estimate carcinogen doses in smokers. The chemical structures of all compounds discussed in this section are illustrated in Figure 5.2.

Polycyclic Aromatic Hydrocarbons

Phenanthrene metabolites. Phenanthrene is the simplest PAH with a bay region (the region of a molecule between positions 4 and 5), a feature closely associated with the carcinogenic activity of PAHs (Figure 5.2). Phenanthrene, however, is inactive as a carcinogen (LaVoie and Rice 1988). Concentrations of phenanthrene in mainstream smoke range from 85 to 620 nanograms (ng) per cigarette (IARC 1986). Studies have quantified the phenanthrene metabolites phenanthrols, phenanthrene dihydrodiols, and *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (*trans, anti*-PheT) in human urine (Hecht 2002b). Levels of phenanthrols in human urine differed between smokers and nonsmokers in some studies but not in others (reviewed in Carmella et al. 2004a). There are sources of phenanthrene exposure other than cigarette smoke, and all people have phenanthrene metabolites in their urine. This finding is well documented in environmental and occupational settings with high exposures to PAH (Grimmer et al. 1993, 1997; Angerer et al. 1997). One metabolite of phenanthrene, *trans, anti*-PheT, results from the diol epoxide metabolic activation pathway common to many carcinogenic PAHs. This metabolite is a promising new biomarker for PAH uptake and metabolic activation and can be readily quantified by gas chromatography (GC)-negative ion chemical ionization–mass spectrometry (MS) (Hecht et al. 2003). Levels of *trans, anti*-PheT are higher in smokers than in nonsmokers (Hecht et al. 2003).

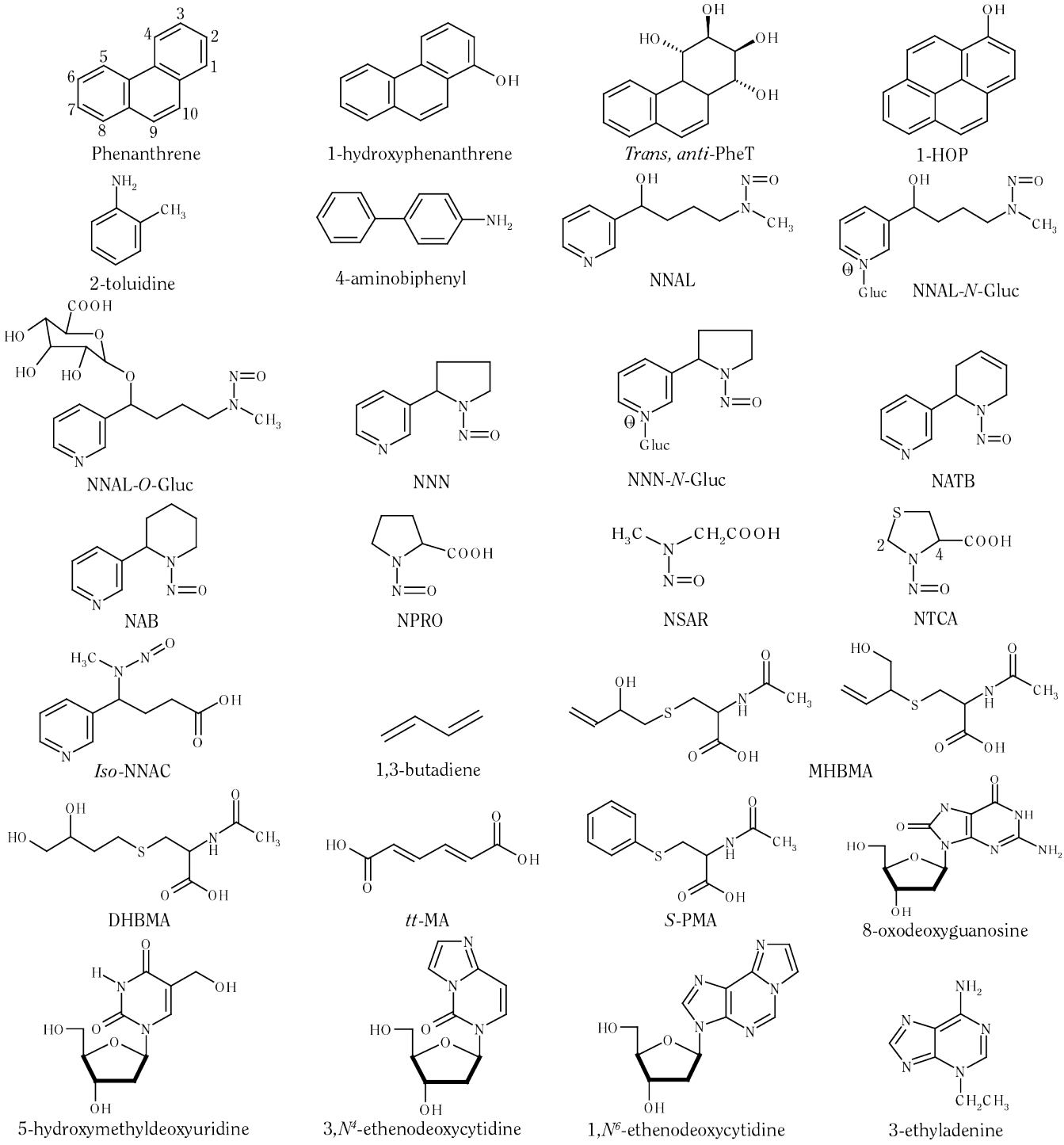
1-hydroxypyrene. Pyrene is a noncarcinogenic component in all PAH mixtures; levels in mainstream cigarette smoke were 50 to 270 ng per cigarette (IARC 1986). The major metabolite of pyrene is 1-hydroxypyrene (1-HOP) glucuronide, which can be measured in urine (Jongeneelen et al. 1985). To quantify 1-HOP in urine, enzymatic hydrolysis is used to release 1-HOP, which is then enriched by reverse-phase chromatography and quantified by high-performance liquid chromatography (HPLC) with fluorescence detection. Studies have described variations of this method (Carmella et al. 2004b). Hundreds of studies of occupational and environmental PAH exposure have measured 1-HOP as a surrogate marker for total PAH exposure. In reviews of the data on the effects of smoking (Jongeneelen 1994, 2001; Van Rooij et al. 1994; Levin 1995; Heudorf and Angerer 2001; Hecht 2002b), most of the studies noted that 1-HOP levels in the urine of smokers were about twice as high as those in the urine of nonsmokers, although some studies have reported greater differences. Levels of 1-HOP may be influenced by genetic polymorphisms in carcinogen-metabolizing enzymes (Alexandrie et al. 2000; Nerurkar et al. 2000; Nan et al. 2001; van Delft et al. 2001).

Other metabolites of polycyclic aromatic hydrocarbons. Studies examining urine biomarkers have measured phenolic metabolites of naphthalene and a variety of PAHs, which show promise as urinary biomarkers of PAH uptake from cigarette smoke (Hecht 2002b; Smith et al. 2002a,b; Serdar et al. 2003). Studies have quantified B[*a*]P metabolites in urine, but the levels are generally low, limiting their routine application in large studies (Hecht 2002b).

Aromatic Amines and Heterocyclic Aromatic Amines

Researchers have quantified aromatic amines, but not their metabolites, in human urine. In one study, levels of 2-toluidine excreted by smokers were 6.3 ± 3.7 (standard deviation [SD]) µg/24 hours and levels excreted by nonsmokers were 4.1 ± 3.2 (SD) µg/24 hours. The difference was not significant (El-Bayoumy et al. 1986). Another investigation reported urine levels of 2-toluidine that were higher in smokers than in nonsmokers (Riffelmann et al.

Figure 5.2 Chemical structures of biomarkers of carcinogen exposure



Note: **1-HOP** = 1-hydroxypyrene; **DHBMA** = dihydroxybutylmercapturic acid; ***iso*-NNAC** = 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid; **MHBMA** = monohydroxybutenylmercapturic acid; **NAB** = *N′*-nitrosoanabasine; **NATB** = *N′*-nitrosoanatabine; **NNAL** = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; **NNAL-*N*-Gluc** = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol-*N*-glucuronide; **NNAL-*O*-Gluc** = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol-*O*-glucuronide; **NNN** = *N′*-nitrosonornicotine; **NNN-*N*-Gluc** = *N′*-nitrosonornicotine-*N*-glucuronide; **NPRO** = *N*-nitrosoproline; **NSAR** = *N*-nitrososarcosine; **NTCA** = *N*-nitrosothiazolidine 4-carboxylic acid; ***S*-PMA** = *S*-phenylmercapturic acid; ***trans, anti*-PheT** = *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene; ***tt*-MA** = *trans,trans*-muconic acid.

1995). There appear to be significant sources of human uptake of 2-toluidine in addition to cigarette smoke. Although these sources are not fully characterized, diet is one likely source. Amounts of 4-ABP excreted by smokers (78.6 ± 85.2 [SD] ng/24 hours) were similar to those excreted by nonsmokers (68.1 ± 91.5 ng/24 hours), and amounts of 2-naphthylamine excreted by smokers (84.5 ± 102.7 ng/24 hours) were similar to those excreted by nonsmokers (120.8 ± 279.2 ng/24 hours) (Grimmer et al. 2000). In another study, Hb adducts appeared to be better biomarkers of exposure to aromatic amines from tobacco smoke than were urinary levels of metabolites (Skipper and Tannenbaum 1990).

Researchers have measured urinary biomarkers of heterocyclic aromatic amines mainly in studies of dietary exposure. Little information is available on the contributions of cigarette smoke to urinary levels of heterocyclic aromatic amines (Hecht 2002b).

N-*Nitrosamines*

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides. In rodents and humans, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides are quantitatively significant metabolites of NNK (Hecht 1998). Both NNAL and NNK are pulmonary carcinogens with particularly strong activity in rats; NNAL also induces pancreatic tumors (Hecht 1998). Glucuronidation of NNAL at the pyridine nitrogen gives NNAL-*N*-glucuronide, and conjugation at the carbinol oxygen yields NNAL-*O*-glucuronide. Both NNAL-*N*-glucuronide and NNAL-*O*-glucuronide exist as a mixture of two diastereomers, and each diastereomer is a mixture of *E*- and *Z*-rotamers (Upadhyaya et al. 2001). The NNAL-*N*-glucuronide and NNAL-*O*-glucuronide isomers are collectively referred to as NNAL glucuronides. (*R*)-NNAL-*O*-glucuronide does not induce tumors in mice (Upadhyaya et al. 1999). The (*S*) isomer has not been tested, but glucuronidation generally deactivates a carcinogenic metabolite in any event.

NNAL and NNAL glucuronides can be readily determined in urine by using GC with nitrosamine-selective detection (Carmella et al. 1993, 1995; Hecht et al. 1999) and by MS methods (Carmella et al. 1993, 1999; Parsons et al. 1998; Lackmann et al. 1999; Hecht et al. 2001; Byrd and Ogden 2003). Typical levels are about 1 nanomole (nmol) of NNAL in 24 hours and 2.2 nmol of NNAL glucuronides in 24 hours, with no detection of unchanged NNK. NNAL and NNAL glucuronides are absolutely specific to exposure to tobacco and have not been detected in the urine of nontobacco users unless they were exposed to secondhand smoke. Because NNAL is not present in cigarette smoke, the origin of NNAL and NNAL glucuronides found in urine is the metabolism of NNK. Most investigations demonstrate a correlation between NNAL plus NNAL glucuronides and cotinine in urine. This finding indicates that NNAL and NNAL glucuronides are a biomarker of uptake of the lung carcinogen NNK and that cotinine is a biomarker of nicotine uptake. Ratios of NNAL glucuronides to NNAL vary at least 10-fold in smokers. This ratio could be a potential indicator of cancer risk, because NNAL glucuronides are detoxification products, whereas NNAL is carcinogenic (Carmella et al. 1995; Richie et al. 1997). In human urine, (*S*)-NNAL-*O*-glucuronide is the predominant diastereomer of NNAL-*O*-glucuronide, and the level of (*S*)-NNAL is slightly higher than that of (*R*)-NNAL (Carmella et al. 1999). (*S*)-NNAL is the more tumorigenic enantiomer of NNAL in the A/J mouse lung (Upadhyaya et al. 1999). NNAL and NNAL glucuronides are released slowly from the human body only after smoking cessation. This finding has been linked to a particularly strong retention of (*S*)-NNAL, possibly at a receptor site (Hecht et al. 1999; Zimmerman et al. 2004). Recent studies indicate that levels of NNAL plus NNAL-glucuronides are not only biomarkers of NNK exposure but also are biomarkers of risk for lung cancer in smokers (Church et al. 2009; Yuan et al. 2009)

N′-nitrosonornicotine, N′-nitrosoanatabine, N′-nitrosoanabasine, and their pyridine-N-glucuronides. Researchers developed a method to analyze NNN, *N′*-nitrosoanatabine (NATB), *N′*-nitrosoanabasine (NAB), and their pyridine *N*-glucuronides (e.g., NNN-*N*-glucuronide) in human urine. NATB and NAB are tobacco-specific nitrosamines that like NNN and NNK are formed by the nitrosation of tobacco alkaloids (Hecht and Hoffmann 1988). Studies show that NATB is not carcinogenic but that NAB is a weak esophageal carcinogen in rats (Hecht 1998). Mean levels of total NNN, NATB, and NAB in the urine of 14 smokers were 0.18 ± 0.22 SD, 0.19 ± 0.20, and 0.040 ± 0.039 picomoles/mg of creatinine, respectively. These compounds have not been detected in the urine of nonsmokers with no exposure to secondhand smoke.

Nitrosamino acids. Researchers have used the *N*-nitrosoproline (NPRO) test to compare endogenous nitrosation in smokers and nonsmokers (Bartsch et al. 1989). The results of clinical studies indicate that the frequency of endogenous formation of NPRO is higher in smokers than in nonsmokers and that it may be enhanced by thiocyanate catalysis (Bartsch et al. 1989; Tsuda and Kurashima 1991; Tricker 1997). However, some population-based studies document similar levels of NPRO in smokers and nonsmokers, because this precursor biomarker for nitrosamine formation is primarily from dietary sources (Tricker 1997).

The major nitrosamino acids present in human urine are *N*-nitrososarcosine, *N*-nitrosothiazolidine 4-carboxylic acid (NTCA), and *trans*- and *cis*-isomers of *N*-nitroso-2-methylthiazolidine 4-carboxylic acid (NMTCA) (Bartsch et al. 1989; Tsuda and Kurashima 1991). NTCA and NMTCA are formed by reactions of formaldehyde or acetaldehyde with cysteine, followed by nitrosation. Some studies demonstrate increased levels of urinary NTCA and NMTCA in smokers (Tsuda and Kurashima 1991). Although some studies show a correlation between total nitrosamino acids and urinary nicotine plus cotinine among smokers (Malaveille et al. 1989), other studies show mixed results (Tricker 1997). Collectively, the available data support the concept that nitrosamines can be formed endogenously in smokers under some conditions.

Studies suggest that 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid is a potential monitor of endogenous nitrosation of nicotine (Djordjevic et al. 1991). However, researchers could not find any evidence for its formation after oral administration of nicotine or cotinine to persons abstaining from smoking (Tricker et al. 1993).

1,3-butadiene. The major urinary metabolites of 1,3-butadiene are monohydroxybutenyl-mercapturic acids (MHBMAs) and dihydroxybutyl-mercapturic acid (DHBMA). Levels of MHBMA were 86.4 ± 14.0 (SD) µg/24 hours in smokers and 12.5 ± 1.0 µg/24 hours in nonsmokers—a significant difference (Urban et al. 2003). Corresponding levels of DHBMA were 644 ± 90 (SD) µg/24 hours in smokers and 459 ± 72 µg/24 hours in nonsmokers, which were not significantly different (Urban et al. 2003). DHBMA does not appear to be specific to exposure to 1,3-butadiene and is probably not a useful biomarker. Hb adducts have also proven useful as markers of long-term exposure to 1,3-butadiene. The long half-lives of these adducts result in an average measurement that is more time weighted than that for some other metabolites (e.g., urinary meta-bolites) (Swenberg et al. 2001; Boysen et al. 2007).

Benzene. One path of benzene metabolism proceeds by ring oxidation, ultimately by ring cleavage to *trans,trans*-muconaldehyde, and finally to *trans,trans*-muconic acid (*tt*-MA), a metabolite widely used as a biomarker of benzene uptake (Scherer et al. 1998). Most studies have found significantly elevated levels of *tt*-MA in the urine of smokers (Scherer et al. 1998; Cocco et al. 2003; Lee et al. 2005). Levels of *tt*-MA were 1.4 to 4.8 times higher in smokers than in nonsmokers, and the additional amount of *tt*-MA excreted by smokers ranged from 0.022 to 0.20 mg/gram of creatinine (Scherer et al. 1998). However, sorbic acid, a food constituent that can be transformed metabolically into *tt*-MA, can contribute to urinary levels of *tt*-MA and thereby decrease its specificity as a biomarker for benzene uptake (Scherer et al. 1998; Pezzagno et al. 1999).

S-phenylmercapturic acid (*S*-PMA) is formed by the metabolism of the glutathione conjugate of benzene oxide and has the potential to be specific for benzene uptake (Stommel et al. 1989; van Sittert et al. 1993; Boogaard and van Sittert 1995, 1996; Qu et al. 2000). In one study, *S*-PMA levels were significantly higher in smokers (1.71 micromoles [µmol]/mole of creatinine) than those in nonsmokers (0.94 µmol/mole of creatinine), whereas *tt*-MA levels were not significantly different (Boogaard and van Sittert 1996). Researchers believe that *S*-PMA and *tt*-MA are the most sensitive biomarkers for low levels of exposure to benzene (Qu et al. 2000, 2003).

Phenol, hydroquinone, catechol, and 1,2,4-trihydroxybenzene are also urinary metabolites of benzene. Studies relating urinary levels of these metabolites to occupational exposure to benzene have mixed results, because background levels of the metabolites are high (Inoue et al. 1988, 1989; Ong et al. 1995, 1996; Qu et al. 2000). Urinary catechol levels did not differ significantly between smokers and nonsmokers (Carmella et al. 1982), and diet has been shown to be a major source of urinary catechol (Carmella et al. 1982).

Products of oxidative damage. The presence of free radicals and oxidants in cigarette smoke can lead to oxidative DNA damage and the subsequent formation of products such as 8-oxodeoxyguanosine, thymine glycol, thymidine glycol, and 5-hydroxymethyluracil. Repair of these modified DNA constituents ultimately leads to their excretion in urine. Researchers have frequently quantified 8-oxodeoxyguanosine in urine of smokers and nonsmokers (Loft and Poulsen 1998; Prieme et al. 1998; Renner et al. 2000). Cigarette smoking usually results in levels of 8-oxodeoxyguanosine in urine that modestly increase to 16 to 50 percent higher than those in nonsmokers, but studies have also reported negative results (Nia et al. 2001; Harman et al. 2003; Mukherjee et al. 2004). Smoking cessation caused a 21-percent decrease in the excretion of 8-oxodeoxyguanosine (Prieme et al. 1998). Longitudinal studies have not shown convincing increases in urinary 8-oxodeoxyguanosine that were attributable to smoking, and a complex pattern of factors may affect background levels of this biomarker in urine (Kasai et al. 2001; Pilger et al. 2001; Mukherjee et al. 2004). Studies on the effects of smoking on urinary levels of 5-hydroxymethyluracil or 5-hydroxymethyldeoxyuridine have obtained mixed results (Pourcelot et al. 1999; Harman et al. 2003). One study showed a correlation between smoking and urinary excretion of 3,N^4-ethenodeoxycytidine, which may result from endogenous lipid peroxidation (Chen et al. 2004a). Studies have also detected 1,N^6-ethenodeoxyadenosine in human urine, but no differences were observed between levels in smokers and those in nonsmokers (Hillestrøm et al. 2004).

Products of alkylating agents. The reaction of alkylating agents with DNA forms alkyladenines, alkylguanines, and other products (Singer and Grunberger 1983). Alkylation at the 3-position of deoxyadenosine or at the 7-position of deoxyguanosine results in products with an unstable glycosidic bond. These products are readily removed from DNA, either spontaneously or by glycosylases, which results in the urinary excretion of 3-alkyladenines and 7-alkylguanines. Studies have more extensively investigated 3-alkyladenines as biomarkers of exposure to alkylating agents, because researchers expected the background levels of 3-alkyladenines in urine to be lower than those of 7-alkylguanines. However, substantial amounts of 3-methyladenine occur in the diet (Prevost et al. 1993; Fay et al. 1997). Nevertheless, two controlled studies demonstrated an increase in the urinary excretion of 3-methyladenine among smokers (Kopplin et al. 1995; Prevost and Shuker 1996). Another study found lower background levels of 3-ethyladenine than those of 3-methyladenine (Prevost et al. 1993). Two studies demonstrated convincing increases in urinary levels of 3-ethyladenine in smokers, indicating the presence in cigarette smoke of an unidentified ethylating agent (Kopplin et al. 1995; Prevost and Shuker 1996). There was no effect from smoking on urinary levels of 3-(2-hydroxyethyl)adenine (Prevost and Shuker 1996). A population-based study found higher levels of both 3-methyladenine and 7-methylguanine in smokers than in nonsmokers, and a second study found no difference in the 3-methyladenine levels (Shuker et al. 1991; Stillwell et al. 1991).

Metals. Studies of urinary cadmium have most consistently demonstrated differences between smokers and nonsmokers. Large studies in Germany and the United States showed increases in urinary cadmium levels with age and smoking (IARC 2004). These results were consistent with those of other studies.

Breath and Blood Biomarkers

Benzene, 1,3-butadiene, and a variety of volatile organic compounds including xylenes, styrene, isoprene, 2,5-dimethylfuran, ethane, and octane were measured in expired air; levels were generally higher in smokers than in nonsmokers (Gordon et al. 2002; Perbellini et al. 2003; IARC 2004). Levels of benzene and 1,3-butadiene in the breath of smokers were 360 and 522 µg/cubic meter (m^3), respectively (Gordon et al. 2002). In another study, mean benzene levels ranged from 58.1 to 81.3 µg/m^3, depending on the cigarette brand (IARC 2004).

Studies have quantified volatile organic compounds, including benzene and styrene, in the blood of smokers; levels were generally higher than those in the blood of nonsmokers. Benzene levels in blood were significantly associated with the number of cigarettes smoked (IARC 2004). Cadmium levels were also higher in the blood of smokers. Measurements of NNAL in blood demonstrated a mean level of 42 femtomoles/milliliter of plasma in smokers; NNAL was not detected in nonsmokers (Carmella et al. 2005). Cigarette smoke induces oxidative damage as determined by elevated blood protein carbonyls (Reznick et al. 1992) and blood protein-bound glutathione (Muscat et al. 2004). F_2-isoprostane levels, which are biomarkers of oxidative damage, were higher in the plasma of smokers than in the plasma of nonsmokers and decreased with vitamin C treatments (Morrow et al. 1995; Dietrich et al. 2002). Hb adducts and DNA adducts in white blood cells are discussed in the next section.

Summary

Quantitative analysis of carcinogens or their metabolites in urine, breath, and blood provides a convenient and reliable method of comparing carcinogen exposure among smokers and between smokers and nonsmokers. The most extensive measurements have been made in urine. Urinary biomarkers of several major types of carcinogens in cigarette smoke are reliable indicators of exposure. These biomarkers include *trans, anti*-PheT and 1-HOP for PAH; total NNAL (NNAL plus NNAL glucuronides) for NNK; MHBMA for 1,3-butadiene; and *tt*-MA and *S*-PMA for benzene. The measurements provide good estimates of minimum doses of relevant carcinogens in smokers and allow comparisons with those in nonsmokers. The total carcinogen dose is generally difficult to calculate because the extent of conversion of a given carcinogen to the measured metabolite is usually unknown and can vary widely among individuals. Nevertheless, the results of these studies are illuminating. They show, for example, that levels of metabolites of benzene (about 1,100 nmol/24 hours of *tt*-MA and 8 nmol/24 hours of *S*-PMA) and 1,3-butadiene (about 340 nmol/24 hours of MHBMA) exceed levels of other biomarkers (e.g., about 3 nmol/24 hours of NNAL plus NNAL glucuronides and 2 nmol/24 hours of 1-HOP). These results are consistent with the levels of benzene and 1,3-butadiene in cigarette smoke, which were higher than those of NNK and PAH.

However, metabolites of benzene and a metabolite of 1,3-butadiene (DHBMA) are also found in nonsmokers in considerable quantities. Comparisons of smokers and nonsmokers demonstrate that total NNAL is the most discriminatory carcinogen biomarker because the only source of the parent carcinogen NNK is tobacco products. Total NNAL is not detected in nonsmokers unless they have been exposed to secondhand tobacco smoke. Therefore, this biomarker is particularly useful for comparing carcinogen uptake in smokers who, for example, use

different tobacco products, because the measurements are not confounded by other exposures such as diet, occupation, or the general environment.

Metabolic Activation and Detoxification of Carcinogens

Most of the carcinogens listed in Table 5.1 require metabolic activation to become intermediate agents, generally electrophiles, which react with nucleophilic sites in DNA to form DNA adducts. All PAHs, heterocyclic compounds, *N*-nitrosamines, aromatic amines, and heterocyclic aromatic amines in cigarette smoke require metabolic activation. Other compounds in Table 5.1 that require metabolic activation are 1,3-butadiene, isoprene, benzene, nitromethane, 2-nitropropane, nitrobenzene, acrylamide, vinyl chloride, and urethane. Detoxification reactions in most cases compete with metabolic activation and also affect the disposition of compounds that do not require metabolic activation, such as ethylene oxide.

An overview of the metabolism of six carcinogens in tobacco smoke that are implicated in the formation of DNA adducts identified in human tissues is presented in Figure 5.3. The six carcinogens are B[*a*]P, NNK, *N*-nitrosodimethylamine (NDMA), NNN, ethylene oxide, and 4-ABP.

The major metabolic activation pathway of B[*a*]P that results in DNA adducts identified in human tissues is the conversion to the highly mutagenic B[*a*]P-7,8-diol-9,10-epoxides (BPDEs). The formation of BPDE occurs in three steps: the metabolism of B[*a*]P to B[*a*]P-7,8-epoxide; hydration of B[*a*]P-7,8-epoxide to give the dihydrodiol B[*a*]P-7,8-diol; and further epoxidation to produce BPDE. One of the four enantiomers is strongly carcinogenic and reacts with DNA to form adducts at N^2 of deoxyguanosine (BPDE-N^2-deoxyguanosine) (Cooper et al. 1983; IARC 1983; Thakker et al. 1985). This adduct was also observed in animals treated with B[*a*]P.

Two other proposed metabolic activation pathways of B[*a*]P exist, but the evidence for their involvement in DNA adduct formation in laboratory animals and humans is not as strong as that for BPDE. One pathway involves the conversion of B[*a*]P-7,8-diol to the corresponding catechol metabolite catalyzed by dihydrodiol dehydrogenase. The catechol can undergo redox cycling to produce a quinone reactive with DNA, and the redox cycling process can produce oxidative damage to DNA (Penning et al. 1999; Yu et al. 2002). Another metabolic activation process occurs when one electron oxidation of B[*a*]P produces unstable depurinating DNA adducts that can lead to apurinic sites and miscoding (Casale et al. 2001). A common mechanism of metabolic activation for a number of PAHs is the formation of diol epoxides in which the epoxide ring is in the bay region of the PAH molecule, similar to that in BPDE (Conney 1982; Thakker et al. 1985; Baird and Ralston 1997). Competing with B[*a*]P metabolic activation processes are detoxification pathways leading to (1) phenols through direct hydroxylation or rearrangement of initially formed epoxides, (2) dihydrodiols through hydration of epoxides catalyzed by epoxide hydrolase, and (3) formation of glutathione, glucuronide, and sulfate conjugates. Researchers have also observed the formation of quinone metabolites from initial hydroxylation at the 6-position, followed by further oxidation (Cooper et al. 1983).

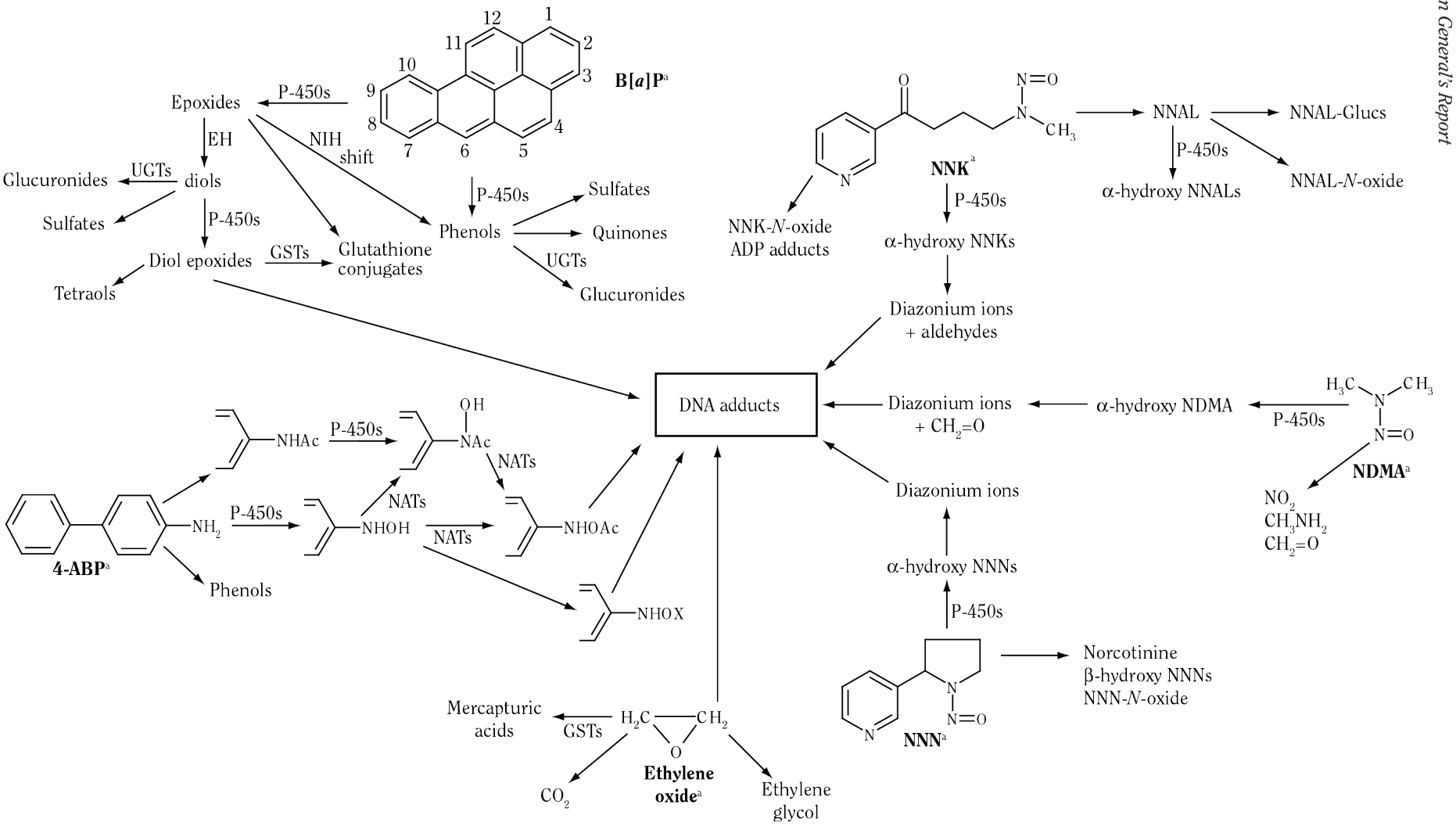
Metabolic activation of NDMA occurs by α-hydroxylation and leads to an unstable α-hydroxymethyl metabolite. This compound spontaneously loses formaldehyde and forms methanediazohydroxide, the same intermediate agent produced in the α-methylene hydroxylation of NNK. Researchers also observed the consequent formation of methyl DNA adducts such as 7-methylguanine, O^6-methylguanine, and O^4-methylthymidine. Denitrosation produces nitrite and methylamine and is a detoxification pathway (Preussmann and Stewart 1984; Hecht and Samet 2007). The metabolism of NNK and NDMA forms aldehydes, whose roles in carcinogenesis are unclear, but studies show that formaldehyde reacts with DNA and protein to form cross-links and other products (Chaw et al. 1980; Beland et al. 1984; Hecht and Samet 2007).

α-hydroxylation of NNN adjacent to the pyridine ring produces the same intermediate agent formed by methyl hydroxylation of NNK, which leads to pyridyloxobutyl (POB)-DNA adducts (Hecht 1998). α-hydroxylation distal from the pyridine ring also produces a reactive diazohydroxide, but its reactions with DNA have not been fully characterized. The acetate esters of the α-hydroxy-NNN metabolites are mutagenic (Hecht 1998; Hecht and Samet 2007). β-hydroxylation of NNN, a minor pathway, and pyridine-*N*-oxidation are detoxification reactions. NNN is also detoxified by denitrosation and oxidation to produce norcotinine, and by glucuronidation of the pyridine ring (Hecht 1998; Stepanov and Hecht 2005; Hecht and Samet 2007).

Ethylene oxide reacts directly with DNA to form 7-(2-hydroxyethyl)guanine and other adducts (IARC 1994; Hecht and Samet 2007). Competing detoxification pathways involve glutathione conjugation and excretion of mercapturic acids (IARC 1994).

4-ABP is metabolically activated by *N*-hydroxylation (Kadlubar and Beland 1985; Hecht and Samet 2007). Conjugation of the resulting hydroxylamine with acetate or other groups, such as sulfate, ultimately produces nitrenium ions, which react with DNA and form adducts

Figure 5.3 Metabolism of six carcinogens in tobacco smoke that produce DNA adducts identified in the lungs of smokers



Source: Adapted from Cooper et al. 1983; Preussmann and Stewart 1984; Kadlubar and Beland 1985; International Agency for Research on Cancer 1994; and Hecht 1998, 1999.
Note: In 4-ABP scheme, X represents conjugates such as glucuronide or sulfate. **4-ABP** = 4-aminobiphenyl; **Ac** = acetyl; **ADP** = adenosine diphosphate; **B[*a*]P** = benzo[*a*]pyrene; **EH** = epoxide hydrolase; **Glucs** = glucuronides; **GSTs** = glutathione-*S*-transferases; **NATs** = *N*-acetyltransferases; **NDMA** = *N*-nitrosodimethylamine; **NIH shift** = National Institutes of Health phenomenon of hydroxylation-induced intramolecular migration; **NNAL** = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; **NNK** = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; **NNN** = *N′*-nitrosonornicotine; **P-450s** = cytochrome P-450 enzymes; **UGTs** = uridine-5′-diphosphate-glucuronosyltransferases.
[a]Carcinogens in tobacco smoke.

mainly at C-8 of guanine. Other aromatic amines, as well as heterocyclic aromatic amines, are predominantly activated metabolically in similar ways. Acetylation of 4-ABP can be a detoxification pathway if it is not followed by *N*-hydroxylation. Ring hydroxylation and conjugation of the phenols result in detoxification.

Two other important carcinogens from cigarette smoke that require metabolic activation are benzene and 1,3-butadiene. DNA adducts of these compounds have not been detected in human samples. However, there is considerable information on their conversion to intermediate agents that react with DNA.

Benzene is metabolized to benzene epoxide, which is in equilibrium with its 7-member ring tautomer oxepin (Scherer et al. 2001; Hecht and Samet 2007). Researchers have observed the reaction of benzene epoxide with DNA to produce 7-phenylguanine. Further metabolism of benzene epoxide-oxepin can occur in a variety of ways. One way is nonenzymatic rearrangement to phenol, which can be further hydroxylated to hydroquinone, catechol, and 1,2,4-trihydroxybenzene. These metabolites can then be conjugated as glucuronides or sulfates. Hydroquinone can be further oxidized to benzoquinone, which can bind to DNA, or hydration catalyzed by epoxide hydrolase can produce benzene dihydrodiol, which can then be converted to catechol or *tt*-MA. Another possibility involves conjugation with glutathione that ultimately produces *S*-PMA. Other pathways of benzene metabolism result in the formation of biphenyl and benzene dioxetane, which can also lead to *tt*-MA (Scherer et al. 2001; Hecht and Samet 2007). Studies have detected nitrobenzene, nitrobiphenyl, and nitrophenol isomers in the bone marrow of mice treated with benzene; these isomers presumably formed from reactions of benzene with endogenously generated nitric oxide (Chen et al. 2004b; Hecht and Samet 2007).

1,3-butadiene is metabolically activated by epoxidation to give a monoepoxide that can be further metabolized to a diepoxide and a dihydrodiol epoxide (van Sittert et al. 2000; Hecht and Samet 2007), which all form DNA adducts. The dihydrodiol epoxide also produces cross-links in DNA and may be the most important of these intermediate agents (Park and Tretyakova 2004; Hecht and Samet 2007). The epoxides can be hydrated to dihydrodiols and conjugated by reactions with glutathione. 1,3-butadiene metabolism can also lead to epoxidation and formation of *N*-terminal Hb adducts, providing a longer-term, "time-weighted" measurement of exposure (Swenberg et al. 2001).

Although details remain to be determined, the major pathways of metabolic activation and detoxification of some of the principal carcinogens in cigarette smoke are well established. Reactive intermediate agents that are critical in forming DNA adducts include diol epoxides of PAH, diazonium ions generated by α-hydroxylation of nitrosamines, nitrenium ions formed from esters of *N*-hydroxylated aromatic amines, and epoxides such as ethylene oxide. Glutathione and glucuronide conjugation play major roles in the detoxification of carcinogens in cigarette smoke.

Enzymology of Carcinogen Metabolism

Introduction

A number of enzyme families are important in both the activation and detoxification of carcinogens in cigarette smoke, including P-450s, GSTs, UGTs, *N*-acetyltransferases (NATs), epoxide hydrolases, and sulfotransferases. The importance of each enzyme to the activation or detoxification of a particular carcinogen depends on characteristics of both the carcinogen (size, polarity, and lipophilicity) and the enzyme (structure, tissue distribution, and regulation of expression). The large number of carcinogens in cigarette smoke and the wide variety of enzymes involved in metabolizing these carcinogens preclude a comprehensive discussion of current understanding of the contribution of each enzyme to every pathway. Therefore, the goals of this presentation are to introduce the families of enzymes involved and to highlight some of the activation and detoxification reactions for specific enzymes and carcinogens.

Cytochrome P-450 Enzymes

P-450s, encoded by *CYP* genes, are microsomal enzymes that catalyze the oxidation of myriad chemicals, including many of the carcinogens in cigarette smoke. Sequencing the human genome has identified 57 *CYP* genes, about 15 of which are considered important in the metabolism of xenobiotics (Nelson 2003; Guengerich 2004). Among the P-450s encoded by these genes, a reasonable argument can be made for the role of six (1A1, 1B1, 1A2, 2A6, 2A13, and 2E1) as important catalysts for the metabolic activation of carcinogens in cigarette smoke. PAHs are metabolized by P-450s 1A1 and 1B1 (Shimada and Fujii-Kuriyama 2004), aromatic amines by P-450 1A2 (Kim and Guengerich 2005), and *N*-nitrosamines by P-450s 2A6, 2A13, and 2E1 (Yoo et al. 1988; Guengerich et al. 1991; Yamazaki et al. 1992; Jalas et al. 2005; Wong et al. 2005a). P-450 2E1 also catalyzes the epoxidation of benzene and 1,3-butadiene (Guengerich et al. 1991; Bolt et al. 2003).

P-450s 1A1 and 1B1 are expressed in a wide range of extrahepatic tissues and catalyze both the activation and detoxification reactions of PAH metabolism (Shimada and Fujii-Kuriyama 2004). In addition, both enzymes are inducible by the PAHs in cigarette smoke (Nebert et al. 2004). Induction of these two enzymes is generally mediated by the aryl hydrocarbon receptor, but differences may exist in the mode of induction for each enzyme. Historically, researchers believed that P-450 1A1 was the predominant P-450 catalyst for the metabolism of PAHs, particularly in the lung. However, the discovery of P-450 1B1 (Sutter et al. 1994) clarified the equal or more predominant role P-450 1B1 may play in the activation of PAHs compared with that of P-450 1A1 (Shimada et al. 1996). Studies show that P-450 1B1, which is heterologously expressed, activates the proximate carcinogen of many PAHs and that in several cases, P-450 1B1 was more efficient than P-450 1A1 (Shimada et al. 1996). For example, (+)-B[*a*]P-7,8-diol was activated to a genotoxic species to a greater extent by P-450 1B1 than by P-450 1A1 (Shimada et al. 1996). In addition, the ratio of the maximum velocity (V_{max}) of an enzyme-catalyzed reaction to the concentration of a substrate that leads to half-maximal velocity (K_m) for the formation of B[*a*]P-7,8-diol was 3.5-fold greater for P-450 1B1 than for P-450 1A1 (Shimada et al. 1999). (The V_{max} to K_m ratio measures an enzyme's efficiency.) In contrast, P-450 1A1 was a better catalyst of B[*a*]P 3-hydroxylation, which is a detoxification pathway (Shimada et al. 1997). Taken together, these data indicate that P-450 1B1 activity, but not P-450 1A1 activity, may contribute to individual susceptibility to B[*a*]P-induced carcinogenesis. However, cigarette smoke has many different PAH carcinogens, and either P-450 1B1 or 1A1 individually or together may be important in their metabolic activation.

As noted previously (see "Cytochrome P-450 Enzymes" earlier in this chapter), both P-450 1A1 and 1B1 are inducible by PAHs. Studies have reported that levels of messenger RNA (mRNA) and protein of both P-450s were higher in the lungs of smokers than in the lungs of lifetime nonsmokers (Willey et al. 1997; Kim et al. 2004a; Port et al. 2004). The levels of P-450 1A1 and 1B1 proteins were correlated in lung microsomes from all participants who smoked. However, the absolute amount of P-450 1A1 in each person was, on average, more than 10-fold greater than the amount of P-450 1B1 (Kim et al. 2004a). Despite the ability of P-450 1B1 to more efficiently mediate the activation of some PAHs, the higher P-450 1A1 levels may result in each enzyme contributing similarly to the total metabolism of PAHs. An equally important factor in determining the role of these P-450s in the activation of PAHs in cigarette smoke is the variability in the induction of P-450s across individuals. Researchers do not know whether the responsible mechanism is common to both P-450s 1A1 and 1B1.

Studies have characterized P-450 1A2 as the best catalyst for aromatic amine *N*-oxidation, which is the first step in the activation of these bladder carcinogens (Butler et al. 1989; Landi et al. 1999; Kim and Guengerich 2005). P-450 1A2 is both constitutively expressed and inducible in the liver. The induction of P-450 1A2 is mediated by the aryl hydrocarbon receptor, and hepatic levels vary more than 60-fold from person to person (Nebert et al. 2004). Cigarette smoking induces the levels of this enzyme in the liver. Researchers have also reported that P-450s 1A1 and 1B1 metabolically activate a number of aromatic amines, including 4-ABP, and may play a role in extrahepatic metabolism (Shimada et al. 1996).

Although hepatic P-450 2A6 catalyzes the metabolic activation of NNK (Yamazaki et al. 1992; Jalas et al. 2005), P-450 2A6 is not a particularly efficient catalyst. The extrahepatic P-450 2A13 might be a more important catalyst of the activation of this carcinogen (Jalas et al. 2005). P-450 2A13 is expressed in the lung (Su et al. 2000) and catalyzes the α-hydroxylation of NNK significantly more efficiently than does P-450 2A6. P-450 2A13 is an excellent catalyst of NNK α-hydroxylation, with a low K_m and a high V_{max}. P-450 2A13 is the sole catalyst of NNK α-hydroxylation in human fetal nasal tissue and is considered equally important in the lung (Wong et al. 2005b). P-450 2E1 has also catalyzed the activation of both NNN and NNK (Yamazaki et al. 1992). However, the catalytic efficiencies of these reactions are poor (Hecht 1998; Jalas et al. 2005). Studies have identified P-450 2E1 as the best catalyst of NDMA metabolism (Yoo et al. 1988; Guengerich et al. 1991) and as an excellent catalyst of the epoxidation and activation of benzene and 1,3-butadiene (Guengerich et al. 1991; Bolt et al. 2003).

Epoxide Hydrolases

Several carcinogens of tobacco smoke, including PAH, 1,3-butadiene, and benzene, are metabolized to epoxides. These epoxide metabolites are substrates for MEH (also known as EPHX1), an enzyme that catalyzes their hydrolysis (Wood et al. 1976; Snyder et al. 1993; Krause and Elfarra 1997; Fretland and Omiecinski 2000). In mammals, at least five epoxide hydrolases were identified. However, four of these predominantly or exclusively catalyze the hydrolysis of endogenous substrates (Fretland and Omiecinski 2000). The fifth, MEH, plays a role in both the detoxification and activation of xenobiotics. Specifically, MEH is involved in the formation of the reactive diol epoxide metabolites of PAHs, and its activity is therefore critical to the carcinogenicity of these compounds (Conney 1982). For example, MEH catalyzes the hydrolysis of

B[*a*]P-7,8-epoxide to B[*a*]P-7,8-diol, which is then oxidized to the ultimate carcinogen BPDE (Levin et al. 1976; Gautier et al. 1996). The importance of this enzyme to PAH carcinogenicity is supported by the observation that MEH-null mice are highly resistant to carcinogenesis induced by 7,12-dimethylbenz[*a*]anthracene (Miyata et al. 1999).

In contrast to its role in the activation of PAHs, MEH detoxifies the epoxides of 1,3-butadiene (Krause and Elfarra 1997; Wickliffe et al. 2003). Studies have reported that several polymorphisms in MEH result in an increased sensitivity to the genotoxic effects of 1,3-butadiene (Abdel-Rahman et al. 2003, 2005). Benzene oxide is also a substrate for MEH (Snyder et al. 1993). However, male mice deficient in MEH are not susceptible to toxic effects induced by benzene (Bauer et al. 2003). The role of benzene oxide in carcinogenesis is unclear.

Glutathione-*S*-Transferases

Another mechanism that may detoxify carcinogenic epoxides is conjugation with glutathione. This reaction can be catalyzed by cytosolic GSTs (Sheehan et al. 2001; Hayes et al. 2005), which are dimeric. Seven classes (alpha, mu, pi, sigma, theta, omega, and zeta) exist in mammalian species (Sheehan et al. 2001), and at least 16 GST subunits exist in humans. However, only four homodimeric enzymes to date have been characterized as catalysts of glutathione conjugation of tobacco smoke carcinogens (Cheng et al. 1995; Norppa et al. 1995; Wiencke et al. 1995; Jernstrom et al. 1996; Sundberg et al. 1998, 2002; Landi 2000; Verdina et al. 2001; Fustinoni et al. 2002; Sørensen et al. 2004a; Hayes et al. 2005). These enzymes are members of four GST classes: alpha (GSTA1-1), mu (GSTM1-1), pi (GSTP1-1), and theta (GSTT1-1). The protein levels of each GST vary significantly from person to person, as well as across tissues within an individual (Rowe et al. 1997; Sherratt et al. 1997; Mulder et al. 1999). Researchers have identified several polymorphisms in the genes encoding these subunits (Hayes et al. 2005). Of particular note with regard to cancer risk in smokers are the **NULL* alleles for *GSTM1* and *GSTT1*, which have decreased detoxification capacity and elevated DNA damage. *GSTA1*, *GSTM1*, and *GSTT1* are expressed in the liver of persons who are not homozygous for either null phenotype; little GSTP1 is present in the liver (Rowe et al. 1997; Sherratt et al. 1997; Mulder et al. 1999). In contrast, the lung expresses higher levels of GSTP1 than those expressed by the other three subunits (Rowe et al. 1997; Sherratt et al. 1997).

GSTA1-1, GSTM1-1, and GSTP1-1 each catalyze the glutathione conjugation of a number of PAH diol epoxides (Jernstrom et al. 1996; Sundberg et al. 1998, 2002). However, the efficiencies and stereoselectivity of each of these enzymes vary with the diol epoxide substrate. For example, GSTM1-1 is a more efficient catalyst of glutathione conjugation of (+)-*anti*-BPDE than is either GSTA1-1 or GSTP1-1 (Sundberg et al. 1997). The GSTA1-1 and GSTP1-1 enzymes have overall K_{cat}/K_m values for catalytic rate or turnover number that are about 3-fold lower than the value for GSTM1-1, but GSTM1-1 is almost 30-fold better as a catalyst for the conjugation of (-)-*anti*-BPDE (Sundberg et al. 1997). The contribution of each GST enzyme to the detoxification of PAH diol epoxides varies with the substrate and across different tissues on the basis of their expression levels. In lung tissue from smokers, levels of (+)-*anti*-BPDE–DNA adducts were dependent on the *GSTM1* genotype (Alexandrov et al. 2002). Persons with the *GSTM1* null genotype had significantly higher adduct levels than did those with the *GST* wild-type genotype. These data support the importance of GSTM1-1 activity in BPDE detoxification in the lung, but they do not exclude a role for GSTA1-1 and GSTP1-1 in the detoxification of this or other PAHs.

GSTM1-1 and GSTT1-1 enzymes play a key role in the conjugation of two 1,3-butadiene epoxide metabolites: 3,4-epoxybutene (EB) and diepoxybutane (DEB) (Norppa et al. 1995; Wiencke et al. 1995; Thier et al. 1996; Bernardini et al. 1998; Landi 2000; Fustinoni et al. 2002; Schlade-Bartusiak et al. 2004). The direct measurement of either GSTM1-1 or GSTT1-1 activity with these epoxide substrates has not been reported. However, several studies of sister chromatid exchange (SCE) in human lymphocyte cultures from persons with the *GSTT1* null genotype support the role of GSTT1-1 in the detoxification of DEB (Norppa et al. 1995; Wiencke et al. 1995; Bernardini et al. 1998; Landi 2000; Schlade-Bartusiak et al. 2004). In conflict with these data, one study reports the increased mutagenicity of DEB in *Salmonella typhimurium* TA1535 expressing *GSTT1-1*, suggesting that the conjugation of this diepoxide is an activation pathway (Thier et al. 1996). The role of both GSTM1-1 and GSTT1-1 in the detoxification of EB is supported by a higher induction of SCE by EB in lymphocyte cultures from persons with either the *GSTM1-1* or the *GSTT1-1* null genotype (Uusküla et al. 1995; Bernardini et al. 1998). Although GSTs play a role in the metabolism of 1,3-butadiene, it remains unclear whether polymorphisms in GSTs modulate the carcinogenic effects of 1,3-butadiene in humans (Fustinoni et al. 2002).

One major excreted metabolite of benzene is *S*-PMA, which is formed from the glutathione conjugate of benzene oxide (Snyder and Hedli 1996). This glutathione conjugate may be generated both enzymatically and nonenzymatically, and it is not clear which pathway predominates. However, a number of studies on benzene exposure and toxicity have suggested a role for either GSTM1-1 or

GSTT1-1 in the conjugation of benzene oxide (Hsieh et al. 1999; Verdina et al. 2001; Wan et al. 2002; Kim et al. 2004b). Researchers have not directly measured which enzyme is the better catalyst of glutathione conjugation of benzene oxide. The in vivo role of GSTT1-1 in benzene oxide detoxification is supported by a report that *S*-PMA levels excreted by persons exposed to benzene who carried the wild-type *GSTT1** allele were higher than those of persons homozygous for the *GSTT1* NULL* allele (Sørensen et al. 2004b).

Ethylene oxide is also detoxified by glutathione conjugation (Brown et al. 1996). Although studies have not directly evaluated the role of specific human GSTs, evidence supports the role of GSTT1-1 as a catalyst of this reaction (Hallier et al. 1993; Fennell et al. 2000). On exposure to ethylene oxide, lymphocytes from persons with the *GSTT1-1* NULL* allele had higher levels of SCE than did those from persons with the wild-type allele (Hallier et al. 1993). In addition, levels of 2-hydroxyethylvaline Hb adducts were higher in smokers than in nonsmokers, because of exposure to ethylene and ethylene oxide in cigarette smoke, and were higher in smokers with the *GSTT1* NULL* allele than in those with the wild-type allele (Fennell et al. 2000).

Uridine-5′-Diphosphate-Glucuronosyltransferases

Conjugation with glucuronic acid is an important metabolic pathway for a number of carcinogens in tobacco smoke (Bock 1991; Hecht 2002a; Nagar and Remmel 2006). (Conjugation is the addition of a polar moiety to a metabolite to facilitate excretion.) The microsomal enzymes, UGTs, catalyze these conjugation reactions. Researchers have identified 18 human UGTs that are members of two families (UGT1 and UGT2) (Tukey and Strassburg 2000; Burchell 2003; Nagar and Remmel 2006). The UGT1A proteins are encoded by a single gene cluster, and expression of the nine members of this subfamily occurs through exon sharing. Exon 1 is unique for each *UGT1A*, whereas exon 2 to exon 5 are shared by all *UGT1As* (Tukey and Strassburg 2000). Thus, all UGT1A proteins are identical in the 245 amino acids of the carboxyl terminus encoded by exon 2 to exon 5 (Tukey and Strassburg 2000; Finel et al. 2005). In contrast, proteins from the UGT2 family are all unique gene products (Riedy et al. 2000; Tukey and Strassburg 2000). The expression of UGTs is tissue specific, and there are large differences in expression among tissues (Gregory et al. 2000, 2004; Tukey and Strassburg 2000; Wells et al. 2004). For example, UGTs 1A1, 1A3, 1A4, 1A6, and 1A9 are highly expressed in the liver; UGTs 1A7, 1A8, and 1A10 are mainly expressed in extrahepatic tissues (Tukey and Strassburg 2000; Gregory et al. 2004; Wells et al. 2004).

Aromatic amines and their *N*-hydroxy metabolites are glucuronidated to facilitate excretion (Bock 1991; Tukey and Strassburg 2000; Zenser et al. 2002). Glucuronidation is a detoxification reaction. Therefore, variations in the expression and catalytic efficiency of the enzymes that catalyze this reaction may influence the carcinogenicity of particular aromatic amines. In general, researchers have suggested that members of the UGT1A family contribute to the glucuronidation of these carcinogens (Orzechowski et al. 1994; Green and Tephly 1998; Tukey and Strassburg 2000; Zenser et al. 2002). However, UGT2B7 also catalyzes their glucuronidation (Zenser et al. 2002). In most cases, data support UGT1A9 as the best catalyst. For the tobacco smoke carcinogen 4-ABP, the relative catalytic efficiency of *N*-glucuronidation is UGT1A9>UGT1A4>UGT1A7>UGT 2B7>UGT1A6, but the catalytic efficiency of all these proteins is approximately equal to that of UGT1A1 (Zenser et al. 2002).

The phenol and diol metabolites of PAHs are primarily eliminated as glucuronide conjugates. Researchers have studied the role of specific UGTs in the metabolism of B[*a*]P (Bock 1991; Guillemette et al. 2000; Fang et al. 2002; Dellinger et al. 2006). Studies with UGT1A-deficient rats have implicated UGT1A enzymes in the detoxification of B[*a*]P (Wells et al. 2004). The glucuronidation of B[*a*]P-7,8-diol and 3-hydroxy-, 7-hydroxy-, and 9-hydroxy-B[*a*]P by heterologously expressed human UGTs has been characterized for a number of UGT1A and UGT2B enzymes (Fang et al. 2002; Dellinger et al. 2006). Among the phenols, UGT1A10 was the most efficient UGT1A catalyst of glucuronidation. UGTs 2B7, 2B15, and 2B17 all catalyzed conjugation of the three B[*a*]P phenols. However, the K_m of the reaction for UGT2B enzymes was 2- to 250-fold higher than that for UGT1A10 (Dellinger et al. 2006). For the carcinogenic (-)-B[*a*]P-7,8-diol, UGT1A10 was a better catalyst of glucuronidation than was UGT1A9, and UGT2B7 did not catalyze detectable levels of glucuronidation (Fang et al. 2002), but UGT2B7 did catalyze the glucuronidation of (+)-B[*a*]P-7,8-diol.

In smokers, glucuronidation also plays an important role in the excretion of the NNK metabolite NNAL (Carmella et al. 2002b; Hecht 2002a). Both *O*-linked and *N*-linked NNAL glucuronide conjugates are formed (Carmella et al. 2002b). In addition, the direct detoxification of the hydroxymethyl metabolite of NNK occurs by glucuronidation in rats (Murphy et al. 1995). However, the contribution of this pathway to NNK detoxification in smokers has not been identified. In vitro studies with fibroblasts both from UGT1A-deficient and control rats have confirmed a role for UGT1A enzymes in the protection of these cells from NNK-induced micronuclei formation (Kim and Wells 1996). Human UGT1A9, UGT2B7,

and UGT2B17 catalyze NNAL-*O*-glucuronidation, with UGT2B17 being the most active, and UGT1A4 catalyzes NNAL-*N*-glucuronidation (Ren et al. 2000; Wiener et al. 2004b; Lazarus et al. 2005). The rate of NNAL *O*- and *N*-glucuronidation by human liver microsomes varies significantly among persons; researchers have suggested that polymorphisms in *UGT2B7* and *UGT1A4* contribute to this variability (Wiener et al. 2004a).

Glucuronidation may also contribute to the detoxification of benzene (Bock 1991). In hepatocytes from rats treated with 3-methylcholanthrene to induce UGTs, phenol glucuronidation increases compared with sulfation. Glucuronide conjugates are more stable than the corresponding sulfates, and researchers have suggested the glucuronidation of phenol as a detoxification pathway (Bock 1991). However, to date, the role of glucuronidation in benzene-induced carcinogenesis has not been characterized and is poorly understood.

N-Acetyltransferases

NATs are cytosolic enzymes that catalyze the transfer of the acetyl group from acetylcoenzyme A to an acceptor molecule (Hein et al. 2000b). This transfer occurs through an enzyme intermediate in which cysteine 68 is acetylated and then deacetylated during the course of the reaction. Humans express two unique enzymes, NAT1 and NAT2, which catalyze both *N*- and *O*-acetylation reactions. Researchers have recognized the polymorphic nature of *NAT2* for more than 40 years and, more recently, have identified more than 35 alleles (Hein et al. 2000b; Hein 2002). *NAT1* is less well studied but is also polymorphic, and more than 25 alleles have been identified (Hein 2002; University of Louisville School of Medicine 2006). Researchers suggest that polymorphisms in both *NAT1* and *NAT2* influence the activation and detoxification of carcinogenic aromatic amines in tobacco smoke (Hein 2002).

The *N*-acetylation of aromatic amines, such as 4-ABP, is a detoxification reaction (Hein 2002). In contrast, *O*-acetylation of the *N*-hydroxy metabolites of arylamines generated by P-450 (e.g., *N*-hydroxy-4-ABP) is an activation reaction leading to DNA adduct formation (Hein et al. 1993, 1995; Hein 2002). NAT1 and NAT2 both catalyze each of these reactions (Hein et al. 1993). However, NAT2 is generally considered the more important catalyst of detoxification, and NAT1 is the more important catalyst of activation (Badawi et al. 1995; Hein 2002). This assumption is based on differences in the catalytic efficiency of the enzymes and their tissue distribution in humans as well as on studies with animal models (Hein et al. 1993; Hein 2002).

Studies with recombinant human *NAT1* and *NAT2* have described differences in the *N*-acetylation of 4-ABP. The apparent affinity of 4-ABP for NAT2 is significantly greater than that for NAT1, and ratios of NAT1 activity to NAT2 activity and clearance calculations support a greater role for NAT2 than for NAT1 in the *N*-acetylation of arylamines (Hein et al. 1993). The characterization of NAT1 as the key catalyst of the *O*-acetylation (i.e., activation) of aromatic amines is more speculative and is primarily driven by the tissue distribution of NAT1 (see the discussion below). No data in the literature report differences between the efficiencies of NAT1- and NAT2-catalyzed *O*-acetylation of aromatic amines. However, more recent studies that engineered *S. typhimurium* strains to overexpress either *NAT1* or *NAT2* reported that NAT1, but not NAT2, catalyzed the genotoxic activation of *N*-hydroxy-4-ABP (Oda 2004). These data provide support for NAT1 as an important catalyst in the activation of this aromatic amine.

The organ and tissue distribution of NAT1 and NAT2 differ markedly (Dupret and Rodrigues-Lima 2005). The NAT2 protein is mainly expressed in the gut and liver; the NAT1 protein is expressed in the liver and a number of other tissues, including the colon and bladder. Researchers believe that aromatic amines in tobacco smoke contribute to smoking-related bladder cancer. Therefore, the potential activation of these compounds in the bladder is important in understanding the etiology of bladder cancer. Researchers have detected NAT1 activity, but not NAT2 activity, in samples of bladder tissue from smokers (Badawi et al. 1995). In addition, DNA adduct levels measured by ^{32}P-postlabeling correlated with NAT1 activity. These data are thus consistent with a role for NAT1 in the activation of arylamines in tobacco smoke.

Epidemiologic studies that demonstrate a modest increase in risk of bladder cancer in persons phenotypically and genotypically identified as having slow acetylation catalyzed by NAT2 further support the role of NAT2 in the detoxification of aromatic amines (Green et al. 2000; Hein et al. 2000a; Gu et al. 2005) (see "Molecular Epidemiology of Polymorphisms in Carcinogen-Metabolizing Genes" later in this chapter). A number of the *NAT2* variant alleles identified in persons with slow acetylation were expressed heterologously and demonstrated a decrease in activity for both the *N*-acetylation of 4-ABP and *O*-acetylation of *N*-hydroxy-4-ABP, primarily because of the instability of the variant enzymes (Hein et al. 1995; Zhu et al. 2002). Both activation and detoxification would be diminished in persons expressing variant NAT2 activity, but NAT1 activity would be maintained. Studies that have characterized NAT1 proteins from a number of variants of this gene have also reported a decrease in enzyme activity (Fretland et al. 2002).

DNA Adducts and Biomarkers

Introduction

Although formation of carcinogen-DNA adducts is a well-characterized phenomenon in laboratory animals, there were no reports of analyses of DNA adducts in smokers before the mid-1980s. In the past 20 years, a large body of literature on DNA adducts in human tissues has emerged with the development of sensitive methods such as HPLC fluorescence, GC–MS, liquid chromatography (LC)–MS, electrochemical detection, ^{32}P-postlabeling, and immunoassay. Researchers have applied all of these methods to analyze DNA adducts, producing data on these biomarkers in molecular epidemiologic studies of cancer susceptibility. Thus, a discussion of DNA adducts in human tissues also includes biomarkers of DNA adduct formation in smokers.

Characterized Adducts in the Human Lung

Available data on characterized DNA adducts in human lung tissue, the tissue most extensively investigated to date, are summarized in Table 5.2. The small number of studies reflects several difficulties in this research. First, DNA from human lung tissue is difficult to obtain. The amounts of DNA available from routine procedures, such as bronchoscopy, are generally too small for analysis of specific DNA adducts. Second, the levels of DNA adducts are generally low: between 1 in 10 million and 1 in 100 million normal DNA bases. Analyzing such small amounts of material is challenging. Nevertheless, methods such as those listed previously and in Table 5.2 were successfully applied. However, because of the limitations noted, the number of participants in most of the studies is small.

The major DNA adduct of B[*a*]P observed in laboratory animals is BPDE-N^2-deoxyguanosine. Acid hydrolysis of DNA containing this adduct releases B[*a*]P-7,8,9,10-tetraol, which can be analyzed by HPLC with fluorescence detection (Rojas et al. 1998). Other BPDE-derived DNA adducts may be hydrolyzed simultaneously. This assay has been applied to lung tissue obtained during surgery (Alexandrov et al. 2002; Boysen and Hecht 2003). Compared with nonsmokers, smokers with the *GSTM1* null genotype displayed higher levels of BPDE-DNA adducts in lung tissue, although this finding is based on a small number of cases (Rojas et al. 1998, 2004). BPDE-DNA adducts were detectable in 40 percent of the smokers with whole lung analyses (Boysen and Hecht 2003) and in all samples with analyses of bronchial epithelial cells (Rojas et al. 2004). When the adduct localization in genes was determined by in vitro studies, one target was seen to be at mutational hot spots in the *P53* tumor-suppressor gene and the *KRAS* oncogene in cells (Tang et al. 1999; Feng et al. 2002).

Several studies have quantified 7-methyldeoxyguanosine in human lung tissue. The source of this adduct in smokers could be NDMA, NNK, or perhaps other methylating agents. Studies have reported mixed results: some show higher adduct levels in smokers than in nonsmokers (Hecht and Tricker 1999; Lewis et al. 2004). One study examined O^6-methyldeoxyguanosine in human lung tissue but was too small to draw conclusions about the effect of smoking (Wilson et al. 1989).

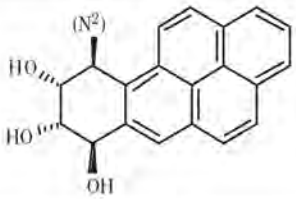
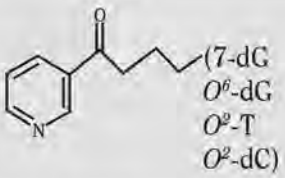
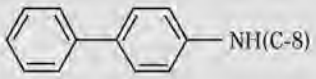
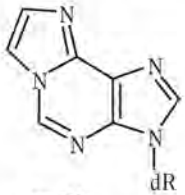
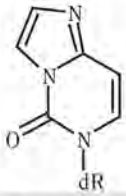
Three small studies provided evidence for ethyl DNA adducts in human lung tissue (Wilson et al. 1989; Blömeke et al. 1996; Godschalk et al. 2002). Levels of both O^6-ethyldeoxyguanosine and O^4-ethylthymidine were higher in smokers than in nonsmokers. Although one source of these adducts could be *N*-nitrosodiethylamine, its level in cigarette smoke is low. As discussed previously, cigarette smoke contains a direct-acting, but chemically uncharacterized, ethylating agent that may be responsible for the presence of these adducts (see "Carcinogens in Cigarette Smoke" earlier in this chapter).

NNK and NNN are metabolically activated to intermediate agents that pyridyloxobutylate DNA. The resulting POB-DNA adducts can be hydrolyzed with acid to yield 4-hydroxy-1-(3-pyridyl)-1-butanone, which can be detected by GC–MS. Application of this method demonstrated higher levels of POB-DNA adducts in lung tissue of smokers than in that of nonsmokers in a small study (Foiles et al. 1991). One study detected 7-(2-hydroxyethyl) deoxyguanosine in human lung tissue (Zhao et al. 1999), and ethylene oxide is the likely source of this adduct. Studies have detected 4-ABP–DNA adducts in human lungs but show no clear effect of smoking on adduct levels (Wilson et al. 1989; Lin et al. 1994).

Researchers have quantified 1,N^6-ethenodeoxyadenosine and 3,N^4-ethenodeoxycytidine in human lungs by ^{32}P-postlabeling (Godschalk et al. 2002). These adducts may result from lipid peroxidation or from metabolic activation of vinyl chloride or ethyl carbamate. No differences were reported between smokers and nonsmokers. Studies of the oxidative-damage product 8-oxodeoxyguanosine in the human lung obtained mixed results regarding a relationship between detection of this product and smoking status (Asami et al. 1997; Lee et al. 1999a).

In summary, data on the quantitation of specific DNA adducts in the human lung are limited. However, some studies document clear evidence for elevated levels of adducts resulting from exposure to specific carcinogens such as B[*a*]P, NNK, or NNN. Several methods used in these studies—HPLC fluorescence, GC–MS, LC–MS, and ^{32}P-postlabeling with modifications for specific adducts—have the potential for application to molecular

Table 5.2 DNA adducts in human lung tissue

Study	Carcinogen	DNA base	Adduct structures[a]	Type of evidence[b]
Rojas et al. 1998, 2004 Boysen and Hecht 2003	Benzo[*a*]pyrene	dG		1
Wilson et al. 1989 Mustonen et al. 1993 Kato et al. 1995 Blömeke et al. 1996 Petruzzelli et al. 1996	*N*-nitrosodimethylamine NNK Others	dG	7—CH_3 O^6—CH_3	2 2
Wilson et al. 1989 Blömeke et al. 1996 Godschalk et al. 2002	*N*-nitrosodiethylamine Others	dG T	7—CH_3CH_2 O^6—CH_3CH_2 O^4—CH_3CH_2	2 2 2
Foiles et al. 1991	NNK *N*′-nitrosonornicotine	dG, T, dC		1
Eide et al. 1999	Ethylene oxide	dG	7—$HOCH_2CH_2$	2
Wilson et al. 1989 Lin et al. 1994	4-aminobiphenyl	dG		2
Godschalk et al. 2002	Vinyl chloride Ethyl carbamate Oxidants	Deoxyadenosine dC	 	2 2
Asami et al. 1997 Lee et al. 1999a	Oxidants	dG	8—oxo	3

Note: **dC** = deoxycytidine; **dG** = deoxyguanosine; **NNK** = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; **T** = thymidine.

[a]Adduct structures show position of attachment to the base (e.g., N^2-, O^6-, or 7- of dG) and the organic moiety derived from the carcinogen.

[b]1 = detection of a released adducted moiety by a specific method; 2 = detection of a nucleoside or base by a relatively nonspecific method (e.g., ^{32}P-postlabeling or immunoassay); 3 = detection of a nucleoside or base by a specific method (e.g., mass spectrometry, high-performance liquid chromatography [HPLC]-fluorescence, or HPLC-electrochemical detection).

epidemiologic studies that relate specific DNA adduct levels to tobacco exposure and cancer risk.

Uncharacterized Adducts in Human Lung Tissue

Studies have extensively applied two main nonspecific methods—^{32}P-postlabeling and immunoassay—to analyze DNA adducts in human lung tissue, as well as in other tissues. Researchers have discussed the advantages and disadvantages of these methods (Kriek et al. 1998; Wild and Pisani 1998; Poirier et al. 2000; Phillips 2002). Major advantages include high sensitivity for analyzing small amounts of DNA, simplicity of analysis, and no need for extremely expensive equipment. Disadvantages include a lack of chemical specificity, particularly in ^{32}P-postlabeling analyses, and difficulty in quantitation. Studies have extensively reviewed the application of these methods to tissues obtained from smokers (Phillips 2002; Wiencke 2002; IARC 2004).

The output of assays using ^{32}P-postlabeling is often a "diagonal radioactive zone" (DRZ), which consists of uncharacterized radioactive components referred to in the literature as hydrophobic or aromatic DNA adducts. In most cases, little if any evidence supports the true chemical characteristics of these adducts. Nevertheless, the intensity of the DRZ is consistently elevated in samples from smokers. Immunoassays have used various methods of detection, including the fluorescent staining of tissue specimens that allows for the location of adducts. Cross-reactivity is a common problem of immunoassays. For example, antibodies raised against protein conjugates of B[*a*]P–DNA adducts cross-react with adducts generated from other PAHs.

Many studies using ^{32}P-postlabeling methods examined DNA adduct levels in the peripheral lung, bronchial epithelium, or cells obtained by bronchial lavage of smokers. Most of the studies found that adduct levels were higher in smokers compared with nonsmokers (Győrffy et al. 2004; IARC 2004). Investigations that attempted to draw quantitative relationships between the extent of smoking and adduct levels had inconsistent results (IARC 2004).

Adducts in Other Tissues

Numerous studies have evaluated DNA adduct formation in fetuses and in various tissues and fluids of smokers, including samples from the larynx, oral and nasal mucosa, bladder, cervix, breast, pancreas, stomach, placenta, and cardiovascular system, and samples of sputum, sperm, and blood cells. Researchers have comprehensively reviewed these studies (Phillips 2002; Weincke 2002; IARC 2004), most of which used ^{32}P-postlabeling and immunoassay techniques.

Levels of 7-alkyl-deoxyguanosines determined by ^{32}P-postlabeling in laryngeal DNA were higher in smokers than in nonsmokers (Szyfter et al. 1996), and they correlated with the DRZ in these samples. Studies used immunoassay also to detect 4-ABP–DNA adducts in laryngeal tissue (Flamini et al. 1998). Other studies examined the DRZ by ^{32}P-postlabeling (IARC 2004).

Researchers have detected 1,N^2-propanodeoxyguanosine (PdG) adducts derived from acrolein and crotonaldehyde in the DNA of gingival tissue of smokers and nonsmokers; adduct levels were higher in smokers (Nath et al. 1998). Adducts detected by ^{32}P-postlabeling in oral and nasal tissue were also higher in smokers than in nonsmokers. Use of immunoassay techniques revealed that levels of BPDE-DNA, 4-ABP–DNA, and malondialdehyde-DNA adducts in human oral mucosal cells of smokers were higher than those for nonsmokers (IARC 2004).

Using ^{32}P-postlabeling, researchers found 4-ABP–DNA (C-8 deoxyguanosine) adducts in exfoliated urothelial cells and bladder biopsy samples (IARC 2004). In studies using antibodies to 4-ABP–DNA, levels detected in biopsy specimens from the bladder of smokers were higher than those for nonsmokers (IARC 2004). Studies using ^{32}P-postlabeling of bladder DNA from smokers and nonsmokers yielded mixed results; some studies showed higher adduct levels in smokers (IARC 2004).

Using GC–MS, Melikian and colleagues (1999) documented that BPDE-DNA adducts were higher in cervical epithelial cells of smokers than in those of nonsmokers. An immunohistochemical analysis using antibodies to BPDE-DNA adduct in human cervical cells also showed higher adduct levels in smokers than in nonsmokers (Mancini et al. 1999). ^{32}P-postlabeling consistently showed higher adduct levels in cervical tissues of smokers than in those of nonsmokers (IARC 2004).

The ^{32}P-postlabeling of DNA from breast tissue yields the characteristic DRZ from smokers. Researchers also investigated adduct levels by using antibodies against BPDE-DNA; results were generally mixed with respect to smoking status (IARC 2004). Studies that used ^{32}P-postlabeling to measure adduct levels in pancreatic and stomach tissues reported a correlation with smoking status (IARC 2004).

Some studies indicate the presence of smoking-related DNA adducts in human placenta, but the overall relationship of placental DNA adducts to smoking is weak (IARC 2004). Analyses of sperm DNA also reported mixed results with respect to smoking status (IARC 2004).

Many studies have examined DNA adducts in blood cells (IARC 2004). The common use of blood cells in these studies is obviously related to the ease of clinically obtaining these samples. From this viewpoint, blood cell DNA is advantageous for biomarker studies. A disadvantage of

using blood cells is that adduct levels in blood cells are not necessarily directly related to levels of DNA adducts in the tissues in which smoking-related cancers occur. The collective results of the studies are somewhat inconsistent with respect to the effects of smoking on levels of DNA adducts. This inconsistency probably results in part from competing sources of adduct formation such as diet, occupation, and the general environment. Another factor is the lifetime of the blood cells investigated; longer-lived cells appear to provide more consistent results with respect to smoking (IARC 2004). Studies comparing levels of blood cell–DNA adducts in smokers with or without cancer had mixed results (IARC 2004).

A meta-analysis of the relationship of DNA adduct levels in smokers to cancer, determined by ^{32}P-postlabeling, used data from case-control studies of lung cancer (five studies), oral cancer (one study), and bladder cancer (one study). Six studies measured adducts in white blood cells, and one study used normal lung tissue. Among current smokers, adduct levels for case patients were significantly higher than those for control participants (Veglia et al. 2003).

Protein Adducts as Surrogates for DNA Adducts

Researchers have proposed that levels of carcinogen-Hb adducts and carcinogen-albumin adducts be used as surrogates for the measurements of DNA adducts discussed in the preceding section (Osterman-Golkar et al. 1976; Ehrenberg and Osterman-Golkar 1980). Although these proteins are not considered targets for carcinogenesis, all carcinogens that react with DNA are also thought to react with protein to some extent. Advantages of Hb adducts as surrogates include the ready availability of Hb in blood and the long lifetime of the erythrocyte in humans (approximately 120 days), which provides an opportunity for adducts to accumulate. Other researchers have comprehensively reviewed studies on protein adducts in smokers (Phillips 2002; IARC 2004).

The Hb adducts of aromatic amines have emerged as highly informative carcinogen biomarkers. Levels of these adducts are consistently higher in smokers than in nonsmokers, particularly for 3-ABP–Hb and 4-ABP–Hb adducts. Adduct levels decrease with smoking cessation and are related to the number of cigarettes smoked (Maclure et al. 1990; Skipper and Tannenbaum 1990; Castelao et al. 2001). Adducts that form with the amino terminal valine of Hb are also informative. Important examples include adducts derived from ethylene oxide, butadiene, acrylonitrile, and acrylamide (Bergmark 1997; Fennell et al. 2000; Swenberg et al. 2001). Ethylated *N*-terminal valine of Hb is also higher in smokers than in nonsmokers (Carmella et al. 2002a).

Summary

Overwhelming evidence indicates that DNA adduct levels are higher in most tissues of smokers than in corresponding tissues of nonsmokers. This observation provides bedrock support for the major pathway of cancer induction in smokers that proceeds through DNA adduct formation and genetic damage. DNA adducts studied can generally be divided into two classes: nonspecific adducts, which are detected by ^{32}P-postlabeling and immunoassay, and specific adducts, which are detected by structure-specific methods. Studies of nonspecific DNA adducts are far more common than studies of specific DNA adducts, which are still scarce and are limited mainly to human lung tissue. Strong evidence exists for the presence of a variety of specific adducts in the human lung, and in several cases, adduct levels are higher in smokers than in nonsmokers. Measuring levels of Hb adducts by MS provides a simple and perhaps more practical approach for assessing carcinogen exposure of the cell. In several instances, levels of specific adducts are substantially higher in smokers than in nonsmokers. Collectively, the results of these biomarker studies demonstrate the potential for genetic damage in smokers from the persistence of DNA adducts. The propagation of this genetic damage during clonal outgrowth is consistent with the accumulation of multiple genetic changes observed in lung cancer progression.

Molecular Epidemiology of Polymorphisms in Carcinogen-Metabolizing Genes

Introduction

Genetic polymorphisms may play a role in tobacco-related neoplasms. Researchers have established cigarette smoking as a major cause of lung cancer: more than 85 percent of lung cancers are attributable to smoking (Ries et al. 2004). However, not all smokers develop lung cancer, and lung cancer can arise in lifetime nonsmokers. This variation in disease has stimulated interest in molecular epidemiologic investigations of genetic polymorphisms, including carcinogen-metabolizing enzymes that may lead to variations in susceptibility to the carcinogens in tobacco smoke (Table 5.3). Considerable data exist on genetic polymorphisms in cancers other than lung cancer, but the discussion here focuses only on lung cancer and bladder cancer, two of the most heavily investigated cancers.

Table 5.3 Selected gene polymorphisms evaluated by molecular epidemiology investigations for relationship to lung cancer through variation in susceptibility to carcinogens in tobacco smoke

Metabolic genes	Nucleotide change	Amino acid change	Enzymatic activity
CYP1A1	T→C (*MSPI*)	NA	Increased
	A→G	Ile462Val	Increased
CYP2E1	T→A (*DRAI*)	NA	Increased
	G→C (*RSAI*)	NA	Increased
CYP2A13	C→T	Arg257Cys	Decreased
GSTM1	Deletion	NA	None
GSTP1	A→G	Ile105Val	Decreased
GSTT1	Deletion	NA	None
NAT2	T→C	Ile114Thr	Decreased
	C→T	Lys161Lys	Decreased
	A→G	Lys268Arg	Decreased
	G→A	Arg197Gln	Decreased
	C→T	Tyr94Tyr	Decreased
	G→A	Gly286Glu	Decreased
MEH	T→C	Tyr113His	Decreased
	A→G	His139Arg	Increased

Note: **NA** = not applicable.

Studies have identified polymorphisms in phase I and II enzymes. Phase I enzymes, such as P-450s, generally add an oxygen atom to a carcinogen, and phase II enzymes, such as GSTs or UGTs, modify the carcinogen by making it highly water soluble for more facile excretion. These enzymes are involved in the activation and detoxification of carcinogens and may be associated with a differential ability to process carcinogens. Researchers have hypothesized that the accumulation of active carcinogen metabolites and hence increased DNA adduct formation add to lung cancer risk. Studies of cases with autopsy of cancer-free lung tissue indicate that polymorphisms in *CYP1A1* and *GSTM1* genes may be associated with higher DNA adduct levels, suggesting that variations in metabolic pathways can play a role in individual response to carcinogen exposure (Kato et al. 1995). Numerous studies have extended this line of analysis to investigate whether this differential ability to metabolize carcinogens leads to differential lung cancer risk. Overall, data from the study of these polymorphisms have generated inconsistent results. These inconsistencies may be explained in part by the combination of a small sample size and variable frequencies of the polymorphic alleles within different ethnic populations. A summary of some of the specific gene polymorphisms investigated is provided in Table 5.3. A recent review summarizes the effects of genetic polymorphisms on lung cancer (Schwartz et al. 2007), and specific examples are discussed here.

CYP1A1 Gene

Researchers hypothesize that interindividual variations in the ability to activate carcinogens such as PAH through the *CYP1A1* gene may lead to differential carcinogenic effects. Studies describe at least two variant polymorphisms in the *CYP1A1* gene. The first is a T3801C base change in intron 6, which results in a new *MSPI* restriction site (Kawajiri et al. 1990). (A restriction site is a site in the gene that is cleaved by a specific restriction enzyme.) The second polymorphism is an A2455G base change in exon 7, which results in an Ile to Val amino acid change (Hayashi et al. 1991). Although these polymorphisms appear to be linked, study results are inconsistent, and wide disparities exist among populations.

Studies of Japanese and Chinese populations associate both of the *CYP1A1* variant polymorphisms with an increase in lung cancer risk. Nakachi and colleagues (1991) were the first to report an association of the **MSPI* polymorphism with lung cancer risk. For patients with lung cancer, the frequency of harboring the homozygous

variant genotype was more than two times higher than that for control participants. Among patients with squamous cell carcinoma (SCC), the homozygous variant genotype was associated with an increased risk of developing lung cancer, especially in those with a lower cumulative dose of cigarette smoke. At low levels of exposure to cigarette smoke, the odds ratio (OR) for developing lung cancer among persons with the homozygous variant genotype was 7.31 (95 percent confidence interval [CI], 2.13–25.12). This increased risk was persistent, but of a lesser magnitude, at higher levels of exposure to cigarette smoke (Nakachi et al. 1991). Okada and colleagues (1994) reported similar findings.

Studies have also associated the *ILE462VAL* polymorphism of *CYP1A1* with lung cancer risk in Japanese and Chinese populations. Again, the homozygous variant **VAL/*VAL* genotype was associated with lung cancer at lower cumulative doses of cigarette smoke (Nakachi et al. 1993; Yang et al. 2004; Ng et al. 2005). One explanation posited for this relationship with the dose level in smokers has been that the relevant enzyme is saturated at high doses but not at low doses of cigarette smoke (Vineis et al. 1997). The effects of genetic variability and differential enzymatic activity are more apparent at low doses, when saturation has not been reached.

Results have been inconsistent outside Asian populations. Individual studies often lack statistical power to detect an association (Shields and Harris 2000). Also, *CYP1A1* polymorphisms are common in Asian populations (30 percent of the population) (Nakachi et al. 1993), but are far less common among Europeans and North Americans (<10 percent of the population) (Warren and Shields 1997). A study of African Americans and Mexican Americans showed a twofold increase in the risk of lung cancer among light smokers with the **MSPI* variant genotype (Ishibe et al. 1997). However, a Brazilian study showed an increase in risk with the **ILE/*VAL* polymorphism but not with the *MSPI* polymorphism (Hamada et al. 1995). A more recent study suggested that in Latinos, the **MSPI* variant genotype was associated with an overall inverse OR of 0.51 (95 percent CI, 0.32–0.81), which reflected the inverse interaction with smoking (Wrensch et al. 2005). Reports from Finland, Norway, and Sweden show a lack of association between either of the *CYP1A1* polymorphisms and lung cancer risk (Tefre et al. 1991; Hirvonen et al. 1992; Alexandrie et al. 2004). A meta-analysis provides little support for this association (Houlston 2000).

Because of the small sample sizes in these studies, Vineis and colleagues (2003) conducted an analysis of pooled data from the International Collaborative Study on Genetic Susceptibility to Environmental Carcinogens, which included raw data from 22 case-control studies totaling 2,451 cases and 3,358 controls. This data set thus comprised approximately one-half of the case-control studies published at that time. Researchers found an association in Whites between the *CYP1A1* homozygous **MSPI* variant and lung cancer risk after adjustment of values for age and gender (OR = 2.36; 95 percent CI, 1.16–4.81). The association held for both SCC and adenocarcinomas (Vineis et al. 2003). However, this association failed to reach statistical significance among Asians in this analysis. Moreover, studies such as the research conducted by Nakachi and colleagues (1991, 1993) discussed previously in this section were not included, making the Asian data difficult to interpret.

CYP2E1 Gene

The *CYP2E1* gene is involved in the metabolic activation of NDMA, as well as several other tobacco smoke carcinogens. Le Marchand and colleagues (1998) performed a population-based, case-control study with 341 lung cancer cases and 456 controls. These researchers found that *CYP2E1* polymorphisms were associated with a decrease in risk of lung adenocarcinoma. A Chinese study (Wang et al. 2003c) confirmed this finding. However, the presence of at least one variant *CYP1A1 *MSPI* allele was associated with an increased risk of SCC, both alone (2.4-fold increase in risk) and in combination with *GSTM1* deletion (3.1-fold increase in risk) (Le Marchand et al. 1998). These researchers suggest that the associations between *CYP1A1* and *CYP2E1* polymorphisms and subsets of lung cancer indicate a specificity of PAHs to induce SCC and of nitrosamines to induce adenocarcinomas.

CYP2A13 Gene

The *CYP2A13* gene is expressed primarily in the respiratory tract and participates in the metabolic activation of *N*-nitrosamines such as NNK. Researchers have identified a polymorphism in *CYP2A13* in which a C→T transition leads to an Arg→Cys substitution at position 257. The variant 257CYS protein, the product of this gene, has one-half to one-third the capacity of the 257ARG protein to activate NNK (Su et al. 2000; Zhang et al. 2002). In a study of 724 lung cancer patients and 791 control participants, Wang and colleagues (2003a) demonstrated that the variant *CYP2A13* genotype (**C/*T* or **T/*T*) was associated with a reduced risk for lung cancer, particularly for adenocarcinomas (OR = 0.41; 95 percent CI, 0.23–0.71). The reduction in risk did not reach statistical significance for SCC or other histologies of lung cancer. The reduced risk for adenocarcinomas was apparent only in smokers, and in light smokers rather than in heavy smokers (Wang et al. 2003a). This finding again indicates that genetic polymorphisms may play a greater role when the carcinogen dose is low and does not saturate enzymatic capacity.

GSTM1 Gene

Study reports have noted large variations in enzymatic activity for several GSTs. About 50 percent of the White population is homozygous for a deletion in the *GSTM1* gene that leads to null expression (Seidegard et al. 1988). The GSTM1 enzyme is important in detoxifying carcinogens, and numerous studies have investigated the possible association of the *GSTM1* null genotype with lung cancer risk.

Some studies have found an association between the *GSTM1* null mutation and lung cancer across many populations. In a Japanese population, the *GSTM1* null genotype was positively correlated with SCC of the lung but not with adenocarcinomas (Kihara et al. 1993). A similar analysis in a Finnish population also correlated the *GSTM1* null genotype with SCC (Hirvonen et al. 1993). Analyses of Scottish (Zhong et al. 1991), Norwegian (Ryberg et al. 1997), and Turkish populations (Pinarbasi et al. 2003) had similar findings. A U.S. study also suggested that the *GSTM1* null genotype was associated with a modest elevation in lung cancer risk, which increased among heavy smokers (Nazar-Stewart et al. 2003). However, some studies have not shown a significant association between the *GSTM1* null genotype and lung cancer risk for SCC or overall for lung cancer (London et al. 1995; Rebbeck 1997). A meta-analysis of data from 12 case-control studies comprising 1,593 cases and 2,135 controls showed a moderate increase in the risk of lung cancer across all histologies with the *GSTM1* null genotype (OR = 1.41; 95 percent CI, 1.23–1.61) (McWilliams et al. 1995). A more recent meta-analysis of 43 studies including more than 18,000 persons showed a smaller but statistically significant OR of 1.17 (95 percent CI, 1.07–1.27) (Benhamou et al. 2002).

Kihara and colleagues (1994) analyzed data on 178 Japanese patients with lung cancer and 201 healthy control participants and found that the *GSTM1* null genotype was associated with an overall increase in lung cancer risk (OR = 1.87; 95 percent CI, 1.21–2.87). The strongest association was for SCC (OR = 2.13; 95 percent CI, 1.11–4.07). With stratification by the amount of smoking, the proportion of *GSTM1* null genotype increased progressively in the SCC group from 50 percent in light smokers to 72 percent in heavy smokers (Kihara et al. 1994). One study suggested that higher intakes of cruciferous vegetables reduced lung cancer risk among persons with the *GSTM1* genotype (highest versus lowest tertile for amount of smoking; OR = 0.61; 95 percent CI, 0.39–0.95) but not among persons with the *GSTM1* null genotype (highest versus lowest tertile; OR = 1.15; 95 percent CI, 0.78–1.68) (Wang et al. 2004b). However, several other studies have shown a greater protective effect in persons with the *GSTM1* null genotype who consumed cruciferous vegetables (London et al. 2000; Spitz et al. 2000). One hypothesis is that these participants were less able to eliminate protective isothiocyanates by conjugation with glutathione. In a case-control study, Cheng and colleagues (1999) analyzed data from 162 patients with SCC of the head and neck and 315 healthy control participants. They found that 53.1 percent of the case patients and 42.9 percent of the control participants were null for *GSTM1* ($p < 0.05$), whereas 32.7 percent of case patients and 17.5 percent of control participants were null for *GSTT1* ($p < 0.001$).

Thus, the effect of *GSTM1* alone may not be dramatic. However, it appears to be magnified by gene-environment and gene-diet interactions, and the effects were significantly greater as exposure to cigarette smoke increased. In addition, the high frequency of *GSTM1* polymorphisms observed across all ethnicities may contribute to the importance of this variant as a risk factor for developing lung cancer (Brennan et al. 2005).

CYP1A1 and *GSTM1* in Combination

Studies of the effect of combined *CYP1A1* and *GSTM1* variant genotypes hypothesized that increased PAH activation and decreased PAH detoxification in tobacco smokers might lead to an increase in lung cancer risk. Numerous studies have explored this association. Perhaps the studies with the strongest support for this association come from Japan, although they are generally limited by small sample sizes.

Combination of the *CYP1A1* variant genotype and the *GSTM1* null genotype enhanced the risk of smoking-related lung cancers in a Japanese population. Hayashi and colleagues (1992) demonstrated this finding with the **ILE/*VAL* polymorphism. These investigators found an increased frequency of the homozygous **VAL/*VAL* genotype combined with the *GSTM1* null genotype in lung cancer patients compared with control participants (8.5 percent versus 2.2 percent, respectively). Nakachi and colleagues (1991) reported similar results with both the **MSPI* and **ILE/*VAL* polymorphisms and the *GSTM1* null genotype. The case-control study found that for light smokers, either of the two *CYP1A1* susceptible genotypes combined synergistically with the deficient *GSTM1* genotype to create a high risk for lung cancer (OR = 16; 95 percent CI, 3.76–68.02 for **MSPI*, and OR = 41; 95 percent CI, 8.68–193.61 for **ILE/*VAL*). Eighty-seven percent of the light smokers who developed lung cancer had at least one of the three homozygous variant genotypes. The investigators suggested that particularly when the cigarette dose is low, *CYP1A1* and *GSTM1* may be an important determinant of susceptibility to lung cancer (Nakachi et al. 1991).

Kihara and colleagues (1994) also demonstrated a synergistic effect. Persons with these variant genotypes in both *CYP1A1* and *GSTM1* had a much higher risk of lung cancer than did those with the variant *CYP1A1* and wild-type *GSTM1* (OR = 21.9; 95 percent CI, 4.68–112.7 versus OR = 3.2; 95 percent CI, 0.37–24.0). Studies in Scandinavian populations (Alexandrie et al. 1994; Anttila et al. 1994), as well as U.S. populations (García-Closas et al. 1997), support an increase in the risk of lung cancer with the combination of variant *CYP1A1* and *GSTM1* genotypes. Using data from the International Collaborative Study on Genetic Susceptibility to Environmental Carcinogens database, Vineis and colleagues (2004) found a statistically significant effect of the **MSPI* variant on lung cancer risk in Whites (OR = 2.6; 95 percent CI, 1.2–5.7) with evidence for an interaction between the *MSPI* and *GSTM1* null genotypes (OR = 2.8; 95 percent CI, 0.9–8.4).

GSTP1 Gene

Studies have reported polymorphisms in the *GSTP1* gene family of phase II enzymes with high expression in the lung. One *GSTP1* polymorphism includes an A→G base change that leads to an isoleucine→valine substitution, which results in lower enzymatic activity toward 1-chloro-2,4-dinitrobenzene (Watson et al. 1998) but higher activity toward PAH diol epoxides (Sundberg et al. 1998). Several studies showed no statistically significant association between *GSTP1* polymorphisms and lung cancer risk (Harris et al. 1998; Katoh et al. 1999; To-Figueras et al. 1999; Nazar-Stewart et al. 2003). However, in the study with the largest sample size of 1,042 cases and 1,161 controls, the *GSTP1* homozygous variant genotype was associated with a higher lung cancer risk at any level of exposure to smoke than was the wild-type genotype (Miller et al. 2003).

The combination of the *GSTM1* null genotype and the *GSTP1 *G/*G* genotype may increase lung cancer risk (Ryberg et al. 1997; Kihara et al. 1999; Perera et al. 2002). In a study of 1,694 cases and 1,694 controls, double variants in *GSTM1* and *GSTP1*, as well as in *GSTP1* and *TP53*, were associated with an increase in lung cancer risk among persons aged 55 years or younger (adjusted OR [AOR] = 4.03; 95 percent CI, 1.47–11.1 for the M1-P1 double variant, and AOR = 5.10; 95 percent CI, 1.42–18.30 for the P1-P53 double variant) (Miller et al. 2002). Another study included 350 persons younger than age 50 years with a diagnosis of lung cancer who were identified from the metropolitan Detroit Surveillance, Epidemiology, and End Results program. The study compared these patients with 410 control participants matched by age, race, and gender. The results indicated that African Americans carrying at least one **G* allele at the *GSTP1* locus were 2.9 times more likely to develop lung cancer than were African Americans without a **G* allele (95 percent CI, 1.29–6.20). African Americans with either one or two genotypes that carry risk at the *GSTM1* and *GSTP1* loci were at higher risk of developing lung cancer than were African Americans who had fully functional *GSTM1* and *GSTP1* genes (OR = 2.8; 95 percent CI, 1.1–7.2 for *GSTM1,* and OR = 4.0; 95 percent CI, 1.3–12.2 for *GSTP1*). No significant single-gene associations were observed between *GSTM1*, *GSTT1*, or *GSTP1* and early-onset lung cancer in Whites (Cote et al. 2005).

GSTT1 Gene

Previous results have not supported an association of the *GSTT1* gene with lung cancer risk (To-Figueras et al. 1997; Malats et al. 2000; Stücker et al. 2002; Ruano-Ravina et al. 2003; Wang et al. 2003b). In a study of Chinese living in Hong Kong, the *GSTT1* null genotype was associated with a higher risk of lung cancer than was the functional *GSTT1* genotype (AOR = 1.69; 95 percent CI, 1.12–2.56) only in nonsmokers (Chan-Yeung et al. 2004). A study from Denmark also suggested that the *GSTT1* null genotype is associated with a higher risk of lung cancer (Sørensen et al. 2004a).

NAT2 Gene

Several widely studied polymorphisms for the *NAT2* gene are associated with decreased activity or reduced stability of the enzyme. Phenotypically, these polymorphisms result in slow or fast acetylation. Study results on the association of the *NAT2* gene with lung cancer risk are conflicting. Most studies report no overall increase in risk with the genotype for either slow or fast acetylation (Philip et al. 1988; Martinez et al. 1995; Bouchardy et al. 1998; Saarikoski et al. 2000). However, a few studies report an increase in risk with the genotype for either slow acetylation (Oyama et al. 1997; Seow et al. 1999) or fast acetylation (Cascorbi et al. 1996). In the largest study, of 1,115 lung cancer patients and 1,250 control participants, no association between the *NAT2* genotype and lung cancer risk was observed. However, the study noted a significant interaction with smoking. Among nonsmokers, the genotype for rapid acetylation decreased lung cancer risk more than did the genotype for slow acetylation. This relationship was reversed among smokers, and persons with the genotype for rapid acetylation had a higher risk. The authors hypothesized that for nonsmokers, the NAT2 protein may provide a means for *N*-acetylation, thereby detoxifying aromatic amines and protecting a person against cancer. However, cigarette smoke markedly induces CYP oxidation and could increase the production of reactive

intermediate agents in smokers. In this setting, NAT2 may instead *O*-acetylate these metabolites and thereby produce more reactive metabolites, thus augmenting the cancer risk (Zhou et al. 2002b). A study from Denmark confirmed the associations of the *NAT2* gene with smoking status (Sørensen et al. 2005). However, a study from Taiwan suggested that the *NAT2* genotype for fast acetylation is associated with an increased risk of lung cancer among women who were lifetime nonsmokers (Chiou et al. 2005).

The NAT2 protein plays an important role in the bioactivation and detoxification of the aromatic amines associated with bladder cancer induced by cigarette smoke. In the phenotypic studies, persons with slow acetylation had increased risk of bladder cancer, particularly when they had occupational exposure to arylamines or were cigarette smokers (Green et al. 2000; Johns and Houlston 2000). In the genotype analysis, more than 20 independent studies, many with small sample size have assessed the association of *NAT2* polymorphisms with the risk of bladder cancer. A meta-analysis of published case-control studies conducted in the general population (22 studies, 2,496 cases, and 3,340 controls) examined the relationship of acetylation status (phenotype and genotype) to bladder cancer risk. Persons with slow acetylation had a 40-percent increase in risk compared with risk for persons with rapid acetylation (OR = 1.4; 95 percent CI, 1.2–1.6) (Marcus et al. 2000b). However, studies conducted in Asia generated a summary OR of 2.1 (95 percent CI, 1.2–3.8), studies in Europe generated a summary OR of 1.4 (95 percent CI, 1.2–1.6), and studies in the United States generated a summary OR of 0.9 (95 percent CI, 0.7–1.3).

In addition, a case series meta-analysis of data from a case series of 16 studies of bladder cancer, conducted in the general population and involving 1,999 cases, showed a weak interaction between smoking status and *NAT2* slow acetylation (OR = 1.3; 95 percent CI, 1.0–1.6). The interaction was stronger when analyses were restricted to studies conducted in Europe (OR = 1.5; 95 percent CI, 1.1–1.9) (Marcus et al. 2000a). In a pooled analysis of data from 1,530 cases and 731 controls from four case-control studies plus two case series conducted in Whites in European countries, a significant association was reported between *NAT2* slow acetylation and bladder cancer (OR = 1.42; 95 percent CI, 1.14–1.77) (Vineis et al. 2001). The risk of cancer was elevated in smokers and in persons with occupational exposure to cigarette smoke, and the highest risk was for persons with slow acetylation (Vineis et al. 2001).

In a hospital-based, case-control study of 201 men in northern Italy and a case-control study with 507 White patients with bladder cancer in the United States, findings also suggested that the *NAT2* genotype for slow acetylation was associated with an increased risk of bladder cancer, especially with the joint effects of cigarette smoking and occupational exposure to aromatic amines (Hung et al. 2004; Gu et al. 2005). In a case-control study of bladder cancer in females, exclusive use of permanent hair dye was associated with a 2.9-fold increased risk of bladder cancer among persons with the *NAT2* genotype and slow acetylation but not in those with the *NAT2* genotype and rapid acetylation (Gago-Dominguez et al. 2003). All of these results confirmed that the genotype for *NAT2* slow acetylation is a risk factor for bladder cancer through interaction with smoking or occupational exposure. However, several studies that included populations of Chinese (Ma et al. 2004), northern Indians (Mittal et al. 2004), and Poles (Jaskula-Sztul et al. 2001) reported no association between the *NAT2* genotype and bladder cancer risk.

Microsomal Epoxide Hydrolase

Like NAT2, MEH can act as both an activator and a detoxifier of carcinogens. As a detoxifier, MEH catalyzes the hydrolysis of highly reactive epoxide intermediate agents to less reactive dihydrodiols that are excretable. As an activator, MEH is involved in further metabolism of PAH epoxides. Several identified polymorphisms include a T→C base change in exon 3 leading to a tyrosine→histidine substitution at residue 113, which is associated with a decrease in enzymatic activity, and an A→G base change in exon 4, leading to a histidine→arginine substitution at residue 139, which leads to an increase in enzymatic activity (Hassett et al. 1994). Several reports of studies have noted an increased risk of lung cancer among persons carrying polymorphisms associated with an increase in enzymatic activity. A study of Mexican Americans and African Americans found a greater risk of lung cancer among young Mexican Americans with the exon 4 polymorphism, but not among those with the exon 3 polymorphism. No association was observed among African Americans (Wu et al. 2001). The homozygous variant genotype at exon 4 confers increased enzymatic activity and was again associated with an increase in lung cancer risk in a study in Texas (Cajas-Salazar et al. 2003). A study from Austria suggested an association between the exon 3 polymorphism of the *MEH* gene and a significantly decreased risk of lung cancer (Gsur et al. 2003). The combination of exon 3 and exon 4 polymorphisms that conferred high enzymatic activity also significantly increased the risk (Cajas-Salazar et al. 2003; Park et al. 2005). In a Chinese population in Taiwan, high MEH activity, defined by the corresponding

combination of exon 3 and exon 4 polymorphisms, was associated with an increased risk for SCC (Lin et al. 2000). In a French population, high MEH activity was similarly associated with lung cancer risk (Benhamou et al. 1998).

A study of 974 White patients with lung cancer and 1,142 control participants found no relationship between *MEH* polymorphisms and lung cancer risk overall. However, evidence of gene-environment interactions was observed. Low-activity *MEH* genotypes were a risk factor for lung cancer among nonsmokers (OR = 1.89; 95 percent CI, 1.08–3.28) but were protective among heavy smokers (OR = 0.65; 95 percent CI, 0.42–1.00) (Zhou et al. 2001b). This effect was stronger in SCC than in adenocarcinoma. The researchers hypothesized that this difference may be explained by the dual actions of MEH. In nonsmokers, the presence of low MEH activity may lead to a decreased ability to detoxify environmental pollutants, thus increasing lung cancer risk. In smokers, MEH may participate in activating the PAHs in cigarette smoke. Therefore, low activity is protective for heavy smokers. Similar results were reported in a Slovak study (Habalová et al. 2004).

In a meta-analysis of data from seven published studies that included 2,078 case patients with lung cancer and 3,081 control participants, investigators found no consistent overall association for either the exon 3 or exon 4 polymorphisms with lung cancer risk (Lee et al. 2002c). However, in an analysis of pooled data from eight studies (four published and four unpublished at that time) with 986 case patients and 1,633 control participants, researchers observed a significant decrease in lung cancer risk (OR = 0.70; 95 percent CI, 0.51–0.96) for the exon 3 **HIS/*HIS* genotype. The protective effect of the exon 3 polymorphism seems stronger for adenocarcinomas of the lung than for other histologic types. Researchers found no overall association between MEH activity and lung cancer risk and no consistent modification of the carcinogenic effect of smoking according to the *MEH* polymorphism. However, the risk of lung cancer decreased among lifetime nonsmokers with high MEH activity and among heavy smokers with the exon 3 **HIS/*HIS* genotype (Lee et al. 2002c).

Genes in the Pathway for Metabolism of Reactive Oxygen Species

Studies have identified an alanine→valine substitution at codon 16 of manganese superoxide dismutase (SOD), which may be associated with a less efficient enzyme transport into mitochondria. The **VAL/*VAL* genotype is associated with risk of lung cancer higher than that for the wild-type genotype (AOR = 1.67; 95 percent CI, 1.27–2.20) (Wang et al. 2001a). Other studies also associate the heterozygous variant genotype with an increased risk of lung cancer (AOR = 1.34; 95 percent CI, 1.05–1.70) (Wang et al. 2001a, 2004c). Studies have identified a G→A polymorphism in the promoter region of myeloperoxidase (MPO) that decreases **A* allele transcription. A study of bronchoalveolar lavage fluid and cells from 106 White smokers who had lung cancer showed an association of the variant genotypes with reduced MPO activity in the fluid and reduced levels of smoking-related DNA adducts in bronchoalveolar cells (Van Schooten et al. 2004). The association was stronger in persons having two variant alleles (homozygous variants) than it was in persons having one normal and one variant allele (heterozygous variants).

Findings on lung cancer risk are conflicting. Most studies performed since 1999 suggested that the variant *MPO* genotypes are associated with a decreased risk of lung cancer (Le Marchand et al. 2000; Dally et al. 2002; Feyler et al. 2002; Kantarci et al. 2002; Lu et al. 2002; Schabath et al. 2002). In contrast, two studies found no association between either the heterozygous or homozygous variant genotypes and lung cancer risk (Xu et al. 2002; Chevrier et al. 2003). Another study suggested that the *MPO-G463A* polymorphism associated with a novel estrogen-receptor-binding site modifies the association between the *SOD ALA16VAL* polymorphism and risk of non-small-cell lung cancer (NSCLC) differently by gender. For women carrying *MPO* variant genotypes, the AOR of the *SOD* polymorphism (**VAL/*VAL* versus **ALA/*ALA*) was 3.26 (95 percent CI, 1.55–6.83). No associations were found in men or women who carried the *MPO *G/*G* wild-type genotype (Liu et al. 2004).

Summary

Studies to date suggest a role for genetic polymorphisms in the risk of lung and bladder cancer in smokers and support a possible association between specific genes and smoking status. Investigations continue on the role of multiple genetic variants that occur simultaneously and the interactions between metabolic gene variants and other kinds of heritable variations, such as DNA repair, cell-cycle control, tumor-suppressor genes, and oncogene activity.

DNA Repair and Conversion of Adducts to Mutations

Repair of DNA Adducts

Introduction

Tobacco products and smoke contain many chemicals that can damage DNA. Multiple repair pathways protect a human cell against the mutagenic and carcinogenic activities of these DNA-damaging agents. Pathways involved in the repair of tobacco-related DNA damage include direct base repair by alkyltransferases, excision of DNA damage by base excision repair (BER), or nucleotide excision repair (NER), mismatch repair (MMR), and double-strand break repair (DSBR). The inadequate removal of DNA damage results in increased rates of mutagenesis and, as a consequence, the increased likelihood of a person developing cancer.

O^6-Alkylguanine–DNA Alkyltransferase

Overview

O^6-alkylguanine adducts are repaired by the repair protein O^6-alkylguanine–DNA alkyltransferase (AGT) in a reaction involving transfer of the methyl group from the O^6 position of guanine to a cysteinyl residue on the protein (Pegg 2000). This transfer reaction results in an error-proof repair as it regenerates an unmodified guanine residue in DNA. However, the repair protein is inactivated as the alkylated protein undergoes a conformational change (Daniels et al. 2000) and is degraded (Srivenugopal et al. 1996; Xu-Welliver and Pegg 2002). As a consequence of this repair mechanism, the constitutive levels of AGT determine the initial repair capacity of a cell by this mechanism. Overall capacity of the O^6-alkylguanine repair is determined by the rate of protein synthesis and the amount of alkylation at the O^6 position of guanine.

Substrate Specificity

Mammalian AGT specifically repairs O^6-alkylguanine adducts, and it repairs the larger O^6-alkylguanine residues more readily than does the bacterial protein. In rodents, AGT repairs O^6-methylguanine, O^6-butylguanine, and O^6-[4-oxo-4-(3-pyridyl)butyl]guanine at comparable rates, whereas human AGT repairs the bulky adducts more slowly (Mijal et al. 2004). This ability of the rodent protein to accommodate such large structural differences likely results from the additional amino acid residue (Gly166) in the binding pocket of the rodent proteins (Loktionova and Pegg 2002). Therefore, the steric constraints of an active AGT site determine whether it can efficiently repair a bulky O^6-alkylguanine adduct such as those more commonly resulting from exposure to smoke.

Protecting Against Mutagenicity of Tobacco Carcinogens

Tobacco smoke contains a number of alkylating agents, such as tobacco-specific nitrosamines, which are capable of forming O^6-alkylguanine adducts (Wang et al. 1997; Hecht 1998). AGT protects against the mutagenic and carcinogenic properties of alkylating agents.

In vitro studies. Increased expression of AGT protects against the mutagenic effects of O^6-alkylguanine (Ellison et al. 1989) and alkylating agents (Kaina et al. 1991; Wu et al. 1992; Ferrezuelo et al. 1998a,b). Consistently, alkylating agents are more toxic and mutagenic when coadministered with AGT inactivators such as O^6-benzylguanine or related compounds (Dolan et al. 1990, 1991; Bronstein et al. 1992). The mutagenic activity of O^6-methylguanine or O^6-[4-oxo-4-(3-pyridyl)butyl]guanine is enhanced when cells are pretreated with O^6-benzylguanine (Pauly et al. 1995, 2002). This finding indicated that AGT is important in protecting against the mutagenic activity of these adducts derived from tobacco constituents.

In vivo studies. AGT is depleted in tissues from NNK-treated rats, and AGT levels are depleted in Clara cells (Belinsky et al. 1988). NNK also reduces AGT levels in the lungs and liver of A/J mice (Peterson et al. 2001). Although the liver function recovers to control values within 96 hours after exposure, AGT activity remains depressed in the lung. Consistently, O^6-methylguanine is efficiently repaired in the liver of NNK-treated mice, but it persists for at least two weeks in lung DNA (Peterson and Hecht 1991; Peterson et al. 2001). Notably, levels of O^6-methylguanine in lung DNA are highly correlated with pulmonary tumorigenic activity in A/J mice (Peterson and Hecht 1991; Peterson et al. 2001). These observations strongly suggest that the inefficient repair of O^6-methylguanine, presumably by AGT, is linked to the tumorigenic activity of NNK. This conclusion is supported by the observation from another study that high AGT levels protect against NNK-induced lung tumorigenesis and that NNK is a less potent lung carcinogen in transgenic mice containing the human *AGT* transgene (Liu et al. 1999).

Base Excision Repair

Overview

BER is a major pathway for the repair of small DNA damage, primarily to alkylated and oxidized DNA bases, as well as the repair of apurinic/apyrimidinic (AP) sites and single-stranded breaks (Fortini et al. 2003; Fromme et al. 2004). BER is initiated by a recognition of the damaged DNA by specific DNA glycosylases. Studies have characterized 12 human glycosylases (Table 5.4) (Christmann et al. 2003; Fortini et al. 2003). Each enzyme has different but sometimes overlapping substrate specificities that are subgrouped into type I and type II glycosylases, depending on their mode of action (Christmann et al. 2003). Type I glycosylases catalyze the cleavage of the *N*-glycosidic bond, leaving an AP site. Type II glycosylases remove the damaged base in a similar manner. They contain 3′-endonuclease activity that cleaves the AP site, which generates a single-strand break with a 3′-terminal deoxyribose phosphate. Spontaneous hydrolysis of a glycosidic bond can also directly generate AP sites. AP sites are substrates for DNA AP endonuclease, which cuts the phosphodiester

Table 5.4 Human DNA glycosylases

Study	Glycosylase	Specificity	Subgroup
Chakravarti et al. 1991 O'Connor and Laval 1991 Samson et al. 1991	Alkylpurine DNA glycosylase or methylpurine DNA glycosylase	3-methyladenine, 7-methylguanine, 3-methylguanine, ethenoadenine, hypoxanthine	Type I
Hendrich and Bird 1998 Hendrich et al. 1999	Methyl-CpG binding endonuclease 1	U or T opposite G, preferentially in CpG sites	Type I
Slupska et al. 1996, 1999 Fortini et al. 2003	Adenine DNA glycosylase	A opposite 8-oxoguanine	Type I
Hazra et al. 2002a,b	Nei-like DNA glycosylase 1	Formamidopyrimidines, oxidized pyrimidines, 8-oxoguanine opposite C, G, or T	Type II
Hazra et al. 2002a,b	Nei-like DNA glycosylase 2	5-hydroxyuracil, 5-hydroxycytosine	Type II
Takao et al. 2002	Nei-like DNA glycosylase 3	Fragmented and oxidized pyrimidines	NR
Aspinwall et al. 1997 Hilbert et al. 1997 Miyabe et al. 2002 Fortini et al. 2003	Thymine glycol DNA glycosylase 1	Ring-saturated, oxidized, and fragmented pyrimidines	Type II
Bjørås et al. 1997 Radicella et al. 1997 Rosenquist et al. 1997 Fortini et al. 2003	8-oxoguanine DNA glycosylase 1	8-oxoguanine opposite C, T, or G	Type II
Hazra et al. 1998	8-oxoguanine DNA glycosylase 2	8-oxoguanine opposite A or G	NR
Haushalter et al. 1999 Nilsen et al. 2001	Mismatch-specific uracil DNA glycosylase 1	Uracil opposite G	Type I
Neddermann and Jiricny 1993, 1994 Neddermann et al. 1996	Thymidine DNA glycosylase	Uracil, T, or ethenoC opposite G; T opposite G, C, or T	Type I
Olsen et al. 1989 Muller and Caradonna 1991 Fortini et al. 2003	Uracil DNA glycosylase	Uracil	Type I

Source: Adapted from Christmann et al. 2003 with permission from Elsevier, © 2003.
Note: **NR** = data not reported.

bond and causes the formation of a strand break with a 5′-terminal deoxyribose phosphate (Barzilay et al. 1995).

Once the phosphodiester bond is cleaved, BER can proceed through two pathways: short-patch or long-patch (Figure 5.4). The balance of the two pathways can depend on tissue type (Sancar et al. 2004). In general, short-patch BER dominates when BER is initiated by glycosylases. Long-patch BER is the preferred pathway when BER is initiated with the formation of AP sites through spontaneous hydrolysis or oxidative base loss (Sancar et al. 2004).

In short-patch BER, DNA polymerase β (polβ) inserts a new nucleotide at the lesion site and catalyzes the release of 5′-terminal deoxyribose phosphates by β-elimination (Matsumoto and Kim 1995; Sobol et al. 1996; Prasad et al. 1998). This step is followed by ligation of the remaining break by the ligase III x-ray repair cross-complementation group 1 (XRCC1) complex (Kubota et al. 1996).

Long-patch BER occurs in oxidized or reduced AP sites, 3′-unsaturated aldehydes, or 3′-phosphates, because these modifications are resistant to β-elimination by polβ. Therefore, this damage is further processed by long-patch repair dependent on the proliferating cell nuclear antigen (PCNA) after the insertion of a nucleotide at the lesion site by polβ (Christmann et al. 2003; Fortini et al. 2003). This mechanism displaces the damaged strand, which is followed by DNA synthesis of an oligonucleotide (up to 10 nucleotides) by polδ or polε in concert with PCNA and replication factor C (Stucki et al. 1998). The flap endonuclease 1 (FEN1) recognizes and cleaves off the damaged oligonucleotide flap structure (Klungland and Lindahl 1997). Ligase I catalyzes the final ligation step (Prasad et al. 1996; Srivastava et al. 1998).

Substrate Specificity

Tobacco smoke is rich in reactive oxygen species that can oxidize DNA bases. BER is an important pathway for the repair of oxidized DNA bases, such as 8-oxoguanine and oxidized pyrimidines, and for the repair of single-strand breaks. Tobacco smoke contains *N*-nitrosamines capable of generating small alkylguanine damage that is repaired by this pathway. The small chemical alterations frequently miscode if they are not repaired by BER. Therefore, this pathway is particularly important in preventing mutagenesis.

Protecting Against Mutagenicity and Carcinogenicity of Tobacco Carcinogens

Single-gene knockouts of glycosylases in mice are well tolerated, with only modest increases in rates of spontaneous mutagenesis (Fortini et al. 2003). This observation likely results from an overlapping specificity of the various glycosylases and repair pathways. However, mutagenicity of methyl methanesulfonate (MMS) in lymphocytes from alkylpurine-DNA-*N*-glycosylase–null mice was three to four times higher than that in lymphocytes from wild-type control mice (Elder et al. 1998). Most of the mutations were AT→TA transversions. In addition, 8-oxoguanine DNA glycosylase 1 (OGG1) knockout mice that are aging eventually develop lung cancer (Sakumi et al. 2003). However, in another study, knockout of proteins involved in the steps after removal of the base caused the knockout mice to die at a very young age (Fortini et al. 2003).

Imbalances in the proteins involved in this pathway could have negative consequences. Chinese hamster ovary cells that overexpress alkylpurine DNA glycosylase are more sensitive to both the toxic and mutagenic effects of MMS and have higher numbers of mutations at AT base pairs than do normal Chinese hamster ovary cells (Calléja et al. 1999). This result suggests that an enhanced repair of 3-methyladenine leads to an accumulation of unprocessed AP sites that are also mutagenic. Findings in another study indicate that imbalances and/or polymorphisms in the proteins involved in BER may cause an increase in cancer susceptibility (Fortini et al. 2003).

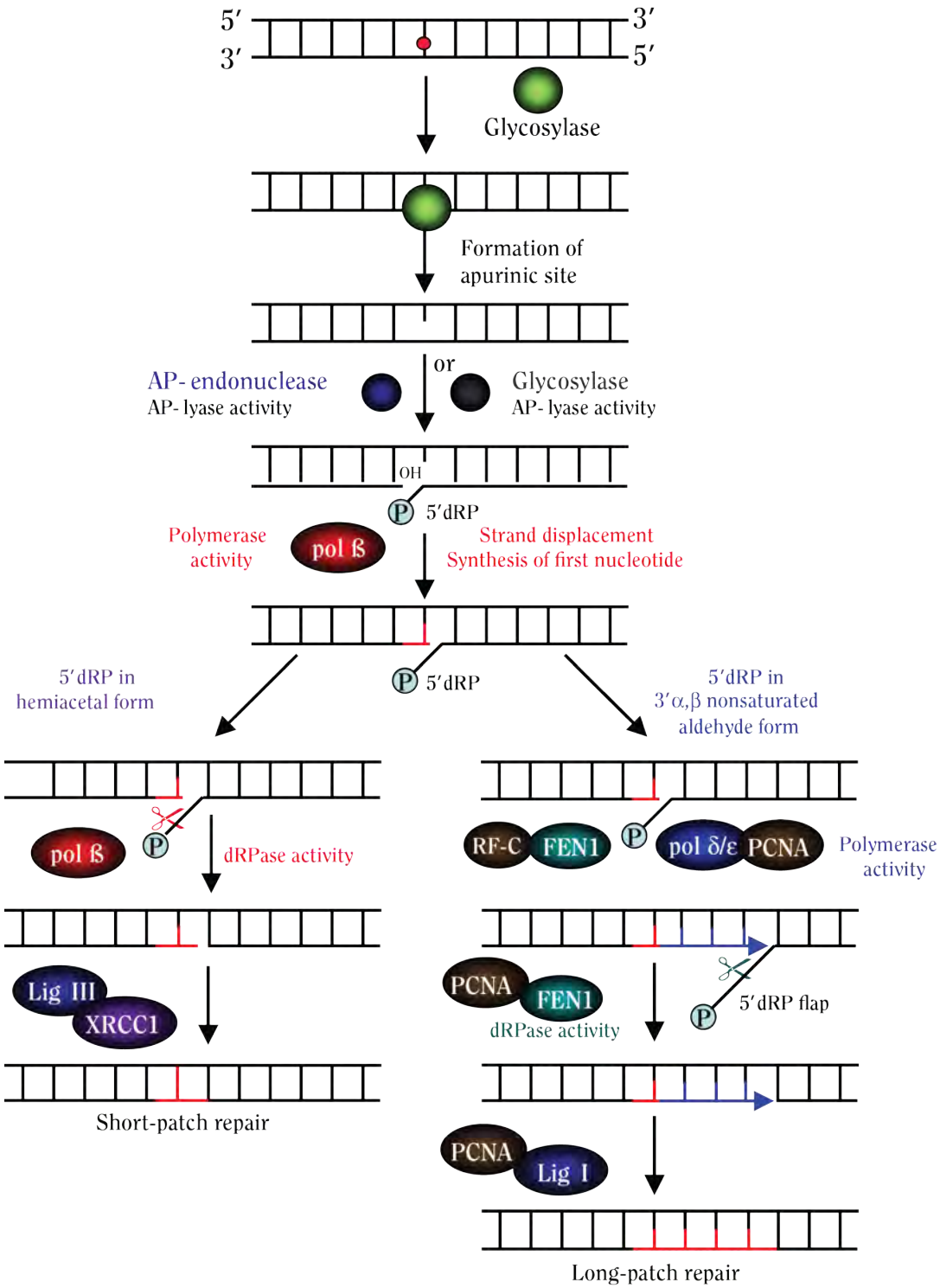
Nucleotide Excision Repair

Overview

NER repairs a wide class of helix-distorting lesions that interfere with base pairing, blocking transcription and normal replication (Petit and Sancar 1999; Sancar et al. 2004). NER is the primary repair mechanism for bulky DNA damage caused by chemicals or ultraviolet (UV) radiation or as a result of protein-DNA cross-links (Sancar et al. 2004). Two NER pathways exist (Figure 5.5): global genomic NER (GGR), which surveys the whole genome for DNA damage, and transcription-coupled repair (TCR), which primarily repairs damage that interferes with transcription. Both pathways involve recognition of DNA damage and excision of the damaged DNA, followed by the synthesis and ligation of new DNA.

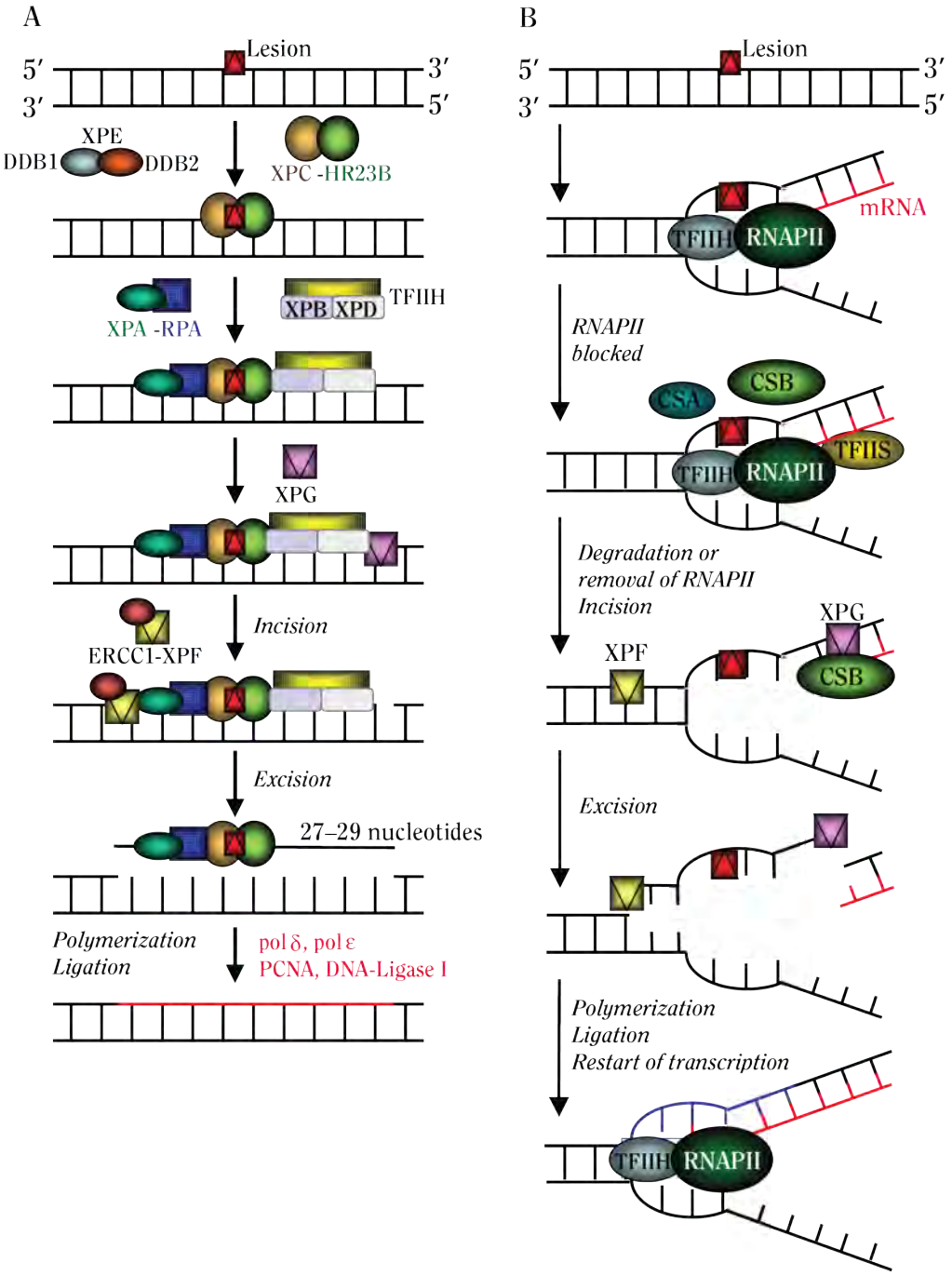
Global genomic nucleotide excision repair. Researchers think that GGR is largely transcription independent, occurring throughout the genome because no gene or strand preference for this repair pathway exists (Hanawalt et al. 2003). Proteins involved in this pathway are presented in Table 5.5. The initial step involves recognition of DNA damage (Figure 5.5). The complex of repair factors in the xeroderma pigmentosum group C (XPC) and the homologous recombinational repair group 23B (HR23B) can directly recognize some DNA damage (Hey et al. 2002). However, in some cases, DNA-binding pro-

Figure 5.4 Mechanism of base excision repair



Source: Adapted from Christmann et al. 2003 with permission from Elsevier, © 2003.
Note: **5′dRP** = 5′-deoxyribose phosphate; **AP** = apurinic/apyrimidinic; **dRPase** = DNA deoxyribophosphodiesterase; **FEN1** = flap endonuclease 1; **Lig** = ligase; **OH** = hydroxide; **P** = phosphate; **PCNA** = proliferating cell nuclear antigen; **pol** = polymerase; **RF-C** = replication factor C; **XRCC1** = x-ray repair cross-complementation group 1.

Figure 5.5 Mechanism of nucleotide excision repair: (A) global genomic repair; (B) transcription-coupled repair



Source: Adapted from Christmann et al. 2003 with permission from Elsevier, © 2003.
Note: **CSA** = Cockayne syndrome complementation group A; **CSB** = Cockayne syndrome complementation group B; **DDB** = DNA binding protein; **ERCC1** = excision repair cross-complementation group 1; **HR23B** = homologous recombinational repair group 23B; **mRNA** = messenger RNA; **PCNA** = proliferating cell nuclear antigen; **pol** = polymerase; **RNAPII** = RNA polymerase II; **RPA** = replication protein A; **TFIIH** = transcription initiation factor IIH; **TFIIS** = transcription initiation factor IIS; **XP** = xeroderma pigmentosum (groups A–G).

Table 5.5 Factors involved in nucleotide excision repair activity in humans

Factor	Proteins	Factor activity	Role in repair
XPA	XPA	DNA binding	Damage recognition
RPA	P70	XPA binding	Damage recognition
	P34	DNA binding	NR
	P11	NR	NR
TFIIH	XPB	DNA-dependent ATPase	Formation of preincision complexes
	XPD	Helicase	NR
	P62	GTP	NR
	P52	NR	NR
	P44	CAK	NR
	CDK7	NR	NR
	CYCH	NR	NR
	P34	NR	NR
XPC	XPC	DNA binding	Molecular matchmaker
	HR23B	NR	Stabilization of preincision complex 1
XPG	XPG	NR	3′ incision
XPF	XPF	NR	5′ incision
	ERCC1	NR	NR

Source: Adapted from Petit and Sancar 1999 with permission from Elsevier, © 1999.
Note: **ATPase** = adenosine triphosphatase; **CAK** = cyclin-dependent kinase-activating kinase; **CDK7** = cyclin-dependent kinase 7; **CYCH** = cytochrome *c*-type biogenesis protein; **ERCC1** = excision repair cross-complementation group 1; **GTP** = guanosine triphosphate; **HR23B** = homologous recombinational repair group 23; **NR** = data not reported; **RPA** = replication protein A; **TFIIH** = transcription initiation factor IIH; **XP** = xeroderma pigmentosum (groups A–G).

tein is required to enhance the DNA distortion before the recruitment of XPC-HR23B (Tan and Chu 2002; Hanawalt et al. 2003). Repair factors XPA and replication protein A are then recruited to the complex to verify that the alteration in the DNA structure results from a lesion, as opposed to a natural variation in DNA structure (Hanawalt et al. 2003).

The DNA is then unwound at the site of the lesion. This step occurs on recruitment of the basal transcription initiation factor IIH (TFIIH) multiprotein complex (Christmann et al. 2003). This complex is likely involved in a further verification of damage and detection of the damaged strand. The helicase subunits of TFIIH, XPB, and XPD then unwind the DNA around the lesion.

Once the DNA has unwound, the lesion is excised at defined sites on either side of the damage (Evans et al. 1997). The 3′-incision is catalyzed by XPG (Habraken et al. 1994; O'Donovan et al. 1994), and the 5′-incision is catalyzed by the excision repair cross-complementation group 1 (ERCC1) XPF complex (Sijbers et al. 1996). The resulting DNA gap is filled in by the PCNA-dependent polymerases: polδ and polε (Aboussekhra et al. 1995; Araújo et al. 2000). The final ligation is performed by DNA ligase I and associated proteins (Aboussekhra et al. 1995; Mu et al. 1995; Araújo et al. 2000). Findings from in vivo studies suggest that the NER machinery is assembled in a stepwise manner from the individual components at the lesion site. After the repair of a DNA lesion, the entire complex is believed by researchers to disassemble (Houtsmuller et al. 1999; Hoeijmakers 2001).

GGR appears to be inducible in humans (Hanawalt et al. 2003). The proteins that recognize DNA damage in GGR are maintained at low levels under normal physiological conditions. However, as a result of genomic stress, the efficiency of GGR is increased through the activation of the *P53* tumor-suppressor gene (Hanawalt et al. 2003). Consequently, the levels of XPC and XPE increase. Repair of PAH adducts depends on the presence of the P53 protein (Hanawalt et al. 2003). These adducts are not repaired in human fibroblasts that lack a functional P53 protein or other gene product (Lloyd and Hanawalt 2000, 2002; Wani et al. 2000).

Transcription-coupled repair. TCR occurs when DNA damage blocks elongating RNA polymerases (Tornaletti and Hanawalt 1999). The transcription-coupled, repair-specific factors belong to two Cockayne syndrome

complementation groups (A and B) and are involved in the displacement of the stalled polymerase (Christmann et al. 2003). At this point, TFIIH is recruited to the lesion, and the subsequent steps in TCR proceed in a manner apparently similar to the steps for GGR. Because of the mechanism of DNA damage recognition, TCR is specific to a DNA strand.

Substrate Specificity

The substrate specificity of NER ranges from small to large distortions in DNA structure. The bulky DNA damage generated by PAHs and aromatic amines in tobacco smoke is repaired primarily by NER (Friedberg 2001). The finding that human NER repairs 8-oxoguanine in vitro more efficiently than thymine dimers or thymine glycol (Reardon et al. 1997) suggests that this pathway may be important for the repair of this mutagenic adduct. In addition, human NER repairs O^6-methylguanine and N^6-methyladenine, as well as A:G and G:G mismatches (Huang et al. 1994).

Protecting Against Mutagenicity and Carcinogenicity of Tobacco Carcinogens

Consistent with the primary role of NER in the repair of bulky DNA damage from PAHs and aromatic amines, cells deficient in NER are more susceptible to the mutagenic and toxic effects of PAHs (Quan et al. 1994, 1995; Lloyd and Hanawalt 2000, 2002; Wani et al. 2000).

Mismatch Repair

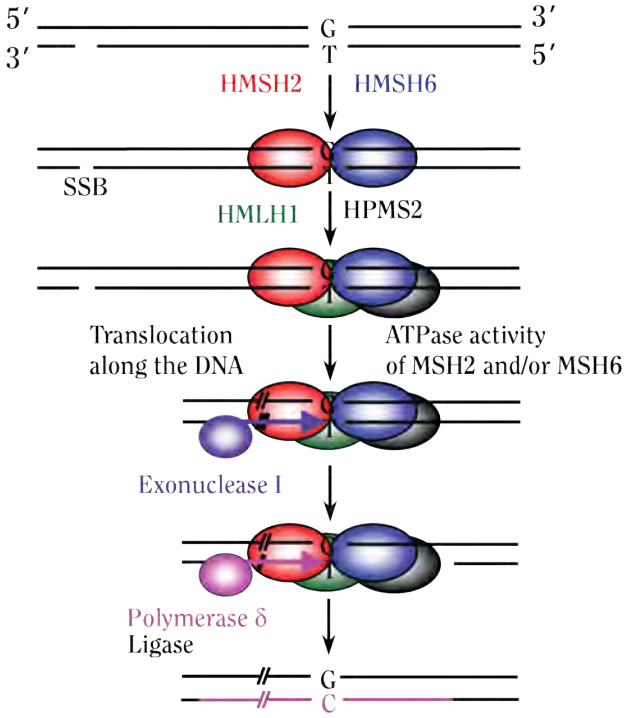
Overview

MMR corrects replication errors (base-base or insertion-deletion mismatches) resulting from DNA polymerase errors. This repair pathway is also involved in the repair of alkylation DNA damage, such as repair of O^6-methylguanine (Duckett et al. 1996), cisplatin-derived 1,2-intrastrand cross-links (Duckett et al. 1996), and adducts derived from B[*a*]P (Wu et al. 1999), 2-aminofluorene, and *N*-acetyl-2-aminofluorene (Li et al. 1996). This pathway can also repair 8-oxoguanine (Colussi et al. 2002).

An overview of MMR is displayed in Figure 5.6. Recognition of DNA damage occurs primarily by the mutSα complex (Christmann et al. 2003). This complex is composed of the mutS homologous proteins MSH2 (Fishel et al. 1993; Leach et al. 1993) and MSH6 (Palombo et al. 1995). Once the heterodimer is bound to the mismatch, it undergoes an adenosine-triphosphate (ATP)-dependent conformational change (Stojic et al. 2004). This complex is involved in determining which strand is the newly synthesized DNA (Christmann et al. 2003). A second heterodimer (mutLα) composed of mutL homologs MLH1 and PMS2 then binds to the mutSα-DNA complex in another ATP-dependent step (Nicolaides et al. 1994; Papadopoulos et al. 1994; Li and Modrich 1995; Stojic et al. 2004). Exonuclease I then excises the DNA strand containing the mispaired base (Genschel et al. 2002), followed by the resynthesis of new DNA by polδ (Longley et al. 1997).

MSH2 can also complex with MSH3 to form mutSβ (Acharya et al. 1996; Palombo et al. 1996). The substrate specificity of this alternate complex is different from that of mutSα (Christmann et al. 2003). The mutSα complex recognizes base-base mismatches, as well as insertion-deletion mismatches (Umar et al. 1994), whereas mutSβ binds only to insertion-deletion mismatches (Palombo et al. 1996; Genschel et al. 1998).

Figure 5.6 Mechanism of mismatch repair



Source: Adapted from Christmann et al. 2003 with permission from Elsevier, © 2003.
Note: For two steps dependent on adenosine triphosphatase (ATPase), see text. **C** = cytosine; **G** = guanine; **HMLH1** = human mutL homolog 1 protein; **HMSH2** = human mutS homolog 2 protein; **HMSH6** = human mutS homolog 6 protein; **HPMS2** = human postmeiotic segregation increased 2 protein; **SSB** = single-stranded DNA binding protein; **T** = thymine.

Protecting Against Mutagenicity and Carcinogenicity of Tobacco Carcinogens

Persistent adducts that escape repair by AGT, BER, or NER may be processed by MMR. Because MMR occurs after DNA replication (Stojic et al. 2004), this is the last opportunity for DNA damage repair before cell division. Consequently, a defective MMR results in an increase in mutagenesis (Schofield and Hsieh 2003). The overall effect of a defective MMR is the likelihood that cells with persistent DNA damage survive with a miscoded DNA. This combination results in a higher cancer risk.

The role of MMR in the toxicity and mutagenicity of alkylating agents is well documented for methylating agents (Karran 2001; Stojic et al. 2004). Cells lacking functional MMR are more resistant to the toxic effects of methylating agents (Koi et al. 1994; Risinger et al. 1995; Umar et al. 1997; de Wind et al. 1999; Karran 2001; Stojic et al. 2004). However, these cells are sensitive to the mutagenic effects of these compounds (Umar et al. 1997; Zhu et al. 1998). These effects are linked to the ineffective MMR of O^6-methylguanine. As a result, a defective MMR is associated with increases in mutagenesis and carcinogenesis mediated by O^6-methylguanine (Hickman and Samson 1999; Pauly and Moschel 2001). Similar effects were reported for ethylating agents (Claij et al. 2003).

Double-Strand Break Repair

Overview

Two pathways exist for DSBRs: homologous recombination and nonhomologous end-joining. Homologous recombination occurs during DNA replication in the S and G_2 phases, whereas nonhomologous end-joining occurs during G_0 and G_1 phases. Homologous recombination uses the sister chromatid as the template for aligning the breaks in the proper orientation and is consequently error free (Hoeijmakers 2001). However, nonhomologous end-joining does not require sequence homology between the two breaks to ligate them and is therefore prone to errors (Hoeijmakers 2001).

Homologous Recombination

The meiotic recombination 11 (MRE11)-RAD50-NBS1 protein complex initiates DSBR by catalyzing the degradation of the DNA in the 5′ to 3′ direction, generating 3′ single-stranded DNA (Figure 5.7). This single-stranded DNA is protected from degradation by a heptameric ring complex of RAD52 proteins (Stasiak et al. 2000). Replication factor A facilitates the assembly of a RAD51 nucleoprotein filament, which consists of RAD51B, RAD51C, and RAD51D, as well as XRCC2 and XRCC3 (Christmann et al. 2003). RAD51 is able to exchange the single strand with the same sequence from the sister chromatid DNA. This double-stranded copy is then used as a template to correctly repair with DNA synthesis machinery. The resulting Holliday structures are subsequently resolved to generate the repaired DNA (Constantinou et al. 2001).

Nonhomologous End-Joining

Nonhomologous end-joining merely links the ends of a DSB together in the absence of a template (Figure 5.8). The break is initially recognized by a heterodimer consisting of the proteins KU70 (Reeves and Sthoeger 1989) and KU80 (Jeggo et al. 1992). This binding protects the DNA from digestion and associates it with the protein kinase catalytic subunit DNA-PK_{cs}, which is dependent on DNA. This complex, in turn, activates XRCC4-ligase IV, which connects the broken DNA pieces together once the MRE11-RAD50-NBS1 complex has processed the break (Maser et al. 1997; Nelms et al. 1998). Researchers think that FEN1 and Artemis also play a role in the processing of DSBs (Christmann et al. 2003).

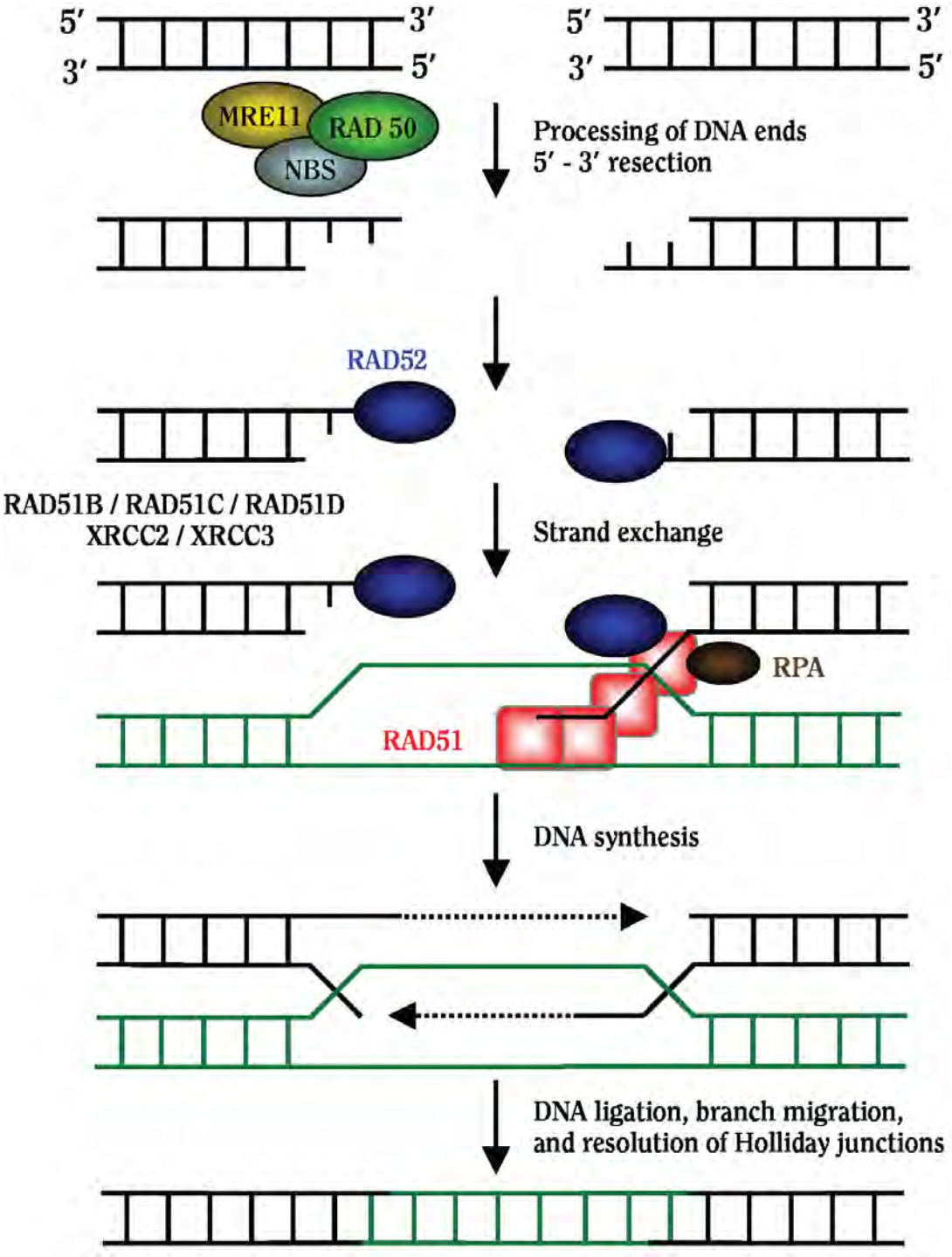
Protecting Against Mutagenicity and Carcinogenicity of Tobacco Carcinogens

Scientists think that DSBs trigger large chromosomal aberrations such as chromosomal breaks and exchanges (Pfeiffer et al. 2000). Studies link an increase in chromosomal aberrations to tobacco exposure (DeMarini 2004). These aberrations are more common in pulmonary lung tumors from smokers than in those from nonsmokers (Sanchez-Cespedes et al. 2001). DSBs are also important in triggering cell death (Rich et al. 2000; Lips and Kaina 2001).

Molecular Epidemiology of DNA Repair

Studies support the substantial interindividual variations in DNA repair capacity (DRC). Researchers hypothesize that common variants in the genes that regulate these protein expressions may modulate repair and influence susceptibility to tobacco carcinogenesis. Two complementary approaches to studying DNA repair as a risk factor for tobacco carcinogenesis are applying functional assays and genotyping variants in gene pathways for DNA repair. Table 5.6 summarizes some relevant genes and their variants in pathways involved in repairing tobacco-induced DNA damage.

Figure 5.7 Proposed mechanism of homologous recombination



Source: Adapted from Christmann et al. 2003 with permission from Elsevier, © 2003.
Note: **MRE11** = meiotic recombination 11; **RAD** = *S. cerevisiae* DNA damage recognition and repair protein; **RPA** = replication protein A; **XRCC2/XRCC3** = x-ray repair cross-complementation groups 2 and 3.

Functional Assays of DNA Damage and Repair to Tobacco Carcinogens

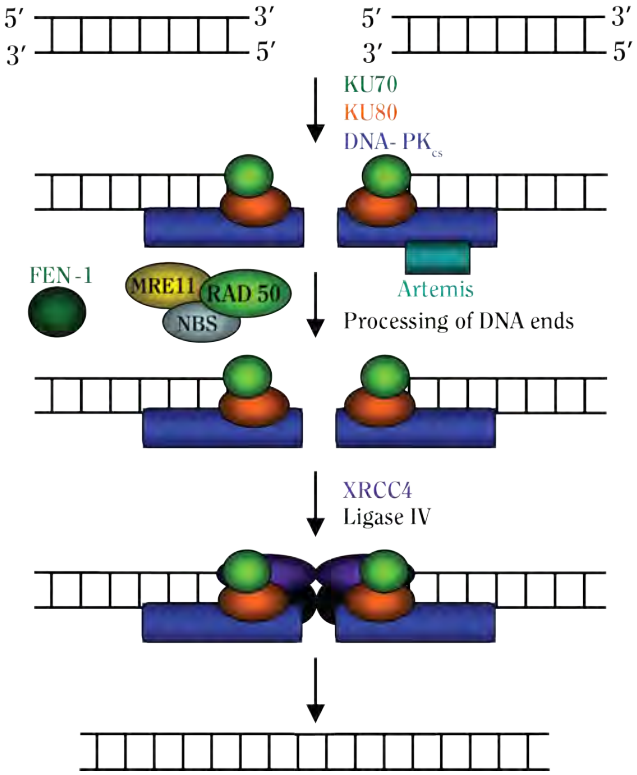
Berwick and Vineis (2000) extensively reviewed the types of functional assays. The review included assays that use a chemical or physical mutagen challenge such as mutagen sensitivity, single-cell microgel electrophoresis (comet assay), and assays of induced adducts and unscheduled DNA synthesis. The review also included the host-cell reactivation (HCR) assay, which measures cellular ability to remove adducts from plasmids transfected into lymphocyte cultures in vitro by the expression of damaged reporter genes.

Host-Cell Reactivation Assay

The HCR assay measures the expression level of damaged reporter genes, which are involved in reactivation of the host cell. The assay uses undamaged host lymphocytes, is fast, and objectively measures intrinsic cellular repair (Athas et al. 1991). In the assay, lymphocytes are transfected with a damaged, nonreplicating recombinant plasmid harboring a chloramphenicol acetyltransferase (CAT) reporter gene (*PCMVCAT*). To study tobacco-related cancers, the mutagen challenge is B[*a*]P (Gelboin 1980). Experimental conditions produce at least one BPDE-DNA adduct per plasmid, completely blocking transcription of the *PCMVCAT* gene without inducing conformational changes in the DNA. This finding is important because conformational change of the plasmid could reduce the transfection rate. Because even a single unrepaired DNA adduct can effectively block *PCMVCAT* transcription (Koch et al. 1993), any measurable *PCMVCAT* activity reflects the ability of the transfected cells to remove BPDE-induced adducts from the plasmids (Athas et al. 1991).

Both lymphocytes and skin fibroblasts from patients with basal cell carcinoma, but not with XP, were found to have lower excision repair rates than those from persons without cancer (Wei et al. 1993). Consequently, the repair capacity of lymphocytes may reflect the overall repair capacity of a person. Spitz and colleagues (2003) showed that case patients with lung cancer had a significantly lower DRC than did control participants and that case patients aged 63 years or younger and lifetime nonsmokers had a lower DRC than that of matched control participants. DRC appears to be highest among case patients and control participants who were current smokers than among those who were former smokers and lifetime nonsmokers. Heavy smokers among both case patients and control participants tended to have more proficient DRC than did light smokers. This finding indicated that cigarette smoking may stimulate DRC in response to the DNA damage caused by carcinogens in tobacco.

Figure 5.8 Proposed mechanism of nonhomologous end-joining



Source: Adapted from Christmann et al. 2003 with permission from Elsevier, © 2003.
Note: **FEN1** = flap endonuclease 1; **MRE11** = meiotic recombination 11; **PK_{cs}** = protein kinase catalytic subunit; **XRCC4** = x-ray repair cross-complementation group 4.

Such an adaptation would be consistent with a baseline DRC that can be mobilized on an increased demand for repair (Eller et al. 1997; Cheng et al. 1998). This adaptation of DRC to smoking, if it exists, appears to be long term rather than transient, because the effect was still present in former smokers but was not stronger in those who had smoked in the 24 hours before the blood sample was drawn (Wei et al. 2000). The finding that long-term heavy smokers with lung cancer had an efficient DRC may also indicate that heavy exposure overwhelms even relatively resistant phenotypes.

Luciferase Host-Cell Reactivation Assay

Researchers have modified the HCR assay by replacing CAT with luciferase (LUC). The cell-extraction procedure is far more simplified for the LUC assay. A luminometer measures LUC optical density, and the laboratory

Table 5.6 Select candidate genes and polymorphisms implicated in repair of tobacco-induced DNA damage

Gene	Nucleotide position	Nucleotide change	Amino acid change	Allele frequency
Alkyltransferases				
AGT, MGMT			Ile143Val	0.05–0.2
Base excision repair				
MBD4	13390	T/C	Ser342Pro	0.05
	13402	G/A	Glu346Lys	0.07
TDG	27090	G/A	Gly199Ser	0.09
MUTYH	18556	G/C	Gly335His	0.44
OGG1	18069	C/G	Ser326Cys	0.25
APEX1	11865	T/G	Glu148Asp	0.25
XRCC1	32584	C/T	Arg194Trp	0.08
	33746	G/A	Arg280His	0.05
	34432	G/A	Arg399Gln	0.31
ADPRT	75787	T/C	Val761Ala	0.38
	77525	C/T	Pro882Leu	0.20
POLD1	24569	G/A	Arg19His	0.12
	27479	A/G	His119Arg	0.16
	27715	A/G	Ser173Asn	0.06
Nucleotide excision repair				
XPC	30197	C/T	Ala499Val	0.24
	42635	A/C	Lys939Gln	0.38
XPD	16232	G/A	Asp312Asn	0.40
	28572	A/C	Lys751Gln	0.33
XPF	34637	T/C	Ser662Pro	0.06
RAD23B	48769	C/T	Ala249Val	0.18
CCNH	23563	C/T	Val270Ala	0.10
XPG	39583	G/C	His1104Asp	0.32
LIG1	29008	A/C	Ala170Ala	0.39
	61130	C/T	Asp802Asp	0.21
	62525	C/G	Ala814Ala	0.30
Double-strand break repair				
Homologous recombination				
RAD51	692 (cDNA)	G/T	Ala143Ser	0.33
XRCC3	26044	T/C	Thr241Met	0.22
RAD52	45137	C/A	Ser346Ter	0.05
RAD54L	1222 (cDNA)	G/C	Arg374Ser	0.11
BRCA2	27113	T/G	His372Asn	0.23
	93268	G/A	Ile3412Val	0.05
RAD50	3374 (cDNA)	G/T	Arg1111Ile	0.25
NBS1	137331	C/G	Gln185Glu	0.46
Nonhomologous end-joining				
KU70	336 (cDNA)	G/A	Glu107Lys	0.06
	454 (cDNA)	T/C	Val146Ala	0.11
	1825 (cDNA)	T/C	Leu603Pro	0.10
KU80	1487 (cDNA)	C/T	Pro485Leu	0.14
LIG4	8 (cDNA)	C/T	Ala3Val	0.07
	27 (cDNA)	C/T	Thr9Ile	0.14
XRCC4	21965	G/T	Ala247Ser	0.08

Note: **cDNA** = complementary DNA.

procedures are shorter. The results for the DRC phenotype from the independent CAT and LUC assays in parallel are highly correlated, with a correlation coefficient of 0.65 ($p < 0.0001$) (Qiao et al. 2002a). This finding suggests that these two assays are comparable.

8-Oxoguanine DNA Glycosylase Activity Assay

The OGG test is another functional repair assay that measures the specific activity of the enzyme 8-oxoguanine DNA *N*-glycosylase in protein extracts prepared from peripheral blood mononuclear cells (Paz-Elizur et al. 2003). The OGG1 enzyme removes 8-oxoguanine from DNA and leaves behind an abasic site that is rapidly cleaved by the AP lyase of OGG1. OGG activity (in units per µg of protein extract) is the amount of fragment cleaved by 1 µg of extract in one hour under standard reaction conditions (Paz-Elizur et al. 2003). In a pilot case-control study of NSCLC, the mean value of OGG activity in the case patients was significantly lower than that in the control participants and was independent of tumor histology, gender, and smoking status (Paz-Elizur et al. 2003). In addition, OGG activity in protein extracts from peripheral blood mononuclear cells correlated well with that from lung tissue in the same patients ($p = 0.003$).

Mutagen Sensitivity Assays

Researchers use cytogenetic assays extensively to measure human exposure and response to genotoxic agents. These assays are based on the unproven hypothesis that the extent of genetic damage in the lymphocytes may reflect critical events in carcinogenesis in the affected tissues. The assays can only indirectly indicate DRC from the cellular damage that remains after mutagenic exposure and recovery and therefore probably reflect nonspecific impairment of the DNA repair machinery (Berwick and Vineis 2000).

Hsu and colleagues (1989) developed the in vitro mutagen challenge assay to demonstrate interindividual differences in susceptibility to carcinogenic agents. This assay counts the frequency of bleomycin-induced breaks in short-term lymphocyte cultures, as a measure of cancer susceptibility. Bleomycin is a clastogenic agent that mimics the effects of radiation by generating free oxygen radicals capable of producing DNA single-strand breaks and DSBs after forming a complex with DNA, ferrous ions, and oxygen that releases oxygen radicals (Burger et al. 1981). Most of the breaks are repaired by BER. Repair is rapid, with a half-life of only a few minutes (López-Larraza et al. 1990).

Mutagen sensitivity is an independent risk factor for lung cancer that has a dose-response relationship with the number of induced chromosomal breaks (Spitz et al. 1995, 2003; Strom et al. 1995; Wu et al. 1995, 1998a; Zheng et al. 2003). The risks associated with mutagen sensitivity, stratified by smoking status, are elevated in all smoking strata but are highest in current smokers and in the heaviest smokers. For studying tobacco-related cancers, a more appropriate mutagen to trigger DNA damage is BPDE; BPDE-induced sensitivity is a risk factor for lung cancer (Wei et al. 1996; Wu et al. 1998a,b).

Comet Assay

The comet assay (single-cell microgel electrophoresis) is applicable to any cell line or tissue from which a single-cell suspension can be obtained (Ross et al. 1995). Singh and associates (1988) have described the methods in detail. For this assay, a cell culture is mixed with agarose gel and attached to a microscope slide. The cells are lysed by submerging the slides in freshly prepared lysis buffer to remove all cellular proteins. To allow for DNA denaturation, unwinding, and expression of the alkali-labile sites, the slides are then placed in an alkali buffer ($pH = 12.0$). To separate the damaged DNA from the intact nuclei, a constant electric current is applied, and the slides are neutralized, fixed, and stored in the dark at room temperature until ready for analysis. During electrophoresis, damaged DNA migrates from the nucleus toward the anode as a result of the constant electric current, which forms the typical "comet" cell. A predetermined number of cells are manually selected, and comet cells are automatically quantified with appropriate imaging software.

Case patients with lung and bladder cancer had significantly higher levels of induced DNA damage after exposure to both BPDE and γ-radiation, which were assessed by the mean "tail moment" in lymphocytes. (The tail moment is the product of lymphocyte tail length and the fraction of the total DNA in the tail.) Higher levels of DNA damage were positively associated with increased risk of bladder and lung cancer (Schabath et al. 2003; Wu et al. 2005). Schmezer and colleagues (2001) showed that case patients with lung cancer were significantly more sensitive to bleomycin and had a reduced DRC (68 percent in case patients and 81 percent in control participants [$p < 0.001$]). Rajee-Behbahani and colleagues (2001) reported a similar DRC finding of 67 percent in 160 case patients with lung cancer and 79.3 percent in 180 control participants. When the data from the cases and controls were considered together, only 18 percent of the case patients were below the median level of sensitivity to bleomycin for all control participants, and 82 percent were in the hypersensitive range.

Mohrenweiser and Jones (1998) have pointed out several lines of evidence documenting that differences in DRC reflect genetic differences. DRC in lymphocyte

subpopulations from an individual exhibited similar repair capacities. Furthermore, intraindividual variations in repair capacity among subpopulations of lymphocytes are significantly smaller than are interindividual variations (Crompton and Ozsahin 1997). The phenotype of reduced repair capacity in the NER pathway is independent of the phenotype for DSBR (Wu et al. 1998a).

Researchers have performed extensive resequencing of DNA repair genes to identify variations that may be associated with a reduced function of their encoded proteins rather than an absence of function. Such polymorphisms could explain interindividual differences in DRC (Spitz et al. 2001; Qiao et al. 2002b; Wu et al. 2003). Although the variant alleles are likely to be associated with only a modest cancer risk, because they exist at a polymorphic frequency, the attributable risks can become substantial. As Berwick and Vineis (2000) noted, studies that compare genetic polymorphisms with results of functional assays will likely be the most valuable type of investigations to clarify the role of a defect in DRC with the development of cancer.

Polymorphisms in *O*^{6}-Alkylguanine–DNA Alkyltransferase

The ability of a cell to withstand alkyl damage is related to the number of AGT molecules in the cell and the rate of de novo synthesis of AGT. AGT levels differed among persons, and protein levels in the liver, colon, and peripheral blood varied 4-, 10-, and 20-fold, respectively (Myrnes et al. 1983; Strauss et al. 1989; Povey et al. 2000). Environmental and genetic factors, but not age, affect expression levels of AGT (Margison et al. 2003). AGT levels are higher in normal lung, colorectal, and placental tissues from smokers than in those from nonsmokers, although these findings are controversial (Slupphaug et al. 1992; Drin et al. 1994; Povey et al. 2000). Exposure to AGT inhibitory aldehydes, such as formaldehyde from occupational exposure, can deplete AGT activity (Hayes et al. 1997). Expression of AGT is higher in NSCLC tumor tissues from smokers than in those from nonsmokers (Mattern et al. 1998). However, small-scale studies examining AGT activity in peripheral lymphocytes have not observed significant differences between smokers and nonsmokers (Vähäkangas et al. 1991; Oesch and Klein 1992; Hall et al. 1993) or between case patients with cancer and control participants (Boffetta et al. 2002). It is not clear how well AGT activity in lymphocytes predicts levels in lung tissue.

The *AGT* gene contains multiple polymorphisms in the 5′ upstream region, which comprises the promoter and enhancer regions, as well as exon 1 (Egyházi et al. 2002; Krzesniak et al. 2004). These genetic variations may account for some of the interindividual variations in expression levels. Eight allelic variants in this region occur at a frequency of at least 0.01. One-half of these variants have a prevalence of 0.1 to 0.6, and this finding suggests a contribution to the variability in AGT expression within populations (Krzesniak et al. 2004). The prevalence of the best-studied enhancer polymorphism, *C1099T*, is 0.09 to 0.12 and is associated with an increase in promoter-enhancer activity in the LUC assay (Krzesniak et al. 2004). However, heterozygotes for this enhancer polymorphism do not appear to be at a markedly lower risk of developing lung cancer (OR = 0.82; 95 percent CI, 0.41–1.67) (Krzesniak et al. 2004). None of the other enhancer-promoter polymorphisms have been examined for their relationship with AGT or cancer risk.

Mutations at or near the cysteine acceptor site can affect AGT activity (Crone et al. 1994). Two polymorphisms have been identified in this region of the protein: codon 143 **ILE/*VAL* and codon 160 **GLY/*ARG*. The variant isoform codon 160 **GLY/*ARG* has exhibited activity to repair bulky alkylated DNA adducts that is significantly less than that of the *GLY* wild-type phenotype (Edara et al. 1996; Mijal et al. 2004). The estimated prevalence of the 143 variant is 0.07 in Whites, 0.03 in African Americans (Kaur et al. 2000), and as high as 0.24 in Swedish populations (Ma et al. 2003). The codon 160 variant allele is likely a mutant, because it was found at a frequency less than 0.01 (Kaur et al. 2000). These researchers reported a marginally significant increase in the risk of lung cancer associated with the codon 143 **ILE/*VAL* genotype (OR = 2.1; 95 percent CI, 1.01–4.7), and no interaction of genotype and smoking dose was seen. More recently, Cohet and colleagues (2004) reported an OR of 2.05 (95 percent CI, 1.03–4.07) for lung cancer among lifetime carriers of the codon 143 and 160 variant alleles who were nonsmokers. The authors suggested that the strongest risk was associated with exposure to secondhand tobacco smoke.

Polymorphisms in the Pathway for Repair of Base Excision

Cross-Complementation Group 1 for Excision Repair

The *XRCC1* gene encodes a protein that functions in BER and involves the excision of the damaged region, followed by repair synthesis that uses the opposite strand as the template. The XRCC1 protein forms scaffolding with DNA ligase III, polβ, and poly (adenosine diphosphate–ribose) polymerase (PARP) to rejoin DNA strand breaks and repair gaps left during BER. Of the estimated 17 variants, 1 is a polymorphism at the *XRCC1* 28152 site

(G→A transition) of codon 399 in exon 10 that results in a nonconservative amino acid substitution of arginine for glutamine. Goode and colleagues (2002) extensively reviewed the conflicting results for lung cancer studies. For example, Divine and colleagues (2001) reported a more than twofold risk for lung adenocarcinoma associated with the **GLN/*GLN* genotype. Zhou and colleagues (2003) found an association that was largely restricted to nonsmokers and light smokers. Some studies failed to find any association between the **GLN/*GLN* genotype and lung cancer risk (Butkiewicz et al. 2001; Ratnasinghe et al. 2001; Popanda et al. 2004; Zhang et al. 2005b). However, Matullo and colleagues (2001a) did find an association for bladder cancer. David-Beabes and London (2001) reported a decreased risk of lung cancer for both African American and White patients. Other studies also report conflicting findings regarding an increased risk associated with the exon 10 **ARG/*ARG* genotype (Lee et al. 2001; Stern et al. 2001).

Another polymorphism at the *XRCC1* 26304 site (C→T transversion) of codon 194 in exon 6 results in a nonconservative amino acid substitution of arginine. Most studies suggest a reduced risk of cancer associated with the 399**ARG* variant allele (Goode et al. 2002). Studies of lung, bladder, head and neck, and gastric cancers all showed inverse associations with the variant allele; some studies included evidence of an interaction with smoking (Sturgis et al. 1999; David-Beabes and London 2001; Ratnasinghe et al. 2001; Stern et al. 2001). Two studies noted no association for esophageal and lung cancers (Butkiewicz et al. 2001; Lee et al. 2001).

A few earlier studies evaluated the functional significance of these polymorphisms. Lunn and colleagues (1999) noted that persons with the 399**GLN* allele were at a significantly higher risk (OR = 2.4) for exhibiting detectable aflatoxin B_1 adducts and a higher frequency of the glycophorin A variant than were carriers of the 399**ARG/*ARG* allele. This study also reported a dose-response relationship between smoking status and presence of the polymorphism for detecting the adducts and the glycophorin A variant (Lunn et al. 1999). However, no significant effects were noted for other *XRCC1* polymorphisms. Duell and colleagues (2000) found elevated SCE frequencies and polyphenol DNA adducts with 399**GLN/*GLN* homozygous genotypes. Abdel-Rahman and El-Zein (2000) noted that persons carrying the **GLN* allele had significantly higher numbers of SCEs in response to NNK treatment than did **ARG/*ARG* genotype carriers. No differences were detected in persons with the codon 194 genotype.

Matullo and colleagues (2001b) found higher DNA adduct levels among lifetime nonsmokers who were healthy and were homozygous for the 399**GLN* allele than among those with the wild-type genotype (15.6 versus 6.78, p = 0.007). Wang and colleagues (2003a) showed that persons with the variant 194**TRP* allele had fewer bleomycin- and BPDE-induced breaks per cell than did those with the wild-type genotype. The *XRCC1* codon 399 is within the BRCT domain (amino acids 301 to 402) that interacts with PARP and is in many proteins with activity involving response to DNA damage and cell-cycle checkpoints. This region also has homology with yeast *RAD4* repair-related genes. Because the role of *XRCC1* in BER brings together DNA polβ, DNA ligase III, and PARP at the site of DNA damage, repair activity of the exon 10 variant may be altered. The codon 194 polymorphism is in the linker region of the *XRCC1 N*-terminal domain separating the helix 3 and polβ involved in binding a single-nucleotide gap DNA substrate (Marintchev et al. 1999). Therefore, this polymorphism is less likely to cause a significant change in repair function.

OGG1 *Gene*

The product of the *OGG1* gene catalyzes the excision of a modified base, 8-oxoguanine, which may be formed by exposure to reactive oxygen species. The reduced ability to excise 8-oxoguanine may lead to an accumulation of oxidation-induced mutations. Studies have identified several polymorphisms at the *OGG1* locus. The most frequently studied polymorphism is a common C→G transversion in exon 7 that results in an amino acid alteration at codon 326 (Ser→Cys). The HOGG1 protein encoded by the wild-type 326**SER* allele exhibited substantially higher DNA repair activity than did the 326**CYS* variant in an in vitro *Escherichia coli* complementation activity assay (Kohno et al. 1998).

Researchers have observed fairly consistent increased risks of *OGG1* polymorphisms (Goode et al. 2002). The largest study was a U.S.-population-based, multiethnic study of lung cancer that identified a significantly increased risk associated with the **CYS/*CYS* genotype (**CYS/*CYS* versus **SER/*SER;* OR = 2.1; 95 percent CI, 1.2–3.7) (Le Marchand et al. 2002). A small, hospital-based study of lung cancer in Japan supported these results (Sugimura et al. 1999). Findings in a third study of lung cancer that also suggested an increased risk had similar findings for comparison of the two homozygote groups (OR = 2.2; 95 percent CI, 0.4–11.8) (Wikman et al. 2000). Analyses of esophageal cancer also showed an increased risk associated with the **CYS/*CYS* genotype (OR = 1.9; 95 percent CI, 1.3–2.6) (Xing et al. 2001). However, overall findings regarding an interaction with smoking were inconsistent.

Polymorphisms in the Pathway for Nucleotide Excision Repair

Xeroderma Pigmentosum A

XPA is an essential DNA-binding protein in the NER pathway that aids in correctly positioning the repair machinery around the damaged areas and in maintaining contact with core repair factors during the repair process. XPA interacts with other proteins, such as replication protein A, TFIIH, and XRCC1/XPF (Volker et al. 2001). Studies have identified an A→G transversion variant in the 5′ noncoding region (Butkiewicz et al. 2000). Researchers investigating this polymorphism in lung cancer reported similar results in two studies. Wu and colleagues (2003) reported that the presence of one or two copies of the **G* allele instead of the **A* allele was associated with a reduced lung cancer risk for all ethnic groups. Furthermore, control participants with one or two copies of the **G* allele demonstrated more efficient DRC, as measured by the HCR assay, than did control participants with the homozygous *A* genotype. In a study in Korea, Park and colleagues (2002) reported that the **G/*G* genotype was also associated with a significantly decreased lung cancer risk when the combined **A/*A* and **A/*G* genotype was used as the reference group. Butkiewicz and colleagues (2004) reported similarly increased risks that had borderline statistical significance for the **A/*A* genotype in all participants and for SCC and adenocarcinomas. For heavy smokers, the risk estimate was 2.52 (95 percent CI, 1.2–5.4). Popanda and colleagues (2004) reported a nonsignificant risk of 1.53 for the **A/*A* genotype compared with the **G/*G* genotype. Thus, all studies confirm the protective effect of the **G/*G* genotype and the enhanced risk for the **A/*A* genotype.

Xeroderma Pigmentosum C

XPC is the step-limiting factor in NER. Researchers have found two single nucleotide polymorphisms (SNPs) in the coding region. *ALA499VAL* is a single **C/*T* nucleotide polymorphism that codes for an amino acid substitution (Ala/Val) at codon 499. Another SNP, *LYS939GLN*, is a single **A/*C* nucleotide polymorphism that codes for an amino acid substitution (Lys/Gln) at codon 939. This variant allele is associated with an increased risk of bladder cancer (Sanyal et al. 2004). There is also a bi-allelic poly AT insertion/deletion polymorphism (*PAT*) in intron 9 of *XPC*. The **PAT* allele has been associated with risk of head and neck cancer (Shen et al. 2001).

Cross-Complementation Group 1 for Excision Repair

The *XRCC1* gene codes for a 5′ incision subunit of the NER complex (Mohrenweiser et al. 1989). The XRCC1 and XPF proteins form a stable complex in vivo and in vitro (de Laat et al. 1999). Although studies have reported no defect in the human *XRCC1* gene, cells from *XRCC1*-deficient mice have an increase in genomic instability and a repair-deficient phenotype (Melton et al. 1998). Five known polymorphisms of the *XRCC1* gene do not cause an amino acid change and are validated in the SNP500Cancer Database of the National Cancer Institute. However, researchers think that a polymorphism with A→C transversion at nucleotide 8092 in the 3′ untranslated region affects mRNA stability (Shen et al. 1998). Studies have implicated the *XRCC1* polymorphism in the risk of adult-onset glioma (Chen et al. 2000) but not in head and neck cancer (Sturgis et al. 2002). Zhou and colleagues (2005) found no overall effect on lung cancer risk, but they did find a lower lung cancer risk in heavy smokers and a significantly higher risk in lifetime nonsmokers (OR = 2.11; 95 percent CI, 1.03–4.31).

Xeroderma Pigmentosum Complementation Group D

XPD (XRCC2) is one of the seven genetic complementation groups that encode for proteins in the NER pathway. The XPD protein has a role in both NER and basal transcription. XPD functions as an evolutionary conserved ATP-dependent helicase within the multisubunit transcription repair factor complex TFIIH. Of the three polymorphisms identified in XPD, two are in exons and the third is silent. The G→A transition in exon 10 at codon 312 results in an amino acid change (Asp→Asn). The transition A→C at codon 751 in exon 23 produces a Lys→Gln change. The amino acid substitution Lys751Gln in exon 23 does not reside in a known helicase/adenosine triphosphatase domain, but it is an amino acid residue identical in human, mouse, hamster, and fish XPD. This finding suggests a functional relevance for such a highly evolutionary conserved sequence (Shen et al. 1998). Goode and colleagues (2002) extensively reviewed numerous reports from case-control studies of lung cancer that have conflicting results. The largest study included 1,092 lung cancer case patients and 1,240 control participants who were spouses or friends (Zhou et al. 2002a). The overall AOR was 1.47 (95 percent CI, 1.1–2.0) for the ASP312ASN polymorphism (*ASN/*ASN versus

*ASP/*ASP), but there was no association for the LYS-751GLN polymorphism (*GLN/*GLN versus *LYS/*LYS). Analyses of the interactions between genes and smoking revealed that the adjusted ORs for each of the two polymorphisms decreased significantly as pack-years[1] increased. The interaction between the ASP312ASN polymorphism and smoking status was stronger than that between the LYS751GLN polymorphism and smoking. The researchers concluded that cumulative cigarette smoking modified the association between XPD polymorphisms and lung cancer risk. Spitz and colleagues (2001) reported AORs for the variant LYS751GLN and ASP312ASN genotypes of 1.36 and 1.51, respectively, although neither estimate was statistically significant. For persons homozygous for the variant genotype at either locus, the AOR was 1.84 (95 percent CI, 1.11–3.04; p = 0.018 for trend).

A recent review and meta-analysis of nine international case-control studies that included 2,886 lung cancer cases and 3,085 controls for codon 312 (*LYS751GLN*) and 3,374 lung cancer cases and 3,880 controls for codon 751 (*ASP312ASN*) did not demonstrate significant associations with either variant genotype (Benhamou and Sarasin 2005). However, for U.S. studies alone, both variants were associated with a significantly increased risk for lung cancer (ORs = 1.43 and 1.25, respectively). Hu and colleagues (2004) conducted another meta-analysis. The combined case-control studies reported a 21-percent higher risk for the *751*C/*C* genotype (OR = 1.21; 95 percent CI, 1.02–1.43) and a 27-percent higher risk for the *XPD 312*A/*A* genotype (OR = 1.27; 95 percent CI, 1.04–1.56) among cases than among controls. Among studies of persons of Asian versus White descent, only the studies of Whites found a significantly higher risk for the *XPD 751*C/*C* genotype (OR = 1.23; 95 percent CI, 1.03–1.47). For the *XPD 312*A/*A* genotype, the risk among Whites compared with other races had borderline statistical significance (OR = 1.22; 95 percent CI, 0.99–1.49) among cases compared with controls. TFIIH transcriptional activity may be tolerant to amino acid changes in the XPD protein, and mutations may destroy or alter the repair function without affecting transcriptional activity. As Lunn and colleagues (2000) suggested, the effects of the **LYS* allele may differ in different repair pathways, as assessed by different repair assays. The overall effect of conservative mutations in *XPD* may be subtle, because they do not alter XPB and XPD helicase activity, and multiple alterations might be needed before any effect is noted.

XPF/ERCC1 *Gene Complex*

ERCC1 forms a complex with *XPF* when it makes a dual incision at the single-strand to double-strand transition at the 5′ end of the damaged DNA strand (Shen et al. 1998). This complex is required to repair interstrand cross-links. A T→C transition at codon 662 results in a serine→proline substitution. Fan and colleagues (1999) reported six SNPs, five in coding regions. Three of the SNPs resulted in nonconserved amino acid differences.

XPG/ERCC5 *Gene Complex*

This complex shows homology with yeast RAD2 and carries out incision at the 3′ end of the lesion in the DNA strand (Harada et al. 1995; Hyytinen et al. 1999). Only two of the seven validated SNPs appear with significant frequency. In one polymorphism, a single nucleotide substitution (G→C) causes an amino acid change (His1104Asp) at codon 1104. In the other polymorphism, a C→G substitution produces an amino acid change from cysteine to serine at codon 529. Sanyal and colleagues (2004) showed that the variant **C/*C* genotype was significantly less frequent in cases of bladder cancer than in controls. Jeon and colleagues (2003) reported similar findings for 310 lung cancer cases, in which the frequency of the variant genotype was less than that for the other two genotypes combined (AOR = 0.54; 95 percent CI, 0.37–0.80). This protective effect was attenuated in heavy smokers. Jeon and colleagues (2003) pointed out that because this SNP is in the C-terminus, it might alter binding to other proteins in the incision complex, thereby affecting DRC.

RAD23B *Gene*

The *RAD23B* gene is an evolutionary, well-conserved gene with 10 exons. The protein complexes with XPC to bind to different types of lesions and recruit the necessary factors for NER. Of the six validated SNPs, four are seen with considerable frequency. One of the most frequently observed polymorphisms is a substitution (C→T) resulting in an amino acid change at codon 249 (Ala249Val).

Genotype-Phenotype Correlations

Amino acid differences, especially at conserved sites in these enzymes, could result in changes in repair proficiency. The next logical step is the challenging task of evaluating the functional relevance of these polymorphisms. A variety of factors that modulate the path from genotype to

[1]Pack-years = the number of years of smoking multiplied by the number of packs of cigarettes smoked per day.

phenotype include protein-protein interaction, posttranslational modification, gene silencing, epigenetic regulation, and environmental factors. Furthermore, proteins involved in DNA repair pathways are often multifunctional, resulting in a variety of phenotypes.

Both of the common genotypes **LYS*/**LYS* 751 and *312 *ASP/*ASP XPD* were associated with a DRC more efficient than that for heterozygotes and with a significantly higher DRC than that for the homozygote mutants (Spitz et al. 2001). These results were confirmed in a different study population with a different mutagen challenge: a UV exposure of 800 joules per square meter that like BPDE invoked NER (Qiao et al. 2002b). Additional validation came from a correlative study that used the comet assay to assess DNA damage and repair (Schabath et al. 2003). These data are consistent with some of the published small-scale studies of these types of genotype-phenotype correlations. Hou and colleagues (2002) noted a significant increase in DNA adduct levels, as measured by ^{32}P-postlabeling, with an increased number of variant alleles in exon 10 ($p = 0.02$) and exon 23 ($p = 0.001$). In addition, persons with the combined exon 10 **A/*A* and exon 23 **C/*C* genotype showed significantly higher levels of adducts than those for persons carrying any of the other genotypes ($p = 0.02$). Lunn and colleagues (2000) reported that possessing the common *XPD* genotype, **LYS/*LYS* 751, was associated with an increased risk of suboptimal DRC, which was reflected in the number of x-ray–induced lymphocyte chromatid aberrations. No association with the **ASN312* allele was found.

However, Møller and colleagues (1998) reported no relationship between the *LYS751GLN* polymorphism and DRC, as measured by HCR or comet assay in 80 participants, including 20 healthy persons. Another study with a small sample of 76 healthy persons found no association between either SCE frequencies or the presence of DNA adducts by *LYS751GLN* genotype (Duell et al. 2000).

For a complex disease such as cancer, multiple genes—each with a small effect—probably act independently or interact with other genes to influence the disease phenotype. Although these data suggest that the polymorphisms have a functional relevance, biochemical and biologic characterizations of the variants are needed to validate these findings.

Polymorphisms in the Pathway for Double-Strand Break Repair

The *XRCC3* gene encodes a protein that acts in the pathway for DSB/homologous recombination repair (DSB/REC repair) and repairs chromosomal damage such as breaks, translocations, and deletions. XRCC3 is a protein related to RAD51, which is a critical component of DSB/REC. Shen and colleagues (1998) identified a C→T substitution in exon 7 at position 18067 of *XRCC3*, a polymorphism that results in a threonine→methionine amino acid substitution at codon 241. David-Beabes and colleagues (2001) found no significant association between the *XRCC3241* polymorphism and lung cancer. This finding was consistent with a smaller study of NSCLC that also found no association after adjustments for age and smoking (Butkiewicz et al. 2001). Wang and colleagues (2003c) reported an elevated but not statistically significant risk of lung cancer associated with polymorphisms of the *XRCC3 *T* allele in African Americans and Mexican Americans, which was evident largely in heavy smokers. Other studies have associated this *XRCC3* polymorphism with an increased risk for melanoma skin cancer (Winsey et al. 2000) and bladder carcinoma (Matullo et al. 2001a). The *THR241MET* genetic variant may also contribute to increases in DNA adducts and bladder cancer risk (Stern et al. 2002).

Summary

The association between common variants in DNA repair genes and the risk for tobacco-induced cancers is the focus of considerable interest, but the results to date are inconsistent. Complementary functional studies are likely to be valuable in addressing these inconsistencies. Molecular epidemiologists now have better access to high-throughput genotyping platforms and an enhanced ability to focus on analyses based on pathways. Haplotype analyses also increase the power to detect relevant associations. In addition, computational algorithms such as PolyPhen and Scale-Invariant Feature Transform correlate with risk estimates, and new analytic tools are being developed.

Conversion of DNA Adducts to Mutations

DNA replication plays a major role in inducing point mutations—substitutions of one base pair for another and small mutations due to insertion or deletion of bases. DNA adducts per se are not mutations and can be removed by various DNA repair mechanisms in cells (Friedberg et al. 1995). When repair is not completed before a replication complex encounters the DNA adducts or other lesions, various events are induced, which are sensed by cell-cycle checkpoint mechanisms that halt cell-cycle progression (Sancar et al. 2004). When the lesion is a strand break, replication causes a DSB that is repaired by homologous recombination or by the erroneous nonhomologous end-joining mechanism. When the lesion is an interstrand

cross-link, the stall of a replication complex triggers the unhooking of the cross-link by endonucleolytic incisions on both sides of the cross-link in one strand. When the lesion is a modified base or the loss of a base, a DNA polymerase often inserts a nucleotide, either correctly or incorrectly, opposite the lesion and extends the DNA strand beyond the site. The modification of a template nucleotide generally impairs its ability to serve as a template in efficiency and fidelity. Therefore, DNA synthesis slows down or is blocked at the site of the adducted template.

Translesion DNA synthesis occurs when a DNA polymerase succeeds in DNA synthesis over the modified template. The synthesis reaction sometimes results in the insertion of an incorrect nucleotide opposite a lesion. This insertion leads to base-substitution mutations, the skipping of the lesion nucleotide template, or the realignment of a growing primer strand on the template strand at the adducted region, which produces frameshift mutations. This step results in the introduction of mutations, and the subsequent replication of the mutated strand establishes the mutation in the genome. This section describes the mechanism of mutation induction by translesion DNA synthesis.

Molecular Analysis of Conversion to Mutations

The strategy for studying the conversion of adducts to mutations is to incorporate a chemically characterized DNA adduct or lesion into a specific sequence (Basu and Essigmann 1988). A DNA adduct can be incorporated into an oligonucleotide sequence by total chemical synthesis. However, a modified oligonucleotide may be prepared by direct reaction with a mutagen, followed by HPLC and/or gel electrophoresis purification. The modified oligonucleotide is then used as a substrate for in vitro and in vivo studies of translesion DNA synthesis, repair, and structure. This experimental approach generally demonstrates a clear relationship between cause and effect. The advantage of this approach is the ability to analyze in detail events in translesion synthesis such as (1) quantification of the effects of blocking DNA synthesis, (2) miscoding frequency, and (3) miscoding specificity.

In vitro studies of translesion synthesis that use purified DNA polymerases complement the in vivo studies. As described in the following section, cells have various types of DNA polymerases and some of them are responsible for translesion synthesis. Therefore, the characterization of in vitro translesion synthesis could help to identify the polymerase responsible for translesion synthesis in cells. In vitro studies can be divided into two phases: insertion and extension steps. The insertion step determines which nucleotide is most efficiently inserted opposite a lesion by a given polymerase. The extension step determines which nucleotide terminus is most efficiently extended opposite a lesion. These experiments characterize the efficiency and fidelity of translesion synthesis by a given polymerase. The results of the in vitro experiments may be reflected in translesion synthesis in cells if the polymerase is involved in the synthesis. The involvement of a candidate polymerase is examined by studying translesion synthesis in a host cell lacking the polymerase of interest. Finally, the involvement is confirmed by a complementation experiment in mutant cells that express the exogenous gene for the polymerase of interest. Thus, translesion synthesis across a given DNA lesion is studied in detail. One limitation of this site-specific experiment is the inability to study the effects on chromosomal aberrations. In addition, the results may not apply to other sequence contexts. Nevertheless, this approach has provided a tremendous amount of information on the mechanism of DNA adduct conversion to mutations.

Translesion Synthesis in Mammalian Cells

DNA polymerases are key players in mutation induction. They introduce mutations during replication and determine the types of mutations generated. Great progress has been made in DNA polymerase studies in the last several years. Many novel DNA polymerases were discovered in prokaryotes and eukaryotes (Hübscher et al. 2002). These DNA polymerases play important roles in various aspects of DNA metabolism. The Y family polymerases (Ohmori et al. 2001) include eukaryotic polη, polκ, and polτ, as well as REV1 and *E. coli* polIV and polV. In addition, polζ is a member of the B family. Researchers think these Y family polymerases are specialized for translesion synthesis (Prakash and Prakash 2002). These discoveries have led to the general idea that these polymerases are responsible for overcoming the blocking effects of DNA adducts and constitute an important mechanism for tolerating unrepaired DNA lesions.

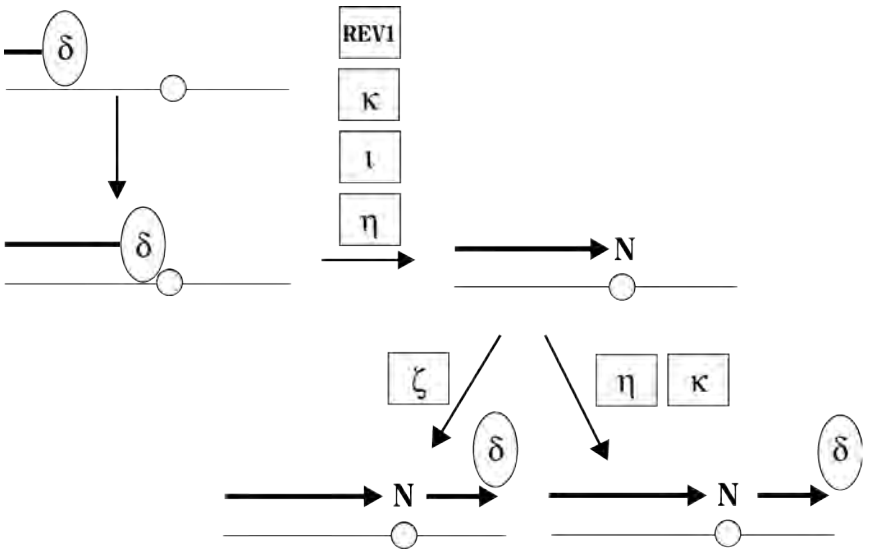
The role of the polη polymerase is most clearly understood. This polymerase is coded by the *XPV* gene, which is defective in persons with XP variant cells (Johnson et al. 1999; Masutani et al. 1999). Although these cells possess NER capability, they carry a predisposition to skin cancer on exposure to sunlight. The discovery that polη is able to bypass the *cis-syn* thymine-thymine dimer efficiently and accurately (Masutani et al. 1999) indicates that this polymerase plays a very important role in protection from the deleterious effects of unrepaired UV photoproducts. In its absence, the unrepaired lesions are bypassed by one or more other polymerases in an error-prone manner leading to skin cancer (Gibbs et al. 1998, 2000). X-ray crystallographic studies reveal that Y family polymerases have loose catalytic pockets enabling them to accommodate

Table 5.7 Translesion-specialized DNA polymerases (pol) and activities on various DNA lesions

DNA adduct	polη	polκ	polζ	polη + polζ	polι + polζ	polδ + polζ	REV1 + polζ
cis-syn TT	+ (a)	– (b)	– (c)		– (c)		
(6-4) TT	– (a)	– (b)	– (c)	+ (d)	+ (c)		
Abasic site	+ (a)	+ (b)	– (c)	+ (e)	+ (c)	+ (f)	+ (f)
Acetylaminofluorene *C*8-dG adduct	+ (a)	+ (b)					
Cisplatin intrastrand dG-dG adduct	+ (a)	– (b)					
1,N^6-ethenodeoxyadenosine	+ (g)	+ (g)					
8-oxodeoxyguanosine	+ (h)	+ (i)					
(+) BPDE-N^2-dG	+ (j)	+ (i)					
(–) BPDE-N^2-dG		+ (i)					

Note: (a) Masutani et al. 2000; (b) Ohashi et al. 2000; (c) Johnson et al. 2000; (d) Johnson et al. 2001b; (e) Yuan et al. 2000; (f) Haracska et al. 2001; (g) Levine et al. 2001; (h) Haracska et al. 2000; (i) Zhang et al. 2000a; (j) Zhang et al. 2000b.
BPDE-N^2-dG = *trans-anti*-benzo[*a*]pyrene-N^2-deoxyguanosine; **dG** = deoxyguanosine; **TT** = thymine-thymine dimer.

Figure 5.9 Model of mechanism for mammalian translesion synthesis



Note: Replicative polymerase δ encounters DNA lesion (open circle) in template, progression of DNA synthesis is blocked, and δ temporarily disengages. Y family polymerases (η, κ, ι, and REV1) are recruited to the sites, and 1 or more polymerases catalyze the insertion of a nucleotide opposite a lesion and the extension from the newly generated terminus. With some DNA lesions, the Y family polymerases can insert a nucleotide (N), but further extension is inhibited. Then, the second translesion polymerase, ζ, catalyzes the extension step. After translesion synthesis, δ resumes replication.

unusual base pairs (Trincao et al. 2001). This structural feature could explain the low fidelity of DNA synthesis on a normal DNA template.

In vitro experiments on translesion synthesis using purified polymerases reveal that each polymerase catalyzes bypass synthesis across various lesions with a different efficiency and fidelity (Table 5.7). The current model of the mechanism for mammalian translesion synthesis is illustrated in Figure 5.9. When a replicative DNA polymerase is inhibited by a lesion, translesion synthesis can be completed by the action of one polymerase or by the cooperation of two polymerases (Prakash and Prakash 2002). Among these polymerases, polζ is unique because it has low ability to insert a nucleotide opposite a lesion but is efficient at extending from unmatched terminal pairs (Johnson et al. 2000). Therefore, researchers think that this polymerase plays a role mainly in extending from a terminus opposite a lesion where another polymerase has inserted a nucleotide and the further extension is blocked.

Factors in Outcome of Translesion Synthesis

Many studies reveal that the efficiency and fidelity of translesion synthesis depend on the host (Moriya et al. 1994, 1996). Some DNA adducts miscode in one host (human cells) but not in another (*E. coli*), and the reverse also occurs (Moriya et al. 1994; Pandya and Moriya 1996). This finding underscores the importance of evaluating translesion events in the appropriate host: human cells. The discrepancy most likely reflects the difference in the activity of the translesion polymerases involved.

Sequence context also plays an important role in determining the outcome of translesion synthesis. Generally, a DNA adduct in iterated sequences, such as monotonous repeats (e.g., GGGGG) and dinucleotide repeats (e.g., GCGCGC), tends to cause frameshift mutations because these sequences misalign easily (Benamira et al. 1992). However, the same adduct induces base-substitution mutations in a different sequence context (Moriya et

Table 5.8 Mutational specificity of selected DNA adducts derived from tobacco smoke

Study	DNA adduct	Mutation specificity
Loechler et al. 1984 Dosanjh et al. 1991 Pauly and Moschel 2001	O^6-methyldeoxyguanosine	G→A
Pauly et al. 2002	O^6-[4-oxo-4-(3-pyridyl)butyl]-deoxyguanosine	G→A
Dosanjh et al. 1991 Pauly and Moschel 2001	O^4-methylthymidine	T→C
Wood et al. 1990 Moriya 1993	8-oxodeoxyguanosine	G→T
Kanuri et al. 2002 Yang et al. 2002	1,N^2-propanodeoxyguanosine from acrolein	G→T
Moriya et al. 1994	3,N^4-ethenodeoxycytidine	C→A, T
Pandya and Moriya 1996	1,N^6-ethenodeoxyadenosine	A→G, T
Lawrence et al. 1990 Cabral Neto et al. 1994 Gibbs and Lawrence 1995	Apurinic/apyrimidinic sites	AP→T, A, G
Moriya et al. 1996 Page et al. 1998	Benzo[*a*]pyrene-7,8-diol-9,10-epoxide-N^2-deoxyguanosine	G→T, A, C
Page et al. 1999	Benzo[*a*]pyrene-7,8-diol-9,10-epoxide-N^6-deoxyadenosine	A→T
Verghis et al. 1997	4-aminobiphenyl-*C*8-deoxyguanosine	G→C

al. 1994). Sequence context influences base-substitution events (Moriya et al. 1996; Page et al. 1998) and translesion efficiency (Latham et al. 1993) (see "Benzo[*a*]pyrene-7,8-Diol-9,10-Epoxide-N^2-Deoxyguanosine stereoisomers" later in this chapter).

Thus, translesion events are determined by the interplay between a DNA adduct, its sequence environment, and the DNA polymerase involved. This finding underscores the importance of conducting experiments with use of a proper sequence context and host.

Conversion of Cigarette-Smoke-Induced DNA Adducts to Mutations

Conversion of DNA adducts induced by cigarette smoke to mutations is summarized in Table 5.8. The discussion that follows provides additional details.

O^6-*Pyridyloxobutyl-Deoxyguanosine*

O^6-POB-deoxyguanosine is formed by a pyridyloxobutylating metabolite of the tobacco-specific *N*-nitrosamines NNK and NNN and is removed by AGT. Therefore, in the presence of this repair enzyme, the adduct induced only a moderate miscoding frequency. The resulting mutations were G→A transitions. In the absence of AGT, the miscoding frequency markedly increased to more than 90 percent (Pauly et al. 2002). The results were similar in *E. coli* and human cells. These results indicate that the frequency of miscoding for this adduct is high. The DNA polymerase involved almost exclusively inserts deoxythymidine monophosphate opposite the adduct, leading to G→A transitions. Thus, repair by the alkyltransferase is extremely critical to the avoidance of mutation induction by this adduct. The biologic characteristics of this adduct are similar to those of O^6-methyldeoxyguanosine (Pauly and Moschel 2001). The DNA polymerase that catalyzes the translesion synthesis and the bypass efficiency of this synthesis remain to be determined.

O^6-*Methyldeoxyguanosine and* O^4-*Methylthymidine*

The O^6-methyldeoxyguanosine and O^4-methylthymidine adducts induce mutations by stable pairing to thymidine (Dosanjh et al. 1993) and deoxyguanosine (Toorchen and Topal 1983), respectively. Accordingly, their miscoding potentials are high (Dosanjh et al. 1991; Pauly and Moschel 2001) and are similar to those of O^6-POB-deoxyguanosine. MMR acts on base pairs containing O^6-methyldeoxyguanosine after replication (Branch et al. 1993) and leads to cell death as a result of a futile MMR. Therefore, MMR mutants are more resistant to methylating agents (Branch et al. 1993) and are more prone to mutation after exposure to these agents (Pauly and Moschel 2001).

8-Oxodeoxyguanosine

8-oxodeoxyguanosine is a representative adduct formed by oxidative damage to DNA, and researchers have extensively studied its mutagenic properties and repair mechanisms (Grollman and Moriya 1993). The miscoding property of this adduct derives from its propensity to assume a *syn* conformation and to pair easily with deoxyadenosine (*anti*), which leads to G→T transversions. To avoid this mutation induction, cells have developed an elaborate postreplication BER mechanism that specifically removes misinserted deoxyadenosine by the action of the DNA glycosylase, adenine-DNA-glycosylase (Parker and Eshleman 2003). Subsequently, when deoxycytidine monophosphat) is inserted opposite the adduct, 8-oxodeoxyguanosine is removed by another BER initiated by OGG1, and a G:C pair is restored.

This adduct is also formed in the nucleotide pool. When 8-oxodeoxyguanosine-triphosphate is inserted opposite a deoxyadenosine template, the misinsertion leads to an A→C transition. To avoid this event, the cellular enzyme MTH1 converts 8-oxodeoxyguanosine triphosphate to 8-oxodeoxyguanosine monophosphate, which is no longer a substrate for DNA synthesis. Thus, cells have developed several layers of defense mechanisms against 8-oxodeoxyguanosine. Therefore, the apparent frequency of mutation induction by this adduct is low in normal cells. However, when *MYH* is inactivated, the frequency of G→T transversions increases drastically (Moriya and Grollman 1993; Hashimoto and Moriya, unpublished data) and mutations in this gene lead to a high incidence of spontaneous human colon cancer (Al-Tassan et al. 2002).

1,N^2-*Propanodeoxyguanosine*

Various unsaturated α,β-aldehydes, such as acrolein and crotonaldehyde, produce the DNA adduct PdG. Acrolein produces two positional isomers: 8-(γ-) and 6-(α-) xy hydroxyl PdG. When positioned in double-stranded DNA, the α adduct is more genotoxic than the γ adduct. The α adduct has significantly more blocking effects, and the γ adduct, but not the α adduct, miscodes with G→T transversions at a frequency of approximately 10 percent (Yang et al. 2002). Most of the miscoding events were induced by polη (Yang et al. 2003). Structural studies reveal that the exocyclic ring of the γ adduct, but not the α adduct, opens in a manner similar to that of the malondialdehyde-induced deoxyguanosine adduct (Mao et al. 1999) when paired to deoxycytidine (de los Santos et al. 2001). This finding may account for the weaker blocking effect and the lack of miscoding, because the ring-opened

γ adduct pairs nicely to deoxycytidine with the Watson-Crick type of (*anti-anti*) conformation. When the γ adduct is inserted in single-stranded DNA and replicates in mammalian cells, the resulting structure miscodes by inducing G→T transversions (Kanuri et al. 2002). In addition, the ring-opened deoxyguanosine adduct forms interstrand G-G cross-links in the sequence 5′CpG (Kozekov et al. 2003), which may also contribute to the genotoxicity of acrolein.

Exocyclic Etheno Adducts

Although the etheno adduct 1,N^6-ethenodeoxyadenosine miscodes efficiently in simian kidney cells, it does not miscode in *E. coli* (Pandya and Moriya 1996). This finding emphasizes the importance of the host. The finding is probably attributable to the difference in the fidelity of the DNA polymerase involved in translesion synthesis. The etheno adduct 3,N^4-ethenodeoxycytidine miscodes efficiently in both hosts (Moriya et al. 1994).

Apurinic/Apyrimidinic Sites

AP sites are generated by the cleavage of a glycosidic bond between a base and a sugar in DNA for various reasons such as (1) the action of a DNA glycosylase and (2) modifications to a base that destabilize the glycosidic bond. These sites do not convey any coding information. Deoxyadenosine is often inserted opposite these sites in *E. coli* (Lawrence et al. 1990), which is known as "the A rule" (Strauss 1991). However, this rule does not appear to be applicable in mammalian cells: various bases are inserted opposite these sites in those cells (Cabral Neto et al. 1994; Gibbs and Lawrence 1995).

Benzo[a]pyrene-7,8-Diol-9,10-Epoxide-N^2*-Deoxyguanosine Stereoisomers*

Studies have extensively characterized the genotoxicity of different stereoisomers of BPDE-N^2-deoxyguanosine (Moriya et al. 1996; Fernandes et al. 1998). A prominent feature is that both the surrounding DNA sequence and the host markedly influence miscoding frequency (Moriya et al. 1996; Fernandes et al. 1998; Page et al. 1998) and miscoding specificity (Kozack et al. 2000). The major adduct, (+)-BPDE-N^2-deoxyguanosine, induces mainly G→T and G→A transversions in 5′-TGC and 5′-AGA sequence contexts, respectively, which researchers hypothesize is attributable to differences in adduct conformations in different sequence contexts (Kozack et al. 2000). The deoxyadenosine adduct (BPDE-N^6-deoxyadenosine) also miscodes with A→T transversions (Page et al. 1999).

4-Aminobiphenyl-C8-Deoxyguanosine

The adduct 4-ABP-C8-deoxyguanosine barely miscodes in *E. coli* by inducing G→C transversions (Verghis et al. 1997). Because researchers observed G→T, G→A, and G→C mutations in an experiment that used a randomly modified single-strand DNA (Verghis et al. 1997), the possibilities of the effects from the sequence context and the involvement of other deoxyguanosine adducts, such as those at N^2, remain to be explored. Furthermore, it appears that a 4-ABP-deoxyadenosine adduct induces A→T transversions (Lasko et al. 1988; Hatcher and Swaminathan 1995).

Assessment of Genotoxicity of DNA Adducts

Genotoxic properties of a DNA lesion can be characterized by using chemically defined substrates. The genotoxicity of a DNA lesion is determined by factors such as the efficiency and fidelity of translesion synthesis and repair. For point mutations, however, the "genotoxic potency" of a DNA adduct can be determined by assessing the bypass efficiency and the miscoding potency. According to this formula, a DNA lesion that is easily bypassed with a high frequency of miscoding events is defined as a highly genotoxic DNA adduct. Furthermore, when the genotoxicity of a DNA lesion is assessed, the information on its abundance in the genome, which reflects the balance between formation and removal, should also be considered. Therefore, conceptually, the total genotoxicity of a DNA adduct could be estimated by determining its genotoxic potency and its abundance in DNA.

According to these criteria, the genotoxicity of the 8-oxodeoxyguanosine adduct, which is a unique case, would be high because it exists in high levels in genomic DNA and is easily bypassed by a DNA polymerase with a high miscoding frequency. However, the apparent genotoxicity is low because of the postreplication repair that is catalyzed by MYH. Therefore, when the postreplication repair is inactivated, this adduct can become a significant genotoxic adduct (Al-Tassan et al. 2002).

Data for the genotoxic effects of DNA lesions derived from tobacco smoke are scarce, and a systematic study is needed. Together with information on the abundance of each lesion, the genotoxicity of tobacco-related DNA adducts might be ranked by using site-specific modified plasmids, introducing them with the use of host cells, and subsequently recovering them for sequence analysis.

Gene Mutations in Tobacco-Induced Cancer

Chromosome Instability and Loss

Lung Cancer

The detection of numerous cytogenetic changes provided the first link to the molecular pathogenesis of lung cancer. Mapping chromosomal sites for rearrangement, breakpoints, and losses revealed both common and distinct changes in both SCLC and NSCLC. In SCLC, breakpoints are commonly seen in chromosomes 1, 3, 5, and 17, although researchers have observed losses of the short arm (p) of chromosomes 3 and 17 and of the long arm (q) of chromosome 5 (Balsara and Testa 2002). Subsequent studies using comparative genomic hybridization showed that deletions on chromosomes 3p, 4q, 5q, 10q, 13q, and 17p were common in SCLC (Petersen et al. 1997). In NSCLC, multiple numeric and structural changes were seen across many chromosomes. The most frequent sites (60 to 80 percent) for chromosome loss were found on chromosomes 3p, 6q, 8p, 9p, 9q, 17p, 18q, 19p, 21q, and 22q (Balsara and Testa 2002). Some of the most common sites for chromosome loss included 3p, 9p, 13q, and 17p. These sites were also detected in nonmalignant bronchial epithelium of current and former smokers and were absent in lifetime nonsmokers (Mao et al. 1997; Witsuba et al. 1997). These findings strongly link tobacco exposure to the development of chromosome damage throughout the aerodigestive tract.

Identification of Tumor-Suppressor Genes

The commonality for specific regions in the genome to lose alleles suggested the presence of tumor-suppressor genes within these loci. The RB gene was the first tumor-suppressor gene linked to lung cancer (Harbour et al. 1988). A loss of function of this gene through either deletion or point mutation occurs in 90 percent of SCLCs, whereas few NSCLCs harbor changes in this tumor-suppressor gene (Table 5.9) (Shimizu et al. 1994). The most frequently inactivated tumor-suppressor gene in lung cancer is *TP53*. *TP53* mutations are found in 70 percent of SCLCs, 65 percent of SCCs, and 33 percent of adenocarcinomas (Greenblatt et al. 1994). (For discussion of specific mutations and their potential relationship to carcinogens in cigarette smoke, see "Relationship of *TP53* Mutations to Smoking and Carcinogens" later in this chapter.)

A frequent deletion within chromosome 3p14 led to the identification of the *FHIT* gene (Zabarovsky et al. 2002). The most common fragile site of the human genome *FRA3B* maps in the *FHIT* gene and may contribute to the susceptibility of this locus to gene rearrangement induced by carcinogens in cigarette smoke. Researchers have observed a loss of the FHIT protein in 50 percent of lung cancers, but somatic mutations are uncommon in the *FHIT* gene. The epigenetic inactivation by methylation of the 5′CpG island located in the promoter region of *FHIT* represents another mechanism for inactivating this gene in lung cancer (see "Gene Promoter Hypermethylation in Cancer Induced by Tobacco Smoke" later in this chapter).

The importance of the inactivation of tumor-suppressor genes *FHIT, RB,* and *TP53* in lung cancer is evident from their functions. The binding of hypophosphorylated *RB* to cyclin-dependent kinase (CDK) 4 or 6 blocks transit of the RB protein through the G_1/S boundary of the cell cycle. Inactivating mutations result in the loss of a functional hypophosphorylated protein associated with a shortening of the G_1 phase of the cell cycle and the enhancement of cell proliferation, a hallmark of the cancer cell (Nevins 1992). The *TP53* gene is central to several critical processes needed to control the response of the cell to exogenous stress from exposure to cigarette smoke. This gene functions as a transcription factor within several pathways and as a sensor of DNA damage (Robles et al. 2002). Thus, the *TP53* gene has an important role in cell-cycle checkpoints, DNA repair, apoptosis, and senescence. A loss of *TP53* function is also an early event

Table 5.9 Frequency of mutation or deletion of tumor-suppressor genes in lung cancer

Gene	Chromosomal location	Frequency (%)	
		Small-cell lung cancer	Non-small-cell lung cancer
RB	13q14	90	15
TP53	17p13	70	50
CHFR	12q24	ND	6
MYO18B	22q12	ND	13
PTEN	10q23	9	17
LKB1/STK11	19p13	ND	35

Note: **ND** = not determined.

in the genesis of SCC that occurs in bronchial dysplasia (Sozzi et al. 1992; Bennett et al. 1993). Studies have also detected *TP53* mutations in peripheral lung tissue from patients with lung cancer, a finding that supports a role for this gene in the early development of adenocarcinomas (Hussain et al. 2001). The *FHIT* gene induces apoptosis mediated by CASPASE-8 and independent of mitochondrial mediators and inhibits cell growth through interactions with the SRC protein kinase (Pekarsky et al. 2004; Roz et al. 2004). A loss of function of the *FHIT, RB*, and *TP53*, genes leads to the immortalization of bronchial epithelial cells, a key step in neoplastic transformation (Reddel et al. 1988) (see "Signal Transduction" later in this chapter).

Tumor-Suppressor Genes Inactivated in Lung Cancer

The search for tumor-suppressor genes inactivated through the two-hit mechanism of the loss of one allele and the mutation of the remaining allele has not recently identified any genes with a frequency of inactivation approaching that seen for the *RB* and *TP53* genes. The discussion that follows describes the involvement of several genes and their functions in subsets of lung cancer (Table 5.9). The mitotic checkpoint gene *CHFR*, which functions in early prophase to regulate chromosome condensation, was mutated in 3 of 53 lung carcinomas (Mariatos et al. 2003). Studies found three somatic mutations in the proapoptotic gene *CASPASE-8* in 2 of 30 lung tumors (Hosomi et al. 2003). *MYO18B* is a candidate tumor-suppressor gene at chromosome 22q12. Of 46 primary NSCLCs, 6 contained somatic mutations within this gene. Restoring *MYO18B* function in cell lines inhibited anchorage-independent growth, thus supporting its function as a tumor-suppressor gene in lung cancer (Nishioka et al. 2002).

The *PTEN* gene is located on chromosome 10. Its gene product is phosphatidylinositol 3′-phosphatase, a protein tyrosine phosphatase that uses the phosphoinositide second messenger, phosphatidylinositol 3,4,5-triphosphate (PIP3), as a physiological substrate (Maehama et al. 2001). Researchers have identified point mutations of the *PTEN* gene in cell lines from 3 of 35 SCLCs and 3 of 18 NSCLCs; there were two homozygous deletions in primary SCLCs (Forgacs et al. 1998). Mutations that impair *PTEN* function result in a marked increase in PIP3 levels and in the constitutive activation of AKT survival, thus signaling pathways that in turn promote hyperplasia and tumor formation. Thus, although it is not common in lung cancer, a *PTEN* mutation or deletion profoundly affects an important signaling pathway in the cell.

Two additional genes with poorly characterized functions and localized to chromosome 3p are altered in lung cancer through deletion or mutation. A specific ATG→AGG mutation in codon 50 of the *ARP* gene was seen in 8 of 20 lung cancers. In addition, researchers observed either exon deletion or intron insertion in the *DLC1* gene in 11 of 30 NSCLCs (Zabarovsky et al. 2002). Frequent deletion involving the short arm of chromosome 19 occurs in lung adenocarcinomas (Sanchez-Cespedes et al. 2001). One gene mapped to this chromosome region is *STK11*, in which germline mutations are causal for Peutz-Jeghers syndrome. This syndrome is characterized by a series of anomalies and increased risk for gastrointestinal and extraintestinal malignancies (Giardiello et al. 1987). Inactivating mutations and/or deletion of the LKB1/STK11 protein were described in about one-third of primary adenocarcinomas (Sanchez-Cespedes et al. 2002; Ji et al. 2007), and these abnormalities were closely associated with mutation of the *KRAS* oncogene in the same tumors (Ji et al. 2007; Matsumoto et al. 2007). The *STK11* gene may function as a growth-inhibiting gene that is activated through phosphorylation by the *ATM* gene, which senses DNA damage (Sapkota et al. 2002), and acts through pathways dependent or independent of the P53 protein to suppress invasion and metastasis (Karuman et al. 2001; Upadhyay et al. 2006; Ji et al. 2007). In addition to inactivating by mutation, epigenetic silencing by promoter hypermethylation has emerged as a major mechanism for inactivating many genes in lung cancer, some of which are described here (e.g., *MYO18B*). (For a detailed discussion, see "Gene Promoter Hypermethylation in Cancer Induced by Tobacco Smoke" later in this chapter.)

Activation of Oncogenes in Lung Cancer

Oncogenes encode proteins that influence cell cycling and promote cancer. They are usually "gain-of-function" mutations of normal genes. Researchers see *KRAS* gene mutations in approximately 30 to 40 percent of adenocarcinomas but rarely in SCCs, SCLCs, or lung tumors from nonsmokers (Slebos et al. 1990; Westra et al. 1996; Ahrendt et al. 2003). Mutations are localized to codons 12, 13, and 61. More than 85 percent occur within codon 12. Nearly 70 percent of the mutations are G→T transversions within codon 12 that change a glycine codon (GGT) to valine (GTT) or cysteine (TGT). Mouse lung tumors induced by B[*a*]P and other PAHs show exclusively G→T transversions in codon 12 of the *Kras* gene. These findings support the hypothesis that activation of this oncogene in lung tumors results from DNA damage leading to base mispairing of these deoxyguanosines. In vitro studies have demonstrated that DNA adducts formed

from the metabolism of B[*a*]P, NNK, and reactive oxygen species can all lead to G→T transversions (Table 5.8) (You et al. 1989; Belinsky et al. 1992). Thus, the activation of carcinogens in tobacco smoke and the pulmonary inflammation that ensues from exposure to particulate matter together can lead to activation of the *KRAS* oncogene. Studies detected *KRAS* gene mutations in 39 percent of atypical alveolar hyperplasia, a putative precursor to adenocarcinoma (Slebos et al. 1996). A similarity in the percentage of precursor lesions and tumors containing *KRAS* mutations supports the importance of this gene in tumor progression in a subset of adenocarcinomas.

The sequence of events leading to activation of the RAS signal transduction pathway is well characterized (Lechner and Fugaro 2000). When the RAS protein is activated through mutations in codon 12, 13, or 61, it binds irreversibly to guanosine triphosphate (GTP) in the cell, which initiates a cascade of protein activations, beginning with v-raf-murine leukemia viral oncogene 1 (*RAF-1*), that transmits a signal from the cell membrane to the nuclear transcription machinery. Ultimately, these signals culminate in the activation of transcription factors including MYC, FOS, and JUN, which in turn influence many cellular activities such as transcription, translation, cytoskeletal organization, and cell-cell interactions. This signal remains active until GTPase (guanosine triphosphatase) dephosphorylates GTP to guanosine diphosphate. Thus, a *RAS* oncogene mutation leads to the disruption of many cellular pathways and provides a strong oncogenic signal for neoplastic transformation (see "Signal Transduction" later in this chapter).

The *MYC* family of genes (*C-MYC*, *N-MYC*, and *L-MYC*) plays a prominent role in the growth of the developing and mature adult lung. Extensive studies have evaluated the expression and amplification of these genes in NSCLC and SCLC (Jänne and Johnson 2000). Most lung cancers express one or more of the *MYC* family of genes, whereas gene amplification is seen in a minority of primary tumors (Table 5.10). Gene rearrangements involving different exons are associated with amplification detected in cell lines but are uncommon in primary tumors (Kinzler et al. 1986; Mäkelä et al. 1991; Sekido et al. 1992). Mechanisms responsible for the increased expression of the *MYC* genes in the absence of gene amplification are not well understood. Increased expression could occur through increased activity in the RAS signaling pathway through either *KRAS* oncogene mutations or effects on the activity of genes in this pathway, such as the activity of mitogen-activated protein kinase (MAPK) (Jull et al. 2001).

Increased gene expression is common in lung cancer but often is not associated with gene amplification. Two genes studied extensively are *EGFR* and *NEU* (*HER-2/NEU* [*ERBB2*]). EGFR is the receptor for the epidermal

Table 5.10 Frequency of gene amplification and increased expression of genes in lung cancer

		Frequency (%)	
Gene	**Tumor histology**	**Amplification**	**Expression**
C-MYC	Small-cell lung cancer	5	25
N-MYC	Small-cell lung cancer	7	3
L-MYC	Small-cell lung cancer	12	33
EGFR	Small-cell lung cancer	0	0
HER-2/NEU	Small-cell lung cancer	<1	0–7
C-MYC	Non-small-cell lung cancer	8	33
N-MYC	Non-small-cell lung cancer	0	ND
L- MYC	Non-small-cell lung cancer	3	ND
EGFR	Non-small-cell lung cancer	9–25	34–62
HER-2/NEU	Non-small-cell lung cancer	2–4	23–58

Note: **ND** = not determined.

growth factor and the HER-2/NEU protein, and the binding of these growth factors to this receptor is associated with increased DNA synthesis, cell proliferation, and differentiation. An increased expression of the *EGFR* gene was not seen in SCLCs but occurred in 34 to 62 percent of NSCLCs (Hirsch et al. 2003b; Suzuki et al. 2005). In addition, an increased expression of this gene was more common in SCC than in adenocarcinoma—82 versus 44 percent (Hirsch et al. 2003b). In contrast, gene amplification was detected in 9 to 25 percent of tumors (Hirsch et al. 2003b; Suzuki et al. 2005). Expression of the *HER-2/NEU* gene was seen in 23 to 58 percent of NSCLCs and in 0 to 7 percent of SCLCs (Shi et al. 1992; Junker et al. 2005; Pelosi et al. 2005). Similar to *EGFR*, *HER-2/NEU* was more commonly expressed in SCC than in adenocarcinoma, and gene amplification was rare in all tumors (<5 percent).

Observation of *EGFR* expression in 34 to 62 percent of NSCLCs led to the development of small molecule inhibitors of the tyrosine kinase domain of the wild-type EGFR protein (Fukuoka et al. 2003; Herbst and Bunn 2003; Lynch et al. 2004; Amann et al. 2005; Baselga and Arteaga 2005). The clinical response of approximately 10 percent of European patients and 30 percent of patients from Japan to treatment with the EGFR inhibitors gefitinib or erlotinib led to a search for the mechanism responsible (Kris et al. 2003; Pérez-Soler et al. 2004). The outcome of these studies was the identification of somatic mutations in the tyrosine kinase domain of the *EGFR* gene in most patients who had demonstrated a clinical response to the drugs (Lynch et al. 2004; Amann et al. 2005). In addition, recent studies suggest that the *EGFR* copy number and *KRAS* mutation may also be involved in determining a response to gefitinib and erlotinib. Subsequent studies have sequenced the *EGFR* gene in thousands of NSCLCs from patients in Asia, Europe, and the United States. These studies found that most mutations were due to either a deletion involving exon 19 or a missense mutation in exon 21. In addition, mutations were two to three times more likely in women than in men and three to five times more likely in nonsmokers than in current or former smokers (Johnson and Jänne 2005). Finally, the prevalence of mutations was 10 percent in tumors of patients from Europe and the United States compared with 30 percent in tumors from persons of Asian background residing in Japan and Taiwan.

The *BRAF* gene encodes a RAS-regulated kinase that can mediate cell growth. *BRAF* mutations were found in 5 of 179 NSCLCs and are almost exclusively confined to adenocarcinomas (Brose et al. 2002; Naoki et al. 2002). Although the mutation is relatively uncommon in lung cancer, its location in either exon 11 or exon 15 altered the phosphorylation of *BRAF* by AKT (Guan et al. 2000). The disruption of AKT-induced *BRAF* inhibition could contribute to malignant transformation.

Oncogene Activation, Tumor-Suppressor Gene Inactivation, and Lung Cancer Survival

Researchers have studied the prognostic impact of commonly altered genes in lung cancer. The effect of an activated *KRAS* oncogene on survival was assessed in 69 patients, including 48 with stage I adenocarcinoma that was completely resected (Slebos et al. 1990). Twelve of 19 patients with a *KRAS* mutation died within the follow-up period (median, 47 months) compared with 22 of 50 patients with a tumor negative for the *KRAS* oncogene. This significant difference in survival was observed even though patients with a *KRAS* mutation had a less advanced disease than those with no mutation. All seven patients with stage III disease were negative for mutations. Rosell and colleagues (1993) conducted a similar study of largely stage I resected adenocarcinomas that again revealed a reduced survival rate independent of lymph node status for patients whose tumor contained a mutated *KRAS* gene. In contrast, a larger study of 127 adenocarcinomas found no difference in survival by *KRAS* mutation status (Keohavong et al. 1996). Overall, data are conflicting with respect to *KRAS* mutations as prognostic factors and further research is needed (Aviel-Ronen et al. 2006).

Studies have examined the effect of *TP53* gene mutations on prognosis in both early- and late-stage lung cancers. After four years of follow-up, the hazard ratio for 106 patients with stage I resected NSCLC with a *TP53* mutation was 2.8 for death, compared with patients who had a wild-type gene (Ahrendt et al. 2003). Four years after surgery, 78 percent of patients with no *TP53* mutation and 52 percent with a *TP53* mutation were alive. A previous study by Tomizawa and colleagues (1999) found a similar survival benefit for patients with stage I NSCLC and no *TP53* mutations, which also confer a poor clinical outcome for those with advanced NSCLC. Independent of chemotherapy or supportive care, median survival duration for patients with stage III or IV NSCLC with or without a *TP53* mutation was 17 versus 39 weeks, respectively (Murakami et al. 2000). Recently, a study of 420 patients with primary head and neck cancer (Poeta et al. 2007) showed that disruptive *TP53* mutations in tumor DNA are associated with reduced survival after surgical treatment of SCC of the head and neck (hazard ratio, 1.7; 95 percent CI, 1.2–2.4; $p = 0.003$).

Together, it is apparent that the inactivation of the *TP53* tumor-suppressor gene and the activation of the *KRAS* oncogene in NSCLCs and other tumors are correlated with exposure to cigarette smoke and contribute

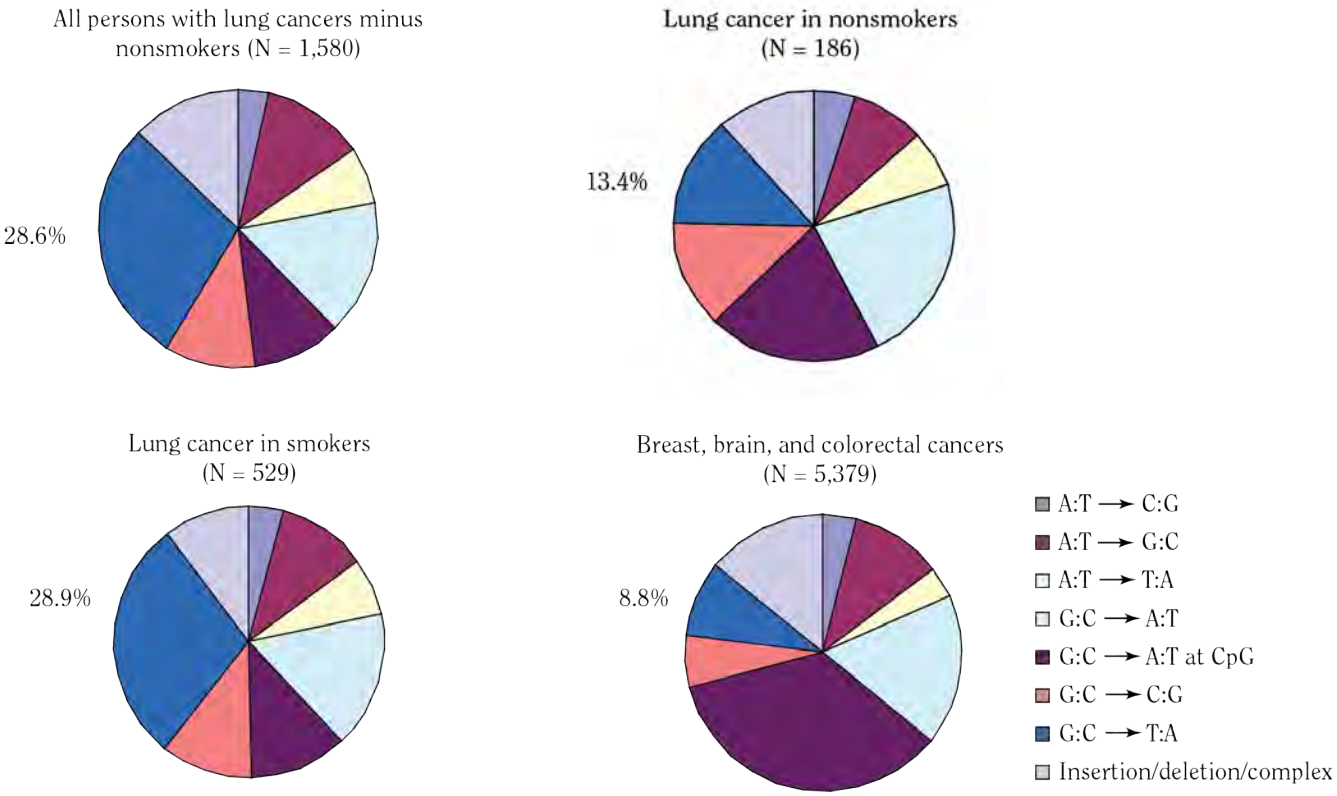
to a phenotype that reduces survival in both early and advanced stages of the disease.

Relationship of *TP53* Mutations to Smoking and Carcinogens

TP53 Mutations in Smoking-Associated Lung Cancers

TP53 gene mutations are found in approximately 40 percent of human lung cancers; *TP53* is the most commonly mutated tumor-suppressor gene in lung cancer (see "Identification of Tumor-Suppressor Genes" earlier in this chapter). These mutations are generally more common in smokers than in nonsmokers (Greenblatt et al. 1994; Hernandez-Boussard and Hainaut 1998; Pfeifer et al. 2002). One study shows that the relative risk of having a *TP53* mutation in lung cancer was up to 13 times higher in lifetime heavy smokers than in lifetime nonsmokers (Le Calvez et al. 2005). G→T transversions are commonly observed in smoking-associated lung cancers (Greenblatt et al. 1994; Hainaut and Hollstein 2000; Hainaut and Pfeifer 2001). The frequency of G→T transversions in lung cancers from smokers is higher than that for lung cancers and most other cancers in nonsmokers (Greenblatt et al. 1994; Husgafvel-Pursiainen and Kannio 1996; Hernandez-Boussard and Hainaut 1998; Bennett et al. 1999; Hainaut and Pfeifer 2001). Mutational patterns for lung cancers from smokers and nonsmokers are shown in Figure 5.10. The difference between 28.9 percent G→T transversion mutations in "designated smokers" (i.e., smoking status

Figure 5.10 Patterns of *TP53* gene mutations and percentage of G→T transversion mutations in human lung cancers



Source: Data are from the R9 version (July 2004) of the International Agency for Research on Cancer *TP53* mutation database (IARC 2006).
Note: Cell lines and metastatic cancers were excluded, as well as cancers with defined exposures other than tobacco (e.g., asbestos, radon, mustard gas, and air pollution) (see database Web site for specifications of exposure data). Nonsmokers included a series of 21 mutations (Le Calvez et al. 2005) not included in the database, in addition to 165 database entries. Data from Gao et al. 1997 were excluded (see Hainaut and Pfeifer 2001 for detailed selection criteria). **N** = total number of mutations.

indicated in literature) and 13.4 percent G→T mutations in nonsmokers has high statistical significance ($p < 0.001$, χ^2 test). The frequency of G→T transversions is higher in lung cancer tumors than in other tumors, except for liver cancers associated with geographic areas with evidence of food contamination from aflatoxins (Greenblatt et al. 1994).

In most internal cancers not strongly linked to smoking, such as breast, brain, and colorectal, the frequency of G→T mutations is 8 to 10 percent (Figure 5.10). Nonsmokers have a higher percentage of G→A transitions (42.5 percent) than do smokers (27.9 percent), a difference that is also statistically significant. Figure 5.10 includes categories of both designated smokers and "all lung cancer cases minus nonsmokers." This category is based on the knowledge that, overall, 90 percent of these lung cancers occur in smokers (Proctor 2001). The proportion of G→T transversions, as well as the overall mutation pattern for all persons with lung cancers, except nonsmokers, is similar to observations of researchers for designated smokers (Figure 5.10). The difference in G→T transversions in smokers versus nonsmokers may be attributable to bias in the database used, which pools data from studies that differ in aims, size, and methods for ascertaining smoking status. However, in a more recent study of a series of 21 mutations that was designed to address this possibility, *TP53* gene mutations were found in 27.5 percent of current smokers, 15.8 percent of former smokers, and 4.8 percent of lifetime nonsmokers (Le Calvez et al. 2005). These observations suggest that the difference in G→T transversions in smokers versus nonsmokers may be larger than that indicated in the database, perhaps due to the misclassification in the database of long-term former smokers as nonsmokers.

To address the issue of whether the major histologic types of lung cancer show differences in *TP53* mutational patterns, researchers analyzed the IARC *TP53* mutation database separately for these tumors (Figure 5.11). The frequencies of G→T transversions in the *TP53* database were 31.4 percent in adenocarcinomas, 27.1 percent in SCCs, 27.5 percent in SCLCs, and 34.7 percent in large-cell carcinomas. Furthermore, the global mutation patterns were similar in the two main histologic types: adenocarcinoma and SCC. Thus, the different types of lung cancer in smokers all show an excess of G→T transversions compared with cancers unrelated to exposure to tobacco smoke.

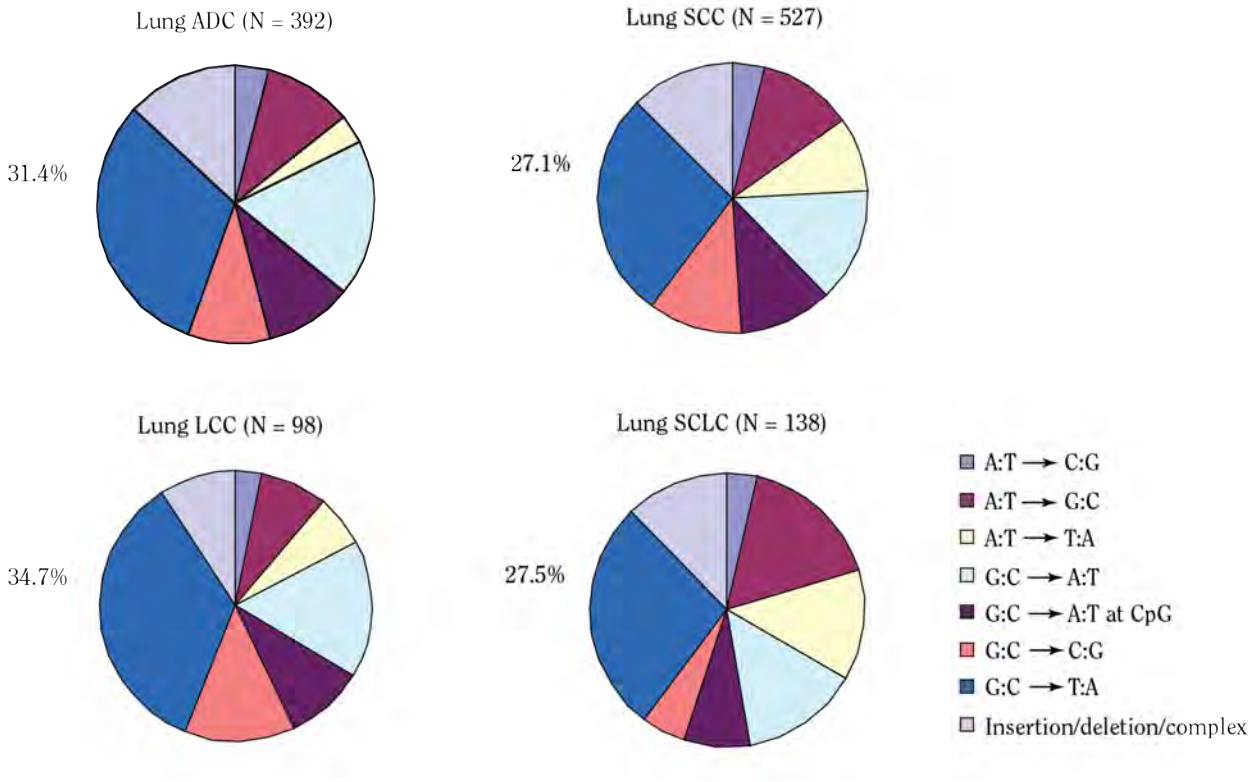
TP53 mutations do not occur at random along the coding sequence. They are typically clustered at mutation "hot spots," which are within the DNA binding domain of the TP53 protein and span codons 120 to 300. Figure 5.12 shows the concordance of codon distribution of G→T transversions (upper panel) along the *TP53* gene in lung cancer with the distribution of adducts in this gene in bronchial epithelial cells treated with BPDE. Hot spots of G→T mutations in cancers of the brain, breast, and colon differ from those in lung cancers (Pfeifer et al. 2002). The codons containing mutation hot spots are important because they may allow determination of which carcinogen caused the mutation. However, hot spot codons may exist solely as a consequence of phenotypic selection in tumors. To address this issue, studies have compared the mutational events in different types of cancers at a number of common hot spot codons. The major lung cancer mutation hot spots at codons 158, 245, 248, and 273 are commonly G→T transversions in lung cancer but are generally other mutation types (almost exclusively G→A) in other internal tumors not associated with smoking (Pfeifer et al. 2002).

G→T Transversions in Lung Cancer

The major product of the diol epoxide BPDE reaction with DNA is BPDE-N^2-deoxyguanosine, which induces mainly G→T transversions, depending on the sequence context, after a DNA polymerase carries out error-prone translesion synthesis past this adduct (Eisenstadt et al. 1982; Chen et al. 1990; Ruggeri et al. 1993; Yoon et al. 2001) (see "Conversion of DNA Adducts to Mutations" earlier in this chapter). Using the UvrABC incision method in combination with a ligation-mediated polymerase chain reaction (LMPCR), scientists mapped the distribution of BPDE and other PAH diol epoxide adducts at the nucleotide level along exons of the *TP53* gene in normal human bronchial epithelial cells treated with diol epoxide (Denissenko et al. 1996; Smith et al. 2000). Frequent adduct formation occurred at guanine positions in codons 156, 157, 158, 245, 248, and 273. These positions of preferential formation of PAH adducts are major mutational hot spots in human lung cancers (Figure 5.12). The only exception is codon 156, where G→T substitution commonly results in a phenotypically silent mutation and is therefore not selected during tumorigenesis.

Researchers analyzed the distribution of BPDE-N^2-deoxyguanosine within *TP53* exons by using stable isotope labeling LC-electrospray ionization tandem MS (Tretyakova et al. 2002; Matter et al. 2004). In this approach, specific guanine nucleobases within *TP53* gene sequences were labeled with ^{15}N so the BPDE adducts originating from these positions could be distinguished from the lesions formed at other sites. Researchers observed an excellent agreement with the data from the UvrABC-LMPCR method (Denissenko et al. 1996). All four diastereomers of BPDE-N^2-deoxyguanosine were formed preferentially at the frequently mutated *TP53* codons 157, 158, 245, 248, and 273. The contributions of individual

Figure 5.11 Patterns of *TP53* gene mutations and percentage of G→T transversion mutations in different histologic types of lung cancer



Source: Data are from the R9 version (July 2004) of the International Agency for Research on Cancer *TP53* mutation database (IARC 2006).
Note: Cancers were classified according to *International Classification of Diseases, Tenth Revision* (*ICD-10*), World Health Organization 1994. The data set excluded lung cancers from nonsmokers. Cell lines and cancers metastatic to the lung were excluded, as well as all cancers with defined exposures other than tobacco (e.g., asbestos, radon, mustard gas, and air pollution). **ADC** = adenocarcinoma (*ICD* C34-8140/3); **LCC** = large-cell carcinoma (*ICD* C34-8012/3); **N** = total number of mutations; **SCC** = squamous cell carcinoma (*ICD* C34-8070/3); **SCLC** = small-cell lung carcinoma (*ICD* C34-8041/3).

diastereomers to the total adducts at a given site varied but were highest (70.8 to 92.9 percent) for (+)-*trans*-BPDE-N^2-deoxyguanosine (Matter et al. 2004).

A mechanistic basis for the selectivity of formation of diol epoxide–DNA adducts in the *TP53* gene is the enhancement of adduct formation by 5-methylcytosine bases present at CpG dinucleotide sequences (Denissenko et al. 1997; Chen et al. 1998; Weisenberger and Romano 1999; Tretyakova et al. 2002; Matter et al. 2004). All CpG sequences in *TP53* coding exon 5 through exon 9 were completely methylated in all of the tissues examined, including the lung (Tornaletti and Pfeifer 1995). In the *TP53* gene of lung cancers, the five major G→T mutational hot spots at codons 157, 158, 245, 248, and 273 (Figure 5.12) consisted of methylated CpGs (Yoon et al. 2001). Methylation at CpG sites may increase the binding of planar carcinogenic compounds at the intercalation step through the hydrophobic effect of the methyl group that can stabilize intercalated adduct conformations (Zhang et al. 2005a). However, the precise mechanism by which cytosine methylation at CpG sites enhances carcinogen binding and mutagenesis still needs to be determined. In contrast, the presence of 5′-neighboring 5-methylcytosine inhibited formation of guanine adducts by NNK metabolites (Rajesh et al. 2005).

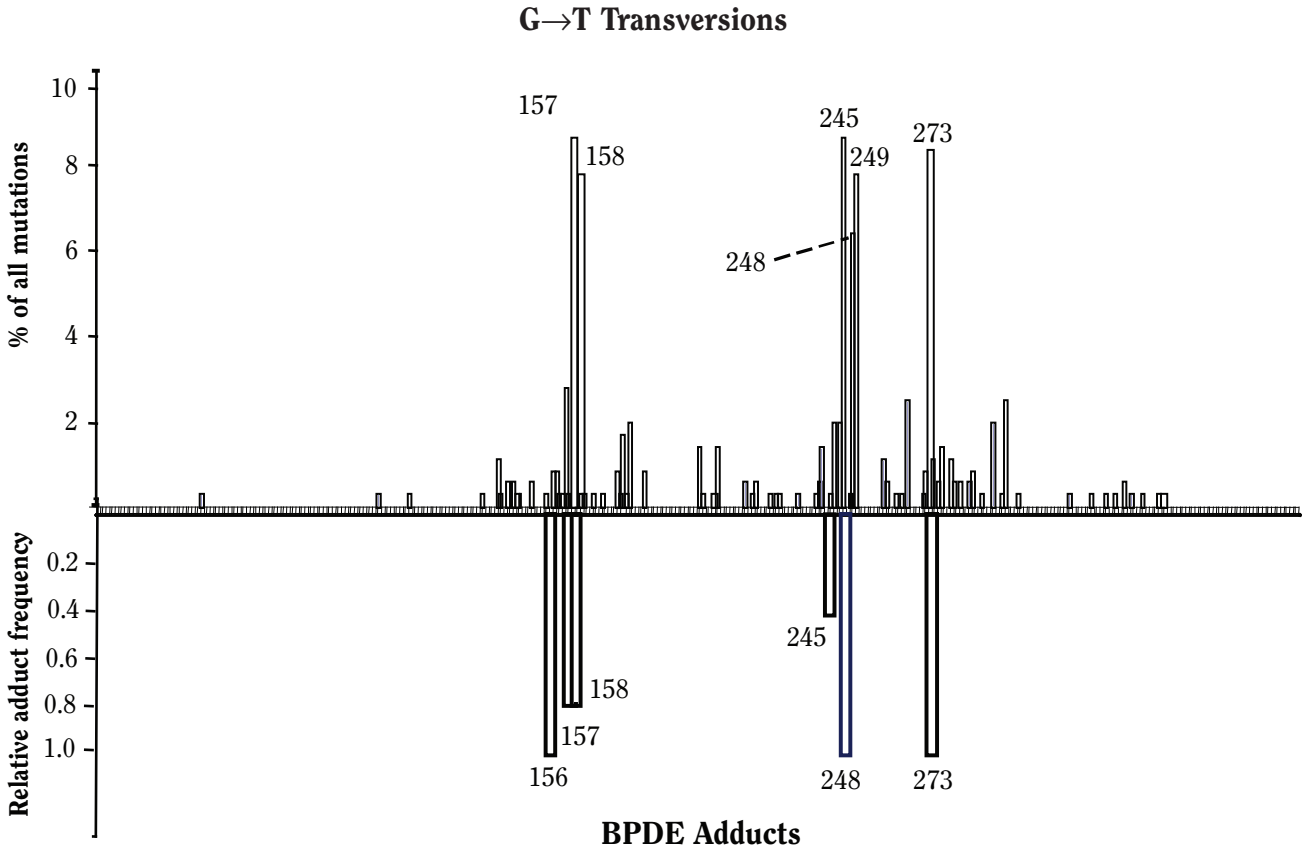
Studies show that the preferential formation of BPDE adducts at methylated CpG sites is reflected in the strongly enhanced mutagenesis at CpG sequences after cells were treated with BPDE. This finding was demonstrated with three different mutated reporter genes,

including two chromosomal genes with methylated CpG sequences (Yoon et al. 2001).

Methylated CpG sites are preferentially modified by several carcinogens, including aromatic amines and aflatoxins (Chen et al. 1998). However, the exact range of compounds that target methylated CpGs is not known. In one study, researchers did not observe a preferential mutagenesis at methylated CpGs by the aromatic amine 4-ABP (Besaratinia et al. 2002). G→T transversions resulting from 8-oxodeoxyguanosine are not specifically targeted to methylated CpG sequences (Lee et al. 2002a). A more recent study demonstrated that the DNA adduction profile of acrolein in the *P53* gene was similar to that of BPDE and other PAH diol epoxides, indicating that this α,β-unsaturated aldehyde reacts at methylated CpG sites and, because of its high concentration in cigarette smoke compared with that of PAHs, could contribute to the *TP53* mutations observed in lung tumors from smokers (Feng et al. 2006).

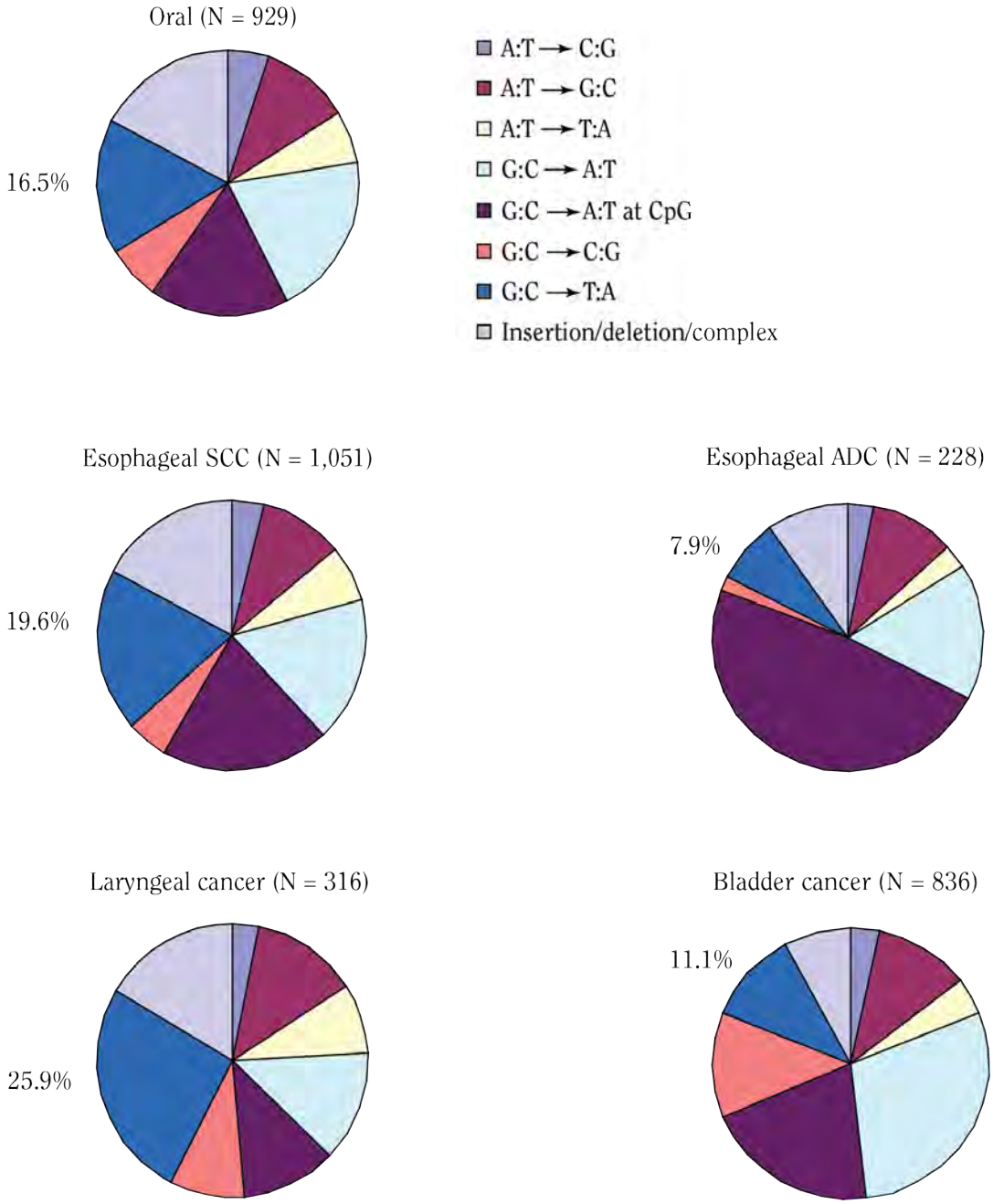
Hussain and colleagues (2001) have shown that exposing bronchial epithelial cells to BPDE produces G→T transversions in the *TP53* gene at lung cancer hot spot codons 157, 248, and 249. Nontumorous lung tissues from smokers with lung cancer carried a high *TP53* mutational

Figure 5.12 Concordance between codon distribution of G→T transversions along *TP53* gene in lung cancers (top) and distribution of adducts of benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE)–DNA adducts in bronchial epithelial cells (bottom)



Source: Adduct data were quantitated from Denissenko et al. 1996 and Smith et al. 2000.
Note: Distribution of G→T mutations is shown along the *TP53* coding sequence, and "hot spot" codons for major mutations are indicated. Mutation data from the International Agency for Research on Cancer *TP53* mutation database were used. Cell lines and cancers metastatic to the lung were excluded, as well as all cancers with defined exposures other than tobacco (e.g., asbestos, radon, mustard gas, and air pollution). Length of bars indicates relative adduct frequency at major hot spots for adducts. For adducts of BPDE, the strongest binding site has a value of 1. Sites with values less than 0.2 are not shown. Numbers correspond to *TP53* codon numbers.

Figure 5.13 Patterns of *TP53* gene mutations and percentage of G→T transversions in smoking-associated cancers other than lung cancer



Source: Data are from R9 version (July 2004) of International Agency for Research on Cancer *TP53* mutation database (IARC 2006).
Note: Oral cancers include cancers of oropharynx, hypopharynx, gum, palate, floor of mouth, and tongue. Cases with defined exposures other than tobacco (e.g., asbestos, radon, mustard gas, and air pollution) were excluded. **ADC** = adenocarcinoma; **N** = total number of mutations; **SCC** = squamous cell carcinoma.

load at these codons, even when another *TP53* mutation was present in the tumor itself. DeMarini and colleagues (2001) studied *TP53* and *KRAS* mutations in lung tumors from Chinese women who were nonsmokers and whose tumors were associated with exposure to smoky coal containing high levels of PAHs and probably other compounds such as acrolein. The tumors showed a high percentage of mutations that were G→T transversions in the *KRAS* oncogene (86 percent) or the *TP53* gene (76 percent). In the *TP53* gene, the mutations clustered at the CpG-rich codons 153 through 158 and at codons 249 and 273.

The site specificity of mutagenesis by PAH diol epoxides implies that targeted adduct formation, in addition to phenotypic selection, is responsible for shaping the *TP53* mutational spectrum in lung tumors. According to the IARC *TP53* mutation database, more than 80 percent of G→T transversions in lung cancers are targeted to guanines on the nontranscribed DNA strand. This observation suggests that a preferential repair of DNA lesions occurs on the transcribed strand. DNA repair experiments analyzing BPDE adducts in the *TP53* gene showed that the nontranscribed strand is repaired more slowly than is the transcribed strand (Denissenko et al. 1998). These findings support the proposal that both the initial DNA adduct levels and a bias in repair of DNA strands may contribute to the mutational spectrum of the human *TP53* gene in lung cancer.

TP53 Gene Mutations in Other Smoking-Associated Cancers

Of four cancer types analyzed, only SCC of the larynx showed a strong similarity with lung cancers. Prevalence of G→T transversions was high (25.9 percent), and many occurred at PAH-target codons 157 and 245. A gradient in the upper respiratory tract reflects the prevalence of *TP53* G→T transversions in cancers of smokers. This prevalence ranges from low in the oral cavity, to intermediate in the larynx, and high in various histologic types of lung cancers. The gradient may reflect the existence of an underlying, parallel gradient in the extent of exposure of respiratory tract cells to carcinogens in tobacco smoke. In oral cancers, studies show that the *TP53* mutation load is proportional to the extent of smoking, with an almost fourfold increase in the prevalence of mutations among heavy smokers compared with nonsmokers (Brennan et al. 1995). In one study of oral and esophageal SCC, however, the frequency of G→T transversions is only slightly higher (16.5 and 19.6 percent, respectively) than those in cancers not strongly related to exposure to tobacco smoke (e.g., breast, colorectal, and brain cancers). The patterns of mutations in both oral and esophageal SCC are similar, perhaps reflecting the importance of common risk factors, such as the combined use of tobacco and alcohol, infections by human papilloma virus (Gillison and Shah 2003), and various lifestyle behaviors such as tobacco chewing or consuming scalding hot beverages, as well as similar histology in oral and esophageal tissues. In contrast, the mutation pattern is different in esophageal adenocarcinomas, with a high prevalence of G→T transversions at CpG sites (Figure 5.13) and a type of mutation that could be associated with the overproduction of reactive nitrogen species due to inflammation (Ambs et al. 1999).

For bladder cancer, the mutation pattern shows an unusually high prevalence of G→A transitions at non-CpG sites. These mutations are not distributed at random, and bladder-specific mutation hot spots can be seen at codons 280 and 285, according to the IARC *TP53* database. Both codons occur within the same primary sequence context (5′AGAG), which raises the possibility that this sequence may be a preferential target site for a carcinogen involved in bladder carcinogenesis. However, aromatic amines, a potent class of bladder carcinogens in tobacco smoke, produce mainly G→T mutations (Besaratinia et al. 2002).

Limitations to the Study of *TP53* Mutations and Smoking-Induced Cancer

Although the study of mutations in the *TP53* gene provides potentially useful leads for understanding mechanisms of tobacco carcinogenesis, this approach also has limitations. As already mentioned, various carcinogen-DNA adducts can produce G→T transversions and even similar spectra of mutations. In addition, most of this research is not population based, and the studies may be biased with respect to the stage of lung cancer represented. Finally, lack of a mutation in the *TP53* gene does not necessarily mean that the tumor is not related to smoking, because other uncharacterized changes could have occurred.

Loss of Mechanisms for Growth Control

Signal Transduction

Introduction

Normally, cell signaling is very tightly regulated and begins with the transduction of the signal through a membrane receptor. The signal is conveyed through a series of intracellular proteins, and the result is the regulation of cellular processes including proliferation and apoptosis. In lung cancer cells, the processes governing these events are frequently deregulated by DNA-damaging mutations induced by cigarette smoke or other alterations in the molecules of numerous signaling pathways. The balance between mechanisms leading to apoptosis (proapoptotic) and those suppressing apoptosis (antiapoptotic) or suppressing increased proliferation will have a major impact on lung tumor growth. Identifying and targeting signaling pathways that lead to therapeutic resistance could help to neutralize a patient's resistance to standard therapies.

Apoptosis

Apoptosis was first described in 1972 (Kerr et al. 1972). The term "apoptosis" is from the Greek word for "falling off." Apoptosis is a natural process that consists of a well-orchestrated cascade of distinct biologic and histologic events (Kerr et al. 1972). These events are critical for eliminating injured or genomically unstable cells while minimizing damage to surrounding normal cells (Martin 2002). The induction of apoptosis prevents the malignant growth of cancer cells (Rich et al. 2000). The deregulation of the mechanisms governing apoptosis is a distinctive characteristic of most cancer cells (Hanahan and Weinberg 2000).

Apoptosis is characterized by morphologic features including membrane blebbing, cell shrinking, and chromosomal condensation. Apoptosis is generally believed to occur through two "effector" mechanisms: extrinsic (death receptor mediated) and intrinsic (mitochondrial mediated) (Hengartner 2000). The extrinsic pathway is regulated by binding a "death receptor molecule" to the cancer cell's membrane receptor (i.e., death receptor). The intrinsic pathway is mediated by rendering the mitochondrial membrane permeable, a phenomenon directly influenced by the ratio of the interaction of proapoptotic and antiapoptotic proteins. In general, researchers believe that the inactivation of apoptosis through the intrinsic pathway is the primary mechanism through which DNA-damaging agents from tobacco smoke act to enhance the survival of lung cancer cells, which is the focus of this section.

Key Apoptotic Regulators

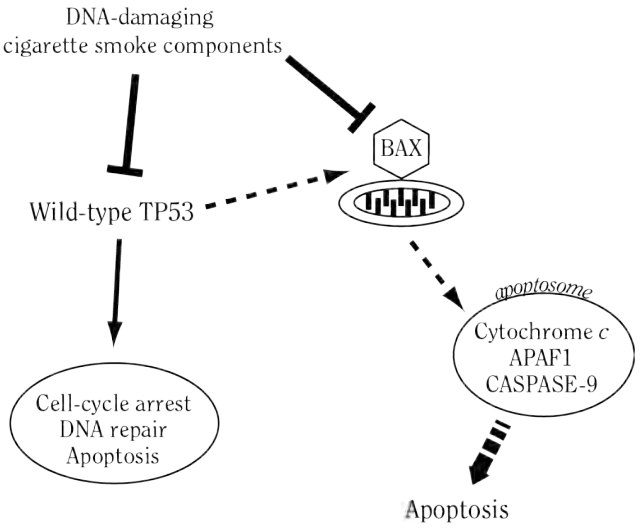
One or more pathways may lead to apoptosis. Stress signals stimulate a pathway that activates proteins to respond to DNA damage. These proteins subsequently phosphorylate, activate, and stabilize the P53 protein. The activated P53 protein drives the transcription of genes associated with cell-cycle arrest, DNA repair, and apoptosis. These genes include the BCL-2 family of proteins, which consists of both proapoptotic and antiapoptotic members. The BCL-2 family of proteins interacts with the outer mitochondrial membrane to regulate the release of cytochrome *c*, which results in the activation of aspartyl and cysteine proteases (caspases) (Igney and Krammer 2002). The caspases are crucial executioners of apoptosis (Meier et al. 2000; Reed 2000). Once stimulated, the caspases activate endonucleases that subsequently cleave the DNA of the targeted cell into nucleosome-sized fragments, which is a common characteristic of apoptosis.

A multitude of signaling molecules mediate the mechanisms that govern apoptosis. An imbalance in proapoptotic and antiapoptotic signaling events contributes to the development and progression of lung cancer. The mechanisms for the deregulation of apoptosis can be categorized into (1) the decrease of signaling associated directly with the induction of apoptosis and (2) the increase of signaling leading to the suppression of apoptosis. This decrease may include mutations induced by cigarette smoke or other smoke-related mechanisms that activate oncogenes or inactivate tumor-suppressor proteins or other proapoptotic proteins. The increase may include mutations induced by cigarette smoke, certain kinases, other antiapoptotic proteins or transcription factors, or overexpression or constitutive activation of growth factors. The end result of this deregulation usually includes a profound resistance to apoptosis.

Regulation of Tumor Suppressors and Proapoptotic Proteins

Decrease of important proapoptotic proteins of the BCL-2 family and tumor suppressors such as the P53 and RB proteins is a characteristic in many types of cancers, including lung cancer. This decrease provides lung cancer cells with a strong ability to resist apoptosis, which leads to a distinct advantage for cell survival (Figure 5.14).

Figure 5.14 Tobacco-associated suppression of proapoptotic proteins and tumor-suppressor proteins



Note: Tobacco-associated suppression of proapoptotic proteins and tumor-suppressor proteins increases cell proliferation and resistance to apoptosis. Two major signaling pathways that are downregulated by DNA-damaging tobacco agents are the TP53 protein and the proapoptotic family of BCL-2 proteins.
APAF1 = apoptotic-releasing factor 1; **BAX** = BCL-2 associated X protein; **CASPASE-9** = cysteine-aspartic acid protease-9.

BCL-2 Family Proteins

In normal cells, stresses initiate apoptosis through the mitochrondrial or intrinsic pathway, and the BCL-2 family proteins are important mediators of the apoptotic response. These proteins are characterized by the presence of one to four conserved BCL-2 homology (BH) domains. The BCL-2 family can be divided into antiapoptotic members: BCL-2, BCL-X_L, and myeloid cell leukemia-1. The proapoptotic BCL-2 proteins are subdivided into two groups: the multidomain BAX subfamily (BAK, BAX, and BOK) and the BH3-only proteins (BAD, BID, and BIM) (Korsmeyer 1995; Hale et al. 1996; Adams and Cory 1998; Huang and Strasser 2000; Cory et al. 2003). The BCL-2 family of proteins appears to directly influence the permeability of the mitochondrial membrane to regulate apoptosis.

The interaction of BAX with the mitochrondrial membrane causes the release of cytochrome *c* into the cytosol, where it binds to apoptotic-releasing factor 1. The binding of cytochrome *c* and apoptotic-releasing factor 1 results in the activation of cysteine-aspartic acid protease-9 (CASPASE-9), which is required to form the "apoptosome" complex that initiates apoptosis. The apoptotic response is critically dependent on the ratio of the expression of proapoptotic and antiapoptotic BCL-2 members (Zha et al. 1997; Korsmeyer 1999; Kroemer 1999; Reed 1999; Huang and Strasser 2000; Lutz 2000; Cheng et al. 2001; Ruvolo et al. 2001). A lack of BAX (Zhang et al. 2000a; Schmitt and Lowe 2002) or an increase of BCL-2 or BCL-X_L (Schott et al. 1995; Walczak et al. 2000; Chipuk et al. 2001) suppresses apoptosis, whereas a decrease of BCL-X_L or BCL-2 enhances apoptosis (Hayward et al. 2003). Dimers containing BAX and BCL-2 inactivate BAX and therefore inhibit apoptosis. In addition, phosphorylation of the BAD protein results in its inactivation, because only the nonphosphorylated form of BAD can antagonize the antiapoptotic BCL-2 or BCL-X_L at the mitochondrial membrane (Hermeking 2003).

Nicotine suppresses the death of lung cancer cells by phosphorylation mediated by the extracellular signal-regulated kinase (ERK) of BCL-2 (Heusch and Maneckjee 1998; Mai et al. 2003). Conversely, NNK inactivates BAD through β-adrenergic receptors and protein kinase C (PKC), which promotes survival of NSCLC cells (Lahn et al. 2004; Jin et al. 2005). Nicotine also stimulates cell survival through the phosphorylation and inhibition of BAD activated by β-adrenergic-receptor–mediated AKT-, PKA-, and/or ERK-dependent pathways (Jin et al. 2004a). These studies show that BCL-2 family members are critical effectors of signaling pathways that promote cancer cell survival in response to components of cigarette smoke—in these cases, through direct receptor binding rather than DNA damage.

P53 Protein

The P53 pathway is clearly involved in cellular life or death. The P53 tumor-suppressor protein can induce the expression of BAX and additional proapoptotic members of the BCL-2 family (Miyashita and Reed 1995; Yin et al. 1997; Oda et al. 2000a,b; Nakano and Vousden 2001). In addition to having direct effects on BCL-2 family proteins, the P53 protein also increases activity of the *APAF1* gene (Robles et al. 2001), which as indicated earlier, is a member of the apoptosome complex and is critical for the activation of CASPASE-9 to initiate apoptosis (Soengas et al. 1999) (see "BCL-2 Family Proteins" earlier in this chapter). Although it is primarily a nuclear protein, P53 may function outside the nucleus by translocating to the mitochondria, where it interacts directly with antiapoptotic proteins such as BCL-2 and BCL-X_L to induce apoptosis (Mihara et al. 2003). The aberrant inactivation of P53 leads to a deregulation of cell-cycle control and a suppression of many crucial proapoptotic pathways (Ford

and Hanawalt 1995; Wang et al. 1995a,b; Offer et al. 1999; Vogelstein et al. 2000; Zhou et al. 2001a). The loss of P53 function markedly decreases the sensitivity of lung cancer cells to apoptosis induced by exposure to tobacco smoke or other stresses (Lowe et al. 1994).

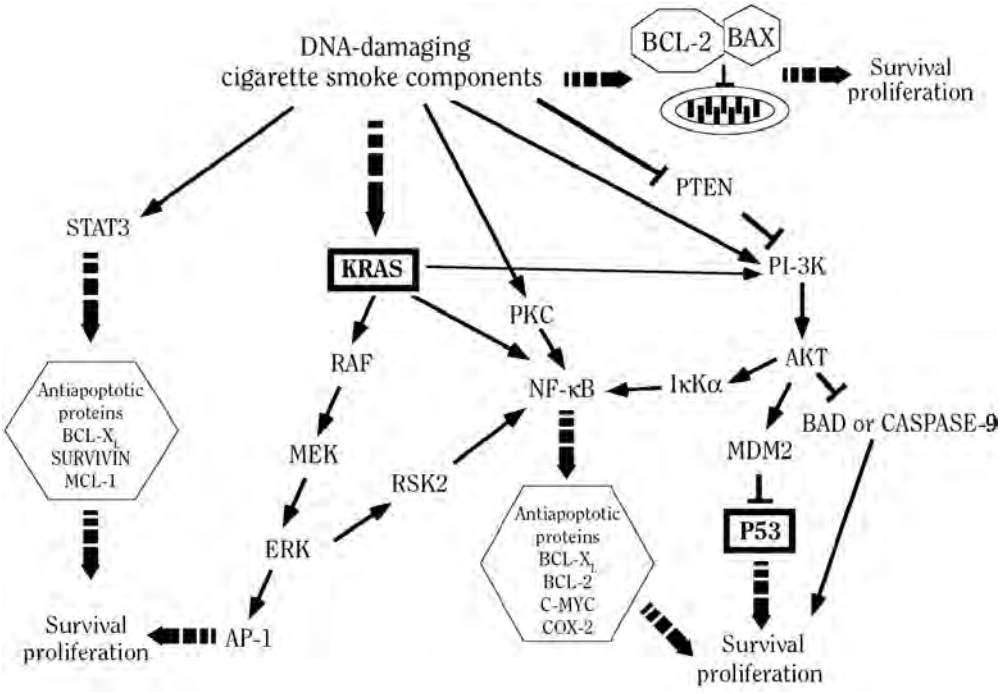
Retinoblastoma Protein

Inactivation of the RB protein results in the release and activation of the transcription factor E2F (Flemington et al. 1993; Helin et al. 1993). Some E2F family members induce expression of the genes important in apoptosis, such as the *P14ARF* gene (DeGregori et al. 1997; Bates et al. 1998). The P14ARF protein is a negative regulator of murine double minute 2 (MDM2), a P53 binding protein. The inhibition of MDM2 leads to elevated P53 levels and apoptosis. The E2F protein can also activate proapoptotic BCL-2 family members and caspases (Nahle et al. 2002; Hershko and Ginsberg 2004).

Regulation of Antiapoptotic Proteins and Effects

Studies document that many genes and signaling proteins are overexpressed or display gain-of-function mutations in lung cancers. These include the *EGFR* gene, signal transduction and activator of transcription, PKC, RAS/MAPK, phosphatidylinositol-3 kinase (PI-3K)/AKT, PTEN, nuclear factor-kappa B (NF-κB), and COX (Figure 5.15).

Figure 5.15 Protein-signaling pathways deregulated in lung cancer



Note: Many protein-signaling pathways deregulated in lung cancer represent a dense interactive network with a range of potential survival-enhancing effects. Tobacco- or cigarette smoke-associated activation of antiapoptotic proteins provides lung cancer cells with a distinct growth advantage. Major protein survival-signaling pathways activated by tobacco carcinogens are illustrated. KRAS and P53 are boxed to emphasize that *KRAS* and *TP53* are the most commonly mutated genes. Mutationally activated KRAS is locked in its active form, resistant to the inactivating effects of GTPase-activating proteins, and cannot hydrolyze guanosine triphosphate to guanosine diphosphate. Similarly, mutated P53 cannot carry out many of its normal protective functions with respect to cell cycle control and apoptosis. **AKT** = protein kinase B; **AP-1** = activator protein-1; **BAD** = BCL-associated death protein; **BAX** = BCL-2 associated X protein; **CASPASE-9** = cysteine-aspartic acid protease-9; **ERK** = extracellular signal-regulated kinase; **IκKα** = I kappa-B kinase alpha; **MDM2** = murine double minute 2 protein; **MEK** = mitogen-activated protein kinase kinase; **NF-κB** = nuclear factor-kappa beta; **PI-3K** = phosphatidylinositol 3-kinase; **PKC** = protein kinase C signaling pathway; **PTEN** = phosphatase and tensin homolog; **RAF** = ν-raf murine leukemia viral oncogene; **RSK2** = P90 ribosomal protein S6 kinase; **STAT3** = signal transducer and activator of transcription 3.

Epidermal Growth Factor Receptor

Exposure of oral cells to cigarette smoke caused an increase in EGFR tyrosine kinase activity (Moraitis et al. 2005). Signaling through EGFR can lead to survival signals that suppress dependent (downstream) apoptotic pathways or stimulate cell proliferation. Some evidence suggests that EGFR signaling can influence the levels and activities of antiapoptotic BCL-2 family members (Kari et al. 2003). The *EGFR* gene is overexpressed and thus constitutively activated in lung cancer cells (Sridhar et al. 2003) and bronchial preneoplastic lesions (Rusch et al. 1995; Kurie et al. 1996; Piyathilake et al. 2002). In addition, a truncated form of the EGFR protein (EGFRvIII) is constitutively active in NSCLC cells (Okamoto et al. 2003). The expression of this mutant form of *EGFR* is associated with an increase in cell transformation and with the constitutive activation of important downstream signaling pathways for survival, including the PI-3K/AKT pathway (Antonyak et al. 1998; Moscatello et al. 1998; Tang et al. 2000).

RAS/Mitogen-Activated Protein Kinase

Activation of the RAS pathway sends a strong antiapoptotic signal, and the constitutive activation of RAS can transform normal cells. Oncogenic RAS protein has a primary role in the development of lung cancer (Johnson et al. 2001a). RAS activates several pathways, including RAF/mitogen-activated protein kinase kinase (MEK)/ERK, PKC, PI-3K/AKT, and NF-κB (Kauffmann-Zeh et al. 1997; Kennedy et al. 1997; Peeper et al. 1997; Baldwin 2001). These pathways are commonly deregulated by RAS in lung cancers (Adjei 2001a,b). RAF/MEK/ERK pathway activation can lead to changes in downstream gene expression through the activation of activator protein-1 (AP-1). AP-1 is a well-characterized transcription factor composed of homodimers and/or heterodimers of the *JUN* and *FOS* gene families (Angel and Karin 1991). AP-1 regulates the transcription of various genes. Many stimuli, including tumor promoters, mediate AP-1 binding to the DNA of genes that govern cellular processes such as inflammation, proliferation, and apoptosis (Angel and Karin 1991).

Phosphatidylinositol-3 Kinase, Phosphatidylinositol 3′-Phosphatase, and Protein Kinase B

PI-3K consists of a family of heterodimeric complexes, each composed of a p110 catalytic subunit and a regulatory subunit that exists primarily as a p85 form (Tolias et al. 1995; Vanhaesebroeck et al. 1997; Wymann and Pirola 1998). This family of proteins is involved in the regulation of proliferation, viability, adhesion, and motility migration in numerous cell types (Carpenter and Cantley 1996; Khwaja 1999; Rameh and Cantley 1999; Blume-Jensen and Hunter 2001; Roymans and Slegers 2001). Cell survival and oncogenic transformation require PI-3K activation (Datta et al. 1999; Stambolic et al. 1999). PI-3K–dependent kinases include 3-phosphoinositide–dependent protein kinase-1 (PDK1) and AKT (PKB). The PI-3K pathway can also be activated by the EGFR protein and by an activated RAS protein (Rodriguez-Viciana et al. 1997). One of the first steps in PI-3K signaling is the activation of PDK1, which phosphorylates and activates AKT (Coffer et al. 1998; Belham et al. 1999). AKT phosphorylates and inactivates several proapoptotic proteins, including BAD and CASPASE-9. Other targets of AKT important in the regulation of apoptosis include glycogen-synthase-kinase-3 (Pap and Cooper 1998), the Forkhead transcription factor FKHRL1 (Brunet et al. 1999), and the mammalian target of rapamycin/p70S6 kinase (McCormick 2004). Furthermore, AKT inactivates P53 by phosphorylating MDM2, which increases the ability of MDM2 to bind to and promote P53 degradation (Ogawara et al. 2002). AKT also suppresses apoptosis by activating NF-κB through AKT phosphorylation of I kappa-B kinase alpha (Ozes et al. 1999; Romashkova and Makarov 1999).

Most NSCLC cells display an increase in PI-3K activity that results in highly active AKT and other downstream mediators (Moore et al. 1998; Brognard et al. 2001). AKT is important in the survival of lung cancer cells and is constitutively activated in most NSCLC cell lines to promote the survival of NSCLC cells under stressful conditions (Brognard et al. 2001). Studies have also found AKT expression in SCLC tumor samples (Lee et al. 2002b; Mukohara et al. 2003), SCLC cell lines (Moore et al. 1998), SCLC tumors (Blackhall et al. 2003), mouse tumors induced by tobacco carcinogens (West et al. 2003), and human bronchial dysplastic lesions (Tsao et al. 2003). (For additional details on AKT activation by components of cigarette smoke through receptor interactions, see "Activation of Cytoplasmic Kinase by Tobacco Smoke" later in this chapter.)

Nuclear Factor-Kappa B

NF-κB is a rapidly induced transcription factor responsive to stress that functions to intensify the transcription of a variety of genes, including those encoding cytokines, growth factors, and acute response proteins (Baldwin 1996). Nicotinic activation of nicotinic acetylcholine receptors (nAChRs) stimulates NF-κB activity downstream of ERK and AKT, which promotes tumor growth and angiogenesis through the vascular endothelial growth factor (VEGF) in vivo (Heeschen et al. 2001, 2002). Moreover, NF-κB activation by exposure to cigarette smoke in

lung cancer cells induces the expression of COX-2 (Anto et al. 2002; Shishodia and Aggarwal 2004). Recent results suggest that nicotine, but not NNK, activates NF-κB–dependent survival of lung cancer cells in addition to their proliferation. These studies illustrate that the activation of NF-κB by nicotine or by cigarette smoke in its entirety through receptor binding can promote tumorigenesis in the lung through many mechanisms, including increased levels of VEGF and COX-2.

Cyclooxygenase

COX-1 and COX-2 were shown to catalyze synthesis of prostaglandins from arachidonic acid. Researchers observed that COX-1 was constitutively expressed in most tissues, whereas COX-2 was inducible and found at elevated levels in various cancers (Koki et al. 2002; Dannenberg and Subbaramaiah 2003; Dubinett et al. 2003). In lung cancers, researchers have found COX-2 expression at most stages of tumor progression (Hida et al. 1998; Huang et al. 1998; Wolff et al. 1998; Hosomi et al. 2000; Anderson et al. 2002; Fang et al. 2003). Others reported high levels of COX-2 in NSCLC and premalignant lesions, but COX-2 expression is less consistent in SCLC (Wolff et al. 1998; Hosomi et al. 2000). Studies show that NNK induces a high expression of COX-2 in rats (El-Bayoumy et al. 1999). Levels of COX-2 mRNA are about four times higher in the oral mucosa of smokers than in that of lifetime nonsmokers (Moraitis et al. 2005). Researchers believe that at least one role of COX-2 in cancer is associated with cell resistance to apoptosis and an increase in metastatic potential (Gupta and Dubois 2001). The supporting evidence shows that COX-2 overexpression coincides with an increased BCL-2 expression (Tsujii and DuBois 1995) and an increased stabilization of the antiapoptotic protein survivin (Li et al. 1998a; Krysan et al. 2004). Lung cancer cells that were induced to express COX-2 demonstrated an increase in survival time (Lin et al. 2001b), and COX-2 inhibitors stimulated apoptosis in lung carcinoma cells (Hida et al. 2000; Yao et al. 2000; Chang and Weng 2001).

Summary

Apoptosis is commonly suppressed in lung cancer, which correlates with increases in cancer cell survival and proliferation. Deregulation of the many pathways for growth control (Figure 5.15) in lung cancer is attributable partly to interactions of carcinogens in cigarette smoke with the *KRAS* oncogene, the *P53* tumor-suppressor gene, and other genes. These pathways represent a dense interactive network with a range of potential effects on cell survival. Mechanisms associated with cigarette smoke that increase resistance to apoptosis include activation of antiapoptotic proteins and/or suppression of proapoptotic and tumor-suppressor proteins.

Cigarette Smoke and Activation of Cell-Surface Receptors in Cancer

Airway Epithelial Cells

Nicotinic Acetylcholine Receptors

Neuronal nAChRs are large membrane-associated proteins that are the first line of contact between cells and components of cigarette smoke such as nicotine and NNK. These proteins were originally described as receptors for acetylcholine (ACh). Their function in the brain has been studied in detail because of their ability to mediate the addictive effects of nicotine. Each receptor is made up of five subunits arranged in a barrel-like structure, creating a pore that allows calcium to enter the cell in response to ligand binding. Nine alpha subunits (α2 through α10) and three beta subunits (β2 through β4) combine with each other to form heteropentamers (combinations of α2 through α6 with β2 through β4) or homopentamers (α7 through α10). Each nAChR consists of 5 subunits, and researchers have identified at least 12 subunits; thus, many functional nAChRs exist. Different ligands, including nicotine, NNK, and ACh, have varying affinities for different nAChRs. Despite this complexity, the primary receptors that mediate the addictive effects of nicotine are α4β2 nAChRs, whereas α7 nAChRs are high-affinity receptors for NNK (Lindstrom 1997, 2003). Moreover, the discovery that mutations in the α4 nAChR subunit lower the threshold for addiction raises the possibility that genetic variations in these receptors could increase susceptibility to nicotine dependence and exposure to carcinogens through smoking (Tapper et al. 2004).

Although nAChRs were originally thought to be limited to neuronal cells, studies have identified functional nAChRs in tissues outside the nervous system. This finding raises the possibility that these receptors may mediate some of the systemic effects of smoking. In lung tissues, researchers have discovered nAChRs in human bronchial epithelial cells, vascular endothelial cells, pulmonary neuroendocrine cells, neuroepithelial bodies, NSCLC cells, and SCLC cells (Tarroni et al. 1992; Maneckjee and Minna 1994; Macklin et al. 1998; Maus et al. 1998; Schuller and Orloff 1998; Wang et al. 2001b; Fu et al. 2003; Schuller et al. 2003; Song et al. 2003a,b; Tsurutani et al. 2005).

The stimulation of nAChRs by components of cigarette smoke has biologic effects on cells that are important for the initiation, progression, and maintenance of cancer.

The activation of nAChRs in lung epithelial cells by nicotine or NNK promotes the survival and proliferation of human mesothelioma and lung cancer cells (Maneckjee and Minna 1994; Schuller and Orloff 1998; West et al. 2002; Schuller et al. 2003; Trombino et al. 2004; Tsurutani et al. 2005). In normal cells, nicotine can stimulate properties consistent with cell transformation and the early stages of cancer formation, such as increased cell proliferation, decreased cellular dependence on the extracellular matrix for survival, and decreased contact inhibition, which is the natural process of arresting cell growth when two or more cells come in contact with each other (West et al. 2003). Furthermore, nicotine stimulation of endothelial nAChRs promotes angiogenesis, another property of cancer (Heeschen et al. 2001, 2002; Zhu et al. 2003). Thus, the induced activation of nAChRs in lung tissues by components of cigarette smoke can promote processes required for development of cancer.

In addition to stimulating nAChRs directly, components of cigarette smoke can indirectly stimulate nAChRs by promoting the growth of tobacco-related cancers that express and secrete ACh, the endogenous ligand for these receptors. SCLC and NSCLC cells synthesize, transport, and release ACh in vitro, which stimulates proliferation of cancer cells through the autocrine activation of nAChRs (Song et al. 2003a; Proskocil et al. 2004). This finding suggests that there are many mechanisms for activation of nAChRs in lung cancer and further emphasizes the importance of these receptors in the biology of tobacco-related cancer.

β-Adrenergic Receptors

The β-adrenergic receptors are neuronal receptors that may play a role in mediating effects of cigarette smoke related to signal transduction. NNK is structurally similar to epinephrine, the endogenous ligand for the β-adrenergic receptor, suggesting that in addition to binding nAChRs, NNK may bind to these receptors. Once bound to β-adrenergic receptors, NNK can stimulate the release of arachidonic acid (Schuller et al. 1999; Weddle et al. 2001). The enzyme COX-2 converts arachidonic acid to prostaglandin E_2, which mediates inflammation and promotes cell survival and proliferation in cancer. This finding is important because cell lines from human lung cancer overexpress the β-adrenergic receptor (Schuller et al. 2001), and several studies suggest that the presence or expression of arachidonic acid is a risk factor for pulmonary adenocarcinomas (Alavanja et al. 1993, 2001). Thus, these studies indicate that the β-adrenergic receptor may be an important mediator of signal transduction pathways associated with exposure to cigarette smoke.

Other Receptors

The ERBB family is another group of EGFRs that indirectly mediate signal transduction associated with cigarette smoke. The four types of ERBB receptors are EGFR (HER-1), HER-2, HER-3, and HER-4. These receptors act in pairs to stimulate downstream signaling pathways that mediate the survival and proliferation of both normal cells and cancer cells. Ligands that bind to ERBB family members include the epidermal growth factor TGFα and amphiregulin. In addition, receptors can be activated in the absence of a ligand through overexpression of the receptors themselves. Both of these mechanisms play a role in activation of these receptors mediated by cigarette smoke.

The hypothesis that ERBB receptors mediate the effects of cigarette smoke on airway epithelial cells emerged from correlative clinical data and mechanisms defined in vitro. Clinical data include many reports of EGFR and HER-2 overexpression in lung cancer (Hendler and Ozanne 1984; Cerny et al. 1986; Veale et al. 1987; Hirsch et al. 2003a; Tan et al. 2003). In addition, some studies have shown that EGFR overexpression and activation in human lung cancers correlate with shorter survival times, suggesting that they play an important role in development of cancer (Kern et al. 1990; Kanematsu et al. 2003; Selvaggi et al. 2004).

Clinical data also support the hypothesis that ERBB expression and activation change with exposure to cigarette smoke or its components. Studies have demonstrated the overexpression of EGFR and ERBB3 in the bronchial epithelium of smokers (Yoneda 1994; O'Donnell et al. 2004). Results of mechanistic in vitro studies, such as the demonstration that NNK-induced transformation of lung epithelial cells is associated with an increase in EGFR expression, support these observations (Lonardo et al. 2002). Moreover, exposure to nicotine alone can increase the expression of EGFR in cervical cancer cell lines (Mathur et al. 2000). Studies also demonstrate that exposure to tobacco smoke increases the activity of EGFR, and metabolites of B[*a*]P induce activation of EGFR and downstream signaling pathways that promote proliferation (Burdick et al. 2003; Moraitis et al. 2005). These studies support the idea that components of cigarette smoke modulate the expression and activation of the ERBB family of receptors.

In addition to increasing the expression of ERBB family members, components of tobacco smoke stimulate cells to produce ligands that activate the receptors. In clinical specimens, studies have described the coexpression of EGFR and its ligand TGFα in human NSCLC. In one study, both EGFR and TGFα were expressed in 38

percent of the cases of NSCLC examined (Rusch et al. 1993). In a second study, 72 percent of SCCs and 34 percent of adenocarcinomas expressed both EGFR and TGFα (Hsieh et al. 2000). This finding may be clinically important because a retrospective analysis showed that the coexpression of EGFR and TGFα is an indicator of a poor prognosis (Tateishi et al. 1990). These studies suggest that the stimulation of ERBB ligands induced by cigarette smoke may be an important mechanism of signal transduction.

Consistent with the clinical data, in vitro studies show that condensate from cigarette smoke stimulates the release of amphiregulin and TGFα from the cell membrane, which leads to the autocrine activation of EGFR and cell proliferation (Richter et al. 2002; Lemjabbar et al. 2003; Moraitis et al. 2005). Several studies demonstrate that cigarette smoke condensate activates matrix metalloproteinases (MMPs), which are enzymes on the extracellular surface of cells that cleave these ligands from the extracellular matrix. Support for these in vitro observations comes from the demonstration that MMP activity is higher in lung tissues from smokers than in those from nonsmokers (Kang et al. 2003; Kangavari et al. 2004).

In addition to stimulating downstream kinases, EGFR activation by cigarette smoke may provide a mechanistic link to the increased inflammation characteristic of smokers by increasing COX-2 activity (see "Activation of Cytoplasmic Kinase by Tobacco Smoke" later in this chapter). In vitro data suggest that autocrine activation of EGFR, by the expression of the *TGF*α and *AREG* genes induced by tobacco smoke, stimulates COX-2 expression (Moraitis et al. 2005). Cigarette smoke also increases COX-2 expression by lung fibroblasts in vitro, and B[*a*]P increases COX-2 expression by oral epithelial cells (Kelley et al. 1997; Martey et al. 2004). Thus, many in vitro studies demonstrate that EGFR activation by components of cigarette smoke can contribute to inflammation through the increased expression and activation of COX-2.

Clinical data support the validity of these in vitro observations. For example, studies document increased levels of COX-2 in the oral mucosa of smokers (Moraitis et al. 2005) and in urothelial tissues from smokers with bladder cancer (Badawi et al. 2002). Moreover, COX-2 is expressed only in neoplastic epithelial cells, not in normal bronchial epithelial cells (Hastürk et al. 2002). COX-2 overexpression in lung cancer is associated with tumor angiogenesis and survival and proliferation of tumor cells (Riedl et al. 2004) and with a poor prognosis in NSCLC (Achiwa et al. 1999; Yuan et al. 2005). Thus, the stimulation of EGFR that leads to COX-2 activity by exposure to cigarette smoke is another mechanism mediated by a growth factor receptor to promote cell survival and proliferation in carcinogenesis.

Activation of Cytoplasmic Kinase by Tobacco Smoke

Activation of cell-surface receptors by components of tobacco smoke stimulates downstream kinases that mediate cancer cell survival, proliferation, and resistance to chemotherapy. The best-described kinases activated by smoking are AKT, ERK, PKC, and PKA. All of these kinases can be activated by cigarette smoke components through nAChRs, but ERBB family members also mediate AKT and ERK activation by cigarette smoke components. In addition, β-adrenergic receptor activation by cigarette smoke components can activate PKA and PKC. Thus, these proteins can be activated by tobacco smoke components through multiple receptor-mediated mechanisms, suggesting that the proteins are important mediators of smoking-induced signal transduction.

Protein Kinase B

The serine/threonine kinase AKT may be the critical effector of signaling induced by cigarette smoke, because AKT is stimulated in response to the activation of nAChRs, β-adrenergic receptors, and the ERBB family of receptors. Moreover, AKT controls many cellular processes that promote cell survival, proliferation, and the resistance of cancer cells to chemotherapy. Clinical data also suggest that AKT activation indicates a poor prognosis in many tobacco-related cancers. Thus, activation of this kinase by components of tobacco smoke can affect many cellular processes important for the initiation, growth, and progression of tumors.

AKT might be important for the initiation as well as the maintenance of tobacco-related cancers. Nicotine and NNK cause rapid AKT activation through different nAChRs (West et al. 2003; Tsurutani et al. 2005). B[*a*]P metabolites activate AKT in breast epithelial cells, although the cellular receptor responsible for the effect has not been identified (Burdick et al. 2003). Furthermore, nicotine-induced AKT activation in normal human bronchial cells or in small airway epithelial cells promotes cell survival, proliferation, and anchorage-independent growth, all of which are properties of transformed cells (West et al. 2003). These studies are important because they suggest that AKT activation by tobacco smoke components may precede the formation of DNA mutations that cause cancer. Thus, AKT activation could serve as a biochemical gatekeeper for lung carcinogenesis by promoting the survival of cells that would normally die from DNA damage.

In addition to promoting AKT-dependent growth and survival of normal epithelial cells, tobacco smoke components have similar effects on cells throughout the phenotypic spectrum of transformation. In a mouse model of NNK-induced lung tumorigenesis, an increase in AKT

activation was associated with an increase in the progression of NNK-induced lung lesions (West et al. 2004b). In human lung cancer cells, nicotine or NNK activated the AKT pathway and stimulated AKT-dependent proliferation through nAChRs (Tsurutani et al. 2005). Moreover, these researchers showed that nicotinic activation of AKT increased survival of lung cancer cells after treatment with chemotherapeutic agents or radiation (Tsurutani et al. 2005). The fact that tobacco smoke components activate AKT and promote the survival of cancer cells is important, and it is supported by the finding that cancer patients who continue to smoke during chemotherapy have a worse prognosis than those who stop smoking (Johnston-Early et al. 1980; Browman et al. 1993; Videtic et al. 2003).

Clinical data and preclinical models support the hypothesis that AKT activation is an early event in carcinogenesis. AKT is activated in preneoplastic lung lesions induced by exposure to NNK (West et al. 2004a) and in dysplastic lung lesions from smokers (Massion et al. 2004). In addition, AKT activation is associated with poor survival in patients with tobacco-related cancers, including lung cancer and pancreatic cancer (David et al. 2004; Hirami et al. 2004; Yamamoto et al. 2004; Tsurutani et al. 2005). Together, these clinical studies support the idea that AKT plays an important role in the formation and maintenance of tobacco-related cancers.

Extracellular Signal-Regulated Kinases

In addition to AKT, ERK may play an important role in smoking-related cancers because it can be activated in response to components of tobacco smoke through both nAChR and ERBB receptors. In normal cells, ERK is activated in response to many extracellular signals and stimulates cell proliferation. In SCLC and pulmonary neuroendocrine cells, NNK-induced activation of nAChR leads to the activation of RAF-1 and its downstream effector ERK (Jull et al. 2001; Schuller et al. 2003). In addition, B[*a*]P metabolites activate ERK (Burdick et al. 2003), and nicotine activates ERK and promotes cell survival (Heusch and Maneckjee 1998). Thus, like AKT, ERK can be activated as an acute response to tobacco smoke components. Because ERK and AKT can promote cell survival and proliferation, early activation of both kinases may contribute to the initiation, promotion, and progression of cancer.

Researchers have also described ERK activation in tobacco-related cancers, thus validating the mechanisms defined in vitro. ERK activation is associated with poor survival in SCLC, which occurs almost exclusively in smokers (Blackhall et al. 2003). The overexpression of *C-MYC*, an oncogene activated by ERK, has been described in lung cancer and promotes proliferation as well as resistance to cell death (Zajac-Kaye 2001). Thus, tobacco smoke components stimulate ERK, which promotes cell proliferation and contributes to the poor prognosis of lung cancer patients with this biochemical alteration.

Protein Kinase C

The PKC kinases also mediate cellular responses to exposure to tobacco smoke. Several isoforms of PKC can promote cell survival, most notably PKCα. Nicotine- and NNK-induced activation of PKCα through the β-adrenergic receptor promotes the survival of lung cancer cells (Schuller et al. 2003). In addition, nicotinic activation of nAChRs activates PKC in human bronchial epithelial cells, as well as in lung cancer cells (Maneckjee and Minna 1994; Carlisle et al. 2004). In SCLC, NNK-induced activation of nAChRs causes PKC activation associated with cell proliferation (Jull et al. 2001). Another PKC isoform, PKCδ, seems to act atypically in NSCLC cells. Activation of PKCδ in NSCLC promotes cell survival and resistance to chemotherapeutic agents (Clark et al. 2003), and nicotine can prevent chemotherapy from inhibiting PKC (Heusch and Maneckjee 1998). Like AKT, nicotinic activation of PKC has ramifications for smokers by contributing to chemotherapeutic resistance. This finding is consistent with the finding that patients with lung cancer who continue to smoke during chemotherapy have a worse prognosis than those who stop smoking.

A clinical study also demonstrates the importance of PKC in tobacco-related cancers. Lahn and colleagues (2004) found that PKCα is overexpressed in a subset of NSCLC. Collectively, the results suggest that the activation of prosurvival PKC isoforms by cigarette smoke is an important mechanism of cell proliferation mediated by nAChRs and β-adrenergic receptors in carcinogenesis.

Protein Kinase A

Another cytoplasmic kinase activated by components of tobacco smoke is PKA. Under normal physiological conditions, PKA is stimulated through the production of cyclic adenosine monophosphate by activated G protein–coupled receptors. Nicotinic activation of PKA occurs through both nAChRs and β-adrenergic receptors (Dajas-Bailador et al. 2002; Jin et al. 2004a). The primary effect of nAChR-mediated PKA activation was an increase in cell proliferation. Nicotinic activation of PKA through β-adrenergic receptors, however, promoted cell survival. Although the data on PKA are limited, they suggest that PKA might be an important mediator of signal transduction, mediating cell survival and proliferation in response to activation by nAChRs and β-adrenergic receptors.

Downstream Targets of Signaling Cascades Mediated by Tobacco Smoke

Activation of cell-surface receptors induced by components of tobacco smoke and the subsequent activation of cytoplasmic kinases stimulate other proteins that dictate cellular responses, such as cell survival and proliferation. Although activated kinases have many downstream targets, the two most studied are the transcription factor NF-κB and proteins in the BCL-2 family. Activation of these proteins by tobacco smoke components through signaling cascades promotes processes involved in initiation, progression, and maintenance of cancers (see "Signal Transduction" earlier in this chapter).

Gene Promoter Hypermethylation in Cancer Induced by Tobacco Smoke

Alternative to Mutation

Gene promoter hypermethylation is an epigenetic change of a gene involving extensive methylation at the 5′ position of C in CpG islands within the promoter region and often extending into exon 1 of regulatory genes (Jones and Baylin 2002; Herman and Baylin 2003). "Epigenetic" refers to alteration in gene expression resulting from changes other than DNA sequence. The end result of this process can be loss of gene transcription and therefore the silencing of gene function.

Inactivation of the *P16* Gene in Lung Cancer

One region on chromosome 9p contains the *CDKN2A* (*P16*) tumor-suppressor gene (Kamb et al. 1994; Merlo et al. 1994). Mutations within the *P16* coding sequence are uncommon in lung cancer (Kamb et al. 1994). In contrast, this gene is inactivated by hypermethylation at prevalences up to 60 percent and 70 percent in adenocarcinomas and SCC of the lung, respectively (Merlo et al. 1995; Belinsky et al. 1998; Kim et al. 2001; Zöchbaur-Müller et al. 2001; Divine et al. 2005). This discovery of inactivation of a tumor-suppressor gene by hypermethylation in lung cancer and identification of such inactivation of the *P16* gene launched an area of research to uncover other genes inactivated by this mechanism. The targeting of *P16* for inactivation is likely attributable to the critical function of this gene in the cell, which is to inhibit CDKs that bind cyclin D1 and phosphorylate the *RB* gene product (Lukas et al. 1995; Weinberg 1995). This regulation is lost if either the *P16* or the *RB* gene is inactivated. The reciprocal relationship between *RB* alterations in SCLC and *P16* alterations in NSCLC supports the premise that dysfunction within the *RB* pathway is a major target in research on the genesis of lung cancer (Swafford et al. 1997).

Critical Pathways Inactivated in Non-Small-Cell and Small-Cell Lung Cancer

More than 50 genes are inactivated by gene promoter hypermethylation in lung cancer, and new genes are still being identified through genomewide screening approaches (Suzuki et al. 2002; Palmisano et al. 2003). The pathways and genes involved are summarized in Table 5.11.

Of particular importance is the DNA repair gene *AGT*, which protects cells from the carcinogenic effects of alkylating agents by removing adducts from the O^6 position of deoxyguanosine (see "Repair of DNA Adducts" earlier in this chapter). Failure to repair this DNA adduct could lead to mutations in genes such as *KRAS* and *TP53*. *AGT* is inactivated by gene promoter methylation in 24 to 48 percent of adenocarcinomas (Esteller et al. 1999; Zöchbaur-Müller et al. 2001; Pulling et al. 2003). SCLC studies conducted for methylation of this gene are limited. Studies have reported an association between *AGT* promoter hypermethylation and a G→A transition mutation at CpG sites within the *TP53* gene in NSCLC (Wolf et al. 2001). In contrast, no association was found between *AGT* gene methylation and a transition mutation in codon 12 of the *KRAS* gene from adenocarcinomas (Pulling et al. 2003).

The RAS superfamily of GTP-binding proteins plays an important role in signal transduction pathways that control cell proliferation, differentiation, and death (Campbell et al. 1998; Downward 2001) (see "Activation of Oncogenes in Lung Cancer" earlier in this chapter). Researchers identified a new family of genes that encode RAS-binding proteins. One of these genes, *RASSF1A*, is located at chromosome 3p21 and inactivated in 30 percent of NSCLCs and in 100 percent of SCLCs (Dammann et al. 2000; Burbee et al. 2001). Attempts to determine the function of this gene are continuing (Agathanggelou et al. 2003). RASSF1A protein forms a heterodimer with NORE1A, which allows it to bind with the proapoptotic protein MST1 (Khokhlatchev et al. 2002). Binding RAS to this complex may mediate RAS-dependent apoptosis. *NORE1A* is also silenced by methylation in NSCLC but not in SCLC (Hesson et al. 2003). Therefore, silencing either *RASSF1A* or *NORE1A* could effectively block apoptosis mediated by RAS activation. Two other RASSF

Table 5.11 Pathways altered through gene silencing by promoter methylation

		Methylation prevalence (%)	
Pathway	**Gene**	**Non-small-cell lung cancer**	**Small-cell lung cancer**
Cell cycle	*P16*	26–70	0
	PAX5α	64–74	ND
	PAX5β	52–61	ND
	CHFR	10–19	ND
DNA repair	*AGT*	27–47	0–19
Apoptosis	*DAPK*	24–48	33
	CASPASE-8	0	35–52
	FAS	ND	40
	TRAIL-R1	ND	40
	FHIT	38–45	ND
RAS signaling	*RASSF1A*	30	100
	RASSF4	20	20
	NORE1A	24	0
Invasion	*E-CADHERIN*	16–19	ND
	H-CADHERIN	43	ND
	TIMP3	19–24	ND
	LAMA3	27–58	65
	LAMB3	20–32	77
	LAMC2	13–32	58
	MYO18B	31	45

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Note: **ND** = not determined.

family members appear to be involved in lung cancer development. Studies show that RASSF2 binds directly to the *KRAS* gene in a GTP-dependent manner and appears to promote both cell-cycle arrest and apoptosis through this interaction (Vos et al. 2003). The expression of this gene is markedly reduced in some lung cancer cell lines, thus suggesting silencing by gene promoter hypermethylation. There is support for this mechanism of inactivation in studies on colon cancer that document hypermethylation of this gene in 70 percent of tumors (Hesson et al. 2005). A third member of this gene family, *RASSF4*, shares 25 percent homology with *RASSF1A* and 40 percent with *RASSF2* and is methylated in approximately 20 percent of NSCLCs and SCLCs (Eckfeld et al. 2004). Thus, the loss of function among members of the *RASSF* gene family is important to the development of lung cancer.

Gene Silencing in Lung Cancer

The most extensively studied gene with respect to timing of methylation in NSCLC is *P16*. Examination of biopsy specimens from premalignant lesions obtained from people without SCLC or from different airways or at different bronchial generations revealed a progressive increase in the prevalence of *P16* methylation as the disease developed. The frequency of *P16* methylation was 17 percent in basal cell hyperplasia, and the frequency increased incrementally over the histologic stages to 60 percent in SCCs (Belinsky et al. 1998). Further studies examined bronchial epithelial cells obtained by bronchoscopy from cancer-free smokers and found that inactivation of the *P16* gene is likely one of the earliest events in lung cancer (Belinsky et al. 2002). Researchers detected *P16* methylation in specimens from 25 of 137 biopsy procedures (18 percent) classified as histologically normal, metaplasia, or mild dysplasia. In contrast, no *P16* methylation was found in biopsy specimens obtained from lifetime nonsmokers. Researchers used an animal model to determine the timing of *P16* methylation in adenocarcinomas. In rats, 94 percent of adenocarcinomas induced by NNK were hypermethylated at the *P16* promoter; this change was frequently detected in precursor lesions to the tumors: adenomas and hyperplastic lesions (Belinsky et al. 1998).

Inactivation of the *AGT* gene appears to be a later event in lung cancer than is the inactivation of *P16*. Only 3 of 40 biopsy specimens (8 percent) from heavy smokers with histologies including normal, hyperplasia, metaplasia, and dysplasia showed methylation of the *AGT* gene (Pulling et al. 2003). In addition, the prevalence of *AGT* methylation increased between stage I adenocarcinoma and stages II to IV. Finally, of the 137 bronchial biopsy specimens studied, 3 percent of the *DAPK* gene and none of the *RASSF1A* genes showed methylation, which suggests that the silencing of these genes likely occurs after *P16* inactivation in SCC (Pulling et al. 2003). In contrast, the inactivation of *DAPK* by methylation in alveolar hyperplasias in a murine model of lung adenocarcinomas suggests a role for this gene in the early development of adenocarcinomas (Pulling et al. 2004).

Gene Promoter Hypermethylation, Prognosis, and Clinical Risk Factors

Numerous studies have evaluated relationships between gene promoter methylation and established clinical risk factors such as smoking dose and tumor stage. In addition, researchers have examined in detail the effect of gene-specific methylation on the survival of patients

with a diagnosis of early-stage lung cancer. Results from investigations of the most commonly studied genes in lung cancer are highlighted here.

P16 methylation was significantly associated with pack-years of smoking and with an independent risk factor that predicts a shorter survival for patients who had resection of a stage I adenocarcinoma (Kim et al. 2001). Several other studies also support *P16* methylation as a prognostic factor for survival of patients who had resection of a stage I adenocarcinoma (Suzuki et al. 2002; Wang et al. 2004a). In contrast, *RASSF1A* methylation in stage III NSCLC was a stronger predictor of poor survival than was *P16* methylation (Wang et al. 2004a). These two genes may differ in that the silencing of *P16* is an early event involved in initiation of tumorigenesis, whereas *RASSF1A* methylation is a later event more likely involved in progression of tumorigenesis. Thus, the methylation of *RASSF1A* may lead to a more aggressive tumor phenotype. This hypothesis is supported by the more frequent involvement of *RASSF1A* methylation in tumors with a vascular invasion, pleural involvement, and a poorly differentiated histology (Tomizawa et al. 2002). Persons who started smoking before 19 years of age were 4.2 times more likely to have methylation of the *RASSF1A* gene than were those who started smoking after 19 years of age (Kim et al. 2003). This research also suggests that for patients with stage I or stage II NSCLC at diagnosis, methylation of this gene is associated with a poorer prognosis (Kim et al. 2003).

Other Tobacco-Related Cancers

In addition to the studies on lung cancer described here, other studies have shown association of cigarette smoking with gene promoter hypermethylation in other tobacco-related cancers, such as the head and neck and bladder. Aberrant promoter methylation is common in head and neck cancer and has been detected by using saliva samples (Rosas et al. 2001). Promoter methylation of the *P16*, *DAPK*, *E-CADHERIN*, and *RASSF1A* genes was associated with smoking and commonly found in head and neck cancer (Hasegawa et al. 2002). *P16* promoter hypermethylation and the loss of P16 protein expression were detected in head and neck SCC; loss of expression correlated significantly with a history of alcohol consumption or tobacco use (Ai et al. 2003). The prevalence of *P15* methylation in the healthy epithelium of patients with head and neck SCC who had long-term smoking and drinking behaviors was significantly higher than that in nonsmokers (Wong et al. 2003). Another study suggested that *P15* gene methylation could be induced by chronic smoking and drinking and could play a role in the early stages of head and neck SCC (Chang et al. 2004). Cigarette smoking was also associated with an increased risk of promoter methylation of the *P16* gene in bladder cancer (Marsit et al. 2006).

Molecular Epidemiology of Cell-Cycle Control and Tobacco-Induced Cancer

Introduction

Cell-cycle checkpoints delay cell-cycle progression, thereby affording adequate time for DNA repair to occur. Such checkpoint signaling also activates pathways leading to apoptosis if the damage cannot be repaired. The introduction of new techniques of profiling gene expression has enabled researchers to comprehensively evaluate activity of proteins in regulating the cell cycle (Singhal et al. 2003). A hallmark of the neoplastic cell is the ability to disrupt the tightly regulated cell-cycle control and enable the cell to bypass checkpoints, especially at the G_1/S and G_2/M boundaries (Hanahan and Weinberg 2000). Persons with defects in cell-cycle checkpoints (acquired or inherited) could therefore exhibit chromosome damage, genomic instability, and increased susceptibility to tobacco carcinogenesis.

In vitro studies show an association between exposure to tobacco carcinogens and the disruption of cell-cycle control (Khan et al. 1999). Furthermore, Jin and colleagues (2004b) provide data showing that NNK promotes cell survival and proliferation through phosphorylation of the proteins BCL-2 and C-MYC. Studies implicate tobacco carcinogens in genetic alterations in the P16-RB and $P14^{ARF}$-P53 pathways, mainly through the formation of DNA adducts. Variations in cell-cycle checkpoints might also be attributed to functional polymorphisms in cell-cycle control genes. The SNP500Cancer Database reports that 27 genes related to the cell cycle are polymorphic, and these include genes that control checkpoints for both the G_1/S and G_2 phases of the cell cycle. However, only a few genes, including *CCND1*, *TP53*, *P21*, and *P73*, have been studied in tobacco-related cancers.

CCND1 Gene

The *CCND1* gene, together with *CDK4*, *P16*, and the tumor-suppressor gene *RB*, comprise a linked system governing the passage of the cell through the cell cycle (Betticher et al. 1997). A common finding in a variety of cancers is the amplification or overexpression of *CCND1*, which contributes to tumor initiation, progression,

and outcome, such as death. A G→A polymorphism at codon 242 in the conserved splice donor region of exon 4 increases alternate splicing (Betticher et al. 1995). The alternate transcript appears to encode for a protein-missing sequence involved in protein turnover, and therefore, the encoded protein may have a longer half-life. This extended half-life, in turn, would facilitate passage of damaged cells through the checkpoint for the G_1/S phase and promote proliferation rather than apoptosis. Researchers have studied the association between the *CCND1* genotype and cancer risk in several tobacco-related cancers. Qiuling and colleagues (2003) reported that the *CCND1 *A/*A* genotype was associated with a significantly increased risk of lung cancer (OR = 1.87; 95 percent CI, 1.01–3.45) compared with that for the **G/*G* genotype. The risk was even higher in young persons and men. A similar finding was reported in cancer of the head and neck (Zheng et al. 2001). These investigators demonstrated that carriers of the **A/*A* genotype, on average, had diagnoses of cancer 3.5 years earlier than did carriers of the**G/*G* genotype. Wang and colleagues (2002) reported that the **A/*A* genotype was associated with a significantly higher risk of transitional cell carcinoma of the bladder than that for the **A/*G* plus **G/*G* genotypes (OR = 1.76; 95 percent CI, 1.09–2.84). However, neither Cortessis and colleagues (2003) nor Yu and colleagues (2003) reported significant associations with either bladder cancer or esophageal SCC, respectively. Spitz and colleagues (2005) demonstrated an increased risk for lung cancer associated with this polymorphism. The risk estimate was 1.35 (95 percent CI, 1.05–1.73) for the **A/*A* and **A/*G* genotypes compared with the **G/*G* genotypes.

P21 Protein

Cell-cycle inhibitor protein P21 (WAF1/CIP1) acts as a checkpoint regulator for the G_1/S and G_2/M phases. Marwick and colleagues (2002) showed a significant increase in P21 mRNA expression in alveolar epithelial cells after exposure to condensate from cigarette smoke and concluded that oxidative stress induced by cigarette smoke modulates the expression of P21.

Three studies of lung cancer have examined the association of cancer risk with a polymorphism of *P21* at codon 31 (*SER31ARG*), but the findings were inconsistent. Själander and colleagues (1996) reported an increased frequency of the variant allele (**ARG*) among patients with lung cancer ($p < 0.004$). Two other studies failed to replicate this finding (Shih et al. 2000; Su et al. 2003). However, Chen and colleagues (2002) reported that the variant allele (**ARG*) was associated with increased risk of bladder cancer.

TP53 Gene

Studies have reported 14 polymorphisms in the *TP53* gene, 3 of which have been widely studied: a G→C polymorphism at codon 72 (proline/arginine), a 16bp insertion in intron 3, and a G→A transition in intron 6. Polymorphisms in codons 21, 36, and 213 are silent. The polymorphism in codon 47 involves a rare allele with a frequency less than 5 percent. The codon 72 polymorphism on exon 4 produces variant proteins with an arginine (CGC) or proline (CCC) at the site. Thomas and colleagues (1999) reported differences between the two variants in their ability to interact with basic elements of the transcriptional machinery and to induce apoptosis. Weston and colleagues (1992) reported an increased frequency of the proline allele in lung adenocarcinoma, which was consistent with findings in a Japanese study of lung cancer (Kawajiri et al. 1993). Jin and colleagues (1995) reported significantly higher risks for the **PRO/*PRO* genotype among patients with lung cancer who were younger than 55 years of age and among patients reporting fewer than 30 pack-years of smoking. In a study of NSCLC, Nelson and coworkers (2005) found that mutation on the **PRO* allele was associated with a significantly worse outcome than that for patients with no mutation or with mutation on the **ARG* allele.

Mutations in intron sequences may initiate aberrant pre-mRNA splicing that results in a defective protein (Hillebrandt et al. 1997) or that may influence mutations in the coding region. Either result would increase the likelihood of a deleterious phenotype (Malkinson and You 1994). Biroš and colleagues (2001) reported a higher percentage of the intron 6 variant allele in patients with lung cancer than in control participants. However, Birgander and colleagues (1995) found no association of the allele with lung cancer. Several studies estimated the pairwise haplotype frequencies for the polymorphisms in exon 4 and introns 3 and 6. The researchers proposed that the *P53* haplotypes were associated with a higher risk for lung cancer (Birgander et al. 1995; Biroš et al. 2001). In one study of 635 pairs of lung cancer patients and control participants, variant alleles of *TP53* exon 4, introns 3 and 6, and their variant haplotypes were associated with an increased risk of lung cancer (Wu et al. 2002). In a meta-analysis of *TP53* polymorphisms and lung cancer risk that included data from 16 case-control studies, Matakidou and colleagues (2003) concluded that persons with the *P53* exon 4 **PRO/*PRO* genotype had a 1.18-fold increase in lung cancer risk (OR = 1.18; 95 percent CI, 0.99–1.41). Other researchers have observed a similar association with polymorphisms of *P53* introns 3 and 6. However, evidence of these associations has not been consistent (Wang et al. 1999; Mabrouk et al. 2003).

P73 Gene

The *P73* gene activates the promoters of several genes that are responsive to the *TP53* gene and participate in cell-cycle control, DNA repair, and apoptosis and inhibit cell growth in a P53-like manner by inducing apoptosis or cell-cycle arrest in the G_1 phase (Nomoto et al. 1998; Cai et al. 2000). Loss of heterozygosity at the *P73* locus is relatively common. Studies have identified an estimated 17 polymorphisms. Two common SNPs at positions 4 (G→A) and 14 (C→T) in the uncoding region of exon 2 of the *P73* gene are in complete linkage disequilibrium and may affect *P73* function by altering the efficiency of translation initiation (Kaghad et al. 1997).

Studies have reported the role of the *P73* G4C14→A4T14 polymorphism in the risk of smoking-related cancer (Ryan et al. 2001; Hamajima et al. 2002; Hiraki et al. 2003; Huang et al. 2003). In NSCLC, the most significant effect observed was among male smokers (OR = 1.87; 95 percent CI, 1.25–2.80) with SCLC, suggesting that this *P73* polymorphism may have an impact on the repair of tobacco-associated DNA damage. A study of 1,054 patients with lung cancer and 1,139 control participants found a dose-response relationship between the frequency of heterozygous or homozygous variant alleles and risk of lung cancer (trend test, $p < 0.001$). ORs were 1.32 (95 percent CI, 1.10–1.59) for the frequency of heterozygous alleles and 1.54 (95 percent CI, 1.05–2.26) for the frequency of homozygous alleles (Li et al. 2004b). The risk of lung cancer was more pronounced in persons younger than 50 years of age, men, light smokers, and patients with SCLC. The variant genotypes were also associated with an increased risk for SCCs of the head and neck that was statistically significant (OR = 1.33) and an even higher risk among current smokers (OR = 1.77) (Li et al. 2004a).

Other Aspects

Carcinogenic Effects of Whole Mixture and Fractions of Tobacco Smoke

Researchers have conducted inhalation studies of cigarette smoke in hamsters, rats, mice, rabbits, dogs, and nonhuman primates. The model systems used in these studies had various problems, including the inability of any study to accurately duplicate human smoking behaviors. Nevertheless, comprehensive reviews of these studies have found a large amount of useful information (IARC 1986, 2004; Coggins 1998; Witschi 2000). Researchers observed the most consistent results on cancer induction in Syrian golden hamsters; whole cigarette smoke and its particulate phase induced malignant tumors and other lesions in the larynx. Tumors were not induced by the gas phase of cigarette smoke.

Findings of studies that induced malignant tumors by inhalation of cigarette smoke and its particulate phase are consistent with those in the substantial amount of literature demonstrating that condensate from cigarette smoke, which lacks volatile constituents of the gas phase, causes benign and malignant tumors when applied to mouse skin and rabbit ears or instilled in rat lungs by intrapulmonary administration (Hoffmann et al. 1978; IARC 1986, 2004). Collectively, these results clearly show that major carcinogenic fractions of cigarette smoke reside in the particulate phase.

Extensive fractionation studies were conducted with cigarette smoke condensate (Hoffmann et al. 1978). Bioassays of the resulting fractions applied to the skin of mice demonstrated that the neutral portion of the condensate has carcinogenic activity and the acidic portion has tumor-promoting and cocarcinogenic activity. The recombined neutral and acidic portions accounted for about 80 percent of the carcinogenic activity of the condensate. Subfractionation of the neutral portion revealed that certain PAHs were the major tumor initiators in this fraction. However, these PAHs alone, in the levels at which they occur, were insufficient to induce tumors. Moreover, when these PAHs were added to the condensate, the tumor yield was higher than with the condensate alone. These results indicate that the combination of PAHs acting as tumor initiators, together with cocarcinogens in the condensate, accounted for the tumorigenicity of the condensate on mouse skin. Researchers identified catechol and alkyl catechols as major cocarcinogens in the condensate, and the weakly acidic portion of the condensate demonstrated tumor-promoting activity (Van Duuren and Goldschmidt 1976; Hecht et al. 1981). Other cocarcinogens in cigarette smoke include undecane, pyrene, fluoranthene, and B[*a*]P (Van Duuren and Goldschmidt 1976). The identity of tumor promoters in cigarette smoke is largely unknown, although simple phenols may contribute weakly (Hecht et al. 1975). Researchers have also observed tumor-promoting activity of cigarette smoke in inhalation experiments with hamsters (IARC 1986). PAH-enriched

fractions of cigarette smoke condensate instilled in the rat lung also resulted in tumor formation (IARC 1986).

These results clearly demonstrate the carcinogenic, tumor-promoting, and cocarcinogenic activity of the particulate phase of cigarette smoke. However, some data indicate that constituents of the gas phase also contribute to tumor induction. Early studies in Snell's mice demonstrated an increase in pulmonary adenocarcinomas in animals exposed to the gas phase alone (IARC 2004). In an exposure model using 89 percent sidestream smoke and 11 percent mainstream smoke, increased multiplicity of lung adenomas was consistently observed in A/J mice exposed to the smoke for five months, followed by a four-month resting period. Tumor response in this model was clearly attributable to the gas phase, because filtration had no effect on multiplicity of lung adenomas (Witschi 2000; IARC 2004). The results of these studies indicate that a volatile carcinogen in cigarette smoke—possibly 1,3-butadiene—produced a tumorigenic response in the A/J mouse lung.

Two other studies not included in the reviews previously cited here demonstrate convincingly that cigarette smoke administered to rats or mice by whole-body exposure for extended periods induces benign and malignant tumors of the respiratory tract (Mauderly et al. 2004; Hutt et al. 2005). When male and female F-344 rats were exposed to smoke from 1R3 research cigarettes or to clean air for six hours per day, five days per week for up to 30 months, the exposure significantly increased the incidence of nonneoplastic and neoplastic proliferative lung lesions in females. The combined incidence of bronchoalveolar adenomas and carcinomas was 14 percent in the high-exposure group (250 mg of particulates per m^3 of air), 6 percent in the low-exposure group (100 mg/m^3), and none in the controls. Mutations in codon 12 of the *KRAS* gene occurred in 4 of 23 tumors. Both males and females had significant increases in neoplasia of the nasal cavity (Mauderly et al. 2004). Female B6C3F1 mice were exposed to smoke for 6 hours per day, 5 days per week, for 925 days (250 mg/m^3) or were sham exposed. The incidence of lung adenoma (28 percent) and lung adenocarcinoma (20 percent) in the mice exposed to smoke were significant (Hutt et al. 2005).

Synergistic Interactions in Tobacco Carcinogenesis

Alcohol

There is persuasive epidemiologic evidence that alcohol consumption and smoking synergistically increase the risk for cancers of the oral cavity, pharynx, larynx, and esophagus (IARC 2004). No single mechanism clearly explains these observations, but several have been proposed and there is reasonable support for some. The most consistent body of evidence relates to the effects of alcohol on the distribution of carcinogenic nitrosamines. Swann and colleagues (1984) demonstrated that alcohol could inhibit the hepatic metabolism and clearance of NDMA, a carcinogen in tobacco smoke. This inhibition occurs because ethanol competitively inhibits hepatic cytochrome P-450 2E1, the main hepatic enzyme responsible for metabolism of NDMA. Consequently, more NDMA reaches extrahepatic tissues where it can be metabolically activated and has the potential to cause cancer. Anderson and coworkers (1992, 1996) demonstrated that coadministration of ethanol and NDMA to A/J mice resulted in an incidence of lung tumors higher than that in mice treated with NDMA alone and that this increase was a consequence of inhibition of hepatic metabolism and not of tumor promotion. Furthermore, administration of ethanol to patas monkeys before they received NDMA resulted in a 14.6-fold increase in O^6-methylguanine in esophageal DNA and other extrahepatic tissues (Anderson et al. 1996).

Another potential mechanism also involves the effects of ethanol on P-450 2E1, but as an inducer of this enzyme. Chronic ethanol consumption is known to induce production of hepatic P-450 2E1, and researchers hypothesized that this induction could lead to increased metabolic activation of carcinogens in tobacco smoke (McCoy et al. 1979). Some *N*-nitrosamines in tobacco smoke—NDMA, *N*-nitrosodiethylamine, and *N*-nitrosopyrrolidine—are all substrates for P-450 2E1. McCoy and colleagues (1981) demonstrated that chronic ethanol consumption in hamsters increased the metabolism of *N*-nitrosopyrrolidine in hepatic and target tissue (e.g., trachea), as well as the carcinogenicity of this nitrosamine, which increases the occurrence of tumors in the nasal cavity and trachea. The carcinogenicity of *N*-nitrosodiethylamine in the rat esophagus was also increased by simultaneous administration of ethanol (Gibel 1967). Overall, however, the effects of ethanol consumption on *N*-nitrosamine carcinogenesis have been mixed, and they appear to depend on the *N*-nitrosamine studied and the protocol used. For example, long-term ethanol consumption had no effect on the carcinogenicity of NNN in the hamster and only modest or no effect in the rat (McCoy et al. 1981; Trushin et al. 1984).

Other mechanisms for the enhancing effect of alcohol consumption on tobacco carcinogenesis have been discussed (Pöschl and Seitz 2004). Persons who abuse alcohol generally have nutritional deficiencies, which could exacerbate the effects of smoking. They commonly have folate deficiency that could contribute to an inhibition of transmethylation, which is important in gene regulation. Zinc deficiency is known to result in enhanced

carcinogenesis in the rat esophagus (Fong et al. 2001). Reduced serum and hepatic levels of vitamin A in persons with long-term alcohol abuse may affect carcinogenesis. Alcohol could also act as a solvent, increasing absorption of tobacco carcinogens (Squier et al. 1986).

Asbestos

Smoking and exposure to asbestos interact synergistically to increase the risk for lung cancer (IARC 2004). The mechanism for this synergy is unknown. Researchers have investigated a number of possibilities, however, and these have been summarized (Nelson and Kelsey 2002). It has been proposed that asbestos fibers serve as a vehicle to deliver tobacco carcinogens to the cell nucleus. Surfactant phospholipids may help to solubilize carcinogenic PAH, increasing their concentrations in the lung epithelium. Studies have also demonstrated that asbestos fibers can induce chromosomal aberrations and extensive deletions, potentially adding to the DNA damage produced by carcinogens in tobacco smoke. Furthermore, asbestos may cause oxidative damage that could be related to inflammation and cell death related to pulmonary fibrosis associated with exposure to asbestos. It seems likely that asbestos fibers could cause proliferation that may increase the probability of mutations attributable to DNA damage by tobacco smoke carcinogens.

Carcinogens as Causes of Specific Cancers

Data from carcinogenicity studies, product analyses, and findings from studies using biochemistry and molecular biology support a significant role for certain carcinogens in tobacco-induced cancer (Table 5.12).

Considerable evidence favors PAHs and NNK as major factors in development of lung cancer. PAHs are strong carcinogens acting locally; thus, fractions of tobacco smoke enriched in these compounds are carcinogenic (Hoffmann et al. 1978; Deutsch-Wenzel et al. 1983; IARC 1983) (see "Carcinogens in Cigarette Smoke" earlier in this chapter). Researchers have detected PAH-DNA adducts in human lungs, and the spectrum of mutations in the *TP53* gene isolated from lung tumors was similar to the pattern of DNA damage produced in vitro by PAH diol epoxide metabolites and in cell cultures by B[*a*]P (Pfeifer et al. 2002; Phillips 2002; Boysen and Hecht 2003; Liu et al. 2005) (see "DNA Adducts and Biomarkers" earlier in this chapter).

NNK is a strong systemic carcinogen in lungs of rodents that induces lung tumors independent of the route of administration (Hecht 1998). NNK was found to be particularly potent in the rat. Significant incidence of lung tumors was induced by total doses as low as 6 mg/kilogram (kg) of body weight or by 1.8 mg/kg as part of a dose-response trend. These doses are comparable to an estimated NNK dose of 1.1 mg/kg in persons who have smoked for 40 years (Hecht 1998). DNA adducts derived from NNK or from the related tobacco-specific nitrosamine, NNN, are present in lung tissue from smokers, and metabolites of NNK are found in the urine of smokers (Hecht 2002b). Epidemiologic data indicate that a systemic carcinogen causes lung cancer in cigar smokers who do not inhale the smoke; this finding is consistent with the tumorigenic properties of NNK (Boffetta et al. 1999; Shapiro et al. 2000).

The changing histology of lung cancer is also consistent with the role of NNK: adenocarcinoma has now overtaken SCC as the most common lung cancer type. This nitrosamine in tobacco smoke produces primarily adenocarcinomas in rodents. However, this outcome has also been attributed to differing inhalation patterns of current cigarette smokers (Travis et al. 1995; Hecht 1998). As nitrate concentrations in tobacco increased from 1959 to 1997, NNK concentrations in mainstream smoke increased and those of B[*a*]P decreased. Researchers attributed these changes to tobacco blends with higher levels of air-cured tobacco, the use of reconstituted tobacco, and other factors (Hoffmann et al. 2001). Other compounds that could be involved in lung cancer include 1,3-butadiene, isoprene, ethylene oxide, ethyl carbamate, aldehydes, benzene, metals, and oxidants, but the collective evidence for each of these substances is not as strong as the evidence for PAHs and NNK (Hecht 1999).

The particulate phase of cigarette smoke causes tumors of the larynx in hamsters, which could be attributed to PAHs (IARC 1986). *TP53* gene mutations identified in tumors of the human larynx support a role for PAHs in the development of this cancer (Pfeifer et al. 2002). *N*-nitrosamines, as well as acetaldehyde and formaldehyde, induce nasal tumors in rodents and are likely candidates for causing nasal tumors associated with smoking (Preussmann and Stewart 1984; IARC 1995c, 1999). On the basis of animal studies, PAH, NNK, and NNN are the most likely causes of oral cancer in smokers (Hoffmann and Hecht 1990). *N*-nitrosamines are the most effective esophageal carcinogens known. NNN causes tumors of the esophagus in rats and is the most prevalent *N*-nitrosamine carcinogen in cigarette smoke (Hecht and Hoffmann 1989; Lijinsky 1992).

NNK and several other *N*-nitrosamines and furan in cigarette smoke are effective hepatocarcinogens in rats (Preussmann and Stewart 1984; IARC 1995b). NNK and its major metabolite NNAL are the only known pancreatic carcinogens in tobacco products. Biochemical data from

Table 5.12 Carcinogens and tobacco-induced cancers

Study	Cancer type	Likely carcinogen involvement[a]
Hoffmann and Hecht 1990 Hecht et al. 1994 Törnqvist and Ehrenberg 1994 Hecht 1999 Hoffmann et al. 2001 Pfeifer et al. 2002	Lung	PAHs, NNK (major), 1,3-butadiene, isoprene, ethylene oxide, ethyl carbamate, aldehydes, benzene, metals
IARC 1986 Hoffmann et al. 2001 Pfeifer et al. 2002	Larynx	PAHs
Preussmann and Stewart 1984 IARC 1995c, 1999 Hecht 1998	Nasal	NNK, NNN, other nitrosamines, aldehydes
Hecht et al. 1986 Hoffmann et al. 1987, 1995, 2001 Hecht and Hoffmann 1988, 1989 Hoffmann and Hecht 1990 Hecht 1998 Vainio and Weiderpass 2003	Oral cavity	PAHs, NNK, NNN
Hecht and Hoffmann 1989 Lijinsky 1992 Hecht 1998 Hoffmann et al. 2001	Esophagus	NNN, other nitrosamines
Preussmann and Stewart 1984 IARC 1995a Hecht 1998	Liver	NNK, other nitrosamines, furan
Rivenson et al. 1988 Hecht 1998 Hoffmann et al. 2001 Prokopczyk et al. 2002	Pancreas	NNK, NNAL
Melikian et al. 1999 Prokopczyk et al. 2001 Phillips 2002	Cervix	PAHs, NNK
IARC 1974 Hoffmann and Hecht 1990 Skipper and Tannenbaum 1990 Skipper et al. 1994 Landi et al. 1996 Probst-Hensch et al. 2000 Hoffmann et al. 2001	Bladder	4-aminobiphenyl, other aromatic amines
IARC 1982	Leukemia	Benzene

Source: Adapted from Hecht 2003 with permission.
Note: **IARC** = International Agency for Research on Cancer; **NNAL** = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; **NNK** = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; **NNN** = *N′*-nitrosonornicotine; **PAHs** = polycyclic aromatic hydrocarbons.
[a]Based on carcinogenicity studies in laboratory animals, biochemical evidence from human tissues and fluids, and epidemiologic data when available.

studies of human tissue provide some support for the role of these carcinogens in smoking-related pancreatic cancer, although the studies did not detect DNA adducts (Rivenson et al. 1988; Prokopczyk et al. 2002, 2005). Biochemical studies demonstrate that both NNK and PAHs can reach the cervix in humans and are metabolically activated in these tissues (Melikian et al. 1999; Prokopczyk et al. 2001). Researchers have detected DNA adducts derived from B[*a*]P and other hydrophobic compounds in cervical tissue from smokers (Melikian et al. 1999; Phillips 2002). Therefore, in combination with the human papilloma virus, these compounds may contribute to development of cervical cancer in smokers (IARC 1995a). 4-ABP and 2-naphthylamine are known human bladder carcinogens, and considerable data from human studies support the role of aromatic amines as the major cause of bladder cancer in smokers (IARC 1974; Skipper and Tannenbaum 1990; Skipper et al. 1994; Landi et al. 1996; Probst-Hensch et al. 2000; Castelao et al. 2001). The most probable cause of leukemia in smokers is exposure to benzene, which occurs in large quantities in cigarette smoke and is a known cause of acute myelogenous leukemia in humans (IARC 1982).

Cigarette smoke causes oxidative damage probably because it contains free radicals, such as nitric oxide, and contains mixtures of hydroquinones, semiquinones, and quinones that can induce reduction and oxidation (redox cycling) (Pryor et al. 1998; Hecht 1999). Smokers have lower levels of ascorbic acid in plasma and, sometimes, higher levels of oxidized DNA bases in white blood cells than do nonsmokers. However, the role of oxidative damage as a cause of specific tobacco-induced cancers remains unclear (Hecht 1999).

Tobacco Carcinogens, Immune System, and Cancer

Cigarette smoke alters a range of immunological functions including innate and adaptive immune responses (Sopori 2002). These effects, acting as tumor-promoting or cocarcinogenic stimuli, could affect tobacco-related carcinogenesis. Cigarette smoking increases the number of alveolar macrophages in the lung, possibly leading to higher levels of oxygen radicals and MPO activity, which are hypothesized to be important in tumor promotion. Investigators examined the effects of smoking on the function of natural killer (NK) cells—a lymphoid cell type involved in surveillance of tumor growth (Lu et al. 2007). They obtained strong evidence that suppression of NK cell activation was related to increased lung metastases in mice exposed to cigarette smoke. Other studies demonstrated that nicotine is immunosuppressive and thus might be responsible for some of the effects of cigarette smoke (Sopori 2002).

Epidemiology of Family History and Lung Cancer

Studies of familial aggregation of lung cancer provide indirect evidence supporting the possibility of an inherited component to tobacco carcinogenesis. A number of published studies showed that significantly more lung cancers were reported in first-degree relatives of probands with lung cancer than were reported in first-degree relatives of healthy control participants. Assuming that the family structure does not differ between cases and controls, this pattern could be explained by shared genes among the family members, shared smoking patterns, or a combination of both factors. By incorporating smoking histories of the probands and the first-degree relatives into a study of familial aggregation, researchers can begin to assess the level of familial risk of cancer while adjusting for tobacco use. However, few studies of familial aggregation incorporate history of involuntary exposure of family members to tobacco smoke. Estimates of the overall proportion of patients with lung cancer who have family history of lung cancer in a first-degree relative range from 6 (Li and Hemminki 2004) to 16 percent (Sellers et al. 1992).

Forty years ago, Tokuhata and Lilienfeld (1963) observed that the number of deaths from lung cancer was higher among relatives of lung cancer case patients than it was among relatives of control participants. These researchers also reported a fourfold excess of lung cancer mortality in nonsmoking relatives of 270 lung cancer probands. The effect among relatives who smoked was less pronounced (twofold). These findings suggest that the risk was not solely attributable to shared smoking patterns in the relatives. Other studies have since demonstrated a familial component of risk for lung cancer. The ORs associated with family history ranged from 1.3 to 7.2 (Ooi et al. 1986; Samet et al. 1986; Wu et al. 1988, 1996; Osann 1991; Shaw et al. 1991; Schwartz et al. 1996; Mayne et al. 1999). For example, in a comparison of 336 lung cancer probands with relatives of control spouses, Ooi and colleagues (1986) reported an association between a family history of lung cancer and a threefold excess risk (OR = 3.09; 95 percent CI, 1.9–5.0). Shaw and associates (1991) found an OR of 2.8 (95 percent CI, 1.2–6.6) for risk of lung cancer among two or more relatives of case patients. Brownson and colleagues (1997) reported a trend for increasing risk associated with the number of first-degree relatives with

lung cancer. The risk was more than twofold for persons with five affected family members.

One approach that evaluates familial aggregation while controlling for the impact of smoking focused on lifetime nonsmokers. Wu and colleagues (2004) evaluated 216 lung cancer probands who were female nonsmokers and reported that family history of lung cancer was associated with a 5.7-fold (OR = 5.7; 95 percent CI, 1.9–16.9) increase in lung cancer risk. Wu and colleagues (1988) noted, after adjustment for exposure to secondhand smoke, a 30-percent increase in risk that was not statistically significant for history of cancers of the respiratory tract. This association was especially evident in mothers and sisters. The risk was also slightly elevated for lung cancer (OR = 1.3; 95 percent CI, 1.0–1.6). Mayne and colleagues (1999) also focused on familial risk in a population-based, case-control study of 437 lifetime nonsmokers and former smokers who had lung cancer and 437 matched control participants. The investigators observed increased risk of cancers of the aerodigestive tract among parents of case patients (OR = 2.78; 95 percent CI, 1.30–5.95) and increased risk of lung cancer among siblings and offspring of case patients (OR = 4.14; 95 percent CI, 0.88–19.46; p = 0.07). They also reported approximately twofold increases in risk of breast cancer among mothers (OR = 2.52; 95 percent CI, 1.21–5.24) and sisters (OR = 2.07; 95 percent CI, 0.99–4.31) of lung cancer patients who were nonsmokers. On the other hand, Kreuzer and colleagues (2002) reported no evidence of familial risk in 234 lung cancer probands who were female nonsmokers.

Other investigators have reported familial aggregation of lung cancer among relatives of case patients who were nonsmokers with early-onset of lung cancer (at ≤60 years of age). Schwartz and colleagues (1996) noted that family members of these case patients (aged 40 to 59 years) had a sixfold increase in risk of lung cancer after adjustments for the age, gender, and race of each relative. In a subsequent study involving 118 population-based probands, Schwartz and colleagues (1999) also showed that family members of case patients younger than 40 years of age who had lung cancer were at increased risk for other cancers. Kreuzer and colleagues (1998) concluded that lung cancer in a first-degree relative was associated with a 2.6-fold increase in risk of lung cancer among young case patients younger than 46 years of age. Elevated risk was not detected in older case patients. A study in Germany of 945 lung cancer cases and 983 controls reported increased risk of lung cancer among first-degree relatives (RR = 1.7; 95 percent CI, 1.1–2.5) and a 4.75-fold increase in risk among relatives of probands younger than 50 years of age who had a diagnosis of lung cancer (Bromen et al. 2000). Radzikowska and colleagues (2001) also noted stronger evidence from a study in Poland for aggregation of cancers among 757 patients with lung cancer who were younger than 50 years of age.

On the other hand, Etzel and colleagues (2003) observed the familial aggregation of lung cancer and smoking-related cancers in late-onset lung cancers in persons older than 55 years of age, but not in early-onset lung cancers. An advantage of this study was the ability to adjust for the smoking status of the relatives. The study noted an excess of cancer among relatives of probands who were current smokers but not among relatives of lifetime nonsmokers. More recently, Li and Hemminki (2004) evaluated familial risks by using data from the Swedish Family Cancer Database and demonstrated that the histologic type of lung cancer in relatives was generally random. These researchers also estimated that 25 percent of familial lung cancers were diagnosed before 50 years of age, which represented about 1.6 percent of all lung cancers before 68 years of age.

However, a cohort study of lung cancer mortality among male twins showed no role for genetic predisposition (Braun et al. 1994). Li and colleagues (1998b) used a parametric likelihood approach and adjustment for shared covariates to study familial association in the age at onset that they hypothesized to be attributable to genetic factors. The analysis indicated that a history of smoking, exposure to secondhand smoke, and chronic obstructive airway disease were all associated with lung cancer risk. After adjustments were made for these factors, there was little evidence of familial aggregation.

In a recent series with high-risk multiplex families, Bailey-Wilson and colleagues (2004) mapped a major susceptibility locus of lung cancer through a genome-wide linkage analysis to chromosome 6q23–25 near the *PARKIN* gene, which carries predisposition for a significantly increased hereditary risk of lung cancer. A study of this locus also indicated presence of gene-environment interaction, and even light smoking by carriers of this gene significantly increased the risk for lung cancer compared with that among heavy smokers. Carriers who did not smoke had much lower risk, comparable to risk for noncarriers. This finding indicated existence of a sensitive group of persons for whom any amount of smoking is deleterious.

Studies have also presented evidence of Mendelian inheritance in lung cancer. Sellers and colleagues (1990) found that the pattern of occurrence of lung cancer was compatible with the Mendelian codominant inheritance of a rare and major autosomal gene. However, a similar study of families with lung cancer probands who did not smoke revealed no evidence for a major gene model and reported that an environmental model best explained the segregation pattern in the data (Yang et al. 1997).

Gauderman and Morrison (2000) determined that when the same data were analyzed but missing data for smoking behaviors were produced from modeling techniques, a single autosomal dominant locus provided a slightly better fit than the codominant model suggested by Sellers and colleagues (1990). In addition to possible etiologic heterogeneity, the inconsistency of these findings may be partly due to the insufficient power for the statistical analysis of limited sample sizes. A reanalysis by Yang and colleagues (1999) found evidence of a major gene with the Mendelian codominant model in the families of probands of nonsmokers younger than 60 years of age. This analysis rejected both the codominant and environmental models and suggested that multiple genetic and/or environmental factors contribute to the age at onset of lung cancer. Therefore, researchers need more complex genetic models for the distribution of age at onset.

Xu and colleagues (2005) completed a segregation analysis on 14,378 persons from 1,561 case families with aggregation of lung cancer. In their modeling, these researchers adjusted for the effects of smoking, gender, and age. This work provided evidence for a model involving multiple gene loci and interactions that contribute to the age at onset of lung cancer.

One caveat that applies to all of these studies is the validity of data on family history, an issue indirectly addressed in an evaluation of family histories of cancer in participants in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (Pinsky et al. 2003). The data showed that in the ratios of reported-to-expected rates of cancer in family members, there were important differences in rates of reporting family history of cancer according to the gender, race, ethnicity, and age of the respondents. These differences were mostly due to underreporting with respect to these covariates. Ziogas and Anton-Culver (2003) found that family histories of cancer reported by probands were more accurate for first-degree relatives and that probands referred through clinics had lower false-positive rates for reporting of family history than did population-based probands. Bondy and colleagues (1994) evaluated the accuracy of cancer diagnosis reported by probands among family members by comparing reported cancer information with documentation available through medical records and death certificates. The study noted high levels of accuracy for cancers of first-degree relatives, as evidenced by agreement between reporting by probands and information in records. Thus, these findings of familial aggregation suggest a role for the inherited susceptibility of lung cancer beyond that associated with familial clustering of smoking behaviors, taking into account family size and structure.

A genomewide association study in 2008 identified a region of strong linkage disequilibrium on the long arm of chromosome 15 as a susceptibility locus for lung cancer (Amos et al. 2008). Studies replicating this association have focused attention on the most likely candidate genes in this region, *CHRNA3* and *CHRNA5,* which encode subunits of the nAChR (Hung et al. 2008). Le Marchand and colleagues (2008) found that carriers of the lung-cancer-associated variants in these genes extract more nicotine and are thus exposed to a higher internal dose of carcinogenic nicotine-derived nitrosamines. SNPs in the same region have also been associated with nicotine dependence and smoking intensity (Caporaso et al. 2009).

Evidence Summary

Although cigarette smoke contains diverse carcinogens, PAH, *N*-nitrosamines, aromatic amines, 1,3-butadiene, benzene, aldehydes, and ethylene oxide are among the most important carcinogens because of their carcinogenic potency and levels in cigarette smoke. Moreover, the major pathways of metabolic activation and detoxification of some of the principal carcinogens in cigarette smoke are well established. Reactive intermediate agents critical in forming DNA adducts include diol epoxides of PAH, diazonium ions generated by α-hydroxylation of nitrosamines, nitrenium ions formed from esters of *N*-hydroxylated aromatic amines, and epoxides such as ethylene oxide. Glutathione and glucuronide conjugation play major roles in detoxification of carcinogens in cigarette smoke.

Familial predisposition and genetic polymorphisms may play a role in tobacco-related neoplasms. Researchers have established cigarette smoking as a major cause of lung cancer; more than 85 percent of lung cancers are attributable to smoking. However, not all smokers develop lung cancer, and lung cancer can arise in lifetime nonsmokers. This variation in disease has stimulated interest in molecular epidemiology of genetic polymorphisms, including genes that regulate the cell cycle and genes for carcinogen-metabolizing enzymes that may lead to variations in susceptibility to the carcinogens in tobacco smoke. Studies to date suggest a role for these genetic polymorphisms in the risk of lung and bladder cancer in smokers, and they support the possibility of interactions between genes and smoking status.

Quantitative analysis of carcinogens or their metabolites in urine, breath, and blood provides a convenient and reliable method of comparing exposure to carcinogens among smokers and between smokers and nonsmokers. Urinary biomarkers of several major types of carcinogens in cigarette smoke are reliable indicators of exposure, and the measurements provide good estimates of minimum doses of relevant carcinogens in smokers and allow comparisons with nonsmokers. The total carcinogen dose is generally difficult to calculate because the extent of conversion of a given carcinogen to the measured metabolite is usually unknown. However, relative carcinogen levels in cigarettes generally correlate with metabolite levels in urine. Comparisons of smokers and nonsmokers demonstrate that total NNAL is the most discriminatory biomarker in that tobacco products are the only source of the parent carcinogen NNK.

Evidence is overwhelming that DNA adduct levels are higher in most tissues of smokers than in corresponding tissues of nonsmokers. This observation provides bedrock support for the major pathway of cancer induction in smokers that proceeds through formation of DNA adducts and genetic damage. Studies of specific adducts are still scarce and are limited mainly to human lung tissue. Strong evidence supports the presence of a variety of specific adducts in the human lung, and in several studies, adduct levels are higher in smokers than in nonsmokers. Collectively, the results of these biomarker studies clearly demonstrate the potential for genetic damage in smokers from the persistence of DNA adducts.

Adducts lead to mutations that drive the process of tumor formation and progression through additional genetic alterations. Chromosomal losses are more common in tumors from smokers. Furthermore, inactivating mutations of the *TP53* tumor-suppressor gene and activating mutations of the *KRAS* oncogene in NSCLCs and other tumors are correlated with exposure to cigarette smoke, and they contribute to a phenotype that reduces survival time in both early and advanced stages of the disease. Different types of lung cancer in smokers all show an excess of G→T transversions compared with cancers that are not related to exposure to tobacco smoke. The site specificity of mutagenesis by PAH diol epoxides implies that targeted adduct formation, in addition to phenotypic selection, is responsible for shaping the *TP53* mutational spectrum in lung tumors. Propagation of these genetic alterations during clonal outgrowth is consistent with accumulation of multiple genetic changes observed in progression of lung cancer.

Gene promoter hypermethylation is an epigenetic change involving extensive methylation at the 5-position of C in CpG islands within the promoter region and often extending into exon 1 of regulatory genes. The end result of this process can be loss of gene transcription and therefore the silencing of gene function. Promoter methylation of several genes including *P16* occurs early in tumor formation. *P16* methylation was significantly associated with pack-years of smoking and was an independent risk factor for shorter survival in patients with early resectable adenocarcinomas. Other genes such as *RASSF1A* may be more frequently methylated in various tumor types from smokers. Methylation of genes, such as *AGT* promoter hypermethylation, may increase G→A transition mutations at CpG sites within the *TP53* gene in NSCLC.

The activation of nAChRs in lung epithelial cells by nicotine or NNK promotes survival and proliferation of cancer cells and also leads to increased angiogenesis. Activation of cell-surface receptors induced by components of tobacco smoke and subsequent activation of cytoplasmic kinases stimulate other proteins that dictate cellular responses, such as cell survival and proliferation. Although activated kinases have many downstream targets, the two most studied are the transcription factor NF-κB and proteins in the BCL-2 family. Activation of key intracellular proteins by tobacco smoke components through signaling cascades promotes processes that are important for initiation, progression, and maintenance of cancer. Apoptosis, the normal mechanism of endogenous cell elimination, is also commonly suppressed in lung cancer by these components. Thus, key genetic and epigenetic events that lead to cancer causation, as well as critical cellular pathways that further growth and development of transformed cells, are directly targeted by components of cigarette smoke individually and in combination as a potent carcinogenic mixture.

Conclusions

1. The doses of cigarette smoke carcinogens resulting from inhalation of tobacco smoke are reflected in levels of these carcinogens or their metabolites in the urine of smokers. Certain biomarkers are associated with exposure to specific cigarette smoke carcinogens, such as urinary metabolites of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and hemoglobin adducts of aromatic amines.

2. The metabolic activation of cigarette smoke carcinogens by cytochrome P-450 enzymes has a direct effect on the formation of DNA adducts.

3. There is consistent evidence that a combination of polymorphisms in the *CYP1A1* and *GSTM1* genes leads to higher DNA adduct levels in smokers and higher relative risks for lung cancer than in those smokers without this genetic profile.

4. Carcinogen exposure and resulting DNA damage observed in smokers results directly in the numerous cytogenetic changes present in lung cancer.

5. Smoking increases the frequency of DNA adducts of cigarette smoke carcinogens such as benzo[*a*]pyrene and tobacco-specific nitrosamines in the lung and other organs.

6. Exposure to cigarette smoke carcinogens leads to DNA damage and subsequent mutations in *TP53* and *KRAS* in lung cancer.

7. There is consistent evidence that smoking leads to the presence of promoter methylation of key tumor suppressor genes such as *P16* in lung cancer and other smoking-caused cancers.

8. There is consistent evidence that smoke constituents such as nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone can activate signal transduction pathways directly through receptor-mediated events, allowing the survival of damaged epithelial cells that would normally die.

9. There is consistent evidence for an inherited susceptibility of lung cancer with some less common genotypes unrelated to a familial clustering of smoking behaviors.

10. Smoking cessation remains the only proven strategy for reducing the pathogenic processes leading to cancer in that the specific contribution of many tobacco carcinogens, alone or in combination, to the development of cancer has not been identified.

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Table 5.2 DNA adducts in human lung tissue

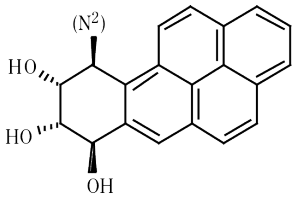
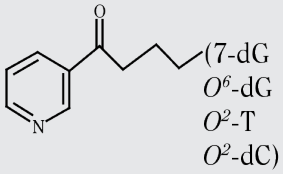
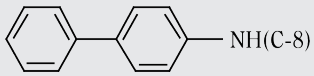
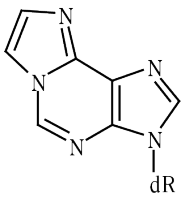
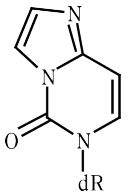
Study	Carcinogen	DNA base	Adduct structures[a]	Type of evidence[b]
Rojas et al. 1998, 2004 Boysen and Hecht 2003	Benzo[*a*]pyrene	dG		1
Wilson et al. 1989 Mustonen et al. 1993 Kato et al. 1995 Blömeke et al. 1996 Petruzzelli et al. 1996	*N*-nitrosodimethylamine NNK Others	dG	7—CH_3	2
Wilson et al. 1989 Mustonen et al. 1993 Kato et al. 1995 Blömeke et al. 1996 Petruzzelli et al. 1996	*N*-nitrosodimethylamine NNK Others	dG	O^6—CH_3	2
Wilson et al. 1989 Blömeke et al. 1996 Godschalk et al. 2002	*N*-nitrosodiethylamine Others	dG	7—CH_3CH_2	2
Wilson et al. 1989 Blömeke et al. 1996 Godschalk et al. 2002	*N*-nitrosodiethylamine Others	dG	O^6—CH_3CH_2	2
Wilson et al. 1989 Blömeke et al. 1996 Godschalk et al. 2002	*N*-nitrosodiethylamine Others	T	O^4—CH_3CH_2	2
Foiles et al. 1991	NNK *N′*-nitrosonornicotine	dG, T, dC		1
Eide et al. 1999	Ethylene oxide	dG	7—$HOCH_2CH_2$	2
Wilson et al. 1989 Lin et al. 1994	4-aminobiphenyl	dG		2

Table 5.2 Continued

Study	Carcinogen	DNA base	Adduct structures[a]	Type of evidence[b]
Godschalk et al. 2002	Vinyl chloride Ethyl carbamate Oxidants	Deoxyadenosine		2
Godschalk et al. 2002	Vinyl chloride Ethyl carbamate Oxidants	dC		2
Asami et al. 1997 Lee et al. 1999a	Oxidants	dG	8—oxo	3

Note: **dC** = deoxycytidine; **dG** = deoxyguanosine; **NNK** = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; **T** = thymidine.
[a]Adduct structures show position of attachment to the base (e.g., N^2-, O^6-, or 7- of dG) and the organic moiety derived from the carcinogen.
[b]1 = detection of a released adducted moiety by a specific method; 2 = detection of a nucleoside or base by a relatively nonspecific method (e.g., ^{32}P-postlabeling or immunoassay); 3 = detection of a nucleoside or base by a specific method (e.g., mass spectrometry, high-performance liquid chromatography [HPLC]-fluorescence, or HPLC-electrochemical detection).