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Review

Genotoxicity of tobacco smoke and tobacco smoke condensate: a review

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Abstract

This report reviews the literature on the genotoxicity of mainstream tobacco smoke and cigarette smoke condensate (CSC) published since 1985. CSC is genotoxic in nearly all systems in which it has been tested, with the base/neutral fractions being the most mutagenic. In rodents, cigarette smoke induces sister chromatid exchanges (SCEs) and micronuclei in bone marrow and lung cells. In humans, newborns of smoking mothers have elevated frequencies of *HPRT* mutants, translocations, and DNA strand breaks. Sperm of smokers have elevated frequencies of aneuploidy, DNA adducts, strand breaks, and oxidative damage. Smoking also produces mutagenic cervical mucus, micronuclei in cervical epithelial cells, and genotoxic amniotic fluid. These data suggest that tobacco smoke may be a human germ-cell mutagen. Tobacco smoke produces mutagenic urine, and it is a human somatic-cell mutagen, producing *HPRT* mutations, SCEs, microsatellite instability, and DNA damage in a variety of tissues. Of the 11 organ sites at which smoking causes cancer in humans, smoking-associated genotoxic effects have been found in all eight that have been examined thus far: oral/nasal, esophagus, pharynx/larynx, lung, pancreas, myeloid organs, bladder/ureter, uterine cervix. Lung tumors of smokers contain a high frequency and unique spectrum of *TP53* and *KRAS* mutations, reflective of the PAH (and possibly other) compounds in the smoke. Further studies are needed to clarify the modulation of the genotoxicity of tobacco smoke by various genetic polymorphisms. These data support a model of tobacco smoke carcinogenesis in which the components of tobacco smoke induce mutations that accumulate in a field of tissue that, through selection, drive the carcinogenic process. Most of the data reviewed here are from studies of human smokers. Thus, their relevance to humans cannot be denied, and their explanatory powers not easily dismissed. Tobacco smoke is now the most extreme example of a systemic human mutagen.
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Keywords: Cigarette smoke; Mutagenicity; Lung cancer; Complex mixture; Tobacco smoke

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Contents

1. Introduction

Tobacco smoking ranks as a major public health problem whose negative impacts have spread around the world. Until recently, the health effects of tobacco smoking were confined largely to developed countries; however, the current promotion and adoption of this habit in developing countries is resulting in an enormous increase in smoking-associated disease and death of global dimensions [1,2]. Worldwide, there is an estimated >1 billion smokers, and ~3 million deaths per year are estimated to be attributable to smoking, with this number rising to ~10 million per year in 30–40 years' time [2]. Estimates suggest that of those people alive today, half a billion will die of tobacco-associated disease [1].

Tobacco smoking is the major risk factor associated with heart disease, which is the primary cause of death in developed countries [3], and smoking is the overwhelming cause of lung cancer, which is the leading cause of cancer deaths worldwide [4]. Currently, cigarette smoking is associated with ~90% of lung cancer cases, resulting in ~1.2 million deaths annually, and it accounts for ~30% of all cancer cases in developed countries [2,4,5]. Recently, the International Agency for Research on Cancer [4] identified tobacco smoking as the cause of cancer at more organ sites than any other human carcinogen. These include cancers of the lung, oral cavity, naso^{-}, oro^{-}, and hypopharynx, nasal cavity and paranasal sinuses, larynx, esophagus, stomach, pancreas, liver, kidney, ureter, urinary bladder, uterine cervix, and

bone marrow (myeloid leukemia). Thus, tobacco is the most extreme example of a systemic carcinogen, and, as this review documents, is must now be considered the most extreme example of a systemic human mutagen.

The mechanisms by which tobacco smoke causes these cancers and other health effects have been studied intensively during the past 20 years, and much has been learned. One mechanism involves the mutagenic activity of tobacco smoke, which has been demonstrated clearly and reviewed 2 decades ago [6–8]. Recent reviews have summarized the studies on smoking-related DNA and protein adducts in human tissues [9] as well as the chemical biomarkers associated with tobacco smoke exposure [10]. This review examines the literature on the genotoxicity of tobacco smoke and tobacco smoke condensate from 1985 onwards in experimental systems as well as the genotoxicity of active tobacco smoking in humans. In addition, some of the mutational mechanisms of tobacco smoke are reviewed within the context of the carcinogenic mechanisms associated with smoking-related tumors.

2. Experimental systems

2.1. Mutagenicity, genotoxicity, and mutation spectra of cigarette smoke condensate (CSC)

As reviewed previously [6–8], CSC is mutagenic in a variety of systems. Most studies of CSC have used CSC generated from various reference cigarettes, such as K1R4F, which was developed jointly by the U.S. National Cancer Institute, the U.S. Department of Agriculture, and the University of Kentucky Tobacco and Health Research Institute [11]. The average mutagenicity of U.S. market and K1R4F mainstream CSCs in the *Salmonella* mutagenicity assay was not significantly different on a revertants/mg condensate or revertants/cigarette basis for a sample of cigarettes representing >70% of the U.S. market [11]. Similar results also were obtained for the K1R5F cigarette [12], indicating that these reference cigarettes are acceptable standards for comparative mutagenicity of cigarettes purchased typically in the U.S.

More recent studies have confirmed and extended the initial observations showing that CSC is mutagenic in *Salmonella* and SOS assays [13–16], induces micronuclei in *Vicia faba* root tips [17], mutations [18,19] and deletions [20] at the $Tk^{+/-}$ locus in mouse lymphoma cells, and *Hprt* mutants in CHO cells [16,21–23]. CSC also can transform human cells in culture [24]; however, it did not induce intrachromosomal recombination in CHO cells [25]. Comparisons of CSC to wood smoke condensate or liquid smoke food flavorings have shown that some flavorings are more mutagenic than CSC [26]; however, the levels of exposure must be considered in evaluating the risk posed by such substances [27,28]. The relative mutagenic potencies of organic extracts of CSC and other combustion emissions rank similarly in *Salmonella*, mouse lymphoma $Tk^{+/-}$, and mouse skin tumor-initiation assays [29], suggesting that the results obtained with these systems are reasonably consistent.

Most of the sister chromatid exchange (SCE)-inducing ability of CSC appears to reside in the neutral and acidic/neutral fractions [30,31], suggesting that PAHs and acidic compounds in CSC are responsible for this activity. Also, the acidic fraction of CSC was the most potent direct-acting fraction that induced deletions and/or nondisjunction in mammalian cells [32]. The acid fraction includes phenolic compounds such as catechol and hydroquinone that may produce free radicals by redox-cycling that could produce the observed clastogenic effects. Reconstruction of a mixture containing a group of PAHs in CSC failed to reproduce the mutagenic activity of CSC, suggesting that PAHs were not contributing substantially to the activity [33]. Nicotine and its metabolites were not mutagenic in *Salmonella*, did not induce SCEs in CHO cells [34], and nicotine did not produce mutagenic urine in rats [35]. Although CSC contains a wide variety of agents exhibiting a wide range of toxicity [36], varying the amounts of 333 ingredients added to typical commercially blended test cigarettes did not alter the mutagenicity, cytotoxicity, or inhalation toxicity of the resulting cigarettes [37,38].

Several lines of evidence indicate that the primary source of mutagenic activity of CSC detected in the *Salmonella* mutagenicity assay is aromatic amine and heterocyclic amine protein pyrolysate products. As reviewed previously [6–8], most of the mutagenic activity of CSC resides in the basic or base/neutral fraction [31,39], which contains the aromatic amines

and heterocyclic amines. Removal of protein and peptides from flue-cured or burley tobacco via water extraction followed by protease digestion reduced the mutagenicity of the resultant CSC by ~80% in TA98 and ~50% in TA100 of *Salmonella* [40]. CSCs produced from tobacco smoke aerosols generated at temperatures below 400 or 475 °C were not mutagenic in TA98 or TA100, respectively, but CSC produced above those temperatures was mutagenic [41]. Heterocyclic amines are pyrolysate products of aromatic amino acids that are produced only at high temperatures, and the findings cited above indicate that these compounds are an important contributor to the mutagenic activity of CSC. Exposure of hamsters to cigarette smoke enhanced the ability of their livers to convert heterocyclic amines to mutagens, suggesting that cigarette smoke, like heterocyclic amines themselves, induces *CYP1A2*, which metabolizes heterocyclic amines [42].

Heterocyclic amines, whose formation would have been reduced greatly by removal of the protein from the tobacco, are much more mutagenic in TA98 than in TA100. This likely explains the greatly reduced mutagenic activity of CSC in TA98 relative to TA100 when protein is removed from the tobacco prior to combustion. In addition, some of the activity detected in TA100 may be due to PAHs, which are more mutagenic in TA100 than in TA98. At the molecular level, the mutation spectrum of CSC in TA98 is identical to that of the heterocyclic amine Glu-P-1, suggesting that this class of compounds is, in fact, most responsible for the frameshift mutagenic activity of CSC detected in TA98 [43]. In contrast, most of the mutations induced by CSC in TA100 were GC → TA transversions (78%), and this resembled most closely the mutation spectrum of the model PAH benzo[*a*]-pyrene [43]. GC → TA transversions, which are the primary class of base substitutions found in lung tumors of smokers (see below), were also induced by CSC at the *HPRT* locus in human MCL-5 cells, which over-express various P450 genes and microsomal epoxide hydrolase [23].

2.2. Cytogenetic effects of CSC and cigarette smoke in vitro

As reviewed previously [6–8] and shown in additional studies [21,22,30,31,44], CSC or cigarette smoke induces SCEs in mammalian cells in culture. Tobacco particulate matter also has been shown to induce structural and numerical chromosome aberrations [8,16,45] and micronuclei [46] in cultured mammalian cells. This last study also suggested that the micronuclei were not due to aromatic compounds but, perhaps, to free radicals, based on the lack of association between micronucleus induction and DNA adducts. The neutral fractions and weakly acidic, semi-volatile components of CSC were the most potent inducers of SCEs in cultured human lymphocytes, and a number of potential candidate compounds were identified, most of which were alkylphenols and benzaldehydes [30,47,48]. Whole smoke also induces micronuclei in V79 cells [49], and these appear to be kinetochore-positive, suggesting that the micronuclei are due to whole chromosome loss [50].

2.3. Cytogenetic effects of cigarette smoke in vivo

As reviewed earlier [8], exposure of rodents to cigarette smoke has generally produced SCEs in the bone marrow. However, such exposure has produced some negative studies [51,52] and one positive study [53] regarding the induction of chromosomal aberrations in lung cells. Nonetheless, this exposure has consistently produced micronuclei in bone marrow [54–60], peripheral blood erythrocytes [55,56], and lung [56].

2.4. DNA damage

Many studies have demonstrated that CSC and cigarette smoke itself can induce DNA strand breaks either in rodents, mammalian cells in culture, or in DNA in vitro [61–73]. Filtered, tar- and aerosol-free cigarette smoke induced accumulation of TP53 protein in mouse cells in culture, which is an indirect indication of DNA damage [74]. Collectively, these studies are consistent with the demonstrated clastogenicity of CSC and cigarette smoke in experimental systems and humans. Several of these studies indicate that reactive oxygen or nitrogen species is the primary cause of the strand breaks. A recent study also has shown that a cigarette smoke extract induced apoptosis in human lung fibroblasts in vitro [75].

2.5. Studies on modified cigarettes and those that primarily heat but do not burn tobacco

Although cigarettes that primarily heat rather than burn tobacco [76] are not in general commercial use, the CSC from such products was not mutagenic in *Salmonella* and did not induce SCEs, chromosomal aberrations, or *HPRT* mutations in CHO cells, nor did it induce unscheduled DNA synthesis in rat cells [16,77–79]. Whole smoke from such cigarettes was also either negative or only slightly mutagenic in *Salmonella* and for SCEs in CHO cells, and it was negative for rat bone marrow chromosomal aberrations, micronuclei, and SCEs [80–83]. Humans who smoked these cigarettes had urine that was ~70% less mutagenic than that from those who smoked standard cigarettes [84–86]. A cigarette whose tobacco was heated electrically produced total particulate matter (TPM) that was ~90% less mutagenic in *Salmonella* than the TPM from a reference cigarette [87]. Consistent with this finding was the observation that this cigarette delivered 50% lower amounts of approximately two-thirds of the 69 chemicals measured in the smoke compared to the levels of these compounds in the smoke from a reference cigarette [88].

In 1990, ~20% of the ~5200 deaths from fires in the U.S. occurred in fires started by cigarettes [89]. The mutagenicity of CSC from a reference cigarette was similar to that from an experimental cigarette with low-ignition propensity [89]. This was achieved by using expanded tobacco and a cigarette wrapper of low porosity that was not treated with sodium potassium citrate. Such a cigarette was developed to be less likely than regular cigarettes to ignite surrounding material. The genotoxicity of CSC from standard cigarettes was similar to that from cigarettes containing an experimental carbon filter that reduces the amounts of certain vapor-phase components of tobacco smoke [90]. However, the whole smoke from this cigarette was less mutagenic in *Salmonella* and induced fewer SCEs in CHO cells than smoke from cigarettes with the usual type of charcoal filter [90]. CSC from dry-ice-expanded tobacco was similar in mutagenic potency in *Salmonella* and in SCE potency in mammalian cells as CSC from tobacco expanded with a traditional expansion agent (Freon-11, also known as trichlorofluoromethane) [91].

Aqueous extracts of tobacco induced chromosomal aberrations and SCEs in CHO cells [92]. Masheri, a pyrolysed tobacco product used primarily in India to clean the teeth, has been shown to induce chromosomal aberrations, SCEs, and *Hprt* mutations in CHO cells as well as mutations in *Salmonella* [93].

2.6. Transplacental effects

Mice born after exposure of pregnant mothers to cigarette smoke had elevated levels of micronuclei in their liver and peripheral blood [94], and such exposures also induced SCEs in mouse fetal liver [95]. Exposure of pregnant Syrian golden hamsters to the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) also induced micronuclei in fetal liver [96]. Exposure of pregnant mice to NNK induced oxidative damage (8-oxo-dG) in the fetuses [97].

2.7. Modulation of genotoxicity of CSC and tobacco smoke

Many studies have examined the ability of various agents to modulate the genotoxicity of CSC or tobacco smoke in vitro or in experimental animals. Studies primarily in rodents exposed to tobacco smoke [98] or with CSC primarily in *Salmonella* [99,100] have identified a variety of agents that either inhibit or modulate the genotoxicity of tobacco smoke and/or CSC. Such agents include *N*-acetylcysteine (NAC), chlorophyllin, phenolic compounds, isothiocyanates, indoles, tetrapyroles, flavonoids, ascobric acid, α-tocopherol, UV light. [101–105]. Much of this work has been reviewed recently [98]. As discussed later, some of these agents have been shown to modulate smoking-associated biomarkers in humans, and a recent study has indicated that, as in humans, *p53* plays a role in tobacco smoke-induced lung tumors in mice [106].

3. Humans

3.1. HPRT mutations

The association between *HPRT* mutant frequencies in peripheral blood lymphocytes and smoking has

been reviewed extensively [107–109], and this literature is not re-reviewed here. However, in general, studies show that smoking increases the *HPRT* mutant frequency in peripheral blood lymphocytes by ∼50%, but the increases did not reach statistical significance in some studies due to the large inter-individual variability. Using the autoradiographic *HPRT* assay, some portion of the *HPRT* mutant frequency was shown to reflect recent smoking exposure as opposed to a permanent increase in *HPRT* mutant frequency [110]. Most importantly, *HPRT* mutant frequencies were similar in lung cancer cases and controls with the same smoking status, indicating that lung cancer per se has little if any effect on *HPRT* mutant frequency in lymphocytes [111]. Although some analyses have found no difference in the mutation spectrum at *HPRT* between smokers and nonsmokers [108], an increase in transversions, in particular, GC → TA, has been noted frequently among smokers [112–115]. This is the primary class of base substitution induced by PAHs, and an excess of this class of mutation in the *HPRT* mutation spectrum of smokers is consistent with an exposure to the PAHs in cigarette smoke.

3.2. Urinary mutagenicity

Urinary mutagenicity in smokers was detected first in 1977 by testing the XAD/acetone-extractable organics from urine in the *Salmonella* (Ames) mutagenicity assay [116]. Several years later, studies using essentially the same methods confirmed and clarified this original observation in smokers [117–120]. The general approach to these studies has remained similar over the years and has involved concentration of the organic compounds from the urine by means of a solid-phase resin followed by elution with an organic solvent and then testing the resulting concentrate in the *Salmonella* mutagenicity assay in the presence of rat liver S9 mix for metabolic activation.

A comparison of three types of resins (C18, XAD-2, and CN) followed by elution with acetone showed that the highest levels of urinary mutagenicity were detected using C18 resin [121]. A study of the stability of stored urine samples showed that no significant loss of mutagenic activity was observed with urine stored frozen as long as 175 days [122]. The use of a *Salmonella* microsuspension assay is generally more sensitive at detecting urinary mutagens from smokers than is the standard plate-incorporation assay [123,124]. Peak mutagenic activity of the urine occurs 4–5 h after the beginning of smoking and decreases to pre-smoking levels in ∼12–18 h [125]. This study suggested that the mutagens are absorbed rapidly (3–5 h) and are eliminated from the body following first-order kinetics; the excretion rate constant for the occasional smoker was $\sim 0.1\ h^{-1}$, and the half-life (T1/2) was ∼7 h. A study in which the SOS Chromotest was used as the indicator assay showed that urine from smokers that was mutagenic in *Salmonella* TA98 was not mutagenic in the SOS Chromotest [126]. Urine concentrates from subjects who smoked tobacco and chewed areca nut induced SCEs and chromosomal aberrations in CHO cells [127].

Snuff users [128] do not have mutagenic urine, and the urine of smokers who smoked cigarettes that heated but did not burn tobacco had levels of urinary mutagenicity that were not different from those of nonsmokers [35,77,78]. However, bidi tobacco rollers have mutagenic urine [129], suggesting that exposure to tobacco per se and not tobacco pyrolysate products may be sufficient to produce mutagenic urine. Urinary mutagenicity generally correlates with the number of cigarettes smoked, and the level of urinary mutagenicity is similar regardless of the level of tar of the cigarettes smoked [121,130]. However, the urine of smokers of black tobacco is twice as mutagenic as is the urine from smokers of blond tobacco, which correlates with the known increased risk for bladder cancer among smokers of black versus blond tobacco [131]. Smoking-associated urinary mutagenicity correlates with external measures of exposure such as daily intake of tobacco condensate, and with internal measures of exposure, such as urinary 1-pyrenol [132]. Among bladder cancer patients, one study showed that there was no association between levels of urinary mutagenicity and tumor status or recurrence of bladder tumors [133].

Although dietary consumption of fried meat can also produce mutagenic urine associated with the heterocyclic amine content of the fried meat [134], experiments with subjects on controlled diets showed that the increased urinary mutagenicity observed in smokers compared with nonsmokers was not due to enhanced mutagenicity by diet-related heterocyclic

amine mutagens in the urine of smokers [84]. Although not reviewed exhaustively here, smoking is generally a confounding factor in occupational studies involving urinary mutagenicity. For example, smoking nurses handling cytostatic drugs had mutagenic urine but those who handled these drugs but did not smoke did not have mutagenic urine [135]. Smoking has been identified as a confounding factor in urinary mutagenicity studies of people in various occupations, including inks and pharmaceuticals [136], tires [137], graphite-electrodes [138], steel [139], benzidine [140], and charcoal production [141].

Indirect evidence suggests that the chemicals responsible for smoking-related urinary mutagenicity are primarily aromatic amines and/or heterocyclic amines. For example, smokers' urine is much more mutagenic in strain YG1024 of *Salmonella*, which over-produces *O*-acetyltransferase, than in strains with less of this activity (TA98) or that over-produce nitroreductase (YG1021) [142,143]. Strain YG1024 shows enhanced sensitivity to specific classes of mutagens: aromatic nitro, amino, and hydroxylamino compounds [142]. One study fractionated the urine of smokers and evaluated the fractions for their ability to induce chromosomal aberrations in CHO cells and concluded that the urine of smokers contained clastogens that may act by the production of active oxygen species [144].

Urinary mutagenicity has been shown to correlate with the levels of a 4-aminobiphenyl-DNA adduct in exfoliated urothelial cells from smokers [145]; however, levels of a 4-aminobiphenyl-hemoglobin adduct showed a more complex association with urinary mutagenicity [146]. Chemical analysis of urine from a smoker with exceptionally high urinary mutagenicity revealed the presence of the mutagen 2-amino-7-naphthol, which is a metabolite of the bladder carcinogen 2-aminonaphthalene (beta-naphthylamine) [147]. Chemical fractionation of mutagenic urine from smokers indicates that much of the mutagenic activity may be due to PAHs and/or heterocyclic amines [148].

Although the concentration of urinary nicotine plus its metabolites correlate with urinary mutagenicity in smokers [149,150], nicotine and its metabolites are not responsible for the mutagenicity of smokers' urine [151]. Smokers also excrete more mutagenic activity and thioether compounds than do nonsmokers; thioether compounds can be considered as nontoxic end products of potentially toxic alkylating agents [151]. A study of coke-plant workers suggested that PAHs were not a primary contributor to the urinary mutagenicity observed among smoking workers [152].

3.3. Chemoprevention of smoking-associated biomarkers

In humans, the administration of *N*-acetyl-L-cysteine (NAC) to smokers reduced significantly the level of urinary mutagenicity [153] as well as the frequency of micronuclei in the mouth floor and in the soft palate [154]. Administration of Vitamins C and E to smokers also reduced the frequency of micronuclei in blood lymphocytes [155]. Higher intakes of vitamin A and selenium were associated with a reduced frequency of SCEs among smokers [156]. In contrast to these studies, administration to smokers of the anti-carcinogenic dithiolethione, oltipraz, had no influence on the levels of urinary mutagenicity [143]. Likewise, supplemental niacin did not decrease the frequencies of *HPRT* mutants or micronuclei in peripheral blood lymphocytes among smokers [157]. Consumption of green tea, but not black tea, reduced the levels of DNA damage in lymphocytes of smokers as measured by the comet assay [158]. Other chemoprevention measures against smoking-associated biomarkers have been reviewed recently [98].

3.4. Genotoxic effects in reproductive tissues/fluids and children of smokers

Based on the autoradiographic *HPRT* assay, pregnant women who smoked either traditional cigarettes [159] or marijuana cigarettes [160] not only had elevated *HPRT* mutant frequencies themselves, but analysis of the cord blood indicated that their children also had elevated *HPRT* mutant frequencies. Sequencing of *HPRT* mutants in cord blood from smoking mothers indicated that most (~86%) of the mutations were V(D)J recombinase-associated exon 2–3 deletions [161]. Although no differences in *HPRT* mutant frequencies were observed between T-lymphocytes from newborns of smokers versus nonsmokers based on the T-cell cloning assay [162], the mutation spectra of these

two groups of children differed significantly, with children of smokers having an increase in ''illegitimate'' genomic deletions mediated by V(D)J recombinase [163]. Thus, the *HPRT* mutation spectrum appears to be altered and the *HPRT* mutant frequency possibly elevated in newborns from mothers who smoke compared to those who do not.

In utero exposure to tobacco smoke also increases translocation frequencies in the newborn, and a significant association was found between the *CYP1A1 MspI* polymorphism in the noncoding region of the gene and chromosome aberration frequencies in the newborns [164]. Suggestive evidence has been obtained that smoking by the mother may cause DNA strand breaks in lymphocytes of newborns [165].

Smoking appears to induce aneuploidy in sperm for certain chromosomes, including 1, 13, and YY disomies [166–168], but not for others, such as XY, XX, or 7 [166,167,169]. However, sperm from smokers displays elevated levels of meiosis II nondisjunction of the sex chromosomes relative to that of nonsmokers [170]. Smoking also appears to induce oxidative damage to sperm DNA as evidenced by elevated levels of 8-hydroxydeoxyguanosine (8-OHdG) in sperm DNA of smokers compared to nonsmokers [171]. In addition, infertile male smokers have higher levels of seminal oxidative stress than infertile nonsmokers [172]. Consistent with these observations is the finding that sperm from smokers had higher levels of DNA strand breaks than did sperm from nonsmokers [173]. Levels of DNA adducts in sperm, as measured by ^{32}P-postlabeling, were also higher in current smokers than among never smokers [174]. These enhanced levels of DNA damage in the sperm of smokers may explain the observation that sperm from smoking males used for in vitro fertilization procedures has a lower probability of resulting in a pregnancy than does sperm from nonsmoking donors [175]. Smoking also can be a confounding factor when assessing aneuploidy and DNA damage in epidemiological studies [176].

With regard to oocytes, smokers have decreased numbers of retrieved oocytes compared with nonsmokers, consistent with the known reduced fertility among smokers [177], and oocytes with diploid complements of chromosomes are more frequent among smokers than nonsmokers; the proportion of diploid oocytes is highly associated with the number of cigarettes smoked per day [177]. In addition, triploid zygotes occur more frequently among smokers than nonsmokers, suggesting preferential digynic fertilization among smokers [177]. These results strongly indicate that cigarette smoking is hazardous to the viability and function of developing oocytes and their resulting embryos, and a recent study suggests that expression of the aromatic hydrocarbon receptor-driven *Bax* gene is required for premature ovarian failure caused by PAH exposure [178]. The early onset of menopause in women smokers may be caused, at least in part, by the apoptotic actions of tobacco smoke-derived PAHs in human oocytes.

The cervical mucus of smokers was more mutagenic than that of nonsmokers when tested in the *Salmonella* (Ames) mutagenicity assay [179], and XAD/acetone extracts of amniotic fluid from smoking mothers induced higher frequencies of SCEs in CHO cells than did extracts from nonsmokers [180]. Consistent with this was the finding that cervical epithelial cells from smokers had higher frequencies of micronuclei than did those from nonsmokers [181].

As reviewed previously [182,183], smoking causes a wide array of reproductive problems, as well as effects on meiotic spindle function, DNA damage (oxidative damage as well as PAH adducts) to spermatozoa and oocytes, as well as gametic transmission of genetic damage. As discussed above, smoking is also associated with second-generation effects. A review showed that among the many studies investigating the association of childhood cancer with paternal smoking, the majority of the studies found no relationship [184]. However, in a case-control study where mothers did not smoke, paternal smoking during the pre-conception period was associated with increased risk of childhood cancer within the child's first 5 years [185]. Earlier data [186], along with those reviewed in this section, provide suggestive evidence for paternal and possible maternal gametic transmission of genetic damage and indirect support for the ability of tobacco smoke to be a germ-cell mutagen.

3.5. Cytogenetic effects

3.5.1. Micronuclei

As reviewed recently [187], many studies have examined the influence of smoking on the frequency of micronuclei in peripheral lymphocytes; however,

mixed results have been obtained. A pooled re-analysis of 24 databases from the HUMN international collaborative project showed that smokers do not have an overall increase in micronuclei frequency in their lymphocytes; however, when the interaction with occupational exposure was considered, smokers of ≥ 30 cigarettes/day had a significant increase in micronuclei frequency compared to nonsmokers [187]. An increased frequency of micronuclei in smokers has been observed to occur preferentially in B lymphocytes and suppressor/cytotoxic T8 lymphocytes [188]. Elevated micronuclei frequencies also have been found in the tracheobronchial epithelium of smokers [189], and smokeless tobacco (e.g., snuff) has generally been found to induce micronuclei in oral mucosa [190–192]. Workers exposed to tobacco while making bidi cigarettes also have been found to have an elevated frequency of MN in buccal epithelium [193].

3.5.2. SCEs

In contrast to micronuclei, SCE frequencies in peripheral lymphocytes are generally elevated among smokers compared to nonsmokers. Numerous studies of SCE frequencies in peripheral lymphocytes in environmentally or occupationally exposed or unexposed populations have found that cigarette smoking induces SCEs and can be a confounding factor in occupational studies [194–214]. The mechanism may not involve free radicals [215]. Of all the cytogenetic endpoints, SCEs are the most sensitive to the effect of smoking. One study even demonstrated that smoking was associated with higher SCE levels in peripheral lymphocytes than in bone marrow [216]. However, among healthy adults, smoking may account for only 19% of the inter-personal variation in SCE frequencies [217]. There is a decrease in SCEs in ex-smokers during the first 78 days after stopping smoking, and the decrease is much slower from the 78th to the 233rd day [218]. Various types of smoking, including bidi cigarettes [219–221] and hookah [222] also induce SCEs.

3.5.3. Chromosomal aberrations

Studies of large populations using cytogenetic banding techniques for chromosomal aberrations have given mixed results, with one study finding that chromosomal aberration frequencies were not increased by smoking [201] and another finding that smoking caused a 10–20% increase [205]. Smaller studies have also given mixed results; however, several have found significant increased frequencies of chromosomal aberrations in lymphocytes from smokers relative to nonsmokers [223–225]. Molecular cytogenetic techniques, such as FISH, also have given mixed results, with some studies finding that smoking (a) did not increase the frequency of unstable or stable aberrations but did increase the frequency of hyperploidy [226], (b) produced a marginal increase in translocation frequency [227], or (c) caused a significant increase in stable aberrations (translocations and insertions) [228]. Smoking can be a confounding factor when measuring chromosomal aberrations in occupational exposure studies [229]. Chromosomal aberration frequencies were elevated among workers in a bidi tobacco plant who were exposed to tobacco particles and volatile constituents via cutaneous and nasopharyngeal routes [230]. One study of marijuana smokers found no increase in chromosomal aberrations in peripheral lymphocytes due to this type of smoking [231].

Mechanistic considerations include the observation that smokers have lower levels of folate in red blood cells than nonsmokers, and this may play a role in the higher levels of chromosomal aberrations detected in smokers relative to nonsmokers [232]. Various studies have found that exposure of peripheral lymphocytes from smokers to various mutagens in vitro results in higher chromosomal aberration frequencies than such exposures cause in lymphocytes from nonsmokers [233–237]. Methyl-purine-DNA glycosylase is elevated in the peripheral blood leukocytes of smokers relative to nonsmokers [238]. Collectively, these studies have suggested that cells of smokers, especially from males, are less able to repair DNA damage and that DNA repair enzyme levels, fragile sites, and telomeric associations can be affected by recent mutagenic exposures such as smoking.

A large international study showed that an elevated frequency of chromosomal aberrations in lymphocytes predicts the risk for cancer independently of exposure to carcinogens, including cigarette smoke [239]. However, many studies have demonstrated an association between smoking and certain genetic changes specifically predictive of various types of tumors. For example, lymphocytes of smokers relative to nonsmokers have higher frequencies of fragile sites

and metaphases with extensive breakage, as well as elevated expression of fragile sites at cancer breakpoints and oncogene sites [216]. Analysis of normal bronchial epithelium using FISH found a significant percentage of trisomy 7 in cancer-free tobacco smokers [240], and LOH involving microsatellite DNA at three specific loci-chromosomes was elevated significantly at chromosomal sites containing putative tumor-suppressor genes in histologically normal bronchial epithelium from chronic smokers [241]. Perhaps most importantly, chromosomal aberrations are present at a much higher frequency in lung tumors from smokers (48%) than from nonsmokers (11%), suggesting that lung cancer in smokers results from genetic alterations distinct from those in nonsmokers [242].

3.5.4. Microsatellite instability

Microsatellite instability in colon tumors [243] and chromosome 9 alterations in bladder tumors [244] have been associated with cigarette smoking. Smoking also has been associated with chromosome instability in lymphocytes as an indication of predisposition to oral premalignant lesions [245] and with mutagen sensitivity of lymphocytes as a predictor of upper aerodigestive tract cancer [246]. Various cytogenetic changes and smoking have been associated with a risk for leukemia and other myelodysplastic syndromes [247–251]. Although smoking-related primary lung cancers rarely show microsatellite instability, they frequently exhibit loss of heterozygosity (LOH) [252].

3.5.5. DNA strand breaks and oxidative damage

A higher frequency of DNA strand breaks has been detected by the comet assay in lymphocytes [253–257], buccal cells [258], and urothelial cells [259] of cigarette smokers than in nonsmokers. Oxidative damage as measured by the levels of 7-hydroxy-8-oxo-2′-deoxyguanosine (8-oxo-dG) in lymphocytes/leukocytes was not increased in low or moderate smokers relative to nonsmokers [260–262]. However, smoking was observed to increase the levels of 8-oxo-dG in lymphocytes/leukocytes of heavy smokers or in lung tissue or urine of smokers [263–266]. As noted in the section on experimental systems, in vitro studies, including some in human cells, have also found that cigarette smoke or its components induce DNA damage or oxidative damage. Collectively, these studies suggest that smoking induces oxidative DNA damage, most likely as a result of the up-regulation of the primary defense antioxidative system and/or complementary DNA repair system.

3.6. Mutations in smoking-associated tumors

Smoking is associated with cancer at 11 organs [4], and mutations in some of these smoking-associated tumors have been identified in both oncogenes and tumor-suppressor genes. The gene found mutated most frequently in smoking-associated lung tumors is *TP53*, and the details of this observation have been reviewed extensively [267–270]. Briefly, *TP53* mutations are more common in smokers than nonsmokers, and the frequency of *TP53* mutations shows a direct relationship to the number of cigarettes smoked. *TP53* mutations are found in pre-neoplastic lesions of the lung, indicating that they are early events that are linked temporally to DNA damage from smoking.

The *TP53* mutation spectrum in lung tumors of smokers contains 30% GC → TA transversions, whereas only 10% of the *TP53* mutations in lung tumors of nonsmokers or in other tumors are of this type. This percentage (30%) is exceeded (76%) only by an almost pure PAH exposure resulting from the use of smoky coal in poorly vented homes [271]. The elevated GC → TA transversion frequency in smokers reflects the type of DNA damage and resulting mutations produced by PAHs, which are an important carcinogenic component of cigarette smoke. There is a high degree of correspondence between the mutational hotspots and the sites of DNA adducts remaining after cells are exposed to various diolepoxides and have undergone a period of DNA repair [272,273]. Other analyses have found some discrepancies in the correspondence between sites of PAH adduction and mutations in the *TP53* gene in the lung tumors of smokers [274]. Nonetheless, the mutations in the tumors are targeted at methylated CpG sites [275], and there is a bias for most of the mutated guanines of the GC → TA mutations to be on the nontranscribed DNA strand in lung tumors from smokers, which is due to the preferential repair of DNA adducts on the transcribed strand. Together, these and other data indicate strongly that the *TP53* mutations in lung tumors of smokers are due to the direct DNA damage resulting from the carcinogens in cigarette smoke.

Although this conclusion has been questioned and other factors invoked to account for the observed mutation spectrum [276,277], additional analyses have largely supported the view that the frequency and site specificity of GC → TA transversions in the *TP53* gene of smoking-associated lung tumors are best explained by a direct mutagenic effect of PAHs (and possibly other mutagens) in cigarette smoke [278–280]. As noted previously [43], CSC induces primarily GC → TA transversions in *Salmonella* TA100, consistent with PAHs being responsible for this class of mutation.

Mutations in the *KRAS* gene (codons 12, 13, or 61) occur in ∼30% of lung adenocarcinomas of smokers and are primarily GC → TA transversions as seen in *TP53* [281–284]. There is also a correspondence between the site at which the majority of a particular type of PAH adducts are formed (the first position of codon 12) and the high frequency of GC → TA transversions at this position in smoking-associated lung tumors [274]. These mutations are associated with smoking and occur less frequently in nonsmokers [284,285]. As with *TP53*, *KRAS* mutations occur early in carcinogenesis of the lung [283,286], and the percentage of mutations in the *KRAS* gene of smoking-associated lung tumors that are GC → TA transversions (66%) is exceeded (86%) only by an almost pure PAH exposure resulting from the use of smoky coal in poorly vented homes [271].

Mutation in *TP53* and other genes, such as *KRAS*, *FHIT*, *Bcl-2*, *BAX*, and unknown genes at specific chromosomal locations, also have been identified as mutated in smoking-associated tumors, including bladder, oral cavity, pancreas, larynx, esophagus. [284,287–301]. Collectively, these observations are consistent with the mutagenic effects of CSC and tobacco smoke as demonstrated in experimental systems.

3.7. Influence of genetic polymorphisms on smoking-associated biomarkers

There is now a large literature on the effects of polymorphisms of various genes on smoking-associated biomarkers, such as urinary mutagenicity and cytogenetic effects, as well as on cancer per se [302]. However, most of these studies have a small number of subjects, have not been repeated, or the repeated studies have frequently produced contradictory results. In addition, where an effect has been seen, the effect is frequently quite modest. Thus, few firm conclusions can be made regarding the influence of polymorphisms on the response of smokers for various smoking-associated biomarkers. Nonetheless, a brief overview (not comprehensive) of this topic is presented here so that the reader can have a sense of what genes and polymorphisms have been examined in relationship to smoking, cancer, and various smoking-associated biomarkers.

Smoking increases the activities of certain enzymes in the lung, such as aryl hydrocarbon hydroxylase (AHH) and ethoxycoumarin *O*-deethylase (ECDE), and a decrease in other enzyme activities in the lung, such as glutathione *S*-transferase (GST) [303]. Among lung cancer patients, nearly all of whom smoked, those who were homozygous for a polymorphism in the promoter region of the myeloperoxidase gene had a 61–70% reduced risk for lung cancer compared to those who were heterozygous or wild-type [304,305]. This enzyme metabolizes PAHs to mutagenic forms, and a lower level of this enzyme should reduce the ability to convert the nonmutagenic parent PAHs in cigarette smoke to mutagens. Thus, this polymorphism may provide a protective effect to smokers.

Several polymorphisms among the cytochrome P450 genes have been found to influence risk for cancer among smokers. A slight increase in breast cancer risk was seen in smokers with either the *1/*2 or the *2/*2 genotype of *CYP1A1*, and a greater increase was found in women who started smoking before 18 years of age [306]. The frequency of *TP53* and *KRAS* mutations in lung cancer among heavy smokers was significantly higher in people carrying the rare homozygous alleles of either the *MspI* or *Ile-Val* polymorphism of the *CYP1A1* gene [307]. A *PstI* polymorphism in the 5′-flanking region of the *CYP2E1* gene has been shown to affect the transcription of the gene and has been linked to an increased susceptibility to lung cancer in smokers [308]. Support for this observation has been found from in vitro studies in human cells with the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) that showed that polymorphisms in *CYP2E1* can modulate the genotoxic response of cells to this compound [309].

A review of case-control studies published since 1990 on the effects of polymorphisms among the *CYP1A1*, *1A2*, *1B1*, *2B1*, *2A6*, *2D6*, *2E1*, *2C9*, *2C19*, *17*, and *19* alone or in combination with detoxifying enzymes on cancer risks among smokers concluded that some *CYP* variants were associated with increased risks for cancer of the lung, esophagus, and head and neck [310]. The risk was often increased in individuals who also had *GSTM1* deficiency. The overall effects of common *CYP* polymorphisms were found to be moderate in terms of penetrance and relative risk, with odds ratios ranging from 2 to 10. The *CYP2A6* is responsible for the majority of the inactivation of nicotine and also activates nitrosamines to mutagens [311]. A common polymorphism in this gene decreases the risk for dependence on nicotine and lowers cigarette consumption [311], apparently lowering the risk for cancer due to cigarette smoking. Some *CYPA1/GSTM1 0/0* genotype combinations predisposed the lung, esophagus, and oral cavity of smokers to risks for cancer or DNA damage that were even higher than by either gene polymorphism alone; however, further testing of these findings is needed [312].

Although many studies have indicated that being homozygous null for *GSTM1* or having other GST polymorphisms presents an increased risk for lung cancer, especially among smokers [312–318], meta– and pooled analyses for 43 case-control studies involving >18,000 subjects has shown that *GSTM1*-null does not increase risk for lung cancer among smokers or nonsmokers [319]. Nonetheless, one study has reported an association between the *GSTM1*-null genotype and the presence of *KRAS* mutations in lung tumors of smokers [320]. Although one study [321] found that the urine of *GSTM1*-null smokers was 3.5× more mutagenic than that of *GSTM1* + smokers, another [322] found no influence of *GSTM1* genotype on smoking-associated urinary mutagenicity.

Among smokers, those who were null for either *GSTM1* and possibly *GSTT1* were at increased risk for smoking-associated coronary heart disease [323] or atherosclerosis [324]. A recent study has indicated that the GSTT1-null genotype was associated with increased risk for pancreatic cancer [325] and prostate cancer among smokers [326]. The *GSTM1*-null genotype alone imparted increased risk among smokers for rheumatoid arthritis [327], head and neck (larynx) cancer [328–330], bladder cancer [331], and *K-ras* [320] and *TP53* [332] mutations in various lung tumors. These observations require additional study.

Among smokers, the combination of the CYP1A1-Val/Val mutation and *GSTM1*-null genotype imparted increased risk for esophageal cancer [333] and oral squamous cell carcinoma [334]. Although the GSTP1 polymorphism in exon 5 alone did not increase the risk of lung cancer in male Japanese smokers [335], the *GSTP1313A/G* polymorphism alone and in combination with *GSTM1*-null increased the risk for bladder cancer among smokers in a Turkish population [331].

The presence of two rare alleles of the *HRAS1* mini-satellite was associated with increased risk for lung cancer among Native Hawaiians and Japanese but possibly not in Caucasians [336]. A polymorphism in codon 72 of exon 4 of *TP53* did not appear to influence lung cancer risk among smokers [336,337]. At least three polymorphisms of the *TP53* gene and haplotypes of the three loci were associated with an increased risk for lung cancer [338]. Individuals who smoked and had the xeroderma pigmentosum complement group D (*XPD*) codon 312 Asp/Asp genotype were at an increased risk of *TP53* mutations compared to subjects without these polymorphisms [339]. Polymorphisms in other genes also may impart risk for cancer among smokers, including 8-oxoguanine glycosylase I (*OGG1*), which repairs oxidative damage [340]; various nucleotide excision repair or base excision repair genes, such as *ERCC2* and *XRCC1* [341,342], *XPD* and *XRCC1* [343], sulfotransferase (*SULT1A1*) [344], and microsomal epoxide hydrolase (*mEPHX*) [345].

Consistent with cancer epidemiology studies, certain genetic polymorphisms also may modulate cancer-associated biomarkers among smokers. Thus, *CYP2E* and *ALDH2* [346] and *GSTM1*-null [156,347,348] were associated with increased frequencies of SCEs among smokers. The *CYP1A1 MspI* polymorphism imparted increased risk for elevated chromosome aberrations frequencies in newborns of smokers [164] and increased risk for lung cancer [349]. Although the *N*-acetylation phenotype did not appear to influence urinary mutagenicity among smokers [146], the *NAT1*10* genotype was associated with increased frequencies of chromosome aberrations and lung cancer risk among smokers [350]. The *NAT2* rapid acetylator genotype seemed to be

protective against lung cancer in nonsmokers but was a risk factor for heavy smokers [351]. Rapid *NAT2* genotype also was associated with a high mutant frequency for *HPRT* mutations among ever-smoking subjects diagnosed at high pack-years, whereas the slow genotype was associated with high mutant frequency at low pack-years [352]. The *NAT2* slow genotype, combined with the *GSTM1*-null genotype, conferred increased *HPRT* mutant frequencies and risk for lung cancer when the smoking dose was low [352].

3.8. Mechanistic considerations

Tobacco smoke condensate and its fractions were studied extensively for mutagenicity in microbial systems and for tumorigenicity on mouse skin, and fractions that were initiators or promoters were identified [8]. To date, cigarette smoke contains 81 compounds classified as carcinogens by IARC, and compounds with a high Clog *P* (a measure of lipophilicity) are likely to be mutagens and carcinogens [353]. Extensive experimental data and epidemiology [354], led to tobacco smoke being regarded as exhibiting the features of the classic initiation-promotion model of carcinogenesis [355]. Recent data, largely in humans, combined with a modern synthesis of molecular biology and carcinogenesis [356], have provided a more nuanced view of tobacco smoke carcinogenesis, involving multi-step genetic and epigenetic events in a tissue field in tandem with selection [357–362].

At least six major pathways must be disrupted in order for a normal cell to become a tumor cell [356]. These include self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, tissue invasion and metastasis, sustained angiogenesis, and limitless replicative potential. Disruption of these major pathways can occur through genetic or genomic alterations in well-defined genes or through a number of epigenetic processes, including methylation of DNA, post-translational modifications of proteins, and modification of gene expression patterns [356]. Thus, the changes in the cellular environment caused by tobacco smoke may produce a selection pressure that favors the emergence of cells that have acquired the capacity to undergo clonal expansion. This phenomenon can occur simultaneously at several sites within an exposed tissue field, resulting in multi-focal lesions, some of which can progress to cancer (field cancinogenesis).

Evidence indicates that smoking is associated with some of the genetic and epigenetic changes affecting these major pathways, and linkages have been made between smoking and essentially all of these ''hallmarks'' of cancer for the major smoking-associated tumors [363–374]. Some of these changes are also observed in smoking-associated diseases other than cancer, such as atherosclerosis and rheumatoid arthritis, indicating that these diseases share common

Table 1
Tobacco-associated genotoxicity in human organs in which tobacco or tobacco smoke causes cancer

Organ[a]	Genotoxicity[b]	Reference[b]
Oral/nasal	Micronuclei, chromosomal instability, DNA strand breaks	[190–192,258]
Esophagus	*TP53* mutations	[301]
Pharynx, larynx	*TP53* and *P16* mutations, LOH	[300]
Lung	Chromosomal and gene mutations	[268–270,281–284, 367,368,372]
Stomach	No data	
Pancreas	*KRAS* and other mutations	[299]
Liver	No data	
Myeloid organs	Cytogenetic changes	[247–251]
Kidney	No data[c]	
Bladder/ureter	Mutagenic urine, cytogenetic changes, DNA strand breaks	[116–133,244,259]
Uterine cervix	Mutagenic mucous, epithelial micronuclei	[179,181]

[a] From [4].
[b] List of genotoxic endpoints and references is not comprehensive; see text for more information.
[c] Smokers have mutagenic urine, which may play a role in tumors at this site.

pathways with cancer and that these pathways are disrupted by tobacco smoke [375–377].

Tobacco smoke causes cancer at more organ sites than any other human carcinogen thus far documented [4]. As noted by others [9,10], there is clear evidence for the presence of smoking-associated DNA damage and biomarkers in many of these organ sites at which smoking causes cancer. Table 1 summarizes some of the evidence for the mutagenicity of tobacco smoke at these organ sites, supporting a mutagenic mechanism in the carcinogenicity of tobacco smoke.

There is a clear convergence between the molecular changes induced by tobacco smoke, such as DNA damage [9,10] and mutation summarized here, and recent experimental evidence for mechanisms of carcinogenesis. Collectively, these data support a multi-step model of carcinogenesis in which the components of tobacco smoke are the direct cause of some of the cellular changes that accumulate to drive the carcinogenic process. Among the many findings that are consistent with this view is the observation that one of the low-fidelity human DNA polymerases, Pol kappa, is over-expressed in smoking-associated lung tumors [378]. Such a finding suggests that unrepaired DNA damage elicits the recruitment of this low-fidelity DNA polymerase to replicate past the damage, thereby producing mutations.

Convincing evidence of the carcinogenicity of tobacco smoke was first presented more than half a century ago [379], and the evidence now is overwhelming [380]. Fifty years since that first report, extensive molecular data have accumulated that provide insight into the mechanisms by which tobacco smoke is carcinogenic. Most of these molecular data are in humans, not experimental animals. Thus, neither can their relevance be denied nor their explanatory powers be easily dismissed.

As noted previously [10], the combination of an addictive component and mutagenic/carcinogenic component to tobacco smoke, along with extensive advertising and limited regulation, has made tobacco smoke a primary health hazard in modern times. An estimated half a billion people currently living will die from using tobacco [1], and exposure of nonsmokers to environmental tobacco smoke can have genotoxic and carcinogenic consequences [4,381]. Although reduced smoking may have limited value in reducing cancer risk [382], smoking cessation, even well into middle age, could reduce considerably the attributable cancer risk associated with smoking [354]. Thus, efforts should continue apace to reduce overall exposure to this most extreme example of a systemic human mutagen and carcinogen.

Note added in proof

After this paper was accepted for publication, several papers were published that should be noted. A set of studies showed that the presence of >400 tobacco ingredients (flavorings and other additives) has little influence on the mutagenicity, toxicity, or chemistry of the resulting smoke from CSCs containing various combinations of these ingredients [383–385]. Also, assays measuring hyperplasia and/or inflammation were capable of discriminating between CSCs with different tumor-promoting potentials [386].

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