Tobacco and Cancer: Epidemiology and the Laboratory

***Paolo Vineis*[1] *and Neil Caporaso*[2]**

[1]Unit of Cancer Epidemiology, Dipartimento di Scienze Biomediche e Oncologia Umana, 10126 Torino, Italy; [2]Genetic Epidemiology Branch, National Cancer Institute, Rockville, MD 20850 USA

Tobacco smoke contains many mutagenic and carcinogenic chemicals. Both whole tobacco smoke and extracts induce tumors in experimental animals. Work with carcinogen–macromolecule adducts provided evidence for the action of specific chemicals. Molecular epidemiology studies suggested that point mutations in tumor–suppressor genes (e.g., p53) and oncogenes (e.g., *ras*) may be specific both for the type of tumor and for the critical environmental exposure. The consistency among investigations on oncogene/tumor-suppressor gene mutations in lung cancer (and other tobacco-related cancers) in smokers is highly suggestive, although we still lack information about the time sequence between exposure, gene mutation, and cancer onset. Current work that deserves emphasis includes investigations revealing that lungs of smokers contain benzo[*a*]pyrene diol-epoxide–guanine DNA adducts, which are in accordance with the type of mutations found in K-*ras* or p53 genes (G to T transversions). In addition, DNA in human exfoliated bladder cells showed a derivative of 4-aminobiphenyl as a main adduct; there was also an association between smoking habits (amount and type of tobacco) and the levels of both DNA adducts and hemoglobin adducts formed by aromatic amines. Increasing evidence indicates that genetically based metabolic polymorphisms exert a role in modulating individual susceptibility to the action of tobacco carcinogens. Overall, the weight of evidence strongly supports the causal nature of the association between smoking and cancer and falsifies Fisher's hypothesis that the association was due to confounding by genetic predisposition. *Key words*: adducts, biomarkers, cancer, genetic susceptibility, metabolic polymorphisms, oncogenes, tobacco, tumor-suppressor genes, twins. *Environ Health Perspect* 103:156–160 (1995)

Even after the publication of persuasive evidence linking lung cancer to tobacco smoking (*1*), some investigators questioned that the evidence incriminated smoking as a cause of cancer in humans. In particular, R.A. Fisher, an eminent statistician of this century, claimed that the early epidemiologic observations could not be interpreted as proof of a cause–effect relationship, arguing that one could not rule out that a genetic factor both increased the propensity to smoke and the risk of lung cancer (*2*). A key issue of criticism was that exact knowledge of the mechanisms of tobacco carcinogenesis was necessary to establish a cause–effect relationship. Such criticism was at the root of skepticism toward epidemiological evidence and its applications in public health.

The investigation of mechanisms of cancer induction by tobacco should be reviewed in the light of the large amount of epidemiological evidence that has been produced in the last decades (*1*). In particular, molecular techniques in the epidemiology of tobacco-related cancer have been applied in making three general types of measurement: 1) internal exposure, including the dose at the presumed target tissue (DNA); 2) early biological effects, particularly mutations and cytogenetic damage, likely to be predictive of cancer; and 3) variations in individual susceptibility to carcinogens, mainly via metabolic polymorphisms.

Biomarkers of Exposure in Smokers

Among the 3800 chemicals that have been identified in tobacco smoke, a large number of biologically active compounds are included. The most important chemical families of carcinogens are polycyclic aromatic hydrocarbons, aromatic amines, nitroso compounds (including nitrosamines), volatile compounds such as benzene, and radioelements such as polonium-210. Some of these chemicals, in particular aromatic amines and nitrosamines, are potent carcinogens in animal systems, where they induce tumors in several organs. Also, exposure of animals (rats, hamsters, mice, and rabbits) to whole smoke or condensate induces tumors of the respiratory tract or of the skin. The tobacco-specific nitrosamine NNK [4-methylnitrosamino)-1-(3-pyridyl)-1-butanone] exhibits organ specificity (respiratory) in rodent tumor induction, although there is some dependency on species and route of administration (*3*).

Chemical compounds derived from tobacco smoke have been measured in biological specimens of smokers and nonsmokers. Considering only carcinogenic compounds, tobacco-specific nitrosamine adducts have been found to be higher in the blood of smokers compared to nonsmokers (*4*). Adducts are reaction products between chemical carcinogens and macromolecules. Among the class of respiratory carcinogens known as polycyclic aromatic hydrocarbons (PAHs), benzo[*a*]pyrene forms DNA adducts in the lung that are associated with smoking. Alexandrov et al. (*5*) revealed the presence of benzo[*a*]pyrene diol-epoxide–guanine adducts in lung samples from smokers; these adducts in the guanine bases of DNA are in accordance with the main type of mutations (G to T transversions) found in the K-*ras* oncogene and p53 tumor-suppressor gene. A correspondence between smoke-associated DNA adducts *in vivo* and DNA damage induced by cigarette smoke condensate *in vitro* has been found (*6*).

Tobacco smoke (particularly from air-cured tobacco) contains significant amounts of another class of carcinogens, aromatic amines (*7*). Several lines of evidence involving the measurement of adducts indicate that aromatic amines are relevant to bladder carcinogenesis in smokers. Epidemiologic studies have suggested that the type of tobacco associated with the highest risk of bladder cancer (air-cured tobacco) is also richer in arylamines (*7*), and that smokers of air-cured tobacco have higher levels of 4-aminobiphenyl-hemoglobin adducts in their blood, compared to smokers of flue-cured tobacco (*8*). Some arylamines, including 4-aminobiphenyl and 2-naphthylamine, are among the most potent human bladder carcinogens.

The concentration of 4-aminobiphenyl –hemoglobin adducts in both smokers and nonsmokers is modulated by the *N*-acetylation phenotype; i.e., it is higher in slow acetylators who deactivate 4-aminobiphenyl more slowly (*8*). Talaska et al. (*9*) found that the administration of 4-aminobiphenyl to dogs resulted in the formation of a main DNA adduct, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl, in bladder cells. *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl was one of the main DNA adducts in the bladder cancer biopsies of smoking subjects (*10*). These epidemiologic and experimental observations suggest that arylamines such as 4-aminobiphenyl may be responsible for the excess risk of bladder cancer in smokers (*8*). About 50% of bladder cancers arising in men living in Western countries is attributable to smoking (*1*).

A group of chemicals that is also highly relevant for the mechanisms of tobacco carcinogenesis is tobacco-specific nitrosamines. Although it is unlikely that they play a role in bladder cancer (*11*), nitrosamines have been invoked to explain the excess of esophageal cancer in smokers. Nitrosamines

Address correspondence to P. Vineis, Unit of Cancer Epidemiology, Dipartimento di Scienze Biomediche e Oncologia Umana, via Santena 7, 10126 Torino, Italy.

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have an organ-specific activity for the esophagus in experimental animals (*12*), and metabolic activation of the tobacco-specific nitrosamine NNN has been observed in cultured human esophageal cells (*13*). Although tar delivery did not correlate with the amounts of *N'*-nitrosonornicotine (NNN) and NNK in mainstream smoke, such nitrosamines are much more concentrated in air-cured tobacco than in flue-cured tobacco. Cancers of the esophagus, larynx, pharynx, and oral cavity are more strongly associated with air-cured than flue-cured tobacco smoking (*14–16*).

In the case of other cancer sites associated with cigarette smoking, the demonstration of elevated smoking-specific DNA adducts strengthens the biologic plausibility of the association. For example, the determination of DNA adducts in the cervical epithelium of smokers is relevant to the alleged increased risk of cervical cancer (*17*).

The measurement of biomarkers of internal dose (adducts) has been applied also to the study of environmental tobacco smoke exposure. For example, metabolites of the nitrosamine NNK have been measured in the urine of five nonsmokers experimentally exposed to environmental tobacco smoke (*18*). Such evidence concerning a tobacco-specific lung carcinogen is relevant to the proposal that environmental tobacco smoke can cause lung cancer. Also, protein adducts formed by carcinogenic aromatic amines have been measured in nonsmokers in relation to environmental tobacco smoke exposure (*19*). Several amines have been measured in indoor air: a considerable concentration of compounds like anilines and toluidine was reported, even in buildings where smoking was not allowed (*20*).

Mutations in Oncogenes or Tumor-suppressor Genes

Proto-oncogenes are normal cellular genes that, when activated as oncogenes, cause alterations of growth and differentiation, thus enhancing the probability of neoplastic transformation. Tumor-suppressor genes are normal cellular genes that, when inactivated, also cause alterations of growth and differentiation patterns (*21*).

Mutations in specific genes can be used in molecular epidemiology as "fingerprints" of specific exposures, as surrogate endpoints of cancer, or for the further subtyping of cancer to clarify causal relations (*22*). Epidemiologic evidence suggests the association between chemical exposure, oncogene mutation, and cancer onset (*23–33*). Investigations concerning the *ras* oncogene family or the p53 tumor-suppressor gene have considered the association between lung cancer and tobacco smoking. Results of these studies suggest that the mutational spectrum of lung cancer depends on whether the person was a smoker (*23*). For example, K-*ras* mutations have been demonstrated as a feature of non-small-cell lung carcinoma (NSCLC), and have been found to be associated with heavy smoking. In a study on smokers and nonsmokers affected by lung adenocarcinoma, K-*ras* mutations were found more frequently in smokers [odds ratio (OR) = 5.3; 95% CI, 1.1–25] (*24*). All mutations were in codon 12, mostly G to T transversions, as in other investigations on lung cancer (*25*). G to T transversions of *ras* have also been shown in lung tumors induced in mice with benzo[*a*]pyrene (*26*). In a second study on 48 lung cancer patients, K-*ras* mutations were found in 14 specimens (and in 12 out of 21 adenocarcinomas) (*27*). Also in this case, the most common types of mutations were G to T transversions in codon 12. An association with heavy smoking was found (OR = 4.9; 90% CI, 1.2–19.5) (*27*). However, it is premature to draw firm conclusions; an unexplained finding is that the alleged smoking-associated mutation is most frequent in the histologic type least associated with smoking, at least in past studies.

Investigations concerning p53 involved the association of smoking with carcinoma of the head and neck (*28*), lung (*29*), radon-associated lung cancer (*30*), and urinary bladder cancer (*31–33*). Among patients with cancer of the head and neck, Field et al. found overexpression in 67% (*28*); only 1 out of 7 nonsmokers showed overexpression, versus 29/37 smokers (OR = 22; CI, 3.5–135). Of a group of 10 patients who had given up smoking more than 5 years before, 9 had elevated expression of p53. Suzuki and colleagues (*29*) examined 30 non-small-cell carcinomas of the lung and found p53 mutations in 14. The mutations were mainly of the G to T type and were closely associated with smoking habits, with an estimated OR of 5.3 for smokers of 20 cigarettes per day or more, compared to nonsmokers.

With regard to bladder cancer, different p53 mutations have been observed in Japanese subjects with urothelial cancer (associated with cigarette smoking) (*31*) and among Egyptians (associated with schistosomiasis) (*32*). In one study, the overexpression of p53 in bladder cancer has been related to the number of cigarettes smoked: the relative risk of nuclear overexpression increased up to 8.4 for those who smoked more than 2 packs per day (*33*).

Methodologic issues concerning this type of study should be considered thoroughly. It is critical to distinguish between mutations that are related to specific exposures (mutational spectra) and are an integral step in the carcinogenic process and findings that are epiphenomena or simply related to tumor progression. It is probably premature to conclude that *ras* or p53 genes are causally involved in the mechanism of tobacco carcinogenesis, partly because we lack longitudinal evidence on the time sequence of tobacco smoking, gene mutations, and cancer onset. Among the issues that require clarification, one is the possibility that various studies show different mutational frequencies because of a bias in the selection of patients at various stages of development in tumor progression (*34*). Cells showing p53 mutations or overexpression are selected as a consequence of proliferative advantage and clonal expansion; therefore, it is not obvious that the molecular events directly reflect the effect of specific exposures. An even more radical view is that mutations are the expression of replicative errors with limited biological significance and that cancer phenotypes result from aberrant patterns of normal gene expression (*35*).

Individual Susceptibility and Genetic–Environmental Interactions

Genetic susceptibility to lung cancer has been suggested by different types of investigations. Excess lung cancer mortality was found among relatives of lung cancer patients after allowing for covariates. A difficulty with these types of studies is adequately adjusting for age at initiation of smoking, passive smoking, and "familial" but nongenetic exposures. However, the evidence suggests that smoking and family history are separate risk factors (thus falsifying Fisher's hypothesis) (*36*). In three studies, relatives of cases had an OR of 2–2.5 for mortality from lung cancer compared with relatives of healthy controls, after controlling for smoking (*37*). According to segregation analysis, genetic transmission was attributed to a single codominant locus. Also, investigations of twins show that monozygotic twins discordant for smoking behavior have clearly different risks of lung cancer, which argues against Fisher's hypothesis (*38,39*). In the Swedish Twin Registry, based on more than 12,000 twin members, the relative risk for lung cancer among male smokers was 19.7; among monozygotic men, the relative risk for smoking twins compared to nonsmoking co-twins was 7.0; among dizygotic twins, the estimate was 17.0. These data, consistent with a more recent study from an American World War II Veterans Twin Registry (*40*), suggest that smoking, and not genetic make-up, is the chief determinant of lung cancer.

Additional evidence has been provided by the study of metabolic polymorphisms

(i.e., the different ability, based on the individual genetic make-up, to metabolize chemical carcinogens). Traditional "monogenic" susceptibility to cancer is related to rare diseases or small subgroups of frequent diseases (like *BRCA1* in familial breast cancer) and involves rare mutations identified through linkage analysis. "Polygenic" susceptibility involves frequent genetic polymorphisms, entails a low-to-medium elevation of the risk for frequent diseases, and is identified through epidemiologic case–control studies. One example of metabolic susceptibility based on genetic polymorphism is the CYP2D6 (debrisoquine) phenotype, which has been extensively studied. About 6–8% of whites are "poor" debrisoquine metabolizers (i.e., they slowly deactivate the drug). In a few investigations, slow debrisoquine metabolizers have been suggested to have a reduced risk of lung cancer compared with extensive metabolizers (*37*), although not all investigations agree. According to a pooled analysis, a weakly positive relative risk of 2.3 (95% CI, 1.6–3.4) was found for the risk of lung cancer in fast metabolizers (*37*). Studies based on the determination of the genotype, rather than the phenotype, showed discordant results, with two studies reporting an association between the extensive metabolizer genotype and lung cancer and one showing the opposite relationship (*41*). While the precise mechanism for the effect of the CYP2D6 polymorphism is uncertain, the possibilities include activation of the tobacco-specific nitrosamine NNK, or the recently reported role for CYP2D6 in nicotine metabolism (*42*).

In Japanese studies, but not in Western studies, the CYP1A1 and glutathione *S*-transferase (GST) polymorphisms have been shown to be associated with lung cancer risk and, in the latter case, bladder cancer risk. CYP1A1 metabolizes PAHs such as benzo[*a*]pyrene to reactive electrophilic epoxide compounds, which can form DNA adducts. In the study by Nakachi et al. (*43*), a very strong association was found between lung cancer and the presence of both the "null" *GSTμ1* genotype and the mutant "val/val" CYP1A1 genotype. The relative risk was 41.0 (95% CI, 9–194), suggesting that the combination of polymorphisms may increase the individual susceptibility enormously (*43*). While the association of CYP1A1 with lung cancer has not been confirmed in a Finnish investigation (*44*), the "null" *GSTμ1* genotype or its phenotypic expression has been consistently associated with increased risk of cancers of the lung and the bladder (*45–47*). In some investigations, relative risks for cancer of the lung were in the order of 0.2–0.3 in those with "high" *GSTμ1* activity, suggesting strong protection (*45,46*). In one study, the presence of PAH–DNA adducts in the lung was strongly related to the null *GSTμ1* genotype (*48*).

In the case of bladder cancer, extensive literature has been published on the *N*-acetyltransferase (NAT) polymorphism (*49*). NAT is a noninducible enzyme that deactivates carcinogenic aromatic amines such as 2-naphthylamine and 4-aminobiphenyl. The frequency of the slow-acetylator genotype is about 50% in white populations. Slow acetylators exposed to aromatic amines have been reported to have an increased risk of bladder cancer and higher levels of 4-aminobiphenyl adducts (*50,51*). The study of metabolic polymorphisms, which is rapidly developing, requires a thorough evaluation in terms of study design and causal assessment (*52*).

Multistep Nature of Carcinogenesis

After Berenblum proposed that carcinogenesis consists of an early stage called initiation and a late stage called promotion (*53*), several lines of evidence supporting the multistep nature of cancer have been provided. Intermediate stages of cancer can be identified by histological and biochemical techniques, and preneoplastic lesions progress to neoplastic with varying degrees of probability. Studies of experimental carcinogenesis established that different chemicals affect different stages in the carcinogenic process, and cell transformation studies revealed that different phenotypic properties of tumor cells are acquired by a progressive process. Also, the neoplastic conversion of normal cells requires multiple cooperative genes. Although experimentally induced and spontaneous tumors frequently show mutations of specific genes, e.g., *ras* or p53, many of them do not contain such mutations. With the possible exception of the retinoblastoma gene, mutations in the hitherto identified oncogenes or tumor-suppressor genes do not seem to be sufficient to induce cancer. In skin papillomas induced in mice with PAHs such as dimethylbenzanthracene (a constituent of tobacco smoke) a high frequency of H-*ras* mutations (A to T transversions) was detected. These mutations were heterozygous and were interpreted as early lesions involved in tumor initiation; H-*ras* homozygous mutations were identified in the later stage of progression, leading from papillomas to carcinomas (*54*). Unlike early papillomas, later lesions are dysplastic and aneuploid with nonrandom chromosomal changes and progress to carcinomas with additional karyotypic abnormalities. A high frequency of H-*ras* mutations has been found also in esophageal tumors induced experimentally with *N*-nitroso compounds.

The studies by Auerbach et al. (*55*) on autopsy specimens from the bronchi showed changes in the epithelium that were clearly associated with smoking habits, including basal-cell hyperplasia, loss of cilia, and occurrence of cells with atypical nuclei. The frequency of such changes was higher in smokers than in nonsmokers and increased with the amount of smoking, after adjustment for age.

Epidemiology can contribute to the elucidation of mechanisms of carcinogenesis (early or late stages of action) through the study of timing of exposure and cancer onset. In the case of lung cancer, a large amount of literature has been published that clearly suggests that quitting smoking is followed by a "freezing" of the risk until the risk of quitters relative to that of nonsmokers (i.e., the relative risk) approaches the value of one (*1,56*). Also, age at starting smoking is an independent determinant of the risk of lung cancer because an earlier age at start is associated with a considerably higher incidence of cancer (*1,57*). Similar data have been published for bladder cancer. An interesting observation was made comparing the effects of age at start and time since cessation of exposure among smokers of air-cured tobacco and among workers heavily exposed to carcinogenic aromatic amines (benzidine and 2-naphthylamine). The trends were almost overlapping for the two exposures: in both cases, after 10 years or more since cessation of exposure, the relative risk of cancer was reduced by at least 75%, while the relative risk approximately halved when exposure started after age 25, compared to earlier age at start (*10*). These observations further suggest that the bladder carcinogenicity of air-cured tobacco may be attributed to arylamines.

Conclusions

Tobacco smoke contains many mutagenic and carcinogenic chemicals. Both whole tobacco smoke and extracts induced tumors in experimental animals. Work with carcinogen–macromolecule adducts is important because these compounds provide evidence for the specific action of exposures in the carcinogenic process. A general trend in molecular epidemiology studies is the increasing evidence that point mutations in tumor-suppressor genes and oncogenes may be specific both for the type of tumor and for the critical environmental exposure. The consistency among investigations on oncogene/tumor-suppressor gene mutations in lung cancer (and other tobacco-related cancers) in smokers is suggestive, although we still lack information about the time sequence between exposure, gene mutation, and cancer onset. The epidemiological study of time variables (age at start of smoking, time since

cessation, age at cancer onset) provides suggestions about an early/late action of carcinogens. In particular, published investigations suggest that for lung and bladder cancer, smoking acts both at an early stage and a late stage of the process. In the case of bladder cancer, the similarity between smoking and heavy occupational exposure to carcinogenic aromatic amines is particularly suggestive, as far as timing of exposure is concerned.

Highlights of current work that deserves emphasis include investigations on the lungs of smokers revealing that they contain benzo[*a*]pyrene diol-epoxide–guanine DNA adducts, which are in accordance with the type of mutations found in K-*ras* or p53 genes (G to T transversions). K-*ras* and p53 mutations have been found more frequently among smokers than among nonsmokers affected by lung cancer. DNA in human exfoliated bladder cells show a derivative of 4-aminobiphenyl as a main adduct; there is an association between smoking habits (amount and type of tobacco) and the levels of both DNA adducts and hemoglobin adducts formed by aromatic amines.

Fisher's hypothesis that genetic predisposition both induces smoking habits and increases the risk of lung cancer has been refuted on the basis of twin studies. However, there is evidence of genetic susceptibility to lung cancer, independent from smoking habits, attributed to a single codominant locus. In addition, the ability to metabolize tobacco carcinogens, such as tobacco-specific nitrosamines and 4-aminobiphenyl, seems to depend on a genetically based metabolic polymorphism (*43*). At least in such cases, genetic susceptibility does not seem to be a risk factor per se, but, rather, an effect modifier of the exposure to carcinogens. Meanwhile, public health efforts should focus squarely on the central cause for preventable cancer in the Western world, limiting tobacco use.

The example of smoking and cancer is encouraging for molecular epidemiology, but issues concerning the validation of biomarkers and the design of studies should be addressed. Interesting developments have been proposed concerning both aspects. For example, Taioli et al. (*58*) have tested a model for the validation of biomarkers, which provides information on intersubject, intrasubject, and analytical measurement variability. Begg and Zhang (*59*) have proposed specific statistical tools for the analysis of molecular epidemiology studies using case-series like those concerning tumor–suppressor gene mutations. More generally, methodological issues should be clarified before straightforward causal inferences are drawn (*60*).

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