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Tobacco smoking and cancer: The promise of molecular epidemiology

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Abstract

Neoplasic development is a multistage process that includes multiple genetic changes. In this article the authors review studies on molecular epidemiology of tobaco. Current concepts of the multistage carcinogenic model are reviewed, as are their use in observational studies. Finally, benzo[a]pyrene are analyzed as an example.

Key words: smoking; neoplasms; epidemiology, molecular; review

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Resumen

El desarrollo neoplásico es un proceso multietápico que comprende cambios genéticos múltiples. En este trabajo se hace una revisión de los estudios sobre la epidemiología molecular del tabaco. Se revisan los conceptos actuales del modelo carcinogénico multietápico, así como su utilización en estudios observacionales. Finalmente se analiza el ejemplo relativo a los benzopirenos.

Palabras clave: tabaquismo; neoplasmas; epidemiología molecular; revisión

A half-century has passed since researchers identified the epidemic of lung cancer and the first investigations on its causes were implemented. During these 50 years, tobacco smoking has been identified not only as a cause of lung cancer but also of cancers of the oral cavity and larynx –both sites of direct smoke deposition– and of cancers of more remote sites including the stomach, the pancreas, and the urinary bladder.[1] Smoking is also a suspect cause of the acute leukemias, cancer of the liver, and cancer of the uterine cervix.

Active smokers are exposed to both mainstream (MS) and sidestream smoke (SS). Mainstream smoke is inhaled directly by the smoker, while sidestream smoke is emitted from the smoldering cigarette. The compounds found in the smoke are a rich mixture of toxic, mutagenic, and carcinogenic compounds. These compounds deposit in the body upon inhalation, primarily in the lower respiratory tract on the airways and the alveoli, and, to a lesser degree, in the upper respiratory tract. Upon deposition, some of these compounds are absorbed into the cells and then dispersed systemically, as demonstrated by the presence of tobacco components such as nicotine in blood, saliva, and urine.[2]

Multifaceted scientific research has been conducted on tobacco smoking and disease, including epidemiologic studies, and in vivo and in vitro toxicologic

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studies. The principal evidence characterizing tobacco smoking as a cause of cancer has come largely from observational epidemiologic studies of the cohort and case-control design. The case-control studies have been conducted on specific sites of cancer while the cohort studies have involved the follow-up of large numbers of smokers and never smokers. Supporting toxicologic evidence has included the identification of carcinogens in tobacco smoke and in vivo and in vitro experiments which show that tobacco smoke and its components can produce malignant cells and cancers.[3]

The identification of tobacco smoking as a cause of cancer is one of the heralded triumphs of epidemiologic research. This success in part reflects the simplicity of characterizing exposure accurately to tobacco smoke in epidemiologic studies. With a few questions answered by study participants, epidemiologists have been able to accurately characterize cumulative exposure to tobacco smoke and to derive biologically relevant measures of the intensity of exposure. Duration of smoking can be readily determined, perhaps because of the addictive nature of tobacco smoking which assures a relatively fixed pattern of consumption.[4] Age of starting to smoke provides a readily determined anchor point for the smoking history and persons who have stopped smoking can usually provide the age at which quitting was successful for the long term. The number of cigarettes smoked supplies a relative index of exposure and because there is a limited range of the number of cigarettes smoked across the population, there is little potential for misclassification. The usual quantity in a package –20 cigarettes– gives smokers an easy starting point to answer questions about the intensity of their smoking . In the past, smokers seemed to report their smoking history without bias, although more recently, with declining acceptability of smoking in some countries, a trend of underreporting has appeared.[5]

Using straight-forward and easily completed questionnaires on smoking history, epidemiologists have now conducted numerous investigations on tobacco smoking and cancer. This research on tobacco and cancer has largely been accomplished under a simplistic and empiric epidemiological model regarding the relationship between exposure and disease. The findings of these investigations have definitively established tobacco smoking as causing a number of different types of cancer (Table I). Data from the American Cancer Society studies (CPS-I & II) demonstrated increased risk for mortality in current male and female smokers from specific cancers. For most types of cancer caused by cigarette smoking, the risk for smokers rises with the number of cigarettes smoked per day and with the duration of cigarette smoking. Table II illustrates this risk with lung cancer serving as an example. Age of starting smoking also affects risk, primarily by increasing the duration of smoking. Comparing cancer mortality risks in CPS-II to CPS-I in part reflects a trend over time of the younger age of starting to smoke. The consistent evidence for exposure-response relationships supports the causality of the associations of tobacco smoking with cancer, meeting one of the widely applied criteria for causality in interpreting epidemiologic evidence.

The consistency and extent of the evidence has supported conclusions by the US Surgeon General, the

Table I

ESTIMATED RELATIVE RISKS FOR CURRENT CIGARETTE SMOKERS AND TOBACCO-RELATED CANCER MORTALITY FOR MALES AND FEMALES AGED 35 YEARS AND OLDER, CPS-I (1959-1965) AND CPS-II (1982-1986)

	Males				*Females*			
Cancer death	*CPS-I*		*CPS-II*		*CPS-I*		*CPS-II*	
	RR	*(95% CI)*	*RR*	*(95% CI)*	*RR*	*(95% CI)*	*RR*	*(95% CI)*
Lung	11.35	(9.10-14.15)	22.36	(17.77-28.13)	2.69	(2.14-3.37)	11.94	(9.99-14.26)
Larynx	10.00	(3.51-28.51)	10.48	(3.61-30.43)	3.81	(0.78-18.52)	17.78	(3.45-91.74)
Lip, Oral cavity, and Pharynx	6.33	(3.60-11.13)	27.48	(9.96-75.83)	1.96	(1.14-3.39)	5.59	(3.15-9.91)
Esophagus	3.62	(2.02-6.48)	7.6	(3.81-15.17)	1.94	(1.02-3.69)	10.25	(4.94-21.27)
Bladder, other urinary organs	2.90	(2.01-4.18)	2.86	(1.85-4.44)	2.87	(1.74-4.74)	2.58	(1.31-5.08)
Pancreas	2.34	(1.81-3.02)	2.14	(1.62-2.82)	1.39	(1.04-1.86)	2.23	(1.77-3.08)
Kidney	1.84	(1.23-2.76)	2.95	(1.92-4.54)	1.43	(0.89-2.31)	1.41	(0.86-2.30)
Cervix uteri					1.10	(0.83-1.47)	2.14	(1.06-4.30)

From: USDHHS, 1989[1]
RR= relative risk
95% CI= 95% confidence interval

Table II
DOSE RELATIONSHIPS FOR AGE-ADJUSTED MORTALITY RATES (PER 100,000) BETWEEN INCREASING TOBACCO INTAKE AND DURATION OF SMOKING FOR LUNG CANCER MORTALITY IN PARTICIPANTS IN THE AMERICAN CANCER SOCIETY'S CPS-II

			Duration of smoking (years)			
Gender	*Cigarettes per day*	*Age*	*20-39*	*30-39*	*40-49*	*50+*
	1-19	50-59	11.6	18.4	32.2	–
		60-69	10.6	9.6	21.5	20.5
		70-79	–	6.5	14.0	20.5
Men	20-39	50-59	13.5	29.9	44.5	–
		60-69	22.1	13.7	28.8	53.4
		70-79	–	16.1	18.5	28.4
	40+	50-59	23.6	39.4	67.4	–
		60-69	–	32.8	40.3	52.0
		70-79	–	–	35.4	36.3
	1-19	50-59	4.4	10.1	9.5	–
		60-69	4.6	9.3	10.1	16.5
		70-79	3.3	4.0	5.4	7.6
Women	20-39	50-59	10.3	18.7	35.0	–
		60-69	10.8	15.7	20.5	43.3
		70-79	10.6	9.5	17.4	17.0
	40+	50-59	16.4	24.8	28.8	–
		60-69	–	18.1	37.2	28.8
		70-79	–	–	15.9	23.7

From: USDHHS, 1995[6]

World Health Organization, and other institutions on the carcinogenicity of tobacco smoke. These conclusions and the supporting evidence have been the principal basis for implementing widespread programs for smoking prevention and cessation. Thus, throughout the world, the health risks of cigarette smoking are now widely appreciated; in many countries, packages are labeled with the health risks of smoking; and advertising of cigarettes is controlled. Additionally, widespread educational campaigns seek to deter young children from starting to smoke and to motivate adult smokers to quit. In many countries, children's access to tobacco products is also limited by law and the Food and Drug Administration in the United States has proposed regulations now being implemented to control the access of youth to cigarettes.

While the wealth of observational data has proved sufficient for the broad purpose of controlling tobacco-caused cancers, a number of key issues related to tobacco smoking and lung cancer remain to be addressed. Tobacco smoke is known to contain a number of carcinogens but the specific mechanisms by which components of tobacco smoke cause cancer have yet to be characterized. New research approaches to active tobacco smoking and cancer, however, are now showing that there are numerous exogenous and genetic factors determining how exposure to tobacco smoke leads to cancer. Attempts to understand the relationship between tobacco smoke and cancer have resulted in the development of additional models for research which we will integrate into the original epidemiological model in the course of this paper. The two models of interest are the multistage carcinogenic model, which details the steps required for cancer development, and the molecular epidemiology model of biomarkers, which affords a framework for investigating the sequence of changes from normal to malignant cells and identifyig the roles of environmental and genetic factors.

The widely held multistage model of carcinogenesis implies multiple stages at which tobacco smoke could act, but the effects of various smoke components in affecting the postulated multi-stage process remain to be described. As our knowledge of the sequence of genetic changes that leads to cancer has advanced, we have an expanded biologic understanding of the multistage process and the investigational challenge of learning how tobacco smoke effects tumor suppressor genes and oncogenes. Observational evidence has documented familial aggregation indicative of a genetic basis for lung and other cancers; we are also now searching for the genetic factors related to carcinogenesis that determine susceptibility to tobacco smoke. Candidate genes determine rates and pathways of carcinogen metabolism and rate of DNA repair.

The transformation of normal cells into clinical cancers can be conceptualized as a multiple-step process affected by the metabolic activation of procarcinogens into ultimate carcinogens; the transfer of carcinogenic compounds across cell membranes; the susceptibility of the cell to carcinogenic change (i.e., cell replication); DNA binding and repair; factors affecting tumor growth; and immunologic destruction of tumor cells.

Advances in the techniques of molecular and cellular biology, largely applied over the last decade, have now begun to provide insights into such issues. The findings of this research have the potential to identify determinants of susceptibility and to identify the genetic and other changes resulting from tobacco smoke that ultimately result in the transformation from nor-

mal to cancerous cells. The anticipated evidence will likely bring new approaches to the control of tobacco-caused cancers, including molecular screening tools for identifying the earliest stages of disease, and the capability of identifying genetically susceptible individuals. Specific markers may allow the attribution of cancer to tobacco smoke in individuals, thereby providing a causal link that might facilitate compensation through litigation or other means.

In this paper, we focus on this new line of investigation and highlight studies conducted with the "molecular epidemiology" paradigm. The findings of the epidemiologic literature on tobacco smoking and cancer have been well summarized in the reports of the US Surgeon General,[1,5,7] the monograph of the International Agency for Research on Cancer (IARC) on tobacco smoking,[3] and in a recent monograph published by the National Cancer Institute.[6] Rather than reviewing this well-documented literature, we focus on the findings of recent studies that have used the approaches of molecular epidemiology. We offer a brief review of current understanding of carcinogenesis within the context of the multistage model and summarize the evidence on tobacco smoking and cancer within this framework.

Carcinogenic models for tobacco and cancer

The multistage model of carcinogenesis

The multistage model of carcinogenesis represents a sequence of four events leading from normal cells to clinical cancer: initiation, promotion, conversion, and progression (Figure 1), during which multiple genetic events are postulated to take place. A list of definitions for relevant terms involved in the carcinogenic process are given in Table III; this list is designed to supplement the following discussion.

Tumor initiation. Tumor initiation refers to the direct effects and irreversible changes in the cellular DNA induced by initiators. Evidence of initiation includes adduct formation, mutations, and altered gene expression, and, in many cases, tumor initiation can be equated with a mutational event that leads to altered gene expression. The cells chosen for initiation and the site of the subsequent cancer are determined by the specific tumor initiator. Tumor initiation may involve the activation of proto-oncogenes and/or inactivation of tumor suppressor genes. These genes have been instrumental in the elucidation of carcinogenesis.

Proto-oncogenes/Oncogenes. Proto-oncogenes are normal cellular genes. When a carcinogen creates a genetic event that activates a proto-oncogene, it becomes an oncogene. As an oncogene it will dysregulate cell growth and differentiation pathways, promoting neoplastic development and enhancing tumor growth. For example, the protein products of the *ras* oncogene are involved in signal transduction pathways and transduction of growth factors. Genetic events leading to activation of proto-oncogenes include base-substitution mutations, chromosomal translocations, and gene amplification.[9-12]

Tumor suppressor genes. Tumor suppressor genes can be considered anti-cancer genes, suppressing cell proliferation and hence tumor growth. A genetic event that deactivates the tumor suppressor gene results in a continuous signal or an abnormal signal for cell proliferation and possible growth of a neoplasm. Both alleles of a tumor suppressor gene must sustain mutations to inactivate the function of these gene products.

p53 is the most commonly studied and altered tumor suppressor gene to date.[13] The site of mutagenicity on the target oncogene or tumor suppressor gene depends on the specific carcinogen. Loss of the tumor suppressor gene (i.e., *p53*) function results in unregulated growth and an increased probability of neoplastic transformation and cancer.

Tumor promotion. Tumor promotion is a gradual process requiring prolonged exposure to the promoting agent. It occupies the greater part of the latent period

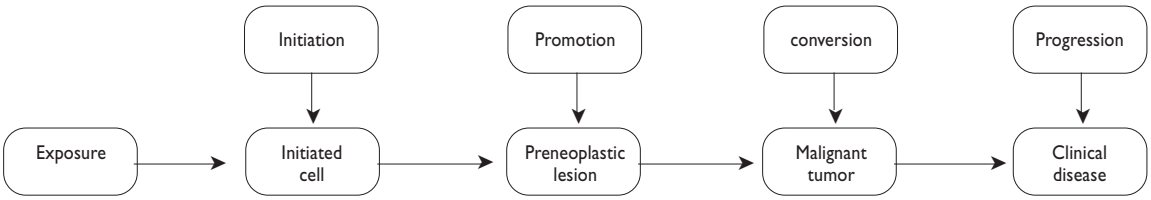

FIGURE 1. MULTISTAGE CARCINOGENIC MODEL (HARRIS, 1991)[8]

Table III
SELECTED TERMINOLOGY RELEVANT TO THE MULTISTAGE CARCINOGENIC PROCESS

Term	*Definition*
Initiators	Tumor initiators are carcinogens or other compounds that begin the carcinogenic process by binding to and damaging cellular DNA, inducing the first genetic event in the multistage process, resulting in an initiated cell
Promoters	Tumor promoters are carcinogens or other compounds that promote the expansion of the initiated cell. They either increase the expression of growth and differentiation of the initiated cell (stimulating cell division), or they selectively induce toxicity to the noninitiated cells in the surrounding tissue
Procarcinogen	Procarcinogens refer to carcinogens which need to be activated metabolically to exert their effects as ultimate carcinogens
Proto-oncogene	A proto-oncogene is a normal cellular gene that becomes an oncogene when activated. As an oncogene, it disregulates cell growth and differentiation pathways, promoting neoplastic development and tumor growth
Tumor suppressor gene	An anti-cancer gene, deactivated tumor suppressor genes result in a continuous or abnormal signal for cell proliferation and possible growth of a neoplasm
Initiation	Tumor initiation is the first stage of the multistage carcinogenic process. Initiation refers to the direct effects and irreversible change in the cell DNA induced by initiators
Promotion	Tumor promotion occurs when initiated cells are selectively expanded into visible benign tumors. Promotion is the second stage of the multistage carcinogenic process, does not require a genetic event, and may be reversible
Conversion	Conversion is the third stage of the multistage process. In this stage, benign tumors are transformed into malignant cells through genetic event(s)
Progression	Progression is the fourth stage of the multistage carcinogenic process. In this stage, the malignant tumor further grows into *clinically* detectable tumors
Genetic event	Genetic events can be DNA mutation, single base pair substitutions, gene mutations, gene amplification, gross chromosomal changes, chromosomal rearrangements, or aneuploidy. A significant genetic event in the carcinogenic process is one that distorts either the expression or the biochemical function of the targeted gene

of carcinogenesis and is partially reversible. Tumor promotion occurs when initiated cells are selectively reproduced and therefore expand clonally into visible benign tumors or loci on neoplastic cells. Initiated cells are selectively reproduced through factors that influence cell proliferation, such as altered growth and resistance to cytotoxicity.

Tumor conversion. To progress from a benign tumor to a malignant tumor, initiated cells must also undergo one or more additional genetic events. Again, activation of proto-oncogenes can enhance the probability of neoplastic transformation as an early or late event. The activation of proto-oncogenes, such as *ras*, or the inactivation of tumor suppressor genes, such as *p53*, have both resulted in malignant tumors. However, conversion may also be an early event in the multistage process, occuring as early as the initiation of the single cell.

Tumor progression. Once preneoplastic lesions have been transformed into neoplastic lesions, tumor progression is defined as the growth and further progression of the tumor to clinically detectable tumors. It requires continued clonal proliferation of the genotypically or phenotypically altered cells. This late stage also includes metastasis to other organs.

Cell proliferation. Cell proliferation contributes to the establishment of the multiple genetic alterations and events that occur. Cell proliferation not only converts adducts to mutations, it decreases the amount of time available for DNA repair processes to remove DNA adducts; subsequently, the unrepaired promutagenic DNA adducts lead to replication errors, possibly resulting in mutations. Initiation can occur after a single, brief exposure to a potent initiating agent, but at least one mitotic cycle is needed for a mutation to become irreversible. Cell replication enhances the effectiveness of mutagens either through committing errors in replication or by converting DNA adducts into mutation. While carcinogens and various compounds can damage DNA, it is cell proliferation that converts these reversible events into irreversible and permanent events.[14]

DNA repair. Before cell replication turns reversible events into irreversible events, there is a window of opportunity where covalent binding of carcinogen adducts to DNA can be repaired. This DNA repair is im-

portant in the carcinogenic process, for it can prevent the initiation of carcinogenesis. If cell proliferation is activated or increased, the time available for DNA repair will be decreased and the opportunity for tumor initiation will be increased. Furthermore, if DNA repair is ineffective or faulty, the probability of tumor initiation is also increased.[14,15]

Summary. Neoplastic development is a multistage, progressive process that involves multiple genetic changes. The constant and long term exposure to compounds from tobacco smoke in active smokers can lead to the multiple genetic events that are needed to cause cancer. The increasing risk for cancer with increasing duration of active smoking is consistent with the multistage model since there is a higher probability for the necessary multiple genetic events to occur for neoplastic development with a longer smoking history. Likewise, the risk for cancer in ex-smokers decreases with lengthening time since quitting.[5] When genetic events that are needed for neoplastic development do not occur due to the absence of exposure, cancer risk declines.

The total carcinogenic activity of tobacco smoke reflects the combination of initiators, promoters, and compounds activating cell proliferation. Human exposures to tobacco smoke consist of simultaneous and repetitive exposure to the initiating agents and tumor-promoter mixtures that are found in tobacco smoke. These mixtures likely induce repetitive rounds of DNA damage, tumor promotion, and clonal expansion (Figure 2).

Because exposures to these compounds are simultaneous, it is difficult to distinguish when in the multistage carcinogenic process specific genetic events occur. However, with the identification of molecular biomarkers, the investigation of carcinogenic mechanisms has been enhanced. With biomarkers, researchers can isolate and monitor specific genetic events from time of exposure to the disease state. The identification of genetic events incurred by different compounds at varying times and sites is gradually allowing us to piece together the mechanistic picture of active smoking and cancer.

The molecular epidemiology model

Biological markers are instrumental in assessing exposures for epidemiological studies, as well as in diagnosing diseases in the clinical setting. They consist of markers of internal dose, biologically effective dose, early biologic effect, and altered structure or function (Figure 3). Briefly, *markers of internal dose* measure toxic chemicals absorbed and/or distributed in the body. *Markers of biologically effective dose* measure toxicological damage such as DNA and protein adducts. *Markers of early biologic effect* are measures of irreversible damage such as mutations in oncogenes or tumor suppressor genes. *Markers of altered structure and function* such as gene amplification and genomic instability represent the ramifications of the irreversible damage that occurred under early biologic effect. As the name suggests, they measure altered structure and function of the gene and gene product, while *markers of susceptibility* distinguish individuals who may be more susceptible to the carcinogenic effects of the exposure.[15-17]

The identification of biological markers helps to describe the multistage carcinogenic process. Associations of tobacco compounds with markers of biologic effective dose, early biologic effect, and altered structure and function indicate compounds that may be responsible for creating the irreversible genetic events needed for the multistage carcinogenic process. Furthermore, the sequence and progression of the markers identified shed light on the mechanistic aspect of the genetic event created.

Tobacco smoke carcinogenesis

Tobacco smoke compounds

Over 4 000 individual compounds have been identified in tobacco smoke, including toxic, mutagenic, and carcinogenic compounds. While carcinogens directly cause cancer, toxic and mutagenic agents also contribute to the carcinogenic process leading indirectly to cancer. For example, ciliatoxic compounds such as formaldehyde and acrolein are not carcinogens themselves but they may contribute to the carcinogenic process by inhibiting lung clearance and thereby prolonging contact of smoke compounds with the respiratory epithelium. Toxic agents such as hydrogen cyanide and ammonia function similarly.[18] Prolonged exposure to tobacco smoke, received by active smokers, provides adequate contact with compounds for the initiation and eventual completion of the multistage carcinogenic process.

Carcinogens in tobacco smoke have been determined by the International Agency for Research on Cancer. Forty-three compounds found in tobacco smoke are classified as human carcinogens with sufficient evidence, according to IARC's 1986 monograph on tobacco smoking.[3] These carcinogens include polyaromatic hydrocarbons, heterocyclic hydrocarbons, N-nitrosamines, aromatic amines, aldehydes, inorganic compounds, and radio-elements such as polonium.

Tobacco compounds, and more specifically the human carcinogens found in tobacco smoke, cannot

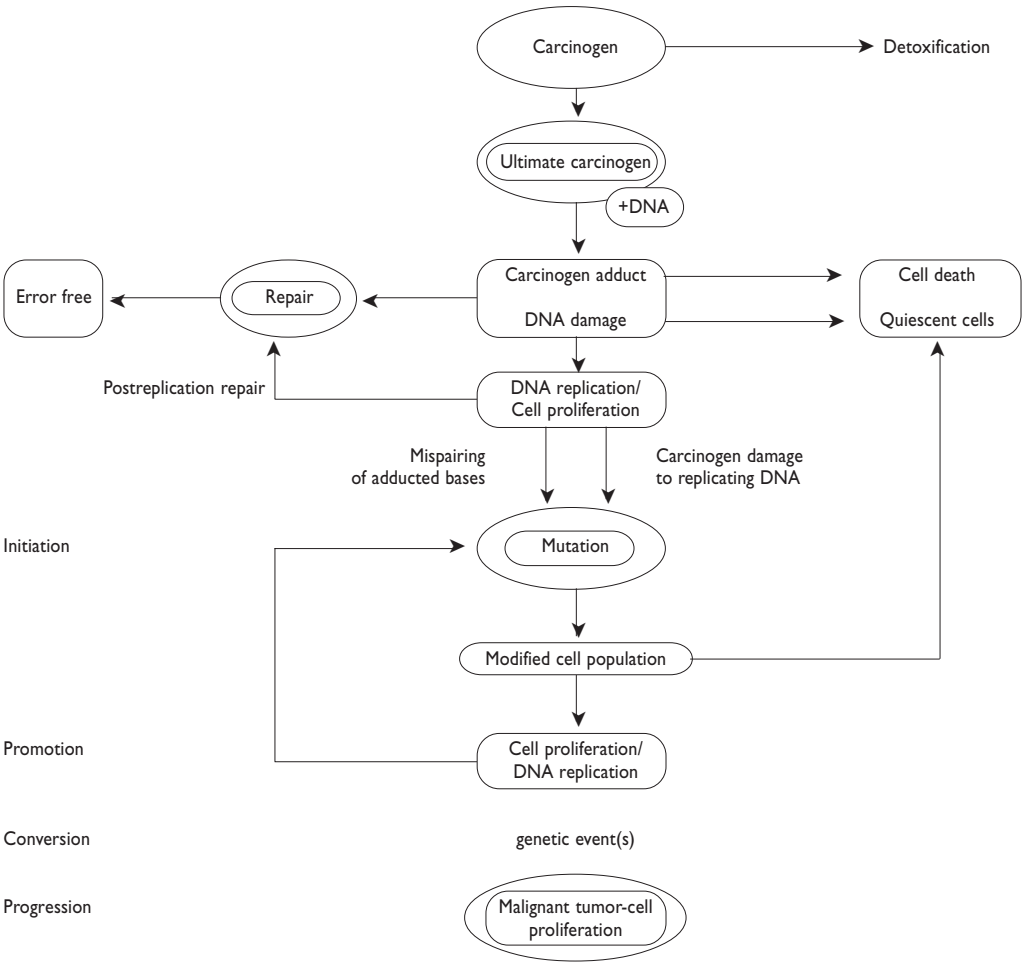

FIGURE 2. ROLES THAT CELL PROLIFERATION AND DNA REPAIR PLAY IN THE MULTISTAGE CARCINOGENIC PROCESS OF INITIATION, PROMOTION, CONVERSION, AND PROGRESSION (ADAPTED FROM IARC, 1992)[14]

be classified simply and rigidly by their actions in the multistage model. Carcinogens may interact with other carcinogens, as well as with outside agents, to produce multiple effects. The damage caused by specific carcinogens can be more harmful in the presence of other carcinogens, whether from tobacco smoke or from work or environmental exposures, or they may negate the effect of a certain carcinogen. Nevertheless, one method of classifying tobacco smoke carcinogens is by their actions as either initiators or promoters.[7] This classification was originally derived from experimental studies on animals that involved a two-step model. These assays denoted the initial carcinogen painted on an animal as an initiator, able to cause tumors by themselves, and usually a carcinogen. A second compound, not a carcinogen and denoted a promoter, was then painted on top of the initiator, upon which more tumors were produced, thus enhancing the carcinogenic effects of the initiator.[19] The definition of a promoter has since been expanded to include carcinogens. While this system classifies carcinogens, it is important to note that tumor initiators and promoters in this two-step experimental paradigm also include compounds which are not necessarily complete carcinogens.

Tumor initiators and promoters are not limited in their actions to the initiation and promotion stages; they contribute to the development of genetic events throughout the carcinogenic process. Initiators cause

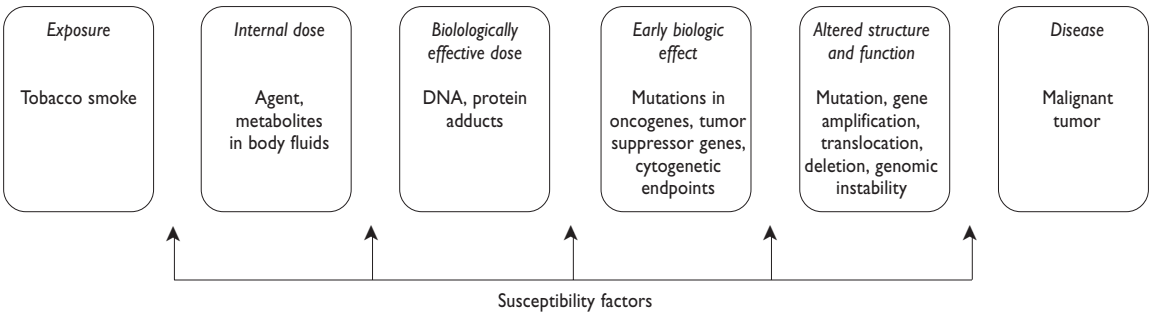

FIGURE 3. BIOLOGICAL MARKERS IN THE MOLECULAR EPIDEMIOLOGICAL MODEL

the genetic changes necessary for carcinogenesis, and promoters propagate these genetic events. This sequence of events may occur many times before the carcinogenic process is completed.

Tumor initiators. Tumor initiators begin the carcinogenic process by binding to and damaging cellular DNA, and inducing the mutations, which are assumed to represent the first genetic event in the multistage process. For chemical compounds, the initiator determines the specific genetic event and the location in the specific gene where it occurs. These genetic events can range from single base pair substitutions, gene mutations, and gene amplification, to gross chromosomal changes, chromosomal rearrangements, and aneuploidy. For the genetic event to be relevant in the carcinogenic process, it must distort either the expression or the biochemical function of the targeted gene.

Genetic events include distortions of proto-oncogenes and tumor suppressor genes. Point mutations activating proto-oncogenes or inactivating tumor suppressor genes have been studied extensively. The identification of genetic events occurring in various proto-oncogenes and tumor suppressor genes helps to determine the specific tobacco compound that created the specific genetic event contributing to neoplastic development. It is important to note that while initiators create the genetic events for the initiation of the multistage carcinogenic process, they also create genetic events at subsequent stages of the carcinogenic process and are not limited to initiation.

Tumor promoters. Tumor promoters were originally defined as compounds with very weak or no carcinogenic activity when tested alone in animals but enhance tumor formation following exposure to an initiator. The current definition of tumor promoters, however, includes all compounds, including carcinogens, that enhance tumor formation. Tumor promoters affect either the initiated cell or its uninitiated neighbors. Some promote the expansion of the initiated cell by modulating and increasing the expression of growth and differentiation of the initiated cell (stimulating cell division), while others selectively induce toxicity to the noninitiated cells in the surrounding tissue. Through either mechanism, the result is the specific expansion of the initiated cells. Unlike initiators, tumor promoters do not necessarily promote tumor growth through a specific genetic event such as a mutation; rather, they act to enhance cell proliferation and clonal expansion of previously mutated cells by altering signal transduction pathways, gene expression, or cellular differentiation.

Molecular epidemiology of tobacco smoke and cancer

Biomarkers of internal dose

Recent epidemiological studies have used biological markers to measure tobacco exposure. Termed *biomarkers of internal dose*, these markers measure prevalence of exposure by identifying toxic chemicals (tobacco products) that have been absorbed and distributed in the body. These chemicals and their metabolites are found in the body in blood, urine, feces, breast milk, amniotic fluid, sweat, hair, nails, saliva, and breath.

Markers of internal dose used to determine tobacco exposure include nicotine, cotinine, thiocyanate, and carbon monoxide. Smoking status in individuals has been examined extensively by measuring nicotine or cotinine (a metabolite of nicotine) levels in the blood or urine. While nicotine and cotinine are both specific to tobacco and tobacco smoke, cotinine is preferred as

a marker because it has a longer half-life, and therefore reflects chronic exposure. Studies using internal dose biomarkers as surrogates for exposure strengthen the associations between smoking and cancer.

Biomarkers of biologically effective dose

Markers of biologically effective dose are direct products of toxicological damage, reflecting the amount of carcinogen that reaches the target macromolecule (DNA or protein).[20] These markers include DNA and protein adducts and constitute the first indication of a biological response to the carcinogen. Markers of biologically effective dose measure mostly recent exposure. They help to identify which compounds in tobacco smoke are likely to be responsible for creating genetic events.

Tobacco carcinogens associated with markers of biologically effective dose. The carcinogens polyaromatic hydrocarbons (PAHs), nitrogen compounds (N-nitroso compounds, nitrosamines, nitrogen oxides), catechols, ethylene, tobacco specific nitrosamines,[21] and quinones, all cause oxyradical damage, resulting in DNA adducts. Specific tobacco adducts include benzo[a]pyrene diol-epoxide-guanine adducts (BPDE), 4-aminobiphenyl-Hb adducts (4-ABP), NNK,NNN-Hb,[22] PAH-DNA adducts, and PAH-protein adducts.[23,24]

In human studies, bronchial tissue DNA adducts have been found in all current and most ex-smokers.[25] An increase in DNA adducts has also been shown in the bladder tissue and oral mucosa of tobacco chewers and smokers.[26,27] The specific adducts formed are determined by the site at which the tobacco compounds are deposited and by how they are metabolized.

Biomarkers of biologically effective dose associated with tobacco-related cancers. PAH-DNA adducts in leukocytes are associated with lung and urinary bladder cancers. Furthermore, 4-ABP-hemoglobin adducts[28] and N-(deoxyguanosin-8-yl)-4-aminobiphenyl adducts are associated with bladder cancer biopsies in smokers.[29] Adducts identified as associated with tobacco-related cancers can serve as another method for measuring exposure to tobacco smoke. More importantly, these adducts help to identify carcinogens that may play roles in the multistage carcinogenic process. They also provide biological plausibility in associating tobacco exposure to specific cancers, such as the identification of DNA adducts in the cervical epithellium of smokers as evidence for the association between tobacco smoke and cervical cancer.[30]

Biomarkers of early biologic effect

If adducts are present at the time of DNA synthesis, they can interfere with DNA transcription and result in mutations.[31] Biomarkers of early biologic effects on DNA reflect irreversible damage which may or may not lead to dysfunction or structural damage. The irreversible damage incurred can be in the form of gene mutations or cytogenetic endpoints and may be one of the multiple genetic events necessary for the multistage carcinogenic process. Well-documented biomarkers of early biologic effect are mutations in oncogenes and tumor suppressor genes. Compounds associated with early biologic effects are possible initiators since they may lead to a genetic event relevant to the multistage carcinogenic process.

Carcinogens associated with markers of early biologic effect. The benzo[a]pyrene diol-epoxide-guanine adducts (BPDE), 4-aminobiphenyl-Hb adducts (4-ABP), NNK, NNN-Hb, PAH-DNA adducts, and PAH-protein adducts all have the potential to lead to specific mutations in their target organs. Nitrosamines result in base substitutions due to mispairing at sites where adducts are formed.

Markers of early biologic effect associated with tobacco-related cancers. Cytogenetic studies have revealed common breakpoints associated with lung cancer, head and neck cancer, esophageal squamous cell carcinomas, and bladder cancers. Mutations in dominant oncogenes have been noted in non-small-cell lung cancer. More specifically, mutations in the *k-ras* oncogene at codon 12 are associated with increased lung cancer incidence.[32] Mutations in the *rb* oncogene are also universally associated with small cell lung cancer.

p53 tumor suppressor gene mutations have been identified with cancers of the esophagus and bladder, and are almost always present in small cell lung cancer and frequent in non-small-cell lung carcinoma.[33-36] The site-specific mutations have been shown in codons 245 through 248 in HNSCC. In fact, over 60% of lung cancers have mutations in their *p53* gene.[37]

Biomarkers of altered structure and function

Biomarkers of altered structure and function are defined as the inactivation or disregulation of a particular gene or protein as a result of the early biologic effect. For example, tumor suppressor genes can be inactivated by mutagenic mechanisms. Therefore, mu-

tations that render the *p53* tumor suppressor gene inactive are markers of altered structure and function. Tobacco compounds associated with markers in this category have an increased probability of playing a role in the carcinogenic process, for they demonstrate true genetic events that may be relevant in the multistage carcinogenic process.

Carcinogens associated with markers of altered structure and function. Exposure to tobacco smoke is associated with micronuclei in peripheral blood lymphocytes and in the oral mucosa. It is also associated with chromosomal aberrations in peripheral blood lymphocytes and sister chromatid exchanges in peripheral blood lymphocytes and bone marrow cells. These aberrations increase with the number of cigarettes smoked and with increasing tar content of the cigarette. Oncogene activation, such as that due to *k-ras* mutations,[38] is also elevated in the serum of smokers.

Markers of altered structure & function associated with tobacco cancers. Loss of heterozygosity (LOH) of the *p53* tumor suppressor gene is seen in over 70% of oral cavity cancers,[39] as well as carcinomas of the head, esophagus,[40] neck,[41] HNSCC,[42] bladder,[43] and lung,[36,44] which are largely related to tobacco smoke. *p53* mutations found in bladder cancer have furthermore been shown to be related to the number of cigarettes smoked.[45] Altered function in six families of proto-oncogenes (*ras, raf, fur, neu, jun* and *myc*) is associated with human lung cancer.[10] *H-ras* oncogene overexpression and sister chromatid exchanges are elevated with increasing packyears of smoking.

The 11q13 breakpoints seen in peripheral blood lymphocytes of smokers are associated with overexpression of the cell cycle regulator protein cyclin D1. Cyclin D1 normally stimulates DNA transcription and cell division. Overexpression of cyclin D1 therefore contributes to tumor proliferation and, hence, to the multistage carcinogenic process. 11q13 amplification is seen in up to 50% of HNSCCs[41] and is also amplified in a subset of lung, bladder, and esophageal cancers.[46,47]

A deletion in the *3p* gene results in altered cell cycle regulation in tobacco cancers and is homologous to a deletion of the ubiquitin-activating enzyme.[48] Ubiquitin plays a role in cell cycle regulation by tagging specific proteins for destruction, such as CCND1 (cyclinD1). Because loss of the ubiquitin-activating enzymes does not lead to the destruction of proper genes, the loss of cell cycle control through the CCND1 gene becomes a possible mechanism for tobacco-induced cancers.

Susceptibility

An understanding of the metabolism of compounds is necessary prior to a discussion of biomarkers of susceptibility. Many markers of susceptibility of current interest relate to an individual's ability to activate or deactivate specific compounds found in tobacco smoke.

Metabolism

Once a procarcinogen or carcinogen is absorbed into a target cell, it is metabolized; metabolism can enzymatically activate the carcinogen and generate an electrophilic metabolite, or it can enzymatically detoxify the carcinogen into a more polar, water-soluble metabolite. Carcinogens requiring enzymatic activation for their carcinogenic effects (as ultimate carcinogens) are termed procarcinogens. For example, N-substituted aryl compounds must be activated metabolically to exert their acute toxic and genotoxic effects. They are generated by N-oxidation of arylamines and by reduction of nitro-aromatic compounds. Ultimate carcinogen metabolites bind to DNA and denote important events in the tumor initiation and malignant conversion stage of carcinogenesis. Ultimate genotoxic metabolites react with DNA and generate promutagenic adducts. Humans vary significantly in their ability to convert environmental carcinogens into metabolites that interact with cellular DNA. Specific organs also differ in their metabolism of compounds.

Metabolism of compounds absorbed by cells leads to either activation or inactivation of carcinogens. A number of compounds, including polyaromatic hydrocarbons, undergo chemical transformation within the body to metabolites considered to be active carcinogens. A majority of transformations are mediated through a mixed-function oxygenase system located predominantly in the microsomal function of the cell. Metabolism of various xenobiotics includes the cytochrome P450 enzymes, epoxide hydrolase, glutathione-S-transferases, and N-acetyl transferase. Methylation, epoxide formation, and alpha-hydroxylation also play roles in metabolic activation of tobacco compounds.[15]

Most carcinogens are activated by oxidative metabolism (e.g., through the cytochrome P450's). Cytochrome P450 IIIA4 is the major liver enzyme involved in oxidative bioactivation of carcinogenic polycyclic aromatic hydrocarbons. The cytochrome P450 IIE1 oxidizes primary alcohols to corresponding aldehydes and activates nitrosamines. P450 IA2 N-oxidizes a number of carcinogenic aromatic amines and activates

4-aminobiphenyl to genotoxic derivatives. P450 IA1 activates cigarette smoke[49] outside the liver, including in the placenta, lymphocytes, skin, and lungs.

Flavin-containing mono-oxygenases are flavoproteins that catalyze mono-oxygenation of amines and sulfur compounds, and the oxygenation of several procarcinogenic compounds such as N-methyl arylamines, 2-aminofluorene, 2-napthylamine, and hydrazine. Other enzymes involved in metabolism are peroxidases, acetyltransferases, N-methyl transferases, sulfotransferases. Peroxidases are capable of activating some procarcinogens including benzo[a]pyrene-7,8-dihydriol to epoxide and ultimate carcinogens. Acetyltransferases are involved in metabolism of carcinogenic aromatic amines.

Glutathione-S-transferases are cytosolic enzymes and usually have protective roles. They catalyze conjugation of potentially deleterious electrophiles, such as benzo[a]pyrene-7,8 oxide and benzo[a]pyrene-7,8 dihydriol-9,10-oxide, with glutathione as the first step of elimination. However, some procarcinogens can also be activated by conjugating with glutathione.

Epoxide hydrolase normally has a protective role removing electrophilic epoxides. However, it, too, can activate compounds such as benzo(a)pyrene by hydrolyzing the benzo[a]pyrene-7,8 oxide to a dihydriole, which is the substrate for P450 oxidation into the ultimate carcinogen 7-8 dihydriol-9,10-oxide.[50]

Biomarkers of susceptibility

An individual's susceptibility to cancer depends on his or her metabolism (uptake, activation, and detoxification) of carcinogenic compounds, capacity for DNA repair, inherited or acquired alterations in proto-oncogenes or tumor suppressor genes, nutritional status, hormonal factors, and immunologic factors. Markers of susceptibility examined to date include enzymes involved in metabolism: aryl hydrocarbon hydroxylase (AHH), N-acetyltransferase (NAT), cytochrome P450, and glutathione S-transferase (GSTM1). Another biomarker of cancer susceptibility, but not involved in metabolism, is mutagen sensitivity, which measures the number of chromatid breaks per lymphocyte.[51]

Carcinogen-metabolizing enzymes as markers of susceptibility. Aryl hydrocarbon hydroxylase (AHH) is an enzyme which converts polycyclic aromatic hydrocarbons (PAHs) to more active carcinogens.[52] Lung AHH activity has been correlated with the level of DNA adducts derived from tobacco smoke.

N-acetyl transferase (NAT) is a cytosolic enzyme involved in the metabolism and deactivation of a variety of carcinogenic aromatic amines such as 2-napthylamine and 4-ABP. The N-acetyl transferase modulates the concentration of 4-ABP-Hb adducts found; slow acetylator phenotypes have higher concentrations of the adduct since they deactivate 4-ABP more slowly.[9] N-acetylation also catalyzes the competing detoxification C-acetylation.

p450 activity varies widely in individuals exposed to polycyclic aromatic hydrocarbons (PAH). In conjunction with AHH, the cytochrome P450 1A1 catalyzes the oxidation of polycyclic aromatic hydrocarbons such as benzo[a]pyrene. Racial comparisons of P450 IA1 genotype distributions have shown the high-risk genotype is limited to individuals of Asian descent, supporting the concept of genetic susceptibility to tobacco-induced cancers.[26,53] The cytochrome P450 IIE1 family has been found to activate nitrosamines such as N-nitrosodimethylamine. The GSTM1 null genotype has also been associated with increased sister chromatid exchanges, PAH-DNA adduct levels in lung tissue from autopsy specimens and *p53* mutations caused by PAH's.[54]

Markers of susceptibility associated with tobacco-related cancers. Lung cancer patients who smoke exhibit high levels of aryl hydrocarbon hydroxylase. Lung cancer is also seen in smokers with low lymphocyte GSTM1 activity.[55,56] Individuals who possess the high risk P450 IA1 genotype and a null GSTM1 genotype have a higher risk of lung cancer than those with either marker alone.[57] Deficiency of GSTM1 has also been associated with bladder and larynx cancer,[58] although this relationship is not yet confirmed.[59-61] Bladder cancer is also associated with a decrease in N-acetyltransferase (slow acetylator phenotype), while 'fast' acetylator phenotypes are associated with colon cancer.[27]

Individuals found to be mutagen sensitive are significantly associated with risk of adenocarcinoma and squamous cell carcinoma. Approximately 55% of lung cancer cases have also been shown to be mutagen-sensitive.[62] While mutagen sensitivity can be used as a predictor of cancer development, the specificity of chromatid breaks that defines mutagen sensitivity does not identify specific compounds in tobacco smoke with the cancer that develops.

Adding the markers of susceptibility to the molecular epidemiological model, we complete the model for smoking and cancer (Figure 4). Susceptible markers indicate which individuals are more like-

ly to experience genetic events that may play roles in the carcinogenic process.

An integrated model-benzo[a]pyrene

The findings on benzo[a]pyrene, a prototypical carcinogen in tobacco smoke, illustrate how molecular biomarkers can be used to examine mechanisms in the multistage carcinogenic model. A component of tar, the polyaromatic hydrocarbon (PAH) benzo[a]pyrene is found in quantities of approximately 20-40 ng per cigarette, and is one of the most potent mutagens and carcinogens known. Active smokers are exposed to this carcinogen upon inhalation of the tobacco smoke, which is subsequently deposited in the lungs. Here, benzo[a]pyrene is metabolized and activated to become the ultimate carcinogenic metabolite [(+)-anti-7b,8a-dihydroxy-9a,10a-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene], otherwise known as BPDE (Figure 5). Many of the susceptibility markers are involved in this process, including the activating enzymes cytochrome p450s, epoxide hydrolase, and peroxidase, and deactivating enzyme glutathione S-transferase.

BPDE binds to DNA and forms benzo[a]pyrene diol epoxide adducts (BPDE adducts). As stated previously, adduct formation is one of the first steps in the carcinogenic pathway and is identified as a marker of biologically effective dose; this marker also verifies an individual's exposure to tobacco smoke, and specifically to benzo[a]pyrene. Adducts formed may or may not result in genetic events and contribute to the carcinogenic pathway; however, BPDE adducts attach onto the *p53* tumor suppressor gene, notably at codon 157 in lung cells, creating a mutation on this gene. Mutations in the *p53* gene are identified as markers of early biologic effect. This action damages the *p53* tumor suppressor genes, incurring a loss of heterozygosity (LOH).[64]

The resulting loss of *p53* function is a marker of altered structure and function. This genetic event leads to the disfunction in the *p53* gene, allowing initiated tumor cells in the lung to grow. When applied to the multistage model, this genetic change occurs after initiation, contributing to the progression and late stage of carcinogenesis. Researchers have concluded that, in fact, BPDE adducts attack the lung cells and transform them into malignant cells. The *p53* mutations aid in this transformation and further assist in the uncontrolled growth of the malignant cells to produce disease. *p53* protein alterations provide a selective advantage for clonal expansion of preneoplastic and neo-

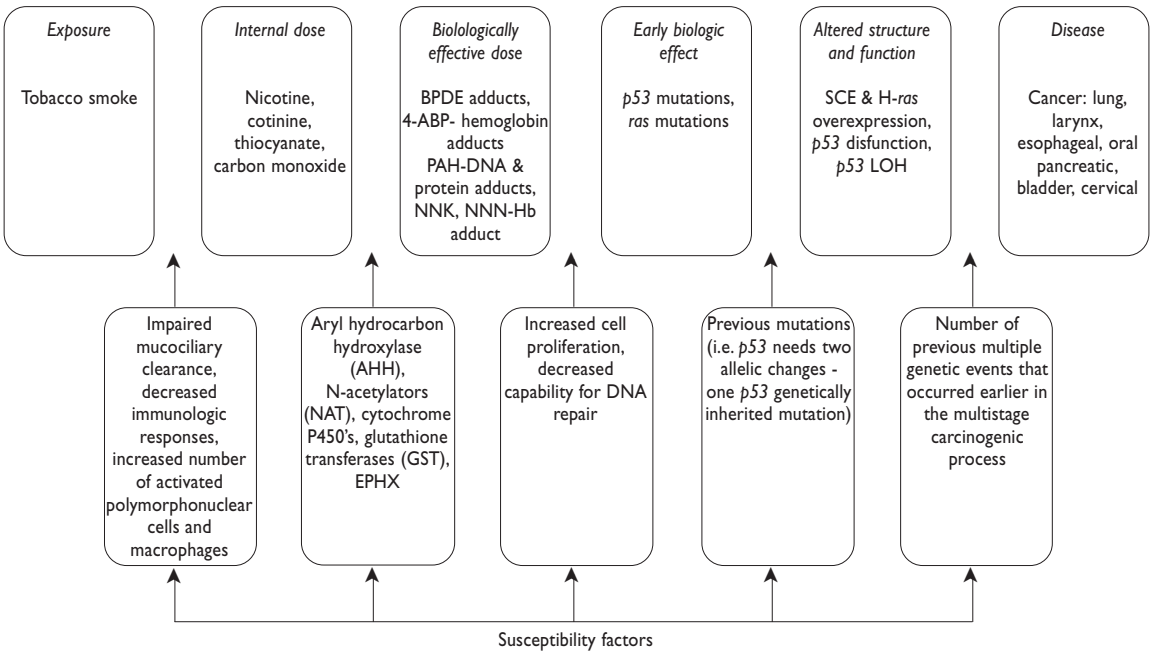

FIGURE 4. EXAMPLES OF TOBACCO SMOKE BIOMARKERS

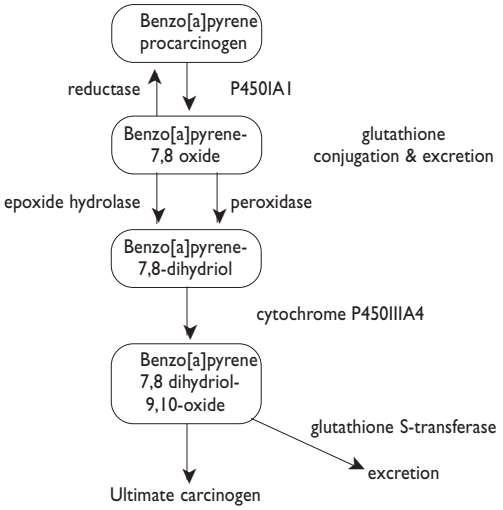

FIGURE 5. BENZO[A]PYRENE METABOLISM AND ACTIVATION INTO AN ULTIMATE CARCINOGEN (ADAPTED FROM AMOS ET AL, 1992)[63]

plastic cells. Researchers have so far identified the *p53* genetic event (codon 157) as playing a role in conversion and contributing to the progression of the neoplasm.

Further isolations of genetic events can determine what is needed for initiation and progression. As more biomarkers are identified, the probability of identifying genetic events that help explain the carcinogenic mechanisms of tobacco smoke and benzo[a]pyrene is increased.

Conclusion

Biological markers allow us to identify specific compounds which are responsible for genetic events in the multistage carcinogenic process. Adduct formation (biological effective dose), mutations (early biologic effect), and loss of function (altered structure and function) represent the actions of specific compounds. Susceptibility markers determine which individuals are more likely to experience genetic events and subsequently cancer. The sequence of biological markers that leads to possible genetic events can be integrated into the multistage carcinogenic model. The integration of the two models demonstrates how identification of biomarkers not only enables us to identify genetic events but elucidates the mechanism and specific compounds contributing to cancer formation.

The identification of additional compounds in tobacco smoke is on going. Current studies are concerned with determining carcinogenic effects from specific individual compounds. This information has revealed much of what is contained in the 'black box' model. Both the molecular epidemiological and the multistage carcinogenic models fit in the black box and have been integrated in Figure 6. Biological markers help identify the mechanisms involved in forming the needed genetic events. The multistage model shows where and when these genetic events occur.

Examples of carcinogens associated with biological markers and markers associated with cancers have been illustrated in this paper. However, this is not a comprehensive list of present knowledge, but serves only as an example of the present knowledge available. While extensive research has been performed on

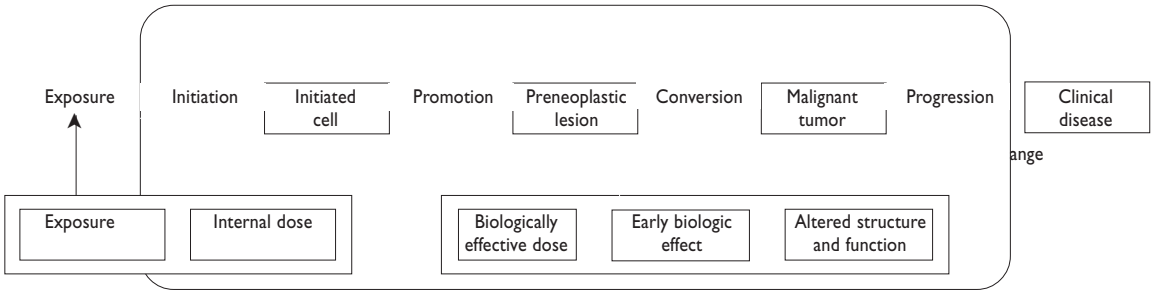

FIGURE 6. INTEGRATION OF MOLECULAR EPIDEMIOLOGICAL BIOMARKERS WITH THE MULTISTAGE MODEL OF CARCINOGENESIS AND THE EMPIRIC EPIDEMIOLOGIC BLACK BOX MODEL

certain markers, sample sizes and extensions to the population-level are still limited. To verify these marker associations, researchers need to perform epidemiological studies using the molecular epidemiology approach.

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