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The first goal of this project is to determine the frequency of genetic polymorphisms for carcinogen metabolism and the p53 mutational spectra in a previously conducted breast cancer case-control study. We have determined that the NAT2 slow acetylator genotype and cigarette smoking is a risk factor for postmenopausalCaucasion women. Analyses for NAT1 is now in progress. We also have found that the alcohol dehydrogenase gene increases alcohol-related breast cancer risk. The sequencing for the p53 mutational spectra is in progress. To corroborate the epidemiological data, over 30 breast cell strains have been established and metabolism is being studied in relation to genotypes. Finally, to identify smoking related risk, we have been studying smoking behavior and addiction. Thus far, genetic polymorphisms in the dopamine transporter and dopamine receptors have been associated with smoking. For the latter, in African Americans, the data indicates that the genetic polymorphism might predict the success of smoking cessation therapy.

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INTRODUCTION

The first goal of this project is to determine the frequency of genetic polymorphisms for carcinogen metabolism and the p53 mutational spectra in a previously conducted breast cancer study designed to assess nutritional risk factors, seeking to identify risk factors related to inheritable susceptibilities and chemical etiologies. The workscope was subsequently expanded to include the same goals, but for other epidemiological studies of breast cancer, and to perform studies of breast metabolism, p53 and smoking (including smoking cessation). The DOD grant allows us to examine a variety of risk factors (hormonal and non-hormonal; environment and diet; carcinogens and anticarcinogens) in relationship to p53 mutations and breast cancer with genetic polymorphisms as effect modifiers. The frequency of genetic polymorphisms themselves in relation to breast cancer and to p53 mutations are being determined.

A population-based case-control study of breast cancer was conducted between 1986 to 1991; blood and tissue have been stored. There were 371 postmenopausal and 301 premenopausal women with breast cancer and 438 and 316 age-matched controls, respectively. Genotyping for GSTM1, CYP1A1, CYP2D6, CYP2E1, APOE, aldehyde dehydrogenase, glutathione-*S*- transferase theta (GSTT) and N-acetyltransferase 1 and 2 is being determined for all subjects. The p53 mutational spectra is being determined for informative cases, who will be identified by single stranded conformational polymorphism analysis and immunohistochemical staining. Persons with mutations will be categorized by mutation and hypothesized chemical etiology will be compared to persons with other types of p53 mutations (four for each case) and also to controls without cancer (ten for each case). Odds ratios and logistic regression will address the association of genetic polymorphisms and exposures as a risk for p53 mutation and breast cancer, adjusting for other risk factors. We also will examine effect modification for other risk factors by genetic polymorphisms.

The current workscope was expanded to perform additional studies relating to findings in the first year of the award, specifically as they relate to smoking, smoking-related carcinogens and breast cancer. Thus, we are culturing human breast epithelial cells and examining the rate of adduct formation from cigarette-smoke carcinogens, as well as the p53 and apoptosis response. Interindividual variation will specifically be addressed. The purpose of these studies is to corroborate our epidemiological findings. We will also reproduce our findings in additional epidemiological studies. Finally, we will examine nicotine addiction and genetic risk factors for addictive behaviors, in the context of a smoking cessation project, in order to identify smoking cessation strategies that

will reduce the incidence of breast cancer in susceptible populations.

BODY

1. Collection of Tissue Samples and Tissue Preparation

- Tumor blocks for 93 cases have been obtained and sectioned, and the DNA has been extracted. An additional 300 have been identified. IRB approval is pending from the hospitals. Some institutions have not been as cooperative as expected, so that there may be as many as 50 blocks not available. We have also had to identify a local pathologist who will go to the hospitals to supervise the block collection. Collection of tissues and interruptions in staff have delayed the collection of these tissues. We expect to request an extension, at no additional cost to the Department of Defense, for one year, in order to have enough time to complete the project.

- A mechanism for receiving fresh breast tissues from autopsy cases and reduction mammoplasties is ongoing. We have received over 42 tissues to date, and culturing is now routine from both autopsy and surgical donors. Additionally, we have collected 150 frozen breast tissues from autopsy and surgical donors, many of the former who have also donated liver. All surgical cases have completed an epidemiological questionnaire.

- DNA has been extracted from over 500 smokers and non-smokers enrolled in a study of tobacco addiction in collaboration with Georgetown University. Outcome data at one year is now available for the ability to quit after smoking cessation counseling.

- Blocks are now being received for a multiracial study of breast cancer in collaboration with MD Anderson Cancer Center. Six hundred cases have been identified who were diagnosed from 1983 to 1993 and have had epidemiological questionnaires completed. These women include 400 Caucasians, 100 African Americans and 100 Hispanics. To date, 14 blocks have been received.

2. Genetic Polymorphism analysis

- Our initial focus was to study tobacco smoking as a risk factor for breast cancer. While smoking is generally considered not to be a risk factor for breast cancer, based on numerous epidemiological studies, it

was our hypothesis that smoking would indeed be a risk factor in some women, but not others. When studied together as a homogenous population, the risk would not be observable. Thus, to test this hypothesis, we studied risk in the *N*-acetyltansferase gene (*NAT2*), because this gene functions as a detoxification pathway for aromatic amines, for which there is ample experimental evidence to suggest that aromatic amines would be a human breast carcinogen. The *NAT2* genetic polymorphism, which predicts rapid or slow acetylation, was tested in 304 breast cancer cases and 327 community controls. Neither smoking or the *NAT2* gene by themselves were risk factors, but when the women were stratified by smoking risk based on acetylation status, in postmenopausal women, smoking carried a risk of up to 4.4 (95% C.I.=1.4, 10.8) in slow acetylators, which was consistent with several different types of analyses for this dataset. There was no similar findings for premenopausal women. A manuscript was published summarizing these findings in the Journal of the American Medical Association attached as Appendix A.

NAT1 genotyping has been completed for postmenopausal women and premenopausal women (Appendix B). The genotypic frequency is similar to previous reports in the literature. Quality control analysis is being completed and the data will be analyzed.

- A commonly accepted risk factor for breast cancer is alcohol consumption, and the findings are more frequently reported in premenopausal rather than postmenopausal women. It is currently unknown what might be the carcinogenic agents in alcoholic beverages. One candidate is ethanol, because ethanol is oxidized to acetaldehyde, which is mutagenic and carcinogenic in laboratory animals. The principle pathway for ethanol oxidation is through alcohol dehydrogenase. In order to study the risk of alcohol drinking in the context of ethanol metabolism, we studied the alcohol dehydrogenase 3 gene (*ADH3*). In this study, we found that women who would be predicted to have an increased capacity to form acetaldehyde ($ADH3^{1-1}$), had an odds ratio of 3.0 (95% C.I.=1.3, 6.6) in high drinkers compared to low or nondrinkers. Compared to women who would have a decreased capacity ($ADH3^{2-2}$), there was a 3.3-fold risk (95% C.I.=0.9, 12.9). For more detailed tables, see Appendix C. This work has resulted in an oral presentation at the American Association of Cancer Research Annual Meeting.

- Apolipoprotein E is involved in the production of VLDL and other parts of cholesterol metabolism. Several studies have related low cholesterol levels to breast cancer risk. The apoE gene is polymorphic, where some variants raise cholesterol levels and others lower them. We therefore measured apoE genotypes in both the pre- and postmenopausal women. The statistical analysis is continuing.

- Our previous results indicated that a polymorphism in cytochrome P450IAI is related to breast cancer in postmenopausal women with low tobacco use. There also was a non-significant trend for GSTM1 in younger postmenopausal women. Both of the enzymes are involved in the activation and detoxification, respectively, of polycyclic aromatic hydrocarbons. The status of the genotyping for GSTM1, CYP1A1 and GST-T in premenopausal women is shown in Appendix D. Upon completion of quality control samples, the data will be analyzed in the context of breast cancer risk. Another enzyme involved in this pathway is microsomal epoxide hydrolase. There are two polymorphic sites that result in a decrease of activity by 40%. The measurement of MEH in pre-and postmenopausal women is almost complete. The current status is presented in Appendix E.

- Cytochrome P450IID6 has been associated with lung cancer and breast cancer. Its metabolic substrate is unknown, but it may be a tobacco-specific nitrosamine. We are measuring the activity of this gene by PCR. Assays (4 different polymorphic sites) are almost complete for pre- and postmenopausal women. The current status is presented in Appendix F.

- We assessed a *CYP2E1* genetic polymorphism (intron 6; *Dra*I restriction enzyme site) as a risk factor for breast cancer in both pre- and postmenopausal women. Because *N*-nitrosamines are metabolically activated by cytochrome P450IIE1 (*CYP2E1*), the risk among women smokers was investigated. The allelic frequencies for the premenopausal (D allele=0.91 and C allele=0.09) and postmenopausal (D allele=0.93 and C allele=0.07) women were similar to previous reports in the literature. There was no statistically significant association for the *CYP2E1* and breast cancer risk for pre- or postmenopausal women (adjusted OR=1.04, 95% C.I.= 0.48, 2.24 and OR = 1.01, 95% C.I. = 0.0.55, 1.84, respectively). When women were categorized as non-smokers versus smokers (smoking more than one cigarette per week for more than one year),

premenopausal women with one or two C alleles, who had a history of smoking, were found be at increased risk (unadjusted O.R. = 7.00, 95% C.I. = 0.75, 14.53 and adjusted O.R. = 11.09, 95% C.I. = 1.51, 81.41), although the number of study subjects with this genotype was small. The small number of study subjects with a C allele precluded a meaningful classification by level of smoking within smokers, but categorization of smokers into two groups (above and below the median) also suggested the increase risk. Premenopausal women with the DD genotype and postmenopausal women with any genotype were not at increased risk. Breast cancer risk was not related to the *CYP2E1* genotype in either premenopausal non-smokers or smokers (adjusted O.R. = 0.66, 95% C.I. = 0.20, 2.17 and O.R. = 2.13, 95% C.I. = 0.0.60, 7.59, respectively) or postmenopausal non-smokers or smokers (O.R. = 0.90, 95% C.I. = 0.34, 2.35 and O.R. = 1.02, 95% C.I. = 0.46, 2.23, respectively), although the difference in the odds ratios for premenopausal non-smokers and smokers suggests an increased risk for smokers. While there are limitations to this study, particularly related to the small number of subjects with the DC or CC genotype, the study suggests that some women might be susceptible to tobacco smoke because of a *CYP2E1* polymorphism. However, these results are preliminary and must be replicated. A manuscript summarizing this data was published in Molecular Carcinogenesis appears in Appendix G.

3. P53 Mutational Spectra Analysis

- Blocks from 91 individuals have been obtained and have been sectioned. P53 immunohistochemistry staining has been done for them. We have identified appropriate controls for sequencing to ensure quality control and no contamination of wild-type DNA. We have identified these controls from lung cancer samples. There are 20 controls that contain mutations in each of the 4 exons of interest. We have also prepared blocks of cell lines with known p53 mutations, which also will be used as controls. The methods to perform the SSCP and sequencing are now being optimized and finalized for these samples. At the present time, we are considering increasing the analysis to include exons II - XI to increase the specificity and power.

4. Ancillary Studies

- We have developed the technique in our laboratory, based upon previously published methods, to isolate

breast epithelial cells and culture them in a sterile environment. Thus far we have established over 40 cell strains. In these cells, we have determined that 4-aminobiphenyl is metabolically activated through cytotoxicity experiments, and are now defining optimal timing and dose response relationships. Both metabolites of 4-ABP and parent 4-ABP are active in producing cell death, suggesting the presence of NAT1 and CYP1A2 in these cells. Preliminary genotyping for NAT2 does not show a difference in metabolic activity, but additional samples and genotyping for NAT1 is underway. We are also now identifying the p53 induction and apoptosis in relation to the exposures. Our data indicates that p53 is induced after exposure to 4-ABP. The current data is presented in Appendix H.

DNA adducts will be measured using the postlabeling ADAM procedure and radiolabeled compounds will also allow us to measure adducts using accelerator mass spectroscopy. Chemical standards have been synthesized to calibrate the assay and optimizing labeling conditions is in progress. The current data is presented in Appendix I.

- An understanding of why people smoke cigarettes can have an important impact upon smoking prevention and cessation. People smoke cigarettes to maintain nicotine levels in the body, and nicotine has been implicated in the stimulation of brain reward mechanisms via central neuronal dopaminergic pathways. We recruited smokers (n=283) and nonsmokers (n=192) through local media for a case-control study of smoking. Following informed consent and a behavioral questionnaire, smokers underwent a single minimal contact session of smoking cessation counseling, and then were followed for up to one year. Thus far, we have found that there is an interaction for polymorphisms with the dopamine transporter gene and the dopamine D2 receptor for smoking risk (P=0.001) and the combination of the two genotypes reduces the risk of smoking by more than half. This manuscript has been submitted to Nature Medicine. The data is presented in Appendix J.

In this study, we also evaluated the association of smoking and smoking cessation with a dopamine D4 receptor 48 base pair variable nucleotide tandem repeat polymorphism, where the 7 repeat allele (D4.7) reduces dopamine affinity. The frequency of the dopamine D4 receptor genetic polymorphism using PCR was determined and individuals were classified by the number of repeat alleles (2-5 repeats as "S" and 6-8 repeats as "L").

Persons with those genotypes including only S alleles (homozygote S/S) were compared with those with at least one L allele (heterozygote S/L and homozygote L/L). Chi Square Tests of Association, Fisher's Exact Test. The data show that African Americans (n=72) who had at least one L allele had a higher risk of smoking (OR=7.7, 95% C.I.=1.5, 39.9; P=0.006), shorter time to the first cigarette in the morning (P=0.03) and earlier age at smoking initiation (P=0.09), compared with homozygote S/S genotypes. Following smoking cessation counseling, none of the African American smokers with an L allele were abstinent at two months, compared with 35% of the smokers who were homozygote S/S (P=0.02). The analysis of Caucasians (n=403) did not suggest a similar smoking risk for the D4 genotypes (O.R. = 1.0; 95% C.I. = 0.6, 1.6; P=0.90), or smoking cessation (P=0.75). While the number of African Americans is small, this study is consistent with the hypothesis that the L alleles increase the risk of smoking because these individuals are prone to use nicotine to stimulate synaptic dopamine transmission. A single minimal contact session of cessation counseling is ineffective in African Americans smokers who have at least one L allele. This manuscript has been submitted to the Journal of the American Medical Association. The data is presented in Appendix K.

CONCLUSIONS

The findings of an association of smoking and breast cancer in Caucasian women with the slow NAT2 acetylation genotype is very important because approximately 50% of women are slow acetylators. This results in a large attributable risk. The findings need to be reproduced and examined in other races. We are currently doing that. Laboratory studies also need to corroborate this finding by examining the metabolic potential in rapid and slow acetylators. Recent studies showing that breast cells contain acetyltansferase activity and our studies described above are consistent with the epidemiological data, but adduct studies also are needed. The development of the ADAM procedure will provide data for intermediate endpoints, which presumably reflect breast cancer incidence. Thus, the application of this procedure for aromatic amine adducts in cell strains and parent tissues may provide important corroborative data for the epidemiological findings. Finally, the p53 mutational spectra will also provide data on intermediate endpoints and also possibly identify the effects of acetyltansferase on ultimate outcome.

The findings of increased alcohol-related breast cancer depending on the alcohol dehydrogenase gene has important

implications for risk. First, if corroborated, the data indicate that acetaldehyde, which is the reactive metabolite of ethanol, is related to breast carcinogenesis. Second, the data indicate that tissues distant from the liver are affected. Third, the data identifies a particular subpopulation of women who might be more susceptible to alcohol.

As follow-up to smoking related risk, the ability to prevent smoking addiction and increase smoking cessation has the greatest potential impact from a public health and individual health perspective. The identification of polymorphisms in the dopamine receptor genes and dopamine transporter genes may be able to identify optimal prevention strategies. More importantly, the data suggests which type of smoking cessation therapy might be optimal for African Americans.

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APPENDIX A

Preliminary Communication

Cigarette Smoking, *N*-Acetyltransferase 2 Genetic Polymorphisms, and Breast Cancer Risk

Christine B. Ambrosone, PhD; Jo L. Freudenheim, PhD; Saxon Graham, PhD; James R. Marshall, PhD; John E. Vena, PhD; John R. Brasure; Arthur M. Michalek, PhD; Rosemary Laughlin, PhD; Takuma Nemoto, MD; Kari A. Gillenwater; Anita M. Harrington; Peter G. Shields, MD

Objective.—To determine if *N*-acetyltransferase 2 (*NAT2*) polymorphisms result in decreased capacity to detoxify carcinogenic aromatic amines in cigarette smoke, thus making some women who smoke more susceptible to breast cancer.

Design.—Case-control study with genetic analyses. DNA analyses were performed for 3 polymorphisms accounting for 90% to 95% of the slow acetylation phenotype among whites.

Setting and Participants.—White women with incident primary breast cancer (n=304) and community controls (n=327).

Results.—Neither smoking nor NAT2 status was independently associated with breast cancer risk. There were no clear patterns of increased risk associated with smoking by NAT2 status among premenopausal women. In postmenopausal women, NAT2 strongly modified the association of smoking with risk. For slow acetylators, current smoking and smoking in the distant past increased breast cancer risk in a dose-dependent manner (odds ratios [95% confidence intervals] for the highest quartile of cigarettes smoked 2 and 20 years previously, 4.4 [1.3-14.8] and 3.9 [1.4-10.8], respectively). Among rapid acetylators, smoking was not associated with increased breast cancer risk.

Conclusions.—Our results suggest that smoking may be an important risk factor for breast cancer among postmenopausal women who are slow acetylators, demonstrate heterogeneity in response to carcinogenic exposures, and may explain previous inconsistent findings for cigarette smoking as a breast cancer risk factor.

JAMA. 1996;276:1494-1501

CIGARETTE SMOKING is a risk factor for many human cancers, at organ sites with both direct and indirect contact with tobacco smoke.[1] Most epidemiologic studies have not found a clear association between smoking and breast cancer risk[2-12]; some report elevated breast cancer risk,[13-21] while others report decreased risk.[22-24] No study has considered genetic variability in susceptibility to cigarette smoke carcinogens.

For editorial comment see p 1511.

Mutagens from cigarette smoke come into direct contact with breast epithelial cells. Nipple fluid aspirated from smokers contains nicotine metabolites[25] and is mutagenic.[26] Tobacco-related carcinogen–DNA adducts in human breast tissue have been identified.[27,28] Aromatic amines found in tobacco smoke, such as 4-aminobiphenyl and β-naphthylamine, could be mutagenic and carcinogenic because they are metabolically activated and cause DNA damage in human breast epithelial cells,[29,30] transform cultured mouse mammary epithelial cells,[31] and induce mammary tumors in laboratory animals.[32,33]

Aromatic amines are detoxified and/or bioactivated by xenobiotic metabolizing enzymes, including *N*-acetyltransferase 2 (NAT2). The activity level of this enzyme determines the rates of detoxification and activation of aromatic amines in

From the National Center for Toxicological Research, Division of Molecular Epidemiology, Jefferson, Ark (Dr Ambrosone); Departments of Social and Preventive Medicine (Drs Ambrosone, Freudenheim, Graham, Marshall, Vena, Michalek, and Laughlin and Mr Brasure) and Surgery (Dr Nemoto), State University of New York at Buffalo; and Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, Md (Mss Gillenwater and Harrington and Dr Shields).

This work was a collaborative effort by the Department of Social and Preventive Medicine, State University of New York at Buffalo, and the Laboratory of Human Carcinogenesis, National Cancer Institute. It is solely the responsibility of the authors and does not necessarily represent the views of the National Cancer Institute.

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humans.[34] For NAT2, phenotypic and genotypic assays are used to classify individuals as rapid or slow acetylators.[35-38] Slow NAT2 acetylators are at increased risk for urinary bladder cancer, particularly with occupational exposure to aromatic amines.[39,40] NAT2 is also involved in *O*-acetylation of amine metabolites, and rapid NAT2 acetylation of heterocyclic amines formed in cooking meat may be related to colon cancer risk.[41-43] Examination of the NAT2 phenotype in breast cancer has shown inconsistent results,[44-48] but phenotypes may be altered by disease or treatment status. We hypothesized that polymorphisms in NAT2 may result in decreased capacity to detoxify carcinogenic aromatic amines in cigarette smoke, thus increasing susceptibility to breast cancer.

SUBJECTS AND METHODS

Study Population

These analyses are based on data from an earlier case-control study (1986-1991) of 617 premenopausal and 933 postmenopausal white women in New York.[49] Protocols for the initial study and the nested study of genetic polymorphisms were reviewed by the State University of New York at Buffalo Institutional Review Board. Written informed consent for interview and medical record review was obtained from participants. The criteria for postmenopausal status in women under the age of 50 years were natural menopause, bilateral oophorectomy, or irradiation to the ovaries; in women aged 50 years and older, the criterion was cessation of menstruation.

Cases, identified from all major hospitals in Erie and Niagara counties, were women with incident primary, histologically confirmed breast cancer. Controls were frequency-matched to cases by age and county of residence. Women under 65 years of age were randomly selected from the New York State Motor Vehicle Registry, while those 65 and over were selected from Health Care Financing Administration rolls. Of premenopausal women contacted, 66% of eligible cases (n=301) and 62% of eligible controls (n=316) participated, and of postmenopausal women, 54% of cases (n=439) and 44% of controls (n=494) participated. After informed consent was obtained, an in-person interview was administered to assess medical, reproductive, and lifetime smoking history and usual food consumption 2 years prior to the interview. While efforts were made for interviewers to be blinded to case-control status, in many cases, due to the nature of the interview, this was not possible. However, during the interview, interviewers and investigators were not aware of the genetic hypotheses.

About 45% of premenopausal and 63% of postmenopausal women interviewed provided blood samples, with written informed consent for blood analyses. In premenopausal women, there were no statistically significant ($P<.05$) differences in socioeconomic, hormonal, reproductive, or dietary factors between those giving and not giving blood, and in postmenopausal cases, only slight differences. Postmenopausal controls providing samples had a greater mean number of pregnancies (3.5 vs 2.9, $P<.01$) and fewer years of smoking (30 vs 33, $P=.14$) than those who did not. There were more never-smokers among controls who consented to phlebotomy (67% vs 62%) ($P=.07$). DNA analyses were performed for cases and controls whose specimens had adequate DNA.

Laboratory Methods

Whole blood was collected in plain red-top tubes and transported within 3 hours, on ice, to the laboratory, where it was processed immediately. Specimens were centrifuged and serum was pipetted into vials. Two aliquots of clots (1 mL each) were preserved and stored at −70°C. For this study the 1-mL blood clots were thawed, mechanically disrupted (Brinkman Instruments Polytron, Westbury, NY) for 10 seconds, and digested with proteinase K (Life Technologies, Grand Island, NY) at 55°C for 8 to 12 hours in 5 mL of buffer containing 10-mmol/L Tris hydrochloride, pH 7.8; 50-mmol/L ethylenediaminetetraacetic acid (EDTA); and 0.5% sodium dodecyl sulfate. Phenol (5 mL) was added to the sample, which was mixed gently for 1 hour. After centrifugation, the aqueous phase was transferred to a clean tube. The phenol phase was mixed with additional buffer (2 mL of 10-mmol/L Tris hydrochloride, pH 8.0, and 0.1-mmol/L EDTA) and centrifuged, and the aqueous phases were combined. The sample was extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1 vol/vol), and DNA was precipitated with sodium acetate (pH 5.2; final concentration, 0.3 mol/L) and 2.5 volumes of ethanol. Yields of DNA were 5 to 20 μg per sample. Genomic DNA (30 ng) was amplified by polymerase chain reaction (PCR) in the presence of primers specific for *NAT2*[36] (5'-TCTAGCATGAATCACTCTGC and 5'-GGAACAAATTGGACTTGG), buffer (10-mmol/L Tris hydrochloride, pH 8.3; 50-mmol/L potassium chloride; and 3-mmol/L magnesium chloride), 0.15-mmol/L 2'-deoxynucleoside-3'-triphosphates (Pharmacia, Piscataway, NJ); and 2.5 U of *Taq* polymerase (Perkin Elmer, Norwalk, Conn) in a total volume of 100 μL. An aliquot (18 μL) was then subjected to restriction fragment length polymorphism (RFLP) analysis for the $C^{481}T$ (*Kpn*I) (New England Biolabs, Beverly, Mass), $G^{590}A$ (α*Taq*I) (New England Biolabs), and the $G^{857}A$ (*BamH*I) (New England Biolabs) polymorphisms, according to the manufacturer's instructions (nomenclature for *N*-acetyltransferases as published[50]). These 3 polymorphic sites predict 90% to 95% of slow acetylation phenotype among whites.[36,38] Agarose gel electrophoresis (2.2% for the $C^{481}T$ and $C^{857}A$; 4% for the $C^{590}A$; NuSieve: Agarose, 3:1, FMC Bioproducts, Rockland, Me) was used to detect RFLP patterns. Each individual was classified as a rapid acetylator (carrying 0 or 1 slow acetylator mutation) or slow acetylator (carrying 2 slow acetylator mutations).[36,37] Assays were performed and interpreted by 2 of the authors (A.H. and P.G.S.), who were blinded to subject status. A second analysis was done to confirm the original findings. In the second analysis, 33 samples were excluded because of faint electrophoretic bands due to suboptimal DNA quality.

Statistical Analyses

Bivariate analyses were used to examine the association between NAT2 status and breast cancer risk. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression and adjusted for potential confounding factors including age, education, body mass index (weight in kilograms divided by the square of height in meters with weight being the reported value 2 years prior to interview), age at menarche, age at first pregnancy, reported family history of breast cancer (mother and/or sister), and age at menopause. Hormone replacement therapy was not associated with breast cancer risk, and its inclusion in the model did not affect risk estimates. Unadjusted and adjusted ORs were similar; adjusted ORs are shown. The P for trend was calculated as level of significance of the β coefficient (indicative of the amount of increase in risk per unit change in the independent variable) for each continuous variable in the logistic regression model with relevant adjusting variables.

Smoking effect in relation to breast cancer risk was examined within strata of NAT2 genotypes. Association between risk and recent smoking (cigarettes smoked 2 years previously) and smoking in the distant past (cigarettes smoked 20 years previously) was assessed. Past smoking was assessed due to probable latency for breast carcinogenesis and possible damage to breast epithelial cells during breast growth. Packs per average year was calculated as weighted average of daily use, and

total duration of smoking was assessed. Pack-years, an estimate of total smoking exposure, was calculated as weighted average of daily use multiplied by total years of smoking. We had smoking information of 2, 10 (data not shown), and 20 years before the interview. The weighted average was the number of cigarettes smoked at each time period multiplied by 6 years and divided by the total number of years of smoking exposure. Smoking-related variables were stratified into quartiles based on approximately uniform distribution of smoking in controls, with never-smokers as referent category. When we assessed smoking 2 and 20 years previously, we excluded persons not smoking then but having previously smoked. Thus, the sample size varied among smoking variables.

Postmenopausal controls consenting to phlebotomy smoked less than nonparticipating controls. To eliminate this nondifferential bias and supplement assessment of acetylator status effects, 2 additional approaches were used. One was a case-series design in which smoking-related variables were regressed on acetylator status among cases only, comparing genotypes in terms of the degree to which smoking was a risk factor. The resultant OR was the ratio of risk associated with smoking for slow vs rapid acetylators, a supported method for studies incorporating genetic markers. Analyses based only on cases may offer better precision for estimating gene-environment interactions than those based on case-control data.[51,52]

The other approach was a model in which a subset of cases and subset of controls were matched on smoking by randomly selecting equal numbers of cases and controls within smoking quartiles (before stratification by genotype) with intent to eliminate selection bias related to smoking. Odds ratios were set to 1.0 at each quartile of exposure for smoking-related variables. Data were stratified by acetylator status, and associations between smoking and risk were evaluated.

RESULTS

Prevalence of genotypes as determined by assessment of each of the 3 *NAT2* mutant alleles (ie, $C^{481}T$, $G^{590}A$, and $G^{857}A$) among cases and controls is shown for premenopausal and postmenopausal women (Table 1). Interpretable PCR assays resulted in genetic data for 233 premenopausal and 398 postmenopausal women (83% and 71%, respectively) (Figure 1). There were no statistically significant differences between persons with and without successful PCR amplification for demographic, dietary, smoking, or reproductive variables (data not shown). Fifty-seven percent of premenopausal and 55% of postmenopausal women were classified as slow acetylators (Table 2). Slow acetylation status did not increase breast cancer risk.

The Student *t* test was used to assess statistical mean differences between rapid and slow acetylators in cases and controls for selected demographic, reproductive, and smoking-related variables (Tables 3 and 4). In premenopausal women, no statistically significant differences between these variables were seen for cases or controls, and in postmenopausal women, there were no statistically significant differences between rapid and slow acetylators for demographic or reproductive variables (Table 3). In postmenopausal controls, no statistically significant differences by genotype for smoking variables were seen, but cases differed in smoking histories by NAT2 genotype (Table 4): Slow acetylators smoked more at all times, suggesting a relationship to disease status among postmenopausal women with the slow acetylation genotype.

Among postmenopausal women, cigarette smoking and NAT2 genotype interacted with breast cancer risk. A statistical test for interaction that regressed NAT2 status, packs of cigarettes smoked per year, and their product on risk revealed that neither acetylator status ($P=.71$) nor smoking status ($P=.80$) was independently associated with breast cancer risk, but an interaction between acetylator status and smoking status contributed to postmenopausal breast cancer risk ($P=.05$).

After stratifying by NAT2 genotype, we evaluated associations between

Table 1.—*N*-Acetyltransferase 2 Polymorphisms for Cases and Controls: Western New York Breast Cancer Study, 1986 to 1991*

	Premenopausal			Postmenopausal		
	Case	Control	Total	Case	Control	Total
Rapid acetylators						
WT/WT	7	9	**16**	13	17	**30**
WT/$C^{481}T$	26	22	**48**	40	45	**85**
WT/$G^{590}A$	17	18	**35**	26	29	**55**
WT/$G^{857}A$	1	0	**1**	1	9	**10**
All	**51** (43)	**49** (43)	**100** (43)	**80** (43)	**100** (47)	**180** (45)
Slow acetylators						
$C^{481}T/C^{481}T$	26	24	**50**	42	49	**91**
$C^{481}T/G^{590}A$	27	26	**53**	43	48	**91**
$C^{481}T/G^{857}A$	4	2	**6**	5	4	**9**
$G^{590}A/G^{590}A$	9	13	**22**	13	8	**21**
$G^{590}A/G^{857}A$	2	0	**2**	2	4	**6**
$G^{857}A/G^{857}A$	0	0	**0**	0	0	**0**
All	**68** (57)	**65** (57)	**133** (57)	**105** (57)	**113** (53)	**218** (55)

*WT indicates wild-type allele in which the $C^{481}T$, $G^{590}A$, and $G^{857}A$ slow acetylator mutations are absent. Slow acetylators carry 2 of any of these mutations. All others are classified as rapid acetylators. Values in parentheses represent percentages of rapid and slow acetylators.

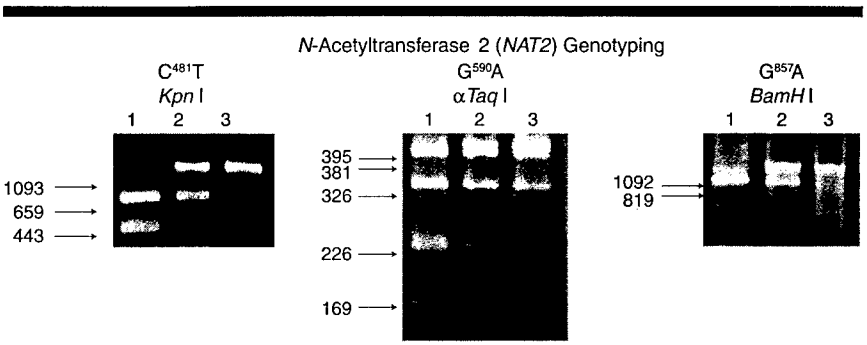

Figure 1.—Detection of NAT2 slow acetylator mutations in polymerase chain reaction (PCR) products. Genomic DNA was amplified by PCR[36] and digested by restriction enzymes. Slow acetylator mutations were examined: $C^{481}T$, by use of *Kpn* I, yields 659- and 443-bp (base pair) bands for wild-type alleles and a single 1093-bp band for the mutant alleles (left); $G^{590}A$, by use of α*Taq*I, yields 381-, 326-, 226-, and 169-bp bands for wild-type alleles and 395-, 381-, and 326-bp bands for the mutant alleles (center); and $G^{857}A$, by use of *BamH*I, yields 819- and 283-bp bands for the wild-type alleles (283-bp band not shown) and a single 1092-bp band for the mutant alleles (right). Thus, for each polymorphic site, homozygous wild-type (lane 1), heterozygote (lane 2), or homozygous mutant (lane 3) patterns are shown. The presence of 2 mutant alleles predicts the phenotypic slow acetylators.

Table 2.—*N*-Acetyltransferase 2 (*NAT2*) Polymorphisms and Breast Cancer Risk: Western New York Breast Cancer Study, 1986 to 1991*

NAT2 Genotype	Cases, No. (%)	Controls, No. (%)	Total, No. (%)	OR (95% CI)
		Premenopausal		
Rapid acetylators	51 (43)	49 (43)	100 (43)	1.0 . . .
Slow acetylators	68 (57)	65 (57)	133 (57)	0.9 (0.7-2.0)
		Postmenopausal		
Rapid acetylators	80 (43)	100 (47)	180 (45)	1.0 . . .
Slow acetylators	105 (57)	113 (53)	218 (55)	1.3 (0.8-1.9)

*The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated with unconditional logistic regression, with rapid acetylators (individuals with <2 mutant alleles) as the reference category (ellipses), adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer, and age at menopause for postmenopausal women.

Table 3.—*N*-Acetyltransferase 2 Polymorphisms and Demographic and Reproductive Characteristics for Cases and Controls: Western New York Breast Cancer Study, 1986 to 1991*

	Premenopausal			
	Cases		Controls	
Characteristic	Rapid Acetylators (n=51)	Slow Acetylators (n=68)	Rapid Acetylators (n=49)	Slow Acetylators (n=65)
Age, y	47 (4)	46 (3)	47 (3)	47 (4)
Education, y	14 (3)	14 (3)	13 (3)	14 (3)
Age at menarche, y	13 (1)	13 (2)	13 (2)	13 (2)
Age at first pregnancy, y	24 (6)	24 (5)	22 (4)	23 (4)
Body mass index, kg/m²	25 (6)	24 (5)	25 (6)	24 (5)
	Postmenopausal			
	Cases		Controls	
	Rapid Acetylators (n=80)	Slow Acetylators (n=105)	Rapid Acetylators (n=100)	Slow Acetylators (n=113)
Age, y	62 (7)	63 (7)	64 (8)	63 (7)
Education, y	13 (3)	12 (3)	12 (3)	12 (3)
Age at menarche, y	13 (2)	13 (2)	13 (1)	13 (2)
Age at menopause, y	47 (6)	48 (6)	47 (6)	47 (6)
Age at first pregnancy, y	25 (5)	24 (5)	23 (4)	23 (4)
Body mass index, kg/m²	26 (4)	26 (6)	26 (4)	26 (6)

*Values are expressed as mean (SD). $P<.05$ for the Student *t*-test for the difference between the means of rapid and slow acetylator cases and controls.

smoking-related variables and breast cancer risk. In premenopausal women, no clear patterns were identified by acetylator status (Table 5). Risk was elevated in rapid acetylators who had smoked 20 years before, although ORs and trend test were not statistically significant.

In postmenopausal slow acetylators, strong associations between smoking and breast cancer risk were seen (Table 5). For them, smoking 2 and 20 years previously increased breast cancer risk in a dose-dependent manner (OR [95% CI] for the highest quartile for smokers vs never-smokers, 4.4 [1.3-14.8] and 3.9 [1.4-10.8], respectively). Although breast cancer risk was elevated with total years of smoking, smoking intensity appeared more important than duration (Figure 2). Packs per average year significantly elevated breast cancer risk among postmenopausal slow acetylators, with a 3-fold risk for smoking more than 1 pack per day ($P<.01$). Total pack-years of smoking were also associated with elevated risk in this group (Table 5).

Among postmenopausal rapid acetylators, adjusted risk was not increased by smoking. For cigarettes smoked 20 years previously, there was reduced risk at the highest quartile of use (OR, 0.30; 95% CI, 0.01-0.80). Neither smoking intensity nor smoking duration was associated with increased risk in postmenopausal rapid acetylators (Figure 2).

As explained previously, a case-series analysis was used to assess associations among smoking, NAT2 status, and postmenopausal breast cancer risk. In this analysis, NAT2 status was the dependent variable in a logistic regression model in which rapid acetylator cases served as comparison group to slow acetylator cases. The resultant ORs do not reflect actual risk of breast cancer, but rather the ratio of risk for slow vs rapid acetylators. Smoking-related variables were regressed on acetylator status; the effect of smoking on risk appeared to vary according to NAT2 genotype. Heavy smokers with breast cancer were more likely to be slow acetylators (Table 6). Risk was almost 7 times greater for women who had smoked more than a pack a day 20 years previously if they were slow vs rapid acetylators (OR, 6.6; 95% CI, 1.7-25.4), and risk associated with beginning smoking at or before the age of 16 years was more than 4 times greater for slow vs rapid acetylators (OR, 4.5, 95% CI, 1.3-14.9). When pack-years smoked were evaluated, risk was almost 3 times greater for slow acetylators in the highest quartile of use.

As previously explained, we evaluated the effect of acetylator status on the association between smoking and breast cancer risk in a model designed to eliminate overall association between smoking and risk, in which postmenopausal cases and controls were matched on smoking, with an OR of 1.0 for breast cancer associated with each quartile of cigarette use. Cases and controls were then stratified by NAT2 genotype, and divergent patterns related to smoking were noted between genotypes. Smoking at each time period had a null or negative effect on breast cancer risk in rapid acetylators, while for slow acetylators, cigarette smoking was associated with increased breast cancer risk (Table 7). Risk associated with age at which smoking began was also modified by genotype; slow acetylators who began smoking at or before the age of 16 years were at highest breast cancer risk. For rapid acetylators, there was an inverse association between risk and earlier initiation of smoking. While these estimates of risk are derived from contrived subsets of data, they illustrate heterogeneity in risk by genotype of the study population in response to cigarette smoking. Mantel-Haenszel tests were performed on the unadjusted categorical data, and trend tests shown are consistent with results from the other methods of analysis. When analyses were repeated using different random-number generators to match on smoking, associations among NAT2 status, smoking, and breast cancer risk were similar.

COMMENT

Cigarette smoking appears to be a risk factor for breast cancer among postmenopausal, but not premenopausal, white women with the NAT2 slow acetylation genotype. Among slow acetylators, smoking intensity, rather than smoking duration, most greatly affected breast cancer risk. Smoking at a young age also appeared to confer risk. Among

rapid acetylators, neither intensity nor duration of smoking increased risk. When examined alone, neither NAT2 genotype nor cigarette smoking was independently associated with breast cancer risk. Differences in results for premenopausal and postmenopausal women could be related to different etiologic pathways or intrinsic differences in the disease. Also, premenopausal women, being younger, may have had fewer years of exposure to tobacco, or not enough elapsed time for the entire carcinogenic process to develop.

Laboratory studies indicate that aromatic amines are mammary mutagens and carcinogens in rodents and in humans.[29-33,53-56] Aromatic amines are bioactivated and/or detoxified by xenobiotic metabolizing enzymes, including cytochrome P4501A2 (CYP1A2) and *N*-acetyltransferases (NAT1 and NAT2). While NAT2 is the focus herein, these other enzymes might also play a role in breast cancer. Both CYP1A2 and NAT1 are polymorphic, although the genetic basis for the former is unknown. *N*-Acetylation of aromatic amines by NAT2 appears to be a detoxification step catalyzed by hepatic NAT enzymes, a pathway competing with that for *N*-oxidation by CYP1A2, whereby reactive *N*-hydroxy metabolites may enter the circulation, undergoing further activation and binding to DNA in target tissues.[34] It has been suggested that NAT1 is more active in breast tissue than NAT2,[57,58] but the importance of hepatic detoxification should not be underestimated.

The role of aromatic amines in breast cancer may be analogous to that in urinary bladder cancer.[59] Cigarette smoking is a risk factor for bladder cancer,[60] and slow acetylators have higher circulating levels of 4-aminobiphenyl-hemoglobin adducts, reflecting decreased clearance of reactive arylamine metabolites.[37,61,62] NAT2 slow acetylators have an increased bladder cancer risk, presumably because of decreased liver detoxification of aromatic amines.[39,40,63] Risk of bladder cancer associated with smoking may vary by NAT2 status,[64] although results have been inconsistent.[65,66] Animal studies indicate that mammary and bladder tissues have similar sensitivities to reactive intermediates.[53] Aromatic amines are rodent mammary carcinogens if activated in the liver.[32] Thus, slow acetylators may have less capacity to detoxify aromatic amines, leading to an increased concentration of reactive intermediates. In the breast, further activation may occur,[57,67] resulting in promutagenic carcinogen-DNA adducts and carcinogenesis.

Table 4.—*N*-Acetyltransferase 2 Polymorphisms and Smoking Characteristics for Cases and Controls: Western New York Breast Cancer Study, 1986 to 1991*

	Premenopausal			
	Cases		Controls	
Characteristic	Rapid Acetylators	Slow Acetylators	Rapid Acetylators	Slow Acetylators
Daily cigarettes 2 y ago	22 (7)	22 (15)	23 (16)	25 (15)
Daily cigarettes 20 y ago	18 (7)	17 (11)	18 (12)	20 (14)
Age smoking began, y	17 (5)	18 (4)	16 (3)	17 (4)
Total duration of smoking, y	22 (9)	19 (9)	23 (8)	22 (10)
Packs per average year	272 (147)	288 (216)	292 (179)	336 (244)
Pack-years	19 (14)	19 (17)	21 (16)	25 (23)
	Postmenopausal			
	Cases		Controls	
	Rapid Acetylators	Slow Acetylators	Rapid Acetylators	Slow Acetylators
Daily cigarettes 2 y ago	21 (11)	22 (10)	19 (9)	19 (13)
Daily cigarettes 20 y ago	16 (8)†	24 (12)†	21 (12)	19 (14)
Age smoking began, y	21 (6)	20 (5)	21 (9)	21 (9)
Total duration of smoking, y	30 (13)	33 (13)	33 (13)	31 (14)
Packs per average year	285 (156)†	397 (204)†	276 (170)	290 (207)
Pack-years	29 (18)†	43 (27)†	27 (20)	29 (22)

*Values are expressed as mean (SD).
†$P<.01$ for the Student t test for the difference between the means of rapid and slow acetylator cases and controls.

Table 5.—Breast Cancer Risk and Cigarette Smoking by Acetylator Genotype: Western New York Breast Cancer Study, 1986 to 1991*

	Premenopausal						Postmenopausal					
	Rapid Acetylators			Slow Acetylators			Rapid Acetylators			Slow Acetylators		
Quartile	Case	Control	OR (95% CI)	Case	Control	OR (95% CI)	Case	Control	OR (95% CI)	Case	Control	OR (95% CI)
Daily cigarettes 2 y ago												
None	18	25	1.0 ...	27	31	1.0 ...	43	50	1.0 ...	41	59	1.0 ...
≤15	2	5	0.7 (0.1-4.6)	6	7	0.6 (0.1-2.5)	6	7	1.8 (0.5-6.5)	6	12	0.8 (0.3-2.5)
16-20	10	4	4.5 (1.0-20.6)	12	4	3.5 (0.9-14.2)	8	11	1.0 (0.3-3.0)	21	11	3.2 (1.3-7.8)
>20	4	5	0.7 (0.1-4.2)	4	10	0.4 (0.1-1.7)	5	3	2.1 (0.4-10.4)	11	5	4.4 (1.3-14.8)
P for trend			.72			.49			.51			<.01
Cigarettes 20 y ago												
None	18	25	1.0 ...	27	31	1.0 ...	43	50	1.0 ...	41	59	1.0 ...
≤15	8	8	1.6 (0.5-5.5)	12	10	0.9 (0.3-2.7)	13	12	1.5 (0.6-3.8)	10	19	0.9 (0.4-2.2)
16-20	10	10	2.2 (0.7-6.8)	14	12	1.1 (0.4-3.2)	13	20	1.1 (0.5-2.6)	23	18	2.3 (1.0-5.0)
>20	2	2	2.6 (0.3-22.3)	4	3	1.2 (0.2-6.3)	3	11	0.3 (0.1-0.8)	17	7	3.9 (1.4-10.8)
P for trend			.35			.63			.21			<.01
Pack-years												
None	18	25	1.0 ...	27	31	1.0 ...	43	50	1.0 ...	41	59	1.0 ...
≤183	9	7	1.8 (0.5-6.5)	13	7	1.7 (0.5-5.1)	4	12	0.4 (0.1-1.6)	5	9	0.9 (0.3-3.0)
184-365	11	7	2.5 (0.7-8.6)	10	10	0.8 (0.3-2.4)	10	10	1.5 (0.5-4.2)	10	14	1.1 (0.4-2.7)
>365	10	7	2.1 (0.5-7.9)	11	10	1.2 (0.4-3.8)	14	23	0.9 (0.4-2.1)	36	23	2.8 (1.4-5.5)
P for trend			.51			.75			.98			<.01

*Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer, and age at menopause among postmenopausal women. Included in each analysis are lifetime nonsmokers (referent [ellipses]) and those who were smoking during that time period; excluded are those who quit smoking before that time period. *P* for trend was calculated from logistic regression with the independent variable as a continuous variable.

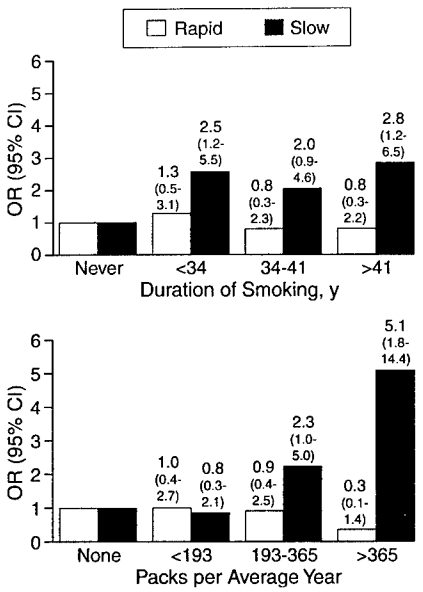

Figure 2.—Odds ratios (ORs) and 95% confidence intervals (CIs) for breast cancer by duration (top) and intensity (bottom) of cigarette smoking among postmenopausal women with rapid and slow acetylation genotypes: Western New York Breast Cancer Study, 1986-1991.

Table 6.—Case Series Analysis of *N*-Acetyltransferase 2 Status and Cigarette Smoking Among Postmenopausal Women: Western New York Breast Cancer Study, 1986 to 1991*

Quartile	No. of Slow Acetylators	No. of Rapid Acetylators	OR (95% CI)	*P* for Trend
Daily cigarettes 2 y ago				
None	41	43	1.0 ...	
≤15	6	6	0.8 (0.2-2.8)	.05
16-20	21	8	2.7 (1.0-6.9)	
>20	11	5	2.5 (0.7-8.3)	
Daily cigarettes 20 y ago				
None	41	43	1.0 ...	
≤15	10	13	0.7 (0.3-1.9)	<.01
16-20	23	13	1.6 (0.7-3.8)	
>20	17	3	6.6 (1.7-25.4)	
Duration of smoking, y				
Never smoked	41	43	1.0 ...	
≤18	10	8	1.5 (0.5-4.2)	.10
19-25	8	3	2.6 (0.6-10.8)	
>25	46	25	1.9 (1.0-3.6)	
Pack-years				
None	41	43	1.0 ...	
≤15	5	4	1.1 (0.3-4.8)	<.01
16-20	10	10	1.0 (0.3-2.7)	
>20	36	4	2.7 (1.2-5.8)	
Age at smoking initiation, y				
Never smoked	41	43	1.0 ...	
>18	31	25	1.2 (0.6-2.5)	.01
17-18	17	8	2.2 (0.7-7.3)	
≤16	16	4	4.5 (1.3-14.9)	

*Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, and family history of breast cancer, with rapid acetylators as the comparison group. Resultant ORs do not reflect actual risk of breast cancer, but rather the ratio of risk for slow vs rapid acetylators. Included in each analysis are lifetime nonsmokers (referent [ellipses]) and those who were smoking during that time period; excluded are those who quit smoking before that time period. *P* for trend was calculated from logistic regression, with the independent variable as a continuous variable.

Table 7.—Smoking-Matched Case-Control Analysis of Postmenopausal Breast Cancer Risk and Cigarette Smoking by Acetylator Genotype: Western New York Breast Cancer Study, 1986 to 1991*

	Rapid Acetylators			Slow Acetylators		
Quartile	Cases	Controls	OR (95% CI)	Cases	Controls	(95% CI)
Daily cigarettes 2 y ago						
None	43	42	1.0 ...	41	42	1.0 ...
≤15	6	6	1.9 (0.5-7.1)	6	6	1.1 (0.3-3.8)
16-20	6	11	0.6 (0.2-2.0)	16	11	1.7 (0.6-4.3)
>20	2	3	0.5 (0.1-3.6)	6	5	1.6 (0.4-5.9)
P for trend		.28			.44	
Daily cigarettes 20 y ago						
None	43	36	1.0 ...	41	48	1.0 ...
≤15	13	10	1.3 (0.4-3.7)	10	13	1.0 (0.4-2.7)
16-20	13	20	0.8 (0.3-1.9)	23	16	2.1 (0.9-4.7)
>20	3	11	0.2 (0.04-0.8)	15	7	2.6 (0.9-7.4)
P for trend		.02			<.01	
Age at smoking initiation, y						
Never smoked	43	40	1.0 ...	41	44	1.0 ...
>18	19	20	1.1 (0.5-2.6)	27	26	1.0 (0.6-2.7)
17-18	8	11	0.6 (0.2-1.7)	16	13	1.4 (0.6-3.5)
≤16	4	13	0.2 (0.01-1.0)	16	7	3.2 (1.1-9.1)
P for trend		<.05			.09	

*Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, and family history of breast cancer. Included in each analysis are lifetime nonsmokers (referent [ellipses]) and those who were smoking during that time period; excluded are those who quit smoking before that time period. *P* for trend was calculated from Mantel-Haenszel tests with unadjusted categorical data.

The case-series and case-control analyses reported herein indicate that effects may be strongest for smoking at an earlier age (ie, age at which smoking began, number of cigarettes smoked 20 years previously), consistent with the hypothesis that environmental insults may be most deleterious during breast development.[68] The apparently stronger effects of heavier smoking, rather than duration of smoking, may also reflect carcinogenic doses administered at an earlier age. Risk associated with smoking 2 years previously may be increased only because heavy smoking occurred at a younger age, and risk relates to exposure earlier, rather than later, in life. Cigarette smoking is clearly a risk factor for lung and bladder cancer, and genetic polymorphisms in carcinogen-metabolizing genes may increase risk at low exposures.[37,69] For breast cancer, the association is less straightforward.

Among rapid acetylators, there was weak indication that smoking at an early age and smoking more than 1 pack per day 20 years previously reduced breast cancer risk in comparison to that of slow acetylators. In women who are rapid acetylators, carcinogenic aromatic amines may be quickly detoxified and excreted, and other smoke components may have inverse risk effects. It has been suggested that smoking may have antiestrogenic effects, thus decreasing breast cancer risk.[70-72] In rapid acetylators, induction of other enzyme activity by smoking may lead to increased metabolism, and therefore to reduction of circulating estradiol. In rapid acetylators, this antiestrogenic effect may override carcinogenic potential, reducing breast cancer risk. Dual effects of these opposing forces may account for previous failures to observe associations between smoking and breast cancer risk. This is speculation, and the

inconsistent findings for rapid acetylators may be due to chance.

Other tobacco smoke constituents, such as *N*-nitrosamines and polycyclic aromatic hydrocarbons, may be related to breast cancer. We studied polymorphisms related to polycyclic aromatic hydrocarbon biotransformation, *CYP1A1* and *GSTM1*, regarding cigarette smoking and postmenopausal breast cancer risk.[73] Neither *CYP1A1* nor *GSTM1* was independently associated with risk, but light smokers with the *CYP1A1* minor allele had increased risk.

Potential sources of bias in these data exist, foremost of which is selection bias. Most case nonparticipation was due to physicians' refusals to allow contact with their patients (72%), and postmenopausal nonparticipants were, on average, about 3 years older than participants. The most ill patients may not have been included, limiting generalizability. Among controls, a sample refusing interview (n=117) was compared with a sample of participants (n=372) in a telephone interview prior to data collection. No differences in meat, vegetable, and fruit consumption or in number of cigarettes smoked were found. Thus, nonresponse among controls may not have been related to exposure. Other potential sources of bias in control selection include random selection of controls younger than 65 years from lists of driver's license holders. Among cases, some women may not drive and may be different from selected controls, but more than 90% of women in this state are licensed to drive. Because controls were drawn from the community and not screened for undetected breast cancer, a small number of controls may have had breast cancer.

All of these sources of bias may affect risk estimates. In estimating risk associated with smoking in slow acetylators, there may have been some overestimation of true risk due to selection bias, since the proportion of nonsmokers was greater in controls consenting to phlebotomy than in the study group. We thus corroborated findings using case-series analysis and generating smoking-matched data subsets in which the ORs were lower than in initial case-control analyses and more likely estimate true risk. We concluded that, while point estimates of risk may be biased, such bias does not explain the marked difference in smoking risk patterns among slow vs rapid acetylators. While biases may affect risk point estimates, it is unlikely that they could affect heterogeneity of response by NAT2 acetylation status, creating substantial differences in associations between smoking and risk. Nor is it likely that biases were associated with genotype.

We stratified women by susceptible subgroups and examined the role of genotype in susceptibility to an exogenous exposure, cigarette smoking, not heretofore consistently shown to be related to breast cancer risk. In our data, 57% of the postmenopausal women studied were slow acetylators, similar to findings from previous studies.[37,74] Among these women, it appeared that smoking, particularly at an early age, increased breast cancer risk. Slow NAT2 phenotype prevalence varies worldwide, estimated present in 10% to 20% of Asians, 35% of African Americans, 65% to 90% of individuals of Middle Eastern descent, and about 55% of whites.[61,74-77] Ethnic distribution of NAT2 genotype, in relation to exposure to aromatic amines, could explain, in part, the wide geographic variability in breast cancer incidence.

In these data, there was some weak indication of a negative association between breast cancer risk among women with the rapid NAT2 genotype, which does not suggest that smoking is beneficial. Smoking cessation should be a goal for all women.

These findings require replication. If further investigations reveal similar associations, a portion of unexplained breast cancer etiology will have been elucidated. Our data suggest that, for more than half the white female population of the United States, avoidance of breast cancer may be yet another motivation for avoiding cigarette smoking, particularly at an early age.

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APPENDIX B
NAT1 IN BREAST CANCER CASES AND CONTROLS
GENOTYPIC FREQUENCIES
(Case status remains blinded)

Genotype	Premenopausal	Postmenopausal
4,4	106	247
4,10	42	97
4,11	5	21
4,3	6	13
10,10	9	16
10,11	0	5
10,3	3	2
11,11	0	2
11,3	1	0
3,3	1	0
Total	173	403

APPENDIX C

WESTERN NEW YORK DIET STUDY
LIFETIME ALCOHOL CONSUMPTION

Alcohol Intake	Ca ses	Controls	Adjusted OR	95% CI
		Premenopausal		
Low	54	63	1.00	
High	80	63	1.42	(0.84-2.41)
Total	134	126		
		Postmenopausal		
Low	63	78	1.00	
Medium	57	76	0.98	(0.61-1.62)
High	61	76	1.00	(0.61-1.62)
Total	181	230		

*** Adjusted for age, education, family history of breast cancer, history of benign breast disease, BMI, parity, age at first pregnancy, age at menarche, fruit and vegetable intake, age at menarche and age at menopause (postmenopausal women only).**

312-PS

ALCOHOL, ALCOHOL DEHYDROGENASE (3) AND BREAST CANCER IN PREMENOPAUSAL WOMEN

WESTERN NEW YORK DIET STUDY

Alcohol Intake		Cases	Controls	ORs (95% CI)*	
Total	Low	54	63	1.0 (ref.)	1.0 (ref.)
	High	80	63	1.4 (0.8, 2.4)	1.4 (0.8, 2.4)
ADH_3^{2-2}	Low	6	9	1.0 (ref.)	1.0 (ref.)
	High	15	15	1.1 (0.2, 4.7)	1.5 (0.2, 9.7)
ADH_3^{1-2}	Low	27	29	1.0 (0.3, 4.2)	1.0 (ref.)
	High	23	31	0.7 (0.2, 2.8)	0.6 (0.3, 1.4)
ADH_3^{1-1}	Low	21	25	0.9 (0.2, 3.7)	1.0 (ref.)
	High	42	17	3.3 (0.9, 12.9)	3.0 (1.3, 6.6)

*** Adjusted OR for age, education, family history, history of benign breast disease, BMI, parity, age at first pregnancy, fruit and vegetable intake, age at menopause**

309-PS

WESTERN NEW YORK DIET STUDY
ALCOHOL DEHYDROGENASE 3 POLYMORPHISMS

	Cases	Controls	Adjusted OR	95% CI
		Premenopausal		
2,2	22	24	1.00 (ref.)	
1,2	51	60	0.74	(0.33-1.68)
1,1	63	43	1.60	(0.71-3.64)
		Postmenopausal		
2,2	29	35	1.00 (ref.)	
1,2	90	115	1.02	(0.54-1.95)
1,1	66	83	1.13	(0.58-2.21)

*** Adjusted for age, education, family history of breast cancer, history of benign breast disease, BMI, parity, age at first pregnancy, age at menarche, fruit and vegetable intake, age at menarche and age at menopause (postmenopausal women only).**

311-PS

WESTERN NEW YORK DIET STUDY
LIFETIME ALCOHOL CONSUMPTION BY ADH3 POLYMORPHISM
Postmenopausal Women

Alcohol Intake	Cases	Controls	Adjusted OR*	95%CI
ADH 2,2				
Low	9	9	1.00 (ref.)	
Medium	9	11	0.51	(0.12-2.27)
High	10	15	0.53	(0.14-2.10)
ADH 1,2				
Low	33	42	0.67	(0.21-2.10)
Medium	26	33	0.81	(0.25-2.61)
High	30	39	0.60	(0.19-1.92)
ADH 1,1				
Low	21	27	0.65	(0.20-2.15)
Medium	22	32	0.78	(0.24-2.56)
High	21	22	0.86	(0.25-2.88)

*** Adjusted for age, education, family history of breast cancer, history of benign breast disease, BMI, parity, age at first pregnancy, age at menarche, fruit and vegetable intake, age at menarche and age at menopause (postmenopausal women only).**

Appendix D
CYP1A1, GSTM1 AND GSTT IN BREAST CANCER CASES AND CONTROLS
GENOTYPE FREQUENCIES
(Case status remains blinded)

Genotype	Premenopausal
CYP1A1	
WW	110
WM	17
MM	2
GSTM1	
PRESENT	62
NULL	67
GSTT	
PRESENT	89
NULL	40

APPENDIX E
MICROSOMAL EPOXIDE HYDROLASE IN BREAST CANCER CASES AND CONTROLS
GENOTYPE FREQUENCIES
(Case status remains blinded)

Genotype	Premenopausal	Postmenopausal
MEH4		
HA	145	127
HH	63	82
AA	7	6
MEH3		
HA	16	41
HH	49	105
AA	74	138

APPENDIX F
CYP2D6 GENOTYPING IN BREAST CANCER CASES AND CONTROLS
GENOTYPE FREQUENCIES
(Case status remains blinded)

Genotype	Premenopausal	Postmenopausal
CYP2D6 A		
WW	220	339
WM	10	13
MM	4	0
CYP2D6 B		
WW	152	240
WM	68	155
MM	14	24
CYP2D6 T		
WW	216	316
WM	5	8
MM	0	0

APPENDIX G

MOLECULAR CARCINOGENESIS 17:144–150 (1996)

A Cytochrome P4502E1 Genetic Polymorphism and Tobacco Smoking in Breast Cancer[1]

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Known breast-cancer risk factors account for only part of the variability in breast-cancer incidence. Tobacco smoke is not commonly considered a breast carcinogen, but many of its constituents, such as *N*-nitrosamines, are carcinogenic in laboratory animal studies. Herein, we assessed a cytochrome P4502E1 (*CYP2E1*) genetic polymorphism (a DraI restriction enzyme site in intron 6) as a risk factor for breast cancer in both premenopausal and postmenopausal women. Because *N*-nitrosamines are metabolically activated by CYP2E1, the risk among women smokers was investigated. Caucasian women were enrolled in a case-control study of breast cancer between 1986 and 1991. A subset of the women (219 premenopausal and 387 postmenopausal women) consented to phlebotomy. The allelic frequencies for the premenopausal women (D allele = 0.91 and C allele = 0.09) and postmenopausal women (D allele = 0.93 and C allele = 0.07) were similar to those previously reported. There was no statistically significant association between the *CYP2E1* polymorphism and breast-cancer risk for premenopausal or postmenopausal women (adjusted odds ratio (OR) = 1.04, 95% confidence interval (CI) = 0.48, 2.24, and OR = 1.01, 95% CI = 0.55, 1.84, respectively). When the women were categorized as nonsmokers versus smokers (those who smoked more than one cigarette per week for more than 1 yr), premenopausal women with one or two C alleles who had a history of smoking were found to be at increased risk (unadjusted OR = 7.00, 95% CI = 0.75, 14.53, and adjusted OR = 11.09, 95% CI = 1.51, 81.41), although the number of study subjects with those genotypes was small. The small number of study subjects with a C allele precluded meaningful classification by level of smoking, but categorizing the smokers into two groups (above and below the median) also suggested an increased risk. Premenopausal women with the DD genotype and postmenopausal women with any genotype were not at increased risk. Breast-cancer risk was not related to the *CYP2E1* genotype in either premenopausal nonsmokers or smokers (adjusted OR = 0.66, 95% CI = 0.20, 2.17, and OR = 2.13, 95% CI = 0.60, 7.59, respectively) or postmenopausal nonsmokers or smokers (OR = 0.90, 95% CI = 0.34, 2.35, and OR = 1.02, 95% CI = 0.46, 2.23, respectively), although the difference in the ORs for premenopausal nonsmokers and smokers suggests an increased risk for smokers. While there are limitations to this study, particularly related to the small number of subjects with the DC and CC genotypes, the study suggests that some women may be susceptible to tobacco smoke because of a *CYP2E1* polymorphism. However, these results are preliminary and must be replicated.

Key words: Tobacco, cytochrome P4502E1, genetic polymorphisms, metabolizing enzymes, N-nitrosamines

INTRODUCTION

The effects of reproductive, hormonal, and family histories on breast-cancer risk are well documented but account for no more than 47% of breast cancers [1]. Descriptive studies by geographic region [2] and migratory studies [3] suggest that diet, environment, and lifestyle behavior may affect risk, but a direct relationship with specific exposures has not been clearly established. In particular, tobacco smoke, which is a potent human carcinogen, has not been implicated as a risk factor in case-control or cohort studies of breast cancer [4,5]. Nonetheless, there are experimental data that suggest that tobacco-smoke carcinogens such as *N*-nitrosamines, polycyclic aromatic hydrocarbons, aryl aromatic amines, and heterocyclic aromatic amines are potential human breast carcinogens [6].

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Abbreviations: NMU, *N*-methyl-*N*-nitrosourea; CYP2E1, cytochrome P4502E1; OR, odds ratio; PCR, polymerase chain reaction; CI, confidence interval.

N-nitrosamines, including the direct acting *N*-methyl-*N*-nitrosourea (NMU), cause DNA damage in mouse mammary cells [7]. These cells form promutagenic O^6-methyldeoxyguanosine adducts and have decreasing levels of O^6-alkylguanine-DNA alkyl transferase activity over time [8], perhaps indicating an increased host susceptibility to nitroso compounds. *N*-nitrosamines also have been shown to cause rodent mammary tumors [6,9–11], which are histologically similar to human cancers [7,12] and can metastasize [9,12]. NMU also possesses the ability to transform cultured mouse mammary cells [7,13]. While it was originally believed that *N*-nitrosamine exposure induced a specific GGA→GAA transition in codon 12 of the Ha-*ras*-1 oncogene [10,14], it is more likely that the observed mutation is a result of cell selection for preexisting mutations [15]. Cultured human mammary epithelial cells also undergo unscheduled DNA synthesis after exposure to ethylmethanesulfonate [16], although transformation by NMU and *N*-methyl-*N*-nitrosoguanidine has not yet been shown [17]. Human exposure to *N*-nitrosamines occurs through diet, endogenous formation in the stomach, tobacco smoke, occupation and medical therapies [18].

Cytochrome P4502E1 (CYP2E1) is one of several enzymes responsible for the metabolic activation of *N*-nitrosamines (including tobacco-specific nitrosamines) and other low molecular weight compounds [19–22]. The activity of this enzyme varies widely among individuals, and both phenotypic and genetic polymorphisms have been identified [23–25]. One specific genetic polymorphism in *CYP2E1* is located in intron 6 [24] and is revealed by DraI restriction enzyme digestion. While there are no clear in vitro data showing that the polymorphic alleles affect function (i.e., induction, quantity, or activity), the *CYP2E1* intron 6 polymorphism has been associated with altered protein levels in human liver samples [26] and increased 7-methyl-2′-deoxyguanosine adduct levels in human lung [27]. Moreover, this polymorphism has been associated with lung cancer in a Japanese study [24] and with a modification of smoking-related risk [26], although no effect has been observed in studies of Caucasians in Europe [28–30] and the United States [30] or African Americans in the United States [30]. The polymorphism, however, has not been shown to be associated with either gastric [26] or nasopharyngeal carcinoma [31]. To date, there are no reports of this polymorphism in relation to breast cancer risk, and to our knowledge *CYP2E1* expression in human breast tissues has not ever been studied.

We report here the results of a study of the *CYP2E1* DraI polymorphism in a case-control study of breast cancer among both premenopausal and postmenopausal Caucasian women.

MATERIALS AND METHODS

Study Subjects

The 606 women in this report were a subset of a study described previously [32–35], in which 1545 Caucasian women were enrolled in a case-control study of breast cancer from 1986 to 1991. The selection of study subjects in this report was based upon the availability of DNA suitable for genotyping. The cases were women diagnosed with incident, primary breast cancer; pathology reports were reviewed for confirmation of diagnosis. Women were classified as either premenopausal or postmenopausal based upon menstrual status. Women under the age of 50 were also considered postmenopausal if they had undergone bilateral oophorectomy or irradiation to the ovaries; women under the age of 50 who had a hysterectomy but retained their ovaries were considered premenopausal. The controls were frequency matched to cases by age and county of residence (Niagara and Erie). Controls under the age of 65 were randomly selected from the New York State Motor Vehicle registry, whereas those over 65 were selected from Health Care Finance Administration lists. Eligibility and participation rates have been previously described [32–35]. The study subjects were administered a 2-h interview by trained interviewers who collected data on medical history, usual diet 2 yr before the interview, lifetime cigarette smoking, alcohol consumption, and occupation. The findings on the other genetic polymorphisms examined in this study were reported previously [36,37].

Given the total number of study subjects for which we have *CYP2E1* data (219 premenopausal women (106 cases and 113 controls) and 387 postmenopausal women (166 cases and 221 controls)) and the expected frequency of combined *CYP2E1* DC and CC genotypes of 14% [30] among controls, this study had a 0.63 and 0.85 power to detect a crude odds ratio (OR) of 2.00 ($P < 0.05$, two tailed) for the genotype and breast cancer, respectively. The *CYP2E1* DC and DD genotypes were combined to increase statistical power. The power of this study is significantly less for determining adjusted ORs and for considering risk within smoking categories.

CYP2E1 Genetic Polymorphism Analysis

DNA was extracted from archived blood clots as previously reported (Ambrosone CB, Freudenheim JL, Graham S, Marshall JR, Vena JE, Brasure JR, Michalek AM, Laughlin R, Nemoto T, Gillenwater KA, Harrington AM, and Shields PG, submitted for publication). Polymerase chain reaction (PCR) was used to amplify the region surrounding the *CYP2E1* DraI restriction fragment length polymorphism site. Two different primer sets were used for the premenopausal and postmenopausal groups, as after analyzing the postmenopausal women, we found that a different primer pair produced more susccessful am-

plifications with fewer PCR attempts. The two methods were therefore compared with a set of DNAs (eight family lines encompassing three generations; NIGMS Human Genetic Mutant Cell Repository, Coriell Institute, Camden, NJ) to validate the newer set of primers. There was complete agreement between the two methods; the genotypes were identical in 100% of samples. Further, the same Mendelian inheritance pattern was demonstrated. As both sets of primers yielded the predicted fragment length, there is no reason to believe that the genotyping results were dependent on the primers. While the new primers provided a higher rate of amplification, the total number of samples that could be amplified was similar to that of other assays that we have performed.

For postmenopausal women, genotyping was performed as previously reported [30]. The PCR amplification yielded a 995-bp product, which was then digested with DraI [30]. The final fragments were either 874 and 121 bp or 572, 302, and 121 bp. The analyses of premenopausal women differed by the substitution of primers 5′-GGTATTTCCCCCAAGAA–AGTC-3′ and 5′-CTAACGTGGGGTGACGTGAG-3′ and buffer (GeneAmp PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; and 0.0001% gelatin); Perkin Elmer, Norwalk, CT) in a total volume of 25 μL. The PCR reactions had an initial melting temperature of 94°C for 4 min, followed by 30 cycles of melting at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. An extension period of 4 min at 72°C followed the final cycle. In this case, using the primers for premenopausal women a 530-bp fragment was revealed on a 2.2% agarose gel. Restriction enzyme analysis followed, using the conditions previously reported [30]. The subjects were classified according to the original nomenclature proposed by Uematsu et al. [24], who designated the common allele "D" and the less common allele "C." Agarose gel electrophoresis was interpreted by two independent investigators who were blinded to case-control status, and analysis of at least 10% of the samples was repeated for quality control.

Statistics

Analyses of premenopausal and postmenopausal women were performed separately. ORs with 95% confidence intervals (CIs) were calculated by unconditional logistic regression (SPSS, Chicago, IL). The ORs were adjusted for age, education, body mass index (BMI), age at menarche, age at first pregnancy, family history of breast cancer, and, for postmenopausal women, age at menopause. BMI was computed as weight (kg)/(height)(m)^2 by using reported weight 2 yr before the interview. Family history was considered positive if there was a history of breast cancer in either a mother or a sister. In this report, smokers were defined as those smoking more than one cigarette per week for 1 yr or longer. Therefore, the smokers' category includes both current and former smokers. Smoking status was not further categorized because of the small number of study subjects with the C allele compared with the D allele.

RESULTS

There was a total of 606 Caucasian women from whom we had DNA for genotyping. The allelic frequencies for the D (0.91) and C (0.09) alleles among premenopausal cases and controls were very similar to the frequencies for postmenopausal cases and controls (0.93 and 0.07, respectively). For all these categories, the predicted genotype frequencies based upon allelic frequency were not statistically different from the observed frequencies ($P < 0.001$); they were all in Hardy-Weinberg equilibrium.

The characteristics of the premenopausal and postmenopausal women with available genotyping data are presented in Tables 1 and 2. There were no statistical differences by *CYP2E1* genotypes within cases or controls for any of the risk factors listed in Tables 1 and 2. For the women with available genotyping

Table 1. Characteristics of Premenopausal Subjects by *CYP2E1* Genotypes*

	Cases		Controls	
	DD	DC or CC†	DD	DC or CC†
Number	88	18	95	18
Age (yr)	46 ± 4	47 ± 3	47 ± 3	48 ± 4
Education (yr)	14 ± 3	13 ± 2	14 ± 3	14 ± 2
Age at menarche (yr)	13 ± 2	13 ± 1	13 ± 2	13 ± 2
Age at first pregnancy (yr)	24 ± 5	23 ± 4	22 ± 4	22 ± 4
Total time breast feeding (mo)	3 ± 7	4 ± 6	6 ± 13	9 ± 15
Total time menstruation (mo)	383 ± 56	373 ± 42	371 ± 50	374 ± 45
Body mass index	24 ± 5	25 ± 5	26 ± 5	24 ± 4
Smoking (yr)‡	20 ± 9	20 ± 9	22 ± 9	22 ± 9
Smoking (pack-years)‡§	19 ± 17	17 ± 13	20 ± 20	21 ± 14

*For subjects with DNA suitable for genotyping. All values except Number are means ± standard deviations.
†The DC and CC categories were combined to increase the statistical power.
‡Data for smokers only.
§Pack-years were defined as the predicted average number (calculated from data for smoking 2, 10, and 20 yr before the interview) of packs smoked per day multiplied by the reported numbers of years smoking.

Table 2. Characteristics of Postmenopausal Subjects by *CYP2E1* Genotypes*

	Cases		Controls	
	DD	DC or CC†	DD	DC or CC†
Number	144	22	190	31
Age (yr)	64 ± 7	62 ± 7	63 ± 7	64 ± 7
Education (yr)	12 ± 3	13 ± 3	12 ± 3	12 ± 3
Age at menarche (yr)	13 ± 2	13 ± 2	13 ± 2	13 ± 2
Age at menopause (yr)	47 ± 6	49 ± 6	47 ± 6	47 ± 5
Age at first pregnancy (yr)	24 ± 5	24 ± 5	23 ± 4	23 ± 4
Total mo breast feeding	5 ± 11	2 ± 6	4 ± 7	6 ± 10
Total mo menstruation	378 ± 77	400 ± 70	375 ± 79	379 ± 63
Body mass index	26 ± 5	25 ± 4	25 ± 4	26 ± 5
Smoking (yr)‡	31 ± 4	36 ± 14	31 ± 14¶	37 ± 11¶
Smoking (pack-years)‡§	30 ± 22	39 ± 29	25 ± 19	30 ± 21

*For subjects with DNA suitable for genotyping. All values except Number are means ± standard deviations.
†The DC and CC categories were combined to increase the statistical power.
‡Data for smokers only.
§Pack-years were defined as the predicted average number (calculated from data for smoking 2, 10, and 20 yr before the interview) of packs smoked per day multiplied by the reported numbers of years smoking.
¶$P<0.05$ by Student's t-test comparisons of DD versus DC or CC controls.

data, the number of cigarettes smoked 2, 10, and 20 yr before the interview, total years smoked, and pack-years were not statistically different between cases and controls, although cases tended to smoke less than controls did. For this smoking data, there were no differences between the *CYP2E1* genotypes for premenopausal women when the DC and CC genotypes were analyzed together (data not shown). For postmenopausal women, there was an increase in smoking duration (Student's *t* test, $P < 0.05$) among women who had either the DC or CC genotypes, compared with women with the DD genotype (37 ± 11 and 31 ± 14 yr, respectively; $P < 0.05$).

When *CYP2E1* genotype was regressed on case-control status, there was no overall association between breast cancer and *CYP2E1* genotype (DD versus DC and CC) (Table 3) for either the premenopausal women (OR = 1.04, 95% CI = 0.53, 2.24) or the postmenopausal women (OR = 1.01, 95% CI = 0.55, 1.84).

Cigarette smoking was examined in relation to the *CYP2E1* genotypes, and crude and adjusted ORs were calculated (Table 3). An examination of the relation-

Table 3. *CYP2E1* Genotype and Breast Cancer Risk

Subjects	*CYP2E1* genotype	Cases (%)	Controls (%)	Total (%)	OR (95% CI)
Premenopausal					
All	DD	88 (83)	95 (84)	183 (84)	1.00
	DC or CC	18 (17)	18 (16)	36 (16)	1.08 (0.53, 2.21)†
					1.04 (0.48, 2.24)‡
Nonsmokers	DD	35 (85)	42 (75)	77 (79)	1.00
	DC or CC	6 (15)	14 (25)	20 (21)	0.51 (0.18, 1.48)†
					0.66 (0.20, 2.17)‡
Smokers	DD	53 (81)	53 (93)	106 (87)	1.00
	DC or CC	12 (19)	4 (7)	16 (13)	3.00 (0.91, 9.88)†
					2.13 (0.60, 7.59)‡
Postmenopausal					
All	DD	144 (87)	190 (86)	334 (86)	1.00
	DC or CC	22 (13)	31 (14)	53 (14)	0.94 (0.52, 1.69)†
					1.01 (0.55, 1.84)‡
Nonsmokers	DD	67 (89)	90 (87)	106 (87)	1.00
	DC or CC	8 (11)	13 (13)	21 (12)	0.83 (0.32, 2.11)†
					0.90 (0.34, 2.35)‡
Smokers	DD	77 (85)	100 (85)	177 (85)	1.00
	DC or CC	14 (15)	18 (15)	32 (15)	1.01 (0.47, 2.16)†
					1.02 (0.46, 2.23)‡

*The DC and CC categories were combined to increase statistical power.
†Unadjusted OR (95% CI).
‡OR (95% CI) calculated by unconditional logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer, and, for postmenopausal women, age at menopause.

ship between breast cancer and the *CYP2E1* genotypes in women who either did or did not smoke failed to reveal a statistically significant association for premenopausal or postmenopausal women. In nonsmokers, the adjusted ORs were 0.66 (95% CI = 0.20, 2.17) for premenopausal and 0.90 (95% CI = 0.34, 2.35) for postmenopausal women. For smokers, the adjusted ORs were 2.13 (95% CI = 0.60, 7.59) for premenopausal and 1.02 (95% CI = 0.46, 2.23) for postmenopausal women. However, as shown in Table 4, smoking premenopausal women who had the DC or CC genotypes had an increased risk of breast cancer (adjusted OR = 11.09, 95% CI = 1.51, 81.41), although the number of study subjects with these genotypes was small and consequently the CI was large. For postmenopausal women, there was no association between breast cancer and smoking in either genotype category.

In the premenopausal women, we categorized the smoking women by pack-years of smoking (that is, greater or less than the median). The number of study subjects was quite small, but compared with the nonsmokers, persons with the DC or CC genotype who had smoked less than 16 pack-years had an OR of 13.5 (95% CI = 0.9, 213) and those who had smoked more than 16 pack-years had an OR of 7.7 (95% CI = 0.8, 72). Analysis of premenopausal women by the age at which they began to smoke indicated that women with the DC or CC genotypes, compared with nonsmokers, had ORs of 8.7 (95% CI = 0.8, 99) for women younger than 16 yr and 13.9 (95% CI = 1.3, 153) for women older than 16 yr.

DISCUSSION

Herein we examined breast cancer and a *CYP2E1* DraI polymorphism in Caucasian women by using a population-based case-control study. The CYP2E1 gene product is responsible for the metabolic activation of *N*-nitrosamines [19,22,38], which may play a role in breast cancer [6–11,13]. Although the genetic polymorphism is located in an intron and does not explain the known interindividual variation in this gene, it is a suspected risk factor for lung cancer in Japanese [24,26] and is associated with increased *N*-nitrosamine–related DNA-adducts in the human lung [27]. Nonetheless, there are no other published studies of *CYP2E1* and breast-cancer risk. In this first report, we found no differences in overall risk associated with the DC and CC genotypes compared with the DD genotype. The allelic frequencies of the cases and controls were very similar to those of previous reports [30].

Cigarette smoking has been hypothesized to be related to breast cancer based upon the results of experimental studies [6], although smoking has not generally been related to risk in epidemiologic studies [4,5]. We hypothesized that tobacco smoke might also be a human breast carcinogen because it contains *N*-nitrosamines and other carcinogens. If so, then a subpopulation of women may be susceptible to cigarette smoke because they have a particular genetic susceptibility. Studying breast cancer and cigarette use without considering genetic susceptibility might obscure the relationship because the increased risk in some women might be diluted in a background of women who are not at risk. Indeed, we previously reported that smoking women with the *N*-acetyltransferase 2 (*NAT2*) slow acetylation genotype are at increased risk for breast cancer [36]. For *CYP2E1*, our data indicated that the DC and CC genotypes were positively associated with breast cancer in premenopausal cigarette-smoking women, although because of the small number of study subjects, this finding should be considered preliminary. For postmenopausal breast cancer, there was no increased smoking-related risk for any smoking or genotype category. While there are morphologic, phenotypic, and prognostic differences between premenopausal and postmenopausal breast cancer, the reasons why premenopausal and not postmenopausal breast cancer should be associated with smoking is not clear. Given our previous finding of a modifying

Table 4. Smoking and Breast-Cancer Risk by *CYP2E1* Genotype

	DD			DC or CC*		
Subjects	No. of cases (%)	No. of controls (%)	OR (95% CI)	No. of cases (%)	No. of controls (%)	OR (95% CI)
Premenopausal						
Nonsmokers	35 (45)	42 (55)	1.00	6 (30)	14 (70)	1.00
Smokers	53 (50)	53 (50)	1.20 (0.67, 2.16)†	12 (75)	4 (25)	7.00 (0.75, 14.53)†
			1.43 (0.74, 2.76)‡			11.09 (1.51, 81.41)‡
Postmenapausal						
Nonsmokers	67 (43)	90 (57)	1.00	8 (38)	13 (62)	1.00
Smokers	77 (44)	100 (56)	1.03 (0.67, 1.60)†	14 (44)	18 (56)	1.26 (0.41, 3.89)†
			1.14 (0.72, 1.81)‡			1.31 (0.39, 4.27)‡

*DC and CC categories combined to increase statistical power.
†Unadjusted OR (95% CI).
‡OR (95% CI) calculated by unconditional logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer, and, for postmenapausal women, age at menopause.

effect of the *NAT2* polymorphism on the relation between smoking and breast-cancer risk [36], *CYP2E1* ought to be examined after stratification by *NAT2* status. However, the small number of subjects with the CD and CC genotypes in this study made that analysis uninformative. Further, the risks from carcinogens that are substrates for the products of these genes (aryl aromatic amines and *N*-nitrosamines, respectively) are different, so that there might not be either an additive or a synergistic effect. A larger study, however, would be needed to examine this question.

While this study allowed us to examine extensive, standardized interview data in conjunction with data on genetic susceptibility, the study had significant limitations. First, while this study did not indicate that there was an overall role for the *CYP2E1* DraI genetic polymorphism in breast cancer, the statistical power of the study was too small to reject this hypothesis for premenopausal women when examining crude risk. The study size was suitable for identifying a statistically significant OR of 2.0 for postmenopausal women but 3.0 for premenopausal women. There was insufficient power for examining adjusted risks for premenopausal or postmenopausal women. Thus, smaller risks may have escaped detection in this study. Second, while the study data set suggested that smoking was a risk factor in women with the CD and CC genotypes, the small number of subjects precluded a meaningful analysis by further categorization of those women. If there had been sufficient numbers, an examination of the data by increasing cigarette consumption would have made the data for a smoking-related breast cancer effect more convincing. Further, among smokers, *N*-nitrosamine exposure may vary by dietary exposures (e.g., it is increased by greater beer consumption or consumption of foods containing nitrates). Third, while this study does not provide evidence for a role for the *CYP2E1* DraI genetic polymorphism in postmenopausal breast cancer or in premenopausal breast cancer in nonsmokers, notwithstanding its limitations in statistical power, it did not exclude a role for *CYP2E1*. For example, the wide interindividual variation for *CYP2E1* activity and *N*-nitrosamine activation (due to inducibility or other polymorphisms) may still be related to breast-cancer risk. Fourth, there are important potential sources of bias that may have affected our results. They include low participation among cases and controls, limited availability of DNA for genotyping among participants, and recall differences for exposures between cases and controls. However, it is unlikely that these biases are related to the *CYP2E1* genetic polymorphism, and so while they are important to consider, they are also not likely to have influenced the results of this study. Last, because this study investigated only Caucasian women, it may not be possible to extrapolate the results to women of other races or to the general population.

In conclusion, this study did not find an association between the *CYP2E1* DraI genetic polymorphism and breast cancer risk. Premenopausal women with the DC and CC genotypes may be at increased risk for breast cancer if they are smokers. However, the results of this study must be viewed as preliminary because of the small number of subjects with the C allele, which made difficult examination of exposure levels and examination of modification by *NAT2*. Nonetheless, that genetic susceptibilities, including that related to *CYP2E1*, modify the effect of exogenous environmental and lifestyle exposures on breast-cancer risk remains an important hypothesis. Further investigation in larger studies and other populations is therefore warranted.

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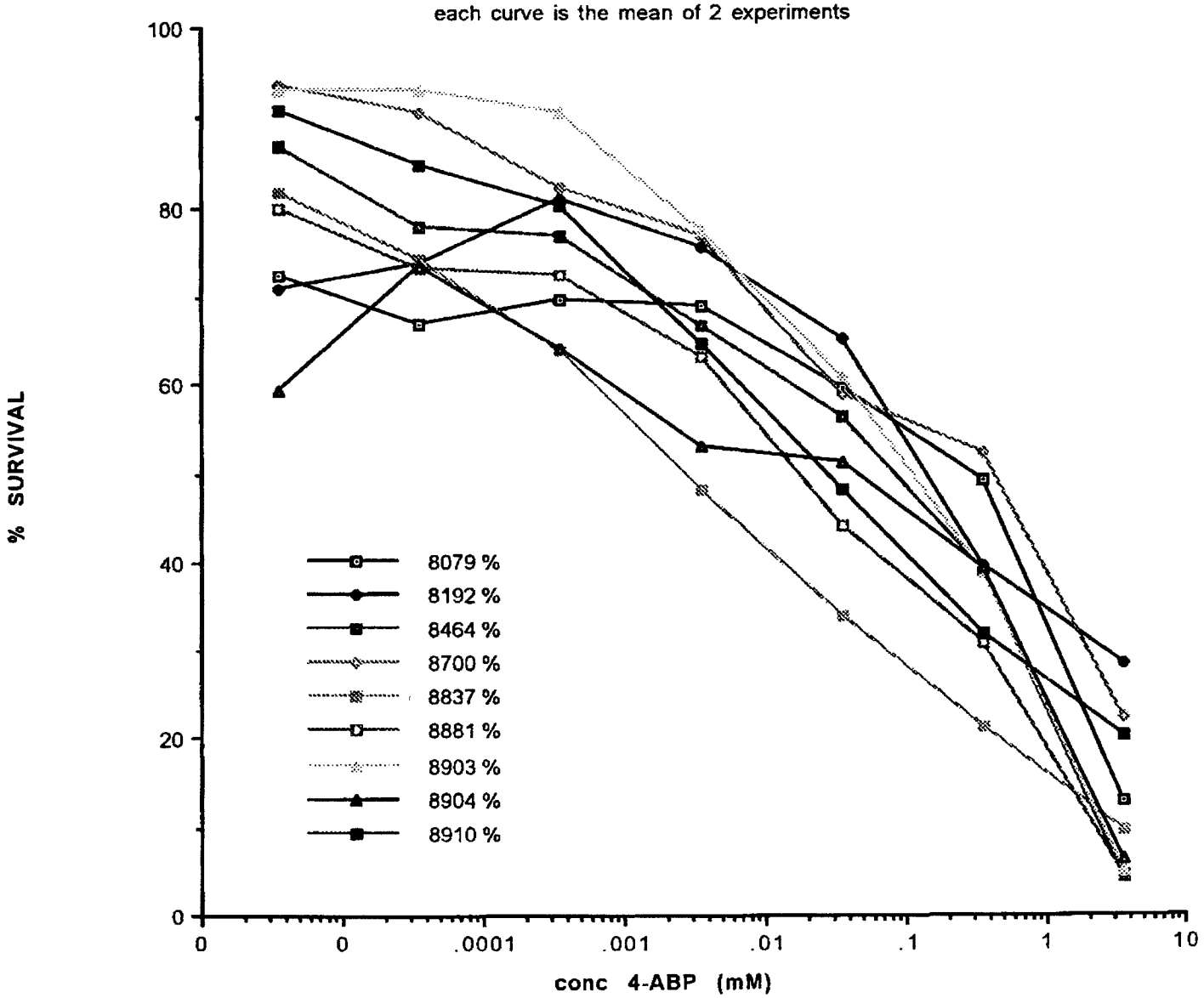
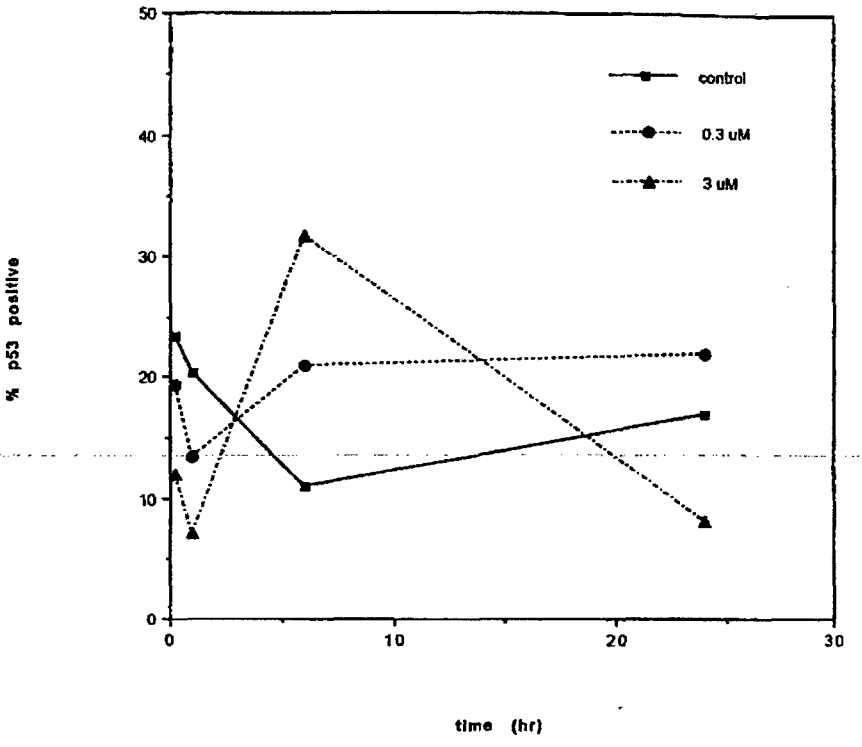
APPENDIX H

Cytotoxicity Curves for 10 primary mammary epithelial cell strains

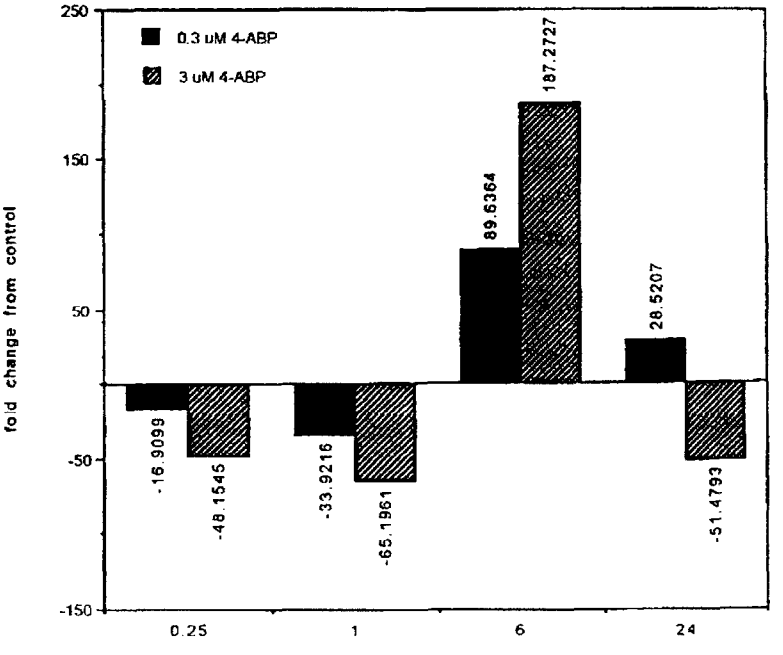
each curve is the mean of 2 experiments

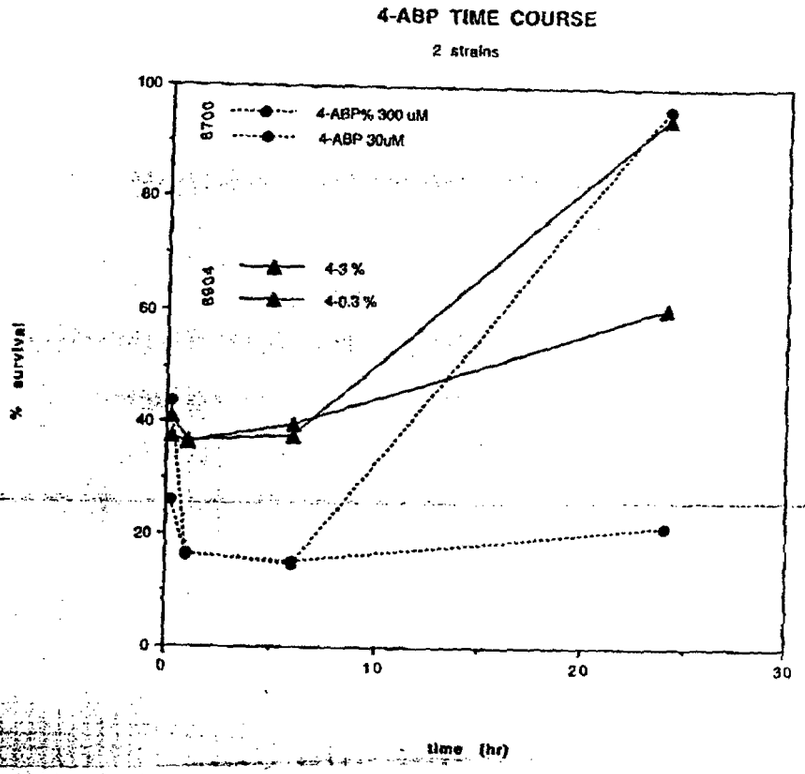


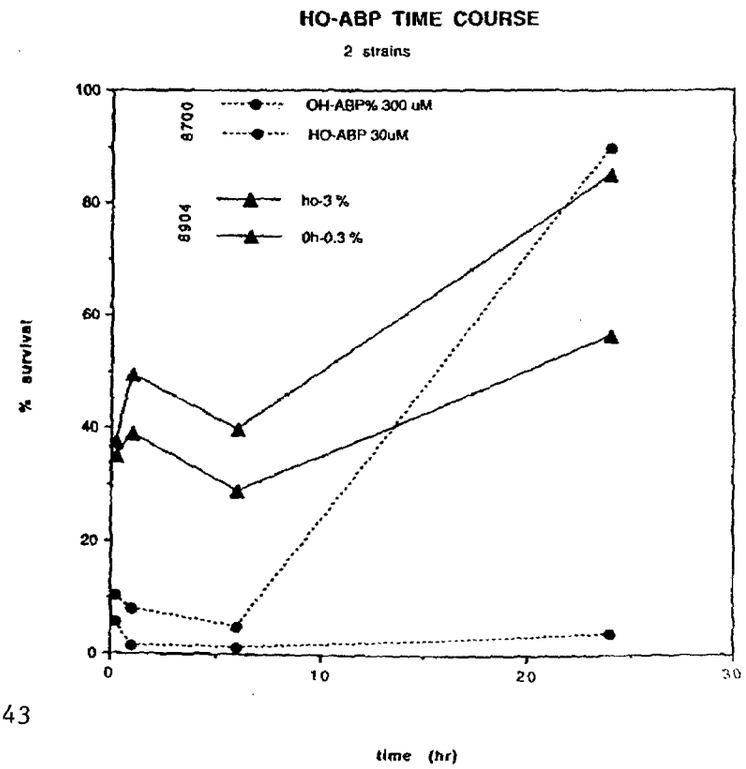
time course of strain 8904 with two doses of 4-ABP



time course for strain 8904 - treatment with 4-ABP at two doses




4-ABP TIME COURSE
2 strains
8700
4-ABP% 300 uM
4-ABP 30uM
8904
4-3 %
4-0.3 %
% survival
time (hr)


HO-ABP TIME COURSE
2 strains
8700
OH-ABP% 300 uM
HO-ABP 30uM
8904
ho-3 %
0h-0.3 %
% survival
time (hr)

APPENDIX I

Amy J. Warren, Peter G. Shields

May 9, 1997

AMINOBIPHENYL-DNA ADDUCT DETECTION USING "ADAM"

Introduction

A continued goal of the LHC is to develop, apply, and validate biomarkers of cancer risk in order to enhance cancer risk assessment, focus cancer prevention strategies, and elucidate mechanisms of carcinogenesis. One indicator for the net effect of exogenous carcinogen exposure and inherited traits for absorption, metabolism, and DNA repair is the carcinogen-DNA adduct. A number of methods exist for the sensitive detection of DNA damage in human tissue. The method of adduct detection currently being developed/adapted in our laboratory is adduct detection by acylation with ^{35}S-methionine (ADAM) (Sheabar *et al.*, *PNAS*, 91:1696-1700, 1994; *Chem. Res. Toxicol.*, 7:650-658, 1994). Current goals are to measure the major DNA adduct of 4-aminobiphenyl (dG-8-ABP) in DNA from human breast tissue in order to determine the *in vivo* relationship of carcinogen-DNA adducts to exposure, metabolic activation, and genetic polymorphisms. In addition, we are interested in measuring adduct levels in DNA from primary breast cultures to study interindividual differences in carcinogen metabolism, metabolizing enzymes, genetic polymorphisms, p53 induction, and apoptosis.

Methodology

- Synthesis of ^{35}S-TBM (the actual labeling reagent) and HPLC purification.
- Acylation of dG-8-ABP with ^{35}S-TBM and HPLC analysis.
- Exploration of alternative labeling reagents. Basic problem of methionine is the amino group which must be protected prior to the acylation reaction. Tried two different methods to deaminate methionine chemically or enzymatically, which would also provide a more reactive compound.

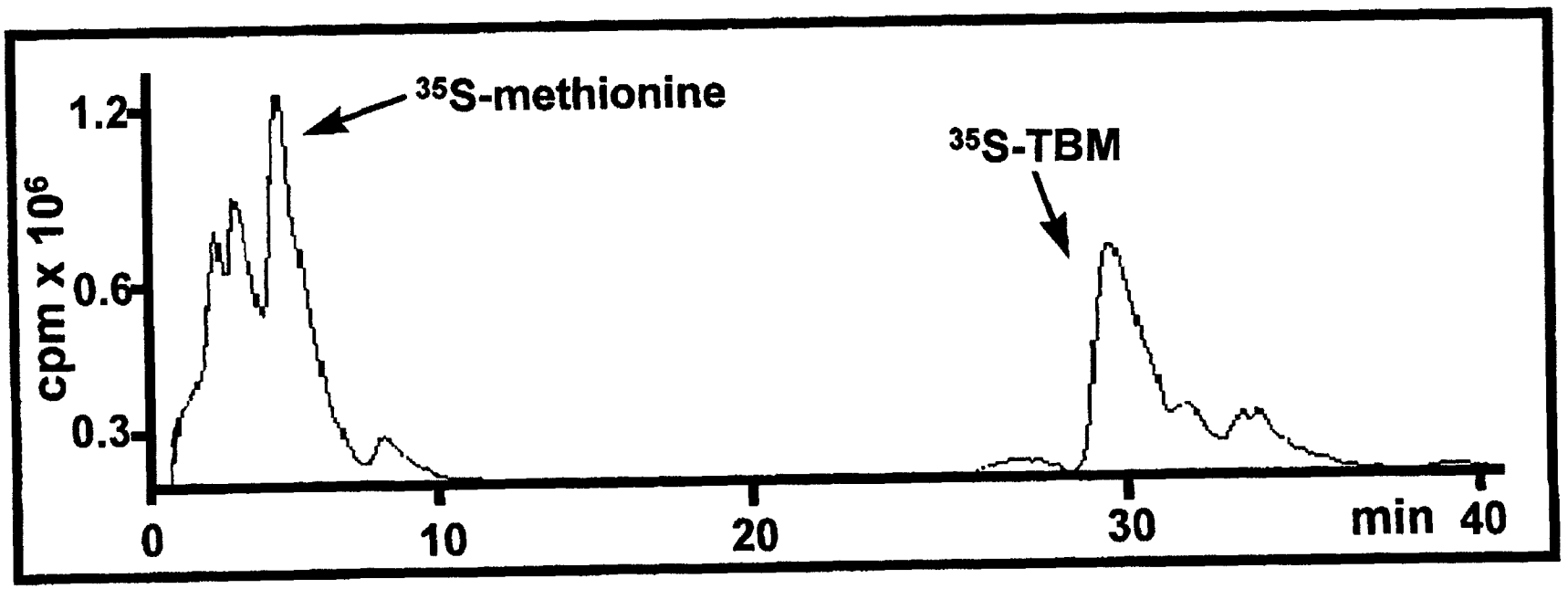
Results

- Reaction of ^{35}S-methionine to produce ^{35}S-TBM (BOC-protected methionine) went with 33.3% efficiency and collected the correct peak this time.
- Acylation using the freshly purified ^{35}S-TBM resulting in 75.6% ± 9.3 (avg of 4 samples) labeling efficiency and 83% of acylated material was represented by the bis compound.
  - Good news: primarily one peak was formed, making this look promising for *in vivo* studies.
  - Bad news: still need to work on washing steps to prevent sample carry over. There is so much radioactive material involved that a much longer wash of the column at the end of each run will be needed (the material appears to be sticking to the column). Although 1 pmol, 100 fmol, 10 fmol, and 1 fmol were all acylated with ^{35}S-TBM, they all ended up with the same number of cpm in the product. Thus, in order to be quantitative, we need to optimize the gradient program more.
- Exploration of methods to deaminate methionine were only partially successful and would require further optimization.

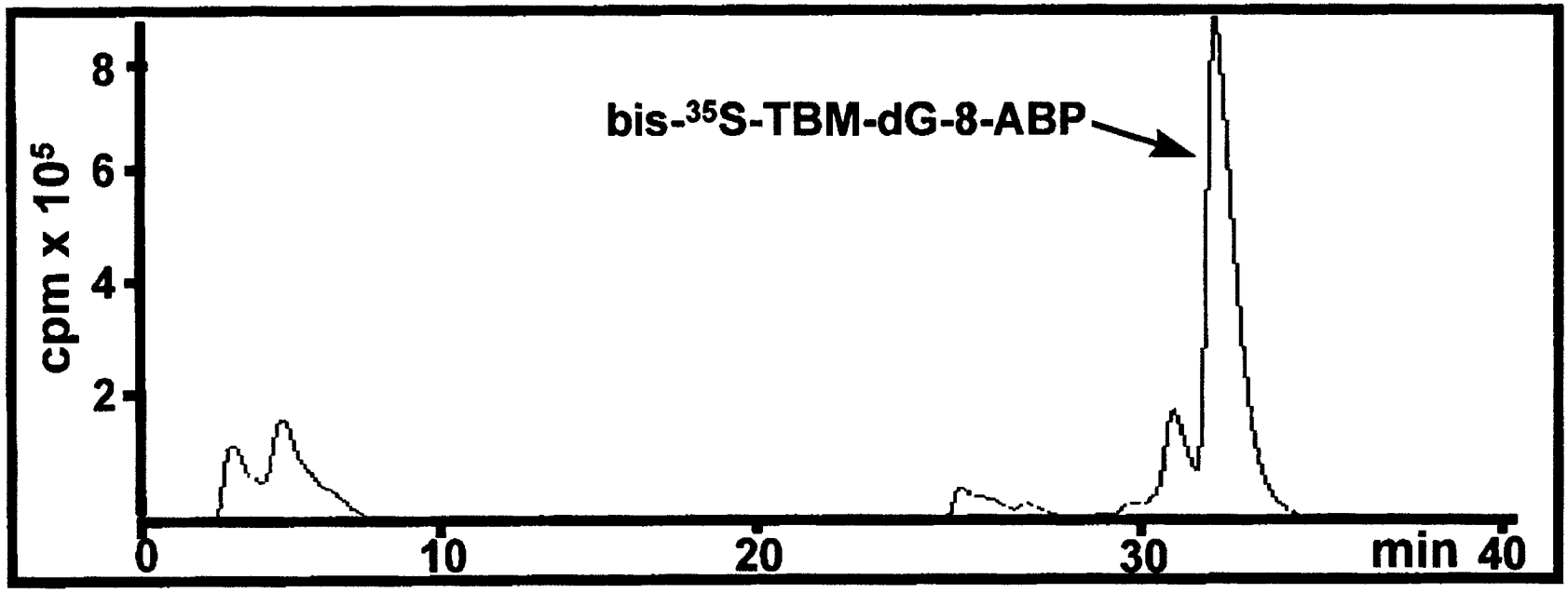
Future Course

- Future work involves repeating the generation of a standard curve of the dG-8-ABP adduct working on more thorough washes of the column between runs to avoid sample carry-over. Bottom line: now that the assay appears to be behaving somewhat, more careful thought will be required to design appropriate internal and external controls to make it not only qualitative, but quantitative.
- Prepare HO-[^{3}H]ABP to use for modifation of calf thymus DNA with ABP at different modification levels—should be most accurate way of checking binding level.
- Synthesize dG-8-[^{3}H]ABP for further optimization of immunoaffinity columns.

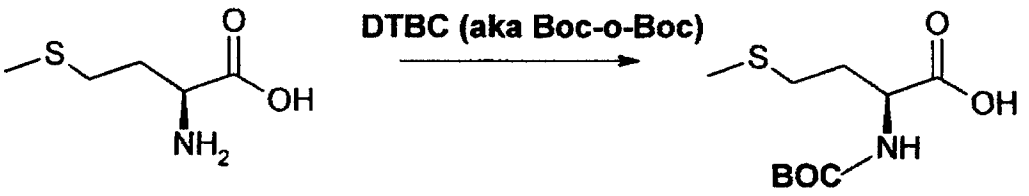
HPLC Analysis of BOC-protected ^{35}S-methionine (^{35}S-TBM)



HPLC Analysis of dG-8-ABP Acylated with ^{35}S-TBM

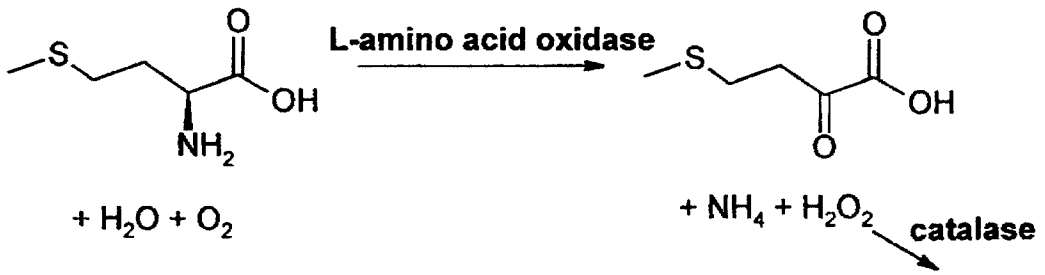


BOC PROTECTION OF METHIONINE
(Step I. of the ADAM method)

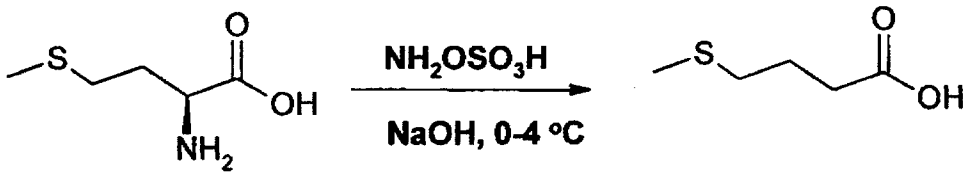


METHODS TO DEAMINATE METHIONINE

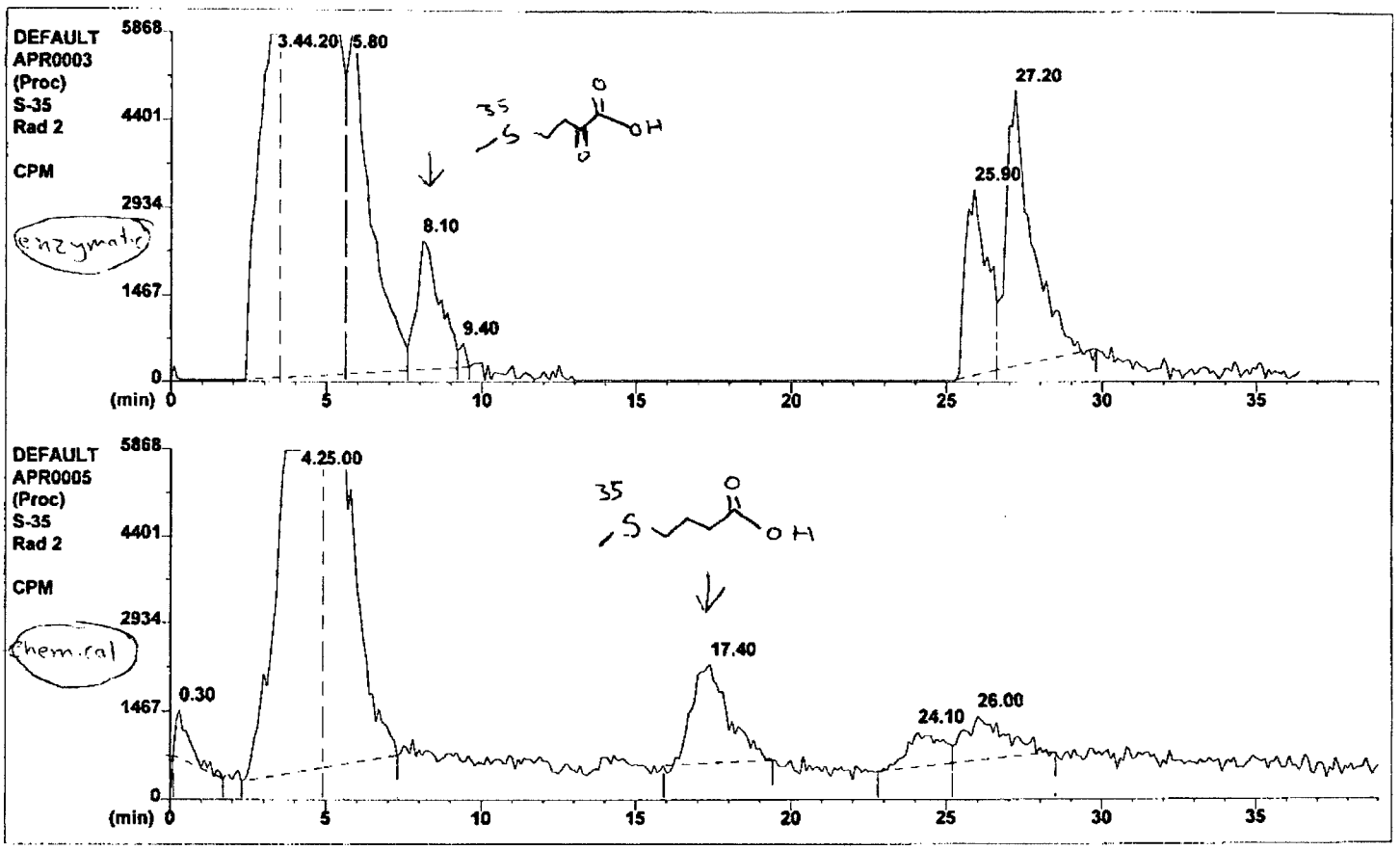
A.



B.



Report Format File: JUSTGRPH



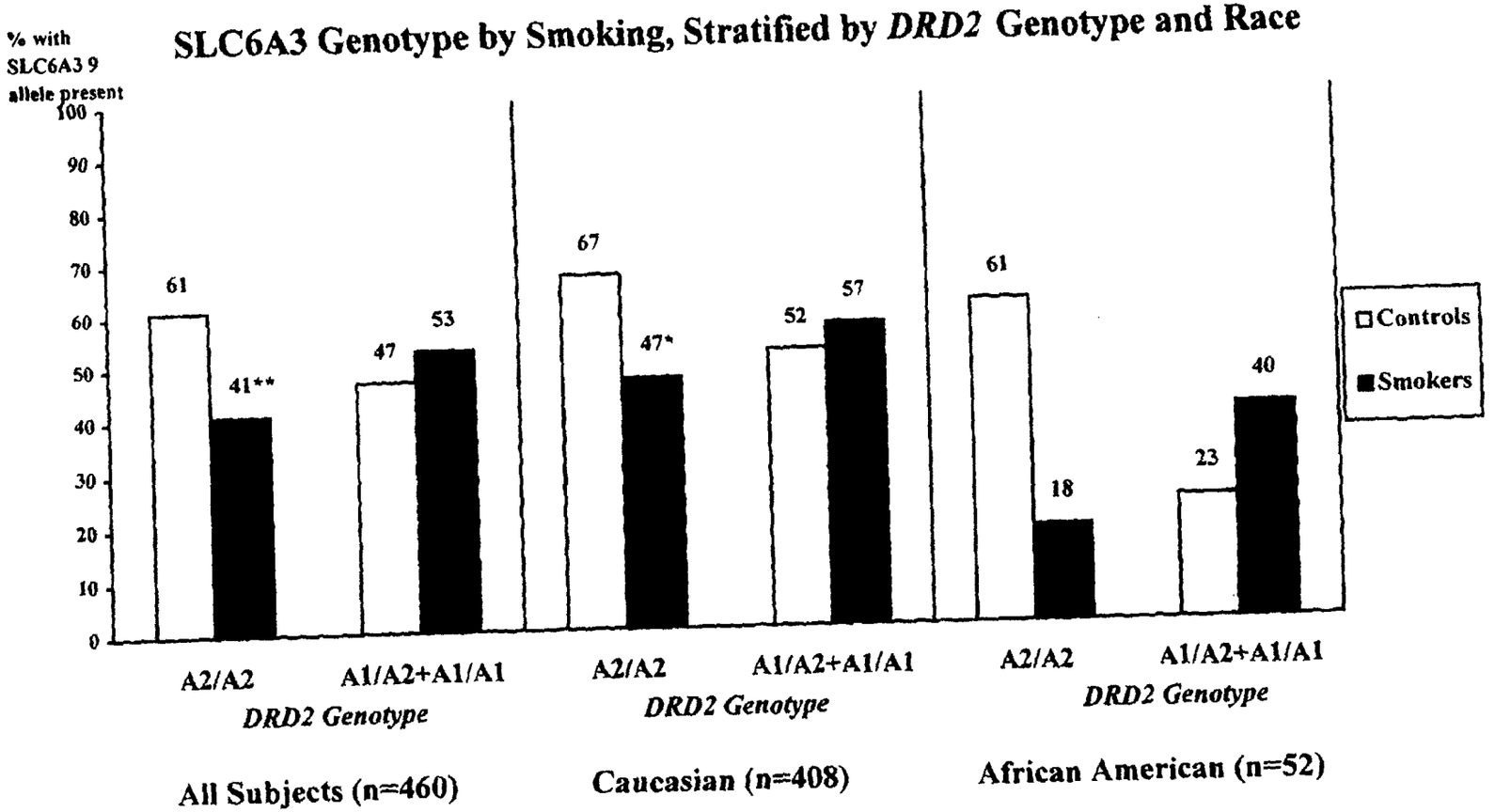
APPENDIX J

Table 1. Associations of *SLC6A3* and *DRD2* Genotypes with Smoking

Sample	Group	*SLC6A3* Genotype		*DRD2* Genotype	
		9/9 + 9/*	* / *	A1/A1+A1/A2	A2/A2
		n (%)	n (%)	n (%)	n (%)
All Subjects (n = 460)	Smokers	106 (46.1)	124 (53.9)	90 (39.1)	140 (60.9)
	Nonsmokers	129 (56.1)	101 (43.9)	79 (34.3)	151 (65.7)
		$\chi^2 = 4.60$, p=0.03		$\chi^2 = 1.13$, p=0.29	
Caucasians (n = 408)	Smokers	98 (48.0)	106 (52.0)	75 (36.8)	129 (63.2)
	Nonsmokers	120 (58.8)	84 (41.2)	66 (32.3)	138 (67.6)
		$\chi^2 = 4.77$, p=0.03		$\chi^2 = 0.88$, p=0.35	
African Americans (n = 52)	Smokers	8 (30.8)	18 (69.1)	15 (57.7)	11 (42.3)
	Nonsmokers	9 (34.6)	17 (65.4)	13 (50.0)	13 (50.0)
		$\chi^2 = 0.09$, p=0.77		$\chi^2 = 0.31$, p=0.58	

* denotes *SLC6A3* allele other than 9

Figure 1.



* p value for chi square = .002; **p value = .001

APPENDIX K

Table 1

Allele Frequency for Smokers and Non-Smokers by Race

	Non-Smokers (%)		Smokers (%)	
Allele	**Caucasians**	**African Americans**	**Caucasians**	**African Americans**
D4.2	44 (13)	11 (23)	49 (10)	6 (6)
D4.3	15 (5)	1 (2)	23 (5)	2 (2)
D4.4	236 (70)	31 (65)	331 (70)	61 (64)
D4.5	2 (1)	3 (6)	4 (0.8)	2 (2)
D4.6	0 (0)	0 (0)	1 (0.2)	0 (0)
D4.7	37 (11)	2 (4)	59 (13)	24 (25)
D4.8	2 (1)	0 (0)	3 (0.6)	1 (1)
Total	336	48	470	96

Table 2

Association of Dopamine D4 Receptor Genotypes and Smoking

Genotype[1]	Non-Smoker (%)	Smoker (%)	P Value
Caucasians			
S/S	132 (79)	183 (78)	0.28[2,3]
S/L	33 (19)	41 (18)	0.90[4]
L/L	3 (2)	11 (4)	
African Americans			
S/S	22 (92)	29 (60)	0.02[2,3]
S/L	2 (8)	13 (27)	0.006[4]
L/L	0 (0)	6 (13)	

[1]S=D4.2, D4.3, D4.4, or D4.5; L=D4.6, D4.7, or D4.8

[2]Fisher's Exact test

[3]P value for S/S versus S/L versus L/L

[4]P value for S/S versus S/L or L/L

Table 3

Association of Dopamine D4 Receptor Genotypes and

Smoking Cessation at 2-Months and 12-Months

	2-Month Follow-Up							12-Month Follow-up						
Genotype[1]	**Abstain**			**Smoking**			**P**	**Abstain**			**Smoking**			**P**
	N	**Row %**	**Column %**	**N**	**Row %**	**Column %**		**N**	**Row %**	**Column %**	**N**	**Row %**	**Column %**	
Caucasians														
S/S	23	15	77	134	85	79	0.78[2,3]	18	15	64	106	85	80	0.12[2,3]
S/L	5	15	17	28	85	16	0.75[4]	7	24	25	22	76	17	0.08[4]
L/L	2	22	7	7	78	4		3	38	11	5	62	4	
Total	30	15		169	85			28	17		133	83		
African Americans														
S/S	9	35	100	17	65	55	0.06[2,3]	5	24	100	16	76	0.6	0.43[2,3]
S/L	0	0	0	8	100	26	0.02[4]	0	0	0	4	100	16	0.29[4]
L/L	0	0	0	6	100	19		0	0	0	5	100	20	
Total	9	23		31	77			5	17		25	83		

[1]S=D4.2, D4.3, D4.4, or D4.5; L=D4.6, D4.7, or D4.8

[2]Fisher's Exact test

[3]P value for S/S versus S/L versus L/L

[4]P value for S/S versus S/L or L/L