

Relationship of H-ras-1, L-myc, and p53 Polymorphisms with Lung Cancer Risk and Prognosis

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Proto-oncogenes (*H-ras-1* and *L-myc*) and tumor-suppressor gene (*p53*) loci have been implicated in lung carcinogenesis. DNA restriction fragment length polymorphisms at these gene loci are being evaluated in a case-control study as markers predictive of risk for cancer or of prognosis when cancer is present. The cases and controls had a cigarette-smoking history of 40 or more pack years or other abnormalities in pulmonary function tests, their ages were closely matched (64 years for cases and 61 years for controls) and the ratio of Caucasians to African Americans was close to unity (cases, 0.95:1.00, controls, 1.00:0.88). The *H-ras-1* gene contains an insertion deletion polymorphism. Inheritance of rare *H-ras-1* alleles, defined by *MspI* digestion, confers a relative risk for lung cancer of 2.0 (95% confidence interval, 0.5-7.3) for Caucasians and 3.2 (0.9-11.6) for African Americans (74 cases, 67 controls). The *L-myc* gene sequence has a restriction site (*EcoRI*) polymorphism between the second and third exons. Inheritance of restriction site-present alleles was reported to confer poor prognosis (presence of lymph node metastases) in Japanese lung cancer patients. This hypothesis was tested in both case-control study subjects (56 cases, 55 controls) and additional surgical cases (40), but no evidence was found to support the hypothesis in the U.S. population. The *p53* gene is a tumor-suppressor gene that can encode either a proline or an arginine in the 72nd residue. No associations was found between the minor allele (proline) and diagnosis of lung cancer (76 cases, 68 controls). Importantly, the observed allelic distributions at each of these genetic loci (*H-ras-1*, *L-myc*, and *p53*) were found to be significantly different between African Americans and Caucasians in a U.S. population.

Introduction

Epidemiological and pharmacogenetic studies have postulated the existence of inherited predisposition for lung cancer (1,2). Furthermore, familial studies suggest a genetic component to this disease, in spite of its now well documented environmental etiology [tobacco smoking (3)]. Few useful genetic markers exist, however, that allow

assessment of individual risk for of human lung cancer. The biomarkers evaluated in the current study consist of proto-oncogenes and tumor-suppressor genes that are implicated in the genesis human lung cancer: *H-ras-1* (4), *L-myc* (5), and *p53* (6). A growing number of investigations by others have attempted to evaluate various potential biomarkers of individual cancer risk, but most of these suffer from inadequate study design. With respect to lung cancer, case-control studies are of limited value if age and tobacco smoking history are not controlled. The studies reported here were undertaken in the context of a formal case-control design that accounts for age, tobacco smoking history, gender, ethnic background, occupational history, and familial history of cancer.

Materials and Methods

Accrual of Study Subjects

The design of this epidemiological case-control study of lung cancer is described in detail elsewhere (7). Briefly,

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patients with histologically confirmed lung cancer who had not received radiation or chemotherapy were recruited at the University of Maryland and Baltimore Veterans Administration Hospitals between 1985 and 1989. Histological diagnosis of lung cancer was confirmed by pathological review. Two control groups were recruited: chronic obstructive pulmonary disease (COPD) patients and patients with cancers at anatomical sites other than the lung and bladder. The COPD patients all had a clinical diagnosis and demonstrated abnormalities in pulmonary function tests (forced expiratory volume in 1 sec [FEV₁] of less than 75% predicted normal and/or FEV₁/forced vital capacity less than 75% predicted) and/or a cigarette smoking history of 40 pack-years or more. The second control group was recruited from among cancer patients with a variety of malignancies including cancers of the colon, esophagus, stomach, and breast and melanoma.

Study recruits were screened for eligibility or exclusion. Exclusion criteria included: treatment in an intensive care unit, blood pressure < 100/60 mm Hg, inability to take oral medication or to be interviewed, general anesthesia within the last 5 days, severe renal or liver disease (creatinine > 4.0 mg/mL or total bilirubin > 3 IU, or serum glutamic-oxaloacetic transaminase or serum glutamic-pyruvic transaminase > 300 IU), previous diagnosis of separate primary malignancy other than basal-cell carcinoma, inability or refusal to give informed consent, or physician refusal. An interview was administered to eligible subjects by a trained study nurse, and data were collected on socio-demographic and anthropomorphic characteristics, recent and remote tobacco use, personal medical history, usual and recent diet, current medications, familial history of cancer, alcohol use, and occupational and residential history. Medical records were also reviewed to abstract other relevant information.

Southern Hybridization for Analysis of the H-ras-1 Gene

High-molecular-weight DNA was subjected to digestion with *MspI/HpaII*. Primary diagnosis of H-ras-1 alleles was made according to criteria previously described, following *MspI* digestion (8). Separation of restriction fragments in the DNA digests by agarose gel (0.8–0.9%) electrophoresis was achieved using constant voltage (40–50 V) for 10–16 hr, so that the dye front migrated a distance of at least 12 cm. Standards for the four common alleles (a1, a2, a3, and a4) as well as standard restriction fragments for the following allelic designations, a1.1, a1.2, a1.3, a2.1, a2.2, a2.3, and a3.5, were used to facilitate allelotyping.

Following electrophoresis, gels were treated with alkali (400 mM NaOH; 600 mM NaCl; 25 min) and neutralized (1.5 M NaCl; 500 mM Tris-HCl; pH 7.5; 30 min), and DNA was immobilized by capillary transfer onto nylon membrane support matrices. Samples were hybridized to the full-length, 6.6kb, ³²P-labeled, human H-ras-1 *Bam*HI-fragment under stringent conditions (NaCl, 100 mM; SDS, 0.5%; dextran sulfate, 10%; 65°C; 16 hr). The membranes were washed under increasingly stringent conditions (final conditions: 37.5 mM NaCl; 3.8 mM HOC [COONa]

[CH₃COO•Na]₂; 0.1% SDS; 65°C; 30 min), and X-ray films (Eastman Kodak Company, Rochester, NY) were used to detect hybridization of the radioactive probe. Allelotyping was based on the original system reported by Krontiris et al. (9); in which common alleles are designated by an integer (i.e., a1, a2, a3, and a4), and other alleles are designated by decimals with respect to the closest smaller common allele (e.g., a1.1, a1.2 for alleles larger than a1 but smaller than a2, and a2.1, a2.2 for alleles larger than a2 but smaller than a3). Similarly, a1.25 is determined to be larger than a1.2 but smaller than a1.3. Authentic, well-characterized alleles from original studies were frequently used in the analysis of test samples.

Polymerase Chain Reaction–Restriction Enzyme Analysis for L-myc

Polymerase chain reaction–restriction enzyme (PCR–RE) analysis for L-myc was carried out on 56 lung cancer patients (clinical diagnosis), 36 COPD patients, and 19 other cancer patients who had been enrolled in the case-control study. Since the allelic frequencies at the L-myc locus were found to differ by race (African American versus Caucasian), further samples were obtained (from trauma victims; 16 Caucasian and 24 African American) to test the hypothesis of an association between race and allelic frequency. Additional lung cancer cases were also obtained at surgery (27 Caucasian and 23 African American) to address rigorously the question of an association between the presence of metastatic disease and the distribution of L-myc alleles in lung cancer. Genomic DNA was amplified using primers that flank the polymorphic *EcoRI* site in the L-myc gene (5'AGTTCACTCACAGGC-CACAT3' → and ← 5'TGCATATCAGGAAGCTTGAG3'). The PCR was performed using a DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, CA), amplified DNA segments were digested with *EcoRI* and subjected to electrophoresis in polyacrylamide gels (8%) (New England Biolabs, Beverly, MA). The gels were treated with an ethidium bromide solution and examined under ultraviolet light. A representative subset of samples was analyzed by Southern hybridization with the 1.9-kb *EcoRI/SmaI* fragment of an L-myc cDNA clone to corroborate the PCR–RE analyses (10).

Polymerase Chain Reaction–Restriction Enzyme Analysis for p53

An easy PCR–RE genotyping test was developed on the basis of previous reports (11,12). The method used here, however, included an important modification: the primer flanking codon 72 in the 5' region contained a single base-pair mismatch (resulting in the formation of a new *AccII* restriction site between exon 2 and exon 3; that is, an A to C change at nucleotide position 11863 of *p53*) to control for completion of digestion. DNA samples were amplified for 35 cycles using a standard protocol (13) either with primers 1 and A (below) or by a hemi-nested strategy using primers 1 and A followed by a further 35 rounds of amplification with primers 1 and B; primer 1 =

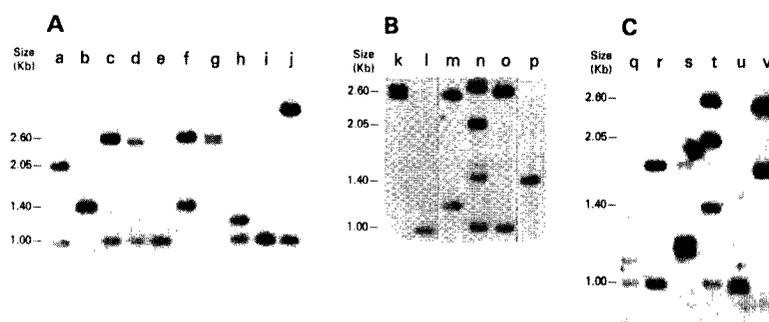


FIGURE 1. Southern hybridization analyses that identified 13 of 23 H-ras-1 alleles in 141 subjects in an epidemiological study of lung cancer. The allelomorphs were diagnosed according to previously defined criteria to be as follows: (blot A) a, a1,a3; b, a2,a2; c, a1,a4; d, a1,a3.5; e, a1,a1; f, a2,a4; g, a3.5,a4; h, a1,a1.2; i, a1,a1; j, a1,a5.2; (blot B) k, a3.5,a4; l, a0.1,a0.1; m, a1.2,a3.5; n, a1,a2,a3,a4 (standard); o, a1,a3.5; p, a2,a2; (blot C) q, a1,a1.2; r, a1,a2.3; s, a1.25,a1.3; t, a1,a2,a3,a4 (standard); u, a0.15,a1; and v, a2.2,a3.3.

5'CCCCAACCCAGCCCCCTAGCAGAGACCTGTGGG-ACGCG3' → (mismatch position underlined), primer 2 = ← 5'TGTCATCTTCTGTCCCTTCCCAGA3' and primer B = ← 5'ACACCGGCGGCCCTGCACCA3'. The resulting PCR products were digested to completion with AccII and analyzed by polyacrylamide gel electrophoresis (12% in Tris-borate:EDTA [45 mM:1 mM] buffer pH 8.3). A small, representative subset of samples was analyzed by dideoxy-chain termination-DNA sequencing (14) to confirm the results of the PCR-RE analyses.

Statistical Analysis

Arithmetic means, standard errors, correlation coefficients, Student's *t*-test stepwise and linear regression were performed using the SAS statistical package (15).

Results

Analysis of the H-ras-1 Polymorphism

Twenty-three discrete restriction fragments (*Msp*I) were found in 141 study subjects for the H-ras-1 proto-oncogene. Resolution of 13 of these restriction fragments is shown in Figure 1, after hybridization with an H-ras-1 clone. Common, intermediate, and rare alleles were determined according to the criteria defined above. The allelic distribution of H-ras-1 *Msp*I fragments was different for African Americans and for Caucasians residing in the

United States. Notably, an allele designated a3.5, a variant of the common a4 allele, was common among African Americans. An excess of rare alleles was observed in lung cancer patients compared to both COPD and nonpulmonary cancer controls (Table 1). In Caucasians, rare alleles were found to be approximately twice as frequent in lung cancer patients as in the COPD or nonlung cancer control groups ($\chi^2 = 6.5$, $p < 0.05$, degrees of freedom (df) = 2); the same comparison in African Americans showed a 3-fold excess of rare alleles in the cases ($\chi^2 = 26.4$, $p < 0.0001$, df = 2). Similar findings were made when lung adenocarcinomas were excluded ($\chi^2 = 9.9$, $p < 0.01$, df = 2, for Caucasians; $\chi^2 = 25.9$, $p < 0.0001$, df = 2, for African Americans).

Since allelic frequency could differ by race (8), Caucasians and African Americans were analyzed independently for relative risk for lung cancer (Table 2). Although small numbers in each group hamper the analysis, an increase in the number of rare alleles for lung cancer (excluding adenocarcinoma) was observed in African Americans. Crude odds ratios for risk based on the number of rare alleles show elevated risk in all individuals with rare alleles and increasing risk based on the number of rare alleles in the four comparisons considered: Caucasians and African Americans, considering all lung cancer cases and excluding cases with adenocarcinoma of the lung. The trend is statistically significant only for non-adenocarcinoma of the lung in African Americans ($\chi^2 =$

Table 1. H-ras-1 allele frequencies in subjects with lung cancer and pooled controls.

Allelotype ^a	Caucasians (n = 78)			African Americans (n = 63)		
	Lung cancer			Lung cancer		
	All lung cancer (n = 41)	Adenocarcinoma excluded (n = 25)	Pooled controls ^b (n = 37)	All lung cancer (n = 33)	Adenocarcinoma excluded (n = 23)	Pooled controls ^b (n = 30)
Common	0.79 (65) ^c	0.74 (37)	0.82 (61)	0.41 (27)	0.39 (18)	0.68 (41)
Intermediate	0.07 (6)	0.08 (4)	0.11 (8)	0.36 (25)	0.35 (16)	0.23 (14)
Rare	0.13 (11)	0.18 (9)	0.07 (5)	0.21 (14)	0.26 (12)	0.08 (5)

^aFrequencies of common, intermediate, and rare alleles for Caucasian populations are given in Weston et al. (22).

^bPooled controls are the sum of chronic obstructive lung disease patients (20 African American, 10 Caucasian) and nonlung cancer patients with cancer (12 African American, 25 Caucasian).

^cNumber of alleles in parentheses.

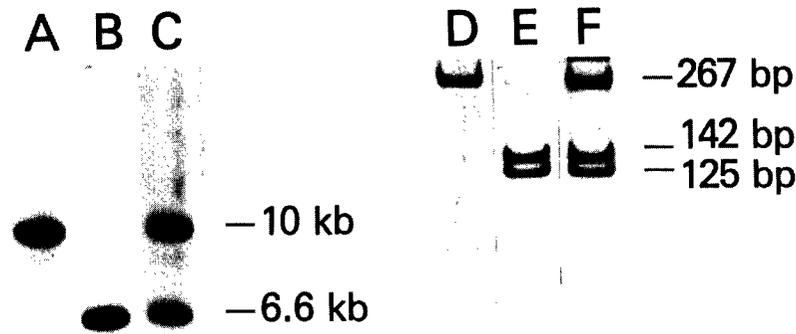


FIGURE 2. Southern blot analysis (A, B, C) and polymerase chain reaction (PCR) restriction fragment length polymorphism analysis (D, E, F) of the *L-myc* proto-oncogene locus. Genomic DNA samples were digested with *Eco*RI, and immobilized DNA was hybridized to a human 1.9-kb *L-myc* fragment (A, B, C). Three possible genotypes are shown; the L-L homozygote (A), the S-S homozygote (B) and the L-S heterozygote (C). Samples of DNA that had been produced by the PCR using primers that flank the polymorphic restriction site were digested with *Eco*RI, electrophoresed in polyacrylamide gels, and stained with ethidium bromide (D, E, F). These figures (D, E, F) were produced by photographing the negative of the ethidium stained-fragment. The corresponding patterns for an L-L homozygote, an S-S homozygote, and an L-S heterozygote are shown in D, E, and F, respectively.

3.9, $df = 1$, $p = 0.05$). Adjustment of these results for other risk factors for lung cancer evaluated in this study, for example smoking (pack years), age, family history of lung cancer in first-degree relatives, and the debrisoquine metabolic phenotype, revealed no important confounding factors.

Analysis of the *L-myc* Polymorphism

Southern blot analysis of human genomic DNA samples that had been digested with *Eco*RI revealed a DNA restriction fragment length polymorphism consisting of two alleles at the *L-myc* locus (Fig. 2A-C). The PCR was also used to reveal this polymorphism in genomic DNA samples when primers that flank the *Eco*RI restriction site were used to amplify a DNA segment of 267 base pairs (Fig. 2D-F). Initially, the two methods were applied to the

same DNA samples, and it was found that the PCR and Southern hybridization of *Eco*RI-digested materials gave consistent results (Fig. 2).

A highly significant difference in allelic distribution between African Americans and Caucasians was found (Table 3); the distributions for all subjects was consistent with the Hardy-Weinberg equilibrium (16). The case-control data show that in the Caucasian population the ratio of restriction site-absent alleles (L-alleles) to restriction site-present (S-alleles) was 0.509:0.491, whereas in the African American population the distribution was 0.277:0.763. Similarly, among a group of trauma victims, allele frequencies were 0.562:0.438 and 0.145:0.855 in Caucasians and African Americans, respectively.

In the lung cancer case-control study, PCR-RE or Southern hybridization analyses for *L-myc* were con-

Table 2. Crude odds ratio for risk of lung cancer with *H-ras-1* rare alleles in different racial groups.

Presence of rare alleles ^a	Lung cancer	Pooled controls	RR ^b	95% CI ^c	χ^2	p
Caucasians ($n = 78$)						
-	34	33	1.0			
+	8	4	2.0	0.5-7.3	1.1	0.29
Caucasians ($n = 62$) (adenocarcinoma excluded)						
-	19	33	1.0			
+	6	4	2.6	0.7-10.4	1.8	0.18
African Americans ($n = 63$)						
-	22	26	1.0			
+	11	4	3.2	0.9-11.6	3.3	0.07
African Americans ($n = 53$) (adenocarcinoma excluded)						
-	14	26	1.0			
+	9	4	4.2	1.1-16.0	4.3	0.04

^a(+) Indicates individuals with rare alleles, (-) indicates common or intermediate frequency alleles.

^bRR = crude odds ratio (relative risk); RR of the subjects with no rare allele is 1.0.

^cCI = Cornfield 95% confidence interval.

Table 3. Frequencies of L-myc alleles in different ethnic groups.

Subjects	Genotype ^a			Total	χ^2	<i>p</i>
	L-L	L-S	S-S			
In case-control study						
Caucasians	13	32	12	57	11.6	0.0007
African Americans	6	18	30	54		
Total	19	50	42	111		
Trauma victims						
Caucasians	4	10	2	16	15.0	0.0001
African Americans	0	7	17	24		
Total	4	17	19	40		

^aDNA restriction fragment length polymorphism (RFLP) pattern determined by Southern blot or polymerase chain reaction-RFLP analysis, L = 10 kb and S = 6.6 kb *Eco*RI fragments of the *L-myc* proto-oncogene.

ducted on a total of 111 subjects; the allelic distributions are given in Table 4. When race and other variables were not controlled for, there was no association between allelic frequency and lung cancer (likelihood ratio $\chi^2 = 1.14$, $p = 0.56$, $df = 2$). In addition, the allelic frequencies were similar in each group (likelihood ratio $\chi^2 = 0.12$, $p = 0.94$, $df = 2$, $n = 55$). When a variety of other study variables were entered into a multivariate statistical model to predict lung cancer, neither race nor the number of restriction site-present alleles was found to be associated with cancer diagnosis. The effect of a number of study variables on the number of alleles was also tested using a multivariate linear regression model: neither lung cancer diagnosis nor any other study characteristic tested predicted the presence of a specific allelotype, with the exception of race ($F = 12.79$, $p = 0.0005$). Thus, African Americans are more likely to have restriction site-present alleles than Caucasians. The distribution of *L-myc* alleles was also unrelated to histologic subtype of lung cancer (data not shown).

There does not appear to be any relationship between clinical stage of lung cancer and *L-myc* alleles in either African Americans or Caucasians. Similarly, within a group of surgically diagnosed Caucasian lung cancer patients with or without lymph node metastasis, no association with *L-myc* genotype was observed. In these latter studies, the number of Caucasian patients was sufficiently large to make valid statistical analyses however, the number of African American patients was too small to evaluate the relationship between lymph node metastasis and the *L-myc* genotype.

Analysis of p53 Polymorphism

Figure 3 shows the strategy for PCR-RE analysis of exon 4 of *p53*. Dideoxy-chain termination DNA sequencing of the PCR products was performed to confirm their authenticity (DNA sequencing analysis was performed on a small subset of samples only). PCR analysis with restriction enzyme digestion was performed on 144 study subjects (76 cases and 68 controls). There was no association between presence of the *p53* genotype and diagnosis of lung cancer (small-cell and non-small-cell combined); however, the observed allelic distribution was significantly different between African Americans and Caucasians ($\chi^2 = 20.8$, $p < 0.0001$, $df = 2$).

Discussion

Molecular epidemiological studies of potential lung cancer risk factors have been described. A case-control study format has been used to evaluate the relationship between risk of developing lung cancer, or prognosis (when lung cancer is present), and genotype at the *H-ras-1*, *L-myc*, and *p53* gene loci. The results of these studies show that individuals with *H-ras-1* rare alleles are at increased risk for lung cancer (especially for nonadenocarcinoma in African Americans); that the *L-myc* genotype is not indicative of lung cancer risk or disease outcome in a U.S. population (prognosis, as measured by performance status, tumor stage, or presence of lymph node metastasis); and that the

Table 4. Allelic distribution of L-myc in a case-control study of lung cancer.

Subjects	Genotype ^a			Total	χ^{2b}	<i>p</i>
	L-L	L-S	S-S			
Lung cancer cases	9	28	19	56	1.14	0.56
All controls	10	22	23	55		
Total	19	50	42	111		
Controls with cancer at sites other than lung	3	8	8	19	0.45	0.80
Controls with COPD	7	14	15	36	1.09	0.58

COPD, chronic obstructive pulmonary disease.

^aDNA restriction fragment length polymorphism (RFLP) pattern determined by Southern blot or polymerase chain reaction-RFLP analysis, L = 10 kb and S = 6.6 kb *Eco*RI fragments of the *L-myc* proto-oncogene.

^bDegrees of freedom = 2.

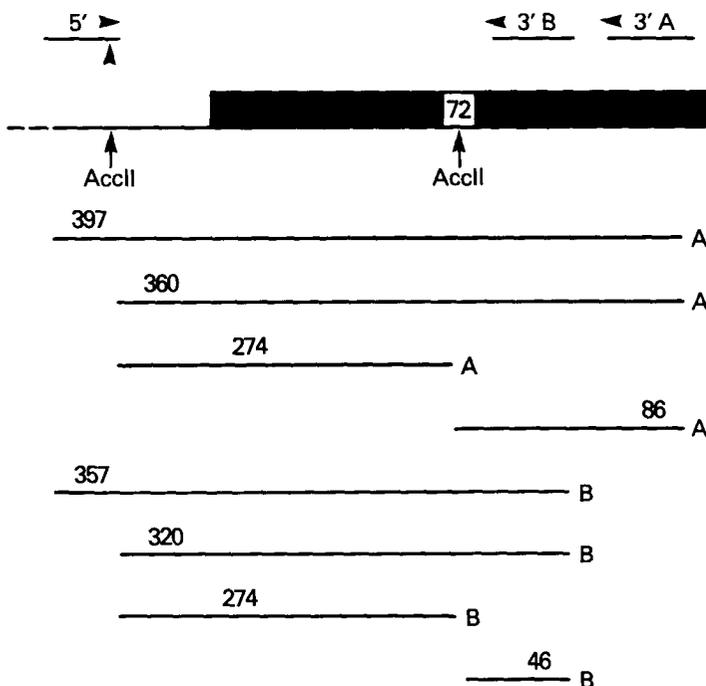


FIGURE 3. Strategy for polymerase chain reaction (PCR)-restriction enzyme analysis of human genomic DNA for the *AccII* polymorphism in exon 4 of *p53*. High molecular-weight DNA isolated from white blood cells of subjects accrued in a case-control study of lung cancer was amplified for 35 cycles using a standard protocol (25). The results of *AccII* digestion are either a single fragment of 360 bp for individuals who are homozygous *AccII* restriction-site absent (CCC or proline genotype), a pair of fragments of 274 bp and 86 bp for individuals who are homozygous *AccII* restriction-site present (CGC or arginine genotype), or three fragments for the heterozygote (360 bp, 274 bp, and 86 bp). The undigested control is 37 bp longer than all of the digested materials because of the presence of the *AccII* site introduced by the 5' primer. Fragments generated with the 3' B primer are 40 bp shorter at the 3' end.

proline variant of *p53* does not appear to predict lung cancer risk. Each of these potentially useful biomarkers of lung cancer risk displayed a strong association with race: that is, allelotypic distributions were dependent on the racial origin of the study subjects (African Americans compared to Caucasians). These data indicate that the *H-ras-1*, *L-myc*, and *p53* polymorphisms should be examined in more detail with regard to their potential as indicators of individual cancer risk, as should the underlying mechanisms that may be responsible for risk of cancer when certain genotypes are inherited. Furthermore, the data indicate the importance of controlling for race as a confounding factor in epidemiological studies.

The underlying mechanistic basis for the contribution of the inheritance of *H-ras-1* rare alleles to risk of lung cancer is not known; however, the variable tandem repeat (VTR) region may be involved in somatic-recombinational events, implying that certain types of VTR are more stable than others (19). This hypothesis may be extended to VTR regions other than that at the *H-ras-1* gene locus. Alternatively, VTR regions may have gene enhancer activity (20,21), therefore, it follows that certain variants may be more active than others. Notwithstanding the absence of a clear mechanistic basis for the observations reported here, they are supported by the results of a number of other studies (22-24), and, although the data in the literature are conflicting (25), not all studies of lung cancer have controlled for the confounding factors of tobacco smoking and racial background of the study subjects.

It was reported previously, by Kawashima et al. (26), that the *EcoRI* restriction site-present allele at the *L-myc* gene locus (S allele) was an indicator of lymph node metastasis (poor prognosis) in Japanese lung cancer patients. This hypothesis and the hypothesis that inheritance of S alleles is indicative of lung cancer risk have now been tested in two independent studies (10,17). Contrary to the results of the study in a Japanese population, neither of the two subsequent studies found associations in Caucasian populations.

L-myc genotyping has been conducted in a variety of cancer groups, mainly of Japanese origin. An association was found in the case of lung (surgical diagnosis) (26), renal (18), gastric (27), and bone cancers (28) between increased frequency of the S allele and advanced disease (metastasis); however, no association was found for lung [clinical diagnosis (26)], colorectal (29), or breast cancers (30). The reasons for these apparently contradictory results are unclear, but a genetic difference related to ethnicity could exist between respective lung cancer populations, even though the allelic frequencies in American Caucasians and Japanese controls do not differ significantly (L:S, 0.562:0.438 versus 0.485:0.515). Future studies which examine this question should control strictly for variation due to race and be designed to eliminate potential bias. Given that allelic frequency varies with ethnicity (L:S, 0.277:0.763 and 0.562:0.438 for African Americans and Caucasians, respectively), if one ethnic group consistently seeks medical attention at a later stage, or is com-

posed of an unusual number of prevalent (versus incident) cases, or is associated with a particular histology or exposure, bias could result. In the present study, the prognostic associations considered were similar in Caucasians and African Americans.

For *p53*, the proline variant of the polymorphism in the coding region is more frequent in African Americans than in Caucasians. No evidence was found, however, for an association between the presence or absence of the proline variant and lung cancer risk.

Tobacco smoke is the major etiological factor in lung cancer, however, not all individuals who smoke cigarettes develop lung cancer, and some individuals with little or no exposure develop lung cancer (31). These observations suggest that other factors may be important in the determination of lung cancer risk. Using a case-control design, we have evaluated three polymorphic genetic loci (*H-ras-1*, *L-myc*, and *p53*) as potential biomarkers of lung cancer risk. Racial variation in allelic distribution was apparent for each of these polymorphisms, which suggests that controlling for racial variation as a confounding factor in epidemiological studies is an important consideration in study design.

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