

Determination of the allelic frequencies of an *L-myc* and a *p53* polymorphism in human lung cancer

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The *L-myc* and *p53* genes have been implicated in lung cancer. Both of these genes have restriction fragment length polymorphisms (RFLPs) that could account for differential expression or activity of variant forms. An *EcoRI* restriction site in the *L-myc* gene was previously reported to be a predictor of poor prognosis in Japanese lung cancer patients. There are several RFLPs in the *p53* gene. In exon 4 there is a polymorphism that codes for either an arginine or proline residue at codon 72. We previously reported the frequency of DNA-RFLPs at these gene loci revealed by *EcoRI* and *AccII* respectively. Here we report results from a study comparing lung cancer cases ($n = 31$) with chronic obstructive pulmonary disease controls ($n = 49$). No association was found between these RFLPs and disease status. Previous observations that the frequencies of these RFLPs varied by race were confirmed. The *p53* arginine allele was found to be more common in Caucasians (0.71) than African-Americans (0.50). The *EcoRI* restriction site present allele in *L-myc* was more frequent in African-Americans (0.71) than Caucasians (0.49). Thus, the allelic frequency for *L-myc* was similar in African-Americans to that reported for Japanese, and the allelic frequency for *p53* was similar in Caucasians to that reported for Japanese.

Introduction

Restriction fragment length polymorphisms (RFLPs*) were recognized as normal variations in DNA sequence more than a decade ago (1-3). DNA-RFLPs and sequence variations arise during evolution as mutational events; however, they are considered to be polymorphisms when the rare allele reaches the level of 1% in the population (i.e. 1 in 100 alleles) (4).

Overt predisposition to cancer occurs when a germ-line mutation in a tumor suppressor gene is inherited (WT-1, Rb, p53 and APC) (5-8). Inheritance of such mutations usually leads to early cancer onset or pediatric cancer, consequently, such mutations remain at low frequency ($\leq 1\%$). However, predisposition to common adult cancers may result from inheritance of genes which may alter susceptibility to cancer given environmental exposures. One example of this are studies that indicate

an association of *H-ras-1* 'rare' allele inheritance and predisposition to cancers of the breast, prostate, lung, testis and melanoma (9-17).

In the current study we have examined the distribution of DNA-RFLPs at the *L-myc* and *p53* gene loci in lung cancer cases and controls for United States African-Americans and Caucasians from a hospital based study in Baltimore, Maryland. A polymorphic *EcoRI* restriction site is located in the second intron of the *L-myc* gene. The presence of this restriction site is revealed by a 6.6 kb fragment when hybridized to a cDNA probe. When the restriction site is absent, a 10 kb fragment is observed (18). The polymorphism can also be detected using the polymerase chain reaction (PCR) and restriction enzyme digestion (19). In a study of Japanese lung cancer patients it was found that individuals with one or two restriction site present alleles were at greater risk of lymph node metastasis than restriction site absent homozygotes (20). In a US population we have tested the hypothesis that inheritance of the restriction site present allele predisposes to lung cancer and is a marker of poor prognosis when lung cancer is present (19,21).

In the case of *p53*, a common polymorphism at codon 72 in the *p53* gene can be found by PCR and restriction enzyme analysis (*AccII* or *Bsp50-1*). The gene products of the possible polymorphic variants differ by the presence of either an arginine (major allele in Caucasians, 0.65), a large, polar amino acid residue or a proline (major allele in African-Americans, 0.61), a small, non-polar amino acid residue (22-24). In the absence of mechanistic studies and without the crystal structures of the variant forms we postulated that their biological effects may be different. Based on the original observation in Caucasians that proline was the minor allele, we have tested the hypothesis that the proline allele is over-represented in lung cancer cases compared to controls; and based on our previously published preliminary data (24), we have also tested the hypothesis that the proline allele is over-represented in adenocarcinoma.

Materials and methods

Design of the case-control study

Subjects were accrued between 1990 and 1992 based on a study carried out between 1985 and 1989 (25). Subjects with histologically confirmed lung cancer were identified at the University of Maryland and Baltimore Veterans' Hospital. Histological typing of lung cancer was performed according to WHO criteria and confirmed by pathological review. In the original study two control groups were selected. Chronic obstructive pulmonary disease (COPD) patients were identified by clinical criteria, and the second group was comprised of individuals with a variety of non-pulmonary cancers. For the current series only one control group was accrued, which consisted of COPD patients. This control group was selected since, in the absence of data, it could not be excluded that either of the RFLPs was associated with non-pulmonary cancer.

A standardized questionnaire was administered. Data were collected on tobacco use, medical history, family history of cancer and occupational exposure as well as socio-economic and demographic parameters and anthropomorphic characteristics. Abstracts of medical records were used to determine clinical staging, performance status, medications administered and results of clinical laboratory tests.

Biochemical reagents

Phenol (nucleic acid grade), agarose, acrylamide and the electrophoresis hardware were products of Bethesda Research Laboratories (Gaithersburg, MD). Restriction

*Abbreviations: RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; COPD, chronic obstructive pulmonary disease.

enzymes and their buffers were obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN (*EcoRI*), US Biochemicals, Cleveland, OH (*AccII*) and Stratagene, LaJolla, CA (*Bsp50-1*). Primers for PCR were prepared using an Applied Biosystems oligonucleotide synthesizer (ABS, Foster City, CA).

RFLP analysis for *L-myc*

RFLP analyses were performed on 80 study subjects (31 lung cancer and 49 COPD patients). DNA was isolated from white blood cells of case-control study subjects by phenol extraction and ethanol precipitation (26). In previous studies DNA samples (10 µg) were subjected to *EcoRI* digestion and Southern hybridization with a ³²P-labeled *L-myc* probe (19,27). Subsequently, a PCR-RFLP method was developed using *L-myc* specific primers (5'-AGTTCACCTCACAGGCCACAT-3' and 5'-TGCATATCAGGAAGCTTGAG-3'). The resulting PCR product (267 bp) was subjected to restriction digestion (*EcoRI*) and fragments were separated on polyacrylamide (8%) or agarose (2%) gels and detected by ethidium bromide staining.

RFLP analysis for *p53*

Primers specific for exon 4 of *p53* (5'-CCCCAACCCAGCCCCCTAGCAGAGACCTGTGGGACGCG-3' and 5'-ACACCGGGCCCTGCACCA-3') were used to amplify a 397 bp fragment containing the polymorphic *AccII* restriction site at codon 72. The forward primer contains a mismatch (bold type) proximal to the 3' end which introduces a new *AccII* restriction site 37 bp from the end of the fragment to serve as a control for completeness of sample digestion (24). The PCR products (79; 31 lung cancer and 48 COPD patients, note one control sample failed to amplify) were digested with either *AccII* or an isoschizomer, *Bsp50-1*. The restriction fragments were separated on gels (as described above) and detected with ethidium bromide.

Statistical analysis

Fisher's exact test was used to compare allelic distributions between cases and controls. Since the major confounding factor in this study was ethnic origin (African-American or Caucasian), three methods were used to examine its effect: restriction (by racial category), stratification (Mantel-Haenszel chi-square) and mathematical modeling using logistic regression. The odds ratios, an estimate of relative risk appropriate for case-control studies, were calculated using standard methods (28). Statistical analyses were performed using the SAS statistical analysis system on a mainframe computer (29).

Results

Genomic DNA was isolated from peripheral white blood cells of 80 subjects (31 lung cancer, 49 COPD controls) enrolled in a case-control study of lung cancer. Demographic characteristics and histories of tobacco use are summarized in Table I. Electrophoretic analysis of genomic products amplified using *L-myc* PCR primers are shown in Figure 1. Each genotypic possibility is represented; *EcoRI* restriction site present homozygote (lane A), *EcoRI* restriction site absent homozygote (lane C) and heterozygote (lane B). The allelic frequencies for all study subjects were compared by race (African-Americans and Caucasians) using a chi-squared test (Table II). The data show a significant difference in *L-myc* allelic distributions between African-Americans and Caucasians ($\chi^2 = 8.7$, $P = 0.01$). Data from an earlier study (1985-1989) (19) were similar with regard to this finding. Chi-squares were observed that indicated significant differences for the *L-myc* allelic distributions between African-Americans and Caucasians. This finding highlights a potentially important confounding factor because without the appropriate control subjects, and by comparison with a previous study on a Japanese population (20), the current study would have identified the African-American population as having a large excess of the 'at risk' *L-myc* genotype.

The data were examined for possible associations with the *L-myc* allelotype. Multivariate analysis (Mantel-Haenszel) was used, and odds ratios were determined using the restriction site absent homozygote (E-/E-) as the referent (Table III). Neither unadjusted odds ratios, nor odds ratios determined by Mantel-Haenszel adjustment for race were significant, indicating that the *L-myc* genotype is not a determinant of lung cancer risk among this US population. This finding is similar to that reported in previous studies of US, Norwegian and Japanese populations

Table I. Demographic characteristics and tobacco use of the study population

	Lung cancer cases	Chronic obstructive pulmonary disease controls
Men	28	46
Women	3	3
African-Americans	13	13
Caucasians	18	36
Age (years, mean ± SE)	61.2 ± 1.7	63.1 ± 1.4
Tobacco use (pack years ^a , mean ± SE)	60.9 ± 8.2	51.1 ± 5.4

^a Pack years = the product of number of years cigarettes smoked and the packs (20 cigarettes) per day.

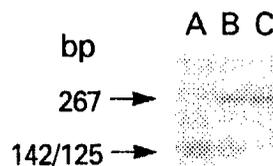


Fig. 1. Detection of *L-myc* RFLP by PCR *EcoRI* digestion. (A) *EcoRI* restriction site present homozygote, (B) heterozygote, (C) *EcoRI* restriction site absent homozygote.

Table II. Frequency of *L-myc* alleles in study subjects by race

	Genotype ^a			χ^2	P
	E-/E-	E-/E+	E+/E+		
African-Americans (n = 26)	4	7	15		
Caucasians (n = 54)	14	27	13	8.7	0.01
Totals	18	34	28		

^aE- = *EcoRI* restriction site absent, E+ = *EcoRI* restriction site present. Overall allele frequencies, E-:E+ = African-Americans 29:71 and Caucasians 51:49.

(19,20,30). However, the presence of the restriction site present allele has been associated with the development of sarcomas, especially osteosarcoma, in Japanese populations (31).

An extended analysis of previously published data (19) that were collected between 1985 and 1989 and those presented above was also performed. Even though the control group was comprised of both non-pulmonary cancer patients as well as COPD patients, no difference was observed between lung cancer cases and controls ($\chi^2 = 1.82$, $P = 0.40$, $df = 2$, $n = 188$).

In the absence of data derived from primary tumors where the lymph node metastasis status of the patient was known, other indicators of prognosis were examined by logistic regression. However, none of these were related to the *L-myc* genotype (tumor stage: $\chi^2 = 6.2$, $P = 0.40$, $df = 6$; grade: $\chi^2 = 10.0$, $P = 0.14$, $df = 4$; or performance status: $\chi^2 = 4.7$, $P = 0.59$, $df = 6$). The results were consistent with previous studies (multivariate analysis) and failed to reveal an association of the *L-myc* genotype with indicators of poor prognosis (19,21,31).

Table III. L-myc allelic distribution in lung cancer cases and controls by race

Diagnosis	Genotype ^a		
	E-/E-	E+/E-	E+/E+
<i>Lung cancer</i> (n = 31)			
African-Americans	2	3	8
Caucasians	3	10	5
Totals	5	13	13
<i>Controls</i> ^b (n = 49)			
African-Americans	2	4	7
Caucasians	11	17	8
Totals	13	21	15
<i>Unadjusted OR</i> ^c	1.0	1.6	2.3
95% CI	---	(0.5-5.3)	(0.7-7.7)
<i>Mantel-Haenszel adjustment for race</i>			
OR	1.0	1.6	1.8
95% CI	---	(0.5-5.5)	(0.5-6.5)

^aE- = EcoRI restriction site absent, E+ = EcoRI restriction site present.

^bControls are chronic obstructive pulmonary disease patients.

^cOR = odds ratio with 95% confidence interval (CI), using the restriction site absent homozygote (E-/E-) as the referent.

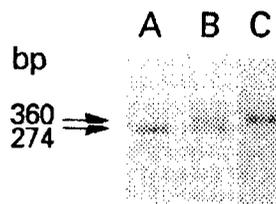


Fig. 2. Detection of p53 RFLP by PCR Bsp50-1 digestion. (A) Restriction site present homozygote (arginine). (B) heterozygote, (C) restriction site absent homozygote (proline).

Table IV. Frequency of codon 72 alleles of p53 in different ethnic groups

Study group	Genotype ^a			χ^2	P
	A/A	P/A	P/P		
Caucasians (n = 54)	31	15	8		
African-Americans (n = 25)	7	11	7	6.0	0.05
Totals	38	26	15		

^aP = proline allele, A = arginine allele.

Overall allele frequencies, arginine:proline: African-Americans 50:50 and Caucasians 71:29.

However, the genotype was strongly associated with race: African-American or Caucasian.

The results of restriction analysis (AccII or Bsp50-1) for PCR-amplified genomic sequences of p53 are shown in Figure 2. In this case the RFLP encodes an amino acid substitution (arginine or proline). Each possible genotype is represented: arginine homozygote (lane A), proline homozygote (lane C) and heterozygote (lane B). The data collected for all study subjects examined between 1990 and 1992 are presented in Table IV. The

Table V. Frequency distribution of p53 alleles in lung cancer cases and controls

Study group	Genotype ^a		
	A/A	P/A	P/P
<i>Lung cancer</i> (n = 31)			
African-Americans	4	5	4
Caucasians	9	7	2
Totals	13	12	6
<i>Controls</i> ^b (n = 48)			
African-Americans	3	6	3
Caucasians	22	8	6
Totals	25	14	9
<i>Unadjusted OR</i> ^c	1.0	1.6	1.3
95% CI	---	(0.6-4.5)	(0.2-3.3)
<i>Mantel-Haenszel adjustment for race</i>			
OR	1.0	1.4	0.9
95% CI	---	(0.5-4.2)	(0.2-3.3)

^aP = proline allele, A = arginine allele.

^bControls are chronic obstructive pulmonary disease patients.

^cOR = odds ratio with 95% confidence interval, using the arginine homozygote (A/A) as the referent.

data collected for a previously published report (1985-1989) (24) were not significantly different to those presented here, data not shown. The chi-square observed for a comparison of the p53 allelic distributions between African-Americans and Caucasians (6.0) was statistically significant ($P = 0.05$). Previous studies in Caucasians have reported p53 allelic frequencies that are almost identical to those reported here for Caucasians (21-24).

When the p53 allelic distributions were compared for lung cancer cases and controls no significant difference was found (Table V). This was true if the current case-control study (conducted between 1990 and 1992) was analyzed alone or together with that performed previously (between 1985 and 1989) (24). Neither the unadjusted odds ratios, nor those calculated using the Mantel-Haenszel summary statistic adjusting for race were significantly different from the referent (Table V). The allelic distributions for lung cancer patients and controls were examined by multivariate analysis, using the arginine homozygote as the referent. The previous study showed a tendency towards increasing frequencies of the proline variant in lung adenocarcinoma patients (24). This trend was not observed in the current study (data not shown).

Discussion

The results of epidemiological studies of lung cancer in two US populations, one Caucasian the other African-American, reported here examine the role of L-myc and p53 polymorphisms. These studies have not revealed any association of the presence of a specific allelomorphic with cancer susceptibility or prognosis. This may be because no such association exists or because the association of the specific allelotype with lung cancer may be too weak to be detected by the current study design. An effect may be dependent on a specific exposure not present in this study population. Additionally, it is possible that in a population (ethnic group) the RFLP may be in linkage disequilibrium with a gene critical to disease development. A recent investigation that examined the relationship of the p53 RFLP in exon 4 with lung

cancer in a Japanese population led to a report which described proline homozygotes as being at 1.7-fold increased risk of lung cancer (32). However, the 'healthy' control group used in this Japanese study was not described and therefore it is not possible to determine whether cases and controls were comparable for age or tobacco smoking exposure—the two major confounders in lung cancer studies. In addition, in this Japanese study the overall frequency of the proline and arginine alleles was similar in cases and controls (0.355:0.645 and 0.347:0.653 respectively) and not significantly different from previous reports of these allelic frequencies in Caucasian populations (22–24).

The studies presented here have confirmed race as an important confounding factor in epidemiological studies involving hereditary factors. Most studies that have characterized common polymorphisms, and polymorphisms in cancer-related genes have focused on majority populations, although it is becoming increasingly accepted that genetic factors may be responsible for variation in cancer incidence among different racial groups. If the distribution of a genetic polymorphism is determined in a specific diseased population a meaningful comparison requires an appropriate control group and not necessarily an existing data base. This area of study is being continually expanded and has recently extended to the analysis of carcinogen metabolizing genes: cytochrome P450s (32–36), *N*-acetyltransferase (35) and glutathione-*S*-transferase (37,38), as well as oncogenes, e.g. *H-ras-1* (11,12,15,21). For many of these genes similar racial variations are being encountered and, as the current study shows, these variations need to be considered in an appropriate context (39).

A complementary approach to identification of genetic risk factors is through functional studies of polymorphic variants in culture systems. This approach has most commonly been used to discern the effects of different somatic mutant forms of *p53* found in tumors (40). However, we have recently examined a polymorphism in codon 47 of *p53* (proline/serine) using this approach (41). Interestingly, the serine variant was found in African-Americans (frequency 0.047, $n = 64$ alleles) but not Caucasians ($n = 138$ alleles); and the functional studies did not reveal a difference between the growth suppressor activity of these variants.

Acknowledgements

The authors thank study nurses Karen Fisher, Daina Buivys, Laureen Gallo and Judy Bobb for interviewing patients and collecting samples. Our thanks also to the following hospitals in Baltimore: University of Maryland, Lock Raven Veterans' Administration, Union Memorial, St Agnes, Mount Sinai, Harbor Center, Mercy Medical Center and Baltimore County General. The editorial assistance of Dorothea Dudek is also appreciated.

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Received on November 12, 1993; revised on December 20, 1993; accepted on December 23, 1993