Genetic Susceptibility Associated with Rare HRAS1 Variable Number of Tandem Repeats Alleles in Spanish Non-Small Cell Lung Cancer Patients¹

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ABSTRACT

The highly polymorphic HRAS1 variable number of tandem repeats (VNTR), which maps 1 kb downstream from the human H-ras1 gene, has been described as an inherited predisposing factor in many human cancers. Here, we investigated the association between the presence of rare HRAS1 minisatellite alleles and lung cancer in the population studied. Four hundred sixty-six HRAS1 VNTR alleles from 233 lung cancer patients and 892 alleles from 446 unaffected controls were typed using PCR-long agarose gel electrophoresis assay of peripheral blood lymphocyte DNA. Rare alleles were differentiated from common alleles (a1, a2, a3, and a4) by shifts in electrophoretic mobility. Odds ratio was calculated to evaluate increased risk of lung cancer associated with the presence of rare HRAS1 alleles. A higher percentage of rare HRAS1 VNTR alleles in lung cancer patients than in unaffected controls (32.7 versus 21.9%) was confirmed. The presence of rare alleles was associated with an increased risk of lung cancer (odds ratio = 1.68; P ≤ 0.0001), indicating a genetic predisposition to lung cancer. No differences based on other clinicopathological variables were observed. Furthermore, a meta-analysis showed a higher distribution of rare alleles in our study of Caucasian Spaniards than was found in other studies of American and Northern European Caucasian populations. We conclude that the presence of rare HRAS1 VNTR alleles may be an inherited predisposing factor in lung cancer. This presence can be easily determined from peripheral blood samples by PCR-based methods. Furthermore, interracial variations in allele frequencies and variations between Caucasian subpopulations suggest that genetic variations may be involved in susceptibility to lung oncogenesis, especially in certain ethnic populations.

INTRODUCTION

As in many other countries, lung cancer has become the main cause of cancer death for men in Spain (1). Lung cancer is the paradigm of a tobacco-induced cancer, whereas lung neoplasms in nonsmokers account for only 5–10% of all lung cancers (2). However, genetic susceptibility to lung oncogenesis may well play a pivotal role because only 10–15% of cigarette smokers develop smoking-related lung neoplasms (3). A comprehensive approach to the underlying genetic mechanisms seems to be mandatory. The multifarious molecular changes in lung cancer development frequently include allelic deletions or loss of heterozygosity. These losses chiefly affect chromosomal region 3p14.2, which contains the common fragile site FRA3B, a hereditary renal carcinoma-associated t(3;8) translocation; the FHIT gene; and also the chromosomal region 11p15.5, which harbors the locus of the c-Ha-ras proto-oncogene (4, 5). Microsatellite instability, mainly on chromosome 3p in early NSCLC⁴ stages, has been observed (6–8), although an inverse correlation between 3p14 deletions and microsatellite instability has also been reported (4). Moreover, alterations on minisatellites or VNTR have also been implicated in lung cancer genesis. In short, minisatellites or VNTR are highly polymorphic structures that are characterized by the tandem iteration of 14–100-bp sequence motifs, dispersed throughout the genomes of higher vertebrates. Several minisatellites associated with specific genes have been involved in diverse diseases, such as HRAS1 in multiple cancer types (9), INS in insulin-dependent diabetes mellitus and polycystic ovary syndrome (10), and EPMI in inherited myoclonus epilepsy (11).

The HRAS1 VNTR region, which maps 1 kb downstream from the canonical polyadenylation signal of the human proto-oncogene H-ras1, consists of four common progenitor alleles, in addition to several rare variants that are thought to derive from germ-line mutations of the nearest common alleles (12). The potential importance of these rare alleles is due to four principal findings. (a) Previous studies have indicated an increased cancer risk for individuals harboring rare alleles at the minisatellite region flanking the H-ras1 proto-oncogene on chromosome 11p15.5 (9, 13–16). Population stratification of 8500 alleles

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⁴The abbreviations used are: NSCLC, non-small cell lung cancer; VNTR, variable number of tandem repeats; CI, confidence interval.
from 23 independent studies showed that aggregation of rare HRAS1 alleles appears twice as frequently in the genomes of cancer patients as in normal unaffected controls. From this meta-analysis, a highly significant association between HRAS1 VNTR alleles and cancer was observed (specifically, HRAS1 VNTR alleles appeared in 1 of 11 neoplasms of the breast, colorectum, and urinary bladder, although no association with lung cancer was observed; Ref. 9). (b) Minisatellite mutation may cause dysregulation of gene expression. In fact, it has been shown that HRAS1 VNTR binds at members of the rel/NF-κB family of transcriptional regulatory factors (17–19). It has also been observed that the rare HRAS1 VNTR alleles show a tendency to bind more avidly to transcriptional regulatory factors (17, 18). (c) Genetic interaction of HRAS1 VNTR has been observed in BRCA1 carriers, in whom the risk of ovarian cancer was 2.11 times greater for BRCA1 carriers harboring one or two rare HRAS1 VNTR alleles. This study was the first to demonstrate the effect of a modifying gene on the penetrance of an inherited cancer syndrome (20). (d) It should be stressed that, besides differing from one another in the number of repeat units, the HRAS1 VNTR sequence derived from the EJ bladder carcinoma cell line revealed regular G for C exchanges through the VNTR at positions 7 and 15 from the 5′ end of the 28-bp repeat motif (21), which provide a sequence-based differentiation of the rare from the common alleles. Parallel analyses have demonstrated that rare alleles possess disorganized internal sequences (22).

This study is a case-control study in which we have investigated whether the HRAS1 VNTR locus modifies the risk of lung cancer in Spanish patients. As an inheritable genetic marker, HRAS1 VNTR alleles can be easily analyzed on peripheral blood lymphocyte DNA samples, thus eliminating the need for tumor tissue.

MATERIALS AND METHODS

Study Population. DNA was extracted from WBCs obtained from lung cancer patients at Hospital Germans Trias i Pujol in Badalona (Barcelona, Spain). The tumors were classified according to the WHO Histological Typing of Lung Tumors and staged according to the tumor-node-metastasis classification of malignant tumors defined by the International Union against Cancer. Two hundred fifty consecutive patients were selected for the study and gave their informed consent; of these, 233 yielded sufficient DNA for analysis. A total of 460 sequential, unrelated blood donors as well as other healthy volunteers were recruited as the controls. Although this was an unmatched case-control study, there were no statistically significant differences between the control subjects and the patients with respect to age, sex, or ethnic background. All study subjects were Spanish Caucasian residents of the surrounding area of Badalona and had no prior history of cancer. Subjects with chronic lung disease were excluded as controls. Of the 460 interviewed controls, 446 yielded sufficient DNA.

Data Collection. Control subjects were interviewed for demographic factors, medical history, health habits such as cigarette smoking, and family history of cancer. All interviewed subjects were asked to provide a blood sample for storage of lymphocytes. Clinical characteristics of the lung cancer patients (including lymph node status in resected patients, distant metastases, histological type, and follow-up) were obtained from the Medical Oncology Service.

DNA Extraction. Three ml of peripheral blood were obtained in the presence of EDTA from the unaffected controls and lung cancer patients. An erythrocyte lysis buffer containing 150 mM NH₄Cl and 10 mM HEPES was used to isolate lymphocytes. After centrifugation, the lymphocytes were separated and digested overnight at 37°C in a buffer containing 10 mM
analyzed by spectrophotometric methods using an Ultraspec phenol/chloroform treatment, and genomic DNA was recovered and 0.25 mg/ml proteinase K. Samples were deproteinized by

$$\text{GGG AAG TCT AT-3}$$

sequences of the primers were: sense, $5'$-GCT CCT GGC CTC GGG AAG TCT AT-3'; antisense, $5'$-AGA GCT AGC AGG GCA TGC CGC T-3'. Amplified products were separated by

$$\text{TAE buffer (pH 8.3), 1.5 mM MgCl}_2, 5% \text{ DMSO, 0.2 mM each dNTP, 0.4 mM sense and antisense primers, 2 units of DNA polymerase [DeepVent (exo-); New England Biolabs, Beverly, MA], and 150 ng of genomic DNA. PCR were run in a final volume of 50 µl on a Perkin-Elmer Corp. (Branchburg, NJ) 9600 Gene Amp PCR thermocycler, using the following temperature program: 94°C for 5 min, followed by 30 cycles each of 1 min at 94°C, 3 min at 68°C, and 3 min at 72°C. A final extension step at 72°C for 4 min was added to terminate the amplification. The sequences of the primers were: sense, 5'-GCT CCT GGC CTC GGG AAG TCT AT-3'; antisense, 5'-AGA GCT AGC AGG GCA TGC CGC T-3'. Amplified products were separated by electrophoresis through 1.2% agarose gels 40 cm long at a constant voltage of 2 V/cm for 15–18 h using 1× TAE buffer [400 mM Tris-acetate and 10 mM EDTA (pH 8.3)]. Molecular weight standards of 50 and 100 and 500 bp were loaded together with PCR products in every six to seven wells. Gels were stained with ethidium bromide and photographed under UV light using a Polaroid DS34 camera system and type 669 film.

**Statistical Analysis.** The association between a rare HRAS1 VNTR allele and lung cancer was first assessed by comparing the proportions of patients and controls with one or more rare HRAS1 VNTR alleles. As in other published studies, alleles were categorized into two sets: common and rare. The statistical significance of the different proportions was measured with the $\chi^2$ test with Yates' correction. The association between HRAS1 VNTR alleles and risk of lung cancer was examined by unconditional logistic regression models and the Mantel-Haenszel method to calculate the odds ratios and associated 95% CIs. The heterogeneity of the odds ratios was evaluated by the method of Breslow and Day (23). In addition, the potential effects of confounding and modifying factors (sex, cigarette smoking, and family history) on the association between HRAS1 VNTR alleles and risk of lung cancer were examined in multivariate models. The $Ps$ presented are based on two-sided tests. The DerSimonian and Laird random effects model (24) was used to examine the relationship between accumulated data from other studies on the association among rare alleles and lung cancer. Pairwise comparisons between the different studies were also performed using the Fisher’s exact test of the frequency of rare HRAS1 VNTR alleles.

**RESULTS**

We compared the frequency of rare HRAS1 VNTR alleles in a population of 233 lung cancer patients and in a population of 446 unaffected controls. Four common alleles ($a1$, $a2$, $a3$, and $a4$) were identified, corresponding to PCR product sizes of 0.926, 1.376, 2.006, and 2.496 kb, respectively. Rare HRAS1 VNTR alleles are shown as the deviation of one or more repeat motifs (28 bp) from the four common alleles. Representative
PCR products on agarose gels stained with ethidium bromide are shown in Fig. 1. Our analyses clearly identified 27 PCR fragments corresponding to alleles differing from those defined as common (Table 1) and a total of 65 genotypes. The sizes of both common and rare alleles were calculated from molecular weight standards, and it was possible to discriminate allele sizes corresponding to 28-bp differences. Our results show that the frequency of lung cancer patients harboring at least one rare allele is 32.7% (75 of 233), whereas the same frequency for unaffected controls is 21.9% (98 of 446). The relative risk of lung cancer associated with the presence of rare alleles was 1.68 (95% CI, 1.6–1.8; \( P < 0.0001 \)). Although the frequency of rare alleles in lung cancer patients was significantly higher than that in the control group (Table 1), this difference was not reflected in the allelotypes. Of the 27 rare alleles found, five were identified solely in the patient group, whereas eight were detected only in the control group.

We also examined the presence of rare HRAS1 VNTR alleles in patients stratified by stage of disease at diagnosis (stage Ia, 14; stage Ib, 60; stage II, 19; stage IIIa, 50; stage IIIb, 43; and stage IV, 47), histological subtype (squamous cell carcinoma, 108; adenocarcinoma, 80; large cell undifferentiated carcinoma, 31; and others, 14) and sex (male, 206; female, 27). None of these differences in HRAS1 VNTR alleles were significant (Table 2).

Stratified analyses were then completed according to history of cigarette smoking and sex for cancer cases and for cancer-free controls. As shown in Table 3, among male smokers, there was an elevated risk of lung cancer associated with rare HRAS1 VNTR alleles (odds ratio = 2.1; 95% CI, 1.7–2.6; \( P < 0.007 \)). Although no association was found for women smokers, probably due to the low number of female patients, there was an increased risk linked with rare HRAS1 VNTR alleles in female nonsmokers.

**DISCUSSION**

PCR amplification of hypervariable loci, including VNTR, has increased the sensitivity for typing hypervariable regions of human DNA showing multiallelic variation. In previous studies, the Southern blot method was used to test the association of rare HRAS1 VNTR alleles and lung cancer. However, Southern blotting is limited in its ability to adequately resolve small differences in allele lengths, especially for the larger alleles and, therefore, may lead to allelic misclassification. Our data indicate that the presence of rare HRAS1 alleles significantly increases the risk of NSCLC, especially among male smokers (odds ratio = 2.13; 95% CI, 1.7–2.6; \( P = 0.007 \)). Conversely, although Heighway et al. (25) found no significant differences in rare alleles among British lung cancer patients when compared

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<th>Table 3</th>
<th>Association between sex and presence of rare HRAS1 VNTR alleles in smokers and nonsmokers</th>
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<tr>
<td>Rare alleles</td>
<td>Lung cancer</td>
</tr>
<tr>
<td>Smokers</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0</td>
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<td>1, 2</td>
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<td>Female</td>
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<td>Nonsmokers</td>
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\(^a\) Smoking history from 40 unaffected controls unavailable.

\(^b\) RR, relative risk; OR, odds ratio; NS, not significant.

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<th>Table 4</th>
<th>Results of different studies of the association between rare HRAS1 alleles and lung cancer</th>
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<td>Ref.</td>
<td>Lung cancer</td>
</tr>
<tr>
<td>This study</td>
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<tr>
<td>Ryberg et al. (28)</td>
<td>385</td>
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<tr>
<td>Ryberg et al. (13)</td>
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<tr>
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<tr>
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<tr>
<td>Heighway et al. (25)</td>
<td>194</td>
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\(^c\) OR, odds ratio.
with a cancer-free control group, they did find a significantly higher frequency of the largest common allele (a4) in NSCLC patients than in controls (29 versus 15%). This finding, however, was not corroborated in a subsequent study (101 NSCLC patients), in which the proportion of subjects carrying the a4 allele was almost identical in NSCLC patients and controls (13 versus 15%; Ref. 26).

Results of the first studies reporting an association between HRAS1 VNTR and neoplasms, including lung cancer (25), were criticized by Peto et al. (27), who adduced that the rare allele frequency was 4.8% in controls and 5.1% in cancer patients (χ² = 0.03; P = 0.86). To date, few studies have focused on the associations between HRAS1 VNTR and lung cancer, and only two Norwegian studies were able to find a significant correlation [odds ratios = 2.26 (P = 0.002) and 1.8 (P = 0.01)], although the frequency of rare alleles among lung cancer patients was still relatively low (13, 28). Interestingly, results of other studies have shown that the frequency pattern of HRAS1 VNTR alleles varies in different ethnic groups. Table 4 summarizes results from six published studies that support the hypothesis of interracial and Caucasian subpopulation variation in multiallelic variation probabilities: the distribution of rare alleles was lowest in British (25) and Norwegian subjects (13, 28); intermediate in Caucasian Americans from Baltimore, Maryland (29); and highest in African-Americans from Baltimore, Maryland (29), and in Caucasian Spaniards in this study. In agreement with the findings of Sugimura et al. (29), in which certain HRAS1 alleles were found more frequently in one race than in others, we have also observed differences in rare and common allele frequencies; in fact, in both lung cancer patients and unaffected controls, the rare allele which appeared most frequently was a1+a4 (Table 1). However, the frequencies observed for the a4 common allele were too low to be considered common in our population. Sixteen of 21 pairwise comparisons by Fisher’s exact test of the frequency of rare HRAS1 VNTR alleles of the population showed significant differences (data not shown). Taken together, the results of all of the individual studies and this meta-analysis yielded a nonparametric estimated Z score of 5.84 (P < 0.001; Fig. 2). Intriguingly, in a recent Norwegian study (12), microsatellite alterations were more often found in NSCLC patients with at least one rare HRAS1 VNTR allele (60%) than in patients with two common alleles (5%). Furthermore, in our laboratory, we observed that the frequency of microsatellite instability in CA repeats at chromosome 3p was relatively high in resected NSCLC tumors and was associated with reduced survival (7, 8). Moreover, our research has revealed shorter survival of lymphoma patients harboring rare HRAS1 VNTR alleles (30). Other potential mechanisms should be taken into account when interpreting our results. A large comprehensive genetic susceptibility study is warranted, in which GSTM1 and CYP1A1 genotypes and microsomal epoxide hydrolase polymorphisms should be analyzed. However, here, we have focused only on the relevance of HRAS1. The oncogenic potential of HRAS1 lies in the fact that it can modify transcription in the mRNA levels of specific oncogene-suppressing and oncogene-inducing genes, with a loss of nm23-H1 and tissue inhibitor of metalloproteinase 1 and an increase of cripeto, M, 94,000 gelatinase/type IV collagenase, osteopontin, and transin/stromelysin transcripts. These modifications lead to tumor progression (31). Here, survival analysis of NSCLC patients showed a tendency to a worse prognosis in patients with at least one rare allele (P = 0.059; median survival, 22 months) than in patients without rare alleles (median survival not reached).

It is worth noting that the rising incidence of lung cancer observed in women worldwide is not representative of the lung cancer index in Spain. Data on incidence obtained from a population-based cancer registry show an incidence rate of 42.5 per 100,000 per year for men and 3.6 per 100,000 per year for women (32). The reasons for this lower incidence rate among women remain unknown. There was no difference in frequency of rare alleles in the control group among healthy men and healthy women (table not shown). However, the frequency of rare alleles in the male lung cancer patients was significantly higher than in the male controls (P = 0.02). Conversely, there was no difference in frequency of rare alleles in female lung cancer patients and female controls (P = 0.22).

Our results prompted us to conclude (a) that rare HRAS1 VNTR alleles may serve as an inherited genetic predisposition marker for NSCLC and (b) that genetic variation within human populations may have some role in determining lung cancer susceptibility.

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