Transcription Deregulation at the 15q25 Locus in Association with Lung Adenocarcinoma Risk

Felicia S. Falvella, Antonella Galvan, Elisa Frullanti, Monica Spinola, Elisa Calabrò, Antonino Carbone, Matteo Incarbone, Luigi Santambrogio, Ugo Pastorino, and Tommaso A. Dragani

Abstract

Purpose: We characterized the candidacy of the six candidate genes mapping in the chromosome 15q25 locus, which was previously reported as associated with lung cancer risk, and confirmed the locus association with lung cancer risk in an Italian population of lung adenocarcinoma patients and controls.

Experimental Design: We did a quantitative analysis of mRNA levels of IREB2 (iron-responsive element-binding protein 2), LOC123688, PMSA4 [proteasome (prosome, macropain) subunit α type 4], CHRN84 (cholinergic receptor nicotinic β 4), CHRNA3 (cholinergic receptor nicotinic α 3), and CHRNA5 (cholinergic receptor nicotinic α 5) genes in paired normal lung and lung adenocarcinoma tissue, and an immunohistochemical localization of CHRNA3- and CHRNA5-encoded proteins. We also examined the association of CHRNA5 D398N polymorphism with lung cancer risk and with CHRNA5 mRNA levels in the normal lung.

Results: Expression analysis of the six candidate genes mapping in the lung cancer risk–associated chromosome 15q25 locus revealed a 30-fold up-regulation of the gene encoding the CHRNA5 subunit and a 2-fold down-regulation of the CHRNA3 subunit in lung adenocarcinoma as compared with the normal lung. The expression of the four other candidate genes resulted either unchanged or absent. The carrier status of the 398N allele at the D398N polymorphism of the CHRNA5 gene was associated with lung adenocarcinoma risk (odds ratio, 1.5; 95% confidence interval, 1.2–2.0) in a population-based series of lung adenocarcinoma patients (n = 467) and healthy controls (n = 739). Analysis of a family-based series of nonsmoker lung cancer cases (n = 80) and healthy sib controls (n = 80) indicated a similar trend. In addition, the same D398N variation correlated with CHRNA5 mRNA levels in normal lung of adenocarcinoma patients.

Conclusions: Our results point to the candidacy of the CHRNA5 gene for the 15q25 locus.

Three genomewide studies have reported an association between the chromosome 15 region and lung cancer risk and smoking habit (1–3). The region, spanning 203 kb, contains six genes [IREB2 (iron-responsive element-binding protein 2), LOC123688, PMSA4 (proteasome (prosome, macropain) subunit α type 4), CHRN84 (cholinergic receptor nicotinic β 4), CHRNA3 (cholinergic receptor nicotinic α 3), and CHRNA5 (cholinergic receptor nicotinic α 5)] that might be good candidates for lung cancer risk. Three of the genes (CHRNA5, CHRNA3, and CHRN84) encode nicotinic cholinergic receptor subunits, whose biological function may underlie the association with smoking habit. PMSA4 encodes a T1A peptidase family member that is a subunit of the proteasome, whereas IREB2 encodes an iron-responsive element-binding protein; the function of LOC123688 is unknown. The strong linkage disequilibrium in this region has led to the identification of several single-nucleotide polymorphisms showing significant association with lung cancer risk (1–3). Because small differences in statistical association values cannot serve as the sole basis for distinguishing the relevant functional polymorphism(s) from a single-nucleotide polymorphism(s) showing association due only to its linkage disequilibrium with the surrounding functional elements, we conducted gene expression analyses of the six candidate genes mapping in the linkage disequilibrium region of chromosome 15q25 in an effort to dissect the functional elements. We also carried out a replica association study focusing on the CHRNA5 D398N polymorphism in lung adenocarcinoma risk and prognosis.

Materials and Methods

Series and DNAs. The population-based case-control series consisted of pathologically documented lung adenocarcinoma, lung
Translational Relevance

The results of this work might be applied to the future practice of cancer medicine, improving cancer prevention based on the prediction of lung cancer risk. Indeed, our manuscript shows that individual risk of lung adenoarcinoma is affected by the individual genetic profile of the chromosome 15q25 locus, thus confirming and extending to the Italian population the previously reported association between this locus and lung cancer risk. Among the genes mapping in this locus, CHRNA5 (cholinergic receptor nicotinic α 5) gene was up-regulated in lung adenocarcinoma, pointing to its candidacy for the chromosome 15q25 locus. These findings represent an important advancement in the understanding of the individual genetic elements that can control lung cancer risk, and they also open the possibility of development of new therapeutics treatments aimed to contrast CHRNA5 overactivity in lung tumor cells.

squamous cell carcinoma patients, and unrelated healthy individuals enrolled at Istituto Nazionale Tumori (Milan, Italy) from 1980 to 2007. Controls were recruited among blood donors (n = 350) or subjects participating in a computed tomography screening for lung cancer prevention (n = 389). Study protocols were approved by the institute ethics committee. About 30% of eligible patients were recruited. Genomic DNA was extracted from peripheral blood with standard methods and quantified using PicoGreen dsDNA Quantitation Kit (Invitrogen). DNAs from patients and controls series, matched for methods and quantified using Picogreen dsDNA Quantitation Kit. The ethics committee. About 30% of eligible patients were recruited.

RNA analysis. Specimens of normal lung parenchyma and lung adenocarcinoma tissue (histopathologically confirmed) were obtained from patients who underwent lung lobectomy at Istituto Nazionale Tumori. RNeasy Midi kit (Qiagen) was used to extract total RNA from 21 matched pairs of nontumor lung parenchyma and lung adenocarcinoma tissue and from 48 additional normal lung samples of lung adenocarcinoma patients. Two RNA pools were prepared from nontumor lung parenchyma and lung tumor tissue.

RNAs from pooled or individual samples were reverse transcribed using the ThermoScript RT-PCR system (Invitrogen) according to the manufacturer's instructions. Quantitative mRNA levels were analyzed by kinetic reverse transcriptase-PCR using Taqman Gene Expression Assays (Applied Biosystems) for the following genes: IREB2 (Hs01021787_m1); similar to RIKEN cDNA C630028N24 gene (LOC123688; Hs01372136_m1); proteasome (prosome, macropain) subunit α type 4 (Hs01002583_m1); CHRNA5 (Hs00181248_m1); CHRNA3 (Hs00609523_m1). The real-time PCR amplification mixture contained aliquots of cDNA template, 10 μL 2X Taqman Universal PCR Master Mix No AmpErase UNG (Applied Biosystems), 1 μL of 20X Taqman Gene Expression Assay Mix (Applied Biosystems), and RNase-free water to a final volume of 20 μL. The human hypoxanathine phosphoribosyltransferase 1 (HPRT1) gene (Hs99999909_m1) was used as housekeeping control. Reactions were run in triplicate (RNA pools) or duplicate (individual samples) on the 7900HT System (Applied Biosystems). Relative changes in mRNA levels were assessed using the comparative cycle threshold (Ct) method, and relative quantities were calculated using a pool of normal lung tissue as calibrator.

Genotyping. Genotyping was carried out by PCR amplification of single-nucleotide polymorphism–containing fragments, followed by pyrosequencing analysis on a P5096MA system (Biotech AB), according to the manufacturer’s instructions using specific primers for CHRNA3 (PCR, 5'-tcttgtaatgtagcgaatag-3'; sequencing, 5'-cttgaatgtagataagtgacag-3') and for CHRNA5 (PCR, 5'-ggctgctgtttactctgc-3'; sequencing, 5'-cttgaatgtagataagtgacag-3').

Immunohistochemistry. Paraffin-embedded tissue sections of lung adenocarcinoma and surrounding normal lung tissue from 16 patients were retrieved from the archives of our Department of Pathology. Histologic sections were immunostained using standard methods after antigen retrieval done in 0.07 mol/L citrate buffer (pH 6) at 95°C for 10 mins. Antibodies used were anti-CHRNA3 (LS-B934; diluted 1:800) and anti-CHRNA5 (LS-C37656; diluted 1:4000) from LifeSpan Biosciences, Inc. Immunoreactive signals were detected with ChemMate DAB (Dako).

**Results**

mRNA levels of CHRNA3 and CHRNA5 are modulated in lung adenocarcinoma. A preliminary gene expression analysis using RNA pools obtained from normal lung and lung adenocarcinoma tissues revealed similar transcript levels of IREB2, LOC123688, and PMSA4 genes, no detectable CHRN8

**Fig. 1.** Quantitative kinetic reverse transcriptase-PCR for CHRNA3 and CHRNA5 genes in paired normal lung and lung adenocarcinoma tissue. The line within each box represents the median fold-change value. Upper and lower edges of each box, 75th and 25th percentile, respectively. Upper and lower bars, highest and lowest values determined, respectively.*, P<0.05; **, P<0.0001.
expression, down-regulated CHRNA3, and up-regulated CHRNA5 expression in lung adenocarcinoma as compared with normal lung. Further analysis focused on CHRNA3 and CHRNA5 gene expression using individual RNA samples from 21 paired nontumor lung and lung tumor specimens (Fig. 1) confirmed the results obtained with the RNA pools. Indeed, we found that CHRNA3 expression levels were about 2-fold lower in lung adenocarcinoma as compared with normal lung in 15 of the 21 pairs ($P < 0.01$), with no detectable expression in tumor samples of six pairs, whereas CHRNA5 gene expression was detected in normal and tumor samples of all 21 pairs, showing about 30-fold up-regulation in lung adenocarcinoma as compared with normal lung tissue ($P < 0.0001$).

Immunohistochemical analysis readily revealed the expression of CHRNA3 and CHRNA5 proteins mainly in the cytoplasm in normal lung and in lung adenocarcinoma tissue (Fig. 2). Immunoreactivity in normal tissue was apparent in alveolar cells (Fig. 2A and B) as well as in bronchiolar cells (Fig. 2C and D). In lung adenocarcinoma, CHRNA3 (Fig. 2E and G) and CHRNA5 (Fig. 2F and H) proteins were detected in tumor cells but not in stromal cells. In agreement with gene expression results, most of the tumors showed a weaker staining with the anti-CHRNA3 antibody (Fig. 2E, left corner; Fig. 2G) than with the anti-CHRNA5 antibody (Fig. 2E, right corner; Fig. 2H), suggesting that differences in expression levels of these two proteins can be detected by immunohistochemical analysis.

**Association of CHRNA5 D398N polymorphism with lung adenocarcinoma risk.** Based on the observed significant gene modulation of the CHRNA3 and CHRNA5 genes in lung adenocarcinoma, we carried out a population-based study on a series of lung adenocarcinoma patients and healthy controls (Supplementary Table 1) to further test the candidacy of these genes for the chromosome 15q25 locus associated with lung cancer susceptibility.

Using two already available DNA pools comprised respectively of 400 cases and 400 age-, sex-, and smoking status–matched controls extracted from our series, we analyzed two coding polymorphisms reportedly associated with lung cancer risk: the rs1051730 single-nucleotide polymorphism, a synonymous change within the CHRNA3 gene (1–3), and the rs16969968 single-nucleotide polymorphism, a D398N polymorphism of the CHRNA5 gene (2, 6). Because the rs1051730 single-nucleotide polymorphism of the CHRNA3 gene showed a slightly weaker statistical association with lung cancer risk as compared with the rs16969968 single-nucleotide polymorphism of the CHRNA5 gene and because of an almost complete linkage disequilibrium between these two single-nucleotide polymorphisms in the European population, we focused our analysis on the D398N polymorphism of the CHRNA5 gene.

### Table 1. Association of CHRNA5 D398N polymorphism rs16969968 with lung adenocarcinoma risk in the population-based case-control series

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Amino acid</th>
<th>Controls</th>
<th>Patients</th>
<th>Odds ratio (95% CI)*</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>Asp/Asp</td>
<td>267</td>
<td>128</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>Asp/Asn</td>
<td>348</td>
<td>226</td>
<td>1.4 (1.0-1.9)</td>
<td>0.0240</td>
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<tr>
<td>AA</td>
<td>Asn/Asn</td>
<td>124</td>
<td>113</td>
<td>1.9 (1.3-2.7)</td>
<td>0.0003</td>
</tr>
<tr>
<td>GA or AA</td>
<td>Asp/Asn or Asn/Asn</td>
<td>472</td>
<td>339</td>
<td>1.5 (1.2-2.0)</td>
<td>0.0020</td>
</tr>
<tr>
<td>Frequency of the rare (A) allele</td>
<td>0.40 †</td>
<td>0.48 †</td>
<td>0.0001</td>
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</table>

*Adjusted for sex, smoking status, and age in decades.
† Nonsignificant deviation from the Hardy-Weinberg equilibrium.
population ($D' = 1.0; r^2 = 0.94; n = 104; genotypes downloaded^6 and ref. 6), only the CHRNA5 polymorphism was analyzed in individuals of the whole series (Table 1). No significant deviation from the Hardy-Weinberg equilibrium was found in either controls or cases. The frequency of the A (398Asn) allele differed significantly between controls and cases (0.41 and 0.48, respectively; $P = 0.0001$). In a small series ($n = 91$) of lung squamous cell carcinoma patients, the frequency of the A (398Asn) allele was identical to that observed in lung adenocarcinoma (not shown).

In lung adenocarcinoma case and control series, the homozygosity status of the A allele showed an odds ratio of 1.9 [95% confidence interval (95% CI), 1.3-2.7] and was significantly associated with lung adenocarcinoma risk ($P = 0.0003$). Comparison of GG (Asp398Asp) homozygous subjects with GA (Asp398Asn) heterozygous subjects (odds ratio, 1.4; 95% CI, 1.0-1.9; $P = 0.024$) or comparison of subjects carrying the A (398Asn) allele versus GG (Asp398Asp) homozygous subjects (odds ratio, 1.5; 95% CI, 1.2-2.0; $P = 0.002$) also showed the significant association of the A allele with lung adenocarcinoma risk. No significant associations of the CHRNA5 D398N polymorphism with patients’ clinical stage or overall survival were detected.

To test the involvement of the 15q25 locus in lung cancer risk in the absence of the environmental risk factor smoking exposure, we carried out a family-based study of nonsmoker lung cancer patients (Supplementary Table 2). Although the detection power of the series is low because of the small sample size (i.e., 80 cases and 80 controls) and no statistically significant association was detected ($P = 0.282$; additive inheritance model), the carrier status of the A (398Asn) allele at CHRNA5 showed an odds ratio of >1.0 with lung cancer risk (odds ratio, 1.3; 95% CI, 0.7-2.8; Table 2). Combining the results of the population-based and family-based association between CHRNA5 polymorphism and lung cancer risk revealed no significant heterogeneity ($P = 0.716$) between the two series, suggesting that, despite the different sizes and power of the series, they detect similar effects. An overall odds ratio of 1.47 (95% CI, 1.16-1.86) was found associated with the A (398N) allele carrier status (Fig. 3).

Table 2. Association of CHRNA5 D398N polymorphism rs16969968 with lung cancer risk in the family-based case-control series

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No.</th>
<th>Odds ratio (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleotide</strong></td>
<td><strong>Amino acid</strong></td>
<td><strong>Controls</strong></td>
<td><strong>Patients</strong></td>
</tr>
<tr>
<td>GG</td>
<td>Asp/Asp</td>
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<td>25</td>
</tr>
<tr>
<td>GA</td>
<td>Asp/Asn</td>
<td>34</td>
<td>36</td>
</tr>
<tr>
<td>AA</td>
<td>Asn/Asn</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>GA or AA</td>
<td>Asp/Asn or Asn/Asn</td>
<td>50</td>
<td>55</td>
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<td><strong>Frequency of the rare (A) allele</strong></td>
<td>0.41</td>
<td>0.46</td>
<td></td>
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</table>

*Adjusted for sex and age in decades.
†No significant deviation from the Hardy-Weinberg equilibrium.

Association of D398N polymorphism with mRNA levels of CHRNA5. Following the results of CHRNA5 up-regulation in lung adenocarcinoma and of the association of its D398N polymorphism with lung adenocarcinoma risk, we investigated the association of such polymorphism with the CHRNA5 mRNA levels in normal lung tissue. We analyzed a total of 69 normal lung mRNA samples selected for their genotype at the D398N single-nucleotide polymorphism to have a similar number of individuals per genotype group (21-26 samples each). Quantitative analysis of CHRNA5 mRNA levels detected a significant gene expression difference related to the D398N genotypes ($P = 8.04 \times 10^{-6}$). Decreasing mRNA levels were observed at increasing dosage of the A (398Asn) allele and gene transcript was about 2.5-fold lower in patients carrying the A allele at homozygosity than patients homozygous for the common G (398Asp) allele (Fig. 4).

Discussion

Our expression analysis of the six genes mapping in the chromosome 15q25 locus previously associated with lung cancer risk (1–3) identified two genes, CHRNA3 and CHRNA5, that seemed to be deregulated in lung adenocarcinoma tissue compared with normal lung tissue. CHRNA3 and CHRNA5 proteins were detected in alveolar and bronchial cells.
of normal lung and in lung adenocarcinoma cells, with expression localized mainly in the cytoplasm.

The CHRNA3 and CHRNA5 genes encode different subunits of nicotinic acetylcholine receptors, which are members of a superfamily of ligand-gated ion channels that mediate rapid signal transmission at synapses. Nicotinic acetylcholine receptors are thought to be (hetero)pentamers composed of combinations of different α and β subunits that are expressed in a tissue-specific manner and whose individual functional roles are not well understood; however, the receptor activity is strongly influenced by the composition of its subunits (7, 8). Because the composition of the receptors depends on the relative expression of the encoding nicotinic acetylcholine receptor subunit genes (9), our findings of different expression of the CHRNA3 and CHRNA5 subunits in lung adenocarcinoma as compared to normal lung could have a biological relevance, for example, by rendering the receptor more active or more responsive to their ligands and thus affecting the signal transduction mediated by the acetylcholine receptor. Indeed, the α5 subunit can alter receptor properties by inducing conformational changes that can modify the receptor functionality, for example, its calcium permeability (8).

We detected a strong up-regulation of the CHRNA5 gene in tumor tissue, in agreement with a recent report (10) of increased expression of the α5 subunit in squamous cell lung carcinomas; however, the same group found no significant modulation of the CHRNA3 gene, in contrast to the down-regulated expression we observed in lung adenocarcinoma samples. This discrepancy might rest in the different tumor histotypes (adenocarcinoma versus SCC) analyzed in the two studies. Another study detected up-regulated CHRNA5 gene expression in non–small cell lung cancer cell lines or in tumor tissue compared with the normal counterpart (11).

Based on the modulation of transcript levels in lung adenocarcinoma, CHRNA3 and CHRNA5 genes seemed good candidates for further investigation for their genetic role in predisposing to lung cancer; however, the complete linkage disequilibrium between these genes and the greater modulation of CHRNA5 in lung adenocarcinoma compared with that of CHRNA3 led us to focus on CHRNA5. We found a significant association of the nonsynonymous D398N polymorphism of the CHRNA5 gene with lung cancer risk in a series of Italian lung adenocarcinoma cases and matched healthy controls. The A (398Asn) allele showed a codominant effect, with odds ratios of 1.4 and 1.9 in heterozygous and homozygous carriers, respectively (Table 1).

The analysis of 80 nonsmoker cases matched with their sib pairs revealed no significant association of the CHRNA5 D398N polymorphism with lung cancer risk. However, the statistical power was limited because of the small size of this series. Our results in nonsmokers are consistent with the lack of association reported in 125 never-smoking cases (3), although we detected an odds ratio of 1.3 versus an odds ratio of ~1 detected by Amos et al. (3). Because we observed no statistically significant heterogeneity between the two series, it is possible that the CHRNA5 D398N polymorphism also modulates lung cancer risk in nonsmokers. In our series, we detected a weak association between the homozygosity, status of the CHRNA5 polymorphism and smoking behavior, suggesting a recessive mode of association of this polymorphism with smoking status.

In addition to the association with lung cancer risk, we found that the D398N CHRNA5 polymorphism was also associated with mRNA levels of the CHRNA5 gene in normal lung tissue (Fig. 4). This association may either underlie a functional role of this polymorphism in CHRNA5 transcriptional regulation or it may reflect linkage disequilibrium of the polymorphism with transcriptional regulatory elements not yet identified in the 15q25 locus.

In conclusion, the significant deregulation of CHRNA5 transcript levels in lung adenocarcinomas, the association of the D398N CHRNA5 polymorphism with lung cancer risk, and the novel association between the same D398N single-nucleotide polymorphism and CHRNA5 mRNA levels pointed to the candidacy of this gene for the 15q25 locus, although candidates of other genes in the linkage disequilibrium region cannot be excluded.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Roberta Ottria and Lucia Gioiosa of the immunohistochemistry service of our department for technical assistance.

References
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