Promoter Polymorphisms and Transcript Levels of Nicotinic Receptor CHRNA5

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Chromosomal locus 15q25, implicated in lung cancer risk and nicotine dependence, shows extensive linkage disequilibrium that complicates identification of causal variation. Cholinergic receptor nicotinic α5 (CHRNA5) has been identified as a lung cancer risk factor. We identified by sequence analysis three haplotypes (delTTC, insATC, and insTGG) in the 5′ promoter region and three at the 3′-untranslated region of CHRNA5. Linkage disequilibrium analysis of the 5′ variants showed that the insTGG haplotype is associated with three tightly linked risk alleles (nicotine dependence, lung cancer, and chronic obstructive pulmonary disease). The three CHRNA5 promoter haplotypes were statistically significantly associated with lung CHRNA5 transcript levels, determined by real-time polymerase chain reaction. In nontumor lung parenchyma from 68 patients who underwent lung lobectomy, the delTTC haplotype was associated with the highest CHRNA5 transcript levels (ΔRQ = 0.88, difference = 0.94 units, P<.001, Welch t test; all statistical tests were two-sided). Luciferase reporter assays in human lung cancer cell lines A549, H460, H520, and H596 also showed that the 5′ region haplotypes were statistically significantly associated with changes in CHRNA5 promoter activity, whereas the 3′-untranslated region variants were not.


Several genetic variants at chromosomal locus 15q25, including cholinergic receptor nicotinic α5 (CHRNA5), were found to be associated with lung cancer risk and nicotine dependence in recent genome-wide association studies (1–3). The same alleles were also associated with increased risk of chronic obstructive pulmonary disease (4,5).

In a previous study (6), we observed increased CHRNA5 transcript levels in lung adenocarcinomas and showed a statistically significant association between the A398 variant of the single-nucleotide polymorphism (SNP) rs16969968 of CHRNA5 and lower transcript levels of this gene in normal lung tissue. Fine mapping and expression analysis in human brain tissue also pointed to the candidacy of CHRNA5 as a risk factor for nicotine dependence and lung cancer (7). Nonetheless, the strong linkage disequilibrium among all CHRNA5 variants complicates assignment of any biological role to a specific variant (1–3). Among these variants, the SNP rs3841324 showed the highest association with CHRNA5 mRNA levels in both brain and lung tissue (7,8).

To unravel the functional variation responsible for modulation of CHRNA5 expression, we integrated genetic information of the 5′- and 3′-untranslated regions (UTR) with expression levels of CHRNA5 in nontumor lung parenchyma of 68 patients who underwent lung lobectomy. Study protocols were approved by the Istituto Nazionale Tumori ethics committee, and written informed consent was obtained from each subject for use of biological material.

To identify genetic polymorphisms associated with CHRNA5 mRNA levels and in linkage disequilibrium with the Asp398Asn polymorphism, genomic DNA of 10 normal lung samples with high (mean relative quantification [ΔRQ] = 1.72) CHRNA5 mRNA levels and 10 samples with low (mean ΔRQ = 0.53) CHRNA5 mRNA levels (6) were amplified by polymerase chain reaction for the 5′ region and 3′-UTR of CHRNA5 (using primer pairs listed in Supplementary Table 1, available online) and sequenced using an automated sequencer (Applied Biosystems, Foster City, CA). Sequences were aligned and compared using Genomatix Dialign software (http://www.genomatix.de).

Sequence analysis of the CHRNA5 5′ region identified SNP rs3841324, an insertion/deletion (ins/del) of 22 base pairs, at position −71 upstream of the transcription start site, as well as three known SNPs: rs503464 mapping at −10, and rs55853698 and rs55781567 mapping at the 5′-UTR. We found that the four variations of the CHRNA5 5′ region define three haplotypes, delTTC, insATC, and insTGG.

SNPs validated in our samples were genotyped in 68 samples previously analyzed (6) for CHRNA5 mRNA levels using either 3% agarose gel electrophoresis (rs3841324) or pyrosequencing analysis on a PSQ96MA system (BioTage AB, Uppsala, Sweden) according to the manufacturer’s instructions (using specific primers listed in Supplementary Table 1, available online). Haplotype analysis showed that in nontumor lung parenchyma, the delTTC haplotype was associated with the highest CHRNA5 transcript levels (ΔRQ = 1.82), whereas the insTGG haplotype was associated with the lowest (ΔRQ = 0.88, diff = 0.94 units, 95% confidence interval [CI] = 0.62 to 1.27, t <.001, two-sided Welch t test; Table 1). Diplototype analysis also showed that individuals homozygous or heterozygous for the delTTC haplotype expressed higher CHRNA5 mRNA levels than reference insTGG homozygous subjects (Figure 1, A).

To place the CHRNA5 promoter haplotypes in the context of the linkage disequilibrium pattern in the 15q25 locus, we evaluated the linkage of the promoter haplotypes with SNP rs16969968 (Asp398Asn) and two other SNPs reportedly associated with nicotine dependence (9), lung cancer (1), and chronic obstructive pulmonary disease (4). Analysis of the HapMap (www.hapmap.org; accession October 30, 2009)
genotyping data of the rs16969968 (Asp398Asn), rs8034191, and rs1051730 in a population of US (Utah) residents of northern and western European ancestry (n = 165) showed that they are in tight linkage disequilibrium \( D' = 1.0, r^2 = 0.89–1.0 \). We found that insTGG is linked to the Asn398 risk allele, which is strongly associated with the C (rs8034191) and T (rs1051730) risk alleles, whereas delTTC and insATC are linked to the context and caveat

Prior knowledge

Cholinergic receptor nicotinic \( \alpha_5 \) (CHRNA5) is located in chromosomal locus 15q25, which has been associated with the risk of lung cancer and nicotine dependence.

Study design

Sequence analysis revealed three variants in the 5' promoter region and three at the 3' untranslated region of CHRNA5. The association between these polymorphisms and CHRNA5 expression levels was examined with real-time polymerase chain reaction of RNA from normal lung tissue of lung cancer patients and with luciferase reporter assays in four lung cancer cell lines.

Contribution

The three promoter variants were associated with statistically significant differences in CHRNA5 expression in the CHRNA5 promoter haplotypes, but the variants at the 3' untranslated region were not.

Implications

Polymorphisms in the CHRNA5 promoter region may modify the binding sites for transcription factors, which can alter CHRNA5 expression levels and the risk of nicotine dependence, lung cancer, and chronic obstructive pulmonary disease.

Limitations

A limitation of the study was the moderate association between the CHRNA5 promoter polymorphisms and CHRNA5 mRNA levels in normal lung tissue and the risk of nicotine dependence, lung cancer, and chronic obstructive pulmonary disease. However, this association is difficult because the 3' untranslated region is difficult to associate with expression levels.
Figure 1. In vitro and in vivo effects of cholinergic receptor nicotinic α5 (CHRNA5) variants on gene expression. A) CHRNA5 mRNA levels as a function of the promoter diplotypes in 68 normal lung tissue samples. Single-nucleotide polymorphisms were genotyped using either 3% agarose gel electrophoresis (rs3841324) or pyrosequencing analysis on a PSQ96MA system (Biotage AB) using specific primers (Supplementary Table 1, available online). Total RNA was extracted from nontumor lung parenchyma using the RNeasy Midi kit (Qiagen, Valencia, CA) and reverse transcribed using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. CHRNA5 mRNA levels were analyzed by quantitative real-time polymerase chain reaction using TaqMan Gene Expression Assays (Hs00181248_m1, Applied Biosystems), as described previously (6). The human hypoxanthine phosphoribosyltransferase 1 gene (Hs99999909_m1) was used as housekeeping control. Relative quantification (RQ) represents normalized expression units assessed using the comparative Ct method and a pool of normal lung tissue as calibrator. The heavy line within each box represents the median RQ value (in log2-transformed units); upper and lower edges of each box represent the 75th and 25th percentiles, respectively; upper and lower bars indicate the highest and lowest values determined, respectively. Asterisks denote statistically significant differences by comparison with the homozygous carriers of the insTGG haplotype, used as reference (mean RQ and number of samples (n) of the diplotypes were: insTGG/insTGG, 0.75, n = 22; insTGG/insATC, −0.25, n = 12; insTGG/delTTC, 0.38, n = 13; insATC/insATC, −0.32, n = 2; insATC/delTTC, 0.23, n = 10; delTTC/delTTC, 1.14, n = 9; P < .001, overall). Differences in mean normalized expression units between patients with insTGG/delTTC, insATC/delTTC, and delTTC/delTTC diplotypes and patients with the insTGG/insTGG diplotype were 0.81 (95% confidence interval [CI] = 0.35 to 1.27, Pdiff = .002), 0.56 (95% CI = 0.28 to 0.84, Pdiff < .001), and 1.73 (95% CI = 0.94 to 2.52, respectively, Pdiff < .001, two-sided Welch t test). Differences in mRNA levels were analyzed by one-way analysis of variance or Welch t test when appropriate. All statistical tests were two-sided. B and D) Schematics of recombinant
Asp398 allele (Table 1). Therefore, risk alleles for nicotine dependence (9), lung cancer (1), and chronic obstructive pulmonary disease (4) are associated with the insTGG haplotype, which is associated with low levels of CHRNA5 mRNA in normal lung tissue (RQ = 0.88; Table 1).

The functional role of the three 5’ region haplotypes was investigated by subcloning 732 base pairs of the proximal promoter into the pGL3-Basic vector upstream of the ATG of the firefly luciferase gene, used as primary reporter to monitor changes in mRNA (Figure 1, B). The respective vectors were transfected together with the Renilla luciferase pRL-TK reporter vector into four lung cancer cell lines (A549, NCI-H460, NCI-H520, and NCI-H596, purchased from American Type Culture Collection, Manassas, VA and passaged three to four times) for analysis by Dual-Luciferase Reporter Assay (Promega, Madison, WI), 48 hours after transfection. Signals were normalized to Renilla luciferase activity and expressed as relative luciferase activity. Data from three replicates of two independent experiments were evaluated (Figure 1, C, and Supplementary Table 2, available online).

Overall, the normalized firefly/Renilla luciferase activity of the three recombinant vectors was increased 94-fold as compared with that of the empty control vector (mean luciferase activity, relative units: recombinant vectors, 1.33; empty control vector, 0.01, difference = 1.31, 95% CI diff = 1.26 to 1.36, Pdiff < .001, two-sided Welch t test), indicating in vitro functional activity of the CHRNA5 promoter.

Luciferase activity was differentially modulated by the insATC and delTCC promoter haplotypes, as compared with the reference insTGG haplotype (Supplementary Table 2, available online). In A549 cells, both the insATC and the delTCC haplotypes showed a statistically significant increase of CHRNA5 promoter activity (P = .001, whereas the delTCC haplotype produced a strong decrease (Pdiff < .001). Finally, in H596 cells, the reference insTGG haplotype showed about the same promoter activity as the insATC haplotype and the delTCC haplotype showed statistically significantly decreased activity (Pdiff = .002, Figure 1, C, and Supplementary Table 2, available online).

Sequence analysis of the CHRNA5 3’- UTR revealed three known SNPs (rs615470, rs8192482 and rs564585), which distinguished three haplotypes, CTA, CCG, and TCA. The same four lung cancer cell lines were transfected with the 3’- UTR subcloned into the pmirGLO vector downstream of the firefly luciferase gene (Figure 1, D). The normalized firefly/Renilla luciferase activities of the three different haplotypes were compared with each other and with the empty control vector and revealed an overall modest effect of the 3’- UTR on firefly luciferase activity in all cell lines, with a decreased activity in A549 and NCI-H460 cells and increased activity in NCI-H520 and NCI-H596 cells (Figure 1, E). Whereas these results do not rule out a minor role for the 3’- UTR in modulating CHRNA5 mRNA levels, luciferase activity did not differ statistically significantly among the three 3’- UTR haplotypes in any of the cell lines, except for a small difference between the CCG (mean luciferase activity, −0.02 units) and TCA (mean, −0.21 units) haplotypes in NCI-H460 cells (difference, 0.19 units, 95% CIdiff = 0.08 to 0.29, Pdiff = .003, two-sided Welch t test).

Because CHRNA5 lies in a tail-to-tail orientation with respect to CHRNA3 and partially overlaps with CHRNA3, we explored the role of CHRNA3 as a possible antisense regulator by determining with reverse transcription–polymerase chain reaction and sequence analysis which transcripts of CHRNA5 and CHRNA3 were expressed in lung tissue. The expression of the two known CHRNA3 and four known CHRNA3 transcripts, described in the Ensembl database (www.ensembl.org; accessed on December 24, 2008), was checked in normal lung tissue (primer pairs listed in Supplementary Table 1, available online) with BIO-X-ACT Short DNA Polymerase (BIOLINE, London, UK). Of the two known CHRNA5 transcripts, only ENST00000299565 was detected, whereas the shorter transcript (ENST00000394802) was not polymerease chain reaction amplified. Polymerase chain reaction analysis of the four CHRNA3 transcripts detected only ENST00000326828, ENST00000394793, and an as yet undescribed shorter CHRNA3 transcript (330 base pairs), resulting from an alternative splice that skipped exon 2 of the ENST00000394793 variant. These two latter CHRNA3 transcripts overlap exon 6 of CHRNA5 (Supplementary Figure 1, A).

Figure 1 (continued).
available online). NCI-H460 and NCI-H520 cells cotransfected with pmirGLO vectors containing CHRNA5 exon 6 and with the pEF6/V5-His vector expressing either of the two full-length CHRNA3 transcripts (ENST00000394793 and the new shorter transcript) showed no modulation of luciferase activity with respect to the three haplotypes as compared with activity associated with the empty pEF6/V5-His vector (not shown). Thus, interactions between CHRNA5 and CHRNA3 transcripts do not appear to play a role in CHRNA5 transcriptional control.

Our in vivo and in vitro results point to the critical role of polymorphisms in the promoter region of CHRNA5 for regulation of transcription. Such polymorphisms can modify the binding sites for transcription factors whose expression levels may change in different cell types, tissues, and pathological conditions. Accordingly, one limitation of the study was that in A549 cells, the promoter haplotypes modulated the CHRNA5 transcriptional activity as observed in vivo, whereas in the other three lung cancer cell lines, the in vitro effects of promoter activity did not parallel the in vivo findings (Figure 1, A and C). It may be possible to explain the discrepancies among cell types by analyzing the activity of different transcription factors acting on the CHRNA5 promoter.

This study identified four genetic variations in the CHRNA5 promoter region that define three haplotypes affecting activity of the promoter of this gene, thus modulating CHRNA5 transcript levels in normal lung tissue. The relationship of these promoter polymorphisms to the mechanisms underlying the risk of nicotine dependence, lung cancer, and chronic obstructive pulmonary disease awaits further study.

Supplementary Data
Supplementary data can be found at http://www.jnci.oxfordjournals.org/.

References

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