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Association of single nucleotide polymorphisms of nicotinic acetylcholine receptor subunits with cervical neoplasia

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Abstract

Aims—Cholinergic signaling, particularly in response to non-physiological ligands like nicotine, stimulates carcinogenesis of a variety of tissue types including epithelia of the cervix uteri. Cholinergic signaling is mediated by nicotinic acetylcholine receptors (nAChRs), which are pentamers formed by subsets of 16 nAChR subunits. Recent literature suggests that single nucleotide polymorphisms (SNPs) of some of these subunits, notably alpha5, are risk factors for developing lung cancer in smokers as well as in non-smokers.

Main methods—We have studied the prevalence of four SNPs in the alpha5, alpha9, and beta1 subunits, which are expressed in cervical cells, in 456 patients with cervical cancers, precursor lesions, and healthy controls from two cohorts in Mexico.

Key findings—A SNP in the alpha9 subunit, the G allele of rs10009228 (alpha9, A>G) shows a significant trend in the combined cohort, indicating that this allele constitutes a risk factor for neoplastic progression. The A allele of the SNP rs16969968 (alpha5, G>A), which correlates with the development of lung cancer, shows a non-significant trend to be associated with cervical

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Conflict of interest statement

None of the authors has any competing interests.

lesions. Two other SNPs, rs55633891 (alpha9, C>T) and rs17856697 (beta1, A>G), did not exhibit a significant trend.

Significance—Our study points to a potential risk factor of cervical carcinogenesis with importance for DNA diagnosis and as a target for intervention.

Keywords

Cervical cancer; Nicotinic acetylcholine receptor; Single nucleotide polymorphism

Introduction

A necessary, but not sufficient, prerequisite for developing cancer of the cervix uteri is infection with high-risk human papillomaviruses (HPVs) (Munoz et al., 2003). The fraction of women infected with HPVs (lifetime cumulative incidence >80%) vastly exceeds the fraction that develops cancer (<1%). Therefore, progression to cancer is determined by additional, poorly understood events. Numerous epidemiological studies of cervical carcinogenesis identified tobacco smoking as the principal behavioral risk factor for this cancer (e.g. Munoz et al., 2003). This epidemiological correlate serves as an important lead to detect the underlying molecular mechanism between lesions that do and do not progress.

Since nicotine and nitrosamines target nicotinic acetylcholine receptors (nAChRs), our research aims to understand the role of nAChRs during cervical neoplasia, as nAChR signaling affects epithelial biology and neoplasia of other cancers (Schuller, 1989; Wessler and Kirkpatrick, 2008). Human nAChRs are pentameric ligand-gated ion channels composed of a combination of 16 subunit proteins (alpha1-7, alpha9-10, beta1-4, gamma, delta, and epsilon). Although the nAChRs' natural ligand is acetylcholine, nAChRs are also activated by nicotine and nitrosamines binding the ligand binding site with a higher affinity than acetylcholine (Wessler and Kirkpatrick, 2008). Epithelial nAChRs are composed of subunits homologous to neuromuscular nAChRs, but exhibit different functions, such as effects on proliferation, apoptosis, adherence, motility, and differentiation (Grando, 2008). Studies of nAChRs in head and neck (H&N) and lung cancers, the sites directly exposed to tobacco smoke, established that nAChR signaling is involved in the etiology of these cancers, as nicotine and nitrosamines are powerful ligands and function as tumor promoters (Schuller, 1989; Maneckjee and Minna, 1990; Grando et al., 1995). Activation of nAChRs in epithelial cells leads to calcium ion fluxes and affects signaling by calcium-calmodulin kinase II and the Ras/Raf-1/MEK1/ERK1/2 and JAK-2/STAT-3 pathways pointing to mechanisms beyond the traditional model for the carcinogenicity of tobacco smoke mediated by mutagenicity.

In spite of the lack of direct exposure, compounds from tobacco smoke reach the cervix with the circulating serum, leading to concentrations of nicotine and nitrosamines in cervical mucus similar to those in the H&N and lung tissues (Prokopczyk et al., 1997). Based on this observation, we are studying mechanistic similarities between cervical and lung and H&N cancers. We have reported that cervical cancer cells express nAChRs, and that biological properties of cervical cancer cells, such as proliferation, are affected by nicotine (Calleja-Macias et al., 2009). The presence of nAChRs in normal cervical tissue also suggests a

functional role of the cholinergic signaling in the physiology of epithelia at the squamocolumnar junction.

Numerous recent studies suggested that single nucleotide polymorphisms (SNPs) of nAChR subunits affect carcinogenesis of the lung. Three genome-wide studies reported association between a region on chromosome 15q25 and lung cancer risk (Thorgeirsson et al., 2008; Hung et al., 2008; Amos et al., 2008). This chromosomal region contains three genes (CHRNA5, CHRNA3, and CHRNB4) encoding the nAChR subunits alpha5, alpha3 and beta4. Exon 5 of CHRNA5 is affected by one SNP, rs16969968, leading to substitution of aspartic acid (D) to asparagine (N) at position 398. rs16969968 was found to confer significant risk for the development of lung cancer. In spite of the investigations cited above, the case for the increased risk of nAChR subunit SNPs in lung cancer is not yet resolved, as the validity of this association has been criticized based on the numbers obtained in another epidemiological study (Yang et al., 2010). It is therefore obvious that this important research has to be extended. Additional studies should also target SNPs other than rs16969968, which may just be the “tip of the iceberg”, as numerous additional nAChR SNPs are known but have not yet been studied regarding their carcinogenic risk.

Here we report a study of the potential risk associated with four SNPs in three nAChR subunits during cervical carcinogenesis. In addition to rs16969968 in alpha5, we concentrated on three SNPs that we identified in sequence databases for alpha9 and beta1, as alpha5, alpha9 and beta1 are among the five principal nAChR subunits that are strongly expressed in cervical cells (Calleja-Macias et al., 2009). Cervical neoplasia progresses from normal cervical epithelia through cervical precursor lesions (cervical intraepithelial neoplasia I to III) to cancer. Here we lumped all grades of precursor lesions into one category (CIN). We found statistically significant support that one of the two SNPs in alpha9 is associated with disease and that there is also a trend for a second SNP to have such an association. We could not reach a conclusive decision about two other SNPs. Our report provides additional evidence on the importance of cholinergic signaling in cervical carcinogenesis and the differential involvement of alternative nAChR subunit alleles, and points to the need for epidemiological studies enrolling larger numbers of subjects. SNPs in nAChR subunits may become useful in genomics based personalized medicine, and some of the known inhibitors of cholinergic signaling may be useful in cancer treatment protocols.

Materials and methods

Patients from Nuevo Leon, Mexico

We analyzed samples from 156 patients from the state of Nuevo Leon, Mexico. Thirty-eight samples were from patients treated for cervical cancer, 36 from patients diagnosed with CIN (lumping cervical intraepithelial neoplasia stages I–III), and 82 samples from normal smears. All patients attended clinics at the Facultad de Medicina, Universidad Autonoma de Nuevo Leon, Monterrey, Mexico. All samples were “archival”, i.e. obtained prior to this study and for diagnostic purposes unrelated to our research and permitted by the Institutional Review Board (IRB) of the Mexican institution. The patients were anonymous to the molecular biologists at the University of California Irvine, who performed the DNA

analyses. For this constellation, the UCI IRB has waived the requirement for IRB review. The same applies to the samples from Guerrero (next paragraph).

Patients from Guerrero, Mexico

We analyzed 300 patients from the state of Guerrero, Mexico. One hundred-and-one samples were from patients with cervical cancer, 100 from patients with CIN, and 99 samples from normal smears. All patients had attended clinics at the Unidad Academica de Ciencias Quimico Biologicas, Universidad Autonoma de Guerrero, Chilpancingo, Guerrero, Mexico and Instituto Estatal de Cancerologia hospital in Acapulco, Guerrero, Mexico. Most women in the populations from Nueva Leon and Guerrero are non-smokers, but we do not have individual information about the smoking habits of the specific patients studied here.

DNA samples

The DNA preparations from all 456 patients were obtained from cervical cells, namely sections from surgically removed tissue or biopsies in the case of the cancer and CIN patients, and cervical smears in the case of the control subjects. Genomic DNA was extracted with the Wizard SV Genomic DNA Purification Systems (Promega, Madison, Wisconsin).

Primers for polymerase chain amplification and nucleotide sequencing

This study addressed four SNPs of genes encoding three subunits of nicotinic acetylcholine receptors: the epidemiologically well studied SNP rs16969968 (alpha5, G>A) (Hung et al., 2008), and three SNPs that we identified in sequence data bases: rs10009228 (alpha9 A>G), rs55633891 (alpha9 C>T), rs17856697 (beta1, A>G) (Database of Single Nucleotide Polymorphisms, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=10009228). The PCR reactions used 100 ng DNA and *Go Taq* polymerase (Promega, Madison, Wisconsin). Genomic segments of exon 5 of the CHRNA5 gene were amplified with the primers SNPNa5F, 5'-TGTAATCAAGCGCCTGCCTCTC-3', and SNPNa5R, 5'-TCAACAACCTCACGGACATCATTT-3', which generated a 521 bp segment. Primer sequences for exon 5 of the CHRNA9 gene were a9ex5F, 5'-TAAATACTACATAGCCACGAT-3' and A9EX5R, 5'-TACCCAGATAGCAAAGCCAATA-3' (569 bp). We used primers B1ex2snpF: 5'-CGCCGCTCGCCCAGGTAAGTGTA-3' and B1ex2snpR: 5'-TGATGAGTTGCGCCAGGATGAGACCA-3' to amplify 234 bp of the CHRNB1 gene. The PCR amplicons were electrophoretically separated, purified with Exo I and alkaline phosphatase (USB, Cleveland, Ohio), and both strands sequenced with the same forward and reverse primers as used for PCR amplification with an ABI Prism 96 capillary 3730xl analyzer. The mutations were analyzed by the ALIGN program at the GENESTREAM network server (<http://www2.igh.curs.fr/bin/align-guess.cgi>).

Statistics

Allele frequencies were compared across the three diagnostic categories (cancer, CIN, control) using a chi-square test for linear trend. Allele frequencies were also compared

between cervical cancer cases and subjects with no positive cytologic diagnosis using a Pearson chi-square or Fisher's exact test.

Results

Alpha5-SNP rs16969968

This SNP could be sequenced in 443 samples. Table 1 summarizes the distribution of the normal allele (G), the SNP (A), and heterozygous carriers (G/A) among 148 cancer cases, CIN lesions and normal smears in patients from Nuevo Leon and 295 individuals from Guerrero. The chi-square test for trend across diagnostic categories (control to CIN to cancer) for frequencies of A or G/A versus G showed a non-significant trend of the alpha5 A allele occurring with higher frequency in cancer tissue ($p=0.31$ for the combined data). When comparing allele frequencies between controls and cancer cases alone, results showed an increase for the alpha5 A allele in cancer tissue ($p=0.35$). The analysis suffered from the low number of homozygous carriers of the A allele (9 out of 443) and inconsistency between the two data sets.

Alpha9-SNP rs55633891

Table 2 documents the distribution of the normal allele (C), the SNP (T), and the heterozygous carriers of rs55633891 in alpha9 in 420 samples. The low prevalence of the T allele (5 out of 420) reduced the power of the analysis. The test for trend across diagnostic groups for frequency of T or C/T vs. C indicated no significant differences for the combined data sets ($p=0.81$). Data from Nuevo Leon and Guerrero were not consistent, as the Nuevo Leon data suggested a higher frequency for the C allele in cancer cases compared to controls ($p=0.01$), whereas no difference in allele frequencies was found in the Guerrero population ($p=0.32$) suggesting that there is no real difference between normal and cancer tissue.

Alpha9-SNP rs10009228

Table 3 shows the analysis of the normal allele (A), the SNP (G), and the heterozygous carriers for rs10009228 in the alpha9 subunit. The table shows substantial representations of samples in all nine pathological and molecular categories (422 samples total). These data revealed a significant trend for an increase in the G allele frequency compared to A or A/G alleles in CIN and cancer cases in Guerrero ($p=0.028$) or combined cohort ($p=0.045$).

Beta1-SNP rs17856697

The A allele of the SNP rs17856697 of the beta1 subunit occurred in homozygous form in 87, 75, and 78 of all cancers, CINs and normal samples, respectively, and heterozygous in 14, 21, and 19 samples of these categories. The G allele was present only in two CIN and two normal samples. While altogether the A allele was higher in the cancer samples than in normal smears ($p=0.19$ for trend; $p=0.20$ for cancer vs. control), there was no significant association with a risk of neoplastic progression (data not shown).

Discussion

Public databases catalog a large number of SNPs in the genes for the 16 nAChR subunits. The cancer relevance of only few of these has been studied in population-based research. In order to address the question whether nAChR SNPs might differentially affect cervical carcinogenesis, we selected SNPs (i) that occur in the five nAChR subunits predominantly expressed in cervical cancers (alpha5, 7, 9, beta1, and epsilon), and (ii) where the rarer allele occurred in studied populations with a prevalence of at least 5%. These considerations led to the selection of the four SNPs rs16969968 (alpha5, G>A), rs10009228 (alpha9 A>G), rs55633891 (alpha9 C>T), rs17856697 (beta1, A>G).

Different alleles of any gene including nAChR subunit genes occur with different frequencies in different ethnic groups (Shiraishi et al., 2009). Therefore, we measured the frequencies of these SNPs in both Mexican cohorts in cases as well as controls, as frequencies established elsewhere do not necessarily apply to these ethnically mixed Caucasian–American Indian populations. We reported the data of both Mexican cohorts separately as well as in the combined cohorts, as they are living about 1000 km apart and allele frequencies may differ due to different ethnic compositions.

Our findings provide evidence of a risk associated with a SNP in the gene encoding the alpha9 subunit, as we measured a significant increase in the G allele frequency of rs10009228 compared to A or A/G alleles in CIN and cancer cases in the Guerrero and combined cohorts with a consistent trend detected in the Nuevo Leon cohort. Our observation is strengthened by the recent findings of the group of one of us (S. A. G.) that the alpha9 subunit with this SNP induces the proliferation and transformation of bronchial cells more strongly than the normal allele (Chikova and Grando, 2011). Also, mechanistic studies that investigated the alpha9 receptor (with the prototype sequence, but not inquiring about differential function linked to its SNP diversity) could demonstrate an important role of alpha9 in carcinogenesis of the breast, including synergies with estrogen signaling (Lee et al., 2010, 2011). In our study, another SNP in alpha9, rs55633891, was not associated with disease. Its evaluation was problematic due to the low prevalence of the T allele. The well-studied SNP rs16969968 in alpha5 subunit gene showed a non-significant association with disease. While the suggested involvement of this SNP as risk factor in lung and H&N cancers made it an important target of our cervical cancer study, the low prevalence of homozygous carriers of the A allele did not allow statistical confirmation of this trend. Lastly, neither allele of SNP rs17856697 of the beta1 subunit was significantly associated with disease.

While high-risk HPV types are a necessary cause of cervical cancer, the search for factors that determine the outcome between regressing and progressing HPV infections is yet inconclusive. We have undertaken this study to direct research interest to cholinergic signaling as a candidate to be a genetic and environmental risk factor of this disease. Prior epidemiological and medical studies had left no doubt that components from tobacco smoke affect cervical neoplasia, and our recent mechanistic research points to cholinergic signaling as a mediator of these effects. Beyond this, acetylcholine receptors may not only affect cervical carcinogenesis after stimulation by tobacco smoking, but polymorphic receptors

may even differentially process auto- and paracrine acetylcholine signals and thereby pass thresholds that normally separate tissue homeostasis from neoplastic differentiation processes. Parnell et al. (Epub ahead of print) recently demonstrated that cancerous cervical cells express the necessary enzymes for ACh biosynthesis and therefore could participate in autocrine as well as paracrine nAChR activation. The evidence that nAChR antagonists suppress basal migration indicates that the nAChRs are activated, presumably autocrine ACh, without exogenous agonists being added. Our findings strengthen the recognition of cholinergic signaling as risk factor of cervical carcinogenesis. We suggest that nAChR genes may be useful to be included in genomic risk factor analyses, and their products could be targeted by pharmaceutical intervention.

Conclusions

Cancer of the cervix uteri is initiated by infection with papillomaviruses and stimulated by cholinergic signaling through nicotinic acetylcholine receptors that are targeted by nicotine and likely also by autocrine pathways. Genomic diversity of nAChR subunits by SNPs apparently contributes to an individual's risk affected by this component of cervical carcinogenesis.

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Table 1

The alpha5-SNP rs16969968.

	G		G/A		A		Total	
	N	%	N	%	N	%	N	%
N. Leon	Control	55	74.3	18	24.3	1	1.4	74
	CIN	24	66.7	11	30.6	1	2.8	36
	Cancer	25	65.8	10	26.3	3	7.9	38
Chi-square test for trend across diagnostic groups for G vs A or G/A: p=0.31; Fisher's exact test for cancer vs. control: p=0.38								
Guerrero	Control	71	74.0	25	26.0	0	0.0	96
	CIN	84	84.0	14	14.0	2	2.0	100
	Cancer	83	83.8	14	14.1	2	2.0	99
Chi-square test for trend across diagnostic groups for G vs A or G/A: p=0.08; Fisher's exact test for cancer vs. control: p=0.11								
Combined	Control	126	74.1	43	25.3	1	0.6	170
	CIN	108	79.4	25	18.4	3	2.2	136
	Cancer	108	78.8	24	17.5	5	3.6	137
Chi-square test for trend across diagnostic groups for G vs A or G/A: p=0.31; Fisher's exact test for cancer vs. control: p=0.35								

Table 2

The alpha9-SNP rs55633891.

	C		C/T		T		Total	
	N	%	N	%	N	%	N	%
N. Leon	Control	66	80.5	16	19.5	0	0.0	82
	CIN	34	97.1	0	0.0	1	2.9	35
	Cancer	37	97.4	1	2.6	0	0.0	38
Chi-square test for trend across diagnostic groups for C vs T or C/T: p=0.003; Fisher's exact test for cancer vs. control: p=0.01								
Guerrero	Control	67	77.9	18	20.9	1	1.2	86
	CIN	81	88.0	9	9.8	2	2.2	92
	Cancer	62	71.3	24	27.6	1	1.1	87
Chi-square test for trend across diagnostic groups for C vs T or C/T: p=0.28; Fisher's exact test for cancer vs. control: p=0.32								
Combined	Control	133	79.2	34	20.2	1	0.6	168
	CIN	115	90.6	9	7.1	3	2.4	127
	Cancer	99	79.2	25	20.0	1	0.8	125
Chi-square test for trend across diagnostic groups for C vs T or C/T: p=0.81; Fisher's exact test for cancer vs. control: p=1.00								

Table 3

The alpha9-SNP rs10009228.

	A		A/G		G		Total	
	N	%	N	%	N	%	N	%
N. Leon	Control	17	20.7	35	42.7	30	36.6	82
	CIN	7	20.0	13	37.1	15	42.9	35
	Cancer	7	18.4	14	36.8	17	44.7	38
Chi-square test for trend across diagnostic groups for G vs A or G/A: p=0.37; Fisher's exact test for cancer vs. control: p=0.43								
Guerrero	Control	13	14.9	53	60.9	21	24.1	87
	CIN	20	21.7	39	42.4	33	35.9	92
	Cancer	14	15.9	39	44.3	35	39.8	88
Chi-square test for trend across diagnostic groups for G vs A or G/A: p=0.028; Fisher's exact test for cancer vs. control: p=0.035								
Combined	Control	30	17.8	88	52.1	51	30.2	169
	CIN	27	21.3	52	40.9	48	37.8	127
	Cancer	21	16.7	53	42.1	52	41.3	126
Chi-square test for trend across diagnostic groups for G vs A or G/A: p=0.045; Fisher's exact test for cancer vs. control: p=0.050								