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Polymorphisms in the promoter region of neutrophil elastase gene and lung cancer risk

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Summary

The neutrophil elastase (NE) gene encodes a powerful serine protease that is involved in the process of normal tissue turnover, natural host defense or tissue damage in acute and chronic inflammatory disorders. Furthermore, NE was suggested as one of the determinant factors of individual susceptibility to lung cancer resulting from imbalance between α 1-antitrypsin (AT) and NE. To determine whether NE plays a role in risk for lung cancer, we screened polymorphisms in the promoter region of the NE gene and assessed the role of the NE polymorphisms in the risk for lung cancer. We confirmed three previously identified polymorphisms which are located at –903, –741, and extra 52 bp STS relative to the transcription initiation site. In addition, two new polymorphisms at –832 (G/T) and –789 (C/T) were identified. Their rare allelic frequencies of new polymorphism are 0.02 and 0.01, respectively, among Caucasians. The prevalence of the NE –903 (T/T) and (T/G) genotypes were 0.88 and 0.12 in controls as compared to 0.96 and 0.04 in lung cancer patients using genomic DNA isolated from 113 Caucasian lung cancer cases and 131 controls. A significant increase in lung cancer risk was observed for expected high NE activity genotypes (OR = 3.2, 95% CI = 1.02–10.3) as compared to low NE activity genotypes. These results were consistent with previous in vitro functional analysis, which reported an approximately two-fold increase enzyme expression with the –903T/–741G allele as compared to the –903G/–741A variant. These results confirm that the NE promoter region polymorphisms may influence in risk for lung cancer.

Keywords

Neutrophil elastase; Lung cancer; Genetic polymorphism; Cancer susceptibility

1. Introduction

Lung cancer is the leading cause of cancer mortality in many developed countries. Cigarette smoking constitutes 80% of the attributable risk in lung cancer. There is significant interest in the potential to predict the small proportion (10%) of smokers who will develop lung cancer as a means to focus on their behavior modification, early detection, and chemoprevention of possible cancer development.

Genetic variation in pathway of protease–protease inhibitor imbalance may be one of the reasons for inter-individual variation among smokers susceptible to lung cancer. Large

differences in the activities of enzymes involved in protease imbalance have been observed among individuals, and it has been shown that enzyme activity-altering polymorphisms may influence individual cancer risk[1,2].

Neutrophil elastase (NE) is a protease which has potent catalytic activity against a broad array of extra-cellular matrix substrates, including the highly resistant elastin that imparts structural stability to the lung tissue [3]. The role of NE in the pathogenesis is controversial because NE is involved in normal host defense or tissue turnover and harmful self-destruction. α 1-Antitrypsin (AT) is a glycoprotein known to inactivate a wide variety of protease including NE. α 1-AT deficiency individuals have a higher risk for lung diseases [1,4,5]. An imbalance between protease and protease inhibitor activity by increasing NE production and decreasing α 1-AT activity in lung tissue is caused by smoking [6] and this imbalance can cause damage to lung tissue. A previous study suggests that individuals with elastolytic damage in lung tissue may have a higher risk for lung cancer than those with normal lung tissue [1]. NE was associated with aggressiveness of the tumor, suggesting its involvement in the invasion of the tumor and association with poor prognosis in non-small cell lung cancer [7].

Taniguchi et al. [2] screened 30 normal DNA samples to identify polymorphisms in the entire coding region and the core expression control region of the NE gene. Among the four polymorphisms that were identified, two polymorphisms (–903 and –741) in the promoter region were associated with lung cancer risk.

The goals of the present study are to identify new polymorphisms in the promoter region of NE gene and determine whether they may be associated with increased risk for lung cancer.

2. Material and methods

2.1. Study populations

To investigate the role of NE polymorphisms in lung cancer risk, subjects were recruited from the H. Lee Moffitt Cancer Center (Tampa, FL). All cases were patients diagnosed with primary lung cancer and were identified between 2001 and 2003. All cases were diagnosed within 1 year prior to recruitment into the study and were histologically confirmed by the Pathology Department at the H. Lee Moffitt Cancer Center. Among total 620 eligible lung cancer patients, 95% (113/119) of case subjects who were asked to participate in the study consented.

We recruited control subjects at the Lifetime Cancer Screening Center affiliated with the H. Lee Moffitt Cancer Center. All control subjects were recruited after an initial screening to determine that they had no previous diagnosis of cancer, and none of the controls recruited into this study were diagnosed with any form of cancer or premalignancy after screening. The control subjects were matched with the same age at diagnosis (± 5 years), and race as the case subjects. Eighty-three percent (131/158) of the control subjects who were asked to participate in the study consented.

A questionnaire that covered demographics and life-long smoking habits was administered to all study subjects. Tobacco use was categorized into pack-years (py) for smokers of cigarettes (1 py equals one pack of cigarettes per day for 1 year). Study subjects who have smoked 100 or fewer cigarettes in their lifetime (the equivalent of 0.014 or fewer py) were categorized as never-smokers.

2.2. PCR-sequencing analysis of the NE gene

A peripheral blood specimen was obtained from all subjects. Genomic DNA was isolated from blood from subjects by incubation overnight with protease K (0.1 mg/ml) in 1% sodium dodecyl sulfate at 50 °C and was extracted with phenol:chloroform and ethanol precipitation as previously described [8]. Protocols involving the analysis of blood specimens were approved by the institutional review board at the H. Lee Moffitt Cancer Center and informed consent was obtained from all subjects.

DNA extracted from 244 subjects was screened for the presence of the polymorphisms in the promoter region of the NE gene by using a PCR-direct sequencing analysis. The core expression control promoter region of the NE gene was PCR-amplified using 100 ng NE sense (5'-GGA AGG ACC AGA GAA GTG C-3') and antisense (5'-CTG CCA AAC CTA GAC CTG AG-3') primers to generate a 397 bp fragment. The standard PCR was performed in a 50- μ l reaction volume containing 50 ng of genomic DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each of the dNTPs, and 2.0 units of Taq polymerase (Eppendorf). The reaction mixtures underwent the following incubations: one cycle of 95 °C for 2 min, 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s, followed by a final cycle of 10 min at 72 °C. The PCR-amplified NE products were directly sequenced in the Molecular Biology Core Facility at the H. Lee Moffitt Cancer Center.

2.3. Statistical analyses

The risks of lung cancer in relation to NE genotypes were estimated using χ^2 -test and unconditional logistic regression to calculate odds ratios (ORs) and 95% confidence intervals (CIs) after with/without adjusting for the following factors: age, sex, and smoking. The χ^2 -test was used for the analysis of allelic prevalence and for a deviation of genotype distribution from the Hardy-Weinberg equilibrium. The allelic frequencies were calculated from the observed genotype frequencies assuming that alleles combine at random according to their frequencies in the set during zygote formation (the assumption being analogous to the Hardy-Weinberg equilibrium). This assumption was verified by a reversed calculation of the expected genotype frequencies from the calculated allelic frequencies. In this study, subjects were categorized into three groups based on the predicted activity of their NE genotype as described previously: the low NE activity genotype (-903TG), intermediate NE activity genotypes (-903TT/-741AG and -903TT/-741AA) and high NE activity genotypes (-903TT/-741GG). The Student's *t*-test (two-tailed) was used for comparing the continuous variable between cases and controls. The statistical computer software SAS (Version 8.0) was used to perform all statistical analyses. All statistical tests were two-sided.

3. Results

A total of 244 Caucasian lung cancer patients and control subjects matched by age at diagnosis, and race were entered into this study. The mean ages of cases and controls were 63 and 64 and approximately 30% of subjects were female (Table 1). The smoking level among controls is similar to cases ($p = 0.83$), but the proportions of never and former smokers among controls are higher than cases ($p = 0.003$; Table 1).

PCR-amplified fragments of NE promoter region of genomic DNA samples from 244 subjects were sequenced for identifying new polymorphisms in the six repetitive tandem motif of the promoter region and for genotyping known polymorphisms in NE gene (Fig. 1). Table 2 shows the location and frequencies of five polymorphisms identified in the samples we analyzed. Among the five polymorphisms we observed, three of them were identified previously [2].

The allelic frequencies of NE -903 (T > G), -741 (G > A), and extra 52 bp polymorphic variants, located from the transcription initiation site, were similar (0.06, 0.27, and 0.01) to those observed in a previous study (0.07, 0.26, and 0.02) [2]. The allelic frequencies for two new polymorphisms of -832 (A > T) and -789 (C > T) among controls were 0.02 and 0.01, respectively. The prevalence of these polymorphisms among controls followed Hardy-Weinberg equilibrium. To determine whether the NE variants contributed to increased lung cancer risk, we examined the frequencies of NE genotypes in lung cancer patients and compared them with control subjects.

Significantly increased risk for lung cancer was observed for subjects with the -903TT genotype (OR = 3.2, 95% CI = 1.03–10.4; Table 2). No significant association between other polymorphisms and lung cancer risk was observed (Table 2). Genotypes of individual subjects were determined by the combined data obtained from analysis of the -903 and -741 polymorphisms. Due to low allelic frequencies, the remaining three polymorphisms were not included for determining combined genotypes. When subjects were stratified based upon predicted NE activity genotypes, a significant increase in lung cancer risk was observed among subjects with either intermediate or high NE activity genotypes as compared to the low NE activity genotype (OR = 3.2, 95% CI = 1.02–10.3) and a near significant increase in lung cancer risk was observed when analyzed separately (intermediate: OR = 3.3, 95% CI = 0.99–10.9; high: OR = 3.2, 95% CI = 1.0–10.8; Table 3).

To examine the association between NE genotypes and lung cancer risk by exposure to the environmental risk factor, smoking, we stratified study subjects by predicted NE activity genotypes and smoking status (Table 3). Ever-smokers (i.e. 100 cigarettes lifetime) were categorized into two groups based upon lifetime smoking history and divided at the median number of pack-years (52 py) of smokers in the control population. The differences in risk associated with the NE genotypes were not modified by smoking history. Due to a low number ($n = 6$) of case subjects, we cannot assess the role of NE polymorphisms among never-smokers (data not shown). Although no significant association between NE genotypes and lung cancer risk was observed in both light and heavy smokers, there were similar trends in both level of smokers. Non-significant risk increases were observed in ever-smokers with either the intermediate or high NE activity genotypes (OR = 2.8, 95% CI = 0.9–9.2).

To assess whether NE genotypes associated lung cancer risk were linked to lung cancer subtypes, cases were stratified according to tumor histological classifications. Non-significant risk increase was observed in the all histological types of non-small cell lung cancer (data now shown). Small cell carcinoma cases were excluded from this analysis, due to a low number ($n = 6$) of subjects.

4. Discussion

Genetic polymorphisms in the genes coding for tobacco carcinogen metabolizing enzymes may influence individual susceptibility to lung cancer. Although neutrophil elastase induces tissue turnover or normal host defense, which are generally considered to represent a beneficial reaction, excessive NE production generated tissue damage in lung tissue leads to susceptibility for lung cancer. During inflammation, neutrophils release elastase, a serine protease capable of cleaving a wide range of substrate, including most of the major protein of connective tissues [9,10]. This mature 218 amino acid glycoprotein has a critical pathophysiological role in a variety of pulmonary disease, including lung cancer [11]. Recently, several studies reported association between mutations in the NE gene and two rare genetic diseases, cyclic neutropenia and severe congenital neutropenia [12–19]. There are 25 single nucleotide polymorphisms (SNPs) listed in the National Center for

Biotechnology Information (NCBI)/SNP database (November 10, 2004), 13 of them are validated. Among 25 SNPs, eight of which are in the intron region and 17 of which are in the locus regions. Except polymorphisms at –903 and –741, allelic frequency and functional effects for these SNPs were not investigated [2].

A few previous molecular epidemiological studies have been performed on protein imbalances in lung cancer risk. Taniguchi et al. [2] reported a significant increase in lung cancer risk for the polymorphisms –903 and –741 and a stronger association with combined genotypes. Yang et al. [1] suggested that individuals who have the α 1-AT deficiency allele have increased lung cancer risk. These results are consistent with functional analysis of transcription activity. This imbalance in lung tissue caused by smoking may stimulate neutrophils to secrete more elastase [6] and inactivate α 1-AT and could induce damage of lung tissue, thereby creating a favorable environment for carcinogenesis. Therefore, NE may play a significant role in the protease imbalance-induced carcinogenic process.

In this study, we observed a significant association between –903 NE polymorphism and lung cancer risk and between predicted NE activity genotypes and lung cancer risk. Similar trend was observed in patients with all histological types of the non-small cell lung cancers. These results are consistent with previous study and are biologically plausible.

In addition to lung cancer, chronic obstructive pulmonary disease (COPD), another smoking-related disease, is also linked with the protease imbalance. Recently, Shapiro et al. [20] reported that a direct role for NE in emphysema and interdependence of the proteases and inflammatory cells that mediate lung destruction in response to smoke. However, the role of NE polymorphisms in risk for pulmonary obstruction is not yet investigated. Since this disease is associated with smoking and an increased four- to six-fold risk for lung cancer [21], it would be interesting to investigate the role of NE polymorphisms in lung cancer risk among subjects who have pulmonary obstructive disease.

Strength of our study is the use of controls matched by smoking level to remove a potential confounding effect. A limitation of this study is the fact that the number of subjects recruited into this study was relatively small, especially for low NE activity genotypes. Another limitation is potential selection bias of subjects, which is commonly encountered in hospital based epidemiological studies [22]. Therefore, these results must be confirmed in larger studies, especially for investigating the role of low NE activity genotypes in lung cancer risk.

In conclusion, the present study has identified new polymorphisms in the promoter region of the NE gene, observed that the NE –903 polymorphism may increase lung cancer risk and that predicted high NE activity genotypes are associated with increased risk for lung cancer.

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Fig. 1.

Location of polymorphisms in the promoter region of neutrophil elastase gene. Sequences show the results from individuals who have heterozygous genotypes for each polymorphism at -903 (T/G), -832 (G/T), -789 (C/T), and -741 (A/G). Rep, repetitive sequence 53 bp; numbers indicate locations from the transcription initiation site.

Table 1

Selected characteristics of study subjects and comparison between cases and controls

	Cases	Controls	<i>p</i> -Value
<i>N</i>	113	131	
Sex (M/F)	73/40	93/38	0.29
Mean age (range)	64 (36–83)	63 (35–80)	0.48
<50 years old	11 (10%)	5 (4%)	
50–59 years	21 (19%)	37 (28%)	
60–69 years	45 (39%)	56 (43%)	
70–79 years	33 (29%)	32 (24%)	
>80 years old	3 (3%)	1 (1%)	
Non-smoker	6/100 (5%)	10/130 (8%)	0.003
Former smoker	41/100 (41%)	80/130 (62%)	
Current smoker	53/100 (53%)	40/130 (30%)	
Smoking (py) mean±S.D.	52.4±27.8	51.5±31.5	0.83
Histology cell type ^a			
Adenocarcinoma	36 (35%)		
Squamous cell carcinoma	34 (33%)		
Large cell carcinoma	22 (22%)		
Small cell	6 (6%)		
Other	4 (4%)		

^a Available for 102 cases.

Table 2

Frequencies of NE genotypes and risk for lung cancer

Location	Genotypes	Controls	Cases	Crude OR (95% CI)	Adjusted OR (95% CI) ^a
-903 (T/G)	TT	110 (86) ^b	96 (96)	3.3 (1.05–10.2)	3.2 (1.03–10.4)
	TG	15 (12)	4 (4)	1.0 (referent)	1.0 (referent)
	GG	0 (0)	0 (0)	na	na
-741 (G/A)	GG	69 (60)	60 (59)	1.0 (referent)	1.0 (referent)
	GA	30 (26)	24 (24)	0.9 (0.5–1.7)	0.9 (0.5–1.7)
	AA	16 (14)	17 (17)	1.2 (0.6–2.6)	1.2 (0.6–2.7)
-832 (A/T)	AA	122 (97)	97 (97)	1.0 (referent)	1.0 (referent)
	AT	3 (2)	3 (3)	1.3 (0.2–6.4)	1.4 (0.3–7.2)
	TT	1 (1)	0 (0)	na	na
-789 (C/T)	CC	115 (99)	99 (99)	1.0 (referent)	1.0 (referent)
	CT	2 (2)	1 (1)	0.6 (0.1–6.5)	0.6 (0.1–6.7)
	TT	0 (0)	0 (0)	na	na
Extra 52 bp	Wild	126 (99)	101 (99)	1.0 (referent)	1.0 (referent)
	Hetero	1 (1)	1 (1)	1.2 (0.1–20.2)	1.3 (0.1–21.8)
	Polymorphic	0 (0)	0 (0)	na	na

na: not available.

^aAdjusted for age, sex, and smoking.^bNumbers in parenthesis refer to percentages.

Table 3

Frequencies of expected NE phenotypes and risk for lung cancer with stratification by smoking

	Phenotypes ^a	Controls	Cases	Crude OR (95% CI)	Adjusted OR (95% CI) ^b
Total	Low	14 (12) ^c	4 (4)	1.0 (referent)	1.0 (referent)
	Intermediate	42 (37)	40 (40)	3.3 (1.01–11.0)	3.3 (0.99–11.1)
	High	59 (51)	56 (56)	3.3 (1.03–10.7)	3.2 (1.0–10.8)
Heavy smoker (52 py or more)	Low	8 (14)	3 (6)	1.0 (referent)	1.0 (referent)
	Intermediate	24 (43)	17 (15)	1.9 (0.4–8.2)	1.6 (0.4–7.2)
	High	24 (43)	29 (59)	3.2 (0.8–13.5)	3.0 (0.7–12.6)
Light smoker (less than 52 py)	Low	4 (8)	1 (2)	1.0 (referent)	1.0 (referent)
	Intermediate	17 (33)	21 (46)	4.9 (0.5–48.4)	6.3 (0.6–70.0)
	High	31 (59)	24 (52)	3.1 (0.3–29.5)	4.1 (0.4–44.2)

^aSubjects were categorized into three groups based on the predicted activity of their NE genotype as described previously: the low NE activity genotype (–903TG), intermediate NE activity genotypes (–903TT/–741AG and –903TT/–741AA) and high NE activity genotypes (–903TT/–741GG).

^bAdjusted for age, sex, and smoking.

^cNumbers in parenthesis refer to percentages.