

Association between -276 C/T Polymorphism of the *ERBB3* Gene and Lung Cancer Risk in a Korean Population

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Abstract. *Background/Aim:* The associations between the polymorphisms in the promoter region of the V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (*ERBB3*) gene and lung cancer risk were investigated. *Materials and Methods:* By direct sequencing, we first found two known polymorphisms (-536 A/G and -276 C/T). Further TaqMan assays and logistic regression analyses were performed in order to characterize the association between the -276 C/T polymorphism of *ERBB3* and lung cancer risk in 425 patients with lung cancer and 411 healthy controls. To examine the potential effects of the -276 C/T polymorphism on *ERBB3* transcription, luciferase reporter assays were performed in non-small cell lung cancer (NSCLC) cell lines. *Results:* The -276 C/T polymorphism was associated with the risk of lung cancer in a recessive model of never-smokers. Interestingly, the T allele conferred significantly lower promoter activity by 32% and 33% than the C allele in H2009 and H358 cell lines. *Conclusion:* *ERBB3* promoter polymorphisms affect *ERBB3* gene expression, and contribute to genetic susceptibility to lung cancer in never-smokers.

Lung cancer is a leading cause of cancer-related death in Korea, and its incidence continues to rise (1). Tobacco smoking plays a critical role in the development of lung cancer, but only 10-15% of all smokers develop lung cancer

(2, 3). Although a significant fraction of lung cancer cases among never-smokers may also be attributable to tobacco, many such cancers arise in the absence of detectable tobacco exposure, and thus the pathogenesis of lung cancer in these patients may follow different cellular and molecular pathways (4, 5).

Lung cancer is a multicellular, multistage process that involves a number of genetic changes in oncogenes and tumor suppressor genes. Single-nucleotide polymorphisms (SNPs) are the most common form of human genetic variation, and may contribute to an individual's susceptibility to cancer (6, 7). Several studies have demonstrated that polymorphisms may affect gene expression and survival, and have suggested possible associations with the risk of lung cancer (8, 9). The recently introduced targeted-agents exhibit a different response according to tumor histological subtypes, and the efficiencies of the treatment modalities for lung cancer depend on the time of diagnosis (10). Accordingly, there is a great need for rapid and efficient early-detection methods, and in order to develop improved molecular biomarkers for the early detection and prediction of response to chemotherapy, it is important to identify genetic alterations, specific to each subtype of lung cancer.

V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (*ERBB3*) is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases. EGFR is known to promote cell growth and to function as an oncogene and is expressed in up to 80% of non-small cell lung cancer (NSCLC) cases (11). *ERBB3* is unique among EGFR family members in that it does not have any intrinsic kinase activity (12). Therefore, *ERBB3* is active only as a heterodimer complex, and *ERBB2*-*ERBB3* heterodimer is the most effective complex for activation of the downstream pathway (13, 14). The overexpression of *ERBB3* has been reported in lung cancer and has been associated with a poor prognosis (15, 16). Furthermore, *ERBB3* protein expression has been

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associated with distant metastasis in lung cancer (17), and more recently, an association was reported between a polymorphism of *ERBB3* and schizophrenia (18, 19). However, the correlation between polymorphisms of *ERBB3* and the risk of lung cancer have not been clarified.

This study was conducted to examine relations between the -276 C/T polymorphism of the *ERBB3* promoter region and the risk of lung cancer in a Korean population.

Materials and Methods

Study subjects. Between August 2001 and August 2010, blood samples were collected from 836 participants, that is, 425 patients with lung cancer and 411 healthy controls without cancer. Patients were recruited from the patient pool at the Genomic Research Center for Lung and Breast/Ovarian Cancers and Seoul National University Medical Center, and controls were randomly selected from a pool of healthy volunteers who had visited the Cardiovascular Genome Center, Genomic Research Center for Allergy and Respiratory Diseases and Seoul National University Medical Center. Detailed information on smoking status, lifestyle, and medical history were collected by a trained interviewer using a structured questionnaire. All study participants provided written consent and were ethnic Koreans; the Institutional Review Boards of each institution approved the study protocol.

Preparation of genomic DNA and direct sequencing. Genomic DNA was extracted from peripheral blood samples using Puregene blood DNA kits (Gentra, Minneapolis, MN, USA), following the manufacture's protocol.

To identify polymorphisms in the promoter of the *ERBB3* gene, human genomic DNA was isolated from the whole blood of 24 patients with lung cancer for direct sequencing, and ~2 kb of the promoter of the *ERBB3* gene at 12q13 were amplified. Polymerase chain reaction (PCR) amplifications were performed in a PTC-225 Peltier Thermal cycler (MJ Research Inc., Waltham, MA, USA) using AmpliTagGold (Roche, Branchburg, NJ, USA). All amplifications were performed using 35 cycles of 30 s at 95°C, 1 min at 64°C and 1 min at 72°C, followed by a single 10-min extension at 72°C. PCR products were purified using a Montage PCR96 Cleanup kit (Millipore, Bedford, MA, USA) and eluted in 20 µl of nuclease-free H₂O. DNA cycle sequencing was carried out using a BigDye Terminator V 3.1 Cycle Sequencing kit (Perkin Elmer, Foster City, CA, USA). For dye terminator removal, Multiscreen SEQ 384-well filter plates were used for dye removal, and sequences were then analyzed on an Applied Biosystems 3700 DNA analyzer (ABI, Foster City, CA, USA). All polymorphisms and sequence alignments were analyzed using Polyphred, which is freely available for academic use.

Genotyping. Genotyping of the -276 C/T polymorphism was performed using the TaqMan fluorogenic 5' nuclease assay (ABI, Foster City, CA, USA) with the following primers and probes: Forward primer sequence, GGCGGTCCGAGGCT; reverse primer sequence, AGAGAGAGAGGGAGGGAGGAA; 4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein (VIC) probe, CGCAATCCCCACTCCA; and 6-carboxyfluorescein (FAM) probe, CCGCAATCCCTACTCCA.

The PCR was conducted in a 5-µl mix, containing 10 ng of genomic DNA, 2.5 µl TaqMan Universal PCR Master Mix, and

Table I. Characteristics of the study subjects.

Variable	Cases (n=425)	Controls (n=411)	p-Value
Age (years)	60.76±10.17	59.63±10.75	0.085
Gender			
Male	307	268	0.036
Female	118	143	
Smoking status			
Smoker	292	127	<0.001
Never-smoker	119	191	
Stage			
1-3a	77		
3b-4	258		
Other	57		
Cell type			
Adenocarcinoma	178		
Squamous carcinoma	123		
Other carcinoma	99		

Other carcinoma includes small cell, large cell, and mixed cell carcinomas or undifferentiated carcinomas.

0.13 µl of 40× Assay Mix. Thermal cycle conditions were as follows: 50°C for 2 min (to activate the uracil N-glycosylase and prevent carry-over contamination), 95°C for 10 min (to activate the DNA polymerase), followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. PCR was performed using 384-well plates, using a Dual 384-Well GeneAmp PCR System 9700 (ABI) and end-point fluorescent readings were obtained using an ABI PRISM 7900 HT Sequence Detection System (ABI). Duplicate samples and negative controls were included to ensure the accuracy of genotyping.

Plasmid construct for the *ERBB3* promoter assay. To examine the potential effects of the -276 C/T polymorphism on *ERBB3* transcription activity, the promoter activities of the C and T alleles were compared. The fragments of the *ERBB3* promoter region (from -664 the +87, translation start site of exon 1 counted as +1) were synthesized by PCR using gDNA with the following primers: forward, 5'-TGGGTGGATGAATTATGGG-3'; and revers, 5'-CTTACCTGCCTGAGAGTTGCC-3'. The PCR products were inserted upstream of the luciferase gene in pGL3-basic plasmid (Promega, Madison, WI, USA). The structure of each construct was verified by sequencing.

Transient transfection and luciferase reporter assay. The NCI-H2009 and NCI-H358 NSCLC cell lines used in this study were purchased from the American Type Culture Collection (ATCC). Cells were plated at a density of 1×10⁵ cells/35 mm in 6-well plates one day before transfection. When cells reached 75% of confluence, they were transfected with 1 µg DNA using 3 µl of Lipofectamine™ 2000 reagent (Gibco BRL, Rockville, MD). The pGL3-basic vector, which lacks both promoter and enhancer, was used as a negative control in each of the transfection experiments. The pGL3-control vector (Promega), containing SV40 promoter and enhancer, was used as a positive control. One hundred nanogram of β-galactosidase luciferase plasmid (Promega) was included in each transfection to check for transfection efficiencies. Cells were collected 48 h after transfection and lysed using 5× lysis buffer (Promega). Luciferase activities were determined using the

Table II. Association between -276 C/T polymorphism and the risk of lung cancer.

Group	Genotype	Cases (%)	Controls (%)	Dominant		Recessive		Co-dominant	
				OR (95% CI) ^a	<i>p</i> -Value ^a	aOR (95% CI) ^a	<i>p</i> -Value ^a	OR (95% CI) ^a	<i>p</i> -Value ^a
Overall*	CC	275 (64.71)	253 (61.56)						
	CT	132 (31.06)	121 (29.44)	0.78 (0.57-1.08)	0.136	0.53 (0.27-1.04)	0.065	0.78 (0.60-1.01)	0.055
	TT	18 (4.24)	37 (9)						
Male [†]	CC	204 (66.45)	169 (63.06)						
	CT	87 (28.34)	70 (26.12)	0.70 (0.46-1.06)	0.088	0.63 (0.29-1.36)	0.244	0.75 (0.54-1.03)	0.073
	TT	16 (5.21)	29 (10.82)						
Female [†]	CC	71 (60.17)	84 (58.74)						
	CT	45 (38.14)	51 (35.66)	0.90 (0.53-1.52)	0.691	0.30 (0.06-1.51)	0.144	0.82 (0.52-1.29)	0.394
	TT	2 (1.69)	8 (5.59)						
Smoker [‡]	CC	192 (65.75)	77 (60.63)						
	CT	83 (28.42)	41 (32.28)	0.78 (0.48-1.17)	0.198	0.91 (0.38-2.15)	0.827	0.82 (0.58-1.17)	0.273
	TT	17 (5.82)	9 (7.09)						
Never-smoker [‡]	CC	77 (64.71)	115 (60.21)						
	CT	41 (34.45)	61 (31.94)	0.79 (0.48-1.27)	0.327	0.11 (0.02-0.87)	0.037	0.70 (0.46-1.05)	0.086
	TT	1 (0.84)	15 (7.85)						

^aORs and *p*-values were calculated by logistic regression and adjusted for *age, gender and smoking status, [†]age and smoking status, [‡]age and gender. Logistic regression models were used to calculate the aORs, 95% confidence interval (CIs) and the corresponding ^a*p*-values of co-dominant (minor allele homozygotes vs. heterozygotes vs. major allele homozygotes), dominant (minor allele homozygotes + heterozygotes vs. major allele homozygotes), and recessive (minor allele homozygotes vs. heterozygotes + major allele homozygotes) models after controlling for age, gender and smoking status. Bold font is used to highlight significant findings.

Luciferase Assay System (Promega), and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), values were normalized against the activity of the β -galactosidase luciferase gene. β -galactosidase assays were performed using a luminescent β -galactosidase Detection Kit II (Clontech, Hampshire, UK), and a minimum of five independent experiments were performed for each plasmid.

Statistical analysis. Allelic frequencies, genotypic frequencies, and genotypic distribution departures from Hardy–Weinberg equilibrium for each SNP were analyzed using the chi-square test or Fisher's exact test. Statistical significance was accepted for *p*-values of <0.05. Genotype-specific risks were calculated as odd ratios (ORs) with associated 95% confidence intervals (CIs) by unconditional logistic regression analysis (SAS Institute, Cary, NC, USA) and adjusted for age, gender, and smoking status.

Results

The study population consisted of 425 patients with lung cancer and 411 normal controls. Table I shows the clinicopathological features of cases and controls. There was no difference in the median age between cases and controls. However, there were significant differences in the gender and smoking status between the two groups. These differences were controlled in the multiple logistic regression analysis with adjustments for age, gender and smoking status.

By direct sequencing of the *ERBB3* promoter region (~2 kb) in 24 samples from patients with lung cancer, we discovered two polymorphisms of the *ERBB3* promoter

region: -536 A/G and -276 C/T, and the minor allelic frequencies of these polymorphisms were 0.063 and 0.167, respectively. We focused on the -276 C/T polymorphism in the *ERBB3* promoter region, which exhibited a more than 10% minor allelic frequency. Further analyses of the -276 C/T polymorphism were then performed on 401 patients with lung cancer and 411 controls. The genotypic distributions of polymorphisms were in Hardy–Weinberg equilibrium (HWE=0.088). Association of the risk of lung cancer with the -276 C/T polymorphism of the *ERBB3* gene was analyzed (Table II). None of the genotypes of -276 C/T polymorphism were associated with the risk of lung cancer in three alternative models. However, the subsequent analysis revealed that the -276 C/T polymorphism was associated with the risk of lung cancer in never-smokers [recessive adjusted odds ratio (aOR)=0.11, 95% confidence interval (CI)=0.02-0.87, *p*-value=0.037].

In order to elucidate mechanisms responsible for the observed associations between the -276 C/T polymorphism and lung cancer, we measured promoter activities using the luciferase assay system and compared the activities of the -276 C/T polymorphism (C and T alleles) in H2009 and H358 cell lines. In H2009 cells, the T allele exhibited significantly lower promoter activity by 32.1% than the C allele (Figure 1A), and similarly, this allele also significantly reduced promoter activity in H358 cells (33%) (Figure 1B).

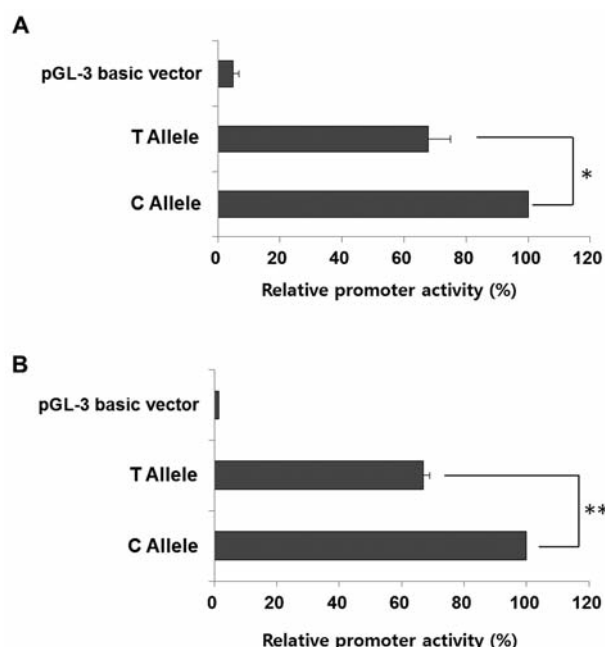


Figure 1. Comparative analysis of transcription activities using constructs of the -276 C/T polymorphism of the V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (*ERBB3*) gene. Promoter activities were measured using a luciferase reporter assay in H2009 (A) and H358 (B) cells. A minimum of five independent experiments were performed for each plasmid. The *p*-values were calculated using Student's *t*-test. **p*=0.006, ***p*<0.001.

Discussion

The *ERBB3* gene maps to human chromosome 12q13, covering 22,702 base pairs, and in normal tissues expresses a 6.2 kb transcript (20). Somatic mutations of *ERBB3* are rare in lung cancer (21, 22), but recently, an association was found between polymorphisms in the exon region of *ERBB3* gene and schizophrenia (18, 19). However, no previous study has reported associations between polymorphisms in the promoter region of *ERBB3* and lung cancer risk.

In this study, we investigated whether polymorphisms of the *ERBB3* oncogene are involved in the carcinogenesis of NSCLC by direct sequencing at the promoter region of the *ERBB3* gene in Korean patients with lung cancer. The results showed that the *ERBB3* promoter polymorphism (-276 C/T) is associated with the risk of lung cancer in never-smokers. Interestingly, our results showed that the recessive model of -276 C/T polymorphism reduced the risk of lung cancer in never-smokers, and in the luciferase reporter assays using NSCLC cell lines, the T allele exhibited a significantly decreased transcriptional activity compared to the C allele. These results suggested that the T allele is a protective allele and the C allele is risky allele in patients with lung cancer.

Polymorphisms often indicate ethnic variations. In the present study, it was found that the frequency of the minor allele -276 C/T among Korean lung cancer patients was 0.198. In comparison, the global minor allelic frequency in the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP>) has been reported to be 0.380.

The limitation of this study was the small size of the TT genotype group in never-smokers, and this small size could lead to random error. However, since this result correlated with transcriptional activity and mRNA expression of *ERBB3* gene, we thought the results had significance because they were adjusted according to multiple variables.

In conclusion, to our knowledge, this is the first study to demonstrate an association between -276 C/T polymorphism of *ERBB3* promoter and the risk of lung cancer. In particular, the T allele was found to be associated with a low risk of lung cancer in never-smokers, and with significantly lower levels of *ERBB3* transcriptional activity than the C allele. Therefore, we suggest that the -276 C/T polymorphisms of the *ERBB3* promoter play an important role in the genetic susceptibility to lung cancer in never-smokers.

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