



Multiple variants of *TERT* and *CLPTM1L* constitute risk factors for lung adenocarcinoma

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ABSTRACT. Recent studies have shown that 5p15.33 is one of the chromosomal regions that is most consistently altered in lung cancer; common variants that are located in this region have been genotyped in various populations. However, the genetic contribution of these variants to carcinogenesis is relatively unknown. A clinic-based case-control study in Shanghai was undertaken on 196 patients with lung cancer and 229 healthy individuals. *TERT* rs2736100 and *CLPTM1L* rs401681 and rs402710 were genotyped using the ABI TaqMan Allelic Discrimination assay. For rs2736100, the G variant and the GG genotype were more frequent, whereas the TT genotype was less frequent in patients with lung adenocarcinoma than in controls. The CT genotype at rs401681 was more common and the TT genotype was rare in patients, and the differences were significant between lung adenocarcinoma patients and controls. This was also true for rs402710. Moreover, the frequency of

the GGCTCT haplotype was higher and the TTTTTT frequency was lower in patients, especially those with lung adenocarcinoma. Aberrant linkage disequilibrium among the three SNPs was found in patients with lung adenocarcinoma. We conclude that multiple variants at 5p15.33 contribute to susceptibility to lung adenocarcinoma.

Key words: *TERT*; *CLPTM1L*; Lung cancer; SNP; Lung adenocarcinoma

INTRODUCTION

The incidence of lung cancer has increased in recent decades and is now the number one cause of cancer mortality in China (Chen, 2009). The majority of patients are diagnosed at advanced stages. Twenty years ago in China, the most common histology was squamous cell carcinoma, followed by adenocarcinoma, small-cell cancer, large-cell cancer, and cancers not otherwise specified (NOS) (Sun, 1987). However, with the increased incidence of lung adenocarcinoma, the distribution of histological types has shifted and adenocarcinoma has become predominant, as is also seen in the USA (Stinchcombe and Socinski, 2009). The etiology of lung cancer is unknown, but the high percentage of smokers in the Chinese population and pollution combine to increase the risk (Molina et al., 2008; Gu et al., 2009; Helland and Brustugun, 2009). However, special attention should be paid to the genetic component of lung carcinogenesis.

Genetic studies of lung cancer have led to the discovery of hundreds of associated genes, such as those encoding p53, BRCA1, and serine/threonine kinase (Reguart et al., 2008; Sreeja et al., 2008; Strazisar et al., 2009), which can partly explain the genetic risk factors. Several lung cancer susceptibility loci have recently been identified in genome-wide association studies (Easton and Eeles, 2008; Thomas et al., 2008; Boezen, 2009; Caporaso et al., 2009; Landi et al., 2009; Chung et al., 2010; Hsiung et al., 2010). 5p15.33 was recently identified as a susceptibility region for lung cancer, and two known genes (telomerase reverse transcriptase [*TERT*] and palate transmembrane 1-like [*CLPTM1L*]) are located in this region. Association studies of several single-nucleotide polymorphisms (SNPs) in these two genes have further confirmed this finding (Kang et al., 2008; McKay et al., 2008; Jin et al., 2009; Rafnar et al., 2009; Zienolddiny et al., 2009; Hsiung et al., 2010; Wang et al., 2008, 2010). It has also been shown that these variants at the *TERT-CLPTM1L* loci are associated with other cancers (Yamamoto et al., 2007). As far as we know, there are no reports available about whether these common SNPs are just disease markers or whether they present functionally significant risk. For cancer, one allele polymorphism could potentially only explain less than 1% genetic risk. Beside functional study, genotype or haplotype analysis could supply some clues for their genetic contribution. In the present study, we compared the 5p15.33 (rs2736100, rs401681 and rs402710) genotypes and haplotypes in 196 patients with non-small-cell lung cancer and 229 healthy controls living in the Shanghai area of China.

MATERIAL AND METHODS

Cancer case-control studies

This study was a case-control analysis of lung cancer. Cancer-free controls (229) were recruited in the west of Shanghai, China, after a physical examination, in 2007-2009, and

the eligible individuals were gender and age (± 3 years) matched. The ethnic background of patients and controls was Han. The initial diagnosis of lung cancer was confirmed histologically within this period, and included adenocarcinoma, squamous cell carcinoma, small-cell carcinoma, and NOS. Large-cell carcinoma and adenosquamous carcinoma were the main constituents of the NOS category. The characteristics of the study population are summarized in Table 1. The Shanghai University of Traditional Chinese Medicine Ethics Committee for Scientific Research approved the study protocol, and written consent was obtained from all participants. The EDTA-blood samples were stored at -20°C before genomic DNA isolation.

Table 1. Demographic characteristics of the case-control study population.

Characteristics	Controls (N = 229)	Cases (N = 196)	P value
Age			
Median age	54.6	55.9	0.926
Mean \pm SD	54.6 \pm 10.2	55.9 \pm 10.3	
Gender			
Male	168	152	0.318
Female	61	44	
Smoking status			
Nonsmoker	119	73	0.002
Smoker	110	123	
Histological cell type			
Adenocarcinoma		96	
Squamous cell		44	
Small cell		16	
Other carcinoma		40	

SNP selection and DNA extraction

The SNPs were selected from the National Center for Biotechnology Information (NCBI). The information on the SNPs is listed in Table 2. DNA was extracted using proteinase K digestion, followed by ethanol precipitation. DNA concentrations were measured with spectrophotometry. The DNA samples were stored at -20°C until use.

Table 2. Primary information on the PCR assays of the three variants.

Gene	Rs No.	Location	Gene region	Mutation	Primers (5'→3')
<i>CLPTMIL</i>	rs402710	1373733	Exon 1	C/T	F: GTGTGCTGTTTTCCCTGCTGACTTA R: CCAGCAATAACAAGACAGAAGAACCAC S: TTTTTTTTTTTTTTATGTTTCCGTGTTGAGTGTCT
	rs401681	1375087	Intron	C/T	F: ACACACCGCCTTACCTTGTAGTTCA R: TCGTAGACTCTTGATAAATTACCAGC S: TTGAAAGCTGCTTCACACCATGAT
<i>TERT</i>	rs2736100	1339516	Intron	G/T	F: GAAAAACCACCACCTGCAAATTATC R: TTAGTTCCAAGCATCTGCCAAAAGC S: TTTTTTTTTTTTTTTTTTTTTTTTACCTGCGGTGAGTGC

F = forward primer; R = reverse primer; S = sequencing primer.

Genotyping

All samples were genotyped with the TaqMan 7900HT Sequence Detection System, according to manufacturer instructions. The assays were carried out in 96-well

plates and included six duplicate samples in each plate for quality control. The genotypes were determined using the Allelic Discrimination Sequence Detection Software (Applied Biosystems, USA). Each assay was performed using 1 ng DNA in a 25- μ L reaction containing the TaqMan Universal PCR Master Mix (Applied Biosystems), forward and reverse primers, and FAM- and VIC-labeled probes designed by Applied Biosystems. The primers and probes used are listed in Table 2. The protocol followed was from the instruction manual from Applied Biosystems. Individual samples with failed sequencing wells were not repeated. Therefore, the numbers of samples successfully genotyped for each polymorphism varied.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was assessed using the goodness-of-fit χ^2 test to compare the observed genotype frequencies with the expected genotype frequencies among the control subjects. The genotype and allele frequencies were calculated using the χ^2 test (2 x 2 contingency tables) and associations are expressed as odds ratios (ORs) with 95% confidence intervals (95% CIs). The linkage disequilibrium between SNPs was estimated by calculating the delta values (Δ) based on the observed and expected haplotype frequencies (Svejgaard et al., 1975).

A value of $P \leq 0.05$ was considered to be statistically significant.

RESULTS

The characteristics of patients with lung cancer are presented in Table 1. There was no difference in the gender distribution of the patients and controls, nor in their average ages; 37.2% of patients were nonsmokers, whereas 52% of controls were nonsmokers ($P = 0.002$).

Three SNPs at these loci were genotyped in patients and controls (196 patients/229 controls). The allele frequencies of the three SNPs in the controls were consistent with HWE. The primary test of association was a comparison of the allele and genotype frequencies between the cases and controls. There were no differences in the frequencies between the controls and patients. When the patients were divided into subgroups according to their histopathology, the frequency of the G allele at rs2736100 was significantly increased in patients with lung adenocarcinoma compared with the frequency in healthy controls (OR = 0.607; 95%CI = 0.432-0.853; $P = 0.004$), but it was not relatively elevated in the other patients. No difference was observed in the frequencies of the other two SNPs between the different cancer subtypes and the control.

The distributions of the genotypes are summarized in Table 3. At rs2736100, the genotype GG was more frequent (OR = 0.530, 95%CI = 0.312-0.901, $P = 0.018$) and TT was less frequent (OR = 2.004, 95%CI = 1.105-3.634, $P = 0.021$) in patients with lung adenocarcinoma but not in the patients with other cancers. The patients with lung cancer carried the CT genotype more often at rs401681 (OR = 0.595, 95%CI = 0.402-0.881, $P = 0.009$) and TT less often (OR = 2.278, 95%CI = 1.066-4.871, $P = 0.030$) than did the controls. When the patients were divided into subgroups, patients with adenocarcinoma accounted for the significant differences between the lung cancer group and controls. At rs402710, the genotype CT was more prevalent in patients with lung cancer (OR = 0.608, 95%CI = 0.410-0.903, $P = 0.013$). The

Table 3. Comparative analysis of genotype frequencies and the risk of lung cancer in the subgroups.

SNP		Control N (%)	Cancer N (%)	Histopathology			
				Adenocarcinoma N (%)	Squamous cell N (%)	Small cell N (%)	Others N (%)
rs402710	C	329 (72.15)	280 (72.54)	146 (76.84)	59 (70.24)	24 (75.00)	51 (63.75)
	T	127 (27.85)	106 (27.46)	44 (23.16)	25 (29.76)	8 (25.00)	29 (36.25)
rs401681	C	329 (72.15)	280 (71.79)	144 (75.00)	62 (72.09)	24 (75.00)	50 (62.50)
	T	127 (27.85)	110 (28.21)	48 (25.00)	24 (27.91)	8 (25.00)	30 (37.50)
rs2736100	G	208 (45.41)	201 (51.28)	111 (57.81) ^a	37 (42.05)	14 (43.75)	39 (48.75)
	T	250 (54.59)	191 (48.72)	81 (42.19) ^a	51 (57.95)	18 (56.25)	41 (51.25)
Genotype							
rs402710	CC	126 (55.26)	96 (49.74)	52 (54.74)	21 (50.00)	8 (50.00)	15 (37.50)
	CT	77 (33.77)	88 (45.60) ^b	42 (44.21)	17 (40.48)	8 (50.00)	21 (52.50)
	TT	25 (10.96)	9 (4.66) ^c	1 (1.05) ^d	4 (9.52)	0 (0)	4 (10.00)
rs401681	CC	126 (55.26)	95 (48.72)	50 (52.08)	22 (51.16)	8 (50.00)	15 (37.50)
	CT	77 (33.77)	90 (46.15) ^e	44 (45.83) ^e	18 (41.86)	8 (50.00)	20 (50.00)
	TT	25 (10.96)	10 (5.13) ^f	2 (2.08) ^b	3 (6.98)	0 (0)	5 (12.50)
rs2736100	GG	48 (20.96)	50 (25.51)	32 (33.33) ^j	7 (15.91)	2 (12.50)	9 (22.50)
	GT	112 (48.91)	101 (51.53)	47 (48.96)	23 (52.27)	10 (62.50)	21 (52.50)
	TT	69 (30.13)	45 (22.96)	17 (17.71) ^j	14 (31.82)	4 (25.00)	10 (25.00)

^aP = 0.004, OR = 0.607, 95%CI = 0.432-0.853. ^bP = 0.013, OR = 0.608, 95%CI = 0.410-0.903. ^cP = 0.018, OR = 2.518, 95%CI = 1.145-5.534. ^dP = 0.003, OR = 11.576, 95%CI = 1.545-86.717. ^eP = 0.009, OR = 0.595, 95%CI = 0.402-0.881. ^fP = 0.030, OR = 2.278, 95%CI = 1.066-4.871. ^gP = 0.040, OR = 0.603, 95%CI = 0.371-0.980. ^hP = 0.008, OR = 5.788, 95%CI = 1.343-24.946. ⁱP = 0.018, OR = 0.530, 95%CI = 0.312-0.901. ^jP = 0.021, OR = 2.004, 95%CI = 1.105-3.634.

frequency of the TT genotype was lower in patients with lung cancer (OR = 2.518, 95%CI = 1.145-5.534, P = 0.018) and the lowest prevalence occurred in the patients with adenocarcinoma (OR = 11.576, 95%CI = 1.545-86.717, P = 0.003).

Because lung cancer is a heterogeneous disorder, the individual's genetic make-up is an important predisposing factor for developing the disease. There is no evidence to suggest that these three variants are themselves causative, so their contributions as risk factors for lung cancer were considered. Here, we also compared the distributions of the haplotypes in patients and controls. The three SNPs rs2736100, rs401681 and rs402710 were connected sequentially. The haplotype GGCTCT was more common in patients than in controls (P = 0.035), whereas TTTTTT was less frequent in patients than in controls (P = 0.040; Table 4), especially in patients with lung adenocarcinoma (P = 0.005 for GGCTCT and P = 0.030 for TTTTTT; Table 5). It seems that the T allele is the protective allele in all three SNPs, whereas the G allele in rs2736100 and the C allele in both rs401681 and rs402710 are risk factors. The combination of these risk factors may partly explain the susceptibility associated with 5p15.33. The two SNPs rs401681 and rs402710 are next to each other in the *CLPTMIL* gene and are strongly linked. The Δ value between rs402710 and rs401681 was 0.288 in controls. However, the linkage with rs2736100 is relatively weaker (the Δ value between rs2736100 and rs402710 was 0.147, and the Δ value between rs2736100 and rs401681 was 0.147). These linkages were reduced in the patient group, as reflected in lower Δ values (0.192 between rs402710 and rs401681; 0.093 between rs2736100 and rs402710, and 0.123 between rs2736100 and rs401681). This aberrant linkage disequilibrium could be another risk factor in the genetic susceptibility to lung cancer.

All data have been analyzed in subgroups according to smokers and non-smokers. No significant difference has been found between these two groups (data not shown), which suggests that genetic susceptibility is the intrinsic carcinogenesis.

Table 4. Comparative analysis of haplotype frequencies and the risk of lung cancer.

Haplotype			Control	Lung cancer	P	OR	95%CI
rs402710	rs401681	rs2736100	N (%)	N (%)			
TT	TT	GG	4 (1.75)	0 (0.00)	0.128		
CC	CC	GG	35 (15.35)	32 (16.58)	0.731	0.912	0.541-1.539
CT	CT	GG	8 (3.51)	16 (8.29)	0.035	0.402	0.168-0.962
CT	CC	GG	1 (0.44)	1 (0.52)	1.000	0.846	0.053-13.613
TT	TT	GT	6 (2.63)	4 (2.07)	0.957	1.277	0.355-4.593
CT	TT	GT	1 (0.44)	0 (0.00)	1.000		
TT	CT	GT	1 (0.44)	1 (0.52)	1.000	0.846	0.053-13.613
CC	CC	GT	64 (28.07)	41 (21.24)	0.107	1.447	0.923-2.269
CT	CT	GT	36 (15.79)	48 (24.87)	0.020	0.566	0.349-0.918
CC	CT	GT	2 (0.88)	3 (1.55)	0.664	0.560	0.093-3.389
CT	CC	GT	1 (0.44)	1 (0.52)	1.000	0.846	0.053-13.613
TT	TT	TT	14 (6.14)	4 (2.07)	0.040	3.091	1.000-9.553
CC	CC	TT	25 (10.96)	19 (9.84)	0.708	1.128	0.601-2.118
CT	CT	TT	30 (13.16)	20 (10.36)	0.377	1.311	0.718-2.392
CC	CT	GG	0 (0.00)	1 (0.52)	0.458		
CT	TT	TT	0 (0.00)	2 (1.04)	0.210		

Table 5. Comparative analysis of haplotype frequencies and the risk of lung adenocarcinoma.

Haplotype			Control	Adenocarcinoma	P	OR	95%CI
rs402710	rs401681	rs2736100	N (%)	N (%)			
TT	TT	GG	4 (1.75)	0 (0.00)	0.324		
CC	CC	GG	35 (15.35)	20 (21.05)	0.214	0.680	0.369-1.252
CT	CT	GG	8 (3.51)	11 (11.58)	0.005	0.278	0.108-0.714
CT	CC	GG	1 (0.44)	1 (1.05)	0.502	0.414	0.026-6.690
TT	TT	GT	6 (2.63)	1 (1.05)	0.678	2.541	0.302-21.393
CT	TT	GT	1 (0.44)	0 (0.00)	1.000		
TT	CT	GT	1 (0.44)	0 (0.00)	1.000		
CC	CC	GT	64 (28.07)	19 (20.00)	0.130	1.561	0.874-2.787
CT	CT	GT	36 (15.79)	23 (24.21)	0.074	0.587	0.326-1.058
CC	CT	GT	2 (0.88)	3 (3.16)	0.154	0.271	0.045-1.651
CT	CC	GT	1 (0.44)	0 (0.00)	1.000		
TT	TT	TT	14 (6.14)	0 (0.00)	0.030		
CC	CC	TT	25 (10.96)	10 (10.53)	0.908	1.047	0.482-2.274
CT	CT	TT	30 (13.16)	6 (6.32)	0.075	2.247	0.903-5.592
CT	TT	TT	0 (0.00)	1 (1.05)	0.294		

DISCUSSION

Consistent with published data, all three SNPs, rs2736100, rs401681, and rs402710, were associated with lung cancer, especially lung adenocarcinoma. The T nucleotide was protective in all three SNPs and the lack of the T nucleotide may be a risk factor for lung adenocarcinoma. The distributions of these genotypes and haplotypes indicate that a combined role of several SNPs better accounts for the association between region 5p15.33 and lung cancer, rather than a single allele. Genotype and haplotype analyses are important because the additive effects of these variants could constitute the genetic risk in this disease.

TERT is the reverse transcriptase catalytic subunit of telomerase. In most replicating eukaryotic cells, telomeres are synthesized and maintained by telomerase. TERT, the rate-limiting determinant of telomerase activity, is tightly repressed in most normal human cells, whereas it is highly expressed in many tumor tissues, including lung cancer (Nishio

et al., 2007; Metzger et al., 2009). It has been established that TERT induction and telomerase activation are critical steps during cellular immortalization and transformation. High telomerase activity or TERT expression predicts disease progression and poor outcomes in patients with lung cancer (Fernandez-Garcia et al., 2008). When telomerase or TERT is inhibited, cancer cells undergo telomere shortening, senescence or apoptosis, and eventually lose their oncogenic potential. However, high TERT expression is not present in all histologies. Low levels of *TERT* mRNA are significantly associated with squamous cell histology (Metzger et al., 2009), whereas adenocarcinoma cells exhibit even higher TERT expression (Zhu et al., 2006). This difference may explain our data, in which a strong association was only observed between the TERT SNP and lung adenocarcinoma. The mutation from T to C at rs2736100 may be associated with the upregulation of TERT expression in lung adenocarcinoma.

In squamous cell lung cancer, lower TERT or telomerase expression may lead to telomere dysfunction. The impact of telomere dysfunction on tumorigenesis depends upon the status of the tumor suppressor p53 (Maser et al., 2007). With abnormal p53 function, the loss of telomere function leads to cancer-relevant genomic changes (Perera et al., 2008). In the presence of functional p53, telomere dysfunction acts as a barrier to tumorigenesis through the elimination of cells by senescence and apoptotic processes. Numerous studies have shown that smoke exposure triggers p53 mutation. Therefore, it is likely that there are different requirements for TERT in the formation and development of these two types of lung cancer. Further studies are required to define the exact roles of the TERT SNPs, telomere dysfunction, and genomic instability in the pathogenesis of squamous cell lung cancer and adenocarcinoma.

CLPTMIL was identified among the genes involved in resistance to the anticancer drug cisplatin in ovarian cancer cells (Yamamoto et al., 2001). Yamamoto found that the expression of *CLPTMIL* was upregulated in all the cisplatin-resistant cell lines examined. The overexpression of *CLPTMIL* in a cisplatin-sensitive cell line resulted in apoptosis, whereas *CLPTMIL* overexpression had no effect on a cisplatin-resistant cell line. The gene is widely expressed in different tissues, including lung tissue. However, its protein structure and function are almost unknown. As far as we are aware, the functions of these two SNPs are totally unknown. The powerful genetic association of *CLPTMIL* with multiple cancers should encourage researchers to define this gene in detail.

CONCLUSION

Our study provides further evidence that 5p15.33 variants are associated with lung adenocarcinoma. Information from our haplotype analysis suggests that a combination of multiple variants underlies this susceptibility to lung cancer. Further studies are required, including a functional study and the identification of more variants in this region.

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