LAPTM4B polymorphism is associated with non-small cell lung cancer susceptibility and prognosis

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Abstract. Lysosome-associated protein transmembrane-4β (*LAPTM4B*) is a novel cancer-related gene that is upregulated in many tumors, and which plays important roles in carcinogenesis. It has two alleles, *LAPTM4B**1 and *LAPTM4B**2. *LAPTM4B**1 contains only one copy of a 19-bp sequence in the first exon, whereas *LAPTM4B**2 contains two tight tandem segments. Previous studies have shown that *LAPTM4B**2 is a risk factor for susceptibility and prognosis of many tumors. The present study investigated the relationship between *LAPTM4B* polymorphism and non-small cell lung cancer (NSCLC) susceptibility and prognosis. We identified *LAPTM4B* genotypes with polymerase chain reaction (PCR) in peripheral blood samples. In the adjusted multivariate logistic regression analysis, we found that *LAPTM4B**1/2, *LAPTM4B**2/2 exhibited 1.48-fold [95% confidence interval (CI), 1.076-2.037] and 2.855-fold (95%CI, 1.722-4.734) increases in the risk of developing NSCLC compared with non-*LAPTM4B**2 carriers. Furthermore, our results showed that overall survival time and disease-free survival time of patients with *LAPTM4B**2 were significantly shorter than in patients carrying *LAPTM4B**1 (P=0.001 and P=0.001, respectively). In addition, multivariate Cox regression analysis revealed that *LAPTM4B**2 was also an independent prognostic factor for NSCLC. These results suggest that *LAPTM4B* polymorphisms may be a prospective marker for evaluating the risk and prognosis of NSCLC.

Introduction

Primary lung cancer is the most commonly diagnosed malignant tumor in the world. It is reported that the number of new lung cancer cases in 2008 was 1.6 million, accounting for 13% of the total cancer cases. Due to its insidious symptoms, late clinical presentation and rapid progression, there are 1.4 million deaths annually making it the leading cause of cancer-related mortality worldwide (1). Non-small cell lung cancer (NSCLC) accounts for ~75-80% of cases (2). It is widely accepted that genetic heterogeneity and environmental factors result in the onset of lung cancer simultaneously. Smoking is the most well-established cause of lung cancer, yet non-smokers take up a very large proportion of lung cancer patients, suggesting the genetic variants also play a role (3-5). It is of utmost importance to identify novel molecules for the detection and diagnosis of lung cancer.

Lysosomal-associated protein transmembrane-4β (*LAPTM4B*), a novel oncogene candidate, was initially identified in hepatocellular carcinoma. It is cloned using fluorescence differential display, rapid amplification of cDNA ends and reverse transcription-polymerase chain reaction (RT-PCR). According to BLAST program analysis, it is located in chromosomes 8q22 and is composed of seven exons separated by six introns (6,7). It was previously reported that *LAPTM4B* protein was markedly overexpressed in various malignant tumors, including pancreatic (8), gallbladder (9), ovarian (10) and cervical cancer (11). Two alleles of the *LAPTM4B* gene have been recognized, designated as *LAPTM4B**1 and *LAPTM4B**2 (GenBank nos. AY219176 and AY219177, respectively). The difference between *LAPTM4B**1 and *LAPTM4B* *2 is the sequence at the 5' untranslated region (UTR) in the first exon. As shown in Fig. 1, *LAPTM4B**1 contains only one copy of a 19-bp sequence whereas *LAPTM4B**2 contains two tight tandem segments.

Previous studies showed that there was an association between *LAPTM4B* polymorphism and increased risk of hepatocellular carcinoma (12), gastric cancer (13), colorectal cancer (14), gallbladder (15) and ovarian carcinoma (16), and breast cancer (17). Two laboratories studied the relationship between *LAPTM4B* polymorphism and susceptibility of lung cancer, however, they drew completely different conclusions (18,19); one reported that *LAPTM4B**2 was associated with NSCLC susceptibility, but the other found no relationship. Less than 200 patients were included in each group, therefore, their conclusions are not very conclusive. Meanwhile, the two groups included all types of histological lung cancer in the samples. It is known that the development of small cell lung cancer and NSCLC has a considerable difference, and *LAPTM4B* polymorphism might influence only a certain histological type, thus it is less rigorous not to divide them. Hence, it is necessary to enrol more patients to rigorously

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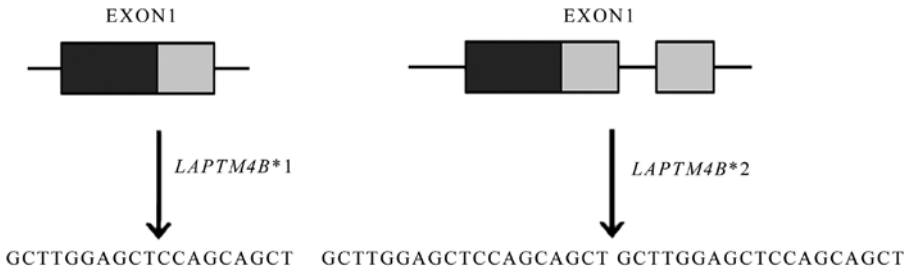

Figure 1. Schematic diagram of lysosome-associated protein transmembrane-4β (*LAPTM4B*) alleles in exon. Allele *1 contains one copy of the 19-bp sequence segment (gray box), while allele *2 contains two tight tandem 19-bp sequence segments. The lower panels display the DNA sequence in the gray boxes.

and deeply explore whether there is a relationship between *LAPTM4B* polymorphism and risk of NSCLC. In addition, *LAPTM4B**2 is also a marker of poor prognosis in gallbladder (20) and hepatocellular carcinoma (21), breast (17) and endometrial cancer (22), while its function in the prognosis of lung cancer patients has not been clarified. The present study was designed to study the effects of *LAPTM4B* gene variants on the susceptibility of NSCLC via a large sample size. Furthermore, the relationship between *LAPTM4B* genotype and prognosis of NSCLC was also analyzed.

Materials and methods

Patients and controls. A total of 392 blood samples of patients were collected from Qilu Hospital of Shandong University between July 2007 and October 2010. All patients underwent surgical resection and were diagnosed as NSCLC by at least two pathologists. For all patients, histological type of lung cancer was determined by the World Health Organization classifications, and pathological staging was based on the international staging system revised in 2009 (23). Also, 437 cancer-free individuals attending the physical examination in Qilu Hospital of Shandong University were recruited as controls. All blood samples were stored at -80˚C for further studies. To analyze the association between NSCLC patients prognosis and *LAPTM4B* polymorphism, we performed a retrospective study. September 30, 2013 was the end date of follow-up, therefore, a total of 101 cases diagnosed as NSCLC before September 31, 2008 were investigated and analyzed. Of these 101 patients, 4 were excluded (3 patients were lost to follow-up, 1 patient died of perioperative complications), thus, 97-long-term follow-up patients were enrolled in this retrospective study. Each patient signed an informed consent according to the Helsinki Declaration and the present study was approved by the Ethics Committee of Qilu Hospital.

DNA extraction. Genomic DNA was extracted from a 1 ml peripheral blood sample obtained from each participant using a RelaxGene Blood DNA System (Tiangen, China) according to the protocol provided by manufacturer. Then, the newly extracted genomic DNA was stored at -20˚C for the subsequent PCR analysis.

DNA genotyping. Polymorphism of *LAPTM4B* was determined by PCR analysis using the specific primers. The primer sequences were: forward, 5'-GCCGACTAGGGGACTGGC GGA-3' and reverse, 5'-CGAGAGCTCCGAGCTTCTGCC-3'

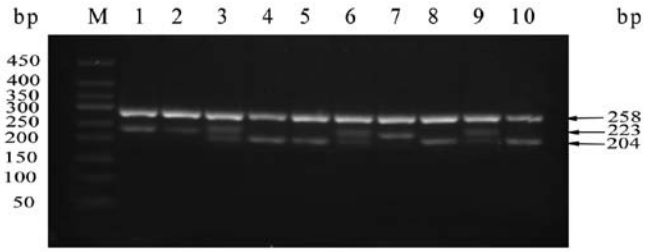

Figure 2. Genotyping of LAPTM4B. Samples were analyzed by separation in 2.5% agarose gel electrophoresis. Lane M, DNA marker (50, 100, 150, 200, 250, 300, 350, 400 and 450 bp). The lower bands represent the target products. Lanes 4, 5, 8 and 10: genotype *1/1; lanes 1, 2 and 7: genotype *2/2; lanes 3, 6 and 9: genotype *1/2. The upper bands show the amplified product of human GAPDH (258 bp) that served as the positive internal control. LAPTM4B, lysosome-associated protein transmembrane-4β.

(7). The PCR was conducted under the standard procedure in a 20 μl reaction mixture, which included 10 μl 2XPCR mix (TransGen, China), 1 μl sense primer, 1 μl reverse primer, 2 μl template DNA and 6 μl ddH_2O. The PCR conditions were: predenaturation at 95˚C for 5 min, 35 cycles of denaturation at 94˚C for 30 sec, annealing at 68˚C for 30 sec, extension at 72˚C for 30 sec, then final extension at 72˚C for 5 min. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was regarded as the positive inner control, which was a 258-bp fragment. The primer sequences were: sense, 5'-TGTCG CTGTTGAAGTCAGAGGAGA-3' and reverse, 5'-AGAACA TCATCCCTGCCTCTACTG-3'. PCR products were analyzed by electrophoresis in a 2.5% agarose gel and visualized with ethidium bromide.

Statistical analysis. Statistical analyses were performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The Chi-squared test or the Fisher's exact was used to test the genotypic frequencies of the patients and controls for Hardy-Weinberg equilibrium and to examine the association between *LAPTM4B* genotype and the patient clinicopathological factors. The relationship between *LAPTM4B* polymorphisms and susceptibility to NSCLC was estimated using unconditional logistic regression method. The Kaplan-Meier method and log-rank test were used to calculate survival curve and to compare the statistical significance of survival differences among patient subgroups. Multivariate Cox regression analysis was carried out to identify the potential prognostic factor of NSCLC patients. P-value <0.05 was considered to indicate a statistically significant difference.

Table I. Distribution of gender and age in case and control groups.

	Controls (n=437) (%)	Cases (n=392) (%)	P[a]
Gender, n (%)			P=0.17
Male	257 (58.8)	212 (54.1)	
Female	180 (41.2)	180 (45.9)	
Age, years, n (%)			**P<0.001**
<60	253 (57.9)	158 (40.3)	
≥60	184 (42.1)	234 (59.7)	

[a]Analysis by Chi-square test. Bold number indicates a statistically significant difference in this analysis.

Table II. Distribution of genotypes and alleles of LAPTM4B in case and control groups.

	Controls n (%)	Cases n (%)	OR (95%CI)[a]
Genotypes			
*1/1	226 (51.7)	158 (40.3)	
*1/2	176 (40.3)	171 (43.6)	**1.48 (1.076-2.037)**
*2/2	35 (8)	63 (16.1)	**2.855 (1.722-4.734)**
Total	437 (100)	392 (100)	
Alleles			
*1	628 (71.9)	487 (62.1)	
*2	246 (28.1)	297 (37.9)	**1.649 (1.316-2.068)**
Total	874 (100)	874 (100)	

[a]Data were calculated by logistic regression analysis and adjusted for age status. OR, odds ratio; CI, confidence interval. LAPTM4B, lysosome-associated protein transmembrane-4β. Bold numbers indicate statistically significant differences in this analysis.

Results

LAPTM4B genotypes. Three different *LAPTM4B* genotypes designated *LAPTM4B**1/1, *LAPTM4B**2/2 and *LAPTM4B**1/2 were identified by PCR-agarose gel electrophoresis analysis. As shown in Fig. 2, 10 representative individuals were chosen to display the polymorphism of *LAPTM4B* genotypes. *LAPTM4B**1/1 displayed a 204-bp fragment, *LAPTM4B**2/2 displayed a 223-bp fragment and *LAPTM4B**1/2 had both fragments. The upper 258-bp band in each lane in Fig. 2 was *GAPDH* as the positive inner control.

LAPTM4B polymorphism and NSCLC susceptibility. We collected 392 NSCLC subjects and 437 cancer-free control subjects for the present study. The main characteristics of the patients and controls are presented in Table I. The mean age of cases and controls was 58.8 and 55.4 years, respectively. We divided the ages into two groups on the basis of the median age. There was no statistical significance in gender between cases and controls, while the age distribution revealed a significant difference (P<0.001).

Table III. Distribution of three genotypes of LAPTM4B in relation to clinicopathological variables in 392 patients with non-small cell lung cancer.

Variables	No. of cases	LAPTM4B genotypes *1/1	*1/2	*2/2	χ^2	P[a]
Gender					1.907	0.358
Male	212	82	91	39		
Female	180	76	80	24		
Age (years)					0.048	0.976
<60	158	64	68	26		
≥60	234	94	103	37		
Pathological type					0.741	0.69
AC	220	85	100	35		
SCC	172	73	71	28		
Differentiation					12.924	**0.012**
Well	109	56	40	13		
Moderately	129	54	58	17		
Poorly	154	48	73	33		
Tumor size (cm)					0.622	0.733
<4	200	77	89	34		
≥4	192	81	82	29		
Lymph node metastasis					3.003	0.223
No	186	83	77	26		
Yes	206	75	94	37		
Classification of TNM					3.155	0.789
I	101	46	42	13		
II	130	53	54	23		
III	124	47	56	21		
IV	37	12	19	6		
Smoking[b]					1.396	0.498
Yes	168	64	79	25		
No	224	94	92	38		

[a]Chi-square test; [b]Yes means smoking for at least one year at the time of sample collection, otherwise no. AC, adenocarcinoma; SCC, squamous cell carcinoma; LAPTM4B, lysosome-associated protein transmembrane-4β. Bold number indicates a statistically significant difference in this analysis.

The genotype and allele frequencies of *LAPTM4B* in cases and controls in the present case-control study are summarized in Table II. We proved that the observed genotype frequencies for this polymorphism were in agreement with the Hardy-Weinberg equilibrium in the controls, suggesting that the control group could represent the whole population. Table II shows that there was a higher proportion of *LAPTM4B**1/2 and *LAPTM4B**2/2 in cases (43.6 and 16.1%, respectively) than in controls (40.3 and 8%, respectively). Odds ratio

Table IV. Univariate Kaplan-Meier survival analysis of overall survival and disease-free survival in 97 patients with non-small cell lung cancer.

Prognostic variables	No. of cases	OS (months)			DFS (months)		
		Mean ± SE	95% CI	P[a]	Mean ± SE	95% CI	P[a]
Gender				0.727			0.954
Male	59	35.2±3.1	29.1-41.4		25.7±3.1	19.6-31.8	
Female	38	35.1±3.7	27.7-42.4		26.0±3.6	19.0-33.0	
Age (years)				0.963			0.933
<60	45	35.0±3.6	27.8-42.1		25.1±3.4	18.5-31.8	
≥60	52	35.3±3.2	29.1-41.6		26.3±3.2	19.9-32.6	
Pathological type				0.172			0.198
AC	63	39.5±4.2	31.2-47.8		23.8±2.8	18.2-29.3	
SCC	34	32.8±2.9	27.2-38.4		29.4±4.0	21.5-37.2	
Differentiation				**0.016**			**0.056**
Poorly	42	27.3±3.2	21.0-33.6		19.1±3.4	12.4-25.8	
Moderately	24	35.7±5.2	25.6-45.8		26.0±4.7	16.9-35.2	
Well	31	44.5±3.8	37.2-51.9		33.8±3.7	26.7-41.0	
Tumor size (cm)				**<0.001**			**<0.001**
<4	53	43.4±3.4	36.7-50.0		34.9±3.4	28.2-41.7	
≥4	44	24.8±2.5	19.8-29.7		15.1±2.3	10.6-19.6	
Lymph node metastasis				**<0.001**			**<0.001**
No	51	47.4±3.1	41.3-53.5		37.6±3.3	31.3-44.0	
Yes	46	21.4±2.4	16.6-26.2		12.9±2.3	8.5.4-17.4	
Classification of TNM				**<0.001**			**<0.001**
I	30	49.9±3.7	42.7-57.1		40.3±3.9	32.8-47.9	
II	35	35.3±3.7	28.1-42.5		25.7±3.8	18.3-33.0	
III	24	22.7±3.9	15.1-30.2		14.7±3.6	7.7-21.7	
IV	8	12.3±2.5	7.4-17.1		4.0±1.6	0.8-7.2	
LAPTM4B genotypes				**0.001**			**0.001**
*1/1	39	45.5±3.6	38.6-52.5		35.2±3.7	28.0-42.3	
*1/2	40	31.3±3.5	24.4-38.1		22.5±3.5	15.6-29.3	
*2/2	18	20.4±4.3	12.1-28.8		12.8±4.1	4.7-20.9	

[a]Log-rank test. OS, overall survival; DFS, disease-free survival; CI, confidence interval; TNM, tumor-node-metastasis; LAPTM4B, lysosome-associated protein transmembrane-4β. Bold numbers indicate statistically significant differences in this analysis.

analysis indicated that *LAPTM4B**1/2, *2/2 were correlated with a significant increased risk of NSCLC compared with *LAPTM4B**1/1 (OR, 1.48; 95% CI, 1.076-2.037; OR, 2.855; 95% CI, 1.722-4.734, respectively). In addition, the frequency of *LAPTM4B**2 was notably higher in cases than in controls (37.9 vs. 28.1%, respectively). *LAPTM4B**2 carriers had a 1.649-fold (95% CI, 1.316-2.068) higher risk of developing NSCLC than non-*LAPTM4B**2 carriers. Our data indicated that *LAPTM4B**2 was likely to be associated with an increased susceptibility to NSCLC in a Chinese population.

LAPTM4B polymorphism and clinicopathological variables. We investigated the distribution of clinical parameters such as gender, age, pathological type, differentiation degree, lymph node metastasis, smoking and tumor-node-metastasis (TNM) stage in different genotypes of *LAPTM4B* in these 392 patients. We found that *LAPTM4B**2 was significantly associated with poor histopathologic differentiation (P=0.012), but not with gender, age, pathological type, lymph node metastasis, smoking and TNM stage (P>0.05), shown in Table III.

LAPTM4B genotype and NSCLC prognosis. We conducted survival analysis in these 97 followed-up patients to examine the impact of *LAPTM4B* polymorphism on NSCLC prognosis. As of September 30, 2013 which was the end date for follow-up, 23 (23.7%) patients were alive and 74 (76.3%) patients had succumbed to the disease. We first performed univariate analysis of clinicopathological variables for prognosis using the Kaplan-Meier method and log-rank test. According to the survival analysis, the 5-year overall and disease-specific survival were 62.7 and 68.1%, respectively. As shown in Table IV, it was of no statistically significant difference in

Table V. Multivariate Cox regression model of overall survival and disease-free survival in 97 patients with non-small cell lung cancer.

	OS			DFS		
Variables	RR	95% CI	P[a]	RR	95% CI	P[a]
Tumor size (cm)						
<4						
≥4	1.184	0.62-2.261	0.61	1.393	0.75-2.587	0.294
Lymph node metastasis						
No						
Yes	2.261	1.241-4.116	**0.008**	2.202	1.242-3.905	**0.007**
Differentiation						
Well						
Moderately	0.967	0.473-1.974	0.926	0.952	0.498-1.821	0.883
Poorly	1.386	0.724-2.652	0.325	1.273	0.702-2.31	0.427
Classification of TNM						
I						
II	1.527	0.783-2.981	0.214	1.288	0.705-2.353	0.411
III	2.478	1.128-5.445	**0.024**	2.102	1.031-4.285	**0.041**
IV	3.969	1.46-10.788	**0.007**	4.502	1.705-11.89	**0.002**
LAPTM4B genotypes						
*1/1						
*1/2 + *2/2	2.025	1.21-3.388	**0.007**	1.678	1.044-2.696	**0.033**

[a]Cox regression test; RR, relative risk; CI, confidence interval; OS, overall survival; DFS, disease-free survival; TNM, tumor-node-metastasis; LAPTM4B, lysosome-associated protein transmembrane-4β. Bold numbers indicate statistically significant differences in this analysis.

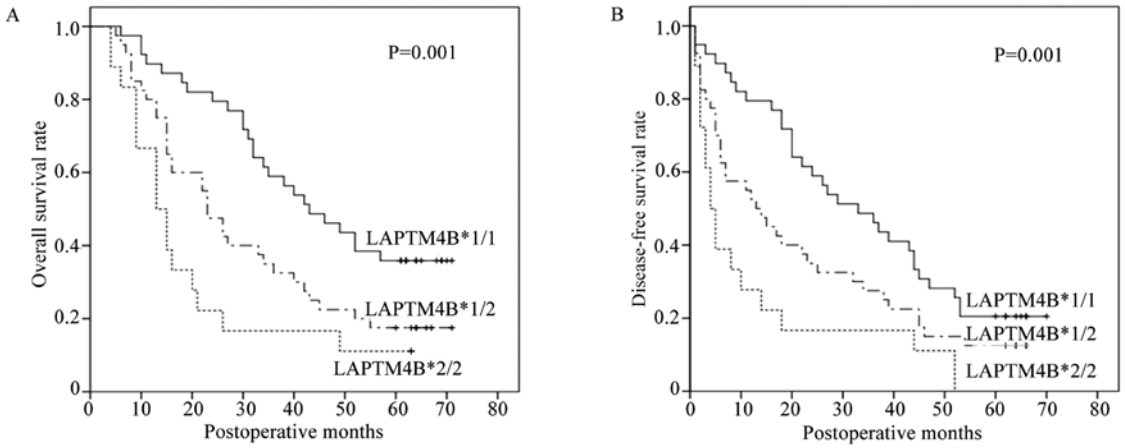

Figure 3. Kaplan-Meier curves of survival related to LAPTM4B polymorphisms in 97 non-small cell lung cancer patients. Patients with LAPTM4B*1/2 and LAPTM4B*2/2 had a poorer prognosis than patients with LAPTM4B*1/1. LAPTM4B, lysosome-associated protein transmembrane-4β.

some clinical parameters, such as age, gender and pathological type (P>0.05). However, the overall 5-year survival rate of patients with *LAPTM4B**2/2 and *LAPTM4B**1/2 (11.1 and 17.5%, respectively, vs. 35.9%, P=0.001) was significantly lower than that of *LAPTM4B**1/1 carriers. In addition, Kaplan-Meier analysis of disease-free survival also demonstrated poor 5-year survival rate in patients with LAPTM4B*2/2 and *LAPTM4B**1/2 (0 and 12.5%, respectively, vs. 20.5%, P=0.001) shown in Fig. 3. Furthermore, poor prognosis was strikingly associated with large tumor size (P<0.01 and P<0.01, respectively), high grade of histopathological differentiation (P=0.016 and P=0.056, respectively), positive lymph node metastasis (P<0.01 and P<0.01, respectively) and high TNM stage (P<0.01 and P<0.01, respectively).

LAPTM4B genotype is an independent prognostic marker for NSCLC patients. To analyze the independent factor of *LAPTM4B* polymorphism on prognosis, a multivariate Cox

regression model adjusted for statistically significant prognostic factors was performed. Table V shows that subjects with the *LAPTM4B**2 allele had, respectively, 2.025-fold (95% CI, 1.21-3.388; P=0.007) increased mortality and 1.678-fold (95% CI, 1.044-2.696; P=0.033) increased recurrence of NSCLC than those carrying *LAPTM4B**1/1 genotype, demonstrating that the *LAPTM4B* genotype was an independent prognostic factor for NSCLC patients. In addition, lymph node metastasis and TNM classification also retained their prognostic significance.

Discussion

In the present study, we detected the polymorphism of *LAPTM4B* genotypes in NSCLC patients, then analyzed the relationship between distribution of *LAPTM4B* genotypes and susceptibility, prognosis of lung cancer. Our finding was that patients with *LAPTM4B**2 showed a higher risk of susceptibility and mortality of NSCLC compared with non-carriers. To our knowledge, this is the first study to prove polymorphism of *LAPTM4B* may act as an indicator for the prognosis of NSCLC. Our results are consistent with the putative role *LAPTM4B* plays in carcinogenesis and tumor progression.

LAPTM4B, a novel oncogene, was first detected in hepatocellular carcinoma and was then found upregulated in various solid malignant tumors (24). *LAPTM4B* is closely related with the biological behaviors of malignant tumors. An increase in *LAPTM4B* expression, as measured by mRNA and protein, was associated with tumor progression and poorer survival in patients with breast (25), pancreatic (8), colon (26), ovary (27) and cervical cancer (11). Furthermore, extensive studies have been performed to account for such outcomes. Studies have shown that upregulation of *LAPTM4B* could promote cell proliferation (28), invasion, migration (29) and may inhibit cell apoptosis (30,31) *in vitro*, while in nude mice the time of tumorigenesis was markedly shortened (29). It was assumed that various signal molecules were associated with cellular malignant transformation after the alteration of *LAPTM4B* protein expression level. It has been confirmed that *LAPTM4B* protein could upregulate some proliferation-promoting transcription factors such as c-Myc, c-Jun and c-Fos, and cell cycle-promoting proteins such as cyclin D1 and E (28). Meanwhile, it could also activate PI3K/AKT signaling pathway to motivate cellular multidrug resistance (31). A recent study clarified that cAMP responsive element binding protein-1 (CREB1) played an important role in *LAPTM4B* transcriptional regulation (32).

The unique region of 19-bp sequence at 5'UTR in the first exon was identified as the difference between *LAPTM4B**1 and *LAPTM4B* *2. The 19-bp difference in the first exon of the *LAPTM4B* gene altered the open reading frame (ORF), so it may influence the structure and function of the protein encoded by it. Previous studies have shown that *LAPTM4B**2 allele played important roles in the susceptibility and prognosis of many tumors. To date, the exact mechanism of the phenomenon has not yet been revealed. It has been reported that a 40 kD protein was encoded by *LAPTM4B**2 allele with an extra 53 amino acids compared with the *LAPTM4B**1 allele encoding a 35 kD protein, which may explain such a difference. Different structures and activities of proteins could alter the cellular metabolism and signal pathway, then induce the malignant transformation. This area requires further study.

The present study was carried out under the strict rules of clinical trial, hence our conclusions are firm. Our data demonstrated that *LAPTM4B**2 is associated with NSCLC susceptibility. This is also the first study to clarify that the polymorphism of *LAPTM4B* genotype is related to NSCLC progression and prognosis. Although the exact molecular mechanisms which underlie the function of *LAPTM4B* in lung carcinogenesis have yet to be fully clarified, *LAPTM4B**2 could be a novel potential marker to estimate susceptibility and prognosis of NSCLC.

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