RESEARCH ARTICLE

Association between a p73 Gene Polymorphism and Genetic Susceptibility to Non-small Cell Lung Cancer in the South of China

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Abstract

Background: This study aimed to identify any association between the p73 gene G4C14-to-A4T14 polymorphism and risk of non-small cell lung cancer (NSCLC) in the south of China. Materials and Methods: We genotyped the p73 gene polymorphism of peripheral blood DNA from 168 patients with NSCLC and 195 normal controls using HRM (high resolution melting) and PCR-CTPP (polymerase chain reaction with confronting two-pair primers). Results: The results of genotyping by HRM and PCR-CTPP were consistent with direct sequencing, the p73 genotype distribution in 168 lung cancer patients being as follows: GC/GC 101 cases (60.1%), GC/AT 59 cases (35.1%), AT/AT 8 cases (4.8%). The carriers of AT/AT genotype had a significantly reduced risk of NSCLC (OR=0.370; 95%CI: 0.170-0.806; *p*=0.010) as compared with non-carriers. However, we found no relations between p73 genotypes and histological type (*p*=0.798, x^2=0.452), tumor stage (*p*=0.806, x^2=0.806), or lymph node metastasis (*p*=0.578, x^2=1.098). Conclusions: Our findings suggest that the p73 G4C14-to-A4T14 polymorphism may be a modifier of NSCLC susceptibility in the Chinese population.

Keywords: NSCLC - p73 gene polymorphism - HRM and PCR-CTPP

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Introduction

NSCLC is the main type of lung cancer, one of the most common malignant tumors of the world, especially in male (Jemal et al., 2011), a serious threat to human lives and health. The incidence of lung cancer is currently increasing in China, a biological complex disease highly relevant to factors such as environment, occupation, smoking , and the genetic factor also plays an important role, the difference between individual cell cycle, DNA repair and apoptosis control may decide to different individual genetic susceptibility to tumor (Zhou et al., 2000; Hu et al., 2005; Cheng et al., 2012).The research of the correlation between gene polymorphism and lung cancer will help to clarify the pathogenesis of lung cancer, including its formation and development, and play an important part of the diagnosis and prognosis of patients with lung cancer.

The p73 gene is a member of p53 family, located at human chromosome 1p36.33, has structural and functional homolog of p53 (Kaghad et al., 1997; Melino et al., 2002). There is more and more evidence to suggest that the p73 gene is vital in the pathogenesis of various cancers, including lung cancer (Uramoto et al., 2006; Liu et al., 2008; Lo et al., 2011). p73 gene encodes a protein, significant similarity with p53 throughout its DNA-binding, transactivation, and oligomerization domains (Kaghad et al., 1997).

The G4C14-to-A4T14 polymorphism lies upstream of the initiating AUG in exon 2, can form a step-loop structure and adjust susceptibility to cancer (Kaghad et al., 1997, Peters et al., 2001; Li et al., 2004). Prior researches on the correlation between the two polymorphisms and other tumors were discussed, but the conclusion was inconsistent (Ryan et al., 2001; Huang et al., 2003; Ni et al., 2004; Li et al., 2004; Pfeifer et al., 2005; Lee et al., 2010). This paper was based on the case-control study, detecting the p73 gene polymorphism by using HRM and PCR-CTPP, at last in combination with DNA sequencing to verify, to explore the association between p73 gene polymorphism and genetic susceptibility to lung cancer in Chinese population.

Materials and Methods

Study subjects and samples

This case-control study consisted of 168 lung cancer

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patients and 195 cancer-free controls. All subjects were consecutively recruited between January 2013 and April 2014 in the Central Hospital of Zhuzhou City (Zhuzhou, China) and Hunan Provincial Tumor Hospital (Changsha, China). All patients were histopathologically confirmed and had no preoperative chemotherapy or radiotherapy. The cancer-free controls were randomly recruited from healthy individuals who underwent routine physical examination in the same regions during the same period when the case patients were selected. At recruitment, written informed consents about the study were obtained from all subjects and each participant was interviewed to collect information regarding demographic factors and medical history. The research was approved by the institutional Review Board of the hospital.

Genotyping of polymorphism

Main reagents were as follows: KI, 0.9% NaCl, chloroform/ isoamyl alcohol (24:1), isopropanol, 70% ethanol, and all reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). SYBR, PCR master mixture and primers were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Blood samples were collected from each participant and genomic DNA was extracted from peripheral blood lymphocytes by phenol-chloroform extraction (Yang 2014). DNA samples were restored in 1.5 ml EP tube at -70°C.

Firstly, we detected samples for the genotype by using HRM. For amplification, primers were shown in Table 1 (Huang et al., 2003; Lee et al., 2010), Real-time PCR was carried out with a 25μl reaction mixture containing SYBR mixture 12.5μl, sterilized water 10.5μl, each primer 0.5μl, DNA template 1μl. An initial denaturation step at 94°Cfor 5 min was followed by 30 cycles of 94°C for 30s, 65°C for 30s, 72°Cfor 30s, and a final extension step at 72°C for 10 min. Melting curves were obtained following a denaturation period for 60s at 95°C and hold 60s at 40°C, at a start temperature of 75°C and a final temperature of 85°C,with a temperature gradient of 0.05°C/s (Li et al., 2011). PCR and melting procedure were detected online with the LightCycler Nano Tutorial instrument (Roche, China), the melting curve (Figure 1) and the accuracy of genotyping data was validated by direct sequencing (Figure 3).

Secondly, we analyzed samples for the p73 G4C14-A4T14 genotype by using another method: PCR-CTPP. The PCR assay was performed in GeneAmp* PCR System 9700 (Applied Biosystems, America). The two-pair primers sequences were also shown in Figure 1, amplifying the region containing the G4C14-A4T14 polymorphism. PCR was performed in a volume of 15μl reaction mixture containing 7.5μl PCR master mixture, 2.5μl ddH_20, 1μl each of four primers (Sangon Biotech, Shanghai, China), and 1μl DNA template. The reaction for amplification was performed in the following conditions: an initial denaturation step for 5 min at 95°C, followed by 35 cycles of 40s at 95°C,40s at 60°C, and 40s at 72°Cand a final elongation for 10 min at 72°C. An aliquot (8μl) of PCR product was visualized on a 2% agarose gel. GC/GC yields two bands of 428bp and 193bp, AT /AT yields two bands of 428bp and 270bp, GC/AT heterozygosity yields three bands: 428, 270, 193bp. The genotyping by PCR-CTPP (Figure 2) was confirmed by DNA sequencing, the result of PCR-CTPP genotyping and sequencing analysis were completely consistent.

Statistical analysis

The comparison of clinical information variables between cases and controls and the distribution differences of genotype and allele was analyzed by x^2-test. The association between p73 gene polymorphism and genetic susceptibility to lung cancer was analyzed by multiple logistic regression analyses, and the P-values, odds ratio (OR), and 95% confidence intervals (CI) were calculated. x^2-tests were used to assess the relationship between the p73 genotypes and the clinicopathological characteristics of lung cancer patients. An association was considered significant at a *P*-value of<0.05, and all statistical tests

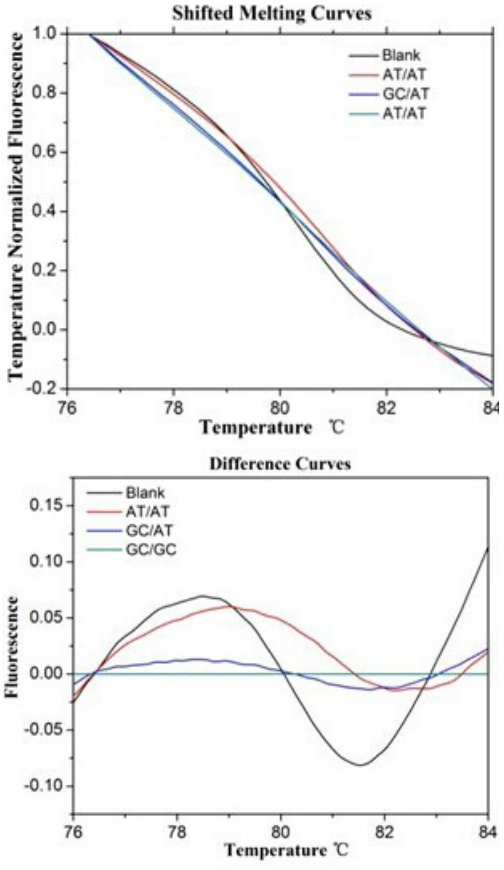

Figure 1. Melting Curve of p73 G4C14-A4T14 Polymorphism

Table 1. Primers of HRM and PCR-CTPP

Primer of HRM (5'-3')	Two pairs of primers of PCR-CTPP (5'-3')
F: CAGGAGGACAGAGCACGAG	F1: CCACGGATGGGTCTGATCC
R: CGAAGGTGGCTGAGGCTAG	R1: GGCCTCCAAGGGCAGCTT
	F2: CCTTCCTTCCTGCAGAGCG
	R2: TTAGCCCAGCGAAGGTGG

were two-sided. These analyses were performed with the SPSS software (version 17.0; SPSS Inc).

Results

In this case-control study, 168 cases and 195 cancer-free controls were recruited, the selected characteristics of the subjects were summarized in Table 2. There were no significant difference in the distributions of age and sex between cases and controls (p=0.822 for age and p=0.367 for sex). However, compared to control subjects, the cases were more likely to be smokers (69.6% in cases *vs* 42.1% in controls), the distributions of smoking status significantly differed between patients and controls (p=0.000). In the total 168 lung cancer cases, 152 (90.5%) were non-small cell lung cancer (98 squamous cell carcinomas, 54 adenocarcinoma).

In concordant with the precious studies(Kaghad et al., 1997; Li et al., 2004; Hu et al., 2005; Zhang et al., 2013), p73 G4C14-A4T14 polymorphism are in complete linkage disequilibrium, only three genotypes, GC/GC,GC/AT,AT/AT were obtained by HRM (Figure 1) and PCR-CTPP (Figure 2). PCR products were validated by DNA sequencing, and the results were consistent with above two methods (Figure 3).

We examined the association between p73 G4C14-A4T14 polymorphism and the susceptibility to lung cancer. The genotype distributions in the cases and controls are shown in Table 3. The observed genotype frequencies for the G4C14-A4T14 polymorphism were in Hardy-Weinberg equilibrium in both cases (p=0.24) and controls (p=0.74). The genotype frequencies of p73 polymorphism were 60.1%(GC/GC), 35.1%(GC/AT), and 4.8%(AT/AT) in cases and 52.3%(GC/GC), 34.9%(GC/AT), and 12.8%(AT/AT) in controls, and the frequencies was significantly different (p=0.006, x^2=7.433). As shown in Table 3, the AT allele was significantly less prevalent in lung cancer cases than in the controls (p=0.016, OR=0.622, 95%CI=0.474-0.927), suggesting that it might be a protective allele against lung cancer. Compared with the subjects carrying GC/GC genotype, the carriers of AT/AT genotype had a significantly decreased risk of lung cancer (p=0.006, OR=0.323, 95%CI=0.139-0.750), the subjects carrying GC/AT genotype also presented a reduced risk for the lung cancer, but there had no significantly difference (p=0.560, OR=0.876, 95%CI=0.562-1.366).

The association between p73 genotype and risk of lung cancer were further examined by clinical pathology variables such as histological types, tumor stage, lymph node metastasis of lung cancer. As shown in Table 4, we found no significantly difference between p73 variant genotypes and histological types (p=0.798), tumor stage (p=0.668), lymph node metastasis (p=0.578) of lung cancer.

Table 2. General Character Analysis of Lung Cancer Cases and Controls

Variable	Cases (n=168) Number	%	Controls (n=195) Number	%	*P*
Age					0.822
≤45	79	47.0	94	48.2	
>45	89	53.0	101	51.8	
Sex					0.367
Male	126	75.0	138	70.8	
Female	42	25.0	57	29.2	
Smoking status					0.000
Yes	117	69.6	82	42.1	
No	51	30.4	113	57.	
Histological type					
Squamous carcinomas	98	58.3			
Adenocarcinomas	54	32.1			
Small cell carcinomas	14	8.3			
Other carcinomas	2	1.2			

*Two-sided x^2-test

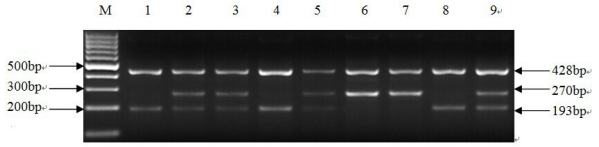

Figure 2. The Analysis for the p73 G4C14-A4T14 Polymorphism by PCR-CTPP. M=100bp marker; Lane 2, 3, 5, 9= GC/AT genotype (428, 270, 193 bp); Lane 1, 4, 8=GC/GC genotype (428, 193 bp); Lane 6, 7=AT/AT genotype (428, 270 bp)

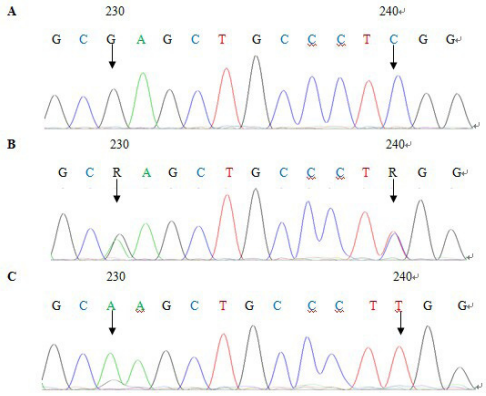

Figure 3. Sequencing Analysis for Genotypes of the P73 G4C14-A4T14 Polymorphism. A) GC/GC genotype, B) GC/AT genotype, C) AT/AT genotype

Table 3. Genotype and Allele Frequencies of P73 among Cases and Controls and Their Association with the Risk of Lung Cancer

Genotype	Cases (n=168) N (%)	Controls (n=195) N (%)	*P*-value	x^2	OR[a] (95%CI)
GC/GC	101 (60.1)	102 (52.3)			1 (reference)
GC/AT	59 (35.1)	68 (34.9)	0.560	0.34	0.876 (0.562-1.366)
AT/AT	8 (4.8)	25 (12.8)	0.006	7.433	0.323 (0.139-0.750)*
GC allele	261 (77.7)	272 (69.7)			1 (reference)
AT allele	75 (22.3)	118 (30.3)	0.016	5.823	0.622 (0.474-0.927)*

[a]ORs were adjusted for age, sex and smoking status; *P<0.05

Table 4. The Correlation Between P73 Gene Polymorphism and Clinical Pathology Parameters of Patients with Lung Cancer

	GC/GC n (%)	GC/AT n (%)	AT/AT n (%)	*P*-value
Histological type(n=152)				0.798
Squamous cell	55 (56.1)	37 (37.8)	6 (6.1)	
Adenocarcinoma	32 (59.3)	20 (37.0)	2 (3.7)	
Tumor stage(n=168)				0.668
I+II	85 (61.6)	47 (34.1)	6 (4.3)	
III+IV	16 (53.4)	12 (40)	2 (6.7)	
Lymph node metastasis(n=168)				0.578
Yes	44 (61.1)	26 (36.1)	2 (2.8)	
No	57 (59.4)	33 (34.4)	6 (6.3)	

*Two-sided x^2-test

Discussion

p73 gene is one of members of p53 family and its encoding protein has structural and functional homology of p53 (Kaghad et al., 1997), excessive expression of p73 protein can affect cell cycle and apoptosis, activate and transcript some target genes of p53 (Zhu et al., 1998; Hu et al., 2005), may be associated with the occurrence and development of human malignant tumors, therefore p73 is considered to be a candidate tumor suppressor gene. p73 initiating AUG start codon of exon 2 exists two completely linked single nucleotide polymorphism (G4C14-A4T14), can form the stem loop structure and affect gene expression (4). The studies of association between p73 G4C14-A4T14 polymorphism and the risk of lung cancer had not been frequent, the research achievements of different populations are also different. Studies have found that the carriers with AT allele genotype have significantly increased risk of lung cancer than GC/GC genotype in the north of China (Zhang et al., 2013) and non-Hispanic whites (Li et al., 2004). On the contrary, another study found that the crowd with AT allele genotype have reduced risk of lung cancer in Chinese population (Hu et al., 2005), was in accordance with our research. Other studies found no significant correlation between the polymorphism and the risk of lung cancer in Korea (Choe et al., 2006) and Japan (Hiraki et al., 2003). All results showed that the correlation of p73 G4C14-A4T114 polymorphism and lung disease might be various in different populations.

At present, there are many kinds of methods to detect gene mutations, such as PCR-RFLP, PCR-CTPP, PCR-SSCP and sequencing. The sequencing method is still the 'gold standard' testing of DNA mutations, but the process is relatively complex and expensive, can't be applied to clinical. High Resolution Melting Curve technology has many characteristics such as high specificity and convenient and high flux, becoming a hot technology in the detection of mutations in recent years (Erali et al., 2008). HRM is based on the physical properties of nucleic acids, a technique different from the conventional PCR, analyzing the difference of nucleic acid melting curve with monitoring saturated dye. The change of melting curve is decided by the sequence and length of amplification product, and the base composition (Michael et al., 2006; Maria et al., 2008; Kramer et al., 2009). This paper detected two completely linked single nucleotide polymorphism (G4C14-A4T14) of p73 gene by HRM, there had little studies to investigate two mutation sites with this method, though it did not affect the smooth of the curve, so we could detect the p73 gene polymorphism by HRM.

This study analyzed the correlation of p73 polymorphism and the risk of lung cancer by detecting the genotypes of 168 cases and 195 controls in south of China with HRM and PCR-CTPP. The carriers of AT/AT genotype had a significantly decreased risk of lung cancer compared with the subjects carrying GC/GC genotype (p=0.006, OR=0.323, 95%CI=0.139-0.750), the subjects carrying GC/AT genotype also showed a trend of decreasing risk for the lung cancer, but there had no significantly difference (p=0.560, OR=0.876, 95%CI=0.562-1.366).

In conclusion, this case-control study found the significant association between the p73 G4C14-A4T14 polymorphism and genetic susceptibility to lung cancer in Chinese population, but still couldn't confirm this correlation because of the sample size was not too much, also the specific role of p73 gene in the development of lung cancer also needs further research.

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