Association of p73 gene G4C14-A4T14 polymorphism and MDM2 gene SNP309 with non-small cell lung cancer risk in a Chinese population

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Abstract. The present study aimed to investigate the association of p73 G4C14-A4T14 polymorphism and murine double minute 2 (MDM2) 309 T/G single nucleotide polymorphisms (SNPs) with the risk of developing non-small cell lung cancer (NSCLC) in Sothern China. The p73 and MDM2 genotypes of peripheral blood DNA from 186 patients with NSCLC and 196 normal controls were detected by polymerase chain reaction (PCR) with confronting two-pair primers (CTPP) and high resolution melting (HRM), respectively. The results of genotyping were consistent with those of direct sequencing. The p73 AT/AT [odds ratio (OR)=0.46; 95% confidence interval (CI)=0.22-0.97] and MDM2 TT (OR=0.48; 95% CI=0.26-0.86) genotypes were associated with a decreased risk of developing NSCLC compared with that of the p73 GC/GC and MDM2 GG genotypes, respectively. In addition, the interaction between the p73 and MDM2 polymorphisms reduced the risk of developing NSCLC in multiple ways (OR=0.13; 95% CI=0.03-0.59) for subjects carrying both the p73 AT/AT and MDM2 TT genotypes. Therefore, the SNP in p73 G4C14-A4T14 and the MDM2 309 polymorphism may be markers of genetic susceptibility to NSCLC in a Chinese population, and there is a possible gene-gene interaction involved in the incidence of NSCLC.

Introduction

Non-small cell lung cancer (NSCLC), the main type of lung cancer, is one of the most common malignant tumors in males worldwide (1). The incidence and mortality of lung cancer is currently increasing in China, and the most common risk factors are involved environmental, occupational and genetic (2-4). Environmental factors, including tobacco smoking, air pollution, contamination in drinking water and food, are passing through the natural barriers such as shin and metabolic elimination; the environmental carcinogens enter cells, damage DNA and destroy the balance among growth, differentiation and apoptosis of the cells, which cause carcinogenesis (3,4).

It has been well established that lung cancer is a complex disease that is highly correlated with environmental pollutants in the general population; in addition, genetic factors are known to plays an important role in this disease (2-4). Studies on the association between gene variants and lung cancer will contribute to clarify the underlying mechanisms of lung cancer, including its development, and will potentially provide therapeutic targets that are important in the diagnosis and prognosis of lung cancer.

p73, one of the p53 family members, is located at the human chromosome 1p36.33, and shares relatively high structural and functional homology with p53 (5,6). As a candidate tumor-suppressor gene, it encodes two different proteins, namely the transcriptionally active full-length TAp73 and the NH_2 terminally truncated dominant-negative ΔNp73 (6), which have remarkable similarity with p53 in their DNA-binding, transactivation and oligomerization domains (5). G4C14 and A4T14, the two single nucleotide polymorphisms (SNPs) of p73 at positions 4 (G>A) and 14 (C>T) (rs2273953 and rs1801173, respectively), are located upstream of the initiating codon AUG in exon 2, and potentially influence the gene expression of p73 by forming a stem-loop structure (5,7). Various studies have investigated p73 polymorphisms and cancer risk in a variety of tumors (8-14), including lung cancer (15-17), but the conclusions of those studies were inconsistent.

The human homolog of mouse double minute 2 (MDM2) is known to act as a major negative regulator of p73. MDM2 is a nuclear phosphoprotein that binds to the N-terminal TA domain of TAp73 and inhibits the transcription of p73 by interrupting its contact with the transcription adaptors/cofactors p300 and CREB-binding protein (18-20). An important polymorphism (T>G, rs2279744), termed SNP309, has been identified to be located in the MDM2 intrinsic p53-response promoter (21-23). Cells carrying the SNP309 GG genotype were observed to exhibit an increased affinity for the transcriptional activator

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specificity protein 1 (Sp1), which resulted in higher messenger RNA and protein expression levels of MDM2 compared with those in cells carrying the SNP309 TT genotype (21,22). Those results demonstrated that SNP309 T/G was associated with a decreased response to DNA-damaging agents and an accelerated tumorigenesis in both hereditary and sporadic cancer patients (22,23), suggesting that SNP309 may contribute to individual differences in cancer susceptibility.

Several studies have shown that disruption of the p53-MDM2 interaction by the suppression of MDM2 expression can lead to p53 activation and tumor growth inhibition (21,22). The p73 and MDM2 genes are involved in the genetics of the p53 signaling pathway in various tumors; however, their role in the tumorigenesis of NSCLC remains to be clarified. In the present study, the interaction between the p73 G4C14-A4T14 and MDM2 SNP309 polymorphisms was found to effect susceptibility to NSCLC in a Southern Chinese population.

Materials and methods

Study subjects and samples. The present case-control study consisted of 186 patients with NSCLC and 196 cancer-free controls. All subjects were consecutively recruited at the Central Hospital of Zhuzhou City (Zhuzhou, China) and Hunan Provincial Tumor Hospital (Changsha, China) between March 2013 and December 2014. All patients were histopathologically confirmed and had not received preoperative chemotherapy or radiotherapy. The cancer-free controls were randomly recruited from healthy individuals who underwent a cancer screening program in the same region and during the same period than the case patients. At recruitment, informed consent forms about the study were signed by all subjects, and each participant was interviewed to collect baseline data, including demographic factors, medical history, current or past tobacco use (yes or no) and alcohol consumption (yes or no). The research protocol was approved by the Institutional Review Board of the Central Hospital of Zhuzhou City and Hunan Provincial Tumor Hospital (Zhuzhou, China).

DNA extraction. Venous blood (5 ml) from each subject was collected into Vacutainer (Hunan Ping'an Medical Machinery Technology Co., Ltd., Hunan, China) tubes containing EDTA and stored at -70˚C. The extraction of genomic DNA was performed using a Dzup (Blood) Genomic DNA Isolation Reagent (Sangon Biotech Co., Ltd., Shanghai, China), in accordance with the manufacturer's protocol.

MDM2 SNP309. Polymorphism in MDM2 was determined by high resolution melting (HRM) (24). The primers used for quantitative polymerase chain reaction (PCR) analysis were T, 5'-CTAGGGCTGCGGGGCCGATT-3'; G, 5'-GCGGGCTGG GCTAGGCTGCGGGGCCGTTG-3'; and common primer C, 5'-ACCCGACAGGCACCTGCGATC-3' (all forward). The PCR volume was 20 μl, and included SYBR-Green PCR Master Mix (Shanghai GeneCore BioTechnologies Co., Ltd., Shanghai, China; 10 μl), primer T (0.4 μl), primer G (0.1 μl), primer C (0.2 μl), double distilled (dd) H_2O (7.3 μl) and venous blood DNA (2 μl). The PCR conditions were an initial denaturation step at 95˚C for 10 min, followed by 40 cycles of 95˚C for 20 sec, 61˚C for 1 min and 72˚C for 30 sec, and a final extension step at 72˚C for 10 min. Melting curves were obtained following a denaturation period at 95˚C for 60 sec and holding for 30 sec at 60˚C, and initial and final temperatures of 65 and 90˚C, respectively, with a temperature gradient of 0.05˚C/sec. PCR and HRM analyses were conducted with the LightCyclerNano System (Roche Diagnostics, Basel, Switzerland), and the melting curve and the accuracy of genotyping data were validated by direct sequencing (Fig. 1A).

p73 G4C14-A4T14 polymorphism. Samples for the p73 G4C14-A4T14 genotype were analyzed using PCR with confronting two-pair primers (CTPP) (11,25). The two-pair primers use were forward (F) 1, 5'-CCACGGATGGGTCTG ATCC-3'; reverse (R) 1, 5'-GGCCTCCAAGGGCAGCTT-3'; F2, 5'-CCTTCCTTCCTGCAGAGCG-3'; and R2, 5'-TTAGCC CAGCGAAGGTGG-3' (11). The PCR volume was 15 μl, and contained 7.5 μl 2X Taq PCR Mastermix (Tiangen Biotech Beijing Co., Ltd., Beijing, China), 2.5 μl dd H_2O, 1 μl each of the above four primers and 1 μl venous blood genomic DNA template. The PCR assay was performed in a GeneAmp PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the PCR conditions included an initial denaturation step for 5 min at 95˚C, followed by 35 cycles of 40 sec at 95˚C, 40 sec at 60˚C and 40 sec at 72˚C, and a final elongation for 10 min at 72˚C (11). An aliquot (8 μl) of the PCR product was analyzed on 2% agarose gel with ethidium bromide (50 bp DNA Ladder Marker; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The GC homozygote is expected to produce two bands of 428 and 193 bp, respectively, while the AT homozygote is expected to produce two bands of 428 and 270 bp, respectively, and GC/AT heterozygosity is expected to produce three bands of 428, 270 and 193 bp, respectively (11,24). The results of PCR-CTPP genotyping and sequencing analysis were consistent (Fig. 1B).

Statistical analysis. Statistical analyses were performed using the statistical software SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). The frequency of polymorphisms in the p73 and MDM2 genes among cases and clinical outcome was statistically analyzed using the χ^2 test. The association between the polymorphisms of these two genes and lung cancer was determined using an unconditional logistic regression model to assess odds ratios (ORs) and 95% confidence intervals (CIs). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of the study subjects. In the present case-control study, the frequency distribution of selected characteristics of the subjects is presented in Table I. There were no significant differences in the distributions of age or sex between cases and controls ($P>0.05$). However, compared with the control subjects, the cases were more likely to be tobacco smokers ($P<0.05$), which indicated that tobacco smoking was a high-risk factor for NSCLC in the present study population.

Distribution of p73 and MDM2 polymorphisms. The genotype and polymorphic allele frequencies of the three polymorphisms

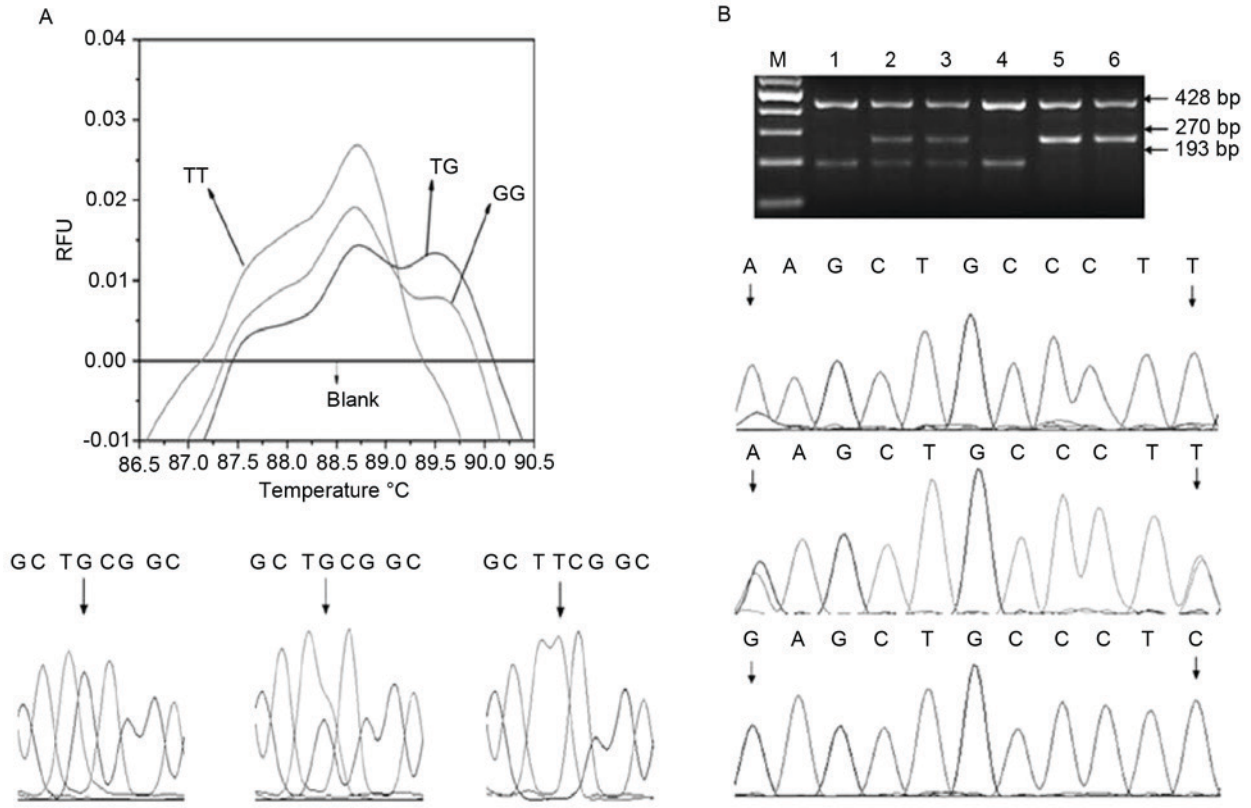

Figure 1. Polymerase chain reaction with confronting two-pair primers, high resolution melting and direct-sequencing results of MDM2 and p73 gene polymorphisms. (A) MDM2 polymorphisms: Three genotypes are indicated by arrows: TT, TG and GG. (B) p73 polymorphisms. Lanes 1 and 4, GC/GC genotype (428 and 193 bp); lanes 2 and 3, GC/AT genotype (428, 270 and 193 bp); and lanes 5 and 6, AT/AT genotype (428 and 270 bp). M, marker; MDM2, murine double minute 2.

(p73 G4C14-A4T14 and MDM2 309T/G SNPs) between the cases and controls are shown in Table II.

The frequencies of the three genotypes (GC/GC, GC/AT and AT/AT) of p73 G4C14-A4T14 polymorphism in NSCLC cases and controls were 50.54, 43.01 and 6.45%, and 50.00, 36.22 and 13.78%, respectively. There were significant differences in the genotype distributions of p73 G4C14-A4T14 polymorphism between cases and controls (P<0.05). The frequencies of the three genotypes (GG, TG and TT) of MDM2 309T/G SNPs in NSCLC cases and controls were 31.18, 51.61 and 17.21%, and 22.45, 51.53 and 26.02%, respectively. A similar result was obtained with the MDM2 309T/G SNPs (P<0.05). Furthermore, the frequencies of the p73 AT and MDM2 G alleles were 27.96 and 56.99%, respectively, among cases, and 31.89 and 48.21%, respectively, among controls. The frequencies the G allele of MDM2 309T/G SNPs were significantly different between cases and controls (P<0.05). By contrast, there was no difference in the frequencies the AT allele of p73 G4C14-A4T14 (P>0.05).

Association between p73 and MDM2 polymorphisms and NSCLC risk. An unconditional logistic regression model was used to estimate the association between genotypes and the risk of developing NSCLC (Table II). The p73 AT/AT genotype was associated with a decreased risk of developing NSCLC (OR=0.46; 95% CI=0.22-0.97), compared with that of the GC/GC genotype. Similarly, the MDM2 TT genotype was also associated with a decreased risk of developing NSCLC

Table I. Demographic characteristics of non-small cell lung cancer cases and controls.

Characteristics	Cases, n (%)	Controls, n (%)	P-value[a]
Total	186	196	
Age, years			0.99
≤45	90 (48.39)	95 (48.47)	
>45	96 (51.61)	101 (51.53)	
Sex			0.49
Male	136 (73.12)	137 (69.90)	
Female	50 (26.88)	59 (30.10)	
Cigarette smoking			<0.01
No	54 (29.03)	115 (58.67)	
Yes	132 (70.97)	81 (41.33)	
Alcohol consumption			0.95
No	122 (65.59)	128 (65.31)	
Yes	64 (34.41)	68 (34.69)	
Histology			
Adenocarcinoma	108 (58.06)	0	
Squamous cell	60 (32.26)	0	
Carcinoma			
Others	18 (9.68)	0	

[a]Two-sided χ^2 test.

Table II. Genotype and allele frequencies of p73 and MDM2 among cases and controls, and their association with the risk of developing non-small cell lung cancer.

Genotype	Cases, n (%) (n=186)	Controls, n (%) (n=196)	P-value	OR (95% CI)[a]
p73 G4C14-A4T14				
GC/GC	94 (50.54)	98 (50.00)	-	1.00 (Reference)
GC/AT	80 (43.01)	71 (36.22)	0.46	1.18 (0.77-1.80)
AT/AT	12 (6.45)	27 (13.78)	0.04	0.46 (0.22-0.97)[b]
AT allele frequency	104 (27.96)	125 (31.89)	-	1.00 (Reference)
GC allele frequency	268 (72.04)	267 (68.11)	0.24	1.21 (0.88-1.65)
MDM2 309 T/G				
GG	58 (31.18)	44 (22.45)	-	1.00 (Reference)
TG	96 (51.61)	101 (51.53)	0.18	0.72 (0.45-1.17)
TT	32 (17.21)	51 (26.02)	0.01	0.48 (0.26-0.86)[b]
G allele frequency	212 (56.99)	189 (48.21)	-	1.00 (Reference)
T allele frequency	160 (23.01)	203 (51.79)	0.02	0.70 (53-0.94)[b]

[a]ORs were adjusted for age, sex, cigarette smoking and alcohol consumption. [b]P<0.05. OR, odds ratio; CI, confidence interval; MDM2, murine double minute 2.

Table III. Risk of lung cancer in association with MDM2 SNP309 and p73 G4C14-to-A4T14 polymorphism.

p73 genotype	MDM2 genotype	Cases, n (%) (n=186)	Control, n (%) (n=196)	P-value	OR (95% CI)[a]
GC/AT + GC/GC	GG + TG	144 (77.42)	132 (67.35)	-	1.00 (Reference)
GC/AT + GC/GC	TT	30 (16.13)	37 (18.88)	0.28	0.74 (0.44-1.27)
AT/AT	GG + TG	10 (5.38)	13 (6.63)	0.42	0.71 (0.30-1.66)
AT/AT	TT	2 (1.07)	14 (7.14)	<0.01	0.13 (0.03-0.59)[b]

[a]ORs were adjusted for age, sex, cigarette smoking and alcohol consumption. [b]P<0.05. OR, odds ratio; CI, confidence interval; MDM2, murine double minute 2.

(OR=0.48; 95% CI=0.26-0.86), compared with that of the GG genotype. However, the heterozygous genotypes for both polymorphisms (p73 GC/AT or MDM2 TG) were not associated with such risk. p73 GC/AT and GC/GC, or MDM2 TG and GG, were combined the into one group for subsequent analysis. The present study examined whether there was a statistical combination effect between the p73 and MDM2 polymorphisms (Table III). It was observed that controls carrying the p73 AT/AT genotype were more likely to carry the MDM2 TT genotype than the cases (7.14 vs. 1.07%, respectively; P<0.05). Furthermore, the OR significantly decreased to 0.13 (95% CI=0.03-0.59) among subjects carrying both the p73 AT/AT and MDM2 TT genotypes.

Discussion

The present study is a case-control study aimed to detect the association between the p73 G4C14-A4T14 and MDM2 SNP309 polymorphisms, alone or under interaction, and the risk of developing NSCLC in a Southern Chinese population. In the present hospital-based analysis, it was demonstrated that both p73 and MDM2 polymorphisms were associated with a decreased risk of developing NSCLC. Furthermore, the interact was detected between the p73 AT/AT and MDM2 TT genotypes using a multiplicative manner.

p73 has structural and functional homology with the p53 gene, and induces cell cycle arrest or apoptosis in response to DNA damage (26,27). A dinucleotide polymorphism at positions 4 and 14 (G4C14-A4T14) is located at exon 2 of the p73 gene, which possibly forms a stem-loop structure to influence p73 translation and gene expression (5). Although the association between the p73 G4C14-A4T14 polymorphism and the risk of developing cancer has been investigated in a variety of tumors, the results were not consistent (8-17,28,29). Niwa *et al* (12) and Zhang *et al* (17) reported that the AT/AT genotype was associated with a significantly increased risk of developing endometrial cancer and lung in Japanese (12) and Northern Chinese (17) populations, respectively. However, Hamajima *et al* (28) did not identify any associations between this polymorphism and the risk of developing digestive tract cancers, including colorectal cancer, in a Japanese population. Similar results were obtained with lung cancer in Korea (29).

On the contrary, Hu *et al* (16) reported that the AT allele of p73 was associated with a significantly decreased risk of developing lung cancer in a Chinese population. In accordance with this result, the present study also observed a significant association between the p73 G4C14-A4T14 polymorphism and the risk of developing NSCLC, and the frequency of the p73 GC allele among the healthy Chinese was 0.707, which was similar to that of the healthy Japanese (12). Besides, there was a significant interaction between p73 G4C14-A4T14 and MDM2 309T/G SNPs in NSCLC in a Southern Chinese population.

The 309T/G SNP is a common SNP in the promoter region of the MDM2 gene, which has been demonstrated to increase MDM2 expression by increasing the binding affinity of Sp1 to the promoter of MDM2 (22,30), and represses the transcriptional activity of p73, thus attenuating its activity in G1 cell-cycle control and apoptosis (18-20). It has been reported that individuals carrying the G allele of MDM2 SNP309 were more sensitized to develop both hereditary and sporadic cancers (22). Various studies have reported an increased risk of developing lung cancer for the carriers of the G allele in Korean and Chinese populations (31,32). Previous studies confirmed that the G allele of MDM2 SNP309 was associated with higher expression levels of MDM2 protein (21). In addition, various studies failed to show a significant association between the G allele of MDM2 SNP309 and the risk of developing breast (33,34) or lung cancer (35). In the present case-control study, it was investigated whether the MDM2 309T/G SNPs were associated with the risk of developing NSCLC in a Southern Chinese population. The results revealed that the frequencies of the three genotypes and the allele distributions were significantly different between the NSCLC patients and the healthy controls. In addition, there was a significant difference between the GG and TT genotypes, and subjects carrying the T allele had a significantly decreased risk of developing NSCLC in the current Southern Chinese population compared with that of patients carrying the GG genotype, which was consistent with the results of Jun *et al* (31). Therefore, these findings further supported the present result that the MDM2 SNP309 T allele may reduce the risk of developing NSCLC. It could be hypothesized that different molecular pathogeneses in different tumors may cause this discrepancy, which would require further study.

The present study examined whether the p73 gene G4C14-A4T14 polymorphism and the MDM2 gene SNP309 were associated with the risk of developing NSCLC in a Chinese population. Compared with the p73 GC/AT + GC/GC and MDM2 GG + TG genotypes, there was a significant difference in the p73 AT/AT and MDM2 TT genotypes with NSCLC in a Chinese population, and the OR was significantly decreased to 0.13 (95% CI=0.03-0.59). Therefore, the present results revealed that there was a significant interaction between p73 G4C14-to-A4T14 and MDM2 309T/G SNPs in NSCLC patients within the present Southern Chinese population. However, the small sample size used in the present study caused broad CIs in the regression model. Thus, further larger population-based studies are required to confirm the association of these two polymorphisms with the risk of developing NSCLC in other populations.

In conclusion, the present study demonstrated a significant association in the decreased risk of developing NSCLC between p73 AT/AT and MDM2 TT genotypes. Furthermore, the p73 G4C14-A4T14 and MDM2 309 T/G SNPs possibly had a interaction on the lower risk of developing NSCLC, and these findings may be a protective factor for NSCLC development in Chinese populations.

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