Correlation of p21 Gene Codon 31 Polymorphism and TNF- α Gene Polymorphism With Nasopharyngeal Carcinoma

Ming-Hsui Tsai,¹ Wen-Chi Chen,^{2,3} and Fuu-Jen Tsai^{2,4*}

¹Department of Otolaryngology, China Medical College Hospital, Taichung, Taiwan

²Department of Medical Genetics, China Medical College Hospital, Taichung, Taiwan

³Department of Urology, China Medical College Hospital, Taichung, Taiwan

⁴Department of Pediatrics, China Medical College Hospital, Taichung, Taiwan

Background p21 (WAF1/CIP1) is a downstream protein from p53 and can arrest the cell cycle at the G1/S phase in response to signal from p53. The most frequently seen polymorphic site is at codon 31, where a base change from AGC to AGA causes an amino acid change from serine to arginine. Tumor necrosis factor- α (TNF- α) is a cytokine that is secreted from macrophages, and is related to a sequence of events in the response to inflammation and cancer formation. The TNF- α gene promoter –308 G/A polymorphism has been reported to be associated with some cancers. In this study, these polymorphisms were proposed to be a candidate genetic marker of nasopharyngeal carcinoma (NPC). The distribution was analyzed in 47 NPC patients and a control group of 119 healthy people. The association of the p21 codon 31 polymorphism with NPC was detected by polymerase chain reaction (PCR) and restriction

analysis by *Bsp* I endonuclease, and calculated by the chi-square test. The TNF- α gene promoter –308 G/A polymorphism was identified by *Nco* I endonuclease. The distribution of the gene p21 codon 31 polymorphisms showed no significant difference between the two groups. The serine form of p21 codon 31 was more prominent in smokers than nonsmokers among the NPC patients ($P < 0.05$). There was no significant difference in the distribution of TNF- α gene promoter –308 G/A polymorphism between control and cancer patients. The results indicate that the gene p21 codon 31 polymorphism and TNF- α promoter –308 polymorphism are not correlated with NPC. However, the difference between smokers and nonsmokers suggests that an environmental factor may be involved in association with the p21 gene in the formation of NPC. *J. Clin. Lab. Anal.* 16:146–150, 2002. © 2002 Wiley-Liss, Inc.

Key words: nasopharyngeal carcinoma; NPC; genetic polymorphism; p21 gene

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an epithelial tumor with a high incidence rate in southern China. NPC is a multifactorial, complex disease involving environmental factors. The etiology of NPC includes Epstein-Barr virus infection, consumption of salted fish and preserved foods, and smoking (1). Genetic involvement in human NPC has been reported, but no major role has been found in serial studies (2,3). Evidence from previous studies suggests that a gene(s) closely linked to the HLA class I loci on chromosome 6p21.2-3 is involved in the development of NPC (4). Recently, a genetic polymorphism was used as a tool in the search for genetic variations and environmental influences in this complex disease gene (5). Hence, this method could be applied to the search for genetic polymorphisms at the short arm of chromosome 6.

P21 (WAF1/CIP1) is a cyclin-dependent kinase inhibitor (CDKI) that regulates the cell cycle (6). The human p21 gene

is localized at chromosome 6p21.2 and consists of three exons of 68, 450, and 1,600 base pairs. The p21 gene encodes a 21 kDa protein with two protein-binding domains: an N-terminal domain which binds and inhibits cyclin-cdk complexes, and a short sequence near the c-terminus (between amino acids 144–151) which binds to the proliferating cell nuclear antigen (PCNA), resulting in the inhibition of DNA replication (7,8). Expression of p21 inhibits PCNA-dependent DNA replication and mismatch repair in vitro and can suppress tumor growth, indicating that it acts as a tumor suppressor gene (9).

*Correspondence to: Fuu-Jen Tsai, M.D., Ph.D., Department of Medical Genetics and Pediatrics, China Medical College Hospital, No. 2 Yu-Der Road, Taichung, 404, Taiwan. E-mail: d0704@www.cmch.org.tw

Received 21 March 2001; Accepted 11 February 2002

DOI: 10.1002/jcla.10032

Published online in Wiley InterScience (www.interscience.wiley.com).

Reported mutations in p21 are rare, although multiple polymorphisms have been reported (10). The most frequently seen polymorphism is in codon 31, with a base change from AGC to AGA resulting in an amino acid change from serine to arginine (11). In 158 patients with brain tumors, the allelic frequency for codon 31 (sequence from AGC to AGA) was 91% and 9%, respectively (10). Reported cancers related to these polymorphisms include brain tumors, colorectal cancer, and esophageal cancers (10–12).

Tumor necrosis factor- α (TNF- α) is a cytokine that is produced by macrophages and monocytes. TNF- α has been postulated to play a role in cancer formation. Results from different cancer cell types in the study of TNF- α revealed both tumor-necrotic and tumor-promoting activities (13). TNF- α has been suggested to play a role in the initiation of an androgen-independent state in prostate cancer through its ability to inhibit androgen receptor activity in prostate cancer (14). DeMiguel et al. (15) found an inverse correlation between TNF- α receptor 2 expression and cell proliferation. Therefore, TNF- α contributes to the cancer progression in some cell types. The TNF- α gene is located at 6q21.3 and a polymorphism involves a single base change from G to A at position -308 in the promoter region of the gene have detected by Wilson et al. (16).

In this study we used a polymerase chain reaction (PCR)-based restriction analysis to investigate the distribution of p21 gene codon 31 polymorphism and TNF- α gene -308 G/A polymorphism between normal control patients and patients with NPC.

MATERIAL AND METHODS

Patient Selection

A total of 47 NPC patients (33 males and 14 females) ranging in age from 27 to 80 years (average age: 49.7 years) were enrolled in this study. A history of cigarette smoking was recorded for 21 of these patients (all male); 26 were nonsmokers. As the control group, 119 healthy volunteers were enrolled in the study. There were 42 males and 77 females in the control group (age range: 42–73 years; average: 55.5 years). All were nonsmokers with no familial history of cancer. Informed consent was obtained from both groups. The genomic DNA was prepared from peripheral blood by use of a DNA Extractor Genomaker kit (Bloosm, Taipei, Taiwan).

PCR

PCRs were carried out in a total volume of 50 μ l, containing 50 ng genomic DNA, 2–6 pmole of each primer, 1 $\times$ Taq polymerase buffer (1.5 mM MgCl₂), and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA). The primer for p21 codon 31 was designed from codon 1 start (5'-GTCAGAACCGGCTGGGGATG-3') to codon 91 (5'-CTCCTCCCAACTCATCCCGG-3'), according to the procedure

described by Li et al. (11). The primer for TNF- α promoter -308 was designed as (5'-AGGCAA TAGGTTTTGAGGGCCAT-3') and (5'-ACACTCCCCATCCTC CCGGCT-3'), according to the procedure described by Galbraith et al. (17). PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (Perkin Elmer). The cycling condition for codon 31 was set as follows: one cycle at 94°C for 5 min, 35 cycles of 94°C for 30 sec, 57°C for 40 sec, 72°C for 40 sec, and one final cycle of extension at 72°C for 7 min. Conditions for the TNF- α gene in PCR were the same as for the p21 gene, except that the annealing temperature was 60°C.

PCR-Based Restriction Analysis

The PCR products of 272 bp (p21) were mixed with 2 U of *Bsp* I (New England Biolabs, Beverly, MA) and a reaction buffer according to the manufacturer's instructions. A restriction site located at the frequently-seen allele of codon 31 (AGC) was designated to form an excisable site. Two fragments of 89 bp and 183 bp were present if the product was digestible. The reaction was incubated for 3 hr at 37°C. Subsequently, 10 μ l of the products were loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was identified as digested (AGC, serine) or undigested (AGA, arginine) homozygotes and heterozygotes (AGC/AGA). The PCR product of 117-bp (TNF- α) was mixed with 2 U *Nco* I (New England Biolabs) and the reaction buffer according to the manufacturer's instructions. The restriction site was designed to be located at the frequently-seen allele of promoter -308 (A) to form a digested site. If two fragments of 97-bp and 20-bp are present, the product can be digested.

The chi-square test was used for the statistical analysis of the polymorphisms. Means and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for the polymorphism of p21 codon 31 and TNF- α gene -308. When the assumption of the chi-square test was violated (i.e., when one cell had an expected count of <1, or >20% of the cells had an expected count of <5), the Fisher's exact test was used. Results were considered statistically significant when the probability of findings occurring by chance was less than 5% ($P < 0.05$).

RESULTS

Using the chi-square test, we compared the distribution of the gene p21 codon 31 polymorphism. The proportions of NPC among different gene types showed no significant difference ($P = 0.904$). The proportions of serine homozygotes/heterozygotes/arginine homozygotes in NPC and normal control populations were 17.7/51.4/31.9% and 14.9/53.2/31.9%, respectively (Table 1). Electrophoresis revealed homozygotes of AGC, AGA, and heterozygotes (AGC/AGA) (Fig. 1). Using the serine/arginine heterozygote at codon 31 of gene p21 as a standard, the

TABLE 1. The distribution of p21 codon 31 polymorphism between healthy control subjects and NPC patients

	C/C	C/A	A/A	Total	P-value
Control					
Female	13	40	24	77	
Male	8	20	14	42	
Total	21	60	38	119	
	(17.7%)	(51.4%)	(31.9%)	(100%)	
NPC					
Female	0	9	5	14	
Male	7	16	10	33	
Total	7	25	15	47	0.904 ^a
	(14.9%)	(53.2%)	(31.9%)	(100%)	

^a χ^2 test = 0.202.

OR for serine homozygote was 0.80, with 95% CI of 2.217 ~ 0.288. The OR for the arginine homozygote was 0.94 (95% CI = 2.141 ~ 0.419). There was a significant difference in the distribution of the gene p21 codon31 polymorphism between smokers and nonsmokers among NPC patients ($P < 0.05$). The frequency of the serine homozygote was higher in smokers than nonsmokers (28.6% vs. 3.8%) (Table 2).

The frequencies of the genotype of the TNF- α gene -308 in the NPC and control group are shown in Table 3. The allelic frequency of the A/G polymorphism was 95–5% in normal individuals and 90.4–9.6% in the NPC patient group. There was no statistical difference in the distribution of allelic frequency between the control group and NPC patients ($P = 0.85$). The distribution of the A/G polymorphism in smokers and nonsmokers showed no statistical difference ($P = 1.0$).

Fig. 1. The analysis of p21 codon 31 polymorphism by 3% agarose electrophoresis resulted in a digested form of homozygotes (183-bp and 89-bp) (lane 1), an undigested form of homozygotes (272-bp) (lane 2), and a form of heterozygotes (lane 3). M: marker; 100-bp ladder.

TABLE 2. The distribution of polymorphisms in gene p21 codon 31 between smokers and non-smokers in NPC patients

	C/C	C/A	A/A	Total	P-value
Control	21	60	38	119	0.166 ^a
NPC patients					
Non-smoker	1	17	8	26	
	(3.8%)	(65.4%)	(30.8%)	(100%)	
Smoker	6	8	7	21	0.040 ^b
	(28.6%)	(38.1%)	(33.3%)	(100%)	

^a χ^2 test = 3.587, comparison between control and non-smoker of NPC patients.

^b χ^2 test = 6.419, comparison between non-smoker and smoker of NPC patients.

DISCUSSION

Sun et al. (18) reported a striking difference between Caucasians and Chinese in the distribution of p21 codon31 polymorphism genotypes. In this study there was a higher percentage of heterozygotes in the Chinese than in the Northern American subjects. Our result was not completely compatible with Sun et al.'s report, as our patient group had a relatively lower incidence of heterozygotes (85% vs. 51 ~ 53%). The distribution of heterozygotes (47.1% of control group) was closer to that in Shih et al.'s report (19), which was obtained from same region as our study.

P21 is situated on the short arm of chromosome 6, and loss of heterozygosity in this region has been reported in patients with colon, lung, ovary, and renal cancers (20–23). Although the overexpression of p21 in urothelial cell carcinoma was not correlated with tumor progression (24), a >50% overexpression of p21 in tumor cells suggested that the expression of the p21 gene may be involved in tumor formation. Therefore, the search for mutations within the p21 sequence has been carried out in colorectal cancer, brain tumors, and esophageal cancers. However, instead of mutations, two different DNA variants have been identified at codon31 and 91. Because the polymorphisms of codon 31 are more frequently seen in p21 than in 91, and the haplo-

TABLE 3. The distribution of TNF- α gene -308 A/G polymorphism in NPC patients*

	A/A	A/G	GG	Total	P-value
Control	124	11	1	136	0.205 ^a
	(82.7%)	(16.7%)	(0.6%)	(100.0%)	
NPC patients	38	9	0	47	0.85 ^b
	(80.9%)	(19.1%)	(0.0%)	(100.0%)	
Smoker	17	4	0	21	1.00 ^c
	(81.0%)	(19.0%)	(0.0%)	(100.0%)	
Non-smoker	21	5	0	26	
	(80.8%)	(19.2%)	(0.0%)	(100.0%)	

*Fisher's exact test.

^aComparison between control and non-smoker of NPC patients.

^bOverall comparison between control and NPC patients.

^cComparison between non-smoker and smoker of NPC patients.

Control group, A:G = 95%:5%; NPC patients, A:G = 90.4%:9.6%.

type frequency is 18–8.5% from serial reports (10–12), this polymorphism is more suitable as a genetic marker to study the possible causes of NPC.

Although no correlation of gene p21 codon 31 polymorphism with NPC has been demonstrated, our data indicate that this polymorphism may be a candidate genetic marker for screening NPC risk in association with smoking. The fact that the serine form was more prominent in smokers within NPC groups suggests that there may be an association. The information will help us to define the environmental factors involved in NPC development. Although the negative associated polymorphism of the p21 gene with NPC is clearly shown in this study, it does not represent other ethnic groups or other factors without such association. Further studies of the risk factors in association with the p21 gene are needed.

The data show that the TNF- α gene -308 A/G polymorphism is not a candidate for use as a genetic marker in screening for NPC. The distribution of the frequency in A/G polymorphism was similar in NPC compared with the control group. Due to the lack of any previous reports on this polymorphism in the NPC, thus other polymorphism over this gene should be further investigated when considering the association.

TNF- α plays a role in some cancer formations, by many pathways. Brenner et al. (25) demonstrated that TNF- α stimulates the activation of oncogene JUN expression, and that collagenase gene expression may be one mechanism by which some of its biological effects are mediated. TNF- α promotes tumor implantation by regulating the secretion of metalloproteinase, and is related to the invasion of ovarian cancer (26,27). Okamoto et al. (28) reported that TNF- α induces transformation of urothelial cells and may involve carcinogenesis (28). Furthermore, the TNF- α gene is located in the central major histocompatibility complex in chromosome 6, and may be associated with disease susceptibility (29,30). The -308 G/A polymorphism may alter TNF- α gene expression (31), and has been associated with various cancers (32,33). Therefore, this polymorphism was chosen in the present study to test for an association with NPC.

In conclusion, there are differences between NPC patients who smoke and nonsmokers in the distribution of the gene p21 polymorphism. Therefore, the arginine form of gene p21 codon 31 may be associated with some determinants of NPC in response to environmental factors.

REFERENCES

- Hildesheim A, Levine PH. Etiology of nasopharyngeal carcinoma: a review. *Epidemiol Rev* 1993;15:466–485.
- Sun Y, Nakamura K, Wendel E, Colburn N. Progression toward tumor cell phenotype is enhanced by overexpression of a mutant p53 tumor-suppressor gene isolated from nasopharyngeal carcinoma. *Proc Natl Acad Sci U S A* 1993;90:2827–2831.
- Sun Y, Hegamyer G, Colburn NH. Nasopharyngeal carcinoma shows no detectable retinoblastoma gene alterations. *Oncogene* 1993;8:791–795.
- NIH/CEPH Collaborative Mapping Group. A comprehensive genetic linkage map of the human genome. *Science* 1992;258:67–86.
- Lorder N. Genetic variations can point the way to disease gene. *Nature* 1999;401:734.
- el-Deiry WS, Tokino T, Waldman T, et al. Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. *Cancer Res* 1995;55:2910–2914.
- Zhang H, Xiong Y, Beach D. Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinases complexes. *Mol Biol Cell* 1993;4:897–906.
- Xiong Y, Zhang H, Beach D. D type cyclins associated with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* 1992;71:505–514.
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature* 1993;366:701–704.
- Koopmann J, Maintz D, Schild S, et al. Multiple polymorphisms, but no mutations, in the WAF1/CIP1 gene in human brain tumors. *Br J Cancer* 1995;72:1230–1233.
- Li YJ, Laurent-Puig P, Salmon RJ, Thomas G, Hamelin R. Polymorphisms and probable lack of mutation in the WAF1-CIP1 gene in colorectal cancer. *Oncogene* 1995;10:599–601.
- Bahl R, Arora S, Nath N, Mathur M, Shukla NK, Ralhan R. Novel polymorphism in p21 waf1/cip1 cyclin dependent kinase inhibitor gene: association with human esophageal cancer. *Oncogene* 2000;19:323–328.
- Rossler K, Suchanek G, Breitschopf H, et al. Detection of tumor necrosis factor- α protein and messenger RNA in human glial brain tumors. Comparison of immunohistochemistry with in situ hybridization using molecular probes. *J Neurosurg* 1995;83:291–297.
- Mizokami A, Gotoh A, Yamada H, Keller ET, Matsumoto T. Tumor necrosis factor- α represses androgen sensitivity in the LNCaP prostate cancer cell line. *J Urol* 2000;164:800–805.
- deMiguel MP, Royuela M, Bethencourt FR, Santamaria L, Fraile B, Paniagua R. Immunoeexpression of tumor necrosis factor- α and its receptors 1 and 2 correlates with proliferation/apoptosis equilibrium in normal, hyperplastic and carcinomatous human prostate. *Cytokine* 2000;12:535–538.
- Wilson AG, Giovane FS di, Blakemore AIF, Duff GW. Single base polymorphism in the human tumor necrosis factor alpha (TNF- α) gene detectable by *Nco*I restriction of PCR product. *Hum Mol Genet* 1992;1:353.
- Galbraith GMP, Pandey JP. Tumor necrosis factor alpha (TNF- α) gene polymorphism in alopecia areata. *Hum Genet* 1995;96:433–436.
- Sun Y, Hildesheim A, Li H, et al. No point mutation but a codon 31 ser $\rightarrow$ arg polymorphism of the WAF-1/CIP-1/p21 tumor suppressor gene in nasopharyngeal carcinoma (NPC): the polymorphism distinguishes Caucasians from Chinese. *Cancer Epidemiol Biomark Prev* 1995;4:261–267.
- Shih CM, Lin PT, Wang HC, Huang WC, Wang YC. Lack of evidence of association of p21WAF1/CIP1 polymorphism with lung cancer susceptibility and prognosis in Taiwan. *Jpn J Cancer Res* 2000;91:9–15.
- Vogelstein B, Fearon ER, Kern SE, et al. Allelotype of colorectal carcinomas. *Science* 1989;244:207–211.
- Lukeis R, Irving L, Garson M, Hasthorpe S. Cytogenetics of non-small cell lung cancer: analysis of consistent non-random abnormalities. *Gen Chrom Cancer* 1990;2:116–124.
- Morita R, Ishikawa J, Tsutsumi M, et al. Allelotype of renal cell carcinoma. *Cancer Res* 1991;51:820–823.
- Sato T, Salto H, Morita R, Koi S, Lee JH, Nakamura Y. Allelotype of human ovarian cancer. *Cancer Res* 1991;51:5118–5122.
- Lin CS, Kuo HH, Chen JY, Yang CS, Wang WB. Epstein-Barr virus nuclear antigen 2 retards cell growth, induces p21(WAF1) expression, and modulates p53 activity post-translationally. *J Mol Biol* 2000;303:7–23.
- Brenner DA, O'Hara M, Angel P, Chojkier M, Karin M. Prolonged activation of JUN and collagenase genes by tumour necrosis factor- α . *Nature* 1999;337:661–663.
- Honchel R, McDonnell S, Schaid DJ, Thibodeau SN. Tumor necrosis

- factor-alpha allelic frequency and chromosome 6 allelic imbalance in patients with colorectal cancer. *Cancer Res* 1996;56:145–149.
27. Mann EA, Hibbs MS, Spiro JD, et al. Cytokine regulation of gelatinase production by head and neck squamous cell carcinoma; the role of tumor necrosis factor-alpha. *Ann Otol Rhinol Laryngol* 1995;104:203–209.
 28. Okamaoto M, Oyasu R. Transformation in vitro of a nontumorigenic rat urothelial cell line by tumor necrosis factor-alpha. *Lab Invest* 1997; 77:139–144.
 29. Hawrami K, Hitman GA, Rema M, et al. An association in non-insulin-dependent diabetes mellitus subjects between susceptibility to retinopathy and tumor necrosis factor polymorphism. *Hum Immunol* 1996; 46:49–54.
 30. Wingerchuck D, Liu Q, Sobell J, Sommer S, Weinshenker BG. A population-based case-control study of the tumor necrosis factor alpha-308 polymorphism in multiple sclerosis. *Neurology* 1997;49: 626–628.
 31. Kroeger KM, Carville KS, Abraham LJ. The -308 tumor necrosis factor-alpha promoter polymorphism effects transcription. *Mol Immunol* 1997;34:391–399.
 32. Warzocha K, Ribero P, Bienvenu J, et al. Genetic polymorphisms in the tumor necrosis factor locus influence non-Hodgkin's lymphoma outcome. *Blood* 1998;91:3574–3581.
 33. Park KS, Mok JW, Rho SA, Kim JC. Analysis of TNFB and TNFA NcoI RFLP in colorectal cancer. *Mol Cells* 1998;8:246–249.