Presence of p53 Mutations in Primary Nasopharyngeal Carcinoma (NPC) in Non-Asians of Los Angeles, California, A Low-Risk Population for NPC[1]

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Abstract

Mutatins of the *p53* tumor suppressor gene are rare in nasopharyngeal carcinoma (NPC) patients who reside in high-risk areas, such as Southeastern China. Among this high-risk group, a pre-existing infection with the EBV and consumption of Cantonese salted fish are closely associated with NPC. We investigated the prevalence of p53 mutations in 28 primary NPC specimens from white (including Hispanic) and African-American patients in Los Angeles, who are at low risk for NPC. Using PCR-based single-strand conformational polymorphism and direct sequencing, we found four mutations (14%) in exons 5–8 of the *p53* gene in four patients. All were C-to-T transition mutations: two were present in exon 5—one at codon 142 [CCT (Pro) → CTT (Leu)] and another at codon 144 [CAG (Gln) → TAG (stop codon)]. The other two mutations were identified in exon 8: one at codon 273 [CGT (Arg) → CAT (His)], a CpG site, and one at codon 271, a silent mutation [GAG (Glu) → GAA (Glu)]. This is the first report investigating the presence of p53 missense mutations in NPC among a low-risk population. Our data indicate that p53 is also an infrequent event among NPC patients at low risk for the disease.

Introduction

Unlike other primary head and neck cancers, the majority of NPCs[7] are poorly or undifferentiated tumors, and they are seen in younger patients in endemic areas (1). There is a consistent male preponderance in incidence, with a male:female ratio of about 2–3:1, and the disease has a marked geographic and ethnic variation in prevalence. Except for a handful of populations (described below), NPC is a rare malignancy with an incidence of less than 1/100,000 population per year in most parts of the world. The high-risk populations include Chinese (especially those in the southern provinces of Guangxi, Guandong, Hunan, Fujian, and Taiwan); the indigenous populations of Southeast Asia; Eskimos and other natives of the Arctic region; and Arab populations of Morocco, Algeria, Tunisia, Sudan, and Saudi Arabia (2). The highest known incidence rate worldwide is found among the Cantonese who reside in central Guandong Province, where males exhibit incidence rates of 25–40/100,000 person-years (3). In contrast, the age-standardized (world population) incidence rate in white and African-American males in the United States during 1973–1986 were 0.5 and 0.8 per 100,000 person-years, respectively (4).

The pathogenesis of NPC is multifactorial, and the molecular mechanism(s) involved in its oncogenesis is not well understood. Specific molecular changes, *i.e.*, frequent loss of heterozygosity at 3p14–16 (5), loss of heterozygosity at 9p21–22 (6), and overexpression of *ras p21* and c-*myc* oncogenes (7) have been associated with NPC. Genetic predisposition is a known risk factor, as indicated by positive associations between the risk of NPC and a susceptibility gene closely linked to the *HLA* locus (8), certain specific HLA-A and -B antigens (9, 10), and specific T-cell receptor gene polymorphisms (11). In addition, NPC has been linked to environmental exposures, including a pre-existing infection with the EBV (12); intake of Cantonese-style salted fish and other preserved foods, especially during childhood (13–15); cigarette smoking (16); and exposure to formaldehyde (17–19).

Mutations in the *p53* tumor suppressor gene are among the most frequently observed genetic changes in human cancers, and epidemiological studies comparing the p53 mutational spectra observed between different regions and populations have been informative in the identification of specific risk factors (20). Although NPC is one of the most prevalent tumors among Cantonese, mutations in the *p53* tumor suppressor gene

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[7] The abbreviations used are: NPC, nasopharyngeal carcinoma; SCC, squamous cell carcinoma; SSCP, single-strand conformational polymorphism.

are rare in NPCs from this high-risk population (21). Yu *et al.* have shown that childhood consumption of salted fish is a major risk factor for NPC among Cantonese, possibly relating to 90% of the cases occurring in that high-risk population (13, 14).

We investigated whether mutations in the *p53* gene play a role in the etiopathogenesis of NPC among individuals whose diet is devoid of Cantonese-style salted fish and who are at low risk for the disease. Thus, we analyzed the p53 mutational spectrum in primary tumor specimens from 28 white (including Hispanic) and African-American patients in the United States with NPC, who were diagnosed at the Los Angeles County/University of Southern California Medical Center in Los Angeles County, California.

Materials and Methods

Subjects. We studied 28 histologically confirmed cases of NPC in African-American and white (including Hispanic) patients, who were diagnosed between 1970 and 1990 at the Los Angeles County/University of Southern California Medical Center. Cases were identified through the Los Angeles County Cancer Surveillance Program (22). The Cancer Surveillance Program is a population-based cancer registry that records all cases of cancer verified microscopically or recorded on a death certificate. For each case, the diagnosis, residence at the time of diagnosis, age at diagnosis, pathology, tumor stage, sex, and ethnicity were available.

The median age at diagnosis of the cases was 50 years (range, 15–77; mean, 46.7). The male:female ratio was 2.4, with 19 males and 8 females. There were 9 African Americans, 11 non-Hispanic whites, and 7 Hispanic whites. The tumors analyzed were paraffin-embedded, surgically obtained diagnostic specimens. All specimens were carcinomas according to the WHO classification (23), and the differentiation status was available in 14 cases: 11 of 14 were poorly or undifferentiated (WHO type III), and 3 of 14 were well to moderately differentiated (WHO type II).

DNA Extraction, SSCP, PCR, and DNA Sequencing. Archival paraffin-embedded specimen sections (8–10 μm thick) were stained with H&E. The specimens were microdissected for tumor tissue, followed by proteinase K digestion and phenol/chloroform extraction (24, 25). We screened for mutations in exons 5–8 of the *p53* gene using nested PCR amplification reactions followed by SSCP analysis. Oligonucleotide primers for the PCR amplification of exons 5–8 were prepared on the basis of the published sequences: PX5LT, 5′-GGAATTCCTCTTCCTGCAGTACTC; PX5RT, 5′-GGAATTCGCCCCAGCTGCTCACC; PX6LT, 5′-GGAATTCCACTGATTGCTCTTAGGT; PX6RT, 5′-GGAATTCACCTCAGGCGGCTCATAG; PX7LT, 5′-GGAATTCCTAGGTTGGCTCTGAC; PX7RT, 5′-GGAATTCAAGTGGCTCCTGAC; PX8LT, 5′-GGAATTCCTATCCTGAGTAGTGGT; and PX8RT, 5′-GGAATTCGCTTAGTGCTCCCTGG (21, 26).

Primary PCR conditions were as follows: in a total volume of 50 μl, genomic DNA was incubated in 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 1 μmol of each primer, 0.2 mM deoxynucleotide triphosphate, and 1.0 unit of Taq polymerase (Boehringer Mannheim). Secondary PCR conditions were as follows: in a total volume of 25 μl, 1 μl of PCR DNA purified using the Mermaid kit (Bio101, La Jolla, CA) was incubated in 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 1 μM of each primer, 0.2 mM deoxynucleotide triphosphate, 2.5 μCi of [α-^{32}P]dCTP (3000 Ci mmol^{-1}), and 0.5 units of Taq polymerase. PCR contamination precautions included separate rooms for pre-PCR preparation and PCR-amplification reactions and the inclusion of non-DNA (negative) controls. In addition, for each exon, positive (mutant p53) and negative (wild-type p53) controls were included in each analysis.

Radiolabeled amplification products were screened by SSCP on a nondenaturing polyacrylamide gel as described (27). In brief, the radiolabeled PCR-amplified products were diluted by adding 1 μl of secondary PCR product to 7 μl of Sequenase stop solution (United States Biochemical Corp., Cleveland, OH) and heat denatured by boiling for 5 min, and 2–3 μl were loaded quickly onto a nondenaturing polyacrylamide gel (6% acrylamide, 0.15% bis-acrylamide, 10% glycerol, and 1× Tris-borate-EDTA). The amplified secondary PCR products demonstrating an aberrant SSCP gel banding pattern were reamplified without radiolabeled nucleotides on 2% agarose gels and isolated for sequencing. After purification with the Mermaid kit (Bio101), the isolated PCR products were sequenced in both directions using the dideoxy chain termination method by Sequenase version 2.0 (United States Biochemical Corp.). Conditions were maintained according to the manufacturer's recommendations. Mutations were confirmed by repeating the analysis from microdissected tissue.

Statistical Analysis. We used the Fisher's exact test and the multinomial test to compare selected demographic and clinical characteristics of NPC patients with and without p53 mutations. The Fisher's exact test was also used to compare the rates of p53 mutation among NPC patients from high- *versus* low-risk populations (28). All quoted *P*s were two tailed.

Results

Twenty-eight primary tumor specimens were screened by PCR-based SSCP for mutations in exons 5–8 (codons 126–306) of the *p53* tumor suppressor gene. We focused our analysis on these exons because they are most conserved evolutionarily and contain 95% of the reported p53 mutations (20, 29).

Of the 28 tumors screened by SSCP for p53 mutations, 4 (14%) showed a shift in SSCP pattern in exons 5 and 8, respectively (Fig. 1). When the results were repeated from microdissected tumor DNA, the same tumor specimens showed similar shifts on SSCP. A representative PCR-SSCP gel for exon 5 of the *p53* gene of a NPC primary specimen is shown in Fig. 1. Both the wild-type control (Fig. 1, *Lanes 1* and *2*) and the sample (Fig. 1*A*, *Lanes 3* and *4*) were double loaded. The mobility shift observed in the specimen (Fig. 1*A*, *Lanes 3* and *4*) in comparison to the pattern for the normal control (Fig. 1*A*, *Lanes 1* and *2*) indicates a p53 mutation. This was confirmed by direct sequencing of the shifted band, where a CAG (Gln)-to-TAG (stop codon) transition mutation was identified (Fig. 1*B*). Direct sequencing of the PCR products demonstrated a G:C-to-A:T transition mutation in each of the four specimens within the conserved regions of the *p53* gene (Table 1). We could not rule out the presence of a polymorphism in the patient with a silent mutation due to lack of normal, *i.e.*, noncancerous, DNA material.

As seen in Table 2, there were no differences in clinical characteristics between the patients who had a missense p53 mutation ($n = 3$) and the remaining group of low-risk patients without a p53 mutation ($n = 25$). Specifically, there were no differences in the histology ($P = 0.46$), staging ($P = 0.92$), or differentiation ($P = 0.70$) of their tumors. Also, there were no statistical differences in the sex (2 of 3 women *versus* 6 of 25; $P = 0.19$), mean age at diagnosis (42.0 *versus* 47.2 years;

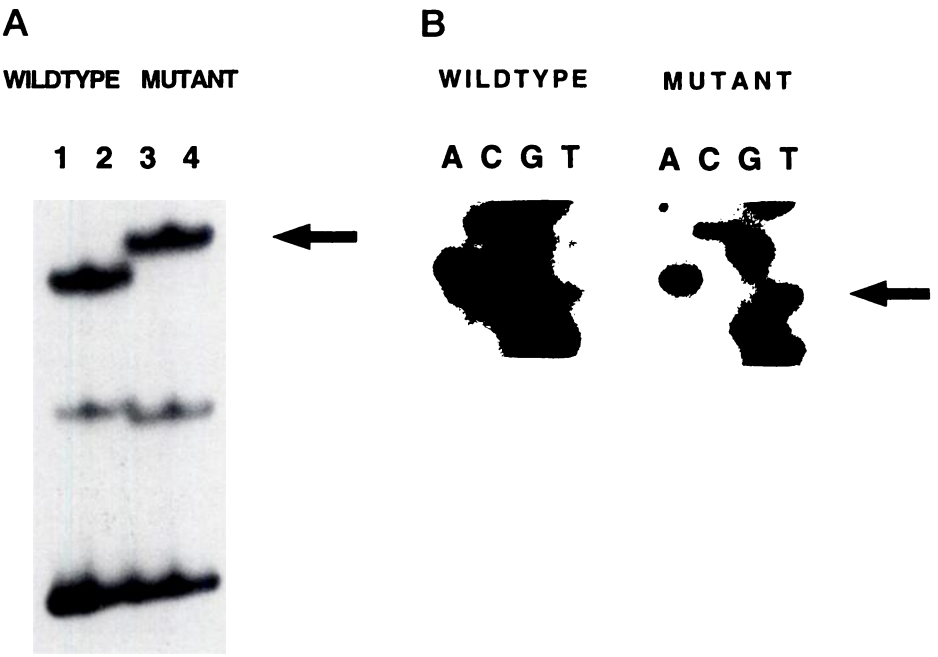

Fig. 1. Representative result of a p53 mutation analysis of a NPC specimen. *A*, autoradiogram of a SSCP gel for exon 5. *Lanes 1* and *2*, wild-type control (double loaded). *Lanes 3* and *4*, one tumor specimen (double loaded) showing a shifted banding pattern. *B*, autoradiogram of the corresponding sequencing gel. *Right*, mutant sequence: a CAG (Gln) → TAG (stop codon) transition mutation at codon 144 in exon 5 of tumor VT-81B. *Left*, wild-type sequence.

Table 1 p53 mutational spectrum in primary NPC specimens

Study	Low risk (LR) *vs* high risk (HR)	Populations	No of mutations: no of specimens	Codon	Exon	Types of mutation
Present study	LR	Whites (including Hispanics) and African Americans in the United States	4:28	142	5	CCT (Pro) to CTT (Leu)
				144	5	CAG (Gln) to TAG (stop codon)
				271	8	GAG (Glu) to GAA (Glu) (silent mutation)
				273	8	CGT (Arg) to CAT (His)
Spruck *et al.* (21)	HR	Chinese (Cantonese)	0:15			
Nasrin *et al.* (30)	HR	Arabs (Saudi Arabia)	1:25	248	7	CCG (Arg) to CAG (Gln)
Sun *et al.* (31)	HR	Chinese (Hunan)	1:12	280		AGA (Arg)-to-ACA (Thr) transversion
		Chinese (Taiwan)	0:10			
Lo *et al.* (32)	HR	Chinese (Cantonese)	0:26			
		Chinese (Guangxi)	1:12	45	4	CCG to CCA (silent mutation)
Effert *et al.* (33)	HR	Chinese, Eskimos, and Arabs	0:18			
Sun *et al.* (34)	HR	Taiwan	0:7			
Chakrani *et al.* (35)	HR	GuangXi	1:20	176	5	TGC (Cys)-to-AGC (Ser) transversion
	HR	Hong Kong	3:21	175	5	CGC (Arg)-to-CAC (His) transition
				177	5	(Two patients with identical mutations) CCC (Pro)-to-CTC (Leu) transition

unpaired *t* test, two-sided $P = 0.59$), or race (2 of 3 African Americans *versus* 7 of 25; $P = 0.23$) between the two groups.

Discussion

The etiopathogenesis of NPC is multifactorial and, despite a vast literature on risk factors associated with NPC, the molecular mechanism(s) involved in its oncogenesis is not well understood. This is the first report investigating the prevalence of missense p53 mutations in NPC among a low-risk population for the disease. Our results indicate that, although its prevalence is low, missense mutations of the *p53* tumor suppressor gene may be involved in the etiopathogenesis of NPC occurring in low-risk areas.

We focused exclusively on United States whites (including Hispanic) and African Americans of Los Angeles County. In this population, dietary exposure to Cantonese salted fish, a probable NPC carcinogen and a common food among the high-risk Cantonese, is nonexistent. We found four mutations (14%), one of which was silent, within the conserved regions of the *p53* gene in four primary tumors among the 28 NPC biopsies examined.

Our data suggest that p53 mutations may be a more frequent event in the low-risk non-Asian population in the United States relative to the higher-risk Southern Chinese, Eskimos, and Arabs. Of the 166 primary NPCs analyzed among the latter group, only 7 missense mutations were found (21, 30–35). However, the two rates of missense p53 mutations (3 of 28 in non-Asians in the United States *versus* 7 of 166 in Southern Chinese, Eskimos, and Arabs) were not significantly different from each other ($P = 0.16$)

With respect to their demographic (sex and mean age at diagnosis) and clinical characteristics (staging, extension, and

Table 2 Histology, staging, differentiation, and mutational status in primary NPC specimens from non-Asians of Los Angeles County

	No p53 mutation (N = 25)	Presence of p53 mutation (N = 3)
Histology		
Squamous cell carcinoma	21	2
Other	4	1
Staging		
Localized/Locoregional	6	1
Distant metastasis	8	1
Unknown	11	1
Differentiation		
Poorly differentiated	11	1
Moderately differentiated	3	0
Unknown	11	2

differentiation), the subgroup of patients positive for p53 mutation did not differ from the remaining group of low-risk patients examined (Table 2). However, due to the small numbers of subjects involved, the power of the present study to detect such differences is low.

Our data are compatible with the hypothesis that cigarette smoking is a risk factor for NPC in whites and African Americans in the United States, as implicated by several epidemiological studies (16, 36–42). Of the three amino acid-changing mutations in our series, two were G:C → A:T transition mutations at non-CpG sites, the type of mutations known to be induced by tobacco-related carcinogens in head and neck cancers (20, 37). The spectrum of p53 mutations observed in head and neck cancers have been found to differ from those observed in smoking-related lung cancer. In contrast with lung cancer, the A:T → G:C (14%) and the non-CpG G:C → A:T (18%) transitions are prominent in head and neck cancers, whereas the G:C → T:A transversions, the most prevalent p53 mutations found in small cell lung cancer, are less common (18 *versus* 40%; Ref. 20). The G:C → A:T p53 transition mutations found in our series also have been observed in lung cancer (37). It should be noted that the same G:C → A:T p53 transition mutation has been linked to other carcinogenic agents such as dietary nitrosamines (43), ionizing radiation (40, 42), and oxygen radical damage to DNA (44–47).

The CCT (Pro)-to-CTT (Leu) transition mutation at codon 142 has not been described in the literature; however, a similar CCT (Pro)-to-TCT (Ser) transition mutation has been found in esophageal cancer (36, 48). The CAG (Gln)-to-TAG (stop codon) mutation at codon 144 has been described in esophageal cancer, stomach cancer, breast cancer, liposarcoma, and lung cancer (36, 49). The codon 273 is a known hot spot for mutations, and the CGT (Arg)-to-CAT (His) mutation found in our series has been described in numerous cancers, including breast, ovary, colon, pancreas, anal, leukemia, lymphoma, lung, and larynx cancer (36, 43). Interestingly, this transition mutation at codon 273 is the homologue of a p53 mutation found in formaldehyde-induced SCCs of the nasal cavities in rats (50). Formaldehyde, a chemical used in several consumer products and found in car exhaust and tobacco, is an established nasal carcinogen in rodents, inducing nasal SCC in a dose-dependent manner in rats. Several recent epidemiological studies have provided limited evidence that formaldehyde might be a human nasopharyngeal carcinogen (17, 19, 50–55). Recio *et al.* identified point mutations in the *p53* gene in 5 of 11 SCCs of the nasal cavity that were induced by chronically exposing the rats to 15 ppm formaldehyde gas for up to 2 years (50). One of the mutations was a transition mutation located at codon 271 [CGT (Arg) to CAT (His)], which is the homologue of codon 273 in humans (50). We identified this latter mutation in one of our specimens. Alternatively, the presence of transition mutations in our series could indicate an endogenous source of mutation in NPC, such as spontaneous deamination of cytosine residues (C) to thymine (T), spontaneous depurination, DNA polymerase infidelity during replication, or other unknown mechanism(s) (43, 45–47).

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