An infrequent point mutation of the p53 gene in human nasopharyngeal carcinoma

(tumor-suppressor gene/nasopharyngeal carcinogenesis/PCR)

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ABSTRACT Point mutations in the p53 gene have been detected in a variety of human cancers; the mutations are clustered in four "hot-spots" located in the coding region of exons 5, 7, and 8, which coincide with the four most highly conserved regions of the gene. We report the finding of a heterozygous G→C mutation at codon 280 (exon 8), position 2, of the p53 gene in a nasopharyngeal carcinoma (NPC) cell line, originating from Guangdong, a province in the People's Republic of China that leads the world in NPC incidence. A survey of nasopharyngeal tissues and NPC biopsies revealed that 1 out of 12 NPC samples from Hunan, another province in the People's Republic of China with high NPC incidence, had the same heterozygous mutation at codon 280 of p53, and none of 10 biopsies from Taiwan showed a mutation within exons 5–8 of the p53 gene. No other alteration of gene structure, including gross rearrangement or loss of heterozygosity or abnormality of gene expression was detected in NPC cell lines or NPC biopsies. We conclude from this study that mutational or other alterations of the p53 gene are not common in nasopharyngeal carcinogenesis and that a codon-280 mutation of p53 may be involved in <10% of NPC cases. This result contrasts with the relatively high frequency of p53 mutations associated with several other human carcinomas and suggests the importance of other genes in NPC genesis.

Nasopharyngeal carcinoma (NPC) occurs in many areas, although the highest incidence is in Southern China and Southeast Asia (1). Epstein-Barr virus (EBV) infection and dietary and environmental factors (i.e., salted fish and medicinal herbs) have been associated with the disease (2–4). It has been proposed that NPC initiation requires EBV expression, but induction of preneoplastic events and maintenance of tumor-cell phenotype require critical cellular genes (5, 6). Previously, we reported the detection of promotion sensitivity and transforming activity after introducing NPC DNA into mouse JB6 cells (7, 8). Recently, we have isolated a transformation-associated sequence from CNE₂ cells that showed measurable transforming activity (9). According to current theories on the origin of human cancer, both activation of oncogenes and inactivation of tumor-suppressor genes are believed to be important in multistage human carcinogenesis. To our knowledge, no investigations on the inactivation of tumor-suppressor genes in NPC have been reported. We therefore investigated the involvement of p53, a potent tumor-suppressor gene, in NPC pathogenesis.

Alteration of the p53 gene (point mutation, deletion, or rearrangement) and/or of its expression have been observed

in diverse human cancers both *in vivo* and *in vitro* (ref. 10 and the references cited therein). The point mutations were found clustered in four "hot-spots" located in exons 5, 7, and 8, which coincide with the four most highly conserved regions of the gene (11); the frequency of these mutations varies in different cancers. Because p53 protein can form a complex with several different DNA tumor virus genes, including the simian virus 40 large tumor antigen (12), the E1B 55-kDa protein from adenovirus type 5 (13), and human papillomavirus types 16 and 18 E6 protein (14), and because NPC is associated with the EBV, and another EBV-associated malignancy, Burkitt lymphoma, has shown frequent p53 mutations (15, 16), the question may be raised as to the role of p53 in NPC pathogenesis. We report here a G→C heterozygous point mutation at codon 280 in NPC cell line(s) and in 1 out of 12 NPC tissue sections from Hunan, China, but not in 10 NPC biopsies from Taiwan. Thus, a subset of NPC might arise from gene cooperation involving EBV genes, one or more specific oncogenes (9), and mutated p53.

MATERIALS AND METHODS

Cell Lines and Tumor Samples. Four Chinese NPC cell lines, designated CNE₁, CNE₂ (from Guangdong), HNE-1, and HONE-1 (from Hunan), were originally established from the tumors of four different patients (17–20). Although the tumors were EBV positive, neither EBV-encoded nuclear antigens nor EBV DNA repeated sequences were detected in the cell lines after several passages in culture (7). The NPC cell lines were grown in culture, and nude mice tumors were generated as described (7, 17–20). Human epidermal keratinocytes (Rhek) and primary human embryonal kidney cell line (293) were cultured as described (21, 22). During this study we discovered through three independent measurements that all four NPC cell lines supplied to us had arisen from a single individual corresponding to CNE₁ or CNE₂.

Nasopharynx tissue was obtained from subjects biopsied at MacKay Hospital in Taipei, for histological diagnosis of NPC. Biopsy samples were obtained from the lesions of 10 histopathologically confirmed NPC cases, 4 adjacent normal tissues, and 8 preneoplastic lesions (3 of chronic inflammation and 5 of lymphoid hyperplasia). All biopsy samples were embedded in optimal-cutting-temperature compounds (Miles) and stored in liquid nitrogen. Twelve paraffin-embedded NPC tissue sections (≈5 μm) were obtained from Hunan Cancer Institute, Hunan. All tissue sections were histopathologically confirmed as low-differentiated squamous carcinoma.

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Abbreviations: EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; LOH, loss of heterozygosity.

RNA Isolation and Single-Stranded cDNA Synthesis. Total RNA was extracted from two immortalized human cell lines (Rhek and 293), cultured NPC cell lines (CNE₁, CNE₂, HNE-1, and HONE-1), and nude mice tumors derived from CNE cells by using RNazol solution (Tel-Test, Friendswood, TX) according to the manufacturer's recommendation. Single-stranded cDNAs were synthesized by avian myeloblastosis virus reverse transcriptase (Seikagaku America, Rockville, MD) as described (23) and amplified by PCR.

Genomic DNA Isolation, PCR Amplification, and Direct Sequencing. Genomic DNA from CNE cells and nude mice tumors derived from them was isolated through CsCl purification (9). The isolation of genomic DNA from biopsies and NPC paraffin section and the subsequent PCR amplification-direct sequencing is detailed elsewhere (24). Briefly, ground biopsies or paraffin-embedded NPC tissue sections were digested in lysis buffer (Applied Biosystems, Foster City, CA) followed by phenol/chloroform extraction and ethanol precipitation of DNA. Five hundred nanograms to 2 μ g of genomic DNA ($\approx$ 50 ng for NPC tissue section) was used for each standard PCR amplification. A sample of amplified fragment was analyzed by electrophoresis in 2% NuSieve (FMC)/1% agarose gels, and the remaining product was purified with Centricon 100 (Amicon) and subjected to single-stranded DNA amplification by using only the excess primer. Amplified single-stranded DNA was purified with Centricon 30 and subjected directly to dideoxynucleotide chain-termination sequencing (24).

Primers. Primers P1 and P2 (11) were used for PCR amplification of the entire coding region of p53. The remaining primers were designed based on the published sequence of p53 (25, 26) and synthesized by Operon Technologies (Alameda, CA). These primers are listed in Table 1.

RESULTS

Normal Expression of p53 mRNA in NPC Cells. To examine whether p53 mRNA was expressed at normal size in NPC cell line(s) CNE₁ and CNE₂, we analyzed with reverse transcriptase-PCR using two primers (P1 and P2) flanking the entire p53 coding region (11). Fig. 1 shows that these cell lines generated fragments of 1.3 kilobases (kb), the size expected for the wild-type open reading frame (11) (lanes 2 and 3). When two pairs of primers flanking mutation hot-spot regions A and B (SHS01/SHS02) and regions C and D (SHS03/SHS04), respectively, were used, fragments showing the expected sizes of 250 and 272 base pairs (bp) according to wild-type sequence (25), were detected (lanes 4 and 5; 6 and 7). Fig. 2 shows the four mutation hot-spot regions as well as the locations and directions of each primer. Northern (RNA) analysis revealed a 2.5-kb transcript in CNE cells with

FIG. 1. Expression of p53 mRNA in NPC cells. Total RNAs were extracted from CNE₁ and CNE₂ cells, reverse-transcribed into cDNAs, and amplified by PCR using one of three pairs of primers. Lanes 2, 4, and 6 were from RNA of CNE₁, and lanes 3, 5, and 7 were from CNE₂. Samples of the PCR products were analyzed on 2% NuSieve/1% agarose gels by using the following pairs of PCR primers: P1 and P2 generate 1300-bp fragment (lanes 2 and 3); SHS01/SHS02 generate 250-bp fragment (lanes 4 and 5); and SHS03/SHS04 generate 272-bp fragment (lanes 6 and 7). ϕ X174 DNA/Hae III marker was also included (lanes 1 and 8).

abundance equal to that detected in Rhek and 293, two human immortalized cell lines (data not shown). These results eliminated the possibility of structural alterations in p53 mRNA arising from deletion, alternative splicing, or rearrangement or of over-expression of the gene.

No Involvement of Loss of Heterozygosity (LOH) or Other Gross Structural Alteration of the p53 Gene in NPC Cell Lines and Biopsies. To extend the above observations on RNA to the DNA level, we examined possible gross deletion or rearrangement of the p53 gene by Southern analysis. Genomic DNA (15 μ g for cell lines and 10 μ g for biopsies) was digested to completion with *Bgl* II on CNE cell lines and nude mice tumors derived from them, biopsies of eight NPCs, three lymphoid hyperplasias, three chronic inflammations, and three adjacent normal tissues. In all samples, two polymorphic bands of 9 kb and/or 12 kb (26) were detected as well as three restriction fragments of 43 kb, 16 kb, and 2.8 kb. No gross deletion or rearrangement was found (data not shown). Due to the limited amounts of DNA isolated from the small NPC biopsies, we could perform Southern analysis on only the six largest samples. When these samples [three from NPC biopsies, two from CNE cell line(s), and one from normal

Table 1. Primers for PCR amplification of the p53 gene in NPC

Name	Sequence	Pair	Flanking region	Fragment size, bp
SHS01	GGAATTCTGTGACTTGCACGTAC	SHS01/02	Codons 121–200	250
SHS02	AGAAGCTTTCTCCACTCGGAT			
SHS03	GGAATTCGACATAGTGTGGTGGTG	SHS03/04	Codons 212–299	272
SHS04	TACTCGAGCTCGTGGTGAGGCTC			
GD01	GGAATTCCTCTTCCTGCAGTAC	GD01/02	Exon 5	224
GD02	AGGGCCCCAGCTGCTCACCATC			
GD03	GGAATTCTCCTAGGTTGGCTCT	GD03/04	Exon 7	142
GD04	ACTTAAGTGGCTCCTGACCTGG			
GD05	GGAATTCCTGAGTAGTGGTAA	GD05/06	Exon 8	158
GD06	GTCGACCTCGCTTAGTGCTCC			
GD09	TTTTCACCCATCTACAGTCCC	GD09/10	Exon 4	310
GD10	CTCAGGGCAACTGACCGTG			
GD13	CACTGATTGCTCTTAGGT	GD13/14	Exon 6	143
GD14	AGTTGCAAACCAGACCTC

FIG. 2. Locations of mutation hot-spots in the structure of p53 cDNA. The four boxes represent the four mutation hot spots (11). The point mutation at codon 280 is indicated, as is the corresponding amino acid substitution. Arrows show approximate locations of the six PCR primers; horizontal arrows point in the direction of nucleotide synthesis. Numbers refer to p53 cDNA codon numbers.

human placenta] were digested with *EcoRI*, *BamHI*, or *HindIII*, all samples showed the same genomic p53 hybridizing fragments as the normal control (data not shown). Thus, no gross deletion or rearrangement of the p53 gene could be detected in NPC cell line(s) or in NPC biopsies.

LOH in the p53 gene has been reported for various tumors (27). We examined whether loss of heterozygosity in the p53 gene occurred in NPC biopsies by analysis of codon 72 and *Bgl* II polymorphisms. Codon 72 (Arg/Pro) polymorphism of the p53 gene can be detected by PCR amplification of genomic DNA with primers GD09/GD10, yielding a 310-bp fragment, followed by *Bst*UI (New England Biolabs) digestion. *Bst*UI cleaves the CGC (Arg) allele, yielding two fragments of 175 bp and 135 bp but does not cleave the CCC (Pro) allele. Fig. 3 shows that DNAs of two NPC biopsies showed heterozygosity at codon 72; the remaining samples showed homozygosity with either CGC or CCC. However, when the DNA of lymphocytes from all eight corresponding NPC patients was analyzed, the digestion pattern was identical (data not shown). These results were further confirmed by PCR-direct sequencing of the 310-bp fragment. The absence of LOH in NPC biopsy 2 (Fig. 3, lane 2) was verified by *Bgl* II polymorphism when compared with adjacent normal tissue from the same patient (data not shown). These results indicated no LOH of the p53 gene in two informative NPC biopsies. Thus, alteration of p53 genomic structure is not seen in NPC cell line(s) or NPC biopsies.

A Heterozygous G → C Point Mutation Found in NPC Cell Line(s) and in an NPC Tissue from Hunan. Having detected no deletion, rearrangement, or alternative splicing of the p53 gene, we next addressed the question of possible gene mutation. Because the mutations of p53 gene found in other human tumors cluster in four hot-spot regions (11), we sequenced the regions encompassing these hot-spots (codon 121–200 and 212–299) (11, 25) by a reverse transcriptase-PCR-direct sequencing technique (Fig. 2). A heterozygous

G → C point mutation at codon 280, position 2 (within hot-spot region D, exon 8) was detected in the CNE cell lines (Fig. 4 Lower Left). Indeed, this is the only mutation detected in CNE₂ cells after sequencing the entire 1.3-kb coding region of p53. When region D of cDNA from two normal control human immortalized cell lines, Rhek and 293, was sequenced, only wild type (AGA) was detected (Fig. 4 Upper). The observed mutation causes a base substitution of the basic amino acid arginine by the neutral amino acid threonine and also a predicted loss of *Bsa* I or *Bsm*AI sites, a prediction confirmed by PCR amplification of exon 8 followed by *Bsa* I digestion (data not shown). The detection of wild-type sequence at codon 280 indicated that one allele was mutated, the other allele was retained as normal, and both alleles were expressed.

If the mutation were important for tumorigenesis, it should be selected for in nude mice tumors derived from these NPC cell line(s). We sequenced these same regions in the nude mice tumors derived from CNE₁ and CNE₂ and found the same G → C heterozygous point mutation expressed at codon 280. The wild-type sequence was also detected (Fig. 4 Lower Right). Throughout this study, both strands were sequenced. In each case, the mutation detected was confirmed by a repeat experiment in which a second sample of cDNA was used as a PCR template and, again, both strands were sequenced. Although differences in morphology, in karyotype, and in degree of tumorigenicity were seen, we discovered [by isozyme phenotypic profile, c-Ha-ras/*Bam*HI polymorphism and microsatellite (λ31-69)/*Hinf*I polymorphism (28)] that all four NPC cell lines had arisen from the same individual, corresponding to CNE₁ or CNE₂. Thus, we conclude that one out of one NPC cell line from Guangdong showed the codon-280 mutation.

The finding of the codon-280 mutation in NPC cell line(s) stimulated us to ask whether the same mutation was also present in NPC tumor biopsies and whether it might be involved in nasopharyngeal tumorigenesis. We, therefore, sequenced all of exons 5, 7, and 8 of 22 nasopharynx biopsies from Taiwan (10 histopathologically confirmed NPCs, 4 samples of paired adjacent normal tissues, 8 samples of nasopharyngeal lesions) and found no mutation in any samples. In addition, we sequenced exons 4 and 6 in the 10 NPC biopsies, and again no point mutation was revealed. Due to limited amounts of NPC tissue section available from Hunan, only exon 8, which contains codon 280, was sequenced. Fig. 5 shows that the same G → C heterozygous point mutation at codon 280 was detected in 1 out of 12 Hunan NPC tissues (Left). For comparison with a negative sample, another NPC biopsy from Hunan, having wild-type sequence, was also included (Fig. 5 Right). We considered three potential sources of error: (i) Contamination from NPC cell line DNA or RNA could have occurred. This possibility can be excluded because neither NPC cells nor cell line nucleic acids were handled during the period in which the Hunan biopsies were analyzed. Furthermore codon-280 mutation was seen in 1 out of 12 biopsies, not 12 out of 12, which would be expected were PCR primers or buffers contaminated. (ii) Normal tissue might contaminate biopsies. Because biopsies contain contaminating normal tissue that would dilute a mutant p53 if

FIG. 3. Codon 72 polymorphism of p53 gene in NPC biopsies. One microgram of DNA was PCR-amplified with primers GD09/GD10, followed by *Bst*UI digestion and gel electrophoresis. Three bands are indicated by arrows. ϕ X174/*Hae* III marker was also shown. DNAs of two NPC biopsies (lanes 1 and 2) showed heterozygosity at codon 72. The remaining samples showed homozygosity with either CGC (lanes 3–7) or CCC (lane 8).

FIG. 4. Expressed G $\rightarrow$ C point mutation at codon 280 of the p53 RNA in NPC cell(s) and tumors derived from them. Total RNAs were isolated from CNE cell line(s), nude mouse tumors derived from them, and from the two human immortalized cell lines Rhek and 293 (normal controls), reverse-transcribed into c-DNAs, and amplified by PCR. As indicated, the wild-type (WT) sequence AGA at codon 280 was detected in Rhek and 293 (Upper), and a heterozygous mutation of G $\rightarrow$ C at codon 280 was detected in CNE cell line(s) (Lower Left) and in the nude mouse tumors derived from them (Lower Right).

present, we conducted a mixing experiment to determine the sensitivity of our assay for the point mutation. When 90% codon-280-negative biopsy DNA from Taiwan was mixed with 10% codon-280-positive DNA from CNE₂ for PCR-direct sequencing, we reproducibly detected the codon-280 mutation. Thus, we conclude that any biopsy consisting of as little as 10% of mutant p53-containing epithelial cells from the NPC would have been detected. (iii) The apparent codon-280 mutation seen in an NPC cell line from Guangdong and in an NPC biopsy from Hunan could represent polymorphism rather than a mutation. This possibility was rendered unlikely by observed loss of growth-suppressor function of p53-Thr²⁸⁰ when over-expressed in recipient cells (data not shown) and by the failure to detect codon p53-Thr²⁸⁰ in any of 14 non-NPC samples measured by us and in any of $\approx$ 50 normal tissues measured by others. Taken together, the above observations indicate that p53 gene mutation is a low-frequency event in NPC carcino-

genesis that occurs in <10% of NPC tumors. To ascertain whether the 8% (1/12) frequency in NPCs from Hunan and the 0% (0/10) frequency in NPCs from Taiwan differ significantly by statistical analysis would require more samples.

DISCUSSION

We report here the detection of a heterozygous G $\rightarrow$ C point mutation at codon 280 of the p53 gene in an NPC cell line that originated from Guangdong province in southern China. This mutation is the only one detected within the entire 1.3-kb coding region of the p53 gene in the NPC cell line CNE₂. It is also the only mutation within exon 8 in one of 12 NPC tissues from Hunan. Although p53 gene mutations have been found in diverse human cancers (10, 11), a mutation at codon 280 has been reported in only one breast cancer cell line with a nonidentical site of mutation (29). Osborne *et al.* (30) recently reported an identical G $\rightarrow$ C homozygous point mutation at codon 280 in a primary breast tumor. This tumor also bore a second mutation at codon 285 (30). Recently we have learned (personal communication) that C. Spruck, Y. Tsai, D. Huang, A. Yang, W. Rideout, and P. Jones have independently measured codon 280 mutation of p53 in CNE₁ and CNE₂ cells obtained from Guangzhou, China.

The observation of two p53 alleles with one allele mutated has been reported for a variety of human cancers including carcinomas of the colon (11), lung (31), esophagus (32), liver (33, 34), and ovary (35) as well as in leukemias (23), even when the sample was composed of 100% cancer cells (35). The mutant p53 gene product has been postulated to override wild-type function by a dominant negative mechanism (36), as indicated by direct inactivation of the wild-type protein by mutant p53 protein through oligomer formation (37, 38), or by conformational adoption of the mutant form (39). In other studies, however, when both wild-type and mutant p53 cDNA constructs were cotransfected into cells, the wild-type form appeared dominant (40, 41). Recent findings in our laboratory (Y.S., K. Nakamura, E. Wendel, unpublished work) show that overexpression of p53 Thr²⁸⁰ in mouse JB6 cells harboring endogenous wild-type p53 causes progression to tumor cell phenotype, suggesting mutant dominance for progression.

FIG. 5. G $\rightarrow$ C point mutation at codon 280 of the p53 gene in NPC paraffin section from Hunan. Genomic DNA was isolated as described (24). About 50 ng of DNA resuspended in double-distilled H₂O was amplified by PCR and directly sequenced (24). A heterozygous G $\rightarrow$ C mutation at codon 280 of p53 gene was detected in one sample (Left) but not in the other sample (Right).

The G → C point mutation at codon 280 causes an amino acid substitution of arginine by threonine. Because p53 is phosphorylated on serine and threonine residues (42, 43), the Arg → Thr substitution may increase the possibility for phosphorylation of the p53 protein. It has been speculated that, like in retinoblastoma, phosphorylation of p53 causes the loss of its tumor-suppressor function (44). Computer secondary-structure prediction of p53 protein shows that the surface probability of codon 280 is very high according to the Emini method (45), implying easy access to molecular interaction, and that the Arg → Thr substitution causes the loss of a turn in the protein secondary structure according to the Garnier-Osguthorpe-Robson method (46) (data not shown), implying a conformational change. Thus, this mutation appears to be in a conformationally significant domain.

We did not detect any point mutations in the 10 NPC biopsies from Taiwan when exons 4–8 of the p53 gene were sequenced, although we cannot rule out mutations in other regions. The possibility for gross gene rearrangement in NPC biopsies as well as cell line(s) was excluded by multiple restriction enzyme digestion followed by Southern analysis. Overexpression or alternative splicing of the genes was also excluded by reverse transcriptase-PCR/Northern analysis in CNE cell line(s). Although the number of informative NPC biopsies was limited, we found no LOH. Overall, we conclude that p53 gene alteration occurs with a low frequency in nasopharyngeal carcinogenesis, with <10% of cases showing the codon-280 mutation. This result distinguishes NPC from such carcinomas as breast, colon, lung, and esophagus (which show a high p53 mutation frequency), putting them in a class with thyroid carcinoma (which shows a low p53 mutation frequency) (47). Whether the high-frequency and low-frequency classes of carcinomas can be distinguished on the basis of dietary or environmental exposure remains to be established. Because p53 mutation is not common, the question is raised whether other genes, such as EBV genes and the recently reported NPC-transforming sequence (9), are important in the genesis of NPC.

The genesis of the G → C transversion in p53 probably does not involve spontaneous mutation, such as deamination of 5-methylcytosine (48). It seems likely that this particular codon-280 mutation of the p53 gene was induced through exposure to environmental carcinogens, such as nitrosamines (4) present in the diet or cigarette smoke but was not induced by aflatoxins that would be expected to produce G → T transversion in codon 249 (33, 34). Further understanding of the possible functional significance of this p53-Thr²⁸⁰ in carcinogenesis awaits evidence of its oncogenic activity in a human model or its activity in antagonizing wild-type p53 tumor-suppressor function.

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