Absence of p53 Gene Mutations in Primary Nasopharyngeal Carcinomas

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ABSTRACT

Alterations in the p53 tumor suppressor gene and Epstein-Barr virus status were investigated in 15 nasopharyngeal carcinoma (NPC) biopsies, 4 xenografts, and 2 cell lines from the Cantonese region of southern China. One other established NPC cell line obtained from a northern Chinese patient was also studied. Restriction fragment length polymorphism analysis revealed a loss of heterozygosity for chromosome 17p, where the p53 gene resides, in only one of 15 NPC biopsies. Polymerase chain reaction-single-stranded conformational polymorphism analysis and direct sequencing failed to detect sequence alterations in exons 5 through 8 of the p53 gene in the 15 tumors and in the 4 NPC xenografts, all of which tested positive for Epstein-Barr virus. In contrast, the 3 NPC cell lines were all negative for Epstein-Barr virus and contained G→C transversions in the p53 gene, with cell lines CNE-1 and CNE-2 harboring identical AGA (arginine) to ACA (threonine) changes at codon 280. These results suggest that p53 inactivation is not a necessary component of nasopharyngeal carcinogenesis in Cantonese but may be important in the establishment of cell lines derived from these tumors.

INTRODUCTION

NPC is a rare malignancy in most parts of the world, where the annual incidence of the disease is generally less than one per 100,000 population in either sex (1). Among the handful of populations that are known to deviate from this low-risk pattern, the highest incidence is observed in southern Chinese who reside in central Guangdong Province and speak the Cantonese dialect; rates of NPC among men in this high-risk population (which are about 2-3 times the female rates) range from 30 to 50/100,000 person-years (1, 2). Causative factors believed to be associated with NPC include a genetically determined susceptibility that is correlated with specific histocompatibility locus antigen haplotypes (3), infection by EBV (4), and life-style factors, including diet (5). There is convincing epidemiological and experimental evidence implicating diet, specifically the intake of salted fish, as the primary cause for the high NPC incidence in the Cantonese. It is estimated that 90% of NPC cases occurring in Hong Kong (whose population is primarily Cantonese) can be attributed to the consumption of salted fish early in life (5).

Tumorigenesis is believed to involve the multistep accumulation of genetic alterations, resulting in the activation of oncogenes and/or the inactivation of tumor suppressor genes (6). RFLP analysis has demonstrated frequent LOH on the short arm of chromosome 3 in NPC tumors, suggesting a possible location for a putative tumor suppressor gene (7). The p53 gene has been implicated as a tumor suppressor gene in many types of human cancers (8). Mutations in the gene are generally concentrated in the four highly conserved regions, within exons 5 through 8, which are known to include the simian virus 40 large-T binding domain (9).

Recently, specific G→T substitutions at codon 249 in the p53 gene were observed in a high proportion of hepatocellular carcinoma cases from regions of Africa and China where dietary aflatoxin is suspected to be a major risk factor (10, 11). Contrary to these reports, hepatocellular carcinoma cases from geographic regions where aflatoxin exposure is rare do not demonstrate the mutational hotspot at codon 249 (12). These observations suggest a direct genetic alteration in humans as a result of exposure to an environmental carcinogen.

The predominant role of salted fish exposure in NPC etiology in Hong Kong (being responsible for 90% of cases in that city) prompted us to search for possible structural alterations in exons 5 through 8 of the p53 gene in 15 NPC biopsies, four xenografts, and one NPC cell line from this region by PCR-SSCP analysis (13). Two additional NPC cell lines established by investigators in the People’s Republic of China were also analyzed. One of the Chinese cell lines (CNE-2) was derived from a Cantonese patient, while the other (CNE-1) came from a patient in the northern Chinese province of Jilin. Supplementary RFLP analysis for LOH on chromosome 17p was also performed on the 15 NPC biopsy specimens.

MATERIALS AND METHODS

Tissues, Cell Lines, and DNA Extractions. Fifteen NPC biopsy specimens and matching blood samples were obtained from patients at the Prince of Wales Hospital, Hong Kong. A portion of the tumor tissue was submitted for histopathologic examination, and the remainder was snap-frozen and kept at −70°C for subsequent DNA analysis. All NPC specimens were undifferentiated carcinomas according to the WHO classification (14). The percentages of tumors in the 15 NPC specimens were not determined, but all 15 DNA samples in a previous study showed complete loss of heterozygosity at either the D3S3 locus or RAF-1 locus on chromosome 3, thus indicating negligible contamination by normal cells in the biopsy specimens (7). Four NPC xenografts (15) (NPC tumors grafted and growing in vivo on athymic nude mice) and one NPC cell line (NPC/HK-1) (16) derived from patients in Hong Kong were established in one of our laboratories. Two other NPC cell lines (CNE-1 and CNE-2) were established by investigators in the People’s Republic of China. One of the Chinese cell lines (CNE-2) was derived from a Cantonese patient (17), while the other (CNE-1) came from a patient in the northern province of Jilin (18). Primary tumor DNA was not available for any of the xenografts or cell lines studied. High-molecular-weight DNA was prepared from tumor specimens, matching blood samples, and cell lines by proteinase K digestion and phenol/chloroform extraction as described (19, 20).

PCR. Oligonucleotide primers used for PCR amplification of exons 5–8 of the p53 gene were prepared based on published sequences (21). PX5LT, 5’GGAATTCCTTCTCTGAGATCTC; PX5RT, 5’GGAATTCGCCAGCTGCTCACC; PX6LT, 5’GGAATTCAGTGCTCTAGG; PX6RT, 5’GGAATTCACCTGAGCGGCCTCAG; PX7LT, 5’GGAATTCCTAGGTTGGCTCTGAC; PX7-
All primers have additional nucleotides creating EcoRI sites at their 5’ ends. Two sets of primer pairs, PX5LT/PX6RT and PX7LT/PX8RT, were used for amplification of exons 5–6 and 7–8, respectively, from genomic DNA. Amplified products were resolved on 1.8% agarose gels and isolated from the gel with GeneClean II (Bio 101, La Jolla, CA). Individual exons for SSCP analysis were amplified in secondary PCR reactions using the GeneCleaned products as templates. Primary PCR conditions were as follows. In a total volume of 50 μl, 1 μg of genomic DNA was incubated in 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 1 μM of each primer, 0.2 mM deoxynucleotide triphosphates, and 1.0 unit of Taq polymerase. Secondary PCR conditions were as follows. In a total volume of 25 μl, 1 μl DNA purified by GeneClean was incubated in 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 1 μM of each primer, 0.2 mM deoxynucleotide triphosphates, 2.5 μCi of [α-32P]dCTP (3000 Ci mmol⁻¹), and 0.5 units of Taq polymerase. NPC biopsy specimens and xenografts were tested for the presence of the EBV genome by PCR as described by Tenedi et al. (22).

SSCP Analysis. SSCP was performed essentially as described (13). Briefly, amplification products were diluted by adding 1 μl of the secondary PCR reaction to 9 μl of Sequenase stop solution (U.S. Biochemicals, Cleveland, OH) and heat-denatured by boiling for 5 min, and 2–3 μl were rapidly loaded onto a nondenaturing polyacrylamide gel (6% acrylamide, 0.15% bisacrylamide, 10% glycerol, 1X Tris-Borate-EDTA). Electrophoresis was carried out at room temperature with a cooling fan at 30 W constant power for 5–6 h, or at 6 W constant power overnight. Gels were subjected to brief drying after electrophoresis and autoradiographed using Amersham Hyperfilm-MP film at -80°C with an intensifying screen. DNA from a grade III transitional cell carcinoma of the bladder containing a known G → A mutation at the second position of codon 146 was used as a mutant control in exon 5 SSCP determination.

DNA Sequencing. Primary PCR products from NPC cell lines (NPC/HK-1, CNE-1, and CNE-2) and NPC tumors (NPC 8 and NPC 38) were digested with EcoRI, cloned into M13 mp19, and sequenced. In all cases, sequencing was performed in both directions using the dideoxy chain termination method by Sequenase version 2.0 (U.S. Biochemicals). Conditions were maintained according to the manufacturer’s instructions.

RFLP Analysis for LOH. DNA from the tumors and the matching WBC were digested with TaqI and analyzed for LOH as described (7, 23). The DNA probes used, their marker loci, and the corresponding chromosomal locations were pEW301 (D17S58) 17pll.2-pl1.1, pYNH37.3 (D17S28) 17pl3.3, respectively; Lane 5. NPC xenograft DNA NPC/HK2117; Lanes 6–8, NPC cell line DNA NPC/HK1, CNE-1, CNE-2, respectively. Arrow, mobility shift observed in CNE-1 and CNE-2 (Lanes 7 and 8).

RESULTS

Fifteen NPC biopsy specimens, 4 xenografts, and 3 established cell lines were analyzed by PCR-SSCP for p53 gene mutations. Our study concentrated on exons 5 through 8 of the p53 gene, since they have been shown to harbor up to 98% of the inactivating mutations of the gene in a variety of human tumors (25).

Fig. 1A shows a representative PCR-SSCP gel of NPC xenografts and cell lines for exon 5 of the p53 gene. Mobility shifts such as those observed in the mutant control, the inactivating mutations of the gene in a variety of human tumors (25).

- Lane 2) and cell line NPC/HK-1 (Fig. 1A, Lane 7) in comparison to the pattern of the normal control (Fig. 1A, Lane 1) indicate the presence of p53 mutations. Direct sequencing showed that cell line NPC/HK-1 carried a C → G point mutation of CTC to GTC at codon 130, substituting a leucine with a valine in the p53 protein (data not shown). The presence of an SSCP band migrating with the mobility of the wild-type band in NPC/HK-1 indicates that this cell line contains both a normal and a mutant copy of the p53 gene. No p53 mutations were detected in exon 5 in the remaining four xenografts and two cell lines tested. The PCR-SSCP pattern of four NPC tumors, one xenograft, and three cell lines for exon 8 of the p53 gene is shown in Fig. 1B. The CNE-1 and CNE-2 cell lines demonstrated mobility shifts indicative of p53 mutations. Both cell lines showed identical G → C transversions at codon 280 of the p53 gene upon sequencing, changing AGA (arginine) to ACA (threonine) (data not shown).

Fingerprinting analysis was performed on CNE-1 and CNE-2 to determine whether the same p53 mutation at codon 280 in these cell lines was due to cross-contamination of the cell cultures. Fig. 2 shows the results of a PCR analysis detecting a GT polymorphism at the D9S59 locus on chromosome 9q. CNE-1 and CNE-2 demonstrated different alleles at this locus, confirming the uniqueness of these cell lines. Therefore the same mutation at codon 280 had occurred independently in the two lines.

We did not detect any mobility shifts by PCR-SSCP in exons 5 through 8 of the p53 gene in the 15 NPC tumor biopsies studied. Tumor purity of the NPC biopsy samples was determined to be nearly 100% based on LOH analysis of chromosome 3p in a previous study (7). The reliability of the SSCP analysis was confirmed by sequencing two randomly chosen biopsy specimens (NPC 8 and NPC 38) for the complete exon 5–8 region. No alteration of the p53 sequence in these biopsy specimens was observed (data not shown). The NPC biopsy specimens were examined for potential loss of heterozygosity at the chromosome 17p region using matched WBC DNA of NPC biopsies (Table 1). Only one (NPC 38) of the 15 informative cases demonstrated loss of an allele at 17p13. Thus, loss of
Fig. 2. Autoradiograph of fingerprinting analysis of NPC cell lines CNE-1 (Lane 1) and CNE-2 (Lane 2) utilizing a GT polymorphism on chromosome 9q.

Table 1 Human nasopharyngeal carcinoma biopsy specimens

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Stage</th>
<th>Chromosome arms and probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPC 3</td>
<td>III</td>
<td>17p13 (D17S34) p144D6</td>
</tr>
<tr>
<td>NPC 8</td>
<td>II</td>
<td>17p11.2-p11.1 pEW301</td>
</tr>
<tr>
<td>NPC 9</td>
<td>III</td>
<td>17p13.3 (D17S28) pYNH37.7</td>
</tr>
<tr>
<td>NPC 10</td>
<td>III</td>
<td>17p11.2-p11.1 pEW301</td>
</tr>
<tr>
<td>NPC 11</td>
<td>II</td>
<td>17p11.2-p11.1 pEW301</td>
</tr>
<tr>
<td>NPC 14</td>
<td>IV</td>
<td>17p11.2-p11.1 pEW301</td>
</tr>
</tbody>
</table>

* All NPC tumor biopsies were characterized as undifferentiated carcinomas. p53 mutations were not detected in any NPC biopsies. Fifteen of fifteen biopsies tested positive for EBV by PCR assay.

DISCUSSION

Our data indicate that mutations in the p53 tumor suppressor gene are not common in NPC in the high-risk Cantonese population in whom the disease is closely associated with the consumption of salted fish. In 15 NPC biopsies tested, only one (NPC 38) demonstrated a reduction to homozygosity of chromosome region 17p within which the p53 gene resides, and none was shown by PCR-SSCP to harbor point mutations in exons 5 through 8 of the p53 gene. Direct sequencing of exons 5–8 in two tumors also showed no mutations, and no evidence of mutations was found in four NPC xenografts. Since the DNA samples were not significantly contaminated with normal tissues as shown by earlier LOH analysis (7), it is unlikely that mutations would have been missed if they were indeed present in the DNA of the tumors studied.

In contrast, all three NPC cell lines examined had p53 point mutations. Cell lines CNE-1 and CNE-2 harbored identical G → C transversions at the second position of codon 280, resulting in an arginine-to-threonine amino acid substitution. This same mutation in p53 has been reported previously in a breast tumor (26).

All NPC biopsy specimens as well as the four xenografts were found to be positive for EBV by PCR (data not shown), but the EBV genome was not detected in any of the three cell lines studied (Table 2).

Table 2 Human nasopharyngeal carcinoma xenografts and cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Stage of disease</th>
<th>Histological type</th>
<th>EBV*</th>
<th>Culturing method</th>
<th>p53 mutation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPC/HK2117</td>
<td>III</td>
<td>Undifferentiated carcinoma</td>
<td>+</td>
<td>Xenograft</td>
<td>ND*</td>
<td>15</td>
</tr>
<tr>
<td>NPC/HK1915</td>
<td>III</td>
<td>Undifferentiated carcinoma</td>
<td>+</td>
<td>Xenograft</td>
<td>ND</td>
<td>15</td>
</tr>
<tr>
<td>NPC/HK666</td>
<td>III</td>
<td>Undifferentiated carcinoma</td>
<td>+</td>
<td>Xenograft</td>
<td>ND</td>
<td>15</td>
</tr>
<tr>
<td>NPC/HK1530</td>
<td>III</td>
<td>Undifferentiated carcinoma</td>
<td>+</td>
<td>Xenograft</td>
<td>ND</td>
<td>15</td>
</tr>
<tr>
<td>NPC/HK1</td>
<td>NA</td>
<td>Well-differentiated squamous cell carcinoma</td>
<td>–</td>
<td>Tissue culture</td>
<td>Exon 5/CTC → GTC codon 130</td>
<td>16</td>
</tr>
<tr>
<td>CNE-2</td>
<td>NA</td>
<td>Poorly differentiated squamous cell carcinoma</td>
<td>+ initially; – after passage 10</td>
<td>Tissue culture</td>
<td>Exon 8/AGA → ACA codon 280</td>
<td>18</td>
</tr>
<tr>
<td>CNE-1</td>
<td>NA</td>
<td>Well-differentiated squamous cell carcinoma</td>
<td>–</td>
<td>Tissue culture</td>
<td>Exon 8/AGA → ACA codon 280</td>
<td>17</td>
</tr>
</tbody>
</table>

* The presence or absence of EBV genome is denoted by + or –, respectively.

** ND, not detected; NA, not available.
and is selected for during culture or whether the mutations were induced during the establishment of cell lines. In this regard, it is significant that p53 mutations are found at a significantly higher frequency in established cell lines (72%) than in primary tumors (39%) (30). For example, p53 mutations were detected in 4 of 8 gastric cell lines, while none were detected in 19 primary tumors (31).

We note that the 15 NPC tumors and 4 xenografts that did not contain p53 mutations all harbor EBV in their genomes, while the 3 NPC cell lines containing mutated p53 alleles tested negative for EBV (Tables 1 and 2). These results parallel those for cervical carcinoma cell lines and tumors in which p53 abnormalities are correlated with an absence of human papillomavirus infection (32, 33). However, unlike human papillomavirus, associations between EBV proteins and p53 or other tumor suppressor gene products remain to be demonstrated. It is worth noting that EBV nuclear antigen-2 contains regions of amino acid sequence homology to human papillomavirus E7, adenovirus E1A, and simian virus 40 large-T antigens in the functional domains for growth transformation (34). EBV has been shown to be associated with cases of Burkitt’s lymphoma (35), but in contrast to the present observations, these tumors are frequently mutated in p53 regardless of the status of EBV infection (36, 37).

Although p53 tumor suppressor gene inactivation does not appear to play a major role in NPC genesis among high-risk Cantonese, RFLP analysis has revealed frequent allelic loss of chromosome region 3p21-14 in these tumors (7). It will be of interest to determine if NPC from low-risk regions proceeds by a similar molecular mechanism: i.e., frequent allelic loss of chromosome 3p but no general involvement of p53 tumor suppressor gene inactivation.

REFERENCES


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