Alterations of the p53 Gene in Nasopharyngeal Carcinoma

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Nasopharyngeal carcinoma (NPC) is a malignancy which is consistently associated with the Epstein-Barr virus (EBV). The structure of the EBV genome in NPC suggests that NPC is a clonal proliferation of epithelial cells which emerges after EBV infection. The disease develops with high incidence in specific populations in discrete geographic locations, implicating possible genetic or environmental cofactors. Mutations of the p53 gene are among the most frequent genetic changes found in a large variety of human tumors. Mutations in p53 have been shown to abrogate the suppressor function of wild-type p53 and thus contribute to the transformed phenotype. To determine if mutation in p53 participates in the development of the malignant clone in NPC, the structure and sequence of p53 in 42 primary, metastatic, and nude mouse-passaged NPC specimens was analyzed. A high frequency (6 of 9) of mutations was detected in the nude mouse-passaged tumors, while only 2 of 15 metastatic and 0 of the 18 primary tumors harbored mutant p53. The p53 mutations included single-point mutations and more extensive changes such as frame shifts, deletion, duplication, or complete loss of coding sequences. These data indicate that alterations of the p53 gene are unlikely to be involved in the initial genetic events leading to the clonal outgrowth in NPC. However, although it is a rare NPC which can be established in nude mice, this growth advantage appears to be conferred on tumors bearing a mutant p53.

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Nasopharyngeal carcinoma (NPC), an epithelial malignancy characterized by marked geographic and population differences in incidence, exemplifies the association of a human solid tumor with viral infection (11). The Epstein-Barr virus (EBV), a ubiquitous lymphotrophic herpesvirus, is consistently detected in NPC, suggesting that EBV infection is critically involved in the induction of this malignancy (10, 34).

Structural analyses of the EBV genome have revealed that the circular intracellular form of the EBV genome can be distinguished from the linear virion form by identification of the restriction enzyme fragment representing the fused termini (33). The terminal restriction enzyme fragments of EBV are heterogeneous in size because of various numbers of copies of the terminal repeat (22, 27). In agarose gel electrophoresis, the terminal fragments of the linear genomes migrate as ladder arrays of fragments. However, in NPC a single restriction enzyme fragment representing the fused termini of the EBV genome is detected (33). The clonality of the viral genomes in NPC suggests, by extension, cellular clonality. The unique ability to assess cellular clonality in this epithelial malignancy makes NPC one of the few carcinomas which is known to be clonal.

The occurrence of NPC at high incidence in discrete genetic populations and in specific areas of endemicity suggests that genetic or environmental cofactors also contribute to the development of NPC. The genetic alterations that contribute to the emergence of a malignant clone from the multiple cells which may be infected by EBV are as yet unknown. Many genes which contribute to malignant progression have been identified cytogenetically. However, cytogenetic studies with NPC are limited because of the difficulties in culturing NPC tissue in vitro (26, 42). Abnormal cellular DNA content, possibly reflecting aneuploidy, was detected in 60% of a large number of pretreatment NPC specimens by using flow cytometry (7). Karyotypic analysis of two epithelial cell lines established from NPC tissue and of the rare NPC samples that can be established in nude mice revealed aneuploidy, but a consistent chromosomal marker was not identified (26, 46, 47).

Gene abnormalities are believed to underlie the development of human cancer. Alterations in oncogenes and suppressor genes in a variety of human cancers have been documented. The importance of suppressor genes in the inhibition of tumor growth is becoming increasingly apparent. The wild-type alleles of suppressor genes have regulatory functions in cell proliferation and differentiation such that their loss or inactivation appears to be oncogenic (37).

The human p53 gene is one of the few well-characterized genes with tumor suppressor function (12, 16, 17). The p53 gene product can be inactivated by virally encoded oncoproteins such as the large T antigen of simian virus 40 and the E6 gene of human papilloma virus (HPV) (28, 29, 38). Genetic mechanisms of p53 inactivation include gross alterations such as deletions or rearrangements and, most frequently, more subtle changes such as single-point mutations (16, 31, 41). Mutations in p53 are among the most frequently detected genetic alterations in human malignancies and have been documented in tumors of the colon, breast, lung, brain, and bladder (2, 31, 40).

NPC is a unique epithelial malignancy in that it develops in discrete genetic populations and geographic pockets, yet it is consistently associated with a single, ubiquitous infectious agent, EBV (10, 11, 34). To date, the role of alterations in oncogenes and suppressor genes in NPC has not been determined. In this study, 42 NPC specimens, including two cell lines established from NPC, were analyzed for alterations of the p53 gene by using restriction enzyme analysis, differential polymerase chain reaction (DPCR), single-stranded conformation polymorphism (SSCP) analysis, and PCR sequencing. The specimens analyzed included primary,
TABLE 1. Sequences of the oligonucleotides used as PCR and sequencing primers

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a The sequence for the p53 primers was taken from the published coding and intron p53 sequence (4). Primers P1 and P2 contain restriction sites (EcoRI and SalI).

metastatic, and nude-mouse passaged NPC. While 0 of the 18 primary tumors and only 2 of the 15 metastatic tumors were found to harbor an altered p53 gene, 6 of 9 nude mouse-supported tumors contained mutant p53.

MATERIALS AND METHODS

Tissues and cell lines. Primary and metastatic NPC obtained at biopsy were preserved at −70°C. The frozen tissues were pulverized and dissolved in 4 M guanidine thiocyanate, and the DNA fractions were extracted twice with phenol and chloroform after dialysis and proteinase K treatment (35). The xenografts passaged in nude mice have been previously characterized and shown to be EBV positive (5, 26, 35). C15 and 2117 were established from primary biopsy material, whereas C17, C18, and C19 were established from metastatic tumors. The CNE-1 and CNE-2 epithelial cell lines, which were established from NPC and can be passaged in nude mice, became EBV negative during culture (46, 47). Du145, ILN, and Dupro are cell lines with characterized mutations in p53 which were established from prostate carcinoma and can be passaged in nude mice. The HT1080 cell line is a human fibrosarcoma with unarranged p53 DNA. The NPC tumor biopsy specimens were classified histopathologically as undifferentiated NPC. One specimen, N18, is a WHO 1 squamous cell NPC which is EBV positive (35). All specimens were screened for EBV DNA and were compared with dilutions of the Raji cell line to determine the EBV genome copies. The biopsy specimens contained between 5 and 50 copies of EBV, which is similar to the number of EBV genome copies in the nude mouse-passaged NPC, which vary between 2 and 30 copies (5). The relative abundance of EBV DNA suggests that the pieces of biopsy analyzed biochemically were predominantly tumor tissue.

Total DNA was digested with EcoRI, transferred to nitrocellulose, and hybridized to a single-stranded RNA probe synthesized from a p53 cDNA which was transferred to the Bluescribe vector (Stratagene, La Jolla, Calif.) (1).

SSCP analysis. The 2.9-kb fragment of genomic DNA, containing exons 5 to 8 of the p53 gene, was amplified under standard PCR conditions (30) by using primers P1 and P2 (Table 1). Amplified fragments were recovered from 1% agarose gels after electrophoresis (GeneClean Kit; Bio 101, Inc., La Jolla, Calif.). For subsequent SSCP analyses, PCR was performed with a fraction of the preamplified 2.9-kb fragment in a reaction containing 200 μM (each) dATP, dGTP, and dTTP; 2 μM dCTP; 0.5 μM each primer; 50 mM KCl; 10 mM Tris Cl; 1.5 mM MgCl2; 0.001% (wt/vol) gelatin; 0.25 U of Taq polymerase; and 1 ml of [32P]dCTP (3,000 Ci/mmol, 10 mCi/ml; NEN, Boston, Mass.) in a 10-μl total volume. By using several different primer pairs (Table 1), 139- to 209-bp fragments within exons 5, 6, 7, and 8 of the p53 gene were amplified in separate PCRs for 30 cycles at 94, 55, and 72°C. One microliter of the PCR product was withdrawn and diluted 100-fold in 0.1% sodium dodecyl sulfate and 10 mM EDTA. Two microliters of this solution was mixed with 2 μl of formamide containing loading dye, heated for 5 min at 94°C, and loaded (1.5 ml per lane) onto a 6% nondenaturing polyacrylamide gel. Electrophoresis was performed at 4°C at 35 W. After being dried, the gel was exposed to Kodak XAR-film at −80°C for 12 to 48 h by using an intensifying screen.

PCR sequencing. Five to 10% of the preamplified and purified 2.9-kb fragments of the p53 gene were subjected to a second asymmetric PCR reaction with primer pairs P3 and P4 (exon 5), P5 and P6 (exon 6), P7 and P8 (exon 7), and P9 and P10 (exon 8) (Table 1) (4). The concentrations of the primers were 0.5 and 0.01 μM, respectively, for the first and second primer of each pair. Both strands were sequenced for each exon. The respective primers were then used to prime the sequencing reactions.

The product of the asymmetric PCR was purified and desalted in 15 μl of water. An aliquot (7.5 μl) was taken for each sequencing reaction. The commercially available Sequenase kit was used for sequencing (United States Biochemical Sequence, Cleveland, Ohio). After electrophoresis on 8% polyacrylamide–5 M urea gels (2 h at 55 W) and drying, the gel was exposed to Kodak XAR film overnight.

Differential PCR. Differential PCR was essentially performed as previously described (30). The primer sequences of the reference genes, beta and gamma interferon, employed in the differential PCR have been published (18, 30). Amplification of sequences in exons 5, 7, and 8 of the p53 gene was performed in conjunction with amplification of single-copy 150- and 170-bp sequences within the beta and gamma interferon genes, respectively. After electrophoresis and ethidium bromide staining of the PCR products, the degree of amplification was determined by densitometric analysis of the negatives obtained.

RESULTS

Restriction enzyme analysis of p53 in NPC. To determine if structural alterations in the p53 gene could be detected by restriction enzyme analysis, total DNA extracted from NPC specimens was digested with EcoRI and hybridized to a single-stranded RNA probe synthesized from a p53 cDNA (Fig. 1) (2). An apparently normal fragment was detected in three of four NPC tumors recently established in nude mice, C15, C18, and C19. However, a hybridizable fragment consistently could not be detected in the C17 tumor, suggesting homozygous deletion of the entire p53 gene. In primary NPC tumors, the normal 15-kb EcoRI fragment representing p53 was detected in 40 specimens. These data indicate that gross rearrangements of the p53 gene are not prevalent in NPC.

Polymerase chain analysis of p53. To screen the DNA samples for deletions in p53 which would not be detected by restriction enzyme analysis, the PCR was employed. A
gamma interferon, could be detected. In C19, exons 7 and 8 were amplified but exon 5 could not be detected (Fig. 2A). Amplification of exon 5 or 7 alone compared with amplification of beta interferon confirmed deletion of exons 5 and 7 in C17 and deletion of exon 5 in C19 (Fig. 2B and C). In contrast, exons 5, 7, and 8 were amplified at equivalent levels to the interferon reference markers in the NPC specimens indicated in Table 2.

These data indicate the loss of the p53 coding sequences in C17 and confirm the complete absence of hybridization in C17 in the Southern blot analyses. In C19, the restriction enzyme fragment appeared to be slightly smaller than the 15-kb normal fragment. This would result from the deletion of sequences 5' to exon 5 through the first amplimer, P1.

Detection of p53 mutations by SSCP analysis. Since point mutation can significantly perturb p53 function, the prevalence of p53 mutation was analyzed in the NPC samples. SSCP analysis (32) was employed to screen DNA samples for changes in exons 5, 6, 7, and 8 in p53, where mutational hot spots exist. The basis of this technique is that radiolabeled PCR products when denatured and allowed to renature in dilute conditions will assume an intramolecular secondary structure unique to that sequence. This approach has been successfully utilized for the detection of point mutations in the ras gene family and the neurofibromatosis type 1 gene (6, 32).

In this study, SSCP analysis was performed by the amplification of the individual exons 5, 6, 7, and 8 by using the 2.9-kb PCR fragment as a template. The amplified fragments representing specific exons were subsequently subjected to nondenaturing gel electrophoresis. To determine the sensitivity of SSCP for detecting p53 mutations, cell lines established from prostate carcinomas with known point mutations were compared with normal blood and the sample DNAs from NPC. Abnormal migration patterns and the appearance of extra bands were consistent and reproducible findings in the mutant samples with distinctly different banding patterns when compared with those of the normal control samples. This analysis of 40 NPC specimens and cell lines revealed abnormal migration in 6 samples.

SSCP analysis of exon 5 revealed abnormal migration in the two prostate carcinoma cell lines, 1LN and Dupro, known to have a deletion of a C residue in codon 138 and identical abnormal migration in a fresh NPC obtained from a lymph node metastasis, N18 (Table 2; Fig. 3A). In this analysis, normal migration was observed in DNA from normal blood and the C15 tumor. Sequence analysis of exon 5 revealed that N18 had the same deletion of the C residue in codon 138 without a detectable normal allele (Table 3; Fig. 3B).

Another type of mutation in exon 5 was detected in C18, a nude mouse-passaged tumor. As shown in Fig. 4A, normal migration was detected in DNA from four specimens of NPC, including metastatic and primary tumors, in comparison with an apparent mutation in C18 and the absence of a normal allele. Sequence analysis of C18 revealed a duplication of codons 149 to 153 (Table 3; Fig. 4B).

Abnormal migration in both exon 6 and 7 was detected in a single specimen, NM6, and NPC passaged in nude mice (35) (Fig. 5 and 6). The abnormal migration for exon 6 in NM6 and in an NPC metastasis, AIL, is compared with normal migration in a primary NPC and a posttransplant lymphoma (Fig. 5A). The altered migration in exon 7 in NM6 is compared with normal migration in a second NPC xenograft, NM1, one primary NPC, and three metastatic NPC (Fig. 6A). Sequence analysis of the NPC, AIL, revealed a
### TABLE 2. p53 DNA structure in NPC

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* SB, Southern blot; Seq, sequence; N, normal; D, deleted; A, abnormal; Dupl, duplication; M, mutation.

** Amplification successful; -, amplification not successful.

nonsense mutation in exon 6 converting a CGA to a termination codon TGA. The sequence of NM6 indicated multiple mutations in codons 210 through 215 in exon 6 and a transition of G for A in codon 234 in exon 7 (Table 3; Fig. 5B and 6B). The SSCP and sequence analysis of exon 6 suggested the absence of a normal allele, whereas in exon 7 NM6 retained a normal allele.

SSCP analysis of exon 8 revealed mutation in DU145, a prostate carcinoma cell line passaged in nude mice, and in two cell lines established from NPC, CNE-1 and CNE-2, also passaged in nude mice (Table 2; Fig. 7). The DU145 cell line has a point mutation, on one allele, in codon 274 with a transversion from G to T, whereas CNE-1 and CNE-2 cell lines had the identical mutation at codon 280 with a transversion from G to C (Table 3; Fig. 6).

Sequence analysis of p53 in NPC. In order to further confirm the adequate sensitivity of SSCP analysis in the detection of p53 mutations, DNA from CNE-2 with a p53 mutation in exon 8 was mixed with normal DNA from the C15 tumor in various proportions from 5 to 50% prior to amplification. Bands with altered migration representing CNE-2 were detectable in mixtures in which the CNE-2 DNA represented 5% of the total DNA (data not shown). In addition, a sampling of NPC specimens which had normal
exon 7 with amplimers for exons 5, 7, 8, and gamma interferon (R). (B) Amplification with exon 7 and beta interferon. (C) Amplification with exon 7 and gamma interferon. 

FIG. 2. Deletion analysis by differential PCR. PCR was performed by using amplimers specific for exons 5, 7, and 8 and beta or gamma interferon as a reference amplification. (A) Amplification with amplimers for exons 5, 7, 8, and gamma interferon (R). (B) Amplification with exon 5 and beta interferon. (C) Amplification with exon 7 and beta interferon. 

migration detected on SSCP were completely sequenced through exons 5, 6, 7, and 8 (Table 2). Wild-type sequence was present in all samples that had been classified as normal according to the results obtained by SSCP analysis. Sequence analysis of mixtures of mutant and normal DNA in the same sample could detect mutations in the sequencing reactions with 20% mutant DNA (data not shown).

The sequence analyses of the NPC specimens revealed that the samples with abnormal banding patterns all contained mutant sequence. Those DNAs with identical migration by SSCP had identical sequence changes (Fig. 3 and 6). Furthermore, it was confirmed that SSCP adequately predicted whether a specimen contained the mutant allele only or whether both a mutant and a wild-type allele were present. Samples displaying both the normal and abnormal banding pattern by SSCP were found to contain the wild type in addition to the mutant sequence, as in the DU145 cell line and exon 7 of NM6 (Fig. 5A and B and 6A and C). Although the PCR can introduce mutations, such mutations are not detected in the sequence of the total PCR. However, to eliminate the possibility that DNAs with introduced mutations were preferentially amplified during PCR, the samples with mutations were sequenced several times from different amplifications.

Tables 2 and 3 summarize the p53 alterations detected in NPC. Despite the rarity of p53 mutations in primary NPC samples, in the nude mouse-passaged NPC tumors, including the CNE-1 and CNE-2 NPC cell lines, six of nine contained mutant p53. The spectrum of mutations included point mutation, frame shift, deletions, duplications, and complete loss of coding sequences.

FIG. 3. Identification of a frameshift deletion in exon 5. (A) SSCP analysis of exon 5 by using the P3 and P4 amplimers. The samples include normal blood (lane 1), C15 (lane 2), N18 (lane 3), 1LN (lane 4), Dupro (lane 5), prostate carcinoma (lane 6), and normal blood (lane 7). (B) Sequence analysis of N18 (M) compared with DNA from normal blood (N). The arrow indicates the deletion of a C in codon 138 (GCC).

FIG. 4. Identification of a duplicated sequence in exon 5. (A) SSCP analysis of exon 5. Normal blood (lane 1), DU145 (lane 2), C18 (lane 3), C18 (lane 4), CL9 (lane 5), CL11 (lane 6), M1 (lane 7) and N12 (lane 8). (B) Sequence analysis of C18 (M) compared with DNA from normal blood (N). The sequence presented in brackets, representing codons 149 to 153, is duplicated.

TABLE 3. p53 mutations in nasopharyngeal carcinoma

<table>
<thead>
<tr>
<th>NPC</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>C17</td>
<td>Deleted for 2.9 kb</td>
</tr>
<tr>
<td>C18</td>
<td>Codon 154 deleted, duplication of codons 149 to 154</td>
</tr>
<tr>
<td>C19</td>
<td>Deletion of sequences 5' to exon 5</td>
</tr>
<tr>
<td>NM6</td>
<td>Codons 210 to 216, multiple point mutations; codon 234, TAC to TGC (Tyr to Cys)</td>
</tr>
<tr>
<td>CNE-1</td>
<td>Codon 280, AGA to ACA (Arg to Thr)</td>
</tr>
<tr>
<td>CNE-2</td>
<td>Codon 280, AGA to ACA (Arg to Thr)</td>
</tr>
<tr>
<td>AIL</td>
<td>Codon 196, CGA to TGA (Arg to termination)</td>
</tr>
<tr>
<td>N18</td>
<td>Codon 138, GCC to GC (frame shift deletion)</td>
</tr>
</tbody>
</table>
The data presented demonstrate that although p53 mutations do occur in NPC, the mutations were primarily identified in the subset of tumors that have been successfully grown in nude mice. In contrast, in direct clinical specimens of the 18 primary tumors and only 2 obtained from metastatic NPC were found to harbor p53 mutations. The absence of p53 mutations suggests that p53 mutation is not a critical event in the initial transformation of the epithelial cells. It is unknown, however, when exactly p53 mutations occur during the neoplastic process. Data obtained from studies of colon carcinomas suggest that they occur as a late event in the molecular carcinogenesis (15). In breast carcinomas p53 mutations do develop in both primary and metastatic disease (9). However, the absence of mutation in p53 indicates that the genetic and environmental factors which contribute to the development of NPC are not manifested in p53 mutations.

In all the NPC specimens passaged in nude mice with mutation in p53, there was a loss of the normal allele and retention of a mutant allele with mutations in two exons in NM6. The spectrum of mutations, including point mutations, frame shifts, deletions, and the 15-bp duplication, suggests that the mutations could have been induced by therapeutic intervention. The C17 and C19 tumors, which have extensive deletions within p53, had been obtained from patients whose tumors had recurred after radiotherapy.

It is possible that the ability of NPC to grow in nude mice identifies those tumors bearing an altered p53 gene. It is difficult to establish NPC tumors in nude mice, although heterotransplantation of metastatic tissues is more successful (5, 21). The nude mouse tumors in which p53 mutations were identified were originally derived from NPC metastases, and mutations in p53 were only identified in metastatic NPC specimens obtained at biopsy. Therefore, the detection of mutant p53 in the specimens passaged in nude mice may indicate that tumorigenicity in nude mice may selectively identify the rare NPC with mutant p53. It is also possible that p53 mutation may develop during passage in nude mice. This would suggest that loss of p53 function in NPC provides a growth advantage in nude mice but apparently not in vivo.

Interestingly, cervical cancer is a malignancy which is also associated with viral infection with detection of HPV in the majority of cervical carcinomas (3, 39). In HPV-associated cervical cancer, mutations in p53 have not been identified, presumably because the p53 gene product is inactivated through the E6 viral gene product (38, 43). In contrast, mutations in p53 have been detected in HPV-negative cervical cancers (8). These data suggest that p53 may be involved in cervical carcinoma and can be inactivated either by somatic mutation in p53 or by interaction with the E6 gene product.

The absence of p53 mutations in most NPC may indicate interference by EBV infection with some aspect of p53 function. If p53 function is inactivated by an epigenetic process, selection for cells with a mutation in p53 would not be necessary. In all NPC in which a single EBV-infected cellular clone has predominated, the absence of p53 muta-
FIG. 7. Mutations in exon 8. (A) SSCP analysis of exon 8. The
specimens presented are CL4 (lane 1), CU145 (lane 2), CIU (lane 3),
E7 (lane 4), and D6 (lane 5). (B) SSCP of exon 8. Primary prostate
carcinoma (lane 1), DU145 (lane 2), CNE-1 (lane 3), CNE-2 (lane 4),
C19 (lane 5), C19 (lane 6). (C) Sequence of mutation in DU145 in
codon 274 (GTT to TTT). (D) Sequence of mutation in CNE-1 in
codon 280 (AGA to ACA).

mutations suggests such a lack of selection. The ability to be
grown in nude mice would then reflect a more complete loss of
p53 function.

In contrast to NPC, p53 mutation was identified in a signifi-
cant percentage of both EBV-positive and EBV-negative
tissues of Burkitt’s lymphoma, suggesting a selection for
loss of p53 function in Burkitt’s lymphoma (14, 19). This
apparent difference between BL and NPC may be related to
differences in the EBV genes which are expressed in the two
diseases. It is believed that in Burkitt’s lymphoma, only the
EBV nuclear antigen, EBNA1, is expressed, whereas in
NPC, EBNA1, the latent membrane proteins, and a gene en-
coded by the BamHI A fragment are expressed (13, 20, 25,
36, 45). It has been shown that expression of latent mem-
brane protein 1 suppresses apoptosis or programmed cell
death (23). It has also been suggested that unmutated p53
may be a mediator of apoptosis (44). Perhaps expression of
latent membrane protein 1 in NPC indirectly interferes with
p53 function by bypassing its role in mediating apoptosis.

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