**p16 Gene Alterations in Nasopharyngeal Carcinoma**

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**Abstract**

In order to investigate the p16 gene alterations in nasopharyngeal carcinoma (NPC), we have examined for mutations and deletions of the p16 gene in samples of NPC including 3 cell lines, 3 xenografts, and 20 primary tumors with matched blood DNA as controls. Using single-strand conformation polymorphism and direct sequencing analysis, no p16 gene mutations were detected in the NPC primary tumors and xenografts. Mutations of the p16 gene were found in three NPC cell lines, but no normal allele was present in these samples. Homozygous deletion of the p16 gene has been identified in 2 (67%) of 3 cases of NPC xenografts and 7 (35%) of 20 cases of primary tumors by comparative multiplex PCR analysis. A homozygous deletion region distal to the p16 locus was observed in a case of NPC primary tumor. Our data document for the first time that alterations of the p16 gene were frequent in NPC and that homozygous deletion was the major mechanism for the inactivation of this gene. These findings suggest that complete inactivation of the p16 gene may play a role in the development of NPC. Moreover, inactivation of other putative tumor suppressor gene(s) outside of the p16 locus within chromosome 9p21–22 may also contribute to the pathogenesis of this disease.

**Introduction**

NPC3 is one of the most common cancers in southern China but is rare in most parts of the world. The incidence of NPC in Chinese living in South China, Hong Kong, Taiwan, and Singapore is 25 times higher than that in Caucasians living in the European and American continents, with the exception of the Alaskan Eskimos (1). Previous studies show that the specific histocompatibility locus antigen haplotypes, EBV infection, and the environmental factors (such as consumption of salted fish at weaning age), all have a close association in the development of this disease (2).

Thus far, little is known of the genetic changes that occur in the tumorigenesis of NPC. Alterations of the common tumor suppressor genes, p53 and RB, are rare in this tumor (3). Cytogenetic studies have revealed several chromosomal abnormalities, particularly losses or structural rearrangements on chromosomes 1, 3, 11, 12, and 17 (5). We previously demonstrated that allelic loss on chromosome 3 was a common genetic alteration that occurred in NPC (6, 7). We have further identified allelic loss on chromosome 9 in 61% of the primary NPCs. A homozygous deletion region at 9p21–22 (between loci D9S161 and D9S162) was found in one of the reported cases of primary tumors (8). This suggested that inactivation of the tumor suppressor gene(s) in 9p21–22 may play an important role in the development of this cancer.

A putative tumor suppressor gene, p16/MTSI/CDKN2, which encodes a cell cycle regulator protein, has recently been mapped to the region of 9p21 (9, 10). The p16 protein binds to and inhibits the catalytic activity of the CDK4/cyclin D complexes. Loss of its function would be expected to lead to uncontrolled cell growth (11). High frequency of homozygous deletion and mutations of the p16 gene have been reported in human cancer cell lines including melanoma, glioma, breast, bladder, lung, head and neck, and leukemia (9, 10, 12). Mutations of the p16 gene have been reported in primary tumors of the head and neck (12) and esophagus in some studies (13, 14), while other researchers have found that p16 gene mutations were rare in primary tumors (15–17). Moreover, homozygous deletion of the p16 gene has been detected in the primary tumors of the bladder (16), brain (18), and lung (19). The presence of homozygous deletion outside the p16 gene locus has been reported by some researchers in malignant mesotheliomas (20) and brain tumors (21), which suggests that there may be other candidate tumor suppressor gene(s) in the 9p21-deleted region. In this study, we intend to investigate whether p16 gene alterations may play a role in the tumorigenesis of NPC. We have examined the deletions and mutations in the DNA samples of NPC, including cell lines, xenografts, and primary tumors.

**Materials and Methods**

NPC Cell Lines, Xenografts, and Primary Tumors. Three NPC xenografts (HK-2117, HK-1915, and HK-666) and one cell line (HK-1) were derived from NPCs of patients in Hong Kong (22, 23). The other two cell lines (CNE-1 and CNE-2) were from patients in South China and in the northern province of Jilin, respectively (24, 25). The primary tumors of these xenografts and cell lines were not available. NP-3 is normal epithelial cell outgrowth from nasopharyngeal mucosal tissue established in our own laboratory. Twenty primary NPC biopsy specimens and matching blood samples were part of specimens after histopathological diagnosis from patients prior to chemotherapeutic or radiotherapeutic treatment at the Prince of Wales Hospital, The Chinese University of Hong Kong (Hong Kong). Another 25 primary NPC tumors without matching blood samples were also available for the analysis of the p16 gene mutations. All of the primary tumors were diagnosed as undifferentiated NPCs according to the WHO classification and reported previously in other studies (6–8).

SSCP Analysis and Direct DNA Sequencing. DNA samples from the 3 cell lines, 3 xenografts, and 45 primary tumors of NPC were screened for mutations in the p16 gene by SSCP analysis. The three exons of the p16 gene were examined using five pairs of primers (exon 1, exons 2A, 2B, and 2C, and exon 3) described by Hussussian et al. (26). All of the primers were synthesized by Oligos Etc., Inc. (Wilsaville, OR) according to the published sequence. The PCR was performed in a 5-μl reaction solution as described previously (27). In brief, both primers (0.25 pmol) were labeled with [γ-32P]ATP (Amersham Corp., Buckinghamshire, United Kingdom) by T4 polynucleotide kinase (Amersham Corp.) and mixed with the reaction solution containing 50–100 ng genomic DNA, 62.5 μM concentrations each of deoxyribonucleotide triphosphates, 1.5 mM MgCl2, 5% DMSO, and 0.25 units of Taq polymerase (Perkin Elmer/Cetus, Norwalk, CT) in 1X PCR buffer. PCR reaction was carried out in a DNA thermal cycler (Perkin Elmer/Cetus), and the amplification cycles were performed as described by Hussussian et al. (26). The PCR products were denatured and were loaded onto two to three gels with different composition (0.5X MDE gel, 6% polyacrylamide gel or with 5% glycerol). Samples showing mobility shift on the gel were reamplified, purified, and then sequenced by Delta Taq Cycle Sequencing Kit (United States Biochemical, Cleveland, OH) as described previously (27).

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3 The abbreviations used are: NPC, nasopharyngeal carcinoma; CDK4, cyclin-dependant kinase 4; SSCP, single-strand conformation polymorphism.
Comparative Multiplex PCR Analysis. All cell lines, xenografts, and 20 primary tumors with matching blood samples were examined for homozygous deletions of the p16 gene by the comparative multiplex PCR analysis (8). The primer pair for exon 2C and one of the control primer pairs for one of the following microsatellite polymorphic markers on chromosome 9 (either D9S162, IFNA, D9S171, D9S161, or GSN) were mixed in a 5-µl PCR reaction solution as described above and amplified for 24 or 27 cycles. The PCR products of the tumor sample were serially diluted and loaded onto the gel along with the PCR products of the normal sample. After autoradiography, the signals of the p16 gene of the tumor and matched blood sample were compared when the signals of the control markers were identical. Homozygous deletion was scored if the signal of p16 was missing or highly reduced in the tumor sample when compared to the corresponding control.

Southern Blotting. DNA of the samples was digested with TaqI, separated on 0.8% agarose gel, and blotted on the Hybond N+ membrane. The DNA probes were labeled with [α-32P]dCTP (Amersham Corp.) using a Megaprime DNA labeling system (Amersham Corp.). After hybridization, the filters were washed and exposed to X-ray film. The probes of the p16 gene were generated from the PCR reaction using the primer pair specific for exon 2 as described by Kamb et al. (9). The same filter was hybridized to β-actin probe as a control.

Reverse Transcriptase-PCR Analysis. The reverse transcriptase-PCR reaction was performed to examine the p16 gene expression in the mRNA of the NPC xenografts and cell lines. The mRNA of the NPC sample was extracted using the Quick Prep Micro mRNA Purification kit (Pharmacia LKB, Uppsala, Sweden). The primers 350F and 530R, described by Hussussian et al. (26), were used for the PCR amplification. The mRNA samples were also amplified by the primers for the G3APD gene (Clontech, Palo Alto, CA) as a control reaction.

Results

Mutations of the p16 Gene in NPC. Using SSCP analysis, we screened for mutations of 3 exons of the p16 gene in 20 primary tumors, 3 xenografts, and 3 cell lines of NPC. No mutations in the coding region of the p16 gene were found in any of the 3 NPC xenografts or 20 primary tumors. Abnormal bands in the SSCP gel for exon 3 of the p16 gene were detected in two primary tumors (24T and 9T). Since the mutations were located in the 3' untranslated region and also occurred in the matched blood of the corresponding patients, such base substitutions may be rare polymorphisms of the p16 gene. Moreover, the absence of p16 gene mutations in the primary NPC tumors was strengthened in an additional 25 cases analyzed. In contrast, all three NPC cell lines (HK-1, CNE-1, and CNE-2) showed mobility shifts in the SSCP gel for exon 2A of the p16 gene while the normal bands were missing (Fig. 1). These suggested that mutated p16 alleles have occurred in these NPC cell lines and that all of these lines are without the wild-type alleles. By direct sequencing, a sequence change (g to a) of the first base in the splicing acceptor site of exon 2 was found in the cell line HK-1. Another two cell lines (CNE-1 and CNE-2), which have previously been demonstrated to be of independent origins (3), showed an identical mutation in the second base (a to c) in the splicing acceptor site of exon 2. It is suspected that such mutations would affect the synthesis of normal p16 protein due to alteration in the splicing site and that complete inactivation of the p16 gene occurred in these cell lines. The results also demonstrated that the p16 gene mutation was rare in primary tumors and xenografts of NPC while occurring frequently in cell lines.

Homozygous Deletion of the p16 Gene in NPC. In order to determine whether the homozygous loss of the p16 gene occurred in NPC, comparative multiplex PCR analysis was performed on all NPC cell lines, xenografts, and 20 primary tumors with matched blood samples. No deletion of the p16 gene was detected in any of the three NPC cell lines. Homozygous deletion of the p16 gene occurred in two of three NPC xenografts. Fig. 2a demonstrated that the PCR products of exon 2 in the p16 gene were absent in the two xenografts (HK-2117 and HK-1915) while the PCR products of D9S171 as internal control were present. Moreover, multiplex PCR was performed with the p16 gene and the IFNA locus; no PCR products of both p16 gene and IFNA were found in HK-1915 (Fig. 2b). This suggested that both loci were deleted in HK-1915. The homozygous deletion of the IFNA locus in HK-1915 was further confirmed by the multiplex PCR analysis with the markers D9S171 and IFNA (Fig. 2c). Moreover, further examination showed retention of the locus D9S162 in HK-1915. These results thus defined the homozygous deletion region between D9S171 and D9S162 in the NPC xenografts HK-1915, while a smaller deletion region flanked by D9S171 and IFNA was found in HK-2117. The homozygous deletion of the p16 gene in these two xenografts was further confirmed by Southern blotting analysis. The 1.3-kb band specific for the p16 gene was absent in these samples.

By reverse transcriptase-PCR analysis, a 225-bp PCR product was detected in the mRNA of normal epithelial cell outgrowth of the nasopharynx (NP-3): three NPC cell lines (HK-1, CNE-1, and CNE-2) and one of the NPC xenografts (HK-666). In contrast, no PCR products were found from NPC xenografts HK-2117 and HK-1915 (Fig. 3). The latter finding demonstrated that there was no p16 gene expression in the two xenografts showing homozygous deletion of the p16 gene, while the mRNA of the p16 gene was expressed in the normal epithelial cells of the nasopharynx and the NPC cell lines.

For the 20 cases of primary tumors, 7 (35%) showed homozygous deletion of the p16 gene (Table 1). The signal intensities of the p16 gene were highly reduced in the tumor samples of these cases, while the control markers showed similar intensities in both normal and
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p16 gene alterations in NPC

The status of the EBV infection and p53 mutations in these NPC samples has been determined previously and are listed in Table 1. We find no correlation of the p53 gene mutation and EBV infection with the p16 gene alterations in these samples.

Discussion

In previous studies, we demonstrated that allelic loss of chromosome 9p was common in NPC and that a homozygous deletion region of 9p21-22 occurred in a case of primary tumor (8). In this report, we examined the p16 gene alterations in NPCs. Complete inactivation of the p16 gene has been found in all 3 NPC cell lines, 2 (67%) of 3 tumor DNAs (Fig. 4, a and b). Among seven cases of primary tumors, T-29 has been reported previously to contain a homozygous deletion between D9S161 and D9S162 (8). It is further noted that the flanking markers D9S171 and IFNA were always retained in all six of the other samples. All of these cases (except T-59) also demonstrated allelic loss on chromosome 9p in a previous report (8). We concluded that the p16 gene was completely inactivated in all seven of these primary NPC tumors. The data showed that homozygous deletion of the p16 gene was common in the primary tumors of NPC (35%) and xenografts (67%).

Interestingly, the signal of the IFNA locus was highly reduced in one case of primary tumors (T-21) when the comparative multiplex PCR reaction was performed with the p16 gene and IFNA locus (Fig. 4c). The homozygous deletion of IFNA in T-21 was further confirmed using the marker D9S171 as control (Fig. 4c). This finding indicated that a homozygous deletion region distal to the p16 gene occurred in T-21. Furthermore, allelic loss of the locus D9S162 in T-21 implied that the homozygous deletion region was situated between the p16 gene and D9S162 in this case.

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cell lines in vitro. Alternatively, cancer cells with pi6 gene mutations were absent in 45 primary tumors. A similar observation has been reported in other cancers such as melanoma and lung cancer (15, 16, 28). This may be due to genetic changes during the establishment of cell lines in vitro. Alternatively, cancer cells with pi6 gene mutations may have a growth advantage to be established as a line in culture. Since the primary tumors of these cell lines were not available, it was not possible to confirm whether such alterations arise in vivo in these three cases.

It was noted that the frequency of homozygous deletion of pi6 in NPC xenografts was higher than that in primary tumors. The frequency for the homozygous deletion in primary NPC tumors was likely to be underestimated due to contamination of non-neoplastic cells in the specimens. Comparative multiplex PCR analysis will not be able to detect the homozygous deletion precisely if the percentage of the non-neoplastic cells is high in the samples. Some researchers have actually grafted the primary tumor into athymic mice, which gave rise to a homogenous population of carcinoma cells for the investigation of the pi6 gene deletion (21, 29). This has proved to be a difficult task in the case of NPC since not many NPC tumors have been successfully grafted in nude mice. It is, therefore, anticipated to utilize the in situ PCR hybridization method to check for the absence of DNA or RNA of the pi6 gene in the NPC cells in these NPC tumors.

Five primary tumors, which were previously shown to have allelic loss on chromosome 9p, did not show any mutation or homozygous deletion of the pi6 gene. It is possible that there were alterations in the promoter of the pi6 gene or alterations in some other target(s) on the short arm of chromosome 9. Cheng et al. (20) demonstrated homozygous deletion outside of the pi6 gene in four malignant mesothelioma cell lines. One of the candidate targets has been suggested to be the pi5 gene (designated as MTS2), located proximal to the pi6 gene (9, 30). The pi5 gene, like pi6, encodes a cyclin-dependent kinase inhibitor protein that binds and inhibits CDK4 function (30). Recently, the deletions of both pi6 and pi5 genes were detected in brain tumors, and it has been suggested that both genes are targets on chromosome 9p21 for inactivation in the development of these cancers (21). On preliminary investigation, we observed that the pi5/MTS2 gene was homozygously deleted in two NPC xenografts (HK-2117 and HK-1915). Moreover, in a primary tumor (T-29), a large homozygous deletion region on chromosome 9p21 (between D9S161 and D9S162) has been observed which might include the pi5 gene (8). Taking these data together, it is possible that the pi5 gene may also be the other target for the inactivation in NPC.

The homozygous deletion region distal to the pi6 gene has also been found in a T-21 tumor. Such deletion may lead to the disruption of the regulatory region of the pi6 gene. Alternatively, there may be other candidate gene(s) on chromosome 9p21 which are involved in the pathogenesis of NPC. In order to confirm whether there are other target(s) for homozygous deletion in NPC samples, more sequence tag site markers between the loci IFNA and D9S171 will be used to identify the most frequent deleted locus in the future.

Recent studies demonstrated that alterations of the cell cycle control-related genes other than pi5/pi6 may act as alternative mechanisms in the development of human cancers. For example, genes like Rb, CDK4, and cyclin D1 (PRAD1) were found to be involved in human cancers (31–33).

In a study on lung cancer cell lines, Otterson et al. (31) pointed out that alterations of the RB gene and the expression of the pi6 gene were inversely correlated. CDK4 gene amplification was reported to be an alternative mechanism for abrogating the growth-regulatory effects of the pi6 gene in glioma cell lines (32). Moreover, cyclin D1 is a putative oncogene and cyclin D1 gene amplification was common in other head and neck cancers (33). All of these gene alterations, like loss of pi6 function, will interfere with the growth-suppressing function of the RB protein in the cancer cells. Summarizing from the above studies, functional inactivation of the RB gene appeared in high frequency in these cancers and might play an important role in the development of these cancers. This has led us to suggest that the RB, CDK4, and cyclin D1 genes may act as the other target(s) for the inactivation in those NPC tumors without pi6 gene alterations or allelic loss on 9p21. Since Sun et al. (4) reported that there was no mutation or deletion of the RB gene detected in NPC (4), it is suspected that the alteration of Rb gene may not play a major role in

Fig. 4. Detection of the homozygous deletion on chromosome 9p in NPC primary tumors by comparative multiplex PCR reaction. D9S161 (a) and D9S171 (b) were used as internal controls for the analysis of homozygous deletion of the pi6 gene. The signal intensities of the pi6 gene in the tumor DNAs were highly reduced compared with their corresponding normal DNAs. c, homozygous deletion of IFNA in NPC primary tumor sample of T-21 while coamplified with the pi5/6 gene or D9S171 marker. N, normal DNA; T, tumor DNA.
the development of NPC. On the other hand, amplification of the CDK4 and/or cyclin D1 gene might have taken place, affecting the cell cycle control pathways in the development of this disease. This will be a subject of our major concern and investigated in the near future.

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