Mutations in the Retinoblastoma-related Gene RB2/p130 in Primary Nasopharyngeal Carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) is an endemic cancer in southern China and northern Africa, and its pathogenesis is not yet well defined at the molecular level. Although the involvement of p53 and of the retinoblastoma gene (RB/p105) in NPC has been well studied, there is paucity of mutational data regarding the retinoblastoma-related gene RB2/p130 in primary tumors and particularly in NPC. We have shown previously that RB2/p130 could be rearranged in a nasopharyngeal cell line. In the present study, we screened by single-strand conformation polymorphism and sequence analysis the retinoblastoma-related gene RB2/p130 for mutations within exons 19–22. Mutations in the RB2/p130 gene were detected in 3 of 10 primary human NPCs from Northern Africa (30%). These findings, along with previous data showing that genetic replacement of RB2/p130 restores a normal growth pathway in the nasopharyngeal cell line Hone-1, strengthen the hypothesis that genetic changes of RB2/p130 may be involved in the development and/or progression of nasopharyngeal cancer and suggest that RB2/p130 could be considered a tumor suppressor gene and may be a candidate for novel gene therapeutic approaches for NPC.

Introduction

The molecular basis of cancer genesis involving tumor suppressor genes is of fundamental importance in cancer research (1). p53 and RB/p105 represent the prototypes of these genes, and their protein products share cell cycle regulatory functions at the G1-S checkpoint (2,3). Wild-type p53 inhibits the growth of tumor cell lines (4), suppresses oncogene-mediated transformations, and reverts the malignant phenotype of tumor-derived cell lines (5). Alterations in the p53 gene, resulting in loss of function, are involved in the development of several types of human cancers, because the normal function of the p53 gene is completely disrupted (6,7). Many mutant forms of p53 have been reported to promote neoplastic transformation (7), and the importance of the p53 gene in determining susceptibility to cancer has been exemplified by its role in the pathogenesis of the Li-Fraumeni syndrome (8).

On the other hand, RB/P105 gene inactivation has been linked to the pathogenesis of a wide range of human tumors (9). The retinoblastoma gene family includes three members: the retinoblastoma gene RB/P105, p107, and RB2/p130 (9). These proteins exhibit different growth-suppressive properties in selected cell lines, suggesting that the different members of the retinoblastoma protein family may complement each other but are not fully functionally redundant (10,11). The RB family members share homology within an important functional domain termed the “pocket-region,” which mediates their binding to different viral and cellular proteins (9). Genomic rearrangements in the RB2/p130 gene have been described in the Hone-1 nasopharyngeal carcinoma cell line as well as mutations in a lung carcinoma cell line and in lung primary specimens (11, 12, 13).3

NPC is an endemic cancer with a very high incidence in southeastern China and North Africa (14). There is a large body of evidence implicating the intake of salted food, acting in concert with EBV infection, as a major cause of the high incidence of NPC in these geographical regions (14–16). Experimental data show that large amounts of nitrates present in salted food, which are converted to carcinogenic nitrosamine, produce adenocarcinomas and undifferentiated carcinomas of the nasal and paranasal sinus cavities in rats (17). However, the NPC pathogenesis is not yet well defined at the molecular level. The involvement of tumor suppressor genes in this cancer, such as p53 and RB/p105, has also been suggested (18–20). Several groups reported that p53 gene mutations could be implicated in NPC genesis. These mutations occur preferentially in exons 5 and 8 of the p53 gene (19, 21). Additional data indicate that p53 protein is overexpressed in NPC (20, 22). Interestingly, previous groups have found no RB/p105 gene rearrangement in NPC (23). However, unlike RB/p105, previous data suggested that RB2/p130 is involved in the oncogenesis of NPC (11). This scenario led us to investigate whether the growth-regulating gene RB2/p130 is altered in NPC tumors.

To better understand the involvement of the RB2/p130 gene in NPC pathogenesis, we undertook a mutational spectrum analysis of the RB2/p130 gene from exons 19 to 22 in 10 NPC primary biopsy specimens from Moroccan patients. The mutations were screened through PCR-SSCP analysis combined with direct DNA sequencing analysis as described before (19).

Materials and Methods

Patient Samples and DNA Extraction. Biopsy samples originated from EBV+ patients from North Africa (10 biopsies in total). All NPC tumor biopsy specimens were classified histopathologically as primary nasopharyngeal tumors (NPCs). None of the patients had received any chemo- or radiotherapy prior to biopsy. The primary tumor biopsies were snap-frozen in liquid nitrogen and stored at −80°C for subsequent DNA extraction as described previously (18).

Amplification of the RB2/p130 Gene by Multiplex-PCR. Specific genomic DNA fragments were amplified by a double-step PCR procedure. For
the first round of amplification, multiplex-PCR was used to amplify the region spanning from exons 19 to 22 of the RB2/p130 gene using four paired oligonucleotides. The paired primers (E19/E19R), (E20/E20R), (E21/E21R), and (E22/E22R) were used, respectively. The amplification mixture and procedures followed were as described previously (18), except that the annealing temperature to amplify the RB2/p130 gene was 55°C. Multiplex PCR products were analyzed on a 1% agarose gel to evaluate the sizes of the PCR products. Subsequently, a second round of amplification was performed. Products of the first amplification were appropriately diluted and reamplified in the presence of [32P]dCTP, using nested primers for exons 19, 20, 21, and 22 for the RB2/p130 gene. The PCR products containing [32P]dCTP were electrophoresed on a 0.5% mutation detection enhancement acrylamide gel (FMC Corp., Rockland, ME) at 8 W constant power for 8 h at 15°C in a 0.6× Tris-Borate EDTA running buffer (TBE). The gel was then dried and exposed to an autoradiographic film at −70°C.

Table 1 shows the nucleotide sequences of the primers used for multiplex-PCR, PCR-SSCP, and sequence analyses for each exon of the RB2/p130 gene that were analyzed.

**Extraction of the Shifted Bands from SSCP Gel and Sequence Analyses.**

The developed film was aligned with the corresponding SSCP gel to identify the aberrantly migrating DNA band. When a band showing a mobility shift was detected through SSCP analysis, the band was excised with a razor blade and placed in a microcentrifuge tube containing 200 μl of H2O and incubated for 5 min at 65°C to remove the DNA from the acrylamide. The isolated DNA was purified, PCR amplified, and sequenced by automated DNA sequencer using the dideoxy terminator reaction chemistry for sequence analysis on the Applied Biosystem Model 373A DNA sequencer.

**Results**

**Identification of RB2/p130 Gene Mutations in NPC.** Because of the small amount of DNA extracted from the NPC biopsies, we performed a multiplex-PCR analysis of the RB2/p130 gene (exons 19 to 22) encompassing part of the B domain, the pocket region, and the COOH-terminal region because the highest frequency of point mutations within the RB/p105 gene are found in these regions (9). Fig. 1 shows an example of the RB2/p130 gene amplification of exons 19 to 22 by multiplex-PCR. We detected the mutations by subjecting each exon to PCR-SSCP analysis, followed by purification of the DNA fragment contained in the shifted band, and direct sequencing of the amplified DNA. This approach was applied to all NPC samples.

PCR-SSCP analysis of exon 19 showed the presence of two different migration patterns: the wild-type SSCP pattern exhibited by the control placenta DNA; and the sample mNPC6 and the mutated pattern found in the samples mNPC7 and mNPC8 (Fig. 2A). The NPC samples that were found to have a migratory pattern comparable with the placenta control were also found to be wild type by sequence analysis. Sample mNPC6 shown in Fig. 2A is a representative example of a specimen found wild type for RB2/p130.

PCR-SSCP analysis of exon 21 showed the presence of two distinct migratory patterns: the wild-type pattern displayed by the control and the mutated pattern exhibited by the samples mNPC8 and mNPC9 (Fig. 2B). The NPC samples displaying a distinctive SSCP migration pattern for the RB2/p130 gene with respect to the control were subjected to automatic sequence analysis.

Table 2 summarizes all mutations detected in the NPC series examined for the RB2/p130 gene. Interestingly, the two tumor samples that exhibited the same pattern for exon 21, mNPC8 and mNPC9, were shown to have the same mutations (Fig. 3C). These mutations were nucleotide insertions causing a frame-shift of the coding sequence occurring at codon 1079 (3304–3305 bp). Other mutations occurred in exon 19 through nucleotide insertions that resulted in a frame-shift of the open reading frame (codons 928 and 957; Table 2; Fig. 3A).

Samples were heterozygous for the mutations, which could be attributable to either the presence of both alleles (the wild-type and the mutated one) in the tumor cells or alternatively, to the presence of the wild-type alleles in the infiltrating lymphocytes commonly found in NPC. In summary, we found that in 3 of 10 tumor samples (30%), the RB2/p130 gene was mutated in the region encompassing exons 19 to 22 (Fig. 3).

**Discussion**

Several investigators have shown that p53 and RB/p105 control the cell cycle at the G1-S checkpoint. Specifically, it was found that rearrangement of these genes resulting in gene inactivation could allow cells to bypass this checkpoint, enabling them to undergo mitosis (1–3). Similarly, cooperation between the two proteins to regulate cellular proliferation is suggested by the fact that DNA tumor viruses simultaneously evolved the ability to repress both p53 and pRB/p105 function to accomplish cellular transformation (1). Furthermore, after cellular DNA damage, a transactivation product of the p53 gene termed cyclin-dependent kinase inhibitor (p21/Waf1) links the response of p53-mediated G1 arrest to the inhibition of phosphorylation of pRb, an indispensable event to enter S phase. This coordination between p53 and pRb/p105 is thought to extend to the induction of apoptosis as well. The E2F1 transcription factor, regulated by complex formation with pRb/p105, cooperates with p53 to mediate apoptosis (24).

Convincing evidence, demonstrating cooperation between p53 and pRb/p105, was found by the study of gene knockout mice. Germ-line mutations in Rb/p105 or p53 predispose the mice to malignancy (25, 26). The null mice for both Rb/p105 and p53 genes developed typical tumors of each individual gene knock-out in addition to novel tumor spectrums when compared with viable RB+/− mice and p53−/− mice (27). This suggested that p53 and pRb/p105 can complement the tumor suppressor function of each other and that some tissues require the inactivation of both proteins for neoplastic transformation.
transforming oncoprotein may provide the cell with a growth advantage, resulting in tumor formation.

Previous studies have led to the conclusion that NPC shows no detectable RB/p105 gene alterations (23). However, rearrangements of the RB2/p130 gene have been found in the NPC cell line Hone-1 (11). The introduction of pRh2/p130 in Hone-1 cells, which expresses pRh2/p130 at a low level, causes a significant reduction in cell proliferation and a change in morphology (11). The Hone-1 cell line expresses the RB/p105 gene normally, and no point mutation has been detected in the common sites for the RB/p105 gene (11, 23). These recent findings hint at a possible involvement of pRh2/p130 in nasopharyngeal carcinogenesis in the presence of a functionally intact RB/p105 gene.

Several groups have studied NPC tumors to identify potential mechanisms of tumor development. Because in certain geographical areas dietary factors are suspected to be important in the development of NPC, it would be interesting to know whether specific chemical carcinogens present as contaminants in certain foods can cause specific RB2/p130 mutations, and whether these carcinogens would be able to act on the DNA either directly or after metabolic modification.

A larger screening of the RB2/p130 gene for mutations in patients from high-risk regions of NPC would help to clarify the relationship between the RB2/p130 gene and NPC genesis. This would also help to clarify the role of dietary factors and/or EBV infections together with tumor suppressor genes mutations in NPC pathogenesis.

Point mutations in the p53 gene have been found to occur in NPC primary tumors at an estimated frequency of 10–14%, clustered within exons 5 and 8 (19, 21). This estimated rate of gene mutation could be lower than the actual rate because NPC tumors are highly infiltrated with lymphocytes (31), and because SSCP analysis is able to detect only 90% of p53 mutants (32).

We report here, for the first time, that among 10 biopsies of primary NPC tumor originating from north Africa, 3 of ten tumors (30%) contained a mutated RB2/p130 gene. Mutations were found to occur preferentially in exons 19 and 21 of the RB2/p130 gene, which are parts of the B and COOH-terminal functional domains, respectively (33). It has been demonstrated that this region is involved in the binding function of the retinoblastoma family proteins to several cellular and viral proteins (9). Furthermore, the analysis of the mutation found in the RB2/p130 gene shows that these mutations caused nucleotide insertions producing frameshift mutations, causing a complete amino acid change in the proteic structure of RB2/p130 (Table 2).

A paradigm is forming that the removal or inactivation of a functional pRh2/p130 protein by way of tumor viral oncoproteins, as in the case of SV40 large T-antigen-associated mesothelioma (34), or by genetic alteration as in the case of NPC, may be a critical event in the malignant transformation of certain cells.

This is the first indication of a possible involvement of the RB2/p130 gene in primary NPC pathogenesis. The hypothesis that mutations in the RB2/p130 gene are involved in NPC pathogenesis must be certainly extended further. A study examining a larger number of NPC biopsies would help to better investigate the role of RB2/p130 in nasopharyngeal tumorigenesis.

Additionally, because identical mutations were found in different

On the other hand, the fact that transforming oncoproteins such as SV40 T-antigen must maintain an intact Rb-family binding domain (LXCXE domain) to transform RB−/− cells indicates that the two other members of the retinoblastoma family, p107 and pRb2/p130, are also strategically important targets for SV40 T-antigen-mediated transformation (29).

In support of the idea that the RB2/p130 gene is a tumor suppressor gene, it has been demonstrated that enhanced expression of pRh2/p130 inhibits tumor growth in vivo (29). In addition, RB2/p130 has been mapped to the human chromosome 16q12.2, an area in which deletions have been found in several human neoplasias including breast, ovarian, hepatic, and prostatic cancers (30). Furthermore, mutations in the RB2/p130 gene were also shown to occur in a human small cell lung cancer cell line (12) as well as primary tumor specimens. This suggests that functional inactivation of one or more of genes in the retinoblastoma family by genomic mutations or by

### Table 2. Mutations in the RB2/p130 gene in NPC biopsies

<table>
<thead>
<tr>
<th>Sample mNPC</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>AGA ⇒ AGC-A [Ins]</td>
<td>Frameshift</td>
<td>Codon 928 (Exon 19)</td>
</tr>
<tr>
<td>7</td>
<td>AAC ⇒ AGA-C [Ins]</td>
<td>Frameshift</td>
<td>Codon 957 (Exon 19)</td>
</tr>
<tr>
<td>9</td>
<td>AAC ⇒ ATA-C [Ins]</td>
<td>Frameshift</td>
<td>Codon 1079 (exon 21)</td>
</tr>
</tbody>
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Fig. 2. Mutations in the RB2/p130 gene detected by PCR-SSCP analysis in NPC samples. A. PCR-SSCP analysis of exon 19 of the RB2/p130 gene from various NPC specimens and genomic placenta DNA as control (CT). B. PCR-SSCP analysis of exon 21 of the RB2/p130 gene from various NPC specimens and genomic placenta DNA as control (CT).
Fig. 3. Sequence analysis of exons 19 and 21 from various NPC specimens and genomic placenta DNA as control.
tumor biopsies, it is worth further investigation of the possible presence of a mutational “hot spot” in NPC that could be useful to develop a rapid diagnostic and/or prognostic tool for these patients.

Acknowledgments

We thank Drs. Eveline May, Pierre May, and Fatima Chakrani for providing the nasopharyngeal tumor samples.

References

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Cancer Res 2000;60:8-12.

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