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Advances in Brief

High Frequency of Promoter Hypermethylation of *RASSF1A* in Nasopharyngeal Carcinoma

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Abstract

We have investigated the genetic and epigenetic changes of a newly isolated tumor suppressor gene on 3p21.3, *RASSF1A*, in nasopharyngeal carcinoma (NPC). Four xenografts, four cell lines and 21 primary tumors were examined. Promoter hypermethylation of the 5′CpG island of *RASSF1A* was detected in 4 of 4 (100%) xenografts, in 3 of 4 (75%) cell lines, and in 14 of 21 (66.7%) primary tumors but not in the normal nasopharyngeal epithelia. Mutations were found in 2 of 21 (9.5%) primary tumors. In the cell lines and xenografts with extensive methylation, no *RASSF1A* gene expression was found. After treatment with 5′-aza-2′deoxycytidine, reexpression and demethylation of the *RASSF1A* gene were detected in a NPC cell line. These findings suggest that promoter hypermethylation may participate in the transcriptional inactivation of the *RASSF1A* gene in NPC. The high incidence of *RASSF1A* alterations suggest that it is the critical target gene on chromosome 3p21.3 involved in the development of NPC.

Introduction

NPC[3] is a serious health problem in southern China because it has an unusually high incidence among our population. The annual male incidence rate in Hong Kong is 24.6/100,000 persons (Hong Kong Cancer Registry, 1997), which contrasts with a frequency of <1/100,000 persons in Caucasians in other countries. The incidence of NPC peaks at the relatively young age of 45 years. We aim to understand the molecular basis of this cancer and thereby expand the prospects for the development of early diagnostic markers and novel therapeutic strategies (1, 2). Over the past decade, molecular and cytogenetic studies have contributed significantly to the identification of genetic and epigenetic changes associated with this cancer (2–6). In addition to the EBV latent infection, inactivation of the *p16* gene by promoter methylation and homozygous deletion is a crucial event in NPC tumorigenesis. Genome-wide studies by allelotyping and CGH have demonstrated high frequencies of genetic abnormalities on 3p, 9p, 11q, 12q, 13q, and 14q in this cancer (3, 4). LOH of 3p was demonstrated in 95–100% of NPCs and in 75% of precancerous lesions (3, 7). These findings suggested that inactivation of the tumor suppressor gene(s) on 3p is a critical and early event in the development of NPC. Deletion mapping and functional studies have targeted the NPC-related tumor suppressor gene(s) to a region at 3p21.3 (3, 8, 9). Recently, Dammann *et al.* (10) have isolated a RAS association domain family protein, RASSF1, which is located in the 120-kb region of minimal homozygous deletion at 3p21.3 in lung cancer. They demonstrated that one of the major transcripts of this gene, *RASSF1A*, is frequently inactivated in lung cancers by promoter hypermethylation. Reexpression of the gene in lung cancer cell lines suppressed the malignant phenotype. These results suggest that the *RASSF1A* gene is the target tumor suppressor at 3p21.3 for human cancers. In this study, we investigated whether *RASSF1A* alterations might play a role in the tumorigenesis of NPC. The frequencies of the promoter hypermethylation and mutation of the *RASSF1A* gene in the NPC samples, including cell lines, xenografts, and primary tumors, were explored.

Materials and Methods

Cell Lines, Xenografts, and Primary Tumor Samples. Four xenografts (xeno-2117, xeno-1915, xeno-666, and xeno-8), two cell lines (C666-1 and CNE-1) derived from undifferentiated NPCs, and two cell lines (HK-1 and CNE-2) derived from differentiated NPCs were examined (5, 11–13). The cell line C666-1 was derived from the xenograft xeno-666 in our laboratory (13). The status of chromosome 3p of these cell lines and xenografts have been investigated previously by karyotyping and CGH analysis (Table 1). Two normal epithelial outgrowths (NO-1 and NO-2) derived from nasopharyngeal mucosal tissue were also included as controls. All cell lines were maintained in RPMI 1640 with 10% fetal bovine serum. Twenty-one microdissected primary NPC specimens from our previous allelotyping study were included (3). All of these tumor biopsies were obtained from NPC patients with consent before treatment at the Department of Clinical Oncology in the Prince of Wales Hospital (Hong Kong, China). Six microdissected samples of normal nasopharyngeal epithelium (NP-1 to -6) from aborted fetuses obtained from the Pathology Tissue Bank in the Department of Anatomical and Cellular Pathology were included as controls (7). High-molecular weight DNA was extracted from the tumor samples according to the conventional methods (9).

SSCP Analysis and DNA Sequencing. DNA samples from the four xenografts, four cell lines, and 21 primary tumors of NPC were subjected to SSCP analysis for screening of *RASSF1A* gene mutation, as described previously (5). The exon-intron boundaries of *RASSF1A* have been revealed by aligning the full-length cDNA sequence to the genomic sequences of the cosmids LUCA12 and LUCA13 using the BLAST-2 program (National Center for Biotechnology Information). All six exons of the *RASSF1A* gene were examined using the 10 pairs of primers listed in Table 2. The shifted bands on the SSCP gels were isolated to elute DNA for sequencing analysis. After reamplification and cloning by using the pMOS*Blue* blunt-ended cloning kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), plasmid DNA was purified and then sequenced by the ABI PRISM DyeDeoxy Terminator Cycle Sequencing Kit and ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

Bisulfite Sequencing. To access the methylation status of the promoter region of *RASSF1A*, four NPC xenografts, four NPC cell lines, two normal nasopharyngeal cell outgrowths, and six samples of microdissected normal epithelia from nasopharynx were subjected to bisulfite sequencing. Genomic

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[3] The abbreviations used are; NPC, nasopharyngeal carcinoma; CGH, comparative genomic hybridization; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism; MS-PCR, methylation-specific PCR; RT-PCR, reverse transcription-PCR; NP, nasopharyngeal epithelial.

Table 1 *Genetic and epigenetic changes of RASSF1A in the xenografts, cell lines, and primary tumors of NPC*

	Mutation	Methylation	3p deletion[a]	RNA expression
Xenografts (n = 4)				
xeno-2117	−ve (P1)[b]	+ve (PM)[b]	? (gain)[c]	+ve
xeno-1915	−ve (P1)[b]	+ve (PM)[b]	−ve	NA[d]
xeno-8	−ve (P1)[b]	+ve	+ve	−ve
xeno-666	−ve (P1)	+ve	−ve	−ve
Cell lines (n = 4)				
C666-1	−ve (P1)[b]	+ve	−ve	−ve
HK-1	−ve	−ve	+ve	+ve
CNE-1	−ve	+ve (PM)[b]	+ve	+ve
CNE-2	−ve	+ve (PM)[b]	+ve	+ve
Primary tumors (n = 21)				
NPC-1	−ve	−ve	+ve (LOH)	NA[d]
NPC-2	−ve (P1, P2)[b]	+ve	+ve (LOH)	NA[d]
NPC-3	−ve	−ve	+ve (LOH)	NA[d]
NPC-4	+ve	+ve	+ve (LOH)	NA[d]
	(Frameshift mutation, 1-bp deletion at nt 829)			
NPC-5	−ve	−ve	+ve (LOH)	NA[d]
NPC-6	−ve	+ve	+ve (LOH)	NA[d]
NPC-7	−ve	+ve	+ve (LOH)	NA[d]
NPC-8	−ve	−ve	+ve (LOH)	NA[d]
NPC-9	−ve	+ve	+ve (LOH)	NA[d]
NPC-10	−ve	+ve	+ve (LOH)	NA[d]
NPC-11	−ve	+ve	+ve (LOH)	NA[d]
NPC-12	−ve	+ve	+ve (LOH)	NA[d]
NPC-13	−ve	+ve	+ve (LOH)	NA[d]
NPC-14	−ve	+ve	+ve (LOH)	NA[d]
NPC-15	−ve	−ve	+ve (LOH)	NA[d]
NPC-16	−ve	−ve	+ve (LOH)	NA[d]
NPC-17	−ve	+ve	+ve (LOH)	NA[d]
NPC-18	+ve	−ve	+ve (LOH)	NA[d]
	(Codon 201 missense mutation, CGC (Arg) to CAC (His))			
NPC-19	−ve	+ve	+ve (LOH)	NA[d]
NPC-20	−ve	+ve	+ve (LOH)	NA[d]
NPC-21	−ve	+ve	+ve (LOH)	NA[d]
	(2/21 = 9.5%)	(14/21 = 66.7%)		

[a] Results of 3p deletions in NPC samples were from previous allelotyping, karyotyping, and CGH studies (Refs. 2–4 and 11–13).
[b] P1, rare polymorphism with sequences changes at codon 53, 56, 57, and 60; PM, partial methylation; P2, rare polymorphism with sequence change at codon 133.
[c] Gain, copy number gain detected by CGH analysis.
[d] NA, not available.

DNAs were modified by bisulfite treatment and purified using CpGenome DNA Modification Kit (Intergen, Purchase, NY) according to the manufacturer's recommendations. To obtain products for sequencing, semi-nested PCR amplification was performed on 100 ng of bisulfite-modified genomic DNA as described by Dammann *et al.* (10). The sequences of the primers were listed in Table 2. The amplified fragments were subcloned. Ten clones of each sample were selected for sequencing.

Methylated-specific PCR. For primary NPC samples, the promoter methylation status of *RASSF1A* was investigated by methylated-specific PCR assay as described previously (14). Genomic DNAs of the microdissected specimens were modified by bisulfite treatment. The primer pairs specific for methylated (MSM-1 and MSM-2) and unmethylated DNA (MSU-1 and MSU-2) are listed in Table 2. One hundred μl of bisulfite-modified DNA from the samples were subjected for PCR amplification. Modified DNA from the NPC cell lines, normal epithelial outgrowths, and six microdissected normal epithelia were included as controls. Controls without DNA were used for each set of assay. The MS-PCR for all samples was repeated to confirm their methylation status. Fifteen μl of PCR products were loaded onto a 10% nondenaturing polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination.

5′-Aza-2′-deoxycytidine Treatment. To determine whether *RASSF1A* expression could be restored by application of a demethylating agent, the NPC cell line C666-1 (which showed complete methylation and no expression of *RASSF1*) was subjected to 5-aza-2′-deoxycytidine treatment. Cells were plated and incubated for 4 days with 1, 3, or 10 μM 5-aza-2′-deoxycytidine (Sigma Chemical Co., St. Louis, MO). The medium and the drug were replaced every 24 h.

RT-PCR. The expression of the *RASSF1A* transcripts in the NPC xenografts and cell lines was examined by RT-PCR analysis. Total RNAs from NPC samples were extracted using the TriZOL reagent (Life Technologies, Inc., Rockville, MD). Total RNAs from two normal nasopharyngeal cell outgrowths were also included. RT-PCR was performed using the primer pairs RTE1F and RTE4R in exon 1α and exon 4 of the *RASSF1A* gene, respectively. The PCR products were then subjected to semi-nested RT-PCR using RTE4R and the internal primer RTE2F in exon 2$\alpha\beta$ (Table 2). The RNA samples were also amplified by the primers of the *β-globin* gene as control.

Results

The status of chromosome 3p in the NPC samples had already been determined by either karyotyping, CGH, or LOH analysis (Table 1). Deletion or LOH of 3p was common. However, no homozygous deletion of the *RASSF1A* gene was found in any of the four xenografts and the four cell lines because all six exons of the gene could be amplified in these homogenous tumor samples.

Mutation of *RASSF1A* in NPC. We have screened all six exons of *RASSF1A* in 21 primary tumors, four xenografts, and four cell lines of NPC for mutations by SSCP analysis. SSCP analysis detected identical mobility shifts of exon 1α in all four xenografts, a cell line (C666-1), and a primary tumor (NPC-2) and its corresponding blood sample by using the primer pairs RAE1-B1/RAE1-B2 and RAE1-C1/RAE1-C2. DNA sequencing confirmed multiple base substitutions at codons 53, 56, 57, and 60. The sequence change in codon 60 (GCC to ACC) leads to an amino acid change from Ala to Thr, whereas those in codon 53 (CGC to CGT), 56 (CCC to CCT), and 57 (GCG to GCA) do not. We believe that these specific sequence changes may be rare polymorphisms of *RASSF1A* in our population. No mutations in *RASSF1A* were found in any of the other three cell lines, HK-1, CNE-1, and CNE-2. Mobility shifts were also detected in 3 of 21 primary tumors. Abnormal bands on SSCP gels for exon 3, exon 4, and exon 5 were found in the NPC cases NPC-2, -18, and -4, respectively. The sequence changes in these tumors and corresponding blood samples of the patients were confirmed further by direct DNA sequencing (Table 1). In case NPC-2, a missense mutation

Table 2 *PCR primer sequences for SSCP, bisulfite sequencing, and MS-PCR assay*

Primer	Sequence	Product size	Annealing temperature	Cycles
SSCP				
Exon 1				
RAE1-A1	5′-CCTGCTAGCGCCCAAAGC-3′	83	55°C	35
RAE1-A2	5′-CAGCTCCCGCAGCTCAAT-3′			
RAE1-B1	5′-ATGTCGGGGGAGCCTGAG-3′	151	58°C	35
RAE1-B2	5′-CACGGCCAGGGACCAG-3′			
RAE1-C1	5′-CTGGAGCGTGCCAACG-3′	150	55°C	35
RAE1-C2	5′-GACGCCCCAGATGAAG-3′			
RAE1-D1	5′-CACACGGCAGCTGGTC-3′	87	58°C	35
RAE1-D2	5′-AAGTCGCCACAGAGGTC-3′			
Exon 2				
RAE2-1	5′-GGGACATTTCCCCGACCT-3′	200	55°C	35
RAE2-2	5′-ATCCTCGCCCTTCCCATAC-3′			
Exon 3				
RAE3-1	5′-CCAGAGGCCATTTTTCAGAG-3′	226	55°C	35
RAE3-2	5′-TAGCTGGGTACCTGCTCCTC-3′			
Exon 4				
RAE4-A1	5′-ACTGACATCTCACCTGGAACC-3′	240	55°C	35
RAE4-A2	5′-CACATGCAGGTGCTTGACA-3′			
RAE4-B1	5′-GCCGCACTTCCTTTTACCT-3′	210	55°C	35
RAE4-B2	5′-GCATGCGTATATACCCTCACA-3′			
Exon 5				
RAE5-1	5′-TGGCCCTGTCTCTGATCATT-3′	231	55°C	35
RAE5-2	5′-TCCTCCTCCAAGCCTTACTG-3′			
Exon 6				
RAE6-B1	5′-CCTGTACACTCCCCTTTTGC-3′	194	58°C	35
RAE6-B2	5′-ACCTGGGGGTACAAGAGGTC-3′			
Bisulfite sequencing				
1st PCR				
RABS-1	5′-GTTTTGGTAGTTTAATGAGTTTAGGTTTTTT-3′		55°C	30
RABS-3	5′-CCCCACAATCCCTACACCCAAAT-3′			
2nd PCR				
RABS-2	5′-ACCCTCTTCCTCTAACACAATAAAACTAACC-3′	205	55°C	30
RABS-3	5′-CCCCACAATCCCTACACCCAAAT-3′			
Methylated specific PCR Assay				
Methylated				
RAM-1	5′-GTGTTAACGCGTTGCGTATC-3′	93	60°C	35
RAM-2	5′-AACCCCGCGAACTAAAAACGA-3′			
Unmethylated				
RAU-1	5′-TTTGGTTGGAGTGTGTTAATGTG-3′	105	60°C	35
RAU-2	5′-CAAACCCCACAAACTAAAAACAA-3′			
RT-PCR				
1st PCR				
RTE1F	5′-ACACGTGGTGCGACCTCT-3′		65°C	35
RTE4R	5′-GATGAAGCCTGTGTAAGAACCGTCCT-3′			
2nd PCR				
RTE2F	5′-CAGATTGCAAGTTCACCTGCCACTA-3′	242	65°C	25
RTE4R	5′-GATGAAGCCTGTGTAAGAACCGTCCT-3′			

(GCT to TCT/G to T) at nt 435 was observed. The mutation leads to an amino acid change (Ala to Ser) at codon 133. Detection of the identical base substitution in the corresponding blood sample of the same patient suggested that it might be a germ-line mutation or rare polymorphism. The sequencing analysis also confirmed a missense mutation of exon 4 at nt 640 (G to A) in case NPC-18. An amino acid change (Arg to His) at codon 201 may occur in *RASSF1A*. No sequence change was found in the corresponding blood sample of this patient. We also detected a frameshift mutation at exon 5 in case NPC-4. A single base deletion at nt 829 (A) was found in the tumor sample but not in the corresponding blood sample.

Methylation of *RASSF1A* in NPC. To investigate the epigenetic changes of *RASSF1A*, we analyzed promoter methylation in four xenografts, four cell lines, two normal NP outgrowths, and six microdissected normal epithelia of nasopharynx by bisulfite sequencing. DNA samples from the two normal NP outgrowths (NO-1 and -2) and six microdissected normal NP epithelia (NP-1 to -6) were completely unmethylated at all 16 CpG sites of the *RASSF1A* promoter (Fig. 1*a*). The absence of methylation was also found in one of the NPC cell lines, HK-1. Two NPC xenografts, xeno-8 and xeno-666, were almost completely methylated at the CpG sites of the *RASSF1A* promoter. The cell line C666-1, which derived from xeno-666, also showed extensive methylation at this promoter region. Partial methylation of the *RASSF1A* promoter was detected in two cell lines (CNE-1 and CNE-2) and in two xenografts (xeno-2117 and xeno-1915). These findings suggest that promoter hypermethylation of the *RASSF1A* gene is a common epigenetic event in the cell lines and xenografts of NPC.

To address the relevance of the promoter hypermethylation of *RASSF1A* in primary tumors, we examined 21 primary NPC samples by MS-PCR. In MS-PCR analysis, we designed the methylated and unmethylated specific primer pairs specific for the 5′ CpG island of *RASSF1A*. Fig. 1*b* shows MS-PCR analysis of the *RASSF1A* promoter on the NPC cell lines, HK1, C666-1, CNE1, and CNE2, the methylation status of which have been revealed by bisulfite sequencing. C666-1 showed amplification in the reaction for methylated sequences, but not for unmethylated sequences. In contrast, HK-1 showed amplification of only the unmethylated sequences. The cell lines CNE1 and CNE2, with partial methylation, showed amplification for both methylated and unmethylated sequences. Some representative examples of MS-PCR analysis of the primary tumors and microdissected normal NP epithelia are also shown in Fig. 1*b*. The methylation status of the NPC samples is summarized in Table 1. Aberrant DNA methylation of *RASSF1A* was found in 14 of 21 (66.7%) primary NPCs but not in the normal NP epithelia. It is

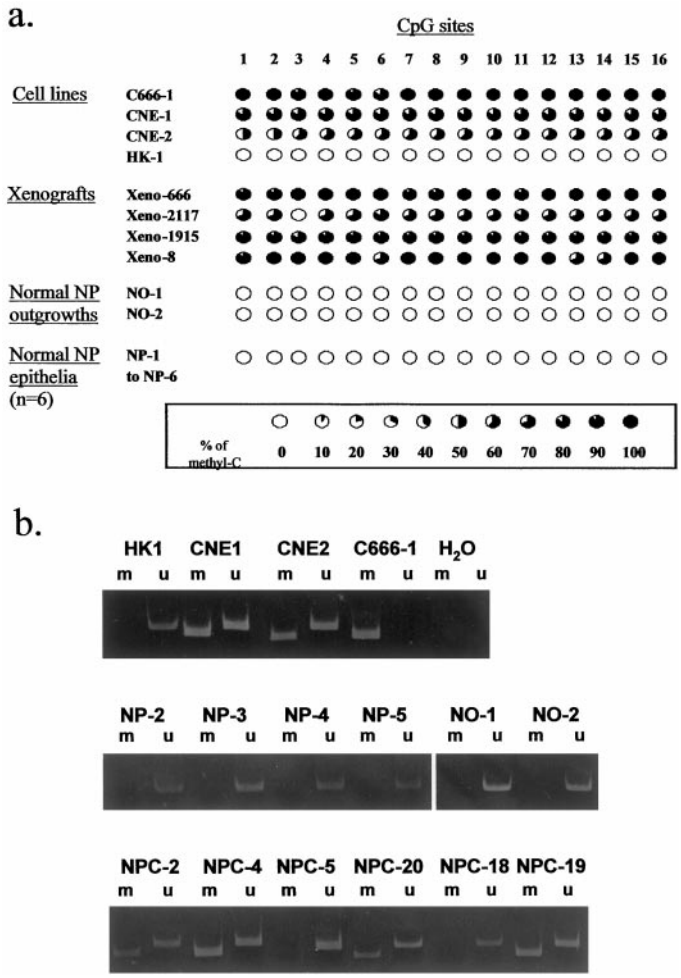

Fig. 1. Methylation analysis of *RASSF1A* in NPCs, normal epithelial outgrowths, and microdissected normal epithelia of nasopharynx. *a*, summary of 5-methylcytosine levels of the *RASSF1A* promoter detected by bisulfite sequencing. Four cell lines (C666-1, CNE-1, CNE-2, and HK-1), four xenografts (xeno-666, xeno-2117, xeno-1915, and xeno-8), two normal epithelial outgrowths (NO-1 and NO-2), and six microdissected normal nasopharyngeal epithelia (NP-1 to -6) were examined. The percentage of 5-methylcytosine for 16 CpG sites of each sample was shown. Each circle indicates a CpG dinucleotide. The numbers of the CpG sites correspond to those listed in Ref. 10. Absence of methylation was found in a cell line HK-1, two normal epithelial outgrowths (NO-1 and NO-2), and all six normal nasopharyngeal epithelia (NP-1 to -6). The cell line C666-1 and two xenografts (xeno-666 and xeno-8) showed extensive methylation in the 16 CpG sites. Partial methylation was observed in xeno-2117, xeno-1915, CNE-1, and CNE-2. *b*, MS-PCR analysis of *RASSF1A* promoter. *U*, presence of unmethylated sequences; *M*, presence of methylated sequences. *Upper figure*, cell line C666-1 was used as a positive control for methylation, and only the methylated sequences was detected. The HK-1 cell line as negative control showed the unmethylated sequences only. Both methylated and unmethylated sequences were observed in the cell lines CNE-1 and CNE-2 with partial methylation of the *RASSF1A* promoter. *Middle figures*, the four normal nasopharyngeal epithelia (NP-2 to -5) and the two normal nasopharyngeal outgrowths (NO-1 and NO-2) were unmethylated. *Lower figure*, examples of promoter hypermethylation in primary NPC. Tumors NPC-2, -4, -19, and -20 were methylated, whereas cases NPC-5 and -18 were unmethylated.

noteworthy that all of these 14 primary tumors have LOH on 3p21 (Table 1 and Ref. 7).

Expression of *RASSF1A* in NPC. We investigated the expression of *RASSF1A* transcripts in a normal nasopharyngeal cell outgrowth, three NPC xenografts, and four NPC cell lines by RT-PCR. The normal nasopharyngeal cell outgrowth NO-1 and the NPC cell line HK-1, which is completely unmethylated at the promoter region, expressed the *RASSF1A* gene (Fig. 2*a*). The expression of *RASSF1A* transcripts was also found in the three cell lines (CNE-1, CNE-2, and HK-1) and a xenograft (xeno-2117) with partial methylation. However, in the two xenografts (xeno-666 and xeno-8) and cell line C666-1, which were completely methylated at the promoter region, no expression of *RASSF1A* transcripts was detected.

To study the consequences of loss of expression of *RASSF1A* in association with promoter hypermethylation in NPC, the cell line C666-1, which showed complete methylation and transcriptional inactivation of *RASSF1A*, was subjected to 5-aza-2′-deoxycytidine treatment. Restoration of the gene expression was observed (Fig. 2*b*). Furthermore, demethylation of the promoter region was detected by MS-PCR, confirming that loss of expression in C666-1 was associated with promoter hypermethylation (Fig. 2*b*).

Discussion

Deletion of the short arm of chromosome 3 is the most common genetic alteration in NPC. Our previous studies have strongly indicated that inactivation of the tumor suppressor gene(s) on this region may be critical and early events for the development of this cancer (2–4, 7, 9). Although multiple tumor suppressor loci have been examined, results of deletion mapping and functional studies have targeted the NPC-associated gene to the 3p21.3 region (2, 3, 8, 9, 15). Frequent allelic loss and the presence of homozygous deletions in this region have been detected in several human cancers, including carcinomas of lung, breast, and ovary, and head and neck cancers (16, 17). Recent studies have focused on the candidate TSGs located within the 630-kb homozygous deletion region defined by a small cell lung cancer cell line GLC20 and a breast cancer cell line HCC1500 (18). Recently, Dammann *et al.* (10) have isolated a tumor suppressor gene, *RASSF1*, in this homozygous deletion region. The RASSF1 protein was found to be associated with the human DNA repair protein XPA,

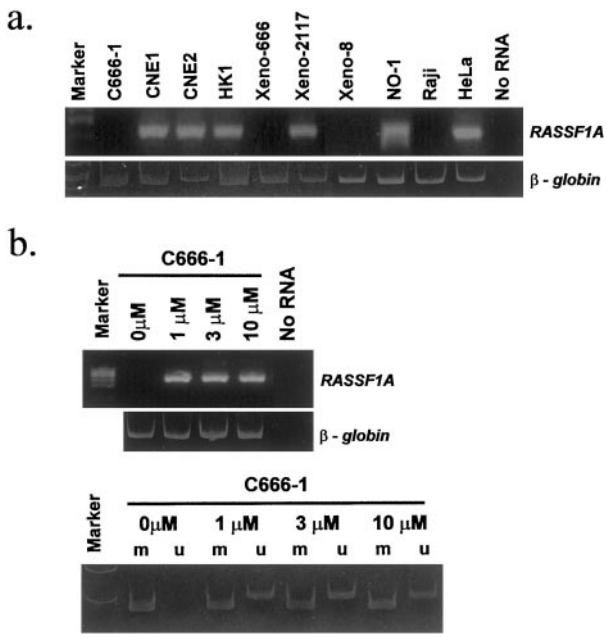

Fig. 2. *a*, semi-nested RT-PCR analysis of *RASSF1A* expression in NPC cell lines and xenografts. A 242-bp PCR product was observed in the *RASSF1A* expressed cell line HeLa but not in the negative control cell line Raji (10). *RASSF1A* expression was detected in a normal nasopharyngeal cell outgrowth (NO-1), a NPC xenograft (xeno-2117), and three NPC cell lines (HK-1, CNE-1, and CNE-2). No PCR product was observed in two xenografts (xeno-8 and xeno-666) and a cell line C666-1. Expression of *β-globin* in these samples was used as a control. *b*, reactivation of *RASSF1A* expression in C666-1 cells treated with 5′-aza-2′-deoxycytidine. The C666-1 cells were treated for 4 days with the indicated concentrations (0, 1, 3, and 10 μM) of 5′-aza-2′-deoxycytidine. *Upper figure*, the reexpression of *RASSF1A* transcripts in the samples of C666-1 treated with 1, 3, and 10 μM 5′-aza-2′-deoxycytidine. RT-PCR analysis detected a 242-bp fragment in these samples. *Lower figure*, the methylation status of the 5′-aza-2′-deoxycytidine-treated C666-1 cells. MS-PCR analysis demonstrated that the unmethylated sequence was detected in the samples treated with 1, 3, and 10 μM 5′-aza-2′-deoxycytidine but not in the untreated C666-1 cells.

and its COOH terminus shows high homology to the Ras effector protein Nore1. Vos *et al.* (19) have shown that RASSF1 binds Ras in a GTP-dependent manner and mediates the apoptotic effects of oncogenic Ras. The RASSF1 amino acid sequence has also been identified as a potential phosphorylation target for ataxia telangiectasia mutated (20). It is suggested that this protein may be involved in the DNA repair system or the Ras pathway. The most striking finding in the study of Dammann *et al.* (10) is the clear demonstration that one of the major transcripts of this gene, *RASSF1A*, is altered in the cell lines and in the primary tumors of lung cancer. The tumor suppressor function of this gene has also been demonstrated by transfection of the full-length *RASSF1A* in lung cancer cell lines. Reexpression of *RASSF1A* in lung cancer cells leads to reduced colony formation, suppressed anchorage-independent growth, and inhibited tumor formation in nude mice (10). Now, we have shown a high incidence of *RASSF1A* alterations in NPC. The data presented in this study support *RASSF1A* as the major target tumor suppressor at 3p21.3 in human cancers.

Homozygous deletion of 3p21.3 has been reported in several breast cancer and lung cancer cell lines and may be one of the major mechanisms for inactivation of *RASSF1A* in these cancers. In NPC cell lines and xenografts, however, no homozygous deletion of *RASSF1A* was detected. SSCP and sequencing analysis also found no mutations in these samples. However, promoter hypermethylation was common in these xenografts and cell lines. We detected three samples (xeno-666, xeno-8, and C666-1) showing extensive methylation and four (xeno-2117, xeno-1915, CNE-1, and CNE-2) showing partial methylation of the *RASSF1A* promoter. Complete absence of *RASSF1A* transcripts in xeno-666, xeno-8 and C666-1 suggested that the gene expression was silenced by promoter inactivation. In C666-1, treatment with 5′-aza-deoxycytidine led to demethylation of the 5′ CpG island of *RASSF1A* and reexpression of its transcripts. This strongly suggests that aberrant hypermethylation of the *RASSF1A* promoter is directly responsible for transcriptional inactivation of its expression in our NPC cell line. Correlation of promoter hypermethylation with loss of *RASSF1A* expression was also shown in the lung cancer cell lines (10). In the present study, we detected aberrant methylation and mutation of *RASSF1A* in 66.7% and 9.5% of primary tumors, respectively. Similar results were also reported in the study of Dammann e*t al.* (10). They found that the promoter was highly methylated in 60% of primary lung tumors. Only 10% of the lung tumors carried missense mutations. These data suggest that promoter hypermethylation is the major mechanism for inactivation of *RASSF1A* in human cancers. Although the incidence of the *RASSF1A* mutation is low in NPC, the identification of the missense and frameshift mutations in primary tumors provides additional support that *RASSF1A* is the target tumor suppressor gene.

The allelic status of chromosome 3p in our NPC samples has been examined by LOH analysis in our previous study. The *RASSF1A* gene is located between the loci *D3S1277* and *D3S1266*. The region flanking these loci was lost in all primary NPC tumors examined (3). As a consequence, it is suggested that *RASSF1A* was inactivated in 71.4% (15 of 21) of primary NPCs. Our previous work has shown that inactivation of the *p16* gene at 9p21 in >50% of primary NPC, whereas EBV latent infection was detected in almost 100% of the tumors (5–7). We believe that inactivation of *RASSF1* and *p16*, together with EBV latent infection, may be the critical events for NPC tumorigenesis.

In summary, we have demonstrated a high frequency of the *RASSF1A* gene aberration in nasopharyngeal carcinoma. In 71.4% of primary tumors, either promoter hypermethylation or mutation of *RASSF1A* was seen. These observations provide evidence that promoter hypermethylation and allelic loss are the major mechanisms for inactivation of this tumor suppressor gene. *RASSF1A* may be one of the key tumor suppressor genes that we have been searching for at 3p21.3 in NPC. Inactivation of its function, resulting in either defective DNA repair or disruption of the RAS pathway, may be closely associated with NPC development.

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