Promoter Hypermethylation of Multiple Genes in Nasopharyngeal Carcinoma[1]

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ABSTRACT

Purpose: **The methylation profile of nasopharyngeal carcinoma (NPC) has been investigated by a candidate gene approach.**

Experimental Design: **Four NPC cell lines, 4 NPC xenografts, 33 NPC primary tumors, and 6 samples of normal nasopharyngeal epithelium were subjected to methylation-specific PCR for analysis of promoter methylation of eight cancer-related genes. These eight genes were *RASSF1A*, *RARβ2*, *DAP-kinase*, *p16*, *p15*, *p14*, *MGMT*, and *GSTP1*. The correlation between methylation status of these genes and clinical features such as stage, local-regional recurrence, distant metastasis, and survival has been analyzed.**

Results: **The incidence of promoter methylation in NPC samples was 84% for *RASSF1A*, 80% for *RARβ2*, 76% for *DAP-kinase*, 46% for *p16*, 17% for *p15*, 20% for *p14*, 20% for *MGMT*, and 3% for *GSTP1*. No methylation of these genes was detected in the six normal nasopharyngeal epithelium samples. All NPC tumor samples in this study displayed aberrant methylation in at least one of these eight genes. No significant correlation between methylation status of these genes and clinical parameters of the patients was found.**

Conclusions: **A high frequency of aberrant methylation of the 5′ CpG island of the *RASSF1A*, *RARβ2*, *DAP-kinase*, and *p16* genes in the present study was noted. Our findings suggest that methylation of the genes in the critical pathways is common in NPC.**

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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 cases per 100,000 persons (Hong Kong Cancer Registry, 1997), which contrasts with a frequency of <1 case per 100,000 persons in Caucasians in other countries. The incidence of NPC peaks at the relatively young age of 45 years. Previous etiological studies demonstrated that the development of NPC might be attributable to a complex interaction of genetic factors, dietary exposure to chemical carcinogen, and EBV infection (1).

The tumorigenesis of NPC is thought to be a multistep process and involves multiple genetic and epigenetic changes. For the past decade, we have focused on the investigation of the molecular basis of this cancer and thereby expanded the prospects for development of early diagnostic markers and novel therapeutic strategies (1, 2).

There is growing evidence demonstrating that alterations in the distribution of 5-methylcytosine are an important factor in multistep carcinogenesis (3, 4). These changes include genome-wide hypomethylation (5, 6) and hypermethylation of CpG sites in 5′-promoter regions leading to genomic instability and inhibition of gene expression, respectively (7–9). Promoter hypermethylation has been proposed to be an alternative way to inactivate tumor suppressor genes in cancer (3, 4). Recent studies showed that this epigenetic change is common in human cancer. Some tumor suppressor genes such as *p16*, *VHL*, and *MLH1* have been found to harbor promoter hypermethylation associated with loss of protein expression in cancer cells (7–9). Several tumor types have also shown aberrant methylation at CpG islands in other genes, including the detoxifying gene *GSTP1* (10), the DNA repair gene *MGMT* (11), and the apoptosis-related and potential metastasis inhibitor gene *DAP-kinase* (12). In NPC, a high frequency of epigenetic inactivation of the tumor suppressor genes *p16* and *RASSF1A* was detected (13, 14). The identification of genes targeted by hypermethylation may provide insights into NPC tumorigenesis. In addition, hypermethylated genes may serve as targets for the development of a novel screening test for cancer (15).

In the present study, we have analyzed the promoter hypermethylation pattern of the human *Ras association domain family 1A (RASSF1A)*, *retinoic acid receptor β-2 (RARβ2)*, *death-associated protein kinase (DAP-kinase)*, *p16 (CDKN2A)*, *p15 (INK4b)*, *p14 (ARF)*, *O^6-methylguanine-DNA methyltransferase (MGMT)*, and *GSTP1* genes in 4 NPC cell lines, 4 xenografts, and 33 primary tumors together with normal nasopharyngeal epithelium. The correlation between methylation status of these genes and clinical features such as stage, local-

[3] The abbreviations used are: NPC, nasopharyngeal carcinoma; MSP, methylation-specific PCR; GST, glutathione *S*-transferase.

Table 1 Summary of primer sequences,[a] annealing temperatures, and PCR product sizes used for MSP

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Annealing temperature (°C)	Product size (bp)
RASSF1A	M:[b] GTGTTAACGCGTTGCGTATC	M: AACCCCGCGAACTAAAAACGA	60	93
	U: TTTGGTTGGAGTGTGTTAATGTG	U: CAAACCCCACAAACTAAAAACAA	60	105
RARβ2	M: GGTTAGTAGTTCGGGTAGGGTTTATC	M: CCGAATCCTACCCCGACG	58	235
	U: TTAGTAGTTTGGGTAGGGTTTATT	U: CCAAATCCTACCCCAACA	58	233
DAP-kinase	M: GGATAGTCGGATCGAGTTAACGTC	M: CCCTCCCAAACGCCGA	58	98
	U: GGAGGATAGTTGGATTGAGTTAATGTT	U: CAAATCCCTCCCAAACACCAA	58	106
p16	M: TTATTAGAGGGTGGGGCGGATCGC	M: GACCCCGAACCGCGACCGTAA	65	150
	U: TTATTAGAGGGTGGGGTGGATTGT	U: CAACCCCAAACCACAACCATAA	60	151
p15	M: GCGTTCGTATTTTGCGGTT	M: CGTACAATAACCGAACGACCGA	60	148
	U: TGTGATGTGTTTGTATTTTGTGGTT	U: CCATACAATAACCAAACAACCAA	60	154
p14	M: GTGTTAAAGGGCGGCGTAGC	M: AAAACCCTCACTCGCGACGA	60	122
	U: TTTTTGGTGTTAAAGGGTGGTGTAGT	U: CACAAAAACCCTCACTCACAACAA	60	132
MGMT	M: TTTCGACGTTCGTAGGTTTTCGC	M: GCACTCTTCCGAAAACGAAACG	61	81
	U: TTTGTGTTTTGATGTTTGTAGGTTTTTGT	U: AACTCCACACTCTTCCAAAAACAAAACA	61	93
GSTP1	M: TTCGGGGTGTAGCGGTCGTC	M: GCCCCAATACTAAATCACGACG	60	91
	U: GATGTTTGGGGTGTAGTGGTTGTT	U: CCACCCCAATACTAAATCACAACA	60	97

[a] References for primer sequences: Ref. 11, 12, 14, and 22–25.
[b] M, methylated-specific primers; U, unmethylated-specific primers.

regional recurrence, distant metastasis, and survival of the patients has been analyzed.

MATERIALS AND METHODS

Cell Lines and Xenografts. 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 (16, 17). Cell line C666-1 was derived from xenograft Xeno-666 in our laboratory (17). These cell lines were maintained in RPMI 1640 with 10% fetal bovine serum. Four NPC xenografts (Xeno-2117, Xeno-1915, Xeno-666, and Xeno-8) were also involved (18). High molecular weight DNA was extracted from the tumor samples according to conventional methods (19).

Tissue Samples. Thirty-three cases of primary tumors (NPC1T–NPC33T) were included. Twenty-five paraffin-embedded tumors and 6 samples of normal tissue (FN1E–FN6E) of the nasopharynx from aborted fetuses were obtained from the Pathology Tissue Bank of the Department of Anatomical and Cellular Pathology at the Princes of Wales Hospital (Hong Kong SAR, People's Republic of China). Eight tumor biopsies were obtained from NPC patients with consent before treatment at the Department of Clinical Oncology at the Prince of Wales Hospital. The latter samples were embedded in OCT compound. All of the specimens were subjected to histological diagnosis by a pathologist (K-F.T.). These tumors were classified as WHO grade II or III (20).

The male:female ratio of the above-mentioned NPC patients was 4.8:1. The age range was 36–68 years (mean age, 52 years). On the basis of Ho's stage classification (21), four patients had stage I disease (12.1%), nine patients had stage II disease (27.3%), eight patients had stage III disease (24.2%), four patients had stage IVA disease (12.1%), and eight patients had stage IVB disease (24.2%). Data regarding the development of local-regional recurrence, distant metastasis, and survival were available on 33 patients with a mean follow-up time of 24 months.

Microdissection and DNA Extraction. For each primary tumor or sample of normal nasopharyngeal epithelium, 40–60 serial sections (5-μm thick) were subjected to microdissection manually or by laser-captured microdissection using a PixCell LCM system (Arcturus Engineering, Mountain View, CA), under the guidance of a pathologist. All sections were lightly stained with hematoxylin. Neoplastic cells of the tumor samples or epithelial cells of the normal nasopharyngeal samples were isolated and collected for DNA extraction. DNA was extracted from isolated tumor cells and normal epithelial cells according to conventional methods (19).

MSP. The methylation status at the promoter region of *RASSF1A*, *RARβ2*, *DAP kinase*, *p16*, *p15*, *p14*, *MGMT*, and *GSTP1* was assessed by MSP as described previously (22). Genomic DNAs from the cell lines, xenografts, microdissected primary tumors, and microdissected normal epithelium were subjected to bisulfite modification by using the CpGenome DNA modification kit (Intergen, New York, NY). Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which is then converted to thymidine during the subsequent PCR step, giving sequence differences between methylated and unmethylated DNA. PCR primers that distinguish between these methylated and unmethylated DNA sequences were used. Primer sequences of all genes for both the methylated and the unmethylated form, annealing temperatures, and the expected PCR product sizes are summarized in Table 1. For PCR amplification, 2 μl of bisulfite-modified DNA were added in a final volume of 25 μl of PCR mixture containing 1× PCR buffer, $MgCl_2$, deoxynucleotide triphosphates, and primers (100 pmol each per reaction), and 1 unit of AmpiTaq Gold (Applied Biosystems, Branchburg, NJ). Amplification was carried out in a 9700 Perkin-Elmer thermal cycler under the following conditions: 95°C for 12 min; 35 cycles of 95°C for 1 min, the specific annealing temperature for each gene for 1 min, and 72°C for 1 min; followed by a final 7-min extension at 72°C. PCR products (15 μl) were loaded onto a 10% nondenaturing polyacrylamide gel, stained with ethidium bromide,

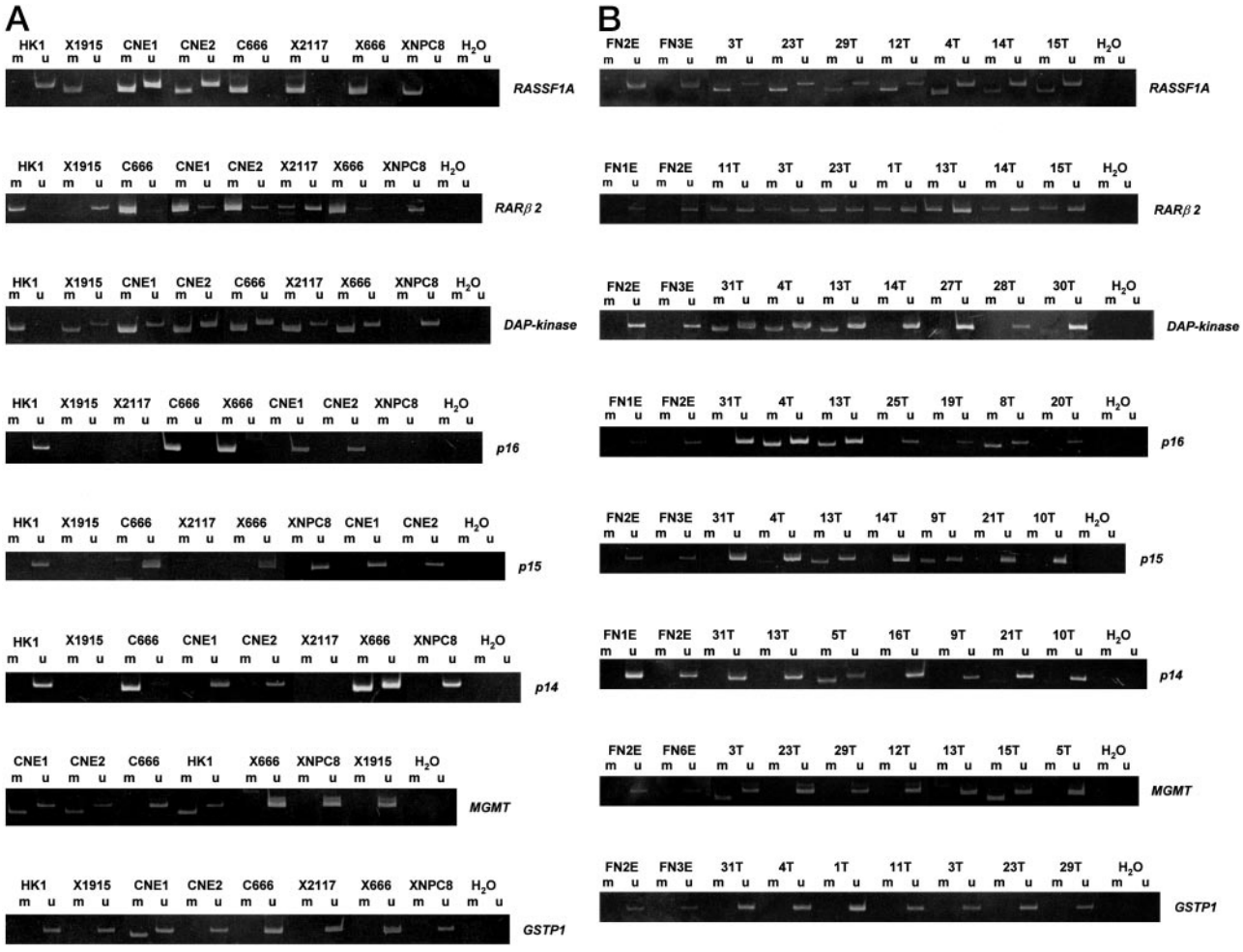

Fig. 1 *A*, MSP analysis of *RASSF1A*, *RARβ2*, *DAP-kinase*, *p16*, *p15*, *p14*, *MGMT*, and *GSTP1* in NPC cell lines (*C666*, *CNE1*, *CNE2*, and *HK1*) and xenografts (*X1915*, *X2117*, *X666*, and *XNPC8*). The PCR products in the *Lanes u* show the presence of unmethylated templates of each gene, whereas the products in *Lanes m* indicate the presence of methylated templates. *H_2O*, water control. *B*, MSP analysis of *RASSF1A*, *RARβ2*, *DAP-kinase*, *p16*, *p15*, *p14*, *MGMT*, and *GSTP1* in normal nasopharyngeal epithelium and primary NPC. The PCR products in *Lanes u* show the presence of unmethylated templates of each gene, whereas the products in *Lanes m* indicate the presence of methylated templates. *FNE*, normal nasopharyngeal epithelia; *T*, primary tumor; *H_2O*, water control.

and visualized under UV illumination. The MSP for all samples was repeated to confirm their methylation status.

Statistical Analysis. All statistical analysis was performed using the SPSS 10.0 statistical software for Windows. The χ^2 test or Fisher's exact test was used to assess the association between various parameters. In the univariate analysis of patient survival, development of local-regional recurrence, and metastasis, Kaplan-Meier survival analysis was used to compare the different patient groups. The significance of these differences was determined by the Breslow test for a $P < 0.05$ or by the Cox model, as appropriate.

RESULTS

Frequency of Methylation in NPC Cell Lines, Xenografts, Primary Tumors, and Normal Nasopharyngeal Epithelia. We determined the frequency of methylation of *RASSF1A*, *RARβ2*, *DAP-kinase*, *p16*, *p15*, *p14*, *MGMT*, and *GSTP1* in 4 NPC cell lines, 4 xenografts, and 33 primary tumors together with 6 normal nasopharyngeal epithelia by MSP. Fig. 1*A* shows MSP analysis in NPC cell lines and xenografts, and Fig. 1*B* shows representative examples of MSP analysis on normal nasopharyngeal epithelia and NPC primary tumors. Table 2 summarizes the promoter hypermethylation frequency of each gene in the NPC samples and in normal nasopharyngeal epithelia. The *RASSF1A* gene was methylated in 84% (31of 37) of the NPC samples. The incidence of promoter hypermethylation in NPC samples was 80% (32 of 40) for *RARβ2*, 76% (31 of 41) for *DAP-kinase*, 46% (19 of 41) for *p16*, 17% (7 of 41) for *p15*, 20% (8 of 41) for *p14*, 20% (8 of 40) for *MGMT*, and 3% (1 of 41) for *GSTP1*. No methylated templates of the six genes were detected by MSP in all six normal nasopharyngeal epithelia.

In the NPC cell lines and xenografts, multiple epigenetic changes were demonstrated (Table 2). Aberrant methylation was

Table 2 Methylation of multiple genes in NPC and normal nasopharyngeal epithelia

Samples	RASSF1A	RARB2	DAP-kinase	p16	p15	p14	MGMT	GSTP1
NPC xenografts (a)	100% (4/4)	50% (2/4)	75% (3/4)	25% (1/4)	0% (0/4)	25% (1/4)	0% (0/4)	0% (0/4)
NPC cell lines (b)	75% (3/4)	100% (4/4)	100% (4/4)	25% (1/4)	0% (0/4)	25% (1/4)	75% (3/4)	25% (1/4)
NPC primary tumors (c)	83% (24/29)	81% (26/32)	73% (24/33)	52% (17/33)	21% (7/33)	18% (6/33)	15% (5/32)	0% (0/33)
Total$^{(a + b + c)}$	84% (31/37)	80% (32/40)	76% (31/41)	46% (19/41)	17% (7/41)	20% (8/41)	20% (8/40)	3% (1/41)
Normal nasopharyngeal epithelia	0% (0/6)	0% (0/4)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)

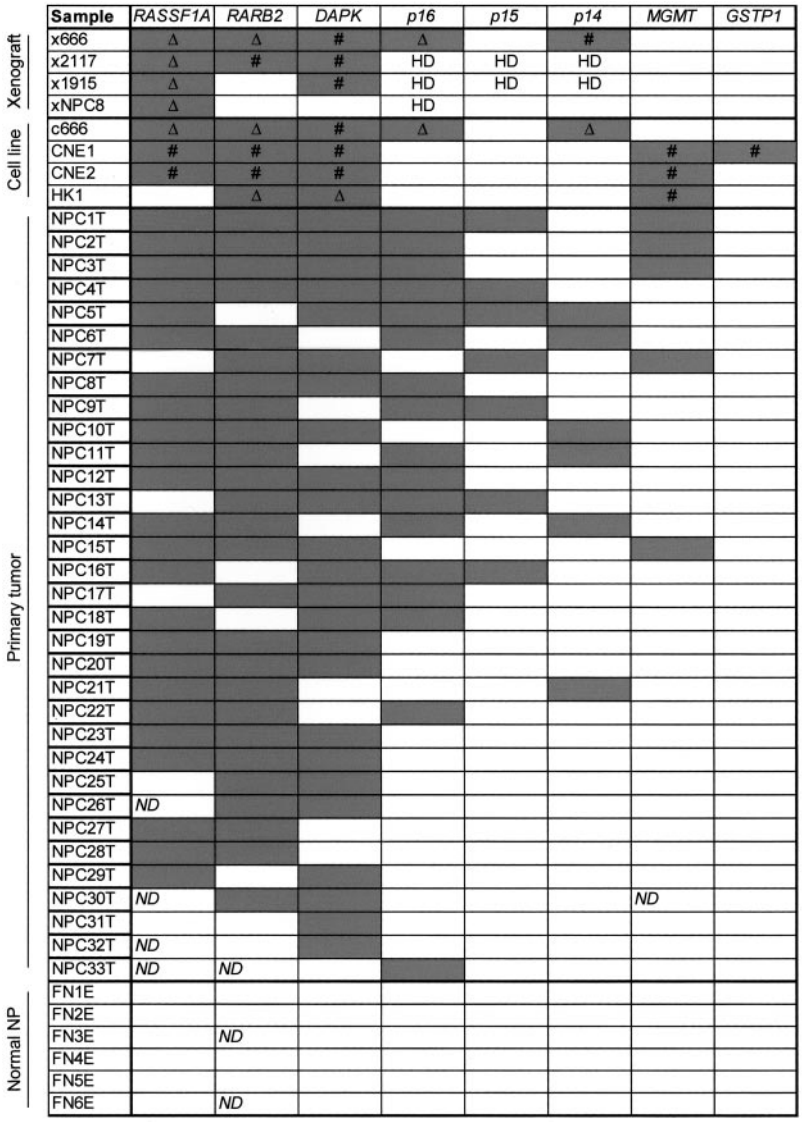

Fig. 2 Summary of methylation of *RASSF1A*, *RARβ2*, *DAP-kinase*, *p16*, *p15*, *p14*, *MGMT*, and *GSTP1* in NPC xenografts, cell lines, primary tumors, and normal nasopharyngeal epithelium. *Filled boxes*, methylated loci; *open boxes*, unmethylated loci. △, only methylated signal detected; *#*, both methylated and unmethylated signal detected; *HD*, homozygous deletion; *ND*, not done.

frequently observed in the *RASSF1A*, *RARβ2*, and *DAP-kinase* genes. Methylation of *p16* and *p14* was also found in C666-1 and Xeno-666. For *MGMT* and *GSTP1* genes, promoter hypermethylation was found in three NPC cell lines. No *p15* methylation was found in NPC cell lines and xenografts (Fig. 2). For the three genes (*p16, p14,* and *p15*) located on chromosome 9p21, no signal of both methylated and unmethylated sequences was detected in Xeno-1915 or Xeno-2117. No signal of *p16* gene was detected in Xeno-8 (Fig. 1*A*).

In the primary tumors, although microdissection was performed in each case, the presence of the remaining nonmalignant cell elements may still contribute to the signal of unmethylated sequence. Thus, the unmethylated sequence of all genes was detected in the NPC primary tumors (Fig. 1*B*). In all 33 primary tumors, at least one of these eight genes showed aberrant methylation in all tumor samples. Methylation of only one gene was found in 10% (3 of 33) of tumors. The percentage of the tumors with methylation in genes 2, 3, 4, 5, and 6 is 18% (6

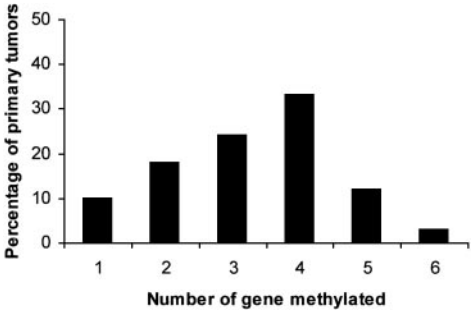

Fig. 3 Percentage distribution of the number of genes methylated in primary tumors.

of 33), 24% (8 of 33), 33% (11 of 33), 12% (4 of 33), and 3% (1 of 33), respectively (Fig. 3). None of the 33 primary tumors showed methylation in all genes examined.

Correlations with Clinical Parameters. Among the NPC samples, the percentage of primary tumors with methylation for specific genes is as follows: (*a*) *RASSF1A*, 83% (24 of 29); (*b*) *RARβ2*, 81% (26 of 32); (*c*) *DAP-kinase*, 73% (24 of 33); (*d*) *p16*, 52% (17 of 33); (*e*) *p15*, 21% (7 of 33); (*f*) *p14*, 18% (6 of 33); (*g*) *MGMT*, 16% (5 of 32); and (*h*) *GSTP1*, 0% (0 of 33; Table. 2). We have correlated the methylation status of each of the genes with clinical characteristics from the NPC patients. We found no significant correlation between the methylation status of each gene and clinical parameters including the stage, development of local-regional recurrence, distant metastasis, and survival. However, methylation of *MGMT* may be associated with the development of the metastasis (Breslow, $P = 0.0623$). We also investigated the clinical correlation of the multiple methylation phenotype in NPC among these 33 primary tumors. In our results, 49% (16 of 33) of the primary tumors were methylated at four or more markers. When we considered this portion of primary tumors as multiple methylation phenotype, no significant correlation between multiple methylation phenotype and stage was found.

DISCUSSION

Transcriptional silencing by hypermethylation of CpG islands in the promoter region is becoming recognized as a common mechanism for the inactivation of tumor suppressor genes (3, 4, 26). Many studies have demonstrated that the CpG islands in the *RB*, *p16*, *VHL*, *APC*, *MLH1*, and *BRCA1* genes are frequently methylated in a variety of human cancers but are usually unmethylated in the corresponding normal tissues (3, 4, 26). Studies showed that when these CpG islands are methylated in cancer cells, expression of the corresponding gene is silenced, and the silencing can be partially relieved by demethylation of the promoter region (4, 26). Recently, the growing list of genes inactivated by promoter hypermethylation provided an opportunity to examine the epigenetic alteration of multiple cancer-related genes in different tumors. In the present study, we determined the frequency of promoter hypermethylation of *RASSF1A*, *RARβ2*, *DAP-kinase*, *p16*, *p15*, *p14*, *MGMT*, and *GSTP1* in NPC. These are genes that are involved in different pathways in cells. Epigenetic inactivation of them may affect all of the molecular pathways involved in cell immortalization and transformation (27). It is possible to find simultaneous inactivation of several pathways by aberrant methylation in NPC.

RASSF1A is a novel tumor suppressor gene that was isolated recently from the lung tumor suppressor locus *3p21.3* (28). The presence of a Ras association domain in RASSF1A suggests that this protein may function as an effector of Ras signaling (or signaling of a Ras-like molecule) in normal cells. Its protein structure also suggests that RASSF1A may participate in the DNA damage response or in DNA damage-induced regulation of other cell signaling events (28, 29). Promoter hypermethylation of *RASSF1A* was found in lung and breast cancers (28, 30–32). Our recent study also demonstrated that the promoter of *RASSF1A* was highly methylated in primary NPCs (67%; Ref. 14). In the present study, we recruited a different cohort of primary NPC samples, and we observed an even higher percentage of *RASSF1A* promoter hypermethylation in the samples (83%). The results suggest that promoter hypermethylation inactivates the critical function of *RASSF1A* in NPC.

Retinoids are known to possess antiproliferative, differentiative, immunomodulatory, and apoptosis-inducing properties. The regulation of cell growth and differentiation of normal, premalignant, and malignant cells by retinoids is thought to result from the direct and indirect effects of retinoids on gene expression. These effects are mediated by the nuclear receptors, including retinoic acid receptor β2 (RARβ2) located at 3p24. Consistent 3p deletion is a unique feature of NPC tumors. From previous studies, hypermethylation of *RARβ2* was common in pancreatic cancers (20%; Ref. 33), breast cancers (23, 34, 35), and lung carcinomas (small cell lung cancer, 72%; non-small cell lung cancer, 41%; Ref. 36). In this study, we found high-frequency hypermethylation of the *RARβ2* promoter in 81% of primary NPC samples. Thus, promoter hypermethylation of *RARβ2* may block or interfere with the retinoid signaling pathways in NPC.

Promoter hypermethylation of *DAP-kinase* was also found in 73% of primary NPCs. This protein is a positive mediator of apoptosis induced by IFN-γ. Sanchez-Cespedes *et al.* (37) observed a positive correlation between methylation of *DAP-kinase* and the presence of lymph node metastases in patients with head and neck cancer. Although we did not find any correlation in our small number of NPC samples, we believe that promoter hypermethylation would inactivate the function of this potential metastasis inhibitor gene in NPC.

The p16 protein is a common tumor suppressor that plays a central role in control of cell proliferation during G_1 (38). Our group has reported previously that mutations of the *p16* gene were found in three NPC cell lines (HK-1, CNE-1, and CNE-2). Homozygous deletion of the *p16* gene has been identified in three NPC xenografts (Xeno-2117, Xeno-1915, and Xeno-8) and 35% of primary NPCs (39). Moreover, aberrant methylation of the 5′ CpG island of the *p16* gene was found in a NPC xenograft (Xeno-666) and in 22% of primary tumors (13). In the present study, the hypermethylation of *p16* promoter was detected in 52% of microdissected primary tumors by a more sensitive method, MSP.

The *INK4a/ARF* locus encodes two cell cycle-regulatory proteins, p16 and p14, which share an exon using different reading frames. Recent work suggests that p14 interacts *in vivo*

with MDM2 protein, neutralizing MDM2-mediated degradation of p53 (40). Promoter hypermethylation of *p14* was found in 18% of primary NPCs in the present study. Epigenetic inactivation of the *p14* gene may thus interfere with the p53 network in a subset of NPC tumors.

The *p15* gene is also an inhibitor of cyclin-dependent kinase 4, which is an important mediator of cell cycle control, especially in a pathway stimulated by transforming growth factor β (41). In the present study, we demonstrated promoter hypermethylation of *p15* in 21% of primary NPCs. Our finding suggests that the *p15* gene may play a role in NPC tumorigenesis.

Of the three genes mentioned above that are located on chromosome 9p21, methylation of *p16* showed the highest rate.

MGMT is a DNA repair protein that removes mutagenic and cytotoxic adducts from O^6-guanine in DNA (42). Frequent methylation of MGMT associated with gene silencing occurs in human cancers (11, 43, 44). GSTs are a family of isoenzymes that play important roles in protecting cells from cytotoxic and carcinogenic agents (45). *GSTP1* hypermethylation was most frequent in prostate, breast, and renal carcinomas. However, we found little promoter hypermethylation of *MGMT* (15%) and *GSTP1* (0%) in our primary tumor samples. Aberrant methylation of *MGMT* and *GSTP1* may occur only in selected tumor types.

In the present study, we did not find any significant correlation between methylation status of the tested genes and clinical characteristics of the NPC patients. Although there may be some correlation between *MGMT* methylation and the development of metastasis, this observation needs to be confirmed by a larger study with more NPC patients.

In conclusion, our study demonstrated a methylation profile of NPC by using a candidate gene approach. Our data stress the high frequency of promoter hypermethylation of multiple cancer-related genes in these samples and demonstrate that methylation may be the most common mechanism of inactivating genes in NPC. The epigenetic silencing of multiple cancer-related genes, including *RASSF1A*, *RARβ2*, *DAP-kinase*, *p16*, *p15*, *p14*, and *MGMT*, may cause disruption of the Ras signaling pathway, the retinoid signaling pathway, the metastasis-related process, cell cycle, p53 network, and DNA repair in NPC.

A number of recent studies also demonstrated the detection of gene promoter hypermethylation in the serum and sputum of lung cancer patients (46, 47). The methylation changes were also used as molecular markers in the serum and saliva from patients with head and neck tumors (37, 48). Promoter hypermethylation may thus be useful as a tumor marker for early diagnosis and disease monitoring. A nasopharyngeal brush biopsy procedure and PCR-based assay for EBV were recently introduced for detection of NPC in a high-risk population (49). In addition to EBV, we believe that the methylation markers identified in the present study may improve the sensitivity and specificity of the detection of NPC in nasopharyngeal brush biopsy samples.

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