High Frequency of Promoter Hypermethylation of the *Death-associated Protein-Kinase* Gene in Nasopharyngeal Carcinoma and Its Detection in the Peripheral Blood of Patients[1]

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ABSTRACT

Purpose: ***Death-associated protein* (*DAP*)*-kinase*** **gene is frequently inactivated by promoter hypermethylation in cancer. The aim of this study was to evaluate the promoter methylation status of the *DAP-kinase* gene in nasopharyngeal carcinoma (NPC).**

Experimental design: **The methylation status was evaluated by methylation-specific PCR (MSP). Thirty-two NPC biopsy specimens, plasma and buffy coat of 12 patients, 5 NPC cell lines, 3 normal nasopharyngeal biopsy tissues, and 2 normal nasopharyngeal epithelial primary cultures were included in this study.**

Results: **There was no promoter hypermethylation in all 3 normal nasopharyngeal tissues and 2 normal nasopharyngeal primary cultures. Hypermethylation was found in 24 (75%) NPC primary tumor biopsies and 4 (80%) NPC cell lines. Of the 24 patients with hypermethylation of DAP-kinase promoter in the primary tumors, 12 patients had their plasma and buffy coat DNA available for MSP study. Hypermethylated DAP-kinase promoter was detectable in 5 patients in the plasma but not in the buffy coat, 2 patients in the buffy coat but not in the plasma, and 1 patient in both plasma and buffy coat. Four patients had no detectable hypermethylated DAP-kinase promoter in both plasma and buffy coat. Hypermethylation of DAP-kinase promoter was found in both early- and late-stage NPC.**

Conclusions: **Our results show that hypermethylation of the DAP-kinase promoter is a common early event in NPC. The high frequency of identification of hypermethylated DAP-kinase promoter in plasma and buffy coat of NPC patients illustrates its potential clinical application as tumor marker for the diagnosis and monitoring of treatment result.**

INTRODUCTION

NPC[3] is common among Chinese, and undifferentiated carcinoma is the main histological type. EBV infection is closely related to the pathogenesis of NPC (1, 2). Telomerase activation is an important mechanism for perpetual proliferation of NPC cells (3). The sequential genetic abnormalities in the development of NPC from normal epithelium are, however, still unclear (4). An allelotyping study of normal nasopharyngeal epithelium showed the presence of a higher incidence of 3p loss in Hong Kong Chinese compared with Chinese from northern China and Caucasians (5). A comparative genomic hybridization study showed a high frequency of chromosomal gain at chromosome band 12p11.2–12 (36%), 12q14–q21 (33%), and chromosomal loss at 3p14–p21 (20%), 11q23–qter (20%; Ref. 6). Genetic abnormalities leading to dysfunction of epithelial adhesion molecules including E-cadherin (7), desmosome (8), and tumor suppressor genes including *p53* (9), *p16* (10), and *RASSF1A* (11) genes are important steps in the development of NPC. Promoter hypermethylation of tumor suppressor genes is one of the key epigenetic changes in many human cancers (12). It has been shown to be an important mechanism in the inactivation of p16 and *RASSF1A* in NPC (10, 11).

DAP-kinase (also known as DAP-2) is an actin-associated, calcium/calmodulin-dependent enzyme with serine/threonine kinase activity (13, 14). DAP-kinase expression may affect apoptosis and contributes to immortalization. It is an essential mediator involved in the IFN-γ-induced programmed cell death in HeLa cells (15). The apoptotic effects of IFN-γ is suppressed in the presence of antisense DAP-kinase mRNA. DAP-kinase is also involved in the tumor necrosis factor-α and Fas-induced apoptosis (15). Furthermore, DAP-kinase is found to be associated with the $p19^{ARF}$/p53-mediated apoptosis in the rodent model (16). The *p53* gene in the embryonic fibroblasts is activated by DAP-kinase in a $p19^{ARF}$-dependent fashion (16). In lung carcinoma cell lines, the metastatic behavior is correlated with the DAP-kinase level (17). *In vitro* metastatic activity of the lung carcinoma cell lines is suppressed if expression of

Received 8/8/01; revised 11/27/01; accepted 11/28/01.
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[1] This study is supported by the Betty and Kadoorie Cancer Research Fund, the Ho Hung Chiu Cancer Research Fund, and a research grant of the University of Hong Kong.
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[3] The abbreviations used are: NPC, nasopharyngeal carcinoma; DAP, death-associated protein; MSP, methylation-specific PCR.

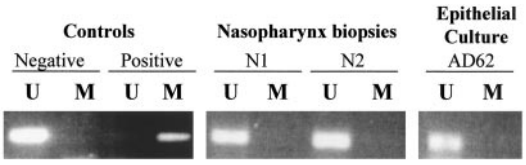

Fig. 1 Representative MSP results of the *DAP-kinase* gene in normal nasopharyngeal biopsy tissues (*N1* and *N2*), normal nasopharyngeal epithelial cultures (*AD62*), and controls (*Negative* and *Positive*). U and M primer sets were used to amplify the unmethylated and methylated sequences, respectively.

DAP-kinase is maintained at a physiological level in animal models (17).

Hypermethylation of DAP-kinase promoter has been demonstrated in human neoplasm-derived cell lines (18), B-cell malignancies (19), primary lung cancer (20, 21), and head and neck cancers (22). In this study, we investigated the methylation status of DAP-kinase in NPC to define the frequency of this epigenetic aberration and its clinicopathological significance.

MATERIALS AND METHODS

NPC Tumor Specimens and Cell Lines. Thirty-two NPC nasopharyngeal biopsy specimens from 28 male and 4 female patients were collected from the Department of Surgery, Queen Mary Hospital, The University of Hong Kong. All patients had radiotherapy treatment for NPC. All specimens were taken before treatment and were histologically evaluated to be undifferentiated carcinoma. Three histologically normal nasopharyngeal biopsy tissues and two normal nasopharyngeal epithelial primary cultures were included as normal controls. Five NPC cell lines (CNE-1, CNE-2, CNE-3, M-1, and SUNE-1) were also studied. The methylation status of *p16* and *RASSF1A* genes of these cell lines have been reported in the literature (10, 11). Twelve patients had plasma and buffy coat available for MSP study.

DNA Extraction and Purification. Blood and tissues were obtained with consent for research purposes. The nasopharyngeal biopsy tissues were immediately frozen in liquid nitrogen and subsequently stored at −80°C until use. The biopsies were treated with proteinase K (0.5 mg/ml) for 36 h at 50°C. High molecular weight genomic DNA was obtained by conventional phenol/chloroform and ethanol extraction (23). For the primary cultures of normal nasopharyngeal tissues and NPC cell lines, DNA was extracted and purified by the Wizard Genomic DNA Purification kit according to the protocol of the manufacturer (Promega Corp., Madison, WI). The peripheral venous blood of patients were collected by EDTA-containing bottle. The plasma was immediately separated by centrifugation at $400 \times g$ for 10 min, and the extracted plasma was transferred to a plain tube for further extraction by centrifugation at $1000 \times g$ for 10 min. The buffy coat fraction was also collected to study the presence of circulating tumor cells in the peripheral blood. The plasma and buffy coat samples were stored at −80°C until further processing. DNA from plasma and buffy coat were extracted by a Puregene Blood kit (Genetra, Minneapolis, MN) using the protocol as recommended by the manufacturer.

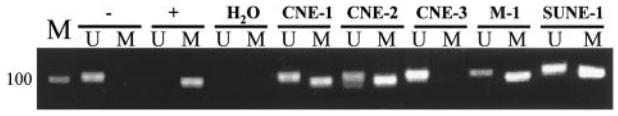

Fig. 2 The MSP results of the five cell lines together with the methylated control, unmethylated normal control, and blank water control.

Bisulfide Modification and MSP. Methylation status of the samples was investigated by MSP as described in the literature (19–22, 24, 25). In brief, 1 μg of the genomic DNA was modified by sodium bisulfide using the CpGenome DNA Modification kit (Intergen, New York, NY) according to the manufacturer's protocol. Modified DNA was amplified by two different primer sets specific to the unmethylated (U) and methylated (M) DAP-kinase sequences, respectively. For the methylated sequence, the forward and backward primers were 5′-GGA TAG TCG GAT CGA GTT AAC GTC-3′ and 5′-CCC TCC CAA ACG CCG A-3′, and those for the unmethylated sequences were forward 5′-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3′ and reverse 5′-CAA ATC CCT CCC AAA CAC CAA-3′. The sense unmethylated and methylated primers correspond to bp 2 and 5 of GenBank sequence no. X76104. The PCR amplification was performed for a total of 35 cycles with an annealing temperature of 60°C. Universal methylated human male genomic DNA (Intergen) was used as the positive control. Genomic DNA purified from peripheral blood of a healthy voluntary donor was used as a negative control. A blank control containing all PCR components except sample DNA was also included in all PCRs. The PCR products were then loaded on a 3.5% agarose gel with ethidium bromide and visualized under UV illumination. The U primers amplify a 98-bp PCR product only on an unmethylated promoter but not on a methylated promoter. The M primers amplify a 106-bp PCR product on a methylated promoter but not on a unmethylated promoter. Specimens with purely unmethylated promoters will have positive PCR products by U primers but not with the M primers. A specimen that contains purely methylated promoter will have PCR products by using M primers but not with the U primers. A specimen that contains heterogeneous tissue of both methylated and unmethylated promoters will have PCR products from both U primers and M primers.

Bisulfite Sequencing. Both U and M products of the MSP were excised from the agarose gel and purified by Geneclean DNA purification kit (BIO101, Vista, CA). The PCR products were sequenced with the forward U and M primers, respectively, using the DNA sequencing kit (Perkin-Elmer Corp, Warrington, United Kingdom) and were analyzed by 377 ABI prism automatic sequencer (Perkin-Elmer Corp., Foster city, CA).

RESULTS

Methylation Status of DAP-Kinase in Normal Nasopharynx. All three normal nasopharyngeal tissues (N_1, N_2, and N_3) and two normal nasopharyngeal epithelium outgrowths (AD2 and AD62) showed no promoter methylation. The representative MSP results of N_1, N_2 and AD62, together with the controls, are shown in Fig. 1.

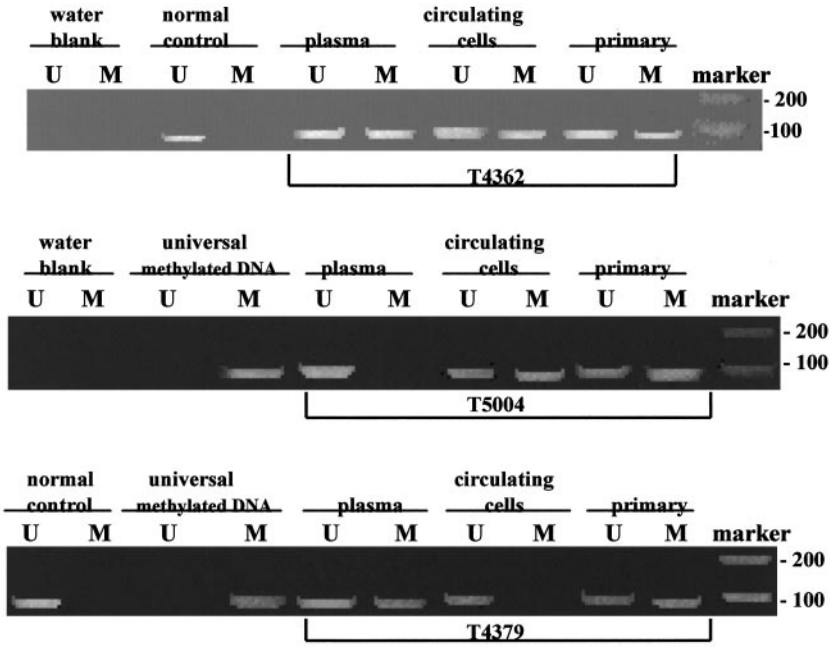

Fig. 3 Representative MSP results of the *DAP-kinase* gene of 3 patients (patient nos. T4362, T5004, and T4379) including the primary tumors, plasma, and circulating cells in the buffy coat. U and M primer sets were used to amplify the unmethylated and methylated sequences, respectively. The methylated control, unmethylated normal control, and blank water control were also included in each PCR.

Methylation of DAP-Kinase in NPC Cell Lines. Five NPC cell lines were examined together with positive and negative controls in the MSP study. Amplification of both unmethylated and methylated sequences was observed in CNE-1, CNE-2, M-1, and SUNE-1. In contrast, only unmethylated sequence could be amplified in CNE-3 (Fig. 2).

Methylation of DAP-Kinase in Primary NPC, Plasma, and Buffy Coat. DAP-kinase promoter was found to be methylated in 24 of 32 (75%) primary tumors. The PCR products obtained with the U and M primers of 2 NPC tumor samples (patient nos. 4305 and 5003) were sequenced. In the MSP product from U primers, all cytosine nucleotides including those within CG dinucleotides were found to be changed to adenosine, indicating the conversion of all cytosine nucleotides to uracil by sodium bisulfide modification of the specimen DNA. In the MSP product from M primers, only the cytosine residues in CG dinucleotides were found to remain as cytosine, which indicated the presence of methylated cytosine in these CG dinucleotides (sequencing tracings not shown). Of the 24 tumors with hypermethylated DAP-kinase promoter, 12 patients had their pretreatment venous plasma and buffy coat available for the MSP study. Of these 12 patients, hypermethylated DAP-kinase promoter was found in 5 patients in the plasma but not in the buffy coat, 2 patients in the buffy coat but not in the plasma, and 1 patient in both plasma and buffy coat. Four patients had no detectable hypermethylated DAP-kinase promoter in both plasma and buffy coat. Representative MSP results of primary tumor, plasma, and buffy coat of 3 patients are shown in Fig. 3.

Clinicopathological Significance of DAP-Kinase Promoter Methylation. The results of statistical analysis of correlation of DAP-kinase promoter methylation status with clinicopathological parameters are shown in Table 1. Hypermethylation of DAP-kinase promoter was found in early-stage NPC. There were no significant clinical correlations of DAP-kinase promoter hypermethylation with sex, age, T stage, N stage, and recurrence. The actuarial 5-year tumor-free survival rate of patients with the presence of DAP-kinase promoter hypermethylation was 53%, and for patients without DAP-kinase promoter hypermethylation, 50% (Wilcoxon; $P = 0.886$).

Of the 12 patients with the hypermethylated *DAP-kinase* gene in the primary tumors who also had peripheral blood available for MSP evaluation, the *DAP-kinase* gene promoter

Table 1 Clinicopathological correlation of DAP-kinase promoter hypermethylation

	Number	DAP-kinase hypermethylation	Statistical significance
Sex			
Male	24	19 (79%)	χ^2, $P = 0.346$
Female	8	5 (63%)	
Ho's T-stage			
T_1	9	6 (67%)	Spearman correlation, $P = 0.65$
T_2	10	8 (80%)	
T_3	13	10 (77%)	
Ho's N-stage			
N_0	5	4 (80%)	Spearman correlation, $P = 0.722$
N_1	10	8 (80%)	
N_2	12	8 (67%)	
N_3	5	4 (80%)	
Ho's stage			
I	1	1 (100%)	Spearman correlation, $P = 0.712$
II	6	5 (83%)	
III	20	14 (70%)	
IV	5	4 (80%)	
Recurrence			
No	21	16 (76%)	χ^2, $P = 0.83$
Yes	11	8 (73%)	

Table 2 Hypermethylated DAP-kinase promoter in primary tumor, plasma, and buffy coat

Patient no.	DAP-kinase hypermethylation[a]			Ho's stage	Recurrence site after treatment	Status at last follow-up
	Primary tumor	Plasma	Circulating cells			
4362	+	+	+	$T_1N_2M_0$	No	Alive, no tumor
4379	+	+	0	$T_3N_3M_0$	Neck and distant	Died of tumor
4621	+	+	0	$T_2N_2M_0$	No	Alive, no tumor
4637	+	+	0	$T_1N_2M_0$	No	Alive, no tumor
4659	+	+	0	$T_1N_1M_0$	No	Alive, no tumor
4932	+	+	0	$T_3N_0M_0$	No	Alive, no tumor
5004	+	0	+	$T_1N_2M_0$	Neck	Alive, no tumor after surgical salvage
5027	+	0	+	$T_2N_2M_0$	No	Died of other cause
4933	+	0	0	$T_3N_3M_0$	Neck and distant	Died of tumor
4957	+	0	0	$T_1N_1M_0$	No	Alive, no tumor
5028	+	0	0	$T_2N_1M_0$	No	Alive, no tumor
5156	+	0	0	$T_3N_1M_0$	Distant	Died of tumor

[a] +, hypermethylation; 0, no hypermethylation.

hypermethylation results are shown in Table 2. For the 1 patient with both plasma and buffy coat found to have detectable hypermethylated *DAP-kinase* gene promoter, there was no recurrence after radiotherapy treatment. Of the 5 patients with detectable hypermethylated *DAP-kinase* gene promoter in plasma but not in the buffy coat, 1 patient developed recurrence and died. Of the 2 patients with detectable hypermethylated *DAP-kinase* gene promoter in buffy coat but not in the plasma, 1 patient developed neck node recurrence and was successfully salvaged by radical neck dissection. Of the 4 patients without detectable hypermethylated *DAP-kinase* gene promoter in both plasma and buffy coat, 2 patients died of recurrences.

DISCUSSION

Hypermethylation of DAP-kinase promoters were found in four of the five NPC cell lines in this study. Hypermethylation was not detected in the normal epithelial outgrowths of the nasopharyngeal tissue. It indicates that promoter hypermethylation of *DAP-kinase* gene is not necessary for normal nasopharyngeal epithelial growth *in vitro*. Furthermore, DAP-kinase promoter methylation was also not induced by *in vitro* culturing of the nasopharyngeal epithelial cells. The presence of both unmethylated and methylated *DAP-kinase* gene in four of five NPC cell lines suggested that heterogeneous subclones of NPC cells were present in these cell lines.

The present study showed a high frequency (75%) of promoter hypermethylation of the *DAP-kinase* gene in NPC tumors but not in normal nasopharyngeal tissues and normal nasopharyngeal epithelial primary cultures. The absence of promoter hypermethylation in histologically normal nasopharyngeal issues and primary cultures showed that DAP-kinase promoters are unmethylated in normal nasopharynx, in contrast with certain genetic aberrations such as chromosome 3p loss that were found to be present sometimes in normal nasopharynx (5).

Although promoter hypermethylation of DAP-kinase is frequently found in NPC, it has no correlation with sex, age, stage, recurrence, and survival. This is in contrast with stage 1 non-small cell carcinoma of lung in which patients with hypermethylation of DAP-kinase have 5-year survival of 56% compared with 92% for those patients without hypermethylation (20). Higher frequency of DAP-kinase promoter hypermethylation was also found in head and neck squamous cell carcinomas with nodal metastasis and in more advanced stage (22). This implies that the prognostic value of promoter hypermethylation of DAP-kinase is disease specific and may be affected by many other factors, *e.g.*, treatment modalities, histology, site, and stage.

Promoter hypermethylation is an important epigenetic mechanism in the development and progression of many human cancers including NPC. Promoter hypermethylation of other genes in NPC has also been found including *p16* (22% of 27 primary tumors) and *RASSF1A* (67% of 21 primary tumors; Refs. 10, 11). Promoter hypermethylation is a potential tumor marker in the diagnosis and monitoring of cancer (22, 25). Although *DAP-kinase* gene hypermethylation does not seem to have prognostic value, it may be one of the potentially useful genes in the clinical monitoring of residual or recurrent disease after treatment. Of those patients with hypermethylated promoter DAP-kinase in the primary tumors, 50% patients had detectable hypermethylated promoter in plasma. The value of hypermethylated promoter DAP-kinase DNA in circulating plasma as a tumor marker needs further evaluation. The presence of detectable hypermethylated promoter DAP-kinase in buffy coat indicates the presence of circulating NPC cancer cells. The presence of circulating tumor cells is an important step in the development of distant metastasis. The possible identification of hypermethylated promoter DAP-kinase promoter in plasma and buffy coat of NPC patients warrants further investigation, particularly in its potential clinical applications in monitoring residual and recurrent tumors after treatment. The early detection of residual and recurrent tumors may allow for a higher chance of successful salvage treatment. Hypermethylated promoter DNA found in some patients with early-stage tumor also indicates its possible useful clinical application in the molecular screening and adjunct in the diagnosis of NPC.

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Clin Cancer Res 2002;8:433-437.