Detection of Hypermethylated RIZ1 Gene in Primary Tumor, Mouth, and Throat Rinsing Fluid, Nasopharyngeal Swab, and Peripheral Blood of Nasopharyngeal Carcinoma Patient

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ABSTRACT

Purpose: The aims of this study are to examine the methylation status of the Fn2 RIZ1 gene in nasopharyngeal (NP) tumors and nasopharyngeal carcinoma (NPC) cell lines and to evaluate the clinical value of methylated RIZ1 gene in body fluid samples of NPC patients.

Experimental design: Methylation status of RIZ1 was evaluated by MSP on CNE-2 and M1 cell lines, 30 tumor biopsies, and their matched body fluid samples, including mouth and throat (M & T) rinsing fluid, NP swabs, plasma, and buffy coat. Normal controls included 8 normal NP biopsies and body fluid samples from 29 healthy volunteers. Sequencing was performed on MSP products from one NP tumor and one M & T rinsing fluid. Transcription of the RIZ1 gene before and after 5-aza-2’-deoxycytidine treatment was examined on CNE-2.

Results: The methylated RIZ1 gene was detected in both CNE-2 and M1, 18 (60%) NP tumors, but not in any of the normal controls. Of 30 matched body fluid samples, methylated RIZ1 DNA was found in 11 (37%) NP swabs, 9 (30%) M & T rinsing fluid, 7 (23%) plasma, and 3 (10%) buffy coat samples. Sequencing analysis confirmed all cytosines to uracils conversion, excepting cytosines in CpG dinucleotides in methylated PCR products. Promoter methylation correlated with loss of RIZ1 mRNA expression, and 5-aza-2’-deoxycytidine treatment restored its expression in CNE-2.

Conclusion: Our results suggest that promoter hypermethylation of the RIZ1 gene is commonly found in NPC. Its detection in body fluid samples of NPC patients but not in normal controls indicates that it is worth to further evaluate its clinical application in assisting screening of NPC and monitoring recurrence after treatment.

INTRODUCTION

Epigenetic silencing of TSGs by promoter hypermethylation has brought attention recently as more TSGs are found to undergo promoter hypermethylation in cancer (1–3). The retinoblastoma protein-interacting zinc finger gene RIZ (PRDM2) is a member, by sequence homology, of a nuclear protein methyltransferase superfamily involved in chromatin-mediated gene expression (4). Two products of the RIZ1 gene are produced: (a) RIZ1; and (b) RIZ2. RIZ1 contains a conserved methyletransferase domain, whereas RIZ2 lacks this domain. Mediation of apoptosis by RIZ1, which causes G2-M cell cycle arrest, has been documented in breast cancer, liver cancer, and microsatellite instability-positive colon cancer cells (5–7). RIZ1 expression, but not RIZ2 expression, is commonly silenced in many types of human tumors, including breast cancer, liver cancer, colon cancer, neuroblastoma, melanoma, lung cancer, and osteosarcoma (5–7). Although mutation of the RIZ1 gene by frameshift mutation and loss of a polymorphic RIZ allelic is common in microsatellite-unstable cancers (8, 9), epigenetic silencing of RIZ1 by methylation was found in 11 of 25 (44%) breast cancer specimens and 20 of 32 (62%) liver cancer (10). Although the RIZ1 gene was found to be hypermethylated in breast and liver cancer specimens (10), methylation status of the RIZ1 gene in NPC has not been reported.

Applications of analysis of free DNA in circulation from tumor cells have been well documented (11, 12). These studies have shown that it is possible to identify tumor-specific alterations, such as loss of heterozygosity, microsatellite instability, and tumor-specific hypermethylation in the salvia, plasma, and serum DNA of patients with various cancers (13–16). We have also found hypermethylated DAP-K promoter DNA in peripheral blood plasma and buffy coat of NPC patients but not in normal controls (17). Tumor-specific methylation of p16, MGMT, and DAP-K genes are also found in the saliva of head and neck cancer patients (16). The high specificity of hypermethylation of many TSGs in cancer but not in normal tissue may be used as tumor marker in helping clinical screening of...
primary cancer of high-risk population and minimal residual cancer after treatment.

Cell cycle regulatory genes involved in growth control are often mutated or deleted in many human cancers but is rarely found in NPC (18, 19). Epigenetic inactivation of cell cycle genes is more commonly found in NPC, including p15, p16, RASSF1A, and DAP-kinase (18, 20). We have reported previously high frequency of DAP-kinase hypermethylation in NPC (17). It would be of interest to know whether epigenetic change of the RIZ1 gene can be found in NPC, which might give additional growth advantage of tumor cells. In this study, we aim to: (a) evaluate the hypermethylation status of RIZ1 in NPC cell lines, NP tumors, and normal NP tissues; (b) test whether loss of its transcription is associated with promoter hypermethylation in the NPC cell line; and (c) evaluate the value of methylated RIZ1 DNA as tumor marker for screening NPC in body fluids, which include NP swab, M & T rinsing fluid, and peripheral blood of NPC patients.

MATERIALS AND METHODS

NPC Cell Lines, NPC Tumor Specimens, Matched M & T Rinsing Fluid, and NP Swabs. Thirty NPC biopsy specimens from 24 male and 6 female patients were collected from the Department of Surgery, at the Queen Mary Hospital, The University of Hong Kong. The clinical American Joint Committee on Cancer 1997 stages were 3 stage I, 11 stage II, 5 stage III, and 11 stage IV. Thirty matched body fluid samples, including M & T rinsing fluid, NP swabs, and peripheral blood samples from NPC patients, were also collected for the present study. M & T rinsing fluid was collected by rinsing the M & T with 20 ml of 0.9% normal saline. NP swab was taken by inserting a cotton tip wooden stick from the nose till touching the NP wall from both right and left nose blindly without any endoscopic guidance as described in detail in our previous publication (21). All tumor specimens were histologically evaluated to be undifferentiated carcinoma. Methylation status of the RIZ1 gene and its transcription was evaluated on two NPC cell lines (CNE-2 and M-1).

DNA Extraction and Purification. The blood and tissues were obtained with consent for research purposes. The NP biopsy tissues were immediately frozen in liquid nitrogen and subsequently stored in −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 (22).

The peripheral venous blood of patients was collected by EDTA-containing tubes. The plasma was immediately separated fromuffy coat fraction by centrifugation at 400 × g for 10 min, and the extracted plasma was transferred to a plain tube for further extraction by centrifugation at 1000 × g for 10 min. The M & T rinsing fluid and NP swabs of patients were collected by bottles containing saline and immediately separated by centrifugation at 400 × g for 10 min. After separation, the remaining cells of M & T rinsing fluid and NP swabs, plasma, anduffy coat fractions were transferred to eppendorf tubes and stored at −80°C until further processing. DNA isolation from M & T rinsing fluid and NP swabs was extracted by a Clontech Nucleospin Blood Mini Kit (Palo Alto, CA) using the protocol as recommended by the manufacturer.

Bisulfide Modification and MSP. Methylation status of the samples was investigated by MSP as described by Du et al. (10). In brief, 1 µg of the genomic DNA was modified by sodium bisulfide using the CpGenome DNA Modification Kit (Intergen, Purchase, NY) using the protocol as recommended by the manufacturer. Modified DNA was amplified by two different primer pairs specific to the unmethylated (U) and methylated (M) RIZ1 sequences, respectively. For the methylated (M-) sequence, the forward and backward primers were 5′-GTGGTGTTATTTGGGCTGACC-3′ and 5′-GCTTCTCGGTCTCT-3′, and those for the unmethylated (U-) sequences were forward 5′-TGTTGGTATTTGGGCTGATT-3′ and backward 5′-ACTATTTCTACCAACCCAAAGA-3′. The PCR amplification was performed for a total of 45 cycles with an annealing temperature of 68°C and 60°C for M-sequences and U-sequences, respectively. Universal methylated DNA was used as the positive control. The PCR products were then analyzed by a 3.5% agarose gel. The amplification by PCR would generate fragments of 177 and 175 bp using M- and U- primer pairs, respectively (10). To test the specificity of the U- and M-primer pairs on NP tissues, we applied the MSP on normal (unmethylated) controls and NPC cell lines.

Sequencing of RIZ1 MSP Products. Both U and M products of the MSP from one NPC tumor biopsy and one M & T rinsing fluid sample were excised from the agarose gel and purified by Concert Matrix Gel Extraction System (Invitrogen, Carlsbad, CA). Subsequently, sequencing reactions were performed separately on the purified U and M products by the DNA sequencing kit (Perkin-Elmer Corp., Warrington, United Kingdom) and analyzed by 377 ABI prism automatic sequencer (Perkin-Elmer Corp., Foster City, CA).

Sensitivity and Specificity of RIZ1 MSP Analysis. Sensitivity of RIZ1 MSP analysis was demonstrated by mixing different copies (10, 25, 50, 60, 70, 80, 90, 100, 250, 500, and 1000) of universal methylated human male genomic DNA (Inthergen) with 1000 copies of unmethylated normal control DNA from one histologically normal NP tissue biopsy (adenoid 1080). A conversion factor of 6.6 pg of DNA/diploid cell was used for copy number calculation. Eight histologically normal NP biopsy tissues, body fluid samples of 20 M & T rinsing fluid and NP swabs, and 18 peripheral blood from 29 healthy subjects were included in this study as normal (negative) controls.

RT-PCR of RIZ1. RNA isolation with TRIzol (Invitrogen) was performed according to the manufacturer’s instructions. The RNA was enriched for polyadenylated RNA molecules using the mRNA Capture Kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. In brief, ~1 µg of total cellular RNA was transferred to a streptavidin-coated PCR tube to immobilize the mRNA, and reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (New England Biolabs) for 1 h at 37°C. Subsequently, PCR was performed with an initial hot start of 2 min at 94°C and followed by amplification of 15 s at 94°C, 15 s at 58°C, and 15 s at 72°C for a total of 40 cycles and a final extension of 10 min at 72°C using forward and backward primers RIZ1 5′-GAA CACTAC TGAGCCTGTGG-3′ (sense) and RIZ2 5′-ACAC- CAATCAGGTTCTT GTC-3′ (antisense). The primers for am-
plification of human glyceraldehyde-3-phosphate dehydrogenase were 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' (sense) and 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3' (antisense).

Re-expression of RIZ1 by 5-Aza-dC Treatment. The NPC cell line, CNE-2, was grown for 4 days in the presence of various concentrations of 5-Aza-dC (1, 5, and 10 μM). RNA and DNA were separately isolated. MSP and RT-PCR were performed afterward as described above.

RESULTS

Sequencing Analysis of Unmethylated and Methylated PCR Products. The U- and M- MSP PCR products obtained from amplification of one tumor biopsy (patient 1152) and one M & T rinsing fluid sample (patient 1128) using either U or M primers were sequenced. The results of U- sequences obtained from amplification using forward U- primer showed all cytosine nucleotides, including those within CpG dinucleotides, were converted to thymines, indicating the complete conversion of all cytosines to uracils by sodium bisulfide modification of the specimen DNA. And the results of M- sequences obtained from amplification using M- primer showed only cytosine residues in CpG dinucleotides remained as cytosines, which indicated the presence of methylated cytosines in these CpG dinucleotides. The representative sequencing results of U- and M- sequences from one tumor sample (patient 1152) are shown in Fig. 1.

Sensitivity and Specificity of RIZ1 MSP Analysis. We examined the sensitivity of this assay by mixing different copies of universal methylated DNA with unmethylated control DNA. Fragments of 177 bp began to be detected with increasing signal intensity from 70 copies of methylated DNA (figure not shown).

All normal controls from eight histologically normal NP tissue biopsies and body fluid samples of 20 M & T rinsing fluid and NP swabs and 18 peripheral blood from 29 healthy subjects showed only unmethylated sequence amplified (Fig. 2). The results suggested that RIZ1 gene promoter was unmethylated in all normal control tissue specimens, including NP tumors biopsies, M & T rinsing fluid, NP swabs, and peripheral blood.

Methylation of the RIZ1 Promoter in NPC Cell Lines, Primary Tumors, and Body Fluid of NPC Patients. Subsequently, we examined the methylation status of the RIZ1 gene on NPC cell lines (M1 and CNE-2). Both M1 and CNE-2 cell lines showed aberrant methylation of the RIZ1 gene; exclusive methylated sequence was obtained in CNE-2 cell lines, whereas both methylated and unmethylated sequences were found in M1 cell line, indicating the presence of heterogeneous clones (Fig. 2). RIZ1 promoter was found to be methylated in 18 of 30 (60%) primary tumors, including 33% (1 of 3) stage I, 64% (7 of 11) stage II, 40% (2 of 5) stage III, and 73% (8 of 11) stage IV tumors (Spearman correlation, P = 0.651). Methylation was unaffected by sex (Fisher’s test, P = 1.00) and age (t test, P = 0.967). Representative MSP results of primary tumor, M & T rinsing fluid, NP swabs, buffy coat, and plasma are shown in Fig. 3 and summarized in Table 1.

MSP analysis was subsequently performed on these 30 matched M & T rinsing fluid, NP swabs, and plasma samples of NPC patients. Among the 12 primary tumors in which the methylated RIZ1 gene was undetectable, their corresponding body fluid samples showed methylation free. Methylation of the RIZ1 gene was found only in the matched body fluid samples among those 18 patients with methylated RIZ1 promoter in their primary tumors. Of all 30 matched body fluid...
Detection of Hypermethylated RIZ1 Gene

Hypermethylation of RIZ1 gene was found in 4 patients in their M & T rinsing fluid but not in the NP swabs, 6 patients in the NP swabs but not in the M & T rinsing fluid, and 5 patients in both M & T rinsing fluid and NP swabs. Methylation of the RIZ1 gene was detectable in 15 (50%) samples from either or both M & T rinsing fluid and NP swabs. Although aberrant methylation was detected in primary tumors, 3 patients had no detectable hypermethylated RIZ1 gene in both M & T rinsing fluid and NP swabs. Methylated RIZ1 gene promoter DNA had lower detectable rate in plasma 7 (23%) and buffy coat 3 (10%) specimens. All plasma and buffy coat samples with methylated RIZ1 gene detected showed promoter hypermethylation in their corresponding primary tumors. Hypermethylated RIZ1 promoter was found in 5 patients in the plasma but not in the buffy coat, 1 patient in the buffy coat but not in the plasma, and 2 patients in both buffy coat and plasma samples. All together, methylation of the RIZ1 gene was detected in 8 (27%) samples from either or both buffy coat and plasma.

Expression of RIZ1 mRNA in NPC Cell Lines Before and After Demethylation Agent 5-Aza-dC Treatment. The expression of RIZ1 was examined on CNE-2 cell line by RT-PCR. No detectable RIZ1 mRNA was detected (Fig. 4A), although mRNA extracted from normal NP frozen tissue biopsies (adenoid 1080), which had no promoter hypermethylation, showed RIZ1 expression. In this experiment, the expression of glyceraldehyde-3-phosphate dehydrogenase was served as an internal index gene to ensure the integrity of mRNA.

Promoters silenced by methylation can be reactivated by treatment with the demethylation agent, 5-Aza-dC, which has been well studied in a variety of tumors (23). We next examined the effect of 5-Aza-dC on RIZ1 expression in CNE-2. By RT-PCR analysis, RIZ1 transcripts from CNE-2 cell line were detectable after treatment with 5-Aza-dC (Fig. 4A). MSP analyses of the RIZ1 gene on CNE-2 after 5-Aza-dC treatment were also studied. The results showed only unmethylated RIZ1 sequence amplified by MSP in CNE-2 cell line (Fig. 4B). These data confirm that methylation plays an important role in causing transcriptional silencing of RIZ1 in CNE-2.

**DISCUSSION**

It has been shown that methylation of RIZ1 promoter is associated with its decreased expression (10). The MSP assay covers eight Cpgs of the RIZ1 gene, all of which have been shown to be methylated frequently in liver and breast cancers as confirmed by DNA sequencing analysis (10). The presence of both unmethylated and methylated RIZ1 gene in M1 cell line but not CNE-2 cell line suggested that heterogeneous subclones of NPC cells were present in the cell line. Methylation of the RIZ1 gene in CNE-2 was found to affect significantly its expression. Treatment of CNE-2 with 5-Aza-dC restored its expression, suggesting that aberrant hypermethylation of the RIZ1 promoter is directly responsible for transcriptional inactivation of its expression in NPC cell line.

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**Table 1**

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*TNM, Tumor-Node-Metastasis.

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**Fig. 3** Representative MSP results of the RIZ1 gene in primary tumor (T), NP swab (NS), M & T rinsing fluid (S), plasma (CS), and buffy coat (CW) from 1 patient with NPC. The methylated control from universal methylated DNA and water blank control (no template control) were included in each PCR amplification.

**Fig. 4** A, RIZ1 transcript in CNE-2 cell line before and after 5-Aza-dC demethylation agent treatment. B, representative MSP results of the RIZ1 gene in CNE-2 cell line after 5-Aza-dC treatment.
Epigenetic silencing of \textit{RIZ1}\footnote{Pref. to \textit{RIZ}} by methylation was found in 11 of 25 (44\%) breast cancer specimens and 20 of 32 (62\%) liver cancer (10). In this study, we have demonstrated the high frequency of \textit{RIZ1} promoter methylation in 60\% NPC tumors. There was no correlation of clinical stage, sex, and age with \textit{RIZ1} methylation. Methylation of \textit{RIZ1} was detected in both early and late stage of tumors, indicating that the inactivation of the \textit{RIZ1} gene might be essential in the early development of NPC and persist throughout the course of this development. Because all 30 NPC patients in this study were followed up to 1 year, we couldn’t evaluate the prognostic value of \textit{RIZ1} methylation.

Metastatic tumor cells may also enter the circulation and can be detected in the buffy coat layer of peripheral blood. NPC cells may detach from the primary tumor and can be collected by NP swabs or M & T rinsing fluid. Tumor DNA may be released after cell death into the nasopharynx or absorbed into the systemic circulation. We have found previously the presence of NP carcinoma cells in cytological swabs of the nasopharynx that can be used as diagnostic adjunct (21). Cytological evaluation is labor intensive, requiring a high degree of experience to accurately identify morphologically suspicious cells. Studies have shown promising applied molecular approach to detect cancer-specific alterations like epigenetic change by hypermethylation, loss of heterozygosity, the presence of Epstein-Barr virus latent membrane protein, and so forth in cancer cells from mouth washes and NP swabs (16, 24, 25). The collection of NP swabs, M & T rinsing fluid, and blood can be easily performed as a screening procedure by family physician or paramedics without the necessity of the more complicated, invasive endoscopic biopsy by a trained otolaryngologist. MSP is a powerful technique in the identification of small quantity of cancer cells or cancer DNA. The PCR amplification has advantages of sensitive and accurate detection of small quantity of cancer cells or cancer DNA within the background of normal cells or normal DNA in tumors or body fluids (26). This is particularly important if methylated DNA is to be used as tumor marker in screening primary NPC of high-risk asymptomatic population or patients with early symptoms suspected of having NPC or in the early detection of minimal residual tumor or asymptomatic stage of recurrence after treatment. From the sensitivity test, the MSP was able to detect a minimum of 70 methylated \textit{RIZ1} gene copies. With this sensitivity, methylated \textit{RIZ1} DNA was detectable in 50\% of the samples from either or both M & T rinsing fluid and NP swabs and in 27\% peripheral blood samples. Specificity of \textit{RIZ1} analysis was demonstrated in this study in which all normal controls, including 8 histologically normal NP tissue biopsies and body fluid samples from 29 healthy subjects and the 12 matched body fluid samples with methylated \textit{RIZ1} gene undetectable in primary tumors, showed methylation free.

Because \textit{RIZ1} methylation is not histological specific for NPC and can be found in other cancers or premalignant conditions other than NPC in the body, the source of methylated DNA in body fluid is therefore not specific for NPC. The NP swab contains DNA from nasal and NP tissues; M & T rinsing fluid DNA comes from whole upper aerodigestive tract, and peripheral blood plasma anduffy coat can come from any organs of the whole body. Methylation marker is a screening tool to assist clinical management and not by itself sufficient for definitive diagnosis. Histopathological examination of biopsy tissue should still be the gold standard for cancer diagnosis that cannot be replaced with molecular tumor marker. The present problem is the difficulty of screening or early detection of primary and minimal residual/recurrent NPC in their asymptomatic stage based on clinical examination. Molecular tools like methylation of the \textit{RIZ1} gene are developed to help in screening of cancer and early detection of residual or recurrent tumor after treatment. In case a positive molecular finding is found, it can help the clinician to further investigate and confirm with histopathological examination. A second cancer other than NPC should also be considered in the differential diagnosis. Treatment of primary and recurrent tumors in their early stage will significantly improve the outcome. With the relatively high sensitivity and specificity of MSP, screening with methylated promoter DNA as tumor marker is still potentially valuable to assist clinical management.

The present study shows the possibility of PCR-based MSP method to detect small quantity of cellular or cell-free DNA in body fluid, including NP swab, M & T rinsing fluid, and circulating blood of NPC patients. Additional large scale prospective studies are worthwhile to fully elucidate its sensitivity and specificity of screening high-risk population and early detection of residual or recurrent NPC after treatment.

\textbf{REFERENCES}


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