Analyses of Promoter Hypermethylation for RUNX3 and other Tumor Suppressor Genes in Nasopharyngeal Carcinoma

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Abstract. Background: Aberrant methylation of cytosine in promoter CpG islands is a recognized contributory process to carcinogenesis. This study explores the methylation profile of RUNX3 in combination with p16, RASSF1A, CDH1 and hMLH1 in nasopharyngeal carcinoma (NPC) patients. Materials and Methods: Genomic DNA was extracted from 19 fresh frozen NPC biopsies, which were then subjected to bisulfite conversion and methylation-specific PCR for analysis of promoter hypermethylation for the five respective genes. Three cell lines, SNU1, RKO and LS174T, were used as controls. Results: The incidences of promoter methylation were as follows: RUNX3 0/19 (0%), p16 6/19 (32%), RASSF1A 13/19 (68%), CDH1 9/19 (47%) and hMLH1 4/19 (21%). Ninety-five percent of the tumor specimens displayed aberrant methylation in at least one of these genes. No significant correlation between methylation status of these genes and clinical parameters was found. Conclusion: Methylation of multiple genes is involved in critical pathways for cancer development in NPC. Promoter hypermethylation for RUNX3 was, however, not present.

Nasopharyngeal carcinoma (NPC) is a malignancy with an unusually variable incidence rate worldwide. This neoplasm is an uncommon disease in most countries with an age-adjusted annual incidence rate of less than 1 per 100,000 population (1). It is, however, endemic in the Southern parts of China, some areas of South-East Asia and the Mediterranean basin (1). The annual incidence of NPC in Southern China ranges from 25-50 per 100,000, while in Singapore, it is the sixth commonest cancer among Chinese males with an age-standardized rate of 12.5 per 100,000 (2). The incidence of NPC peaks between 40-65 years with 83.2% of all histological types comprising an undifferentiated carcinoma (WHO Type III) (2). The development of NPC is attributable to three major well-defined etiological factors: a genetically determined susceptibility in some individuals, an early-age exposure to chemical carcinogens, particularly Cantonese salted fish, and an association with a latent Epstein-Barr virus (EBV) infection (1).

This unique interaction of environmental and genetic factors accompanied by the accumulation of specific genetic and epigenetic changes has allowed the gradual elucidation of a multistep model for the mechanism of tumorigenesis for NPC. This is likely to bring about novel diagnostic and therapeutic techniques into clinical practice.

Although it is known that germ line or somatic mutations can enhance the function of oncogenes or down-regulate the activity of tumor suppressor genes leading to the development of cancer, investigations have shown the presence of epigenetic changes in promoting the development of tumors from the earliest to the latest stage. In addition to genome-wide hypomethylation leading to genomic instability (3, 4), aberrant methylation of cytosine in promoter CpG islands causing transcriptional silencing of vital tumor suppressor genes in cancer cells has been recognized as an important feature of human cancer (5, 6). Using restriction landmark genomic scanning methods, it has been demonstrated that an average of 600 CpG islands (range from 0 to 4,500) are aberrantly methylated in human cancer (7).

Epigenetic inactivation of several tumor suppressor genes leading to gene silencing is common in NPC, implying a "methylator" phenotype in this cancer. In addition to high frequencies of aberrant CpG island promoter hypermethylation for p16 (8) and RASSF1A (9) in NPC,
there are other genes, including CDH1 (10), DAP-kinase (11) and hMLH1 (12), which have been found to be involved in the methylation process. However, there are several tumor suppressor genes, such as RUNX3, which have not yet been evaluated.

In this study, the promoter hypermethylation profile of a panel of five genes, namely RUNX3 (Run-related transcription factor 3), CDKN2A (p16), RASSF1A (Ras association domain family 1A), CDH1 (E-cadherin) and hMLH1 (human mut L homolog), was examined in 19 NPC primary tumors using three cell lines, SNU1, RKO and LS174T, as controls. The correlation between methylation status of these genes and clinical features, such as age, clinical tumor stage, T stage, lymph node status and EBV copy number of the patients, has also been analyzed.

**Materials and Methods**

**Tissue samples.** Nineteen samples of tumor biopsies (NPC 1-19) were obtained from NPC patients with consent at the National University Hospital. All samples were collected before treatment. Specimens were snap-frozen in liquid nitrogen and subsequently stored at –80 °C. Hematoxylin-eosin (HE)-stained sections of all specimens were examined for the presence of tumor cells by a pathologist. These tumors were classified as WHO grade III (13). Detailed clinical information of these patients is summarized in Table I. Measurement of EBV copy number was not carried out for 6 patient samples as blood was not drawn. The age range was 31-76 years (mean age 46 years) and all patients were males. On the basis of Ho’s stage classification (14), there were three patients with Ho’s stage II disease (15.8%), eight patients had stage III (42.1%) and eight had stage IV (42.1%).

**DNA extraction.** Genomic DNA was extracted from frozen tissue biopsies with TRI Reagent (Molecular Research Center, INC.) using the manufacturer’s recommended protocol. Two cancer cell lines SNU1 and LS174T were used as positive and negative controls, respectively, for RUNX3, p16, RASSF1A and CDH1. RKO and LS174T were used as positive and negative controls, respectively, for hMLH1.

**Bisulfite treatment and methylation-specific PCR (MSP).** One µg of sample DNA was treated with sodium bisulfite using the CpGenome DNA Modification kit (Chemicon, Temecula, CA, USA), converting all unmethylated, but not methylated, cytosine to uracil. All bisulfite-modified DNA were resuspended in 20 µl of TE buffer [10 mmol/L Tris-0.1 mmol/L EDTA (pH 7.5)] and stored at –20 °C until subsequent MSP. PCR primers distinguishing between methylated and unmethylated DNA for RUNX3 were used; the primer sequences were: S (sense): 5’-GCCGTAAAGTAGGGCGAGAATA-3’, AS (antisense): 5’-CACGAACTCGCCTACGTAATC-3’ and S: 5’-TGTTAAGATGGGTGGTAGAATA-3’, AS: 5’-CAGAACACTCTGTTAGATC-3’, respectively. The primer sequences of p16 (15), RASSF1A (16) and CDH1 (17) and the expected PCR product sizes had been previously reported. The PCR was performed in 25-µl reaction volumes containing 2.5 µl of bisulfite-modified DNA, 10X PCR buffer, 10 mM each deoxynucleotide triphosphates, 10 µM each primers and 0.125 µl each of HotStarTaq (Qiagen, Hilden, Germany). PCR amplification of the template DNA was performed under the following conditions for RUNX3: methyl specific, 95 °C for 15 min; 38 cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 20 sec; unmethyl specific, 95 °C for 15 min; 38 cycles of 95 °C for 15 sec, 56 °C for 30 sec and 72°C for 20 sec. Methylation analysis was carried out on the remaining four genes with the annealing temperature depending on the primer set to be used. PCR products (5 µl) were loaded onto a 2% non-denaturing agarose gel together with a 100-bp ladder, stained with ethidium bromide, and visualized under UV illumination. The sample was scored as methylated when there was a visible band on the gel with methylated primers and similarly for unmethylated primer sets.

**Statistical analysis.** The number of methylated genes (RUNX3, p16, RASSF1A, CDH1 and hMLH1) were assessed for correlation with the clinical tumor stage, T stage and lymph node status using the polychoronic correlation coefficient. On the other hand, the correlation with age of patients and EBV copy number was measured using the polyserial correlation coefficient. All tests were conducted at the 5% significance level (LISREL 8.72, Scientific Software International, Inc.).

**Results**

**Frequency of methylation in NPC primary tumors.** The frequencies of methylation of RUNX3, p16, RASSF1A, CDH1 and hMLH1 were determined in 19 NPC fresh frozen biopsy specimens. Representative gel electrophoresis results of MSP analysis for RUNX3 are shown in Figure 1A, and
representative examples for the remaining four genes are shown in Figure 1B. The promoter hypermethylation frequency of each gene in the NPC samples and control cell lines is summarized in Figures 2 and 3. No CpG island methylation was detected in the \textit{RUNX3} exon1 region while the gastric cancer cell line SNU1 was positive for methylation. To confirm our results, MSP for \textit{RUNX3} was repeated with another primer set, which also showed an absence of methylation (data not shown). Furthermore, PCR using non-methylation specific primers for \textit{RUNX3} showed bands for all 19 NPC samples. The incidence of promoter hypermethylation for the other four genes were as follows: \textit{p16} 6 out of 19 (32%), \textit{RASSF1A} 13 out of 19 (68%), \textit{CDH1} 9 out of 19 (47%) and \textit{hMLH1} 4 out of 19 (21%). The median number of methylated gene promoters was two (mean = 1.68). Methylation of only one gene was found in 42% (8 out of 19) of tumors. The percentage of tumors showing methylation in two or three genes was 32% (6 out of 19) and 21% (4 out of 19), respectively. None of the primary tumors showed methylation in four or more genes (Figure 4).

**Correlations with clinical parameters.** The correlation between methylation of each gene with age, clinical tumor stage, T stage, lymph node status and EBV copy number was analyzed. No significant association was found among the relationships examined.

**Discussion**

The abnormal hypermethylation patterns of promoter site DNA causing gene silencing in cancer cells plays a pivotal role in the development and progression of cancer. The widespread hypermethylation observed in NPC samples suggests a potential role in the pathogenesis of this disease. Further studies are needed to confirm these findings and to elucidate the specific mechanisms by which these genes are silenced in NPC.

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**Figure 1.** A) Representative MSP analysis of \textit{RUNX3} in NPC biopsy samples. M, PCR products amplified by M primers; U, PCR products amplified by U primers. The first lane shows the 100 bp DNA molecular weight ladder. Tumor cell lines SNU1 and LS174T (LS) as positive and negative controls respectively; H2O, water control. B) Representative results showing promoter methylation amplified by MSP for \textit{p16}, \textit{RASSF1A}, \textit{CDH1} and \textit{hMLH1}. SNU1, positive control for \textit{p16}, \textit{RASSF1A} and \textit{CDH1}; RKO positive control for \textit{hMLH1}; LS174T (LS), negative control for all genes; H2O, water control.

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**Figure 2.** Summary of methylation for \textit{RUNX3}, \textit{p16}, \textit{RASSF1A}, \textit{CDH1} and \textit{hMLH1} in NPC tissue samples. SNU1, RKO and LT174T are cancer cell lines used as controls. Filled boxes: methylated loci; open boxes: no methylation detected; ND: not done.
role in tumorigenesis. In fact, the importance of promoter hypermethylation has been illuminated by the ability of demethylating drugs, such as 5-azacytidine, to reactivate the affected genes and restore production of the corresponding protein in cultured cancer cells (18).

In recent years, the growing list of genes silenced by hypermethylation has provided the opportunity to examine their role in various malignancies. In this study, the frequency of aberrant promoter hypermethylation was determined, with a focus on RUNX3 together with a panel of other genes including p16, RASSF1A, CDH1 and hMLH1. Epigenetic inactivation of these different genes, which may occur simultaneously, affects all the molecular pathways involved in cell immortalization and transformation (19).

RUNX3 plays a role in carcinogenesis. Transforming growth factor-β (TGF-β) is a multifactorial growth factor, crucial in many developmental and physiological processes. Polyomavirus enhancer binding protein/core binding factor (PEBP2/CBF) is a heterodimeric transcription factor composed of α and β subunits and forms an important target for TGF-β superfamily signaling essential for mammalian development (20). The α subunit contains a conserved region known as the Runt domain required for DNA binding and dimerization with the β subunit. Three mammalian runt-related α subunit genes exist, namely RUNX1/AML1, RUNX2/CBFA-1 and RUNX3/PEBP2αC, the former two genes being essential for hematopoietic differentiation and osteogenesis, respectively (20-22). Each RUNX gene forms complexes with Smad2 and Smad3 that transmit TGF-β/activin signals (23). The RUNX3 gene is located on human chromosome 1p36, where high frequency loss of heterozygosity (LOH) has been detected in various cancers. Thus, RUNX3 may be a tumor suppressor candidate in cancer development. This relationship between RUNX3 and carcinogenesis has already been extensively studied in the context of gastric cancer and it has been found that 45-60% of human gastric cancer cells do not express RUNX3 due to hemizygous deletion and hypermethylation of the RUNX3 exon 1 region (22). Furthermore, RUNX3 expression has also been reported to be lost in a variety of other cancer types, such as 19-50% of non-small cell lung cancer tissue samples (24, 25), 70-75% of bile duct and pancreatic cancer cell lines (26), 40-80% of liver cancer cell lines and tissue (27, 28), 50% of breast cancer tissue (29) and 30-50% of sporadic colon carcinoma tissue samples (30). The frequency of RUNX3 in NPC has as yet been unreported. Unexpectedly, an absence of promoter hypermethylation in the RUNX3 exon 1 region was detected. However, it is possible that RUNX3 may participate in the process of carcinogenesis via alternative pathways. It has been reported that methylation-independent inactivation of RUNX3 by protein mislocalization to the cytoplasm due to impaired components of the TGF-β signaling cascade is another mechanism apart from hypermethylation which is causally related to gastric cancer (31). In addition, overexpression of the RUNX3 protein has been found in human basal cell carcinomas implicating RUNX3 functioning as an oncogene (32).

Figure 3. Percentage of distribution of hypermethylated genes in primary undifferentiated nasopharyngeal carcinoma.

Figure 4. The number of methylated gene promoters in undifferentiated nasopharyngeal carcinoma.

p16 is a common tumor suppressor that plays a central role in control of cell proliferation during G1. Loss of p16 function occurs in at least three ways: homozygous deletion, methylation of the promoter and point mutation, the first two mechanisms comprising the majority of inactivation events in most primary tumors. p16 is a major target in carcinogenesis, rivaled in frequency only by the p53 tumor-suppressor gene. Its mechanism of action as a cyclin-dependent kinase inhibitor (CDKI) has been elucidated and involves binding to and inactivating the cyclin-dependent kinase 4 (or 6) complex, and, thus, rendering the retinoblastoma protein inactive. This effect blocks the transcription of important cell-cycle regulatory proteins and results in cell-cycle arrest (33). It has been previously reported, that aberrant methylation of the 5' CpG island of the p16 promoter was detected in 22-26% of primary tumors (8, 34). In this present study, our detection rate of 32% was comparable to previously reported figures.
A new 3p21.3 tumor suppressor gene, the Ras association domain family 1A gene (RASSF1A) from a minimal 120 kb region defined by overlapping homozygous deletions in lung and breast cancers has also been recently identified (35, 36). RASSF1A may participate in the DNA damage response or in DNA damage-induced regulation of other cell signaling events (36, 37). RASSF1A appears to have a high frequency of epigenetic alterations in NPC with methylation frequencies of 66-80% (11, 38). Our study also demonstrated a comparably high methylation rate of 68%.

The down-regulation of CDH1 (E-cadherin) via methylation of the promoter region is observed in a wide variety of tumors from epithelial cells. E-cadherin mediates cell-cell adhesion by association with intracellular molecules of α, β and γ-catenins (39). Reduction of E-cadherin induces cell mobility and promotes tumor cell invasion (40). E-cadherin is decreased or lost frequently in NPC and such loss has been correlated with an advanced stage and poor survival (41). Our methylation result of 47% is slightly less than previously reported values (10, 42).

The hMLH1 gene is located at chromosome 3p21-23, the protein product of which is an important component of the DNA mismatch repair pathway. Hypermethylation of hMLH1 is the most frequent change associated with micro-satellite instability in colon, endometrial and gastric neoplasia (43-45). The reports on this tumor suppressor gene in NPC have been scarce with a 40% hypermethylation rate in one study (12) as opposed to our 21%. Further studying on this gene is required before the methylation frequency can be ascertained.

There are several possible explanations for the differences in our results with the literature reports, including variation in laboratory conditions and patient characteristics. Another possible reason could be the bias due to small sample size in all studies, because of the relative difficulty in obtaining biopsy specimens.

In conclusion, our study has shown that methylation of RUNX3 may not be involved in the carcinogenesis process of NPC, in contrast to p16, RASSF1A, CDH1 and hMLH1, which via genetic silencing by methylation affect the cell cycle, Ras signaling pathway, metastasis-related processes and DNA repair mechanisms. Further identification and studying of potentially new tumor suppressor genes, will add to our current knowledge of the as yet unestablished panel of methylated markers, thus improving detection and suggesting novel therapies for NPC.

Acknowledgements

We wish to thank Kenichi Inoue for technical support.

References


