Gene expression and promoter polymorphisms of DNA methyltransferase 3B in nasopharyngeal carcinomas in Taiwanese people: A case-control study

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Abstract. Overexpression of the DNA methyltransferase 3B (DNMT3B) gene and its effect on carcinogenesis has been demonstrated for various types of cancer. Recently, three single nucleotide polymorphisms (SNPs) of the DNMT3B promoter region, C46359T (-149C>T), -283T>C, and -579G>T have also been reported to be stratification markers that can predict an individual's susceptibility to cancers. In this study, we analyzed expression of DNMT3B in nasopharyngeal carcinoma (NPC) specimens and did not find elevated levels of DNMT3B in tumors using cDNA microarray analysis and RT-PCR. Meanwhile, 259 NPC patients and 250 controls were genotyped for the above three SNPs using a MALDI-TOF based mini-sequencing method. For C46359T (-149C>T), only the T/T genotype was found to be present in both patient and control groups (100% frequency). The frequency of the genotypes, -283CC, -283CT and -283TT, amongst NPC patients versus controls was, respectively, 86.1% versus 84.0%, 13.5% versus 15.6%, and 0.4% versus 0.4% (P=0.589). The allele frequency, -597TT, -597GT and -597GG, for patients versus controls was, respectively, 87.3% versus 84.8%, 12.0% versus 15.2%, and 0.8% versus 0 (P=0.501). The distribution of SNPs among cancer patients either featuring or not featuring cervical metastasis also did not reveal any significant difference. In conclusion, our data indicate that neither overexpression of DNMT3B nor the presence of three DNMT3B SNPs are associated with NPC, which suggests that DNMT3B might not play a role in hypermethylation of many tumor suppressor genes during carcinogenesis of NPC.

Introduction

CpG methylation patterns are established and maintained during development by DNA methyltransferase 1 (DNMT1) and DNMT3 families in mammalian species (1). Global epigenetic alterations such as 'globally hypomethylated; gene specific hypermethylated in DNA' have been found in cancer cells, which has highlighted the idea that DNA methylation plays a significant role in development and progression of cancers (2). Indeed, reports have shown that cell cycle control, apoptosis-related, tumor suppressor and DNA repair genes are hypermethylated in several kinds of cancer cells (2). DNA methylation is mediated by DNA methyltransferases including DNMT1, DNMT3A and DNMT3B which cooperatively establish and maintain genomic methylation patterns (3). DNMT1 is considered to be a maintenance DNA methyltransferase due to the fact that it preferentially methylates hemi-methylated DNA after DNA replication; whereas DNMT3A and DNMT3B function as de novo methyltransferases, which methylate unmethylated and hemi-methylated DNA with equal efficiency (3).

Among these DNMTs, overexpression of DNMT3B was found to be present in several tumors, demonstrating that DNMT3B plays an important role in tumorigenesis (4-7). Recently, three single nucleotide polymorphisms (SNPs) of the DNMT3B promoter [C46359T(-149 C>T), -283T>C and -579G>T] were reported to be crucial for the promoter's activity. This result is in an involved individual's predisposition to certain cancers; including lung cancer, prostate cancer, hereditary nonpolyposis colorectal cancer (HNPPC) and acute leukemia (8-11). Nevertheless, the biological significance of these three DNMT3B SNPs is somewhat controversial as regards individuals of differing ethnicity, and persons with different types of tumors (12-14).
Nasopharyngeal carcinoma (NPC) is a common cancer in the southeast region of Asia including Taiwan (15). Although Epstein-Barr virus has been reported to be associated with NPC, certain other factors, such as environmental or genetic factors, still appear crucial for the development and/or progression of NPC (16). In some epigenetic studies, hyper-methylation of the promoters of many critical tumor suppressor genes has been shown in NPC (17-19). Latent membrane protein-1, an oncprotein derived from the Epstein-Barr virus, can activate the promoter of DNMT1 via the JNK-AP-1 pathway and then induce the downregulation of the E-cadherin gene which increases the migration capability of NPC cells (20,21). However, the relative significance of the expression pattern of DNMT3B and these novel SNPs with regards to the genetic susceptibility of an individual to NPC in Taiwan, to the patients recruited for this study provided written informed radiological examination conducted at the time of presentation, NPC patients, who had a positive nodal status as revealed by chest x-ray, abdominal ultrasonography, and a bone scan. Those computed tomography and/or magnetic resonance imaging, treatment, routine diagnostic work-ups including head-and-neck NPC patients enrolled in this study underwent, prior to their health-examination program, including individuals presenting and/or tumor stage. Control subjects were derived from a condition histopathologically confirmed between September 1990 and November 2005, inclusively. No restrictions applied previously untreated patients with NPC. All had their Hospital, Tao-Yuan, Taiwan. All cases were newly diagnosed, the RNAzol B reagent (Tel-Test Inc., Friendwood, TX), and frozen paired tumor and pericancerous normal tissues using 19 paired samples having a minimum of 80% tumor tissue contamination on gene expression profiling of the tumor sections) were selected for cDNA microarray analysis.

cDNA microarray analysis was performed using total RNA extracted from NPC and paired normal tissues. Information regarding the GMRCL Human 15K set, Version 2 microarray chips used in this study can be downloaded from <http://www.cgmh.org.tw/intr/intr2/c32a0/chinese/corelab_intro/genetics/index_1.htm> in the Minimum Information About a Microarray Experiment (MIAME) format. Briefly, 2 μg total RNA was used for labeling and hybridization with the 3DNA 300Array detection kit (Genisphere, Hatfield, PA) according to the manufacturer's protocol. The experiment was replicated under the dye-swapping microarray strategy in an effort to minimize statistical variances in the data. After hybridization and washing, slides were scanned with a ChipReader confocal scanner (Virtek, Canada) and spot and background intensities were acquired using GenePix Pro 4.1 software (Molecular Devices, Sunnyvale, CA). For within-slide normalization, the local weighted regess (LOWESS) method was used. We averaged four replicated log ratios of each gene (two from duplicates on the slide and two others from the dye-swapping replicate) and performed Student's t-tests on each gene between the indicated groups. Stringent criteria were used to define the statistical significance of each observed change in gene expression, using F statistics (p<0.001), with significance confirmed over 1,000,000 random permutations.

Reverse transcription-polymerase chain reaction (RT-PCR) of DNMT3B. One μg of total RNA purified from tissues samples was reverse transcribed using reverse transcriptor first-strand cDNA synthesis kits (Roche) in a total volume of 25 μl. RT-PCR amplification reactions of DNMT3B and PCNA were performed with 100 ng cDNA, 10% DMSO, 100 μM deoxy-nucleotide triphosphates, Taq DNA polymerase (Roche), and 1 μM primers. The RT-PCR primers for DNMT3B isoforms were as follows: DNMT3B catalytic sense, 5'-AAG CCC ATG CAA TGA TCT CTC TAA CG-3' and antisense, 5'-CAC GTC GGT GTA GTG AGC AGG GAA GC-3'; and human PCNA sense, 5'-CAA TTG GGT ATG CAT CTT GTA ATG TAC-3' and antisense, 5'-GGG TAC ATC TGC AGA CTR ACT GAG TGT CA-3'. The primer and PCR condition was as previously described (23).

DNMT3B genotyping by matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF)-based mini-sequencing analysis. Genomic DNA was extracted from peripheral blood lymphocytes according to the manufacturer's recommendations (Nucleobond, Macherey-Nagel). The DNMT3B -283T/C, -579G/T, C46359T polymorphisms were determined by a MALDI-TOF based mini-sequencing genotyping method as previously described (24,25). The primer sets used for amplification of each SNP region were as described previously (26). In brief, the PCR reactions were performed in a total of 25 μl containing 200 ng genomic DNA, 25 μM each primer, 0.2 mM dNTPs, 75 mM Trs-HCl (pH 9.0), 15 mM ammonium sulfate, 0.1 μg/ BSA, 2.5 mM MgCl2, 1 M betaine and 1 unit of Fast-start Taq polymerase (Roche), which was initiated for
95°C for 5 min, followed by 40 cycles of 95°C for 45 sec, 50°C for 45 sec and 72°C for 45 sec, and finally extended for 10 min at 72°C. The unincorporated dNTPs and primers were removed automatically by MAPIIA (genepure PCR purification system; Brucker, Bremen, Germany). The purified PCR products were used to conduct mini-sequencing reactions by individual mini-sequencing primers as we described previously (26). These were carried out in 20 μl of solution containing 50 ng PCR product and 1 μl of 10 pmol mini-sequencing primer, 0.5 μl of 1 mM of ddNTPs/ddNTP mixture (for -283T/C, dA ddC ddG; -597G/T, dT ddG; C46359, dC dA ddT), 0.5 U of Thermo Sequenase DNA polymerase (Amersham Biosciences), and 2 μl reaction buffer provided by the manufacturer. The reaction was carried out in a multiblock system (MBS) thermocycler (ThermoHybaid) with an initial denaturation step at 96°C for 1 min followed by 50 cycles of 96°C for 15 sec, 50°C for 15 sec, 60°C for 100 sec, and then 96°C for 30 sec. The mini-sequencing reaction products were purified by single-strand binding beads automatically via MAPIIA (Brucker) and analyzed by MALDI-TOF MS. Prior to MALDI-TOF MS analysis, samples were mixed with 0.5 μl of matrix solution (50 mg/ml 3-hydroxypicolinic acid in a 4:5:1 mixture of water, acetonitrile and 50 mg/ml diammionium citrate) and spotted on a 384-well teflon sample plate (PerSeptive Biosystems). MALDI-TOF mass spectra were acquired using a Brucker Autoflex MALDI-TOF (Brucker) and AutoXecute software as validating tools for genotyping data. To confirm the results of MALDI-TOF, 1/10 of the PCR products were randomly selected for DNA auto-sequencing analysis (ABI autosequencer 3730) by 3b-1 sequencing primer (5'-AATTT GAAAT CGCTC GGAGC CTC-3'), 3b-2 sequencing primer (5'-GACCT GGAGC TGTTT GTGGT -149C>T) was found to be present in the DNA of individuals (26). These were carried out in 20 μl of solution randomly selected to determine the DNA sequences of the three SNP (C46359T) of the DNMT3B SNP, the mini-sequencing primer, and the molecular weight of mini-sequencing products are as we described previously (26). The PCR products were randomly selected to determine their DNA sequences by auto-sequencing analysis. The results of DNA sequences of the three DNA fragments covering each of the three DNMT3B SNPs, the mini-sequencing primer, and the molecular weight of mini-sequencing products are as we described previously (26). The PCR products were randomly selected to determine their DNA sequences by auto-sequencing analysis. The results of DNA sequences of the three DNMT3B SNPs confirmed the genotyping results (data not shown).

Contrary to what has been reported in several previous studies, we observed that only the T/T genotype (C46359T, -149C>T) was found to be present in the DNA of individuals from both study groups (100% frequency), whereas the C/C and C/T genotype (C46359T, -149C>T) was not found to be present in either of the two study groups. Our data suggest that there is no association between SNP (C46359T) of the DNMT3B promoter and NPC risk in a Taiwanese population, similar to a finding for gastric cancer in a Japanese population (12).

The frequency of appearance of the genotypes, -283CC, -283CT and -283TT, amongst NPC patients versus controls was, respectively, 86.1% versus 84.0%, 13.5% versus 15.6%, and 0.4% versus 0.4%. The frequency of appearance of the two genotypes, -597TT, -597GT and -597GG, for patients versus controls was, respectively, 87.3% versus 84.8%, 12.0% versus 15.2%, and 0.8% versus 0 (Table II). Our data show that the incidence of these two DNMT3B SNPs for individuals afflicted with NPC did not differ significantly from corresponding values for controls (-283T>C, P=0.589; -597G>T, P=0.501) (Table II).
The risk of suffering NPC and contracting cervical metastasis appears to relate to the presence or absence of either of the two DNMT3B genotypes -283T>C and -579G>T, the odds ratios (ORs) for which were also analyzed (Table III). ORs and their 95% CIs were calculated using the more-common homozygous genotype as the reference group (-283CC and -579TT genotypes, respectively). When comparing the results for the patient group with the corresponding results for the control group, the ORs for NPC for the -283 TT and the TC genotype combined, and the ORs for NPC for the -579 GT genotype combined with the GG genotype were lower for the patient group than was the case for the control group, although such differences did not prove to be statistically significant (respectively, adjusted OR=0.843, 95% CI=0.517-1.384, P=0.493; adjusted OR=0.817, 95% CI=0.494-1.351, P=0.431) (Table III). Of the 259 NPC patients, 160 (61.8%) had cervical metastasis upon radiological examination conducted prior to treatment for NPC, although none of these 160 cervical-metastasis patients were found to show any evidence of distant metastasis during systemic work-up prior to their treatment. Again, the ORs for cervical metastasis for the two genotypes -283 TT and TC combined, and for the -579 GT and GG genotypes combined were observed to be lower within the patient group, but again were not statistically significant when compared with the corresponding results for the reference group (adjusted OR=0.500, 95% CI=0.219-1.143, P=0.100; adjusted OR=0.649, 95% CI=0.275-1.532, P=0.324, respectively) (Table III). Our data thus reveal that there are no significant differences between NPC patients and controls as regards to the frequency or presence of any or all of these three DNMT3B SNPs or the likelihood of the occurrence of carcinogenesis and/or cervical metastasis for NPC patients, at least amongst a Taiwanese population.

Since a previous paper showed that polymorphisms in DNMT3B promoters increase promoter activity of DNMT3B, the transcripts of DNMT3B were also examined by cDNA microarray analysis in 19 NPC biopsies and their normal counterparts whose DNMT3B genotype had been determined. Most of them were -283CC/-597GG; only one was -283CT/-597GT. As regards DNMT3B expression profiles on cDNA microarrays, we found that the fold change of DNMT3B mRNA signal ratio between each paired NPC tumor and pericancerous normal tissue ranged from 0.420 to 1.562 and there was no significant differential expression of DNMT3B were not found in NPC biopsies as compared with their pericancerous normal tissue. Among 19 NPC biopsies, some did not show expression of DNMT3B (n=5) and even showed a tendency toward down-regulation of DNMT3B (n=8) (Fig. 1A). Elevated levels of DNMT3B were confirmed by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR); the data were consistent with results of cDNA microarray profiling (data not shown). Previous reports indicated that several isoforms of DNMT3B were over-
Thus, the isoforms of DNA methyltransferase (DNMT) analysis, reaction (QRT-PCR) (4-7). Using immunohistochemical analysis, oral cancer, by using either immunohistochemical analysis or including lung cancer, endometrial cancer, breast cancer, and their normal counterparts. Overexpression of DNMT3B expressed in tumor cells including DNMT3B4 and Δ-DNMT3B. Thus, the isoforms of DNMT3B were examined by consensus primer sets as previously described (23). Some of the NPC biopsies did not express any DNMT3B isoforms as compared with their normal counterparts (Fig. 1B; T12 and T8). The predominant form of DNMT3B in NPC tumors and normal tissues was DNMT3B; DNMT3B4 was observed in only one tumor (Fig. 1A; T6). Most of them showed expression of different DNMT3B isoforms between normal and tumor (Fig. 1B; T2/N2, T6/N6, T7/N7, and T8/N8). Neither overexpression of DNMT3B nor DNMT3B4 was found in NPC tumors as compared with their normal counterparts.

Discussion

Overexpression of DNMT3B has been found in several cancers, including lung cancer, endometrial cancer, breast cancer, and oral cancer, by using either immunohistochemical analysis or real-time quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) (4-7). Using immunohistochemical analysis, DNMT1, DNMT3A and DNMT3B proteins were found to be highly expressed in a coordinate manner in lung cancers, particularly in smokers (5). Also, overexpression of DNMT3B was found to be an independent prognostic marker for breast cancer, especially for estrogen receptor α-positive breast cancers (27). In addition, DNMT3B overexpression was associated with poor relapse-free survival in the subgroup of patients who received adjuvant hormone therapy (7). However, a role for DNMT3B in NPC is not established. To investigate a role for DNMT3B in NPC, we analyzed the expression of DNMT3B between NPC tumors and their pericancerous counterparts by cDNA microarray and QRT-PCR. Unlike the cancers mentioned above, the expression of DNMT3B did not show significant differences between NPC tumors and their normal counterparts (Fig. 1A). In previous studies, the predominant isoforms of DNMT3B had been identified as contributing to human carcinogenesis (28,29). Overexpression of DNMT3B4, a splice variant of DNMT3B, which may lack DNA methyltransferase activity and compete with DNMT3B3 for targeting to pericentromeric satellite regions, results in DNA hypomethylation in these regions, even in precancerous stages, and plays a critical role in human hepatocarcinogenesis by inducing chromosomal instability (28). Also, a novel DNMT3B subfamily, termed Δ-DNMT3B, has been shown to be the major expression form of DNMT3B in non-small cell lung cancer and may play an important role in the development of aberrant promoter methylation during lung tumorigenesis. To further identify the predominant isoforms of DNMT3B in NPC we analyzed QRT-PCR results. We observed DNMT3B4 in only one tumor sample (Fig. 1B; T6). Furthermore, expression of DNMT3B was not detected in some NPC tumors as compared with their normal counterparts (Fig. 1B; T12 and T8). In other words, unlike the effects of DNMT3B on many types of malignancies, neither DNMT3B overexpression nor DNMT3B4 appear to have a role in NPC tumors.

Recently, a single nucleotide polymorphism of the DNMT3B promoter (C46359T, -149C>T) was reported to be crucial for its promoter activity, although the results were conflicting. However, these results showed that persons with this SNP have a predisposition to certain cancers including lung, prostate, and acute leukemia (9,10,11). However, other studies showed conflicting results, that the C46359T polymorphism of DNMT3B (-149C>T) cannot be used as a stratification marker for prediction of susceptibility to breast and lung tumors, Caucasian HNPCC and gastric cancer (8,12,14). In our study, the CT-genotype was not found in either control or NPC groups from Taiwan. Our finding is very similar to findings from several recent reports, which showed that the polymorphism in C46359T of DNMT3B is not significant either in gastric cardiac adencarcinoma, acute leukemia patients of Chinese populations and gastric cancer patients in Japanese populations (9,12,14). Moreover, the T/T alleles of C46359T were found in 100% of cases in both NPC patients and healthy controls, which is consistent with other studies in Asian populations which show that more than 95% of alleles in C46359T were T/T (9,12,14). These findings are distinct from the results reported for Caucasian populations and might indicate that C46359T of DNMT3B is unable to be a useful stratification marker to predict susceptibility or survival differences of NPC in populations in Taiwan.
Besides C46359T, -283T>C (-283 bp from the transcription start site of exon 1A) and -579G>T (-579 bp from the transcription start site of exon 1B) of DNMT3B were investigated recently because expression of the DNMT3B gene is regulated via different promoters, which exist in different exons (exons 1A and 1B) with different CpG content (30,31). A previous study suggested that the -283C allele (within exon 1A of DNMT3B) may create a potential Sp1 responsive element to enhance promoter activity nearly two fold over -283T, and demonstrated that -283TT was associated with a significantly decreased risk of adenocarcinoma of the lung with an adjusted odds ratio of 0.48 as compared with -283CC (8). Therefore, the diversity in DNMT3B SNPs in different ethnic populations or in different tumor types remains to be elucidated.

In conclusion, our study shows that DNMT3B has only trivially small effects on NPC carcinogenesis because it has been shown not to be overexpressed in NPC specimens. Also, the three reported DNMT3B SNPs (C46359T, haplotype -283T>C and -579G>T) were not associated with higher risk of susceptibility to NPC. Furthermore, an impact of these DNMT3B SNPs on tumor metastasizing tendencies was not observed in populations of Taiwan. Consequently, DNMT3B should not be considered to have a major role in the phenomenon of hypermethylation of many vital genes during NPC carcinogenesis.

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