

Frequent Hypermethylation of *RASSF1A* and *TSLC1*, and High Viral Load of Epstein-Barr Virus DNA in Nasopharyngeal Carcinoma and Matched Tumor-Adjacent Tissues

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Abstract

We examined the promoter hypermethylation of tumor-suppressor genes *RASSF1A* and *TSLC1*, quantitated EBV DNA load in nasopharyngeal carcinoma (NPC) tissues (T tissues), and matched tumor-adjacent tissues outside 0.5 cm (P tissues) and outside 1.0 cm (Z tissues) to evaluate the role of promoter hypermethylation of *RASSF1A* and *TSLC1* as well as viral load in the pathogenesis of NPC. Methylation-specific polymerase chain reaction (PCR) for *RASSF1A* and *TSLC1* and quantitative real-time PCR analysis of EBV DNA were performed on matched T, P, and Z tissues ($n = 28$) as well as chronic nasopharyngitis tissues ($n = 8$). Hypermethylated *RASSF1A* was frequently detected in the T (82%) and P tissues (75%), but less frequently in Z tissues (46%). The average quantities of EBV DNA (copies/ μ g DNA) in matched T, P, and Z tissues were 673,000, 90,000, and 7000. The differences of promoter hypermethylation of *RASSF1A* and EBV viral load among T, P, and Z tissues were statistically significant, with more frequent methylation and higher viral load detected when tissues examined were nearer to the NPC tissues. Our results suggest that aberrant hypermethylation of *RASSF1A* and high EBV load might be important events in NPC pathogenesis, and they may be useful molecular diagnostic markers for this cancer.

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Keywords: NPC, *RASSF1A*, *TSLC1*, EBV, hypermethylation.

have been suggested to play roles in the development of NPC [1,2].

3p21.3 is a chromosome region of particular significance to NPC [3,4]. Loss of heterozygosity (LOH) and comparative genomic hybridization (CGH) analyses indicate a high incidence of allelic loss of 3p in NPC. Microsatellite marker deletion and familial NPC pedigree analyses suggest that the NPC-related locus is 3p21.3 [4–7]. Many tumor-suppressor genes locate on the 3p21.3 region, including the NPC-related tumor-suppressor gene *RASSF1A* [8,9]. Lost or altered expression of this gene has been associated with the pathogenesis of a variety of cancers. Aberrant promoter hypermethylation of *RASSF1A* occurs frequently in NPC [10–13]. Inactivation of *RASSF1A* was found to be related with the hypermethylation of CpG island in its promoter region. 11q22–23 is another important tumor-suppressive region in NPC, involving candidate tumor-suppressor gene *TSLC1*. Expression of *TSLC1* was downregulated by promoter hypermethylation in NPC cell lines [14].

EBV, also referred to as the human herpesvirus-4 (HHV-4), is a double-strand DNA gamma herpesvirus with a genome of 172 kb [15]. EBV is directly associated with human malignancies. EBV infection occurs worldwide and most people become infected during their lifetime. Infection with EBV usually occurs at a very early age, particularly in developing countries, and is closely associated with NPC and Burkitt's lymphoma. In contrast, in developed countries, most people are infected during adolescence or young adulthood, associated with

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy particularly prevalent in Southern China and Southeast Asia. Various studies indicate that the development of NPC is a multistage process involving multiple factors. Genetic predisposition and epigenetic alterations, Epstein-Barr virus (EBV), and environmental factors including dietary habits

Abbreviations: CN tissues, chronic nasopharyngitis tissues; EBER, EBV-encoded small RNA; ISH, *in situ* hybridization; HE, hematoxylin–eosin; M, PCR reaction using primers specific for methylated CpG sites; U, PCR reaction using primers specific for unmethylated CpG sites; NPC, nasopharyngeal carcinoma; P tissues, matched tumor-adjacent tissues outside 0.5 cm; T tissues, nasopharyngeal carcinoma tissues; Z tissues, matched tumor-adjacent tissues outside 1.0 cm

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Table 1. Clinical Information of Patients with Chronic Nasopharyngitis; Summary of MSP Results of *RASSF1A* and *TSLC1*, and EBV DNA Quantities in Chronic Nasopharyngitis Tissues.

Patient Number	Sex	Age (years)	Methylation Status at		EBV (copies/ μ g DNA)
			<i>RASSF1A</i>	<i>TSLC1</i>	
CN1	F	62	–	–	5.040×10^2
CN2	M	38	–	–	6.493×10^2
CN5	M	19	–	–	10.95×10^2
CN10	F	13	–	–	0
CN20	F	52	–	–	20.86×10^2
CN2a	F	51	–	–	0.089×10^2
CN21	F	60	–	–	0
CN50	M	50	–	–	2.564×10^2

CN: patients with chronic nasopharyngitis; (–) no hypermethylation detected.

infectious mononucleosis (IM). Basically, there exist two cell types susceptible to EBV infection: epithelial cells located in human oropharynx/nasopharynx and B-lymphocytes. EBV is strongly associated with NPC, an epithelial-derived cancer [15]. Many evidences indicate that EBV may be one of the most important factors involved in the tumorigenesis of NPC, but the exact role of EBV in NPC pathogenesis is not clear yet.

In this study, we detected the promoter hypermethylation of *RASSF1A* and *TSLC1* genes, quantitatively analyzed the

EBV DNA load in NPC tissues, and matched tumor-adjacent tissues to evaluate the role of promoter hypermethylation of *RASSF1A* and *TSLC1* as well as EBV load in the development of NPC.

Materials and Methods

Specimens

Twenty-eight matched tumor and tumor-adjacent tissues as well as eight chronic nasopharyngitis tissues were obtained from the Pathology Department of Xiangya Hospital (Hunan, China), with the consent of patients according to the regulation of university policy. Detailed information of these patients was summarized in Tables 1 and 2. Specimens were snap-frozen in liquid nitrogen and subsequently stored at -80°C . Hematoxylin–eosin (HE)-stained sections were examined for the presence or absence of tumor cells, as well as tumor cell density. Only samples containing more than 70% of tumor cells were selected for T tissues. P and Z tissues were defined as tissues located 0.5 and 1.0 cm outside of visible NPC lesions, respectively. Histopathologic examination indicated that P tissues had mild, moderate, to severe presence of atypical hyperplastic cells, and were infiltrated by a number of lymphocytes. The Z tissues had a mild presence of atypical hyperplastic cells and were also infiltrated by inflammatory cells [16].

Table 2. Clinical Information of Patients with NPC; Summary of MSP Results of *RASSF1A* and *TSLC1*, and EBV DNA Quantities in Matched T, P, and Z Tissues.

Patient Number	Sex	Age (years)	TNM	Methylation Status at <i>RASSF1A</i>			Methylation Status at <i>TSLC1</i>			EBV (copies/ μ g DNA)		
				T	P	Z	T	P	Z	T	P	Z
29	M	64	IVa	–	–	–	+	–	–	3.110×10^2	4.211×10^2	19.63×10^2
30	M	42	IIb	+	+	+	+	+	+	395.2×10^2	93.80×10^2	0
31	F	31	IIb	+	+	+	–	–	–	9307×10^2	4634×10^2	210.8×10^2
32	M	45	IIb	+	+	+	+	+	–	868.0×10^2	917.0×10^2	353.8×10^2
36	M	68	IVa	+	+	+	+	+	–	665.0×10^2	483.8×10^2	30.78×10^2
40	M	40	IIb	+	+	–	+	+	–	8480×10^2	2188×10^2	19.27×10^2
44	F	50	IVb	+	–	–	+	+	+	2149×10^2	4.350×10^2	0
46	M	30	I	+	+	+	+	+	+	29190×10^2	3.247×10^2	0.816×10^2
48	M	37	III	+	+	–	–	–	–	37580×10^2	331.4×10^2	0
49	M	28	III	+	+	–	–	–	–	4650×10^2	105.4×10^2	17.22×10^2
50	M	26	IIb	–	–	–	–	–	–	9580×10^2	22.23×10^2	3.723×10^2
51	M	32	IIb	+	–	–	+	+	+	6880×10^2	10.62×10^2	8.970×10^2
54	M	54	IIa	–	–	–	+	+	+	17.92×10^2	2.615×10^2	0
56	M	37	IIb	+	+	+	+	+	+	21720×10^2	889.0×10^2	192.8×10^2
58	F	42	IIb	–	–	–	+	–	–	430.5×10^2	37.59×10^2	3.738×10^2
59	F	35	IIb	–	–	–	+	+	+	434.0×10^2	10.38×10^2	3.620×10^2
60	M	41	IIb	+	+	–	+	+	+	153.3×10^2	29.31×10^2	7.110×10^2
62	M	52	IIa	+	+	+	–	–	–	5620×10^2	373.0×10^2	2.201×10^2
64	M	55	IIa	+	+	–	+	+	+	1142×10^2	878.0×10^2	0.256×10^2
61	F	62	IVb	+	+	+	–	–	–	6.960×10^2	3.982×10^2	0
66	M	60	IIa	+	+	–	+	+	+	10190×10^2	0	0
70	M	55	III	+	+	+	+	+	+	98.00×10^2	29.41×10^2	0.107×10^2
71	F	45	III	+	+	–	+	+	+	2851×10^2	2283×10^2	23.97×10^2
72	F	38	IIb	+	+	+	–	–	–	10510×10^2	712.0×10^2	3.460×10^2
73	F	48	I	+	+	+	+	+	+	12330×10^2	1310×10^2	920.0×10^2
76	M	45	III	+	+	+	–	–	–	421.0×10^2	0.268×10^2	0
77	M	55	IVb	+	+	+	+	+	+	1807×10^2	1031×10^2	210.8×10^2
79	F	55	IVa	+	+	–	–	–	–	11040×10^2	760.0×10^2	40.30×10^2

TMN: tumor–node–metastasis stage; (+) hypermethylation; (–) no hypermethylation detected.

EBER-ISH (EBV-Encoded Small RNA–In Situ Hybridization)

Five cases of matched T, P, and Z tissues were randomly selected. ISH was performed using the EBER-ISH kit (PanPath Co., Amsterdam, The Netherlands) according to the manufacturer's instruction. Briefly, after the sections were dewaxed with dimethyl benzene, dehydrated with alcohol, and predigested with pepsin, hybridization solution containing the fluorescein-conjugated EBV nucleic acid probe was applied. After application of alkaline phosphatase–conjugated antibody against fluorescein isothiocyanate, nitroblue tetrazolium chloride/bromochloroindolylphosphate (NBT/BCIP) was applied as the chromogen. Dark brown nuclear staining was interpreted as a positive hybridization signal.

Cell Lines and Culture Conditions

The NPC cell lines were cultured in RPMI 1640 supplemented with 10% FCS (Invitrogen, Carlsbad, CA).

DNA Preparation

Genomic DNA from matched T, P, and Z tissues as well as chronic nasopharyngitis tissues were prepared using an improved method of extracting high-molecular-weight DNA with phenol/chloroform as described in *Molecular Cloning* [17]. Briefly, tissues were ground in liquid nitrogen, dissolved in 500 μ l of Tris/NaCl/EDTA/SDS with proteinase K (100 μ g/ml), incubated at 55°C for 2 hours, heated at 65°C for 15 minutes to inactivate the enzyme, and then extracted by phenol/chloroform followed by precipitation with 70% of ethanol. Finally, pellets of DNA were dissolved in DNase-free water and the concentrations were determined before being stored at –80°C.

Bisulfite Treatment

DNA was subjected to bisulfite treatment [18] according to the protocol of Tao et al. [19], with a little modification. Briefly, 2 μ g of genomic DNA was denatured in 0.3 M NaOH for 10 minutes at 37°C. The denatured DNA were diluted in 300 μ l of freshly prepared solution of 10 mM hydroquinone and 4.8 M sodium bisulfite, and incubated for 4 hours at 55°C in darkness. After the incubation, the DNA samples were desalted through a column provided by genome DNA clean-up system (TaKaRa Bio Inc., Shiga, Japan). The eluted DNA was treated with 0.3 M NaOH for 8 minutes at room temperature, and precipitated with acetate ammonia and ethanol. The bisulfite-modified genomic DNA was resuspended in 20 μ l of TE buffer and stored at –20°C.

Methylation Analysis

Methylation-specific polymerase chain reaction (MSP) was performed according to the TaKaRa HS Taq kit protocol. Primers for MSP were described by Qiu et al. [11] for *RASSF1A*, and redesigned for *TSLC1* according to the reports by Kuramochi et al. [20], Fukuhara et al. [21], and Lung et al. [14]. For each reaction, 1 μ l of sodium bisulfite–treated DNA was added to 24 μ l of reaction buffer (1.25 mM dNTPs, 16.6 mM (NH₄)₂SO₄, 6.7 mM MgCl₂, and 1.5 U of

HS Taq polymerase; TaKaRa Bio Inc.) containing 100 ng of forward and reverse primers specific to the methylated or unmethylated DNA sequences. Conditions for amplification of DNA were 94°C for 4 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds (53°C for *TSLC1*), and 72°C for 30 seconds, with a final extension of 72°C for 5 minutes.

Sequencing of MSP Products of *RASSF1A* and *TSLC1*

Both U (reactions using primers specific for unmethylated CpG sites) and M (reactions using primers specific for methylated CpG sites) products of the *RASSF1A* and *TSLC1* MSP from one T tissue were excised from agarose gels and purified by the PCR product purification system (TaKaRa). Subsequently, sequencing reactions were performed separately on the purified U and M products by 377 ABI PRISM DNA sequencer (Shanghai BoYa Company, Shanghai, China).

RT-PCR

The expression of *RASSF1A* and *TSLC1* in NPC tissues and NPC cell lines as well as chronic nasopharyngitis tissues was examined by RT-PCR analysis. Total RNA was isolated by TRIZOL reagent. Two micrograms of total RNA was subjected to cDNA synthesis using a Reverse Transcription System according to the manufacturer's protocol (Promega, Madison, WI). PCR analysis of *RASSF1A* expression was carried out with primers according to Qiu et al. [11], and primer sequences of *TSLC1* are 5'-ATGAAATGCCTCAACACGCCGTAC-3' and 5'-TCTGCTGCGTCATCGGCTC CT-3'.

Real-Time PCR

EBV DNA copies in matched T, P, and Z tissues as well as chronic nasopharyngitis tissues were measured by real-time quantitative PCR analysis. The PCR primers and DNA probe with fluorescent dye (FAM) and TAMRA were designed according to Lo et al. [22] and synthesized by Dalian Baosheng Life Technologies, Inc. (Dalian, China), and used according to the manufacturer's protocol. In brief, PCR was set up in a total volume of 25 μ l, including 2.5 μ l of 10 \times buffer, 300 nM of each of the primers, 25 nM of the fluorescent probe, 200 nM dNTPs, 3 mM MgCl₂, 2.5 μ l of DNA template, and 1 U of Taq DNA polymerase (Promega). The reactions were performed and analyzed using the Double Fluorescent Quantitative PCR System (Light Cycler; Roche, Morristown, NJ). Parameters for PCR were as follows: 95°C for 5 minutes, followed by 40 cycles of 94°C for 15 seconds and 56°C for 1 minute.

Statistical Analysis

For comparison of hypermethylation level and EBV quantities in matched samples of T, P, and Z tissues, odds ratios were calculated and evaluated using chi-square analysis for hypermethylation data and analysis of variance (ANOVA) for EBV data. We used the logarithm of EBV data for statistical analysis in this study. *P* < .05 was considered as

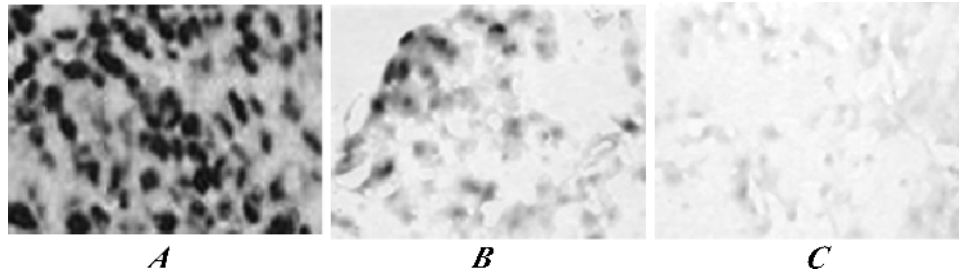


Figure 1. Nuclear staining for EBV by ISH in NPC and matched tumor-adjacent tissues (original magnification, $\times 400$). (A) Strong positive signals were detected in many carcinoma nuclei of T tissues. (B) Weak to strong positive signals were detected in the nuclei of some atypical hyperplastic cells of P tissues. (C) No positive signal was detected in any cell of Z tissues.

statistically significant. The statistical analyses were performed using the Excel software (Microsoft Corp., Redmond, WA) and statistical software SPSS Version 11.0 (SPSS Inc., Chicago, IL).

Results

Detection of EBV Infection in NPC and Matched Adjacent Tissues

EBER-ISH detected many EBV⁺ tumor cells in all five cases of T tissues with strong positive signals, but only scattered EBV⁺ cells in all five cases of P tissues. However, all five cases of Z tissues were EBER⁻ (Figure 1).

Methylation Status and Expression of RASSF1A and TSLC1 in NPC Cell Lines

The methylation status of *RASSF1A* and *TSLC1* in all currently available NPC cell lines ($n = 11$) was examined by MSP. Promoter hypermethylation of *RASSF1A* and *TSLC1* was detected in the majority of cell lines. RT-PCR results showed that the expression levels of both genes

were inversely correlated with their promoter methylation status (Figure 2).

Hypermethylation of RASSF1A in Matched T, P, and Z Tissues

Because the aberrant methylation of *RASSF1A* and *TSLC1* was frequently detected in NPC cell lines, we further investigated the methylation of these two tumor-suppressor genes in NPC tumors and the tumor-adjacent tissues. By MSP, the frequencies of promoter hypermethylation of *RASSF1A* in matched T, P, and Z tissues were 82% (23 of 28), 75% (21 of 28), and 46% (13 of 28), respectively. No promoter hypermethylation of *RASSF1A* was detected in chronic nasopharyngitis tissues (zero of eight). All P tissues with hypermethylation of *RASSF1A* also had methylation in their matched T tissues. All Z tissues with hypermethylated *RASSF1A* also had methylation in matched T and P tissues. The results are summarized in Tables 1 and 2, and the representative MSP results are shown in Figure 3.

A statistically significant difference in hypermethylation levels among matched T, P, and Z tissues was found, and there were a significant difference between P and Z tissues,

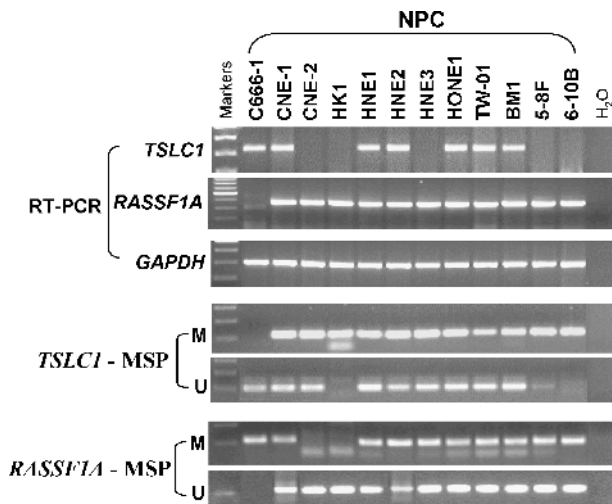


Figure 2. Methylation status and expression of *RASSF1A* and *TSLC1* in NPC cell lines. Promoter hypermethylation of *RASSF1A* and *TSLC1* was detected in the majority of cell lines with expression downregulated or silenced.

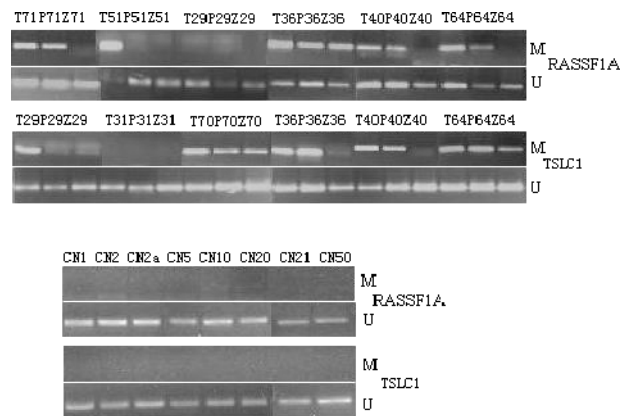


Figure 3. Detection of hypermethylation of *RASSF1A* and *TSLC1* in matched T, P, and Z tissues as well as chronic nasopharyngitis tissues. MSP analysis was performed using primers specific for both promoters. PCR products were visualized after electrophoresis on a 2% agarose gel (T: NPC tissues; P: tumor-adjacent tissues outside 0.5 cm; Z: tumor-adjacent tissues outside 1.0 cm; CN: chronic nasopharyngitis tissues).

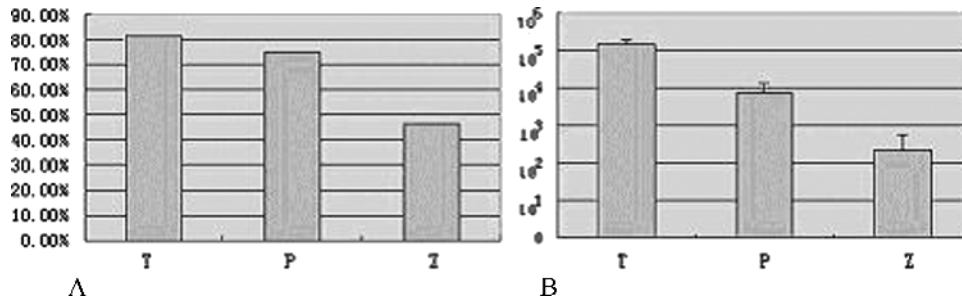


Figure 4. Histogram of frequencies of aberrant promoter hypermethylation of *RASSF1A* (A) and geometric mean of EBV quantities (B) in T, P, and Z tissues of NPC patients. They followed a similar trend from high to low in these samples.

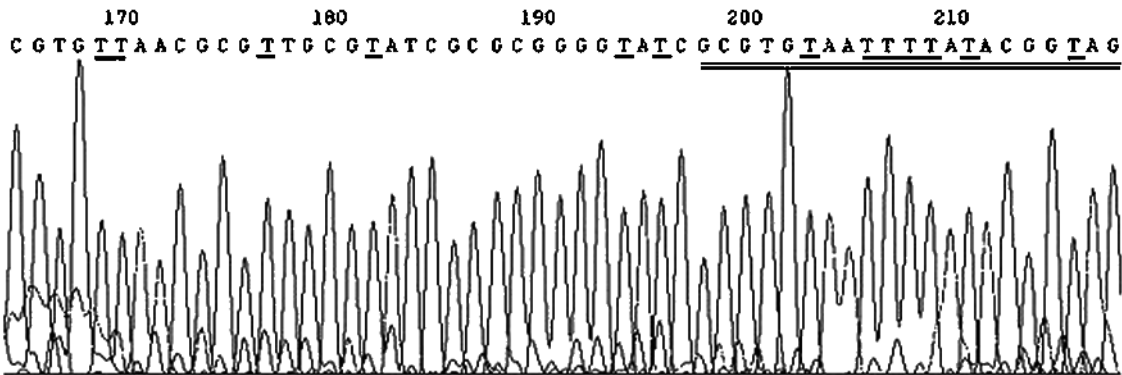
but no significant difference between T and P tissues (Figure 4A). Hypermethylation levels in T and P tissues were significantly higher than that in matched Z tissues ($P = .002$ and $.029$, respectively), and the hypermethylation level in Z tissues was significantly higher than that in chronic nasopharyngitis tissues ($P = .016$).

Hypermethylation of TSLC1 in Matched T, P, and Z Tissues

The frequencies of *TSLC1* promoter hypermethylation in matched T, P, and Z tissues were 68% (19 of 28), 61%

(17 of 28), and 50% (14 of 28), respectively. No promoter hypermethylation of *TSLC1* was detected in chronic nasopharyngitis tissues (zero of eight). All P tissues with hypermethylation of *TSLC1* also had methylation in their matched T tissues. All Z tissues with hypermethylation *TSLC1* also had methylation in the matched T and P tissues. Three cases exhibited aberrant promoter hypermethylation in T tissues and matched P tissues, but not in Z tissues. Two cases exhibited aberrant promoter hypermethylation in T tissues, but not in matched P and Z tissues. Fourteen cases exhibited

M-sequence of RASSF1A from patient 71 T tissues



U-sequence of RASSF1A from patient 71 T tissues

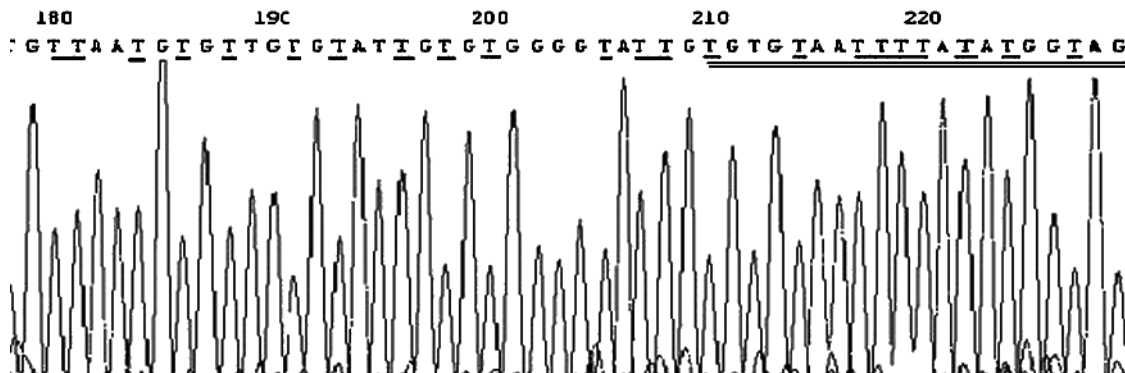


Figure 5. Sequencing analysis of M-MSP and U-MSP products for *RASSF1A* from NPC tissues (patient 71). For M-products, all cytosines were changed to thymines, except cytosines in CpG dinucleotide. The changes are underlined and M- and U-primers of MSP are double-underlined.

aberrant promoter hypermethylation in T, P, and Z tissues. The results were listed in Tables 1 and 2, and representative MSP results are shown in Figure 3.

No statistically significant difference in *TSLC1* hypermethylation among matched T, P, and Z tissues was found.

Sequencing Confirmation of Methylated and Unmethylated PCR Products

The M-MSP and U-MSP products amplified from one T tissue (patient 71) for either M or U primers of *RASSF1A* and *TSLC1* were sequenced. The sequencing result showed that all cytosine residues in the sequence were converted to thymines, except for cytosine residues in CpG dinucleotides, indicating the presence of methylated cytosines in these CpG dinucleotides; the sequencing result of U-PCR product showed that all cytosine nucleotides, including those within CpG dinucleotides, were converted to thymines, indicating complete conversion of all cytosines to uracils by sodium bisulfite and the presence of unmethylated CpG sites. The representative sequencing results of M- and U-sequences of *RASSF1A* were shown in Figure 5.

EBV DNA Quantities in Matched T, P, and Z Tissues as Well as Chronic Nasopharyngitis Tissues

Quantitative analysis of EBV DNA was performed on all matched T, P, and Z tissues. EBV DNA was detectable in 28 (100%) T tissues, and the average EBV DNA quantity was 6730×10^2 copies/ μ g DNA. EBV DNA was detectable in 27 (96%) matched P tissue samples of NPC patients, and the average EBV DNA quantity was 900×10^2 copies/ μ g DNA. EBV DNA was detectable in 21 (75%) matched Z tissues, and the average EBV DNA quantity was 70×10^2 copies/ μ g DNA. EBV DNA was also detectable in six (75%) chronic nasopharyngitis tissues, but the average EBV DNA quantity was only 6×10^2 copies/ μ g DNA (Tables 1 and 2).

A statistically significant difference of EBV DNA quantities among T, P, and Z tissues and chronic nasopharyngitis tissues was discovered (Figure 4B). The EBV DNA quantities in P tissues were significantly lower than those in matched T tissues ($P = .027$), whereas the EBV DNA quantities in Z tissues were significantly lower than those in matched P tissues ($P < .01$). The EBV DNA quantities in chronic nasopharyngitis tissues were lower than those matched tissues in NPC patients ($P < .01$).

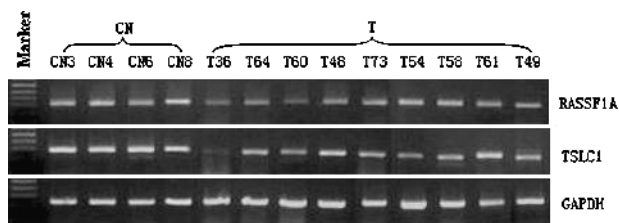


Figure 6. Expression of *RASSF1A* and *TSLC1* in chronic nasopharyngitis tissues and T tissues. Chronic nasopharyngitis tissues expressed a relatively higher level of both genes compared with T tissues (CN: chronic nasopharyngitis tissues; T: NPC tissues).

Expression of *RASSF1A* and *TSLC1* in T Tissues and Chronic Nasopharyngitis Tissues

Expression of *RASSF1A* and *TSLC1* in T tissues and chronic nasopharyngitis tissues was examined by RT-PCR. Figure 6 shows that chronic nasopharyngitis tissues expressed a relatively higher level of both *RASSF1A* and *TSLC1* mRNA when compared with T tissues.

Discussion

NPC is an endemic cancer with high incidence in Southeast Asia and Southern China. Both physical mapping and functional studies have assigned NPC-related tumor-suppressor gene(s) to 3p21.3. *RASSF1A* is a critical tumor-suppressor gene located at this locus, which has been shown to be frequently inactivated by promoter hypermethylation in Hong Kong and Singaporean NPC, both in NPC cell lines and primary tumors [9–11]. Although these studies demonstrated that promoter hypermethylation inactivated *RASSF1A* expression in NPC tissues and cell lines, they could not elucidate whether promoter hypermethylation is indeed involved in NPC tumorigenesis, or just a passenger phenomenon after the development of NPC.

Although our locality (Hunan Province, China) is not located in the highly prevalent area of NPC like Hong Kong, we still detected aberrant promoter methylation of *RASSF1A* in 82% of NPC tissues, similar to the previous reports of NPC tumors from Hong Kong and Singapore. We also detected aberrant methylation in 75% of P tissues and 46% of Z tissues of NPC patients. These results demonstrated the lowest level of *RASSF1A* methylation in Z tissues, a moderate level of methylation in P tissues, the highest methylation level in T tissues, and no aberrant methylation in chronic nasopharyngitis tissues. There are statistically significant differences in hypermethylation levels among T, P, and Z tissues. Our results suggest that: 1) aberrant promoter methylation of *RASSF1A* is a critical event in the development of NPC because no aberrant methylation was detected in chronic nasopharyngitis tissues; and 2) aberrant promoter methylation of *RASSF1A* is an early event during the pathogenesis of NPC because aberrant methylation of *RASSF1A* exists in both Z and P tissues.

In our studies, *RASSF1A* and *TSLC1* genes were both frequently inactivated by promoter hypermethylation in NPC tissues and NPC cell lines. The results confirmed that promoter methylation plays a direct role in silencing both genes in NPC and contributes to NPC pathogenesis.

EBV is a member of the herpesvirus family, widely associated with human tumors. Although the association of EBV with the development of NPC is well-established, the precise role of EBV in the carcinogenic process remains unknown. Humans appear to be the only reservoir for EBV. Infection with the virus can lead to three different outcomes: 1) replication of the virus in epithelial cells permissive for EBV proliferation; 2) latent infection of B-lymphocytes in the presence of competent T-lymphocytes; and 3) stimulation and immortalization of B-cells [15]. Previous studies have demonstrated that EBV appears in many healthy subjects

as well as patients with other cancers [23–25]. In our studies, the difference of EBV DNA levels among T, P, and Z tissues and chronic nasopharyngitis tissues was statistically significant. Further analyses showed that there were significant differences in EBV levels between either of the two abovementioned tissues. The EBER-ISH results also showed the different numbers of latent EBV-infected cell among T, P, and Z tissues. The results indicated that EBV copies were more abundant in regions nearer to the NPC tissues, suggesting either an infiltration of EBV tumor cells to adjacent area or a continuous malignant transformation of nearby epithelial cells. Our study also demonstrated that EBV is present in both NPC and healthy nasopharyngeal tissues, but the EBV load in healthy subjects was far less than that in NPC patients.

Aberrant promoter hypermethylation of *RASSF1A* and EBV quantities in T, P, and Z tissues followed a similar trend from high to low, suggesting a possible correlation between them. The promoter hypermethylation levels of *TSLC1* in matched T, P, and Z tissues are not statistically significant, although a larger-scale study is needed to confirm this observation. There was no evidence for possible correlation between *RASSF1A* and *TSLC1* methylation in this study.

In summary, our results indicate that promoter hypermethylation of both *RASSF1A* and *TSLC1*, and high EBV DNA load were commonly found in NPC tissues. The differences of aberrant promoter hypermethylation of *RASSF1A* and EBV levels between matched T, P, and Z tissues of NPC patients were statistically significant, with both levels being higher in tissues nearer to the NPC tissues. The results suggest that both promoter hypermethylation of *RASSF1A* and EBV infection might have occurred in the early phase of the development of NPC, and there may be a direct correlation between these two events.

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