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Promoter hypermethylation of tumor suppressor genes in serum as potential biomarker for the diagnosis of nasopharyngeal carcinoma

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ABSTRACT

Purpose: Promoter hypermethylation of tumor suppressor genes may serve as a promising biomarker for the diagnosis of cancer. Cell-free circulating DNA (cf-DNA) shares hypermethylation status with primary tumors. This study investigated promoter hypermethylation of five tumor suppressor genes as markers in the detection of nasopharyngeal carcinoma (NPC) in serum samples. *Methods*: cf-DNA was extracted from serum collected from 40 NPC patients and 41 age- and sex-matched healthy subjects. The promoter hypermethylation status of the five genes (*RASSF1*, *CDKN2A*, *DLEC1*, *DAPK1* and *UCHL1*) was assessed by methylation-specific PCR after sodium bisulfite conversion. Differences in the methylation status of these five genes between NPC patients and healthy subjects were compared. *Results*: The concentration of cf-DNA in the serum of NPC patients was significantly higher than that in normal controls. The five tumor suppressor genes – *RASSF1*, *CDKN2A*, *DLEC1*, *DAPK1* and *UCHL1* – were found to be methylated in 17.5%, 22.5%, 25.0%, 51.4% and 64.9% of patients, respectively. The combination of four-gene marker – *CDKN2A*, *DLEC1*, *DAPK1* and *UCHL1* – had the highest sensitivity and specificity in predicting NPC. *Conclusion*: Screening DNA hypermethylation of tumor suppressor genes in serum was a promising approach for the diagnosis of NPC.

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1. Introduction

Nasopharyngeal carcinoma (NPC) is a head and neck cancer that is rare among Caucasians, but prevalent in southern China and Southeast Asia [1]. Epidemiological studies reveal that genetic susceptibility, environmental factors and Epstein–Barr virus (EBV) infection play important roles in NPC carcinogenesis. In endemic regions, Type I non-keratinizing squamous carcinoma is predominant, whereas Type II keratinizing squamous carcinoma is more common in low-incidence areas [2,3]. It has been reported that EBV is generally absent in Type II, but prevalent in Type I NPC, suggesting the existence of a different pathogenesis in Type II NPC [4].

Based on the correlation between EBV and NPC in endemic regions, various EBV detection systems have been established. High antibody titer against EBV viral proteins like early antigen (EA) IgA, viral capsid antigen (VCA) IgA, nuclear antigen1 (EBNA1) IgA, and EBV transcription factor (EB1/Zta) IgG have been identified in NPC patients. However, none of these markers is sensitive and specific enough to predict NPC [5–7]. In one study, the sensitivity of VCA IgA, EA IgA, EBNA1 IgA, and EB1/Zta IgG were found to be 92.7%, 72.7%, 83.6%, and 74.5%, respectively. Though the sensitivity of VCA IgA is the highest, its specificity is only 60.1% [8]. Additionally, the low sensitivity and high false-positive rates of EBV DNA detection reported in some studies make it an unsatisfactory method for the diagnosis of NPC [6,8,9]. It has been suggested that the effectiveness of the combined detection of methylated DNA in plasma and serological EBV antibody is better than either one alone [10]. Detection of DNA hypermethylation hopefully compensates for the shortcomings of the EBV detection system or becomes an alternative choice for the diagnosis of NPC.

DNA hypermethylation, one of the most studied epigenetic events in mammals, has been described in a variety of human cancers and is considered the hallmark of many cancers [11–13]. DNA hypermethylation involves the binding of a methyl group to CpG dinucleotides in the promoter region of a gene by DNA methyltransferases (DNMTs). Aberrant promoter hypermethylation

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may result in expression silencing of tumor suppressor genes [14]. A number of tumor suppressor genes have been found to be frequently methylated in NPC. Among these genes are *RAS* association domain family member 1 (*RASSF1*) [15], cyclin-dependent kinase inhibitor 2A (*CDKN2A*) [16], deleted in lung and esophageal cancer 1 (*DLEC1*) [17], death-associated protein kinase1 (*DAPK1*) [18] and ubiquitin C-terminal hydrolase L1 (*UCHL1*) [19], all of which contribute to NPC pathogenesis by disrupting the normal regulation of apoptosis, DNA repair, cell proliferation, and signal transduction [19,20]. As DNA hypermethylation is one of the earliest molecular alterations during malignant transformation in human epithelial cells and is often present in the precursor lesions of cancers [21–23], analysis of promoter methylation of tumor suppressor genes may serve as a promising method for the detection of NPC.

Cell-free circulating DNA (cf-DNA) in serum and plasma is an emerging target for cancer screening in recent years [24]. The concentration of cf-DNA in the blood of cancer patients was much higher than that from healthy individuals [25]. The high level of cf-DNA in cancer patients is thought to be related to the necrosis or apoptosis of tumor cells during tumorigenesis [25]. Currently, tumor-specific genetic and epigenetic alterations can be detected in serum DNA from patients with various cancers including head and neck, lung, gastric and colorectal cancer [26–29]. Testing cf-DNA has the advantages of high sensitivity, low cost, noninvasiveness, and suitability for routine clinical application.

In this study, we evaluated promoter hypermethylation of five tumor suppressor genes (*RASSF1*, *CDKN2A*, *DLEC1*, *DAPK1* and *UCHL1*) as a means to detect NPC in serum samples. This would provide a useful reference in the detection of methylation for a relatively small number of genes in serum, which could be a powerful tool for the diagnosis of NPC in clinical practice.

2. Materials and methods

2.1. Blood collection and serum separation

Blood samples (3 mL) were collected from 40 newly diagnosed NPC patients before treatment in Cancer Hospital, Shantou University Medical College between July 2011 and January 2012. All patients were diagnosed with primary NPC and no other cancers by clinical and radiological evaluation. Blood samples (12 mL) were also obtained from 41 age- and sex-matched healthy individuals. All subjects were explained the procedure and signed a consent form before joining the study. Ethics approval from the relevant institutions was obtained.

Serum samples were separated from clotted blood by centrifugation at 1900 $\times$ g for 10 min at 4 °C. To remove additional cellular nucleic acids attached to cell debris, the aspirated serum samples were centrifuged at 16,000 $\times$ g for additional 10 min at 4 °C. The separated serum samples were stored at −80 °C.

2.2. DNA extraction and quantification

Cell-free circulating DNA was extracted from serum samples with QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was eluted in 50 μL elution buffer and stored at −20 °C. Extracted DNA samples were quantified by Quant-iT™ PicoGreen® dsDNA Reagent and Kits (Invitrogen, Carlsbad, CA) according to the manufacturer's directions.

2.3. Bisulfite-modification

Bisulfite treatment of DNA (up to 2 μg) was performed using EZ DNA Methylation-Direct Kit (Zymo Research, Orange, CA), following the manufacturer's protocol. The concentration of all bisulfite-modified DNA was adjusted to 1 ng/μL by re-suspending in elution buffer.

2.4. Methylation-specific PCR (MSP)

PCR amplifications for the five genes were carried out in a 15-μL reaction mixture consisting of 0.3 μM of each primer, 0.3 unit of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), 0.2 mM of each dNTP, $MgCl_2$ (2.5 mM for *RASSF1*, *DLEC1*, *DAPK1* and *UCHL1*; 1.5 mM for *CDKN2A*) and GeneAmp 1$\times$ PCR buffer II. The primer sequences and PCR conditions for MSP are shown in Table 1. One microliter of bisulfite-modified DNA was added to each reaction. PCR amplifications were carried out in a 96-well Plate 9700 thermal cycler (Applied Biosystems, Foster City, CA). The PCR conditions were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 30 sec, specific annealing temperature (60 °C for *RASSF1*, *CDKN2A*, *DAPK1* and *UCHL1*; 55 °C for *DLEC1*) for 30 s, and 72 °C for 30 s; and a final 5 min extension at 72 °C. Genomic DNA purified from peripheral blood of healthy individuals, treated or not with *SssI* methyltransferase (New England Biolabs, Beverly, MA), was used as positive control for methylated alleles and unmethylated alleles, respectively. Molecular grade water was used as negative control. The PCR products were visualized in 6% polyacrylamide gels stained with SYBR Green I under UV transillumination.

2.5. Real-time PCR analysis of EBV DNA concentration

The concentration of EBV DNA in the serum of NPC patients and normal controls was measured by real-time quantitative PCR by amplifying a DNA sequence in the *Bam*HI W fragment of EBV genome [30]. The principles and procedures for real-time quantitative PCR were described previously [30]. The *β-globin* gene was used to normalize the quantity of EBV DNA. Water blanks were included as controls in every analysis. Concentration of EBV

Table 1
Summary of primer sequences, annealing temperatures, cycles and PCR product sizes used for MSP.

Gene	Forward primers (5′-3′)	Reverse primer (5′-3′)	Position[b]	Annealing temp (°C)	Cycles	Product size (bp)
RASSF1-M [a]	TCGTATTCGGTTGGAGCG	ACCCCGCGAACTAAAAACG	18151–18260	60	40	110
RASSF1-U	GGGTTGTATTTGGTTGGAGTG	TAACAAACCCCACAAACTAAAAACA	18148–18266	60	40	119
CDKN2A-M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	65499–65648	60	40	150
CDKN2A-U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	65498–65648	60	40	151
DLEC1-M	GATTATAGCGATGACGGGATTC	ACCCGACTAATAACGAAATTAACG	23456–23648	55	40	193
DLEC1-U	TGATTATAGTGATGATGGGATTTGA	CCCAACTAATAACAAAATTAACACC	23457–23649	55	40	193
DAPK1-M	GATAGTCGGATCGAGTTAACGTC	CAAATCCCTCCCAAACGCCGA	47020–47121	60	40	102
DAPK1-U	GGAGGATAGTTGGATTGAGTTAATGTT	CACAAATCCCTCCCAAACACCAA	47016–47123	60	40	108
UCHL1-M	TTTATTTGGTCGCGATCGTTC	CAAACTACAACTATAAAACGCCG	28689–28793	60	40	105
UCHL1-U	GTTTGTATTTATTTGGTTGTGATTGTTT	CCAAACTACAACTATAAAACACCA	28682–28794	60	40	113

[a] M, primers for methylated DNA; U, primers for unmethylated DNA. The CpG sites for methylation detection are underlined.
[b] Positions of primers for *RASSF1*, *CDKN2A*, *DLEC1*, *DAPK1*, *UCHL1* are according to GenBank accession number AC002481.1, AL449423.14, AC144536.4. AL161787.13 and AC095043.3, respectively.

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