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SEPT9, *H4C6*, and *RASSF1A* methylation in nasopharyngeal swabs: A reflection of potential minimally invasive biomarkers for early screening of nasopharyngeal cancer

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Abstract

The potential value of epigenetic DNA methylation in early cancer screening has been demonstrated. Therefore, in this study, we performed QMS-PCR and quantitative reverse transcription PCR on the genes *RASSF1A, H4C6, SEPT9, GSTP1, PAX1, SHOX2*, and *SOX2*, which are common in epithelial cancers. We found hypermethylation in *RASSF1A, H4C6* and *SEPT9*. The mRNA expressions of *RASSF1A, H4C6* and *SEPT9* in tumor group were significantly different from those in the inflammatory group and healthy group ($P < .05$). Receiver operating characteristic (ROC) analysis showed that the area under the curve (AUC) of *RASSF1A, H4C6* and *SEPT9* genes were 0.831, 0.856 and 0.767, respectively. The areas under the AUC curve of *SEPT9 + H4C6, SEPT9 + RASSF1A* and *H4C6 + RASSF1A* are 0.946, 0.912 and 0.851, respectively. The diagnostic ability of dual gene combination is better than that of single gene combination, among which *SEPT9* and *H4C6* combination has the best diagnostic effect. In conclusion, our findings suggest that *H4C6, RASSF1A*, and *SEPT9* methylation occur more frequently in nasopharyngeal carcinoma, and their detection in nasopharyngeal swabs may be a minimally invasive tool for diagnosis of nasopharyngeal carcinoma.

Abbreviations: AUC = area under curve, EBV = Epstein-Barr virus, FFPE = formalin fixed paraffin embedded, GADPH = Glyceraldehyde 3-phosphate dehydrogenase, GSTP1 = Glutathione S Transferase pi 1, H4C6 = H4 clustered histone 6 Gene, IRB = Institutional Review Board, NPC = nasopharyngeal cancer, PAX1 = paired box gene 1, qMS-PCR = quantitative methylation-sensitive, RASSF1A = Ras association domain family 1 A, ROC = receiver operating characteristic, SEPT9 = A member of the septin family known as MSF1, SHOX2 = short stature homeobox 2, SOX2 = SRY-related HMG-box 2.

Keywords: biomarkers, DNA methylation, minimally invasive tool, nasopharyngeal cancer

1. Introduction

Nasopharyngeal carcinoma (NPC) is an aggressive and metastatic head and neck malignancy commonly found in Southern China, South-Central Asia, and the Middle East and North Africa. It affects an estimated 130,000 patients worldwide, with the highest prevalence in Southeast Asia.[1,2] Current studies have shown that there are 3 main factors contributing to NPC: Epstein–Barr virus (EBV) infection, genetic susceptibility, and environmental influences.[3] However, despite the fact that EBV antibody and EBV DNA copy number positivity can increase the diagnosis rate of NPC by 3-fold (21%–79%) and reduce the risk of death by 88%,[4,5] their use in the clinical diagnosis of NPC alone still results in underdiagnosis. The reliability of noninvasive screening using only EBV antibody and EBV DNA-positive results is not clinically satisfactory. Furthermore, although radiotherapy remains the most effective treatment modality for all stages of NPC, its 5-year survival rate is only 70%.[3,6] Therefore, the development of new and potentially effective screening mechanisms holds great value for the clinical management of NPC. Epigenetics, which refers to alterations in the expression of inherited genes unrelated to changes in DNA sequence,[7,8] is considered a hallmark of cancer as it promotes cancer development and progression.[9,10] DNA methylation, one of the key factors affecting the function of NPC, especially tumor suppressor genes, is a valuable diagnostic biomarker and potential therapeutic target. Approximately 1/4 of cellular changes associated with

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oncogene expression are linked to DNA methylation.[11] In recent decades, differential gene expression in NPC due to DNA methylation affecting gene transcription or binding of relevant promoters to corresponding transcription factors has been increasingly reported. In our study, we detected and analyzed the DNA methylation of *RASSF1A* (Ras association domain family 1 A), *H4C6* (H4 clustered histone 6 Gene), *SEPT9* (a member of the septin family known as MSF1), *GSTP1* (Glutathione S Transferase pi 1), *PAX1* (Paired box gene 1), *SHOX2* (Short Stature Homeobox 2), and *SOX2* (SRY-related HMG-box 2) in NPCs. Based on our investigation of common epidermal cell carcinoma-related genes associated with methylation and combining it with our existing research data, we determined the methylation status of the aforementioned genes in nasopharyngeal tissues and corresponding swabs from patients with NPC, chronic nasopharyngitis, and healthy controls. Our results suggest that *SEPT9*, *H4C6*, and *RASSF1A* methylation occur at a high frequency in NPC and their detection in nasopharyngeal swabs may be a minimally invasive tool for diagnosing NPC.

2. Materials and methods

2.1. Study design and sample collection

Study on the selection of highly relevant methylation genes in NPC and a prospective study to validate the clinical efficacy of gene methylation detection in a single hospital using noninvasive clinical methods. Approval for the study was obtained from the Institutional Review Board (IRB) of Ethics Committee at the First Affiliated Hospital of Guangdong Medical University (IRB No: [2023] IIT(4)). Written informed consent was obtained from all participants in the prospective study after they were provided with detailed information and their samples were collected. In the retrospective study, approval from the IRB was obtained, and patients' signed informed consent was not required.

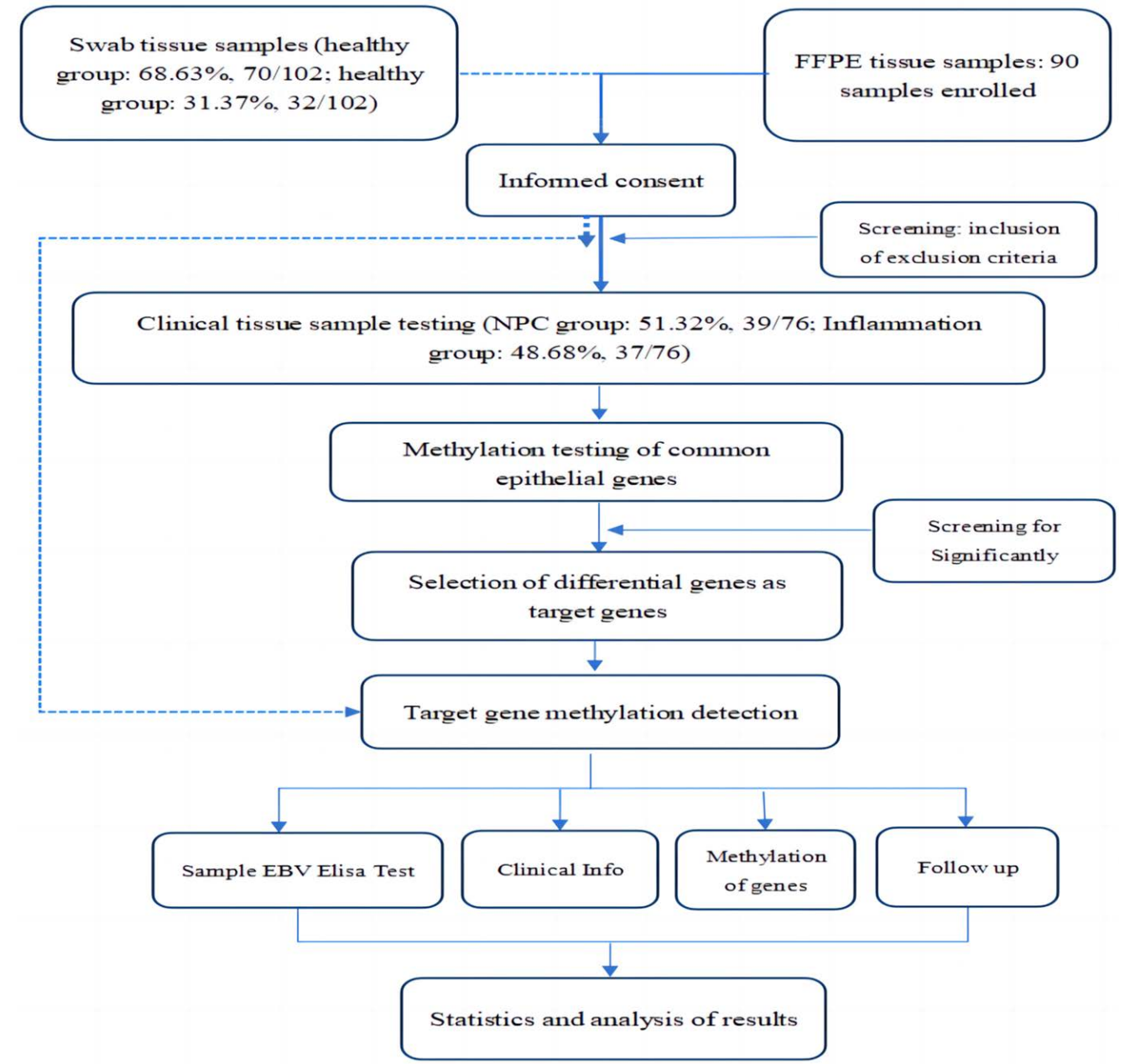

Figure 1. Research idea.

The overall research process is shown in Figure 1. We retrospectively collected formalin-fixed paraffin-embedding (FFPE) tissue samples of nasopharyngeal tissue confirmed by pathological biopsy in the tissue bank of Pathology Department, the First Affiliated Hospital of Guangdong Medical University from January 2019 to December 2021. Cut FFPE tissue into about 20 slices, each with a thickness of 5 μm, and put all slices into 1.5 mL EP tube stored at room temperature for methylation test. A total of 76 samples were obtained from patients who exhibited biopsy-proven NPC, and 39 samples were obtained from patients who were finally diagnosed with chronic nasopharyngitis (n = 37) or other non-NPC tumors (n = 2).

The nasal mucosa exfoliated cells from healthy individuals and suspected patients with nasopharyngeal carcinoma (NPC) were collected through nasal swabs in the Otorhinolaryngology Department after participants signed the written informed consent. The collection procedure was conducted under the guidance of endoscopy by experienced specialists. Endoscopy was used to evaluate the entire nasopharynx in order to identify sites of suspicious tumors. Before the biopsy, a nasopharyngeal brush (Copan Diagnostics, CA)[12] was inserted via the nasopharyngeal cavity and rotated 3 to 5 times over the nasopharyngeal epithelium at the site of the suspected focus. Immediately after sampling, the brush tip (1.5 cm) was cut and placed in 1 mL of PBS buffer (pH = 7.4, non-cation). The samples were then stored at -20°C before DNA extraction, which occurred within 3 days. Two brushing samples were collected for each participant. The endoscope was slowly inserted into the patient nasopharynx, and images and tissues were collected at the lesion site. Biopsy histology confirmed that all patients with suspected nasopharyngeal carcinoma were indeed positive for the disease.

2.2. DNA extraction and bisulfite conversion

FFPE tissue sample was dissolved in 1 mL of turpentine and vortexed thoroughly. Samples were then centrifuged at 16,000 × g for 3 minutes on a benchtop centrifuge and washed 3 times with 70% ethanol. Genomic DNA was extracted using Paraffin DNA Extraction Kit (Magen Biotechnology Co., Guangzhou, China) according to the manufacturer standard protocol. Genomic DNA of nasal mucosa exfoliated cells was extracted using Solarbio Animal Tissues/Cells Genomic DNA Extraction Kit (Solarbio Scie. & Tec. Co., Beijing, China). DNA concentration was determined using a BioSpec Nano spectrophotometer (Shimadzu Europa GmbH, Duisburg, Germany).

Briefly, 500 ηg of gDNA were subjected to bisulfite conversion using TIANGEN Sulfite Conversion Kit (Tiangen Bio-Tech. Co., Beijin, China) according to the manufacturer recommendations.

2.3. Quantitative methylation specific polymerase chain reaction (QMS-PCR)

TaqMan-based QMS-PCR was performed after bisulfite treatment on denatured genomic DNA.[13] The primers and probes for QMS-PCR genes were named *GSTP1m, H4C6m, PAX1m, RASSF1Am, Sept9m, SHOX2m*, and *SOX2m* (The relevant sequences for QMS-PCR are shown in Table 1).

QMS-PCR assay was performed in a certified DNA laboratory, and the operators and staff were blinded to the clinical information, including basic and clinical information on participants and pathologic results. The QMS-PCR reaction consisted of cDNA template, forward/reverse primers, TB Green Premix Ex Taq (Tli RNaseH Plus), and ROX Reference Dye II, in a total volume of 20 μL. Subsequently, the cycling conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 20 seconds, and 60°C for 1 minute. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as the internal control. All assays were run on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems), and the experiments were performed in triplicate.

The GADPH reference gene is not only the indicator for the DNA quantity of the sample but also the baseline for the methylation level determination of the 7 target genes. Two crossing point (Cp) values are from target gene and GADPH. If the Cp value of GADPH (Cp GADPH) is >35 or could not be detected, it was considered an invalid result. The methylation level of the target gene was determined using the difference between the 2 Cp values (ΔCp = Cp target-Cp GADPH). The lower ΔCp values are associated with higher methylation levels of Targetm and vice versa.[14–16]

2.4. Quantitative reverse transcription PCR

Nasopharyngeal tissues were stored in RNAStore (Beijing Cowin Bioscience Co., Ltd.). Total RNA was extracted with RNAiso Plus (Takara Biomedical Technology Co.,Ltd,Beijing). Then consequently, 1 μg of RNA was pretreated with DNase I and reverse transcribed into the first strand. The target DNA was reverse transcribed into first-strand cDNA using Oligo (dT)20VN primer.(dT)20VN primer and HiScript II One Step RT-PCR Kit (Vazyme Biotech Co., Ltd., Nanjing) were used to amplify the target gene (*SEPT9, H4C6, RASSF1A*). The PCR reaction consisted of a cDNA template, forward/reverse primers, TB Green Prem primers, TB Green Premix Ex Taq (Tli RNaseH Plus) and ROX Reference Dye II in a total volume of 20 μL. Cycling conditions were cycling at 95°C for 5 minutes followed by 40 cycles of 95°C for 20 seconds and 60°C for 1 minute. The actin gene (β-actin, ACTB) was used as an internal control (Table 2). mRNA relative expression levels

Table 1

Primers and probes used for qMS-PCR.

Primer/probe	Sequence (5′-3′)
SEPT9	
SEPT9-F	GTTATTCGAGTTGTAAAGGGC
SEPT9-R	CCTCACCTATAAATAAACGCG
SEPT9-Probe	FAM-TTTCGTTGTATCGATATATCGAGTTGCGTACGGTGT-BHQ1
H4C6	
H4C6-F	GTTTAGGAAAGGGAGGC
H4C6-R	TCTCCTCATAAATAAAACCCG
H4C6- Probe	FAM-ACGAAGTTCGTTATTCGTCGTTTG-BHQ1
RASSF1A	
RASSF1A-F	GGGGTAGTTAAGGGGTAGC
RASSF1A-R	CTCGAAAACTATCCCCG
RASSF1A-Probe	FAM-CGCGGGTTAAGTCGCGGTAGA-BHQ1
SOX2	
SOX2-F	GTTGTATATGAAGGAGTATTCG
SOX2-R	ATACGTTGTTCGGCGGGT
SOX2- Probe	FAM-TTCGGATTATAAATATCGGTTTCGGCGGA-BHQ1
SHOX2	
SHOX2-F	TCGAGTTTGCGTTTTTACG
SHOX2-R	ACCCAAAAAACTCGCCG
SHOX2- Probe	FAM-TTTTCGTTCGTTATCGGAGGTTG--BHQ1
GSTP1	
GSTP1-3-F	GTTTAGGGGATTTAGGACGTT
GSTP1-3-R	CGAAATCCCATCCCTCC
GSTP1-3- Probe	FAM-AGTGTCGTTAGCGGTTTTTAGGGGGT-BHQ1
PAX1	
PAX1-3-F	AAGTCGCGAAAGGGTACGG
PAX1-3-R	TAAACAAAAACGCAAAAAAACC
PAX1-3- Probe	FAM-GTCGCGTTTTATTTCGCGGTT--BHQ1
GADPH-F	CAAGCTCATTTCCTGGTATGACA
GADPH-R	GGGAGATTCAGTGTGGTGGG
GADPH-Probe	VIC-CCACCTTACCCTAAACACTACAAC-BHQ1

qMS-PCR = quantitative methylation-sensitive PCR; *SEPT9* = A member of the septin family known as MSF1; *H4C6* = H4 clustered histone 6 Gene;*RASSF1A* = Ras association domain family 1 A;*SOX2* = SRY-related HMG-box 2;*SHOX2* = Short Stature Homeobox 2;*GSTP1* = Glutathione S Transferase pi 1;*PAX1* = Paired box gene 1;*GADPH* = Glyceraldehyde 3-phosphate dehydrogenase

were calculated using the 2-ΔCt × 100 method. All assays were performed on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems) and the experiments were performed in 3 replicates.

2.5. EBV antibodies ELISA assay

3 mL venous blood samples were collected from all patients and centrifuged at 3000 r/min for 10 minutes to separate plasma and serum samples. Serum EBV VCA IgA antibody was detected using the EBV VCA IgA Antibody ELISA Test Kit (Euroimmun Med. Lab. AG, Luebeck, Germany) (The FFPE sample has been examined by the hospital, the other tissue samples were examined by the current investigator, and all valid results were counted).

2.6. Data analysis and statistics

The participants were characterized using descriptive statistics. The positive result of the Target m assay was determined when ΔCp ≤ X and the negative result was ΔCp > X. X was determined by the training set of this study. The ΔCp values of Target were compared using the one-way ANOVA test. Differences in proportions between independent groups were compared using Fisher exact test. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for detecting cancer were calculated. The confidence intervals for sensitivity and specificity were Clopper-Pearson exact intervals, and the confidence intervals for PPV and NPV were the standard logit confidence intervals. Differences in sensitivity and specificity were tested with McNemar χ^2 test, and differences in PPV and NPV with the method described by Leisenring et al Receiver operating characteristic (ROC) curves were used to evaluate the AUCs of Target m and EBV testing, differentiating between normal and cancer. SPSS 26.0 (IBM Corp., Armonk, NY), GraphPad 8.0 and R (version 4.1.2, Vienna, Austria) were used for all statistical analyses. All differences were considered 2-sided, and statistical significance was set at $P < .05$.

Table 2

Primer sets designed for qRT-PCR.

Primer set	Sequence (5′-3′)	Amplicon length (bp)
SEPT9		
SEPT9-F	AGTAGTAGGATAGGTGTTTTGTTTC	147
SEPT9-R	GAACGCTCCGAACGACCCC	
H4C6-F	GGATTATTTGAAGTTAGGAGTTC	176
H4C6		
H4C6-R	TCTCATACCTCAACCTCACG	
RASSF1A		
RASSF1A-F	GGGGTAGTTAAGGGGTAGC	169
RASSF1A-R	CTCGAAAACTATCCCCG	
ACTB-F	CAAGCTCATTTCCTGGTATGACA	189
ACTB-R	GGGAGATTCAGTGTGGTGGG	

qRT-PCR = quantitative reverse transcription PCR; *SEPT9* = A member of the septin family known as MSF1; *H4C6* = H4 clustered histone 6 Gene; *RASSF1A* = Ras association domain family 1 A

Table 3

Sample included in the study.

	FFPE/ Case	Swab/Case
NPC	39	29
Nasopharyngitis	37	0
Health	0	64
Percentage(%)	44.97%	55.03%

NPC = Nasopharyngeal cancer; FFPE = Formalin Fixed Paraffin Embedded; Swab = nasal swab.

3. Results

3.1. Sample of research

At the beginning of this study, a total of 196 samples were collected, consisting of 84 FFPE samples (42.86%) and 102 nasal swab samples (57.14%). After combining the clinical information with the screening of the enrollment, 169 samples were deemed eligible for the study. The subjects and their related information can be found in Tables 3 and 4.

3.2. Expression of high-frequency methylated genes with corresponding mRNAs

We used QMS-PCR to detect hypermethylation in the NPC of *SEPT9, RASSF1A*, and *H4C6* genes, as well as hypomethylation or no methylation of *GSTP1, SHOX2, SOX2*, and *PAX1* genes in 37 nasopharyngitis FFPE samples (Table 5). The comparison with nasopharyngitis and healthy groups showed that NPC subjects exhibited higher methylation levels of *SEPT9* (65.63%/ 20.00%), *RASSF1A (*68.75%/ 4.29%), and *H4C6* (46.88%/ 0%) in swab samples from 93 cases (NPC 29, nasopharyngitis 64). Additionally, using RT-PCR, we investigated the mRNA levels of these 3 genes in NPC, nasopharyngitis, and healthy subjects. The differences in methylation score among groups were computed using Kruskal-Wallis test or Mann–Whitney *U* test, which revealed significant differences in the methylation levels of *SEPT9, RASSF1A*, and *H4C6* in NPC compared to the nasopharyngitis and healthy groups. Furthermore, the

Table 4

Clinical information on subjects.

	NPC (n = 68)	Nasopharyngitis(n = 37)	Health (n = 64)
Age			
>60	55(80.88%)	7(18.92%)	0(0%)
≤60	13(19.12%)	30(81.08%)	64(100%)
Genders			
male	46(67.65%)	22(59.46%)	28(43.75)
Female	22(32.35%)	15(40.54%)	36(56.25%)
Cigarette history			
Yes	18(26.47%)	26(70.27%	4(6.25%)
No	50(73.53%)	11(29.73%)	60(93.75%)
Alcoholic history			
Yes	3(4.41%)	15(40.54%)	3(4.69%)
No	65(95.59%)	22(59.46%)	61(95.31%)
Family history			
Yes	4(5.88%)	1(2.70%)	2(1.56%)
No	64(94.12%)	36(97.30%)	62(98.44%)
TNM			
I/II	10(14.71%)	—	—
III/IV	58(85.29%)	—	—
EBV infection			
Positive(+)	45(66.18%)	10(27.03%)	—
Negative(-)	23(33.82%)	27(72.97%)	—
Tumor size			
<6 cm	30(44.12%)	—	—
≥6 cm	38(55.88%)	—	—
Tumor stage			
T1/ T2	18(26.47%)	—	—
T3/ T4	50(73.53%)	—	—
LNM			
N0	9(13.24%)	—	—
N1 - N3	59(86.76%)	—	—

"—"=Not counted or non-existent; NPC = Nasopharyngeal cancer; EBV = Epstein-Barr Virus; TNM = Tumor Node Metastasis; LNM = lymph node metastasis.

Table 5

Gene methylation in FFPE sample.

	SEPT9	*H4C6*	*RASSF1A*	*SHOX2*	*GSTP1*	*SOX2*	*PAX1*
NPC(n = 39)	66.67%	59.26%	62.96%	24.00%	0	0	22.00%
Nasopharyngitis(n = 37)	30.77%	15.38%	7.69%	13.89%	0	0	8.33%

FFPE = Formalin Fixed Paraffin Embedded; NPC = Nasopharyngeal cancer; *SEPT9* = A member of the septin family known as MSF1; *H4C6* = H4 clustered histone 6 Gene; *RASSF1A* = Ras association domain family 1 A; *SHOX2* = Short Stature Homeobox 2; *GSTP1* = Glutathione S Transferase pi 1; *SOX2* = SRY-related HMG-box 2; *PAX1* = Paired box gene 1.

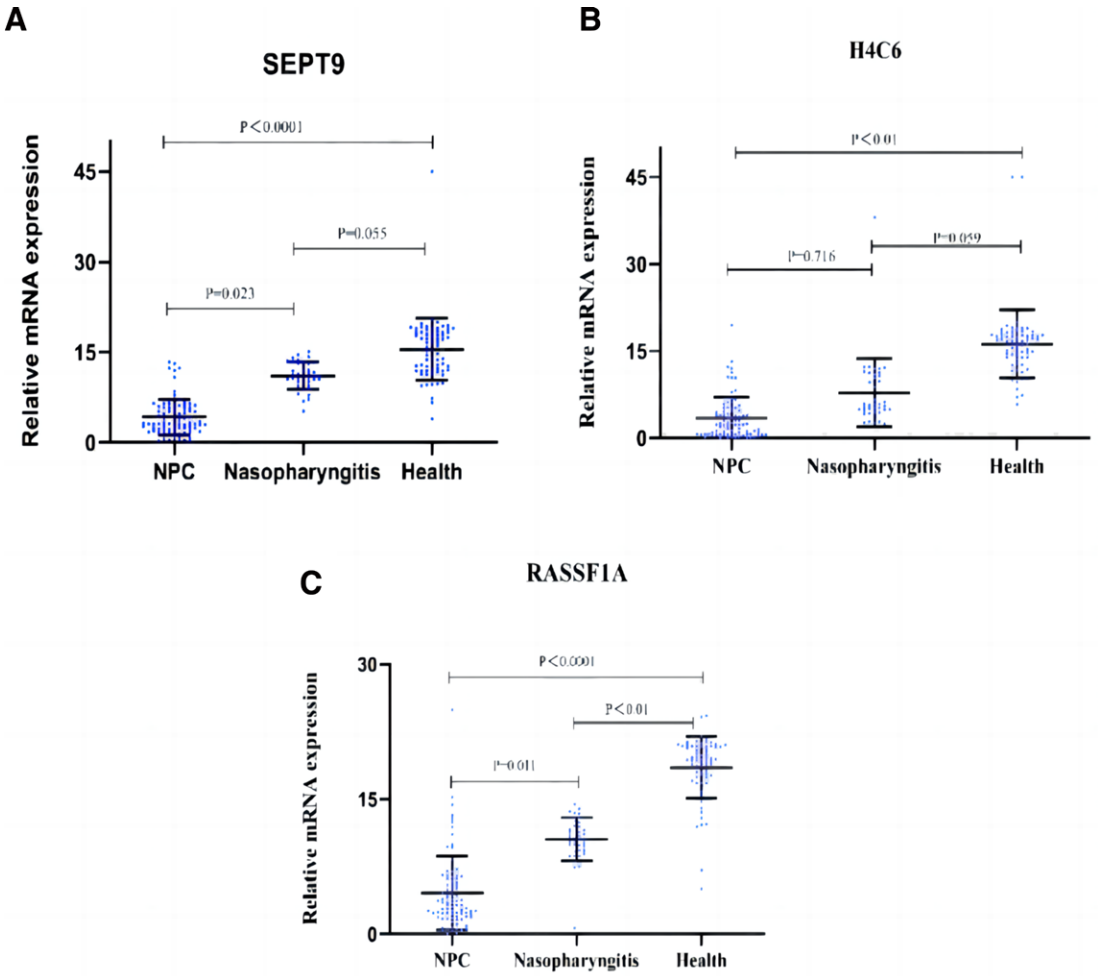

Figure 2. Comparison of mRNA expression of 3 genes in NPC, nasopharyngitis and healthy.

expression of 3-gene mRNAs in NPC was significantly lower than that in the nasopharyngitis and healthy subjects (Fig. 2). For NPC, nasopharyngitis, and healthy subjects, the Pearson r values were –0:478 ($P > .05$), –0:753 ($P > .05$), and –0.931 ($P > .05$).

3.3. Clinical correlates of SEPT9, RASSF1A, and H4C6 methylation

By combining the results of methylation testing of NPC samples with their corresponding demographic data and pathological examinations, we conducted an analysis to determine the correlation between methylation positivity of *SEPT9*, *H4C6*, and *RASSF1A* with clinical and pathological characteristics. Our statistical findings indicate that *SEPT9* and *RASSF1A* were hypermethylated in patients aged 60 years and above (84.62%, 84.62%). Furthermore, *H4C6* showed hypermethylation in 4 specific factors: a history of smoking (77.78%), being female (77.27%), having a small focal area (76.66%), and having no metastasis in the lymph nodes (77.78%). Moreover, upon conducting a statistical analysis of the positive methylation results and clinical and pathological characteristics in all NPC samples, we discovered that the difference in *SEPT9* hypermethylation between stage I/II and III/IV patients was relatively greater in the TNM staging of NPC ($P < .05$). Additionally, the methylation of *RASSF1A* and *H4C6* was found to be higher in the smoking population ($P < .05$, $P < .05$). It is important to note that *SEPT9* and *H4C6* were hypermethylated in EBV-positive patients compared to negative patients ($P < .05$; $P < .001$) (Table 6).

3.4. Diagnostic capacity of SEPT9, RASSF1A, and H4C6 methylation in NPC vs non-NPC controls

To evaluate the clinical performance of methylation in diagnosing NPC, we constructed receiver operating characteristic curves (ROC) and calculated the area under the curve (AUC). The AUC area results for FFPE showed that *SEPT9*, *RASSF1A*, and *H4C6* methylation had a better ability to assess NPC (Table 7, Fig. 3A),

Table 6

Correlation of *SEPT9, H4C6*, and *RASSF1A* methylation with clinical and pathological characteristics of NPC.

Characteristics	All (N = 68)	*SEPT9*			*H4C6*			*RASSF1A*		
		n	Percent(%)	*P* Value	n	Percent(%)	*P* Value	n	Percent(%)	*P* value
Age										
>60	55	34	61.82	.250	35	63.64	.266	30	54.55	.224
≤60	13	11	84.62		9	69.23		11	84.62	
Genders										
male	46	30	65.22	.493	26	56.52	.588	26	58.70	.418
Female	22	14	63.64		17	77.27		13	59.01	
Cigarette history										
Yes	18	11	61.11	.112	14	77.78	.033	11	61.11	.018
No	50	33	66.00		29	58.00		26	52.00	
Alcoholic history										
Yes	3	1	33.33	.663	2	66.67	.584	2	66.67	.418
No	65	42	64.62		40	61.54		37	56.92	
Family history										
Yes	4	1	25	.099	2	50	.410	2	50	.606
No	64	44	68.75		41	64.06		35	54.69	
TNM										
I/II	10	5	50	.021	5	50	.475	4	40	.495
III/IV	58	38	65.52		37	63.79		34	58.62	
EBV infection										
Positive(+)	45	30	66.67	.031	28	62.22	<.001	24	53.33	.206
Negative(-)	23	16	69.57		15	65.22		15	65.22	
Tumor size										
<6 cm	30	20	66.66	.786	23	76.66	.928	17	56.67	.776
≥6 cm	38	25	65.79		21	55.26		22	57.89	
Tumor stage										
T1/ T2	18	11	61.11	.883	11	61.11	.663	8	44.44	.592
T3/ T4	50	34	68.00		31	62.00		29	58.00	
LNM										
N0	9	5	55.56	.727	7	77.78	.959	4	44.44	.856
N1 - N3	59	40	67.80		35	59.32		33	55.93	

EBV = Epstein-Barr Virus; *H4C6* = H4 clustered histone 6 Gene; LNM = lymph node metastasis; NPC = Nasopharyngeal cancer; *RASSF1A* = Ras association domain family 1 A; *SEPT9* = A member of the septin family known as MSF1; TNM = Tumor Node Metastasis.

Table 7

Results of ROC analysis for FFPE (NPC vs Nasopharyngitis).

Gene	Case/control	AUC(95%CI)	*P*
RASSF1A	21/34	0.887 (0.793–0.982)	<.001
H4C6	22/30	0.886 (0.789–0.984)	<.001
SEPT9	17/30	0.892 (0.789–0.996)	<.001
GSTP1	20/28	0.628 (0.469–0.787)	.115
SHOX2	9/19	0.667 (0.424–0.910)	.179
SOX2	9/18	0.583 (0.347–0.820)	.498
PAX1	20/29	0.507 (0.341–0.673)	.935

AUC = Area Under Curve; FFPE = Formalin Fixed Paraffin Embedded; *GSTP1* = Glutathione S Transferase pi 1; *H4C6* = H4 clustered histone 6 Gene; NPC = Nasopharyngeal cancer; *PAX1* = Paired box gene 1; *RASSF1A* = Ras association domain family 1 A; ROC = Receiver Operating Characteristic; *SEPT9* = A member of the septin family known as MSF1; *SHOX2* = Short Stature Homeobox 2; *SOX2* = SRY-related HMG-box 2.

consistent with the results in the swab samples (Table 8, Fig. 3B). The subjects were divided into NPC and non-NPC disorders, and the AUCs of *SEPT9*, *RASSF1A*, and *H4C6* methylation were 0.767, 0.831, and 0.856, respectively (Table 9, Fig. 3C). These results indicate that *SEPT9, RASSF1A,* and *H4C6* methylation provide a potential method to distinguish NPCs from non-NPCs, demonstrating a good diagnostic ability.

3.5. Diagnostic capacity of dual gene combined methylation in NPC vs non-NPC

After analyzing single-gene methylation to assess the diagnostic ability of nasopharyngeal carcinoma, we combined the methylation results of *SEPT9*, *H4C6*, and *RASSF1A* to assess the ability of dual-gene combination to diagnose nasopharyngeal carcinoma. We first assigned a positive value to the combination of positive results for any of the 2 genes, and a negative value to the combination of negative methylation results for both genes. Based on the above data, we constructed subject operating curves to evaluate the ability of dual-gene diagnosis of nasopharyngeal cancer and non-nasopharyngeal cancer. The results showed that the areas under the AUC curves of *SEPT9* + *H4C6*, *SEPT9* + *RASSF1A*, and *H4C6* + *RASSF1A* were 0.946, 0.912, and 0.851, respectively. In all cases, the diagnostic ability of the dual-gene combination was superior to that of the single gene, with the combination of *SEPT9* and *H4C6* being the most effective (as shown in Table 10 and Fig. 4). These findings suggest that combined dual-gene methylation can provide a better, valuable, and potential method for nasopharyngeal cancer screening.

4. Discussion

Although each type of cell has a unique DNA methylation patterning, aberrant epigenetic alterations have been recognized as a common hallmark of human cancers. Some studies have demonstrated that *SEPT9, RASSF1A* is highly methylated in a wide range of tumors, such as cervical cancer, colorectal cancer, etc. Our clinical study further confirms the normality of this methylation occurring in NPC on the basis that the samples were expanded. While clinically validating *SEPT9* and *RASSF1A* gene methylation for the diagnosis of nasopharyngeal carcinoma, our study identifies and analyzes for the first time the association between *H4C6* gene methylation and NPC

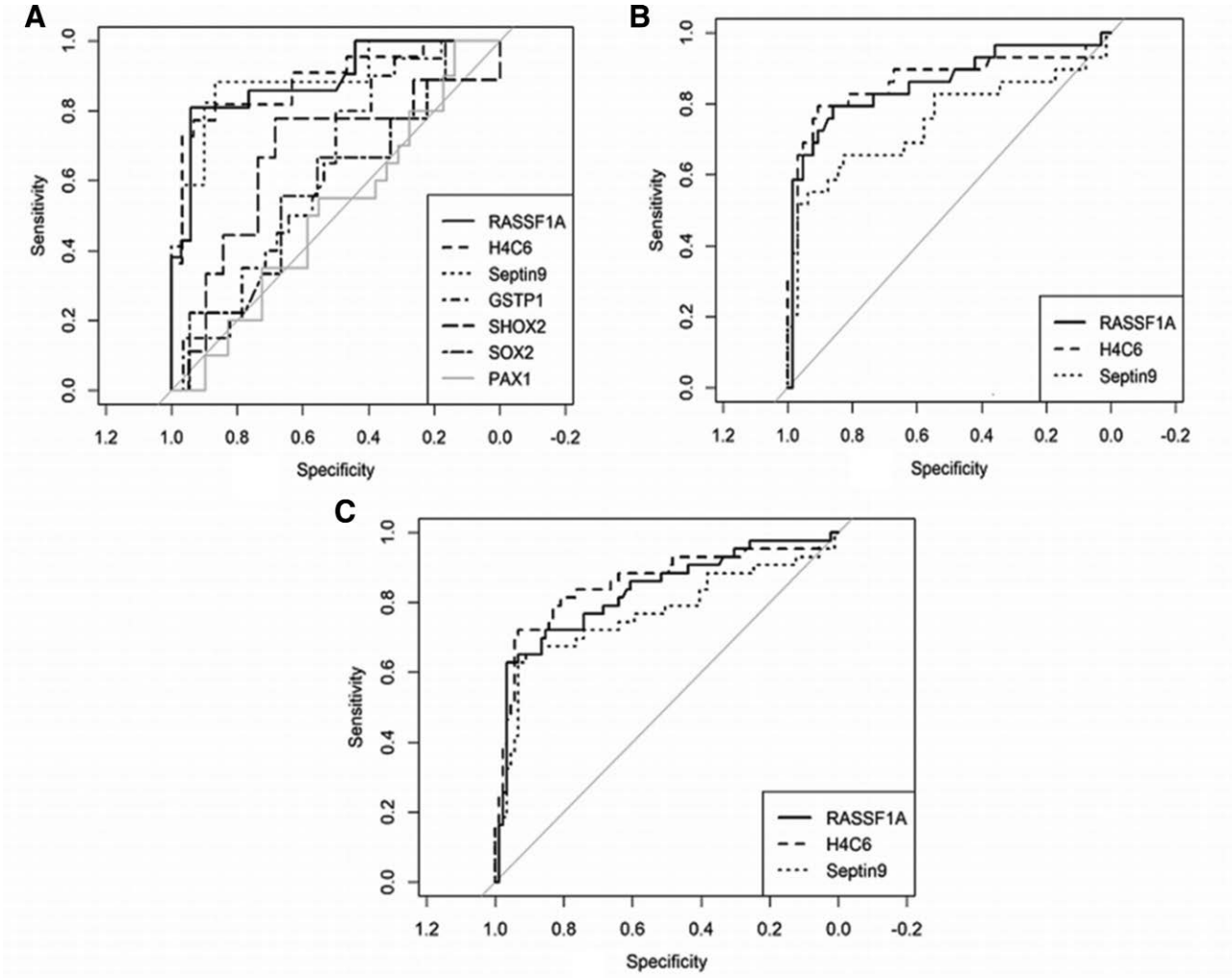

Figure 3. ROC curves for methylation diagnostic reliability tests; (A) *SEPT9*, *RASSF1A*, *H4C6*, *PAX1*, *GSTP1*, *SHOX2*, and *SOX2* methylation in NPC and nasopharyngitis for diagnosing NPC; (B) *SEPT9*, *RASSF1A*, and *H4C6* methylation in NPC and Health to diagnose NPC; (C) *SEPT9*, *RASSF1A*, and *H4C6* methylation in NPC and non-NPC to diagnose NPC. ROC = receiver operating characteristic, SEPT9 = A member of the septin family known as MSF1, SHOX2 = short stature homeobox 2, SOX2 = SRY-related HMG-box 2.

Table 8

Results of ROC analysis for Swab (NPC vs Health).

Gene	AUC(95%CI)	Cutoff	Sensitivity %	Specificity %	PPV %	NPV %	*P* value
RASSF1A	0.857	0.075	79.3	85.9	71.9	90.2	
	(0.763–0.953)		(60.3–92.0)	(75.0–93.4)	(53.2–86.3)	(79.8–96.3)	.002
H4C6	0.865	3.480	79.3	90.6	79.3	90.6	
	(0.766–0.964)		(60.3–92.0)	(80.7–96.5)	(60.3–92.0)	(80.7–96.5)	<.001
SEPT9	0.748	3.130	65.5	82.8	63.3	84.1	
	(0.622–0.875)		(47.3–80.1)	(71.3–91.1)	(43.8–80.1)	(72.7–92.1)	.003

AUC = area under curve; *H4C6* = H4 clustered histone 6 gene; NPC = nasopharyngeal cancer; NPV = negative predictive value; PPV = PositivePredictive Value; *RASSF1A* = Ras association domain family 1 A; ROC = receiver operating characteristic; *SEPT9* = a member of the septin family known as MSF1; Swab = nasal swab.

with the aim of exploring the potential of gene methylation as a new biomarker for early detection of NPC. Here, targeting new genes we found that *H4C6* was frequently methylated in 68.75% of NPC tissues, which is a higher frequency of methylation compared to other genes reported to be hypermethylated in NPC[17]. *H4C6* methylation levels were also significantly higher in the nasopharyngeal cancer group than in the non-nasopharyngeal cancer control group ($P < .001$). The mRNA levels of *H4C6* were reduced in all nasopharyngeal cancer subjects compared to the healthy population ($P < .01$), suggesting a strong association between DNA methylation and *H4C6* transcriptional silencing in NPC

In addition, we found that the methylation status of *SEPT9*, *H4C6*, and *RASSF1A* in nasal swabs from recruited subjects was significantly correlated with that in paired NPC tissues, and as a Squamous cell carcinoma, nasal swabs can reach NPC cells. In addition, cancer cells can be readily be released from the tumor mass and acquired when cell-cell adhesion, which often occurs in malignant tumors, is destroyed. Therefore, analysis of *SEPT9, H4C6*, and *RASSF1A* methylation in nasal swabs may

Table 9

Results of ROC analysis in NPC vs non-NPC.

Gene	AUC (95%CI)	Cutoff	Sensitivity %	Specificity %	PPV %	NPV %
RASSF1A	0.831 (0.750–0.912)	2.096	72.1 (56.3–84.7)	85.4 (76.3–92.0)	70.5 (54.8–83.2)	86.4 (77.4–92.8)
H4C6	0.856 (0.778–0.935)	3.480	81.4 (66.6–91.6)	80.9 (71.2–88.5)	67.3 (52.9–79.7)	90.0 (81.2–95.6)
SEPT9	0.767 (0.668–0.867)	10.987	67.4 (51.4–80.9)	86.5 (77.6–92.8)	70.7 (54.5–83.9)	84.6 (75.7–90.7)

AUC = area under curve; *H4C6* = H4 clustered histone 6 Gene; NPC = nasopharyngeal cancer; NPV = Negative Predictive Value; PPV = PositivePredictive Value; *RASSF1A* = Ras association domain family 1 A; ROC = receiver operating characteristic; *SEPT9* = A member of the septin family known as MSF1.

Table 10

Results of ROC analysis in NPC vs non-NPC.

Dual Gene	AUC	95% CI	Specificity	Sensitivity	*P*
H4C6 + RASSF1A	0.851	0.761–0.941	96.00%	63.89%	<.001
SEPT9 + RASSF1A	0.912	0.843–0.981	87.50%	93.48%	<.001
SEPT9 + H4C6	0.946	0.877–1.000	100%	86.20%	<.001

AUC = area under curve, H4C6 = H4 clustered histone 6 Gene, NPC = nasopharyngeal cancer, RASSF1A = Ras association domain family 1 A, ROC = receiver operating characteristic, SEPT9 = A member of the septin family known as MSF1

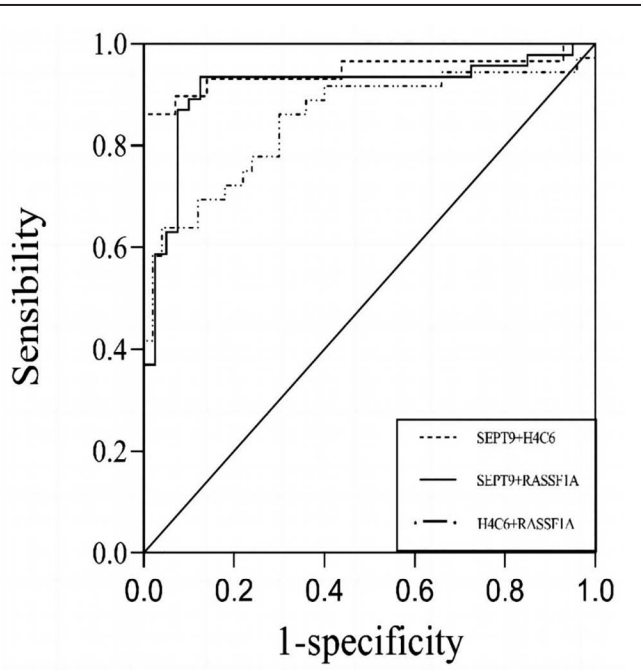

Figure 4. ROC curves for a dual-gene combined methylation diagnostic reliability test. ROC = receiver operating characteristic.

provide a minimally invasive method to assist in the diagnosis of NPC or to monitor recurrence after treatment. In particular, nasal swabs have the potential to become a population screening tool for NPC due to their simplicity and convenience, especially in communities in Guangdong Province. If the test is positive, in patients with NPC/ nasopharyngitis, pathologic examination is performed without finding cancer cells, and the results are used as an indication for stable follow-up or may be an effective means of preventing direct or worsening NPC in such patients. As shown in a previous study, hypermethylation of FAM19A4/mir124-2 was found in cervical scrapings of HPV-positive women with normal cytology at the time of sampling, who developed cervical cancers 5 and 10 years later[18], and Eads et al found frequent methylation of specific genes present in nondegenerative Barrett esophagus and associated adenocarcinomas[19]. Other reports showed hypermethylation of p16 in the gastric mucosa of 5 patients with gastric dysplasia, who developed gastric cancer 5 years later[20]. Similarly, p16 methylation was detected in oral epithelial dysplasia lesions in patients approximately 45 months prior to clinical and pathologic diagnosis of oral squamous cell carcinoma[21]. Notably, our study demonstrated that combined dual-gene methylation was superior to single-gene methylation in screening nasopharyngeal and non-nasopharyngeal cancers, and combined dual-gene methylation may provide rich information for clinical screening and diagnosis of nasopharyngeal cancer. Thus, methylation imbalance is not only a consequence of malignant transformation. It may serve as a predictive marker for normal samples in "at-risk" individuals. This is particularly important and helpful in clinical practice if a single methylation marker can be used to screen patients suspected of having NPC or at high risk of NPC with early symptoms. In conclusion, we validated *SEPT9, RASSF1A* and predicted a high risk of developing NPC in patients with NPC complaints carrying methylated *H4C6*, and we will subsequently follow up these subjects for a final diagnosis.

Our pilot study, although limited in sample size, demonstrated the potential of *SEPT9*, *H4C6*, and *RASSF1A* methylation-based approaches in diagnosing NPC. However, it is important to note that the conclusions drawn from this study still need confirmation through a larger cohort, especially when it comes to the diagnostic accuracy of *H4C6* gene methylation. In addition, the lack of follow-up examinations in our study is a limitation. Moving forward, we plan to address these limitations by collecting longitudinal clinical samples for a prospective study. This study aims to validate the diagnostic potential of *SEPT9* and *RASSF1A* methylation for NPC diagnosis while also evaluating the predictive ability of *H4C6* methylation in localized high-risk subgroups.

5. Conclusion

Our data indicate that *SEPT9*, *H4C6*, and *RASSF1A* methylation are commonly seen in NPC. Detecting these methylations in nasopharyngeal swabs could offer a minimally invasive and informative method for early NPC detection.

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