



# Combination of *RERG* and *ZNF671* methylation rates in circulating cell-free DNA: A novel biomarker for screening of nasopharyngeal carcinoma

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## Abstract

Nasopharyngeal carcinoma (NPC) is a prevalent malignancy in Southeast Asia, hence, identifying easily detectable biomarkers for NPC screening is essential for better diagnosis and prognosis. Using genome-wide and targeted analyses based on next-generation sequencing approaches, we previously showed that gene promoters are hypermethylated in NPC tissues. To confirm whether DNA methylation rates of genes could be used as biomarkers for NPC screening, 79 histologically diagnosed NPC patients and 29 noncancer patients were recruited. A convenient quantitative analysis of DNA methylation using real-time PCR (qAMP) was carried out, involving pretreatment of tissue DNA, and circulating cell-free DNA (ccfDNA) from nonhemolytic plasma, with methylation-sensitive and/or methylation-dependent restriction enzymes. The qAMP analyses revealed that methylation rates of *RERG*, *ZNF671*, *ITGA4*, and *SHISA3* were significantly higher in NPC primary tumor tissues compared to noncancerous tissues, with sufficient diagnostic accuracy of the area under receiver operating characteristic curves (AUC). Interestingly, higher methylation rates of *RERG* in ccfDNA were statistically significant and yielded a very good AUC; however, those of *ZNF671*, *ITGA4*, and *SHISA3* were not significant. Furthermore, the combination of methylation rates of *RERG* and *ZNF671* in ccfDNA showed higher diagnostic accuracy than either of them individually. In conclusion, the methylation rates of specific genes in ccfDNA can serve as novel biomarkers for early detection and screening of NPC.

**Abbreviations:** AUC, accuracy of the area under ROC curves; ccfDNA, circulating cell-free DNA; DEC, methylation-dependent enzyme control; HPV, human papilloma virus; *ITGA4*, integrin subunit alpha 4; mCRC, metastatic colorectal cancer; NNE, noncancerous nasopharyngeal epithelial; NPC, nasopharyngeal carcinoma; OD, optical density; qAMP, quantitative analysis of DNA methylation using real-time PCR; qPCR, quantitative PCR; *RERG*, RAS-like estrogen regulated growth inhibitor; ROC, receiver operating characteristic; SEC, methylation-sensitive enzyme control; *SHISA3*, Shisa family member 3; *ZNF671*, zinc finger protein 671.

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**KEY WORDS**

circulating cell-free DNA, DNA methylation, nasopharyngeal carcinoma, qAMP, screening biomarker

## 1 | INTRODUCTION

Nasopharyngeal carcinoma is a prevalent malignancy in Southeast Asia, especially in southern China, and it critically endangers public health.<sup>1</sup> Unfortunately, as the early stages of NPC are relatively asymptomatic, many patients are diagnosed when the disease is at an advanced stage. The identification of patients with early-stage NPC through screening could potentially improve treatment outcomes. A better prognosis relies on early diagnosis. Therefore, screening biomarkers for early detection of the disease are essential.

Aberrant DNA methylation has been reported as a crucial carcinogenic mechanism in various cancers, including NPC,<sup>2-6</sup> and promoter hypermethylation has been detected from an early stage of cancer development.<sup>7,8</sup> The detection of promoter methylation is valuable for cancer screening. A good compliance of patients or high-risk people requires more simple and noninvasive screening methods.

Circulating cell-free DNA derived from tumor cells and is characterized by neoplastic properties.<sup>9</sup> It could provide valuable information for early detection of cancer by using a convenient and minimally invasive method. Tumor-associated methylation alterations in ccfDNA have been detected in cancer patients using samples from peripheral blood-based "liquid biopsy."

There are many techniques for quantitatively detecting the DNA methylation status of specific genes. Most of the earlier methodologies to quantify DNA methylation used bisulfite treatment. However, longer assay times and a complex chemical reaction were the common limitations of this method that hindered their application in routine clinical screening. Alternatively, numerous bisulfite-free methods have been developed, which are highly sensitive and simple to perform. One such method is qAMP, involving methylation-sensitive and/or methylation-dependent restriction enzyme digestion of nucleic acids.<sup>10-15</sup>

In our previous study, we had explored and confirmed the presence of highly methylated promoter CpG islands in 7 candidate genes by genome-wide and targeted analyses using next-generation sequencing approaches, and the ratios of the methylation rates (NPC/NNE) were, in descending order, *ITGA4* (11.8), *REMG* (9.1), *ZNF671* (8.9), *SHISA3* (7.3), *ZNF549* (4.2), *CR2* (3.9), and *RRAD* (2.2).<sup>4</sup> Additionally, qAMP was used to further detect *REMG* methylation rates in NPC primary tumor tissues and NNE tissues.<sup>16</sup> Here, we examined methylation rates of the top 4 candidate genes (*ITGA4*, *REMG*, *ZNF671*, and *SHISA3*) in ccfDNA by qAMP, as well as tissue DNA, to evaluate them as prospective NPC screening biomarkers.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and collection of clinical samples

Seventy-nine histologically diagnosed NPC patients and 29 noncancer patients were recruited to the Department of Otolaryngology – Head and Neck Surgery, First Affiliated Hospital of Guangxi Medical University, China. This study was undertaken in accordance with the Declaration of Helsinki, and the protocol was approved by institutional ethics review committees at the First Affiliated Hospital of Guangxi Medical University, China (ethical approval no. 2009-07-07), and at Mie University, Japan (ethical approval no. 1116). All study participants provided written informed consent before they participated in the study.

Primary NPC tumor biopsies were obtained from NPC patients. The diagnoses were made by experienced pathologists according to the WHO classification. The NPC patients were investigated by MRI, clinically staged according to the 8th edition of the American Joint Committee on Cancer/UICC staging system. Noncancerous nasopharyngeal epithelial tissue biopsies obtained from noncancer patients were used as controls. The characteristics of patients from whom tissue samples were obtained are shown in Table 1. For plasma preparation, peripheral blood (5 mL) was collected from the ulnar vein into EDTA tubes before any therapeutic treatment, including radiotherapy. The characteristics of patient plasma samples are shown in Table 2. The blood was centrifuged at 1900 *g* for 10 minutes at 4°C to collect plasma, which was then centrifuged at 16 000 *g* for 10 minutes at 4°C to remove additional cellular debris and contamination by genomic nucleic acids derived from damaged blood cells. Biopsy samples and plasma were stored at –80°C, until further use.

### 2.2 | Tissue DNA and ccfDNA extraction

Total RNA-free genomic DNA was extracted from the biopsy samples with RNase treatment using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Tissue DNA was eluted with ultra-clean water at a final elution volume of 50  $\mu$ L. Plasma samples were analyzed to determine the OD at 414 nm ( $OD_{414}$ ) using NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific). A hemolysis cut-off value was fixed at 0.13 ( $OD_{414}$ , 10 mm pathlength equivalent) with reference to the report by Pizzamiglio et al.<sup>17</sup> Our preliminary data showed that hemolysis decreased the detectable methylation rates, because of DNA contamination from blood cells (Figure S1). Nonhemolytic plasma samples were chosen

**TABLE 1** Characteristics of patients who provided nasopharyngeal carcinoma (NPC) or noncancerous nasopharyngeal epithelial (NNE) tissue samples

	NPC (n = 65)	NNE (n = 22)
Age (y)	45.4 ± 14.2	43.1 ± 13.5
Sex, male/female	46/19 (70.8/29.2)	13/9 (59.1/40.9)
Histological subtype <sup>a</sup>		
Keratinizing squamous cell carcinoma	0 (0.0)	
Nonkeratinizing carcinoma	65 (100.0)	
TNM classification <sup>b,c</sup>		
Tumor size		
T1	9 (25.0)	
T2	12 (33.3)	
T3	11 (30.6)	
T4	4 (11.1)	
Lymph node metastasis		
N0	12 (33.3)	
N1	10 (27.8)	
N2	6 (16.7)	
N3	8 (22.2)	
Metastasis		
M0	36 (100.0)	
M1	0 (0.0)	
Cancer stage <sup>b,c</sup>		
I-II	12 (33.3)	
III-IV	24 (66.7)	

Data are shown as mean ± SD or n (%).

<sup>a</sup>According to the WHO histological classification of tumors of the nasopharynx.

<sup>b</sup>According to the 8th Edition of the American Joint Committee on Cancer/UICC Staging System for Nasopharyngeal Cancer.

<sup>c</sup>Missing data from 29 patients due to lack of follow-up.

for DNA extraction. Circulating cell-free DNA was extracted using the QIAamp MinElute ccfDNA Mini Kit (Qiagen) according to the manufacturer's instructions. Two milliliters of plasma samples (1 mL/column) from each subject was used for DNA extraction. Each column of ccfDNA was eluted with 30 µL ultra-clean water.

### 2.3 | Quantification of DNA methylation rate by qAMP

Gene promoter methylation rates were quantified by qAMP. Isolated DNA was treated with a methylation-sensitive and/or a methylation-dependent restriction enzyme using the EpiTect Methyl II DNA Restriction Kit (Qiagen). Tissue DNA (250 ng) or eluent of extracted ccfDNA (45 µL) was used to analyze 6 genes, including 4 candidate genes and 2 quality control genes. Both SEC and DEC were set up to test the digestion efficiencies of the restriction enzymes. Following the

**TABLE 2** Characteristics of patients who provided nasopharyngeal carcinoma (NPC) or noncancerous nasopharyngeal epithelial (NNE) plasma samples

	NPC (n = 26)	NNE (n = 13)
Age (y)	48.3 ± 13.6	42.5 ± 10.9
Sex, male/female	15/11 (57.7/42.3)	4/9 (30.8/69.2)
Histological subtype <sup>a</sup>		
Keratinizing squamous cell carcinoma	0 (0.0)	
Nonkeratinizing carcinoma	26 (100.0)	
TNM classification <sup>b,c</sup>		
Tumor size		
T1	1 (6.25)	
T2	3 (18.75)	
T3	5 (31.25)	
T4	7 (43.75)	
Lymph node metastasis		
N0	3 (18.75)	
N1	1 (6.25)	
N2	10 (62.5)	
N3	2 (12.5)	
Metastasis		
M0	15 (93.75)	
M1	1 (6.25)	
Cancer stage <sup>b,c</sup>		
I-II	2 (12.5)	
III-IV	14 (87.5)	

Data are shown as mean ± SD or n (%).

<sup>a</sup>According to the WHO histological classification of tumors of the nasopharynx.

<sup>b</sup>According to the 8th edition of the American Joint Committee on Cancer/UICC Staging System for Nasopharyngeal Cancer.

<sup>c</sup>Missing data from 10 patients due to lack of follow-up.

enzymatic digestion, qPCR was carried out using the RT<sup>2</sup> SYBR Green ROX qPCR Mastermix (Qiagen) and EpiTect Methyl II PCR Primer Assay (Qiagen) on a StepOnePlus Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific). The data analysis template was used to calculate methylation rates and data quality control. The threshold cycle values from the qPCR were calculated by the data analysis spreadsheet supplied by the manufacturer, and a "Pass" or "Fail" result was determined for the SEC and DEC assays. Pass meant that the restriction enzymes were active and digested the DNA efficiently. Methylation rates for target genes were used for statistical analysis only when both SEC and DEC assays yielded a Pass result from the qPCR data.

### 2.4 | Statistical analysis

All statistical analyses were undertaken using the SPSS version 22.0 software package (IBM). Characteristics of patients who provided

samples are presented as means  $\pm$  SD (Tables 1 and 2). The Mann-Whitney  $U$  test was used to compare statistical differences in methylation rates of candidate genes between NPC and NNE groups. Methylation rates of each group are represented by box-and-whisker plots. Receiver operating characteristic (ROC) curves were generated to confirm the accuracy of diagnosis by methylation rates. Sensitivity and specificity were also computed. The value of the area under curve (AUC) of ROC plots represents discrimination between the groups. The values of 0.9-1.0, 0.8-0.9, 0.7-0.8, 0.6-0.7, and 0.5-0.6 represent the diagnostic accuracy of excellent, very good, good, sufficient, and fail model, respectively.<sup>18,19</sup> All statistical tests were 2-sided and a  $P$  value of less than .05 was considered statistically significant.

### 3 | RESULTS

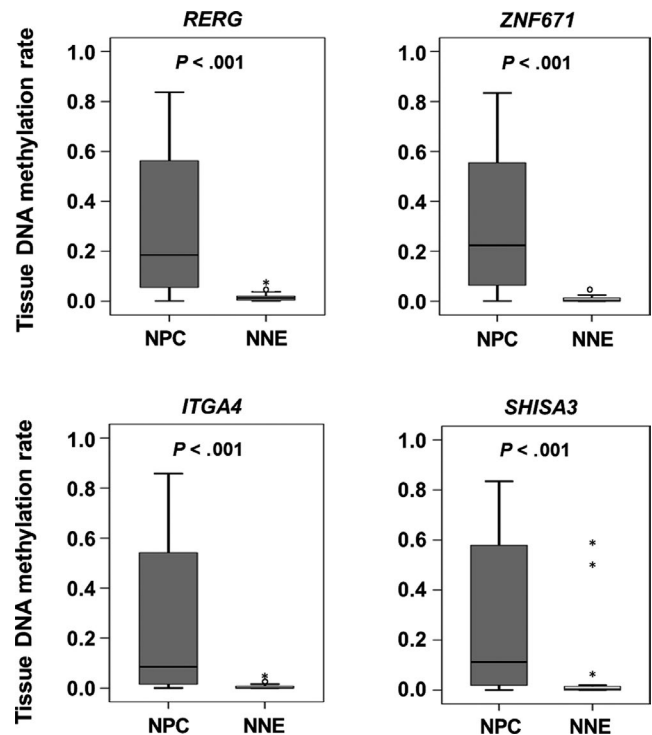
#### 3.1 | *RERG*, *ZNF671*, *ITGA4*, and *SHISA3* are hypermethylated in NPC primary tumors

The methylation rates of candidate genes, *RERG*, *ZNF671*, *ITGA4*, and *SHISA3*, in NPC and NNE tissues were quantified by qAMP (Figure 1). The methylation rates of these candidate genes were significantly higher in the NPC primary tumor tissues than those in the NNE tissues ( $P < .001$ ).

Interestingly, the methylation rates of *RERG*, *ZNF671*, *ITGA4*, and *SHISA3* of tissues were significantly higher in both early-stage (stage I-II) and late-stage (stage III-IV) NPC patients compared with NNE patients (Figure S2). However, there was no significant difference in tissue DNA methylation rates between patients with early-stage and late-stage disease. These results suggest that those hypermethylated candidate genes might be useful for NPC early detection and screening, rather than clinical application.

#### 3.2 | Methylation rates of candidate genes in tissue DNA as scientific bases for NPC screening biomarkers

Individual methylation rates of the candidate genes from tissue samples of NPC and NNE patients are shown in Figure 2 (left panels). Receiver operating characteristic curves were plotted to evaluate whether the methylation rates of the 4 candidate genes could serve as scientific bases for assessing NPC screening biomarkers. The AUC of ROC plots (Figure 2, right panels) as well as sensitivity and specificity for *RERG*, *ZNF671*, *ITGA4*, and *SHISA3* methylation rates in tissue DNA are shown in Table 3. The ROC curve analyses (Figure 2, right panels) showed that *ZNF671* had "excellent" diagnostic efficacy, with AUC of 0.946. *RERG*, *ITGA4*, and *SHISA3* had "very good" diagnostic efficacies with AUCs of 0.885, 0.871, and 0.809, respectively, which significantly distinguished NPC tissues from noncancerous tissue samples. In particular, *RERG* and *ZNF671* showed high AUC and resulted in good sensitivity and specificity.



**FIGURE 1** DNA methylation rates of *RERG*, *ZNF671*, *ITGA4*, and *SHISA3* in nasopharyngeal carcinoma (NPC) primary tumors. Methylation rates in NPC and noncancerous nasopharyngeal epithelial (NNE) tissues were measured by quantitative analysis of DNA methylation using real-time PCR. Data are shown as box-and-whisker plots of *RERG* methylation rates in NPC ( $n = 63$ ) and NNE ( $n = 21$ ), *ZNF671* methylation rates in NPC ( $n = 59$ ) and NNE ( $n = 19$ ), *ITGA4* methylation rates in NPC ( $n = 55$ ) and NNE ( $n = 18$ ), and *SHISA3* methylation rates in NPC ( $n = 58$ ) and NNE ( $n = 19$ ).  $P$  value by Mann-Whitney  $U$  test

#### 3.3 | Differential methylation rates of candidate genes in plasma ccfDNA samples

Circulating cell-free DNA was extracted from NPC and NNE patient plasma, and the methylation rates of candidate genes were quantified by qAMP (Figure 3). The methylation rates of *RERG* were significantly higher in the NPC group than in the NNE group ( $P < .001$ ). *ZNF671*, *ITGA4*, and *SHISA3* showed a trend of higher methylation rates in ccfDNA samples from NPC patient plasma, although they offered no statistically significant difference between NPC and NNE groups ( $P = .059$ , .101, and .393, respectively).

#### 3.4 | Methylation rates in ccfDNA can serve as NPC screening marker

Individual methylation rates of candidate genes in ccfDNA samples from NPC and NNE patients are shown in Figure 4 (left). The AUC of ROC curves for methylation rates of candidate genes in plasma ccfDNA (Figure 4, right panels) as well as sensitivity and specificity are shown in Table 4. The ROC curve analyses (Figure 4, right panels) showed that *RERG* had "very good" diagnostic accuracy, with a

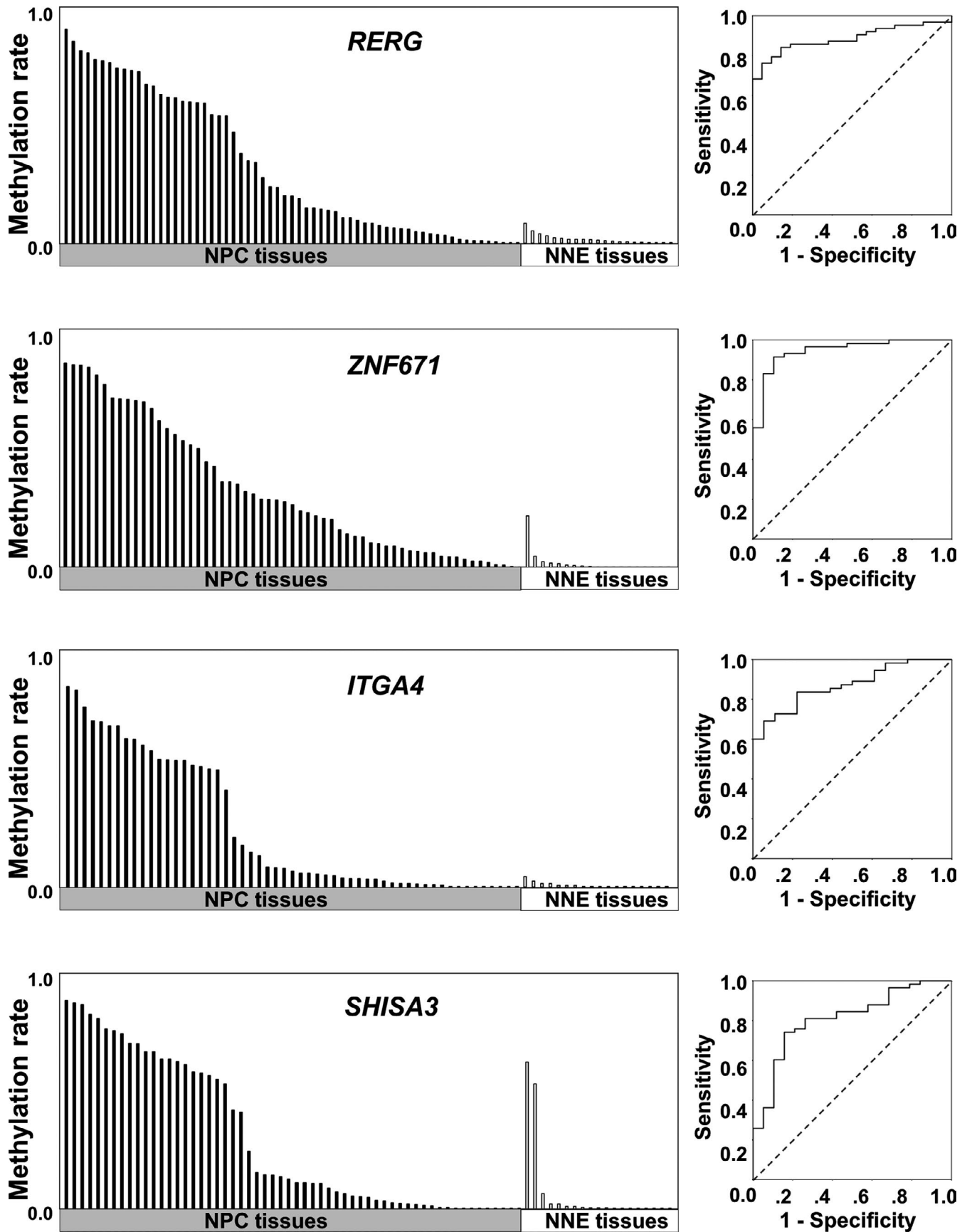


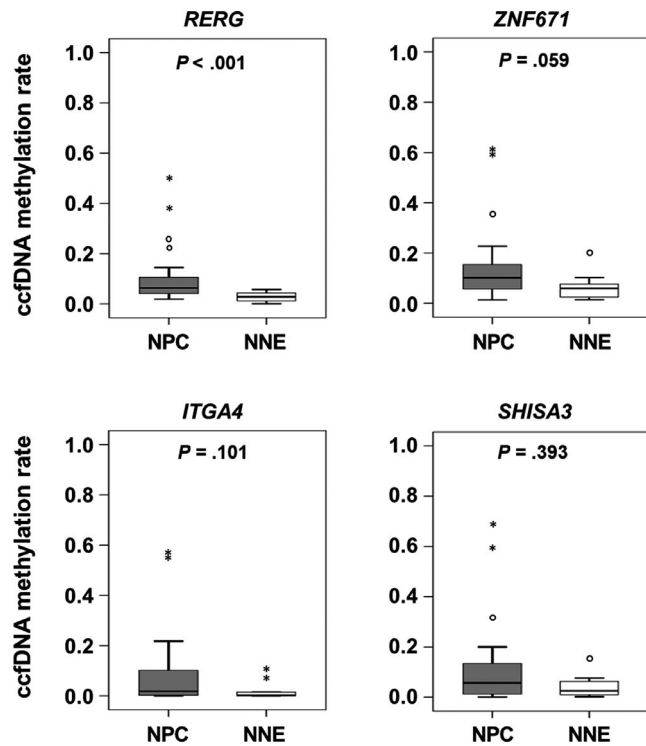
FIGURE 2 Individual methylation rate and receiver operating characteristic curve for screening nasopharyngeal carcinoma (NPC) by tissue DNA methylation rate. Methylation rates of *RERG*, *ZNF671*, *ITGA4*, and *SHISA3* in NPC and noncancerous nasopharyngeal epithelial (NNE) tissues were measured by quantitative analysis of DNA methylation using real-time PCR

**TABLE 3** Areas under receiver operating characteristic curves (AUC) for methylation rates of *REGG*, *ZNF671*, *ITGA4*, and *SHISA3* in tissue DNA

Methylated genes	AUC (95% CI)	Cut-off value (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
<i>REGG</i>	0.885*** (0.815-0.955)	5.19	76.2	95.2	81.0
<i>ZNF671</i>	0.946*** (0.891-1.000)	2.48	91.5	89.5	91.0
<i>ITGA4</i>	0.871*** (0.790-0.951)	3.05	69.1	94.4	75.3
<i>SHISA3</i>	0.809*** (0.700-0.918)	2.01	74.1	84.2	76.6

Abbreviation: CI, confidence interval.

\*\*\* $P < .001$ .



**FIGURE 3** DNA methylation rates of *REGG*, *ZNF671*, *ITGA4*, and *SHISA3* in plasma circulating cell-free DNA (ccfDNA) samples. Methylation rates in nasopharyngeal carcinoma (NPC) and noncancerous nasopharyngeal epithelial (NNE) plasma ccfDNA were measured by quantitative analysis of DNA methylation using real-time PCR. Data are shown as box-and-whisker plots of *REGG* methylation rates in NPC ( $n = 25$ ) and NNE ( $n = 13$ ), *ZNF671* methylation rates in NPC ( $n = 17$ ) and NNE ( $n = 10$ ), *ITGA4* methylation rates in NPC ( $n = 24$ ) and NNE ( $n = 10$ ), and *SHISA3* methylation rates in NPC ( $n = 21$ ) and NNE ( $n = 10$ ).  $P$  value by Mann-Whitney  $U$  test

high AUC of 0.855, which is statistically significant ( $P < .001$ ), thus distinguishing NPC from noncancer plasma samples. Furthermore, it showed 60.0% sensitivity, 100.0% specificity, and 73.7% accuracy (Table 4). *ZNF671* had a “good” AUC at 0.724, with a  $P$  value of .056, obtained by comparison between NPC and noncancer plasma samples. *ITGA4* and *SHISA3* had “sufficient” AUC but did not yield a significant difference between NPC and NNE groups ( $P = .096$  and .375, respectively).

Additionally, we tried to estimate the use of methylation rates of combinations of 2 genes as a viable tool for NPC screening. The

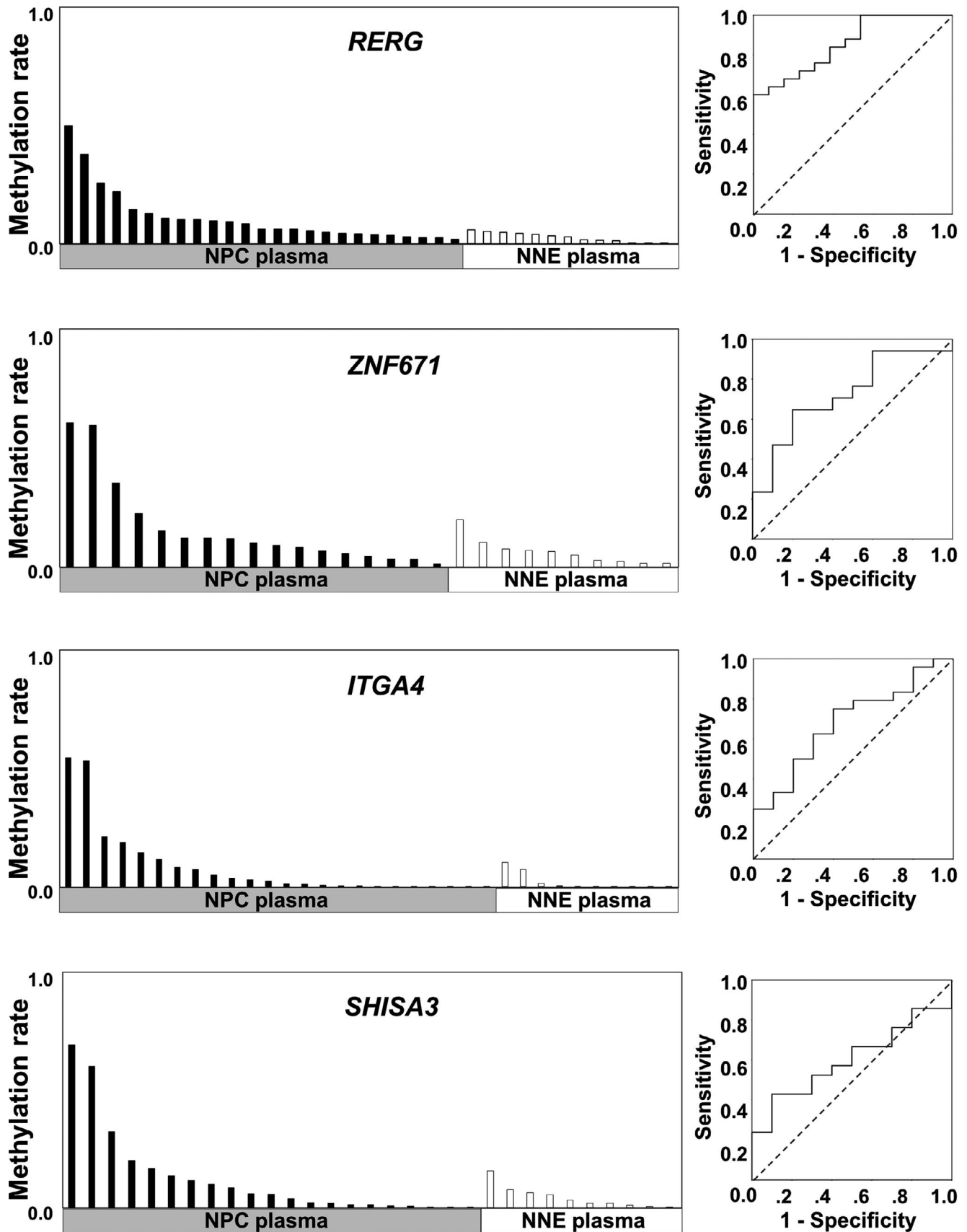
methylation rates that were above the cut-off value were used as a parameter to select genes from the set of candidate genes, whose AUCs were further analyzed (Table 5). The best combination was *REGG* and *ZNF671*, with an “excellent” AUC of 0.900, which was statistically significant ( $P < .001$ ), with 93.8% sensitivity, 80.0% specificity, and 88.5% accuracy.

## 4 | DISCUSSION

In this study, *REGG*, *ITGA4*, *ZNF671*, and *SHISA3* showed significantly higher methylation rates in tissue DNA of early-stage NPC than in NNE, revealing the ability of those candidate genes to assist in early diagnosis of NPC (Figure S2). *REGG* is a member of the Ras superfamily of small GTPases. Previous studies have reported that hypermethylation-silenced *REGG* was found in colorectal adenocarcinoma<sup>20</sup> and NPC.<sup>16</sup> Epigenetic silencing of tumor suppressor *ZNF671* was reported in renal cell carcinoma,<sup>21,22</sup> urothelial cancer,<sup>23</sup> HPV-related cervical cancer,<sup>24</sup> ovarian cancer,<sup>25</sup> oropharyngeal carcinoma,<sup>26</sup> and NPC.<sup>27</sup> Aberrant methylation of *ITGA4* was shown to be a useful diagnostic biomarker for HPV-positive cervical cancer<sup>24</sup> and HPV-associated oropharyngeal squamous cell carcinoma,<sup>28,29</sup> and it was also validated as an early detection marker of colorectal tumors.<sup>30</sup> *SHISA3* promoter methylation provides a predictive prognostic marker for patients with colorectal cancer<sup>31</sup> and laryngeal squamous cell carcinoma.<sup>32</sup> Moreover, Zhang et al reported that hypermethylated *SHISA3* promotes NPC invasion and metastasis.<sup>33</sup> These abovementioned reports support our results of candidate genes as epigenetic markers in cancers, including NPC.

Moreover, ROC curve analysis confirmed that the methylation rates of *REGG*, *ITGA4*, *ZNF671*, and *SHISA3* in tissue DNA are able to distinguish NPC patients from NNE patients significantly, and provide satisfied diagnostic efficacies for NPC screening (Table 3). In particular, *REGG* and *ZNF671* showed good sensitivities and specificities. Although tissue DNA could not serve as a population-based screening method due to the invasive operation, our results support the 4 genes as scientific bases for NPC screening biomarkers.

Interestingly, *REGG* in ccfDNA showed significantly higher methylation rates in NPC patients compared to the NNE group (Figure 3). The trends of higher ccfDNA methylation rates of *ZNF671*, *ITGA4*, and *SHISA3* were also observed in the NPC than in the NNE group,



**FIGURE 4** Individual methylation rate of *RERG*, *ZNF671*, *ITGA4*, and *SHISA3* along with receiver operating characteristic curves for screening nasopharyngeal carcinoma (NPC) by plasma circulating cell-free DNA (ccfDNA) methylation rate. Methylation rates of the genes in NPC and noncancerous nasopharyngeal epithelial (NNE) ccfDNA were evaluated by quantitative analysis of DNA methylation using real-time PCR

**TABLE 4** Areas under the receiver operating characteristic curves (AUC) for methylation rates of *REGG*, *ZNF671*, *ITGA4*, and *SHISA3* in circulating cell-free DNA for nasopharyngeal carcinoma screening

Methylated gene	AUC (95% CI)	P value	Cut-off value (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
<i>REGG</i>	0.855 (0.738-0.973)	<.001	5.96	60.0	100.0	73.7
<i>ZNF671</i>	0.724 (0.526-0.921)	.056	8.08	64.7	80.0	70.4
<i>ITGA4</i>	0.683 (0.492-0.875)	.096	0.28	75.0	60.0	70.6
<i>SHISA3</i>	0.600 (0.401-0.799)	.375	8.03	42.9	90.0	58.1

Abbreviation: CI, confidence interval.

**TABLE 5** Areas under receiver operating characteristic curves (AUC) for combinations of methylation rates of *REGG*, *ZNF671*, *ITGA4*, and *SHISA3* in circulating cell-free DNA for nasopharyngeal carcinoma screening

Methylated genes <sup>a</sup>	n	AUC (95% CI)	P value	Sensitivity (%)	Specificity (%)	Accuracy (%)
<i>REGG</i> or <i>ZNF671</i>	26	0.900 (0.771-1.000)	<.001	93.8	80.0	88.5
<i>REGG</i> or <i>ITGA4</i>	34	0.817 (0.675-0.958)	.004	83.3	60.0	76.5
<i>REGG</i> or <i>SHISA3</i>	30	0.815 (0.662-0.968)	.006	70.0	90.0	76.7
<i>ZNF671</i> or <i>ITGA4</i>	24	0.811 (0.624-0.998)	.012	93.3	55.6	79.2
<i>ZNF671</i> or <i>SHISA3</i>	22	0.731 (0.521-0.941)	.071	61.5	77.8	68.2
<i>ITGA4</i> or <i>SHISA3</i>	29	0.792 (0.624-0.959)	.013	80.0	66.7	75.9

Abbreviation: CI, confidence interval.

<sup>a</sup>According to the cut-off values in Table 4, each gene was assumed to be methylated. At least 1 gene was methylated in the combination of 2 genes, and then the combination was estimated as screening-positive.

which were consistent with our tissue results, but these differences in ccfDNA were not statistically significant (*ZNF671*,  $P = .059$ ). The difference in methylation rates of ccfDNA between NPC and NNE groups was lower than the differences observed in tissue DNA. For example, *ITGA4* has been reported to serve as 1 of the methylated biomarkers for mCRC. Average *ITGA4* methylation rate (and range) was 0.5% (0-9) for normal tissues and 51.7% (2-96) for matched tumor tissues, whereas the ccfDNA methylation rate was 0.2% (0-0.2) for healthy subjects and 33.2% (0-99) for mCRC samples.<sup>34</sup> Therefore, the diagnostic effectiveness (accuracy) is higher in tissue DNA methylation rates than in ccfDNA methylation rates, as previously reported by Usadel et al.<sup>35</sup> The frequency of detecting methylated genes in serum/plasma was approximately half to two-thirds compared with that in tumor tissues. One reason could be that ccfDNA might come from not only cancer cells but also normal cells.<sup>36,37</sup> Additionally, this discrepancy between sample types could be attributed to possible biological or technical bases, such as intratumor epigenetic heterogeneity, limited DNA shedding into circulation, or suboptimal plasma specimen handling resulting in hemolysis.<sup>34</sup> The nonhemolytic plasma used in our study can prevent nucleic acid contamination from blood cells. The frequency of methylation in plasma DNA in our study could be quite reasonable, and the discrepancy between sample types could be attributed to a possible biological basis.

As observed from our results, methylation rates of *REGG* in ccfDNA by qAMP showed 60% sensitivity, 100% specificity, and “very good” AUC at 0.855 (Table 4). Higher sensitivity can

be achieved if the screening is undertaken with more than 1 biomarker.<sup>34</sup> We have shown here that the combination of *REGG* and *ZNF671* methylation rates in ccfDNA can be used as a biomarker to screen for NPC with an “excellent” diagnostic accuracy (93.8% sensitivity, 80% specificity, and AUC of 0.900; Table 5); its diagnostic accuracy level is the same as tissue *ZNF671* (AUC 0.946) and better than other genes of tissue DNA (Table 3). Although our pilot study has the limitation of a small sample size, the combination of ccfDNA methylation rates brings a hope of blood-based assays for population-based NPC screening.

In summary, the 4 candidate genes *REGG*, *ZNF671*, *ITGA4*, and *SHISA3* showed significantly higher methylation rates in tissue DNA from NPC patients, providing a scientific basis for assessing DNA methylation as a biomarker for NPC early detection. The combination of ccfDNA methylation rates of *REGG* and *ZNF671* showed “excellent” diagnostic accuracy. We found that qAMP is a convenient and promising screening method for verifying NPC-specific epigenetic markers in a large sample size. The detection of ccfDNA methylation rates for a combination of genes by qAMP could be a useful biomarker for blood-based NPC screening.

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## CONFLICT OF INTEREST

The authors have no conflict of interest.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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