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The clinical significance of *HOXA9* promoter hypermethylation in head and neck squamous cell carcinoma

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Background: The purpose of the current study was to assess the association between *HOXA9* (homeobox A9) promoter methylation and head and neck squamous cell carcinoma (HNSCC) and its diagnostic value.

Methods: Quantitative methylation-specific PCR (qMSP) was applied to measure *HOXA9* promoter methylation levels in 145 paired HNSCC and corresponding normal tissue samples. Data from the Cancer Genome Atlas (TCGA) database (n = 578; 528 HNSCC and 50 normal) were also analyzed.

Results: Significantly higher levels of *HOXA9* promoter methylation were detected in HNSCC, compared with normal, tissues (our cohort: P = 1.06E-35; TCGA cohort: P = 3.06E-39). Moreover, *HOXA9* methylation was significantly increased in patients with advanced tumor (T) stage, lymph node metastasis, and advanced clinical stage. Areas under the receiver characteristic curves (AUCs) based on our cohort and TCGA data were 0.930 and 0.967, respectively.

Conclusion: In summary, our study reveals that *HOXA9* promoter hypermethylation contributes to the risk of HNSCC and its progression and metastasis. Additionally, *HOXA9* hypermethylation is a potential biomarker for the early diagnosis and screening of patients with HNSCC.

KEYWORDS
diagnosis, HNSCC, *HOXA9*, metastasis, methylation

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1 | INTRODUCTION

Worldwide, head and neck cancer is the sixth most common malignancy and fifth leading cause of cancer-related death.[1] More than 90% of head and neck tumors are squamous cell carcinomas (HNSCC) arising from the epithelial mucosal membranes of the upper aerodigestive tract (oral and nasal cavity, oropharynx, hypopharynx, and larynx).[2] Besides established risk factors (tobacco and alcohol abuse), high-risk human papillomavirus (HPV) infection has recently been reported as an independent risk factor for a subset of HNSCC.[3,4] According to recently epidemiological data from the American Cancer Society, 64 690 new cases of HNSCC and at least 13 740 deaths are projected to occur in the United States in 2018,[5] which is consistent with a continued rising trend over recent years.[6,7] As early-stage HNSCC is often symptomless, the majority of HNSCC patients are diagnosed with advanced stage disease, including lymphatic metastasis and distant metastasis, and the 5-year survival rate remains <50%,[8] while outcomes and quality of life can be remarkably improved if HNSCC is detected at an early stage.[9] In addition, biopsy by laryngoscope, which often requires general anesthesia, is considered the gold standard method for HNSCC diagnosis; the lack of a similarly valuable, non-invasive and cost-effective method for screening and early diagnosis of this disease is considered the major obstacle to improving the prognosis of HNSCC. Hence, identification of effective biomarkers for early HNSCC is an urgent priority for individual diagnosis and therapy.

Head and neck squamous cell carcinoma is a complex disease that can affect multiple sites and is caused by intricate interactions among genetic susceptibility, epigenetic modification, and environmental factors.[10] Emerging evidence indicates that epigenetic inactivation of tumor suppressor genes (TSGs) resulting from promoter methylation is involved in the onset and progress of various cancers, including esophageal squamous cell carcinoma,[11] cervical cancer,[12] lung cancer,[13] and breast cancer.[14] Aberrant methylation events are frequent, chemically stable, and relatively early molecular changes during carcinogenesis,[15,16] which have potential as biomarkers for cancer screening and early diagnosis.[17,18]

Homeobox (HOX) genes are a highly conserved family of 39 transcription factors that are grouped into four clusters, HOXA through HOXD.[19,20] HOX genes regulate and determine different cell types during embryonic development.[20,21] In addition, there is increasing evidence that HOX genes have important functions in regulation of the delicate balance between cell proliferation and differentiation during cancer development.[22,23] The *HOXA9* gene, mapping to chromosome 7p15.2, is a member of this large family, and its abnormal expression is involved in the emergence of numerous solid and hematopoietic malignancies. *HOXA9* is frequently activated in hematopoietic malignancies[24]; however, it can be downregulated in solid tumors,[25,26] particularly squamous cell carcinoma. Recent studies have revealed that hypermethylation of the *HOXA9* promoter leads to its transcriptional inactivation in several of cancers, including those of the lung,[28] breast,[29] cervix,[25] and bladder[30]; however, the relationship between *HOXA9* and HNSCC remains unclear.

In the present study, we investigated the association between *HOXA9* promoter methylation in 145 HNSCC patients and its potential diagnostic value. Furthermore, data from 578 samples available from the Cancer Genome Atlas (TCGA) database were analyzed to validate our findings.

2 | MATERIALS AND METHODS

2.1 | Specimens and clinical data collection

Head and neck squamous cell carcinoma tissues (n = 145) and corresponding non-tumor tissues were collected at the Department of Otolaryngology-Head and Neck Surgery and Oral and Maxillofacial Surgery at Ningbo Lihuili Hospital between November 2012 and August 2017. Before surgery and tissue collection, all patients provided written informed consent. All specimens were freshly obtained and preserved in RNA-fixer Reagent (Bioteke, Beijing, China) at −80°C until use. Final diagnoses were confirmed histopathologically. Histological grade was defined according to the National Comprehensive Cancer Network oncology guidelines. No patient received chemotherapy or radiotherapy before surgery. None of the patients had a history of hereditary cancer. Tumors were staged according to the TNM classification (7th edition) of the Union for International Cancer Control. Age, gender, smoking behavior, histological classification, tumor location, T classification, lymph metastasis, and tumor stage were extracted from medical records for all cases. The study was approved by the Human Research Ethical Committee of Ningbo Lihuili Hospital.

2.2 | DNA extraction and bisulfite conversion

Genomic DNA was extracted from frozen tissues using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). A Nanodrop 2000 spectrophotometer (Thermal Scientific Co. Ltd., Wilmington, USA) was used to measure DNA concentration, and quality. extracted DNA was bisulfite-converted subsequently using the EZ DNA Methylation-Gold Kit, according to the manufacturer's protocol (Zymo Research, Orange, CA, USA). This procedure converts unmethylated cytosines to uracil, while the methylated cytosines are unaffected.

Bisulfite-converted DNA was stored in tris-ethylenediaminetetraacetic acid buffer for subsequent methylation analysis.

2.3 | Quantitative methylation-specific polymerase chain reaction (qMSP)

Methylation levels of the *HOXA9* promoter (chr7:27206577-27206704) in 145 HNSCC and paired adjacent tissue samples were determined using a qMSP assay as described previously.[31] *ACTB* was chosen as the internal control[28] and human methylated DNA (Zymo Research, Orange, CA, USA) served as the positive control. The qMSP primer sequences were as follows: *HOXA9*, 5′-TGATTATTTTTGTTTTAGGAGTCGT-3′ (forward) and 5′-TAAAAAAATTTATTTCTCACCCGTT-3′ (reverse); *ACTB*, 5′-TGGTGATGGAGGAGGTTTAGTAAGT-3′ (forward) and 5′-AACCAATAAAACCTACTCCTCCCTTAA-3′ (reverse). PCR conditions for both methylated (M) and unmethylated (U) primer pairs comprised initial denaturation at 95°C for 10 minutes, followed by 45 cycles of 20-seconds denaturation at 95°C, 20-seconds annealing at 60°C, and 30-seconds extension at 72°C. Products were stored at 4°C. The percentage of methylated reference (PMR) was calculated to determine the *HOXA9* promoter methylation level.[32]

2.4 | The Cancer Genome Atlas (TCGA) data mining study

The Cancer Genome Atlas (TCGA), supervised by the National Cancer Institute's Center for Cancer Genomics and the National Human Genome Research Institute, is a large-scale cancer genome project which provides researchers with multi-dimensional maps of the key genomic changes, clinicopathological information, and survival data in 33 types of cancer (http://cancergenome.nih.gov/)[33] DNA methylation profiles (Illumina Human Methylation 450K) and details of the clinicopathological characteristic of patients providing the 528 HNSCC tissues and 50 non-tumor tissues in TCGA cohort (Project Id: TCGA-HNSC) were downloaded from the University of California Santa Cruz (UCSC) Xena browser (www.xena.ucsc.edu). The average β values of two Illumina Human Methylation 450K BeadChip probes (cg02643054 on chr7:27206544 and cg00905524 on chr7:27206907), close to the qMSP amplification fragment (chr7:27206577-27206704), were used to evaluate *HOXA9* methylation in this dataset.

2.5 | Statistical analysis

All statistical analysis was performed using Statistical Program for Social Sciences (SPSS) 20.0 software (Chicago, IL, USA) and GraphPad Prism 6.0 (La Jolla, CA, USA), which were also used to generate figures. For comparisons between groups, independent Student's *t* test, paired Student's *t* test, and one-way analysis of variance (one-way ANOVA) tests were employed, as appropriate. Receiver operating characteristic (ROC) analysis was used to assess the diagnostic value of *HOXA9* methylation for HNSCC. A two-tailed *P* value <0.05 was defined as statistically significant.

3 | RESULTS

In the current study, 145 HNSCC and corresponding non-tumor tissue samples were collected to investigate the association of *HOXA9* promoter methylation and HNSCC. Illumina Human Methylation 450K data from 528 patients with histologically confirmed HNSCC, including tumor tissues in all cases and matched adjacent normal tissues in 50 cases, were available from TCGA project, and two Methylation 450K CpG sites (cg02643054 and cg00905524) located near the tested fragment (chr7:27206577-27206704) were chosen to verify our findings (Figure 1).

In our study cohort, we found that the *HOXA9* methylation levels in tumor tissues were significantly higher than those in paired

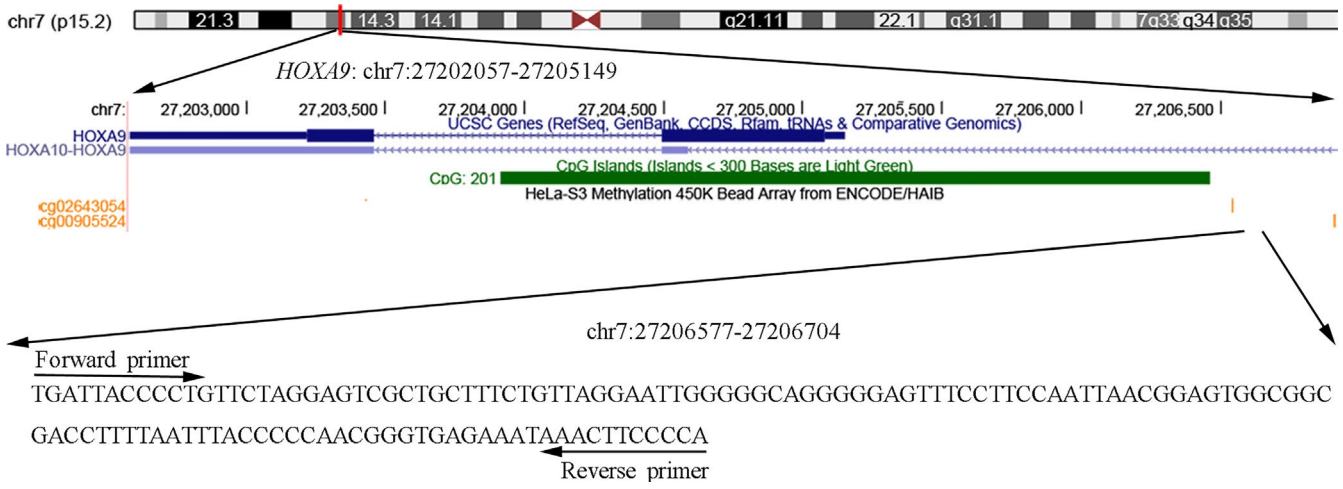

FIGURE 1 Genomic position of the quantitative methylation-specific PCR (qMSP) amplified fragment shown in the UCSC genome browser (human 2009 assembly; GRCh37/hg19)). Two available CpG probes (cg00905524 and cg02643054) in the Illumina Human Methylation 450K also map to the *HOXA9* promoter, close to the qMSP amplified fragment

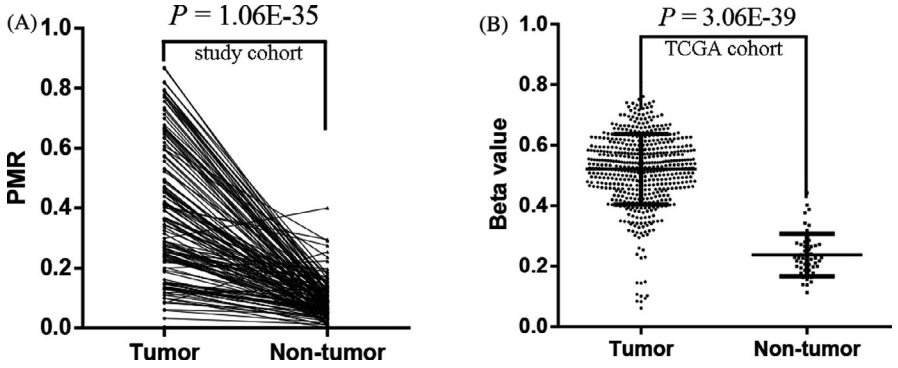

FIGURE 2 Comparisons of *HOXA9* promoter methylation levels in HNSCC tumor and adjacent non-tumor tissues. A, our study cohort: *P* = 1.06E-35; B, TCGA cohort: *P* = 3.06E-39

TABLE 1 Association of *HOXA9* promoter methylation with clinicopathological characteristics of HNSCC patients in our study cohort

Characteristics	N	Mean ± *SD*	*P* value
Gender			
Female	30	0.407 ± 0.228	0.698
Male	115	0.389 ± 0.222	
Age			
<60 y	78	0.397 ± 0.235	0.786
≥ 60 y	67	0.387 ± 0.209	
Smoking behavior			
No	28	0.359 ± 0.233	0.376
Yes	117	0.400 ± 0.221	
Histological classification			
Well and Moderately	126	0.379 ± 0.220	0.063
Poorly	19	0.481 ± 0.228	
Tumor location			
Oral cavity	21	0.360 ± 0.224	0.881
Oropharynx	6	0.393 ± 0.273	
Hypopharynx	26	0.413 ± 0.120	
Larynx	92	0.394 ± 0.228	
T classification			
T1 + 2	82	0.350 ± 0.214	0.008[a]
T3 + 4	63	0.448 ± 0.223	
Lymph metastasis			
No	97	0.360 ± 0.213	0.012[a]
Yes	48	0.458 ± 0.231	
Tumor stage			
Stage I + II	64	0.333 ± 0.211	0.004[a]
Stage III +IV	81	0.439 ± 0.222	

[a]The difference in *HOXA9* promoter methylation between these groups was significant.

adjacent normal tissues: median PMR with interquartile range, 0.359 (0.221, 0.556) vs 0.075 (0.043, 0.116), *P* = 1.06E-35 (Figure 2A). Subsequently, we determined *HOXA9* methylation levels using data from TCGA database. As shown in Figure 2B, this analysis validated our finding that there was a significant difference in *HOXA9* methylation levels in tumor compared with adjacent non-tumor tissues(median β value with interquartile range, 0.529 (0.457, 0.594) vs 0.226 (0.186, 0.268), *P* = 3.06E-39).

Based on these findings, we analyzed the association between *HOXA9* methylation levels, its expression levels, and the clinicopathological characteristics of patients with HNSCC, including age, gender, smoking behavior, histological classification, tumor location, T classification, lymph metastasis, and tumor stage. As shown in Table 1, *HOXA9* hypermethylation in human HNSCC tissues was associated with T classification (*P* = 0.008), lymph metastasis (*P* = 0.012), and tumor stage (*P* = 0.004). Importantly, these findings were replicated by analysis of TCGA data. *HOXA9* was significantly hypermethylated in patients with advanced pathologic tumors (*P* = 0.041), advanced pathologic nodal category (*P* = 0.047), and advanced pathologic stage (*P* = 0.009) (Table 2). Additionally, a borderline significant difference in *HOXA9* methylation status was identified between male and female patients in TCGA data (*P* = 0.06). This may be explained in part by differences in smoking habits, with female patients mainly being non-smokers; however, no statistically significant correlation was identified with any other clinicopathological characteristic in either our study cohort or the TCGA cohort.

Sensitivity and specificity are objective and easy to understand; however, they are often affected by the use of different threshold values. We examined the diagnostic value of *HOXA9* promoter methylation in HNSCC using ROC curve analysis, which is a synthesized index that reflects the accuracy of diagnostic test. An area under the ROC curve (AUC) close to 1.0 signifies that the test has almost perfect discrimination. The maximum Youden index was used as a cutoff point. In our study cohort, *HOXA9* hypermethylation yielded an AUC of 0.930 (95% CI: 0.901-0.959), a sensitivity of 79.3%, and a specificity of 93.8%, with a cutoff value of 0.185 (Figure 3A). In the TCGA cohort, *HOXA9* hypermethylation yielded an AUC of 0.967 (95% CI: 0.952-0.983), a sensitivity of 93.6%, and a specificity of 92.0% with a cutoff value of 0.343 (Figure 3B).

4 | DISCUSSION

Hypermethylation, causing the transcriptional silencing of the promoters of tumor suppressor genes (TSGs), occurs in various

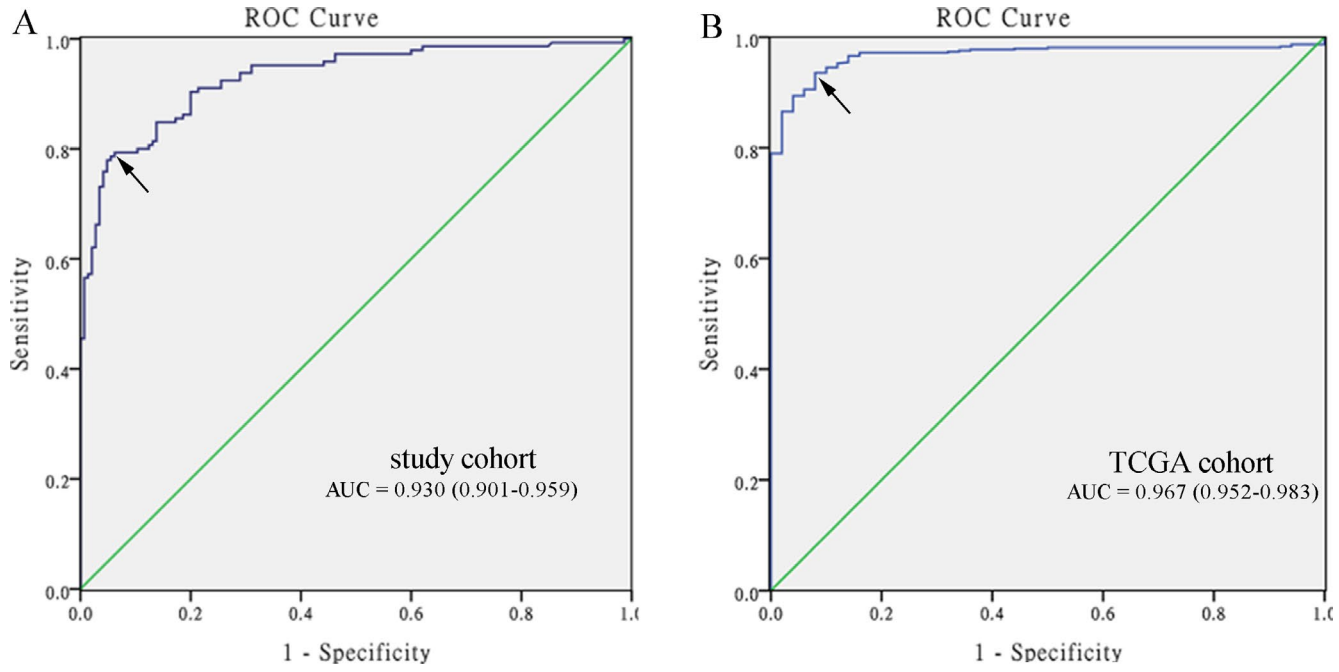

FIGURE 3 Receiver operating characteristic (ROC) curves to assess the diagnostic value of *HOXA9* promoter methylation in HNSCC patients. A, in our study cohort, the area under the curve (AUC) was 0.930. B, in TCGA cohort, the AUC was 0.967. The arrows indicate cutoff points

malignancies as part of the process of carcinogenesis.[34] Compared with other molecular markers, DNA hypermethylation is a common and early event during the progression of various tumors and it is chemically and biologically more stable than RNA or the majority of proteins.[35] Given these advantages and the development of technology for their detection, methylation biomarkers have great potential for use in early screening and diagnosis of cancer.[36] *HOXA9* functions as a tumor suppressor gene that suppresses breast tumor growth and metastasis.[23] Furthermore, methylation of the *HOXA9* promoter is associated with progression and prognosis in numerous cancers. [28,29]

In the current study, we recruited 145 HNSCC patients to investigate the association of *HOXA9* methylation with HNSCC and its potential for use in detection of HNSCC. Our results showed that methylation levels of the *HOXA9* promoter were significantly higher in HNSCC than adjacent non-tumor tissues. Similarly, further bioinformatics analyses of TCGA data confirmed that *HOXA9* methylation was higher in HNSCC compared with normal tissues. Taken together, these findings suggest that *HOXA9* methylation is a risk factor for HNSCC and has potential value for its diagnosis.

Subsequently, we also determined the association between *HOXA9* promoter methylation and the clinicopathological characteristics of patients with HNSCC. Tumor invasion and clinical stage are vital factors for assessing prognosis in patients with cancer and are among the most common tools used for that purpose.[37,38] In our qMSP study, we demonstrated a significantly elevated frequency of *HOXA9* promoter methylation in patients with advanced tumor stage and advanced clinical stage, which was confirmed by analysis of TCGA data. Overall, these results suggest that *HOXA9* methylation may be involved in the progression and metastasis of HNSCC. Given the well-developed lymphatic network in the neck region, HNSCC has a high propensity to undergo lymph node metastasis[40]; however, because of the high incidence of occult lymph node metastasis,[41] the accurate diagnosis of lymph node metastases remains challenging. Analyses of both our cohort and TCGA data showed that *HOXA9* promoter methylation levels were significantly higher in HNSCC patients with lymph node metastasis compared with those without, providing a potential means to distinguish HNSCC patients with lymphatic metastasis. Additionally, an almost statistically significant difference in *HOXA9* methylation status was found between male and female patients in TCGA data ($P = 0.06$), which may be partly attributable to differences in smoking habits, with female patients mainly being non-smokers.

Therapeutic procedures and prognosis differ substantially between early- and late-stage HNSCC. Early-stage HNSCC patients receive minimally invasive surgery or irradiation alone, with good outcomes, while late-stage patients receive aggressive therapy, such as expanded surgery and/or concomitant chemoradiotherapy, resulting in dismal survival rates and poor quality of life.[42,43] Screening for HNSCC depends on clinical symptoms and imaging examinations (laryngoscopy, computed tomography, magnetic resonance imaging, and positron emission tomography) and histopathological examination[44]; however, owing to the non-specificity of symptoms in early-stage disease and ineffective conventional cancer-related biomarkers,[45] the early detection of HNSCC remains unsatisfactory. As they occur early in carcinogenesis and have other advantageous characteristics, abnormal methylation patterns represent potential markers for early detection of cancer and can even be non-invasively detected in various body

TABLE 2 The association of *HOXA9* promoter methylation with clinicopathological characteristics of HNSCC patients in TCGA cohort

Characteristics	N	Mean ± *SD*	*P* value
Gender			
Female	142	0.506 ± 0.107	0.06
Male	386	0.526 ± 0.120	
Age			
<60 y	260	0.524 ± 0.120	0.602
≥60 y	267	0.518 ± 0.114	
Smoking behavior			
No	122	0.528 ± 0.107	0.44
Yes	393	0.519 ± 0.120	
Alcohol history			
No	165	0.511 ± 0.134	0.225
Yes	352	0.526 ± 0.108	
Histological grade			
G1 + 2	374	0.520 ± 0.111	0.779
G3 + 4	132	0.523 ± 0.125	
Tumor site			
Oral cavity	392	0.526 ± 0.109	0.241
Oropharynx	9	0.547 ± 0.076	
Hypopharynx	10	0.494 ± 0.168	
Larynx	117	0.504 ± 0.138	
HPV status			
Positive	41	0.534 ± 0.142	0.119
Negative	74	0.495 ± 0.117	
Pathologic tumor category			
Tis/T1/T2	190	0.504 ± 0.120	0.041[a]
T3/T4	276	0.527 ± 0.116	
Pathologic nodal category			
No	180	0.504 ± 0.119	0.047[a]
Yes	240	0.528 ± 0.117	
Pathologic stage			
StageI + II	104	0.490 ± 0.116	0.009[a]
StageIII + IV	352	0.525 ± 0.118	

[a]The difference of *HOXA9* promoter methylation between these groups was significant.

fluids (blood, bronchial aspirates, brushing, saliva, and urine).[46,47] In the present study, we constructed ROC curves and calculated the AUC to determine the diagnostic value of *HOXA9* methylation for HNSCC. The AUC values were close to 1.0, signifying near perfect diagnostic power. The AUC for our study was 0.930 and that based on TCGA data was even more encouraging at 0.967, indicating that *HOXA9* promoter methylation has excellent diagnostic accuracy for HNSCC.

The current study had some limitations. A number of methods have been developed to assess biomarkers in biological fluids to non-invasively identify early HNSCC.[49,50] Due to a lack of blood and/or saliva samples, we were unable to explore the diagnostic value of non-invasive detection of HNSCC methylation. In addition, emerging evidence indicates that a panel of several methylation genes could improve cancer diagnosis,[51] while our study focused on a single gene, which may not completely satisfy the requirements for clinical application. Therefore, future investigation is needed to determine whether a panel for analysis of the *HOXA9* promoter combined with other epigenetic biomarkers will be of higher diagnostic value, particularly using liquid biopsies.

5 | CONCLUSION

In conclusion, *HOXA9* promoter hypermethylation is associated with the risk for HNSCC and its progression and metastasis. Additionally, *HOXA9* hypermethylation has potential for use as a biomarker for the early diagnosis and screening of patients with HNSCC.

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