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Development of a Nomogram Based on Clinicopathological and Biological Features to Predict Neck Lymph Node Metastasis in Hypopharyngeal Squamous Cell Carcinoma

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Abstract

Background: Clinicopathological and biological features are associated with neck lymph node metastasis (LNM) of hypopharyngeal squamous cell carcinoma (HSCC). However, there is no complete nomogram combining multiple factors that can be used to accurately predict the neck LNM status for HSCC patients. **Purpose:** To guide the selection of surgical methods and radiotherapy areas for hypopharyngeal cancer. In this study, a nomogram was developed to combine these risk factors to predict neck LNM and guide the treatment of HSCC. **Material and Methods:** This retrospective study included 117 patients (training cohort, 64 patients; trial cohort, 53 patients). Biological characteristics of HSCC patients were assessed using immunohistochemical staining, and data of patient age, gender, and preoperative computed tomography (CT) scan reports were collected. Significant risk factors in univariate analysis were further identified to be independent variables in multivariate logistic regression analysis, which were then incorporated in and presented with a nomogram by using the rms package in R software. Receiver operating characteristic (ROC) curves and calibration curves were used to validate the discrimination and accuracy in the training and validation cohorts, respectively, and clinical usefulness was verified in decision curve analysis curves. **Results:** All variables with P-values < 0.2 in the univariate analysis were selected for multivariate logistic regression analysis to further identify independent risk factors for neck LNM. In multivariate logistic regression analysis, variables with P-values < 0.2 were identified as independent risk factors and then used to construct the nomogram. In total, five independent predictors, including the maximum tumor diameter in CT, tumor cell diffe-

rentiation, LNM status in CT, Stathmin1 expression level, and lymphatic vessel invasion were included in the nomogram. The area under the ROC curve (AUC) was 0.916 (95% confidence interval [CI], 0.833 - 1.000) and AUC of 0.928 (95% CI, 0.864 - 1.000) in internal validation and the external validation. **Conclusions**: Both the internal validation in the training cohort and the external validation in the validation cohort showed that the nomogram had good discrimination, accuracy, and excellent clinical usefulness. The nomogram based on clinicopathological and biological features developed in this study has strong predictive power and could be used to predict neck LNM of HSCC in clinical practice.

Keywords

1. Introduction

Hypopharyngeal squamous cell carcinoma (HSCC) is one of the most common malignant tumors of the head and neck and has a poor prognosis. Numerous studies have confirmed that the high risk of neck lymph node metastasis (LNM) is the main reason for the poor prognosis of this cancer [1] [2]. The two main methods of treating HSCC, in addition to resection and irradiation of the primary mass, are routine neck lymph node dissection and irradiation [3], and at the same time increase the incidence of surgical complications and radiation damage such as local dysfunction, pharyngeal fistula, neck edema, and others [4]. Several studies of head and neck malignancies found that more than 50% of the patients who underwent neck lymph node dissection were found not to have developed tumor metastases in postoperative pathology [5] [6]. Therefore, identifying HSCC patients at high risk for neck LNM is critical for proper lymph node management to improve the therapeutic effect of these patients.

With respect to possible risk factors for predicting neck LNM of HSCC, there have been many reports in the last few decades. Imaging examination is the most common method to evaluate neck LNM in cases of HSCC. Horváth *et al.* compared the results of computed tomography (CT), magnetic resonance imaging, ultrasound, and other routine preoperative examinations of neck LNM with postoperative pathology records and showed that none of these imaging examinations could accurately determine neck LNM of the tumor [5]. Monroe *et al.* reported that assessing neck LNM based on tumor thickness and size from imaging examination still resulted in a high percentage (80%) of unnecessary neck lymph node dissections [7]. These findings mean that the assessment of neck LNM of HSCC is not very accurate when examined by conventional imaging methods.

Some clinicopathological characteristics and biological molecules have also

been widely reported to be associated with neck LNM of HSCC. In 2016, Jang *et al.* analyzed the risk factors for neck LNM in 295 patients with head and neck tumors and showed that neck LNM of HSCC was influenced not only by the size of the primary tumor but also by biological factors such as lymphovascular infiltration or the timing of tumor cell multiplication [8]. Chen *et al.* experimentally demonstrated that Stathmin1 (STMN1) promoted neck LNM of HSCC by participating in the epithelial-mesenchymal transition (EMT) process [9]. Lymphatic vessels are an important pathway for lymphatic metastasis of tumors. Lymphatic vessel invasion is regarded as the most reliable marker to predict LNM [8], and can be recognized by podoplanin, the endothelial marker of lymphatic vessels [9] [10]. In addition, tumor cell proliferation and vascular invasion have also been reported to be associated with neck LNM of malignant tumors [11] [12] [13] [14].

However, although the above factors have been reported to be associated with neck LNM of HSCC, so far it is not possible to use any single imaging examination, clinicopathological characteristic, or biological molecular marker to predict neck LNM of HSCC. Neck LNM of HSCC is a result of the combined effect of many risk factors. Thus it will be more valuable to explore a method that can combine all the risk factors for neck LNM of HSCC to predict neck LNM. Consequently, we aimed to combine the risk factors for neck LNM of HSCC to develop a nomogram and predict neck LNM and provide personalized precision treatment for HSCC patients.

2. Materials and Methods

2.1. Study Population and Ethics Approval

All the patients in this study were selected in accordance with the following criteria: all patients were confirmed to be free of distant metastases, and all patients underwent radical hypopharyngeal cancer resection with bilateral neck lymph node dissection; all patients were pathologically confirmed to have HSCC postoperatively and neck LNM status was pathologically evaluated postoperatively; no preoperative chemotherapy or radiotherapy was administered. Clinical data before the operation and paraffin-embedded specimens for immunohistochemical staining (IHC) were available. Eligible patients (n = 64) were recruited from the Second Hospital of Shandong University (Jinan, China) between January 2015 and December 2020 for risk factor selection and nomogram construction (training cohort). A second cohort (n = 53) was recruited from the Shandong Provincial Hospital between January 2018 and March 2021 and used for external validation (validation cohort). The risk factors analyzed included some clinicopathological characteristics such as gender, age, tumor cell differentiation, maximum tumor diameter in CT images, LNM status in CT images, lymphatic vessel invasion, vascular invasion, and biological markers such as STMN1 and Ki-67.

This study was approved by the Ethics Committee of the Second Hospital of Shandong University and the Shandong Provincial Hospital.

2.2. IHC Staining and Evaluation of Staining Results

IHC staining was used to evaluate the expression status of STMN1 to evaluate EMT, CD31 to evaluate vascular invasion status, D2-40 to evaluate lymphatic vessel invasion status, and Ki-67 to evaluate tumor cell proliferation capacity. The detailed procedure of IHC was as follows:

IHC staining was performed on 4-μm paraffin tissue sections mounted on slides and dried for 8 h at 60°C. The slides were deparaffinized in xylene and dehydrated conventionally, then pressure-cooked in sodium citrate buffer (pH 6.0) (LBP Med-Sci, Guangzhou, China) to facilitate antigen retrieval. After natural cooling, endogenous peroxidase was blocked with 3% hydrogen peroxide. The sections were subsequently incubated with rabbit anti-STMN1 polyclonal antibody (dilution 1:75) (Boster-Bio, Wuhan, China), or rabbit anti-Ki-67 polyclonal antibody (dilution 1:50) (Boster-Bio, Wuhan, China), or rabbit anti-D2-40 polyclonal antibody (Boster-Bio, Wuhan, China) and rabbit anti-CD31 polyclonal antibody (dilution 1:75) (Boster-Bio, Wuhan, China) overnight at 4°C. After washing with PBS (LBP Med-Sci, Guangzhou, China), the sections were incubated for 30 min with the two-step method followed by the poly-HRP anti-mouse/rabbit detection system (LBP Med-Sci, Guangzhou, China). A DAB detection kit (Talent-Bio, Xiamen, China) was used for 5 - 10 min to show immunolabeling, resulting in a brown precipitate. Finally, the sections were re-stained with hematoxylin (Solarbio, Beijing, China), differentiated in hydrochloric acid alcohol, and sealed with neutral balsam (Solarbio, Beijing, China).

IHC staining of STMN1 is mainly localized to the cytoplasm of tumor cells [15]. The staining results were evaluated using the IHC scoring method previously applied by Chen *et al.* [9]. Scores higher than 6 were recorded as high expression, and scores lower than 6 were recorded as low expression. Anti-D2-40 antibody was used to stain lymphatic vessels, and in stained lymphatic vessels, the presence of tumor embolus represented lymphatic vessel invasion, otherwise, lymphatic vessel invasion was considered absent. Similarly, the presence of tumor embolus in vasculature stained by CD31 represented vascular invasion. Ki67 staining was restricted to the nuclei of tumor cells [16]; regions with the highest number of positive tumor nuclei were selected for analysis, and Ki67 index was calculated as the number of stained cell nuclei/by the number of all nuclei) $\times$ 100% [17]. If the Ki67 index was <50%, Ki67 was considered to represent low expression, while if the Ki67 index was ≥50%, Ki67 was considered to represent high expression.

All IHC staining results were confirmed by two senior pathologists without seeing any of the patients' clinicopathological information. Any disagreement between the two pathologists was resolved by consensus after discussion with a third pathologist.

2.3. Feature Extraction, Risk Factor Screening for Neck LNM, and Statistical Analysis

Data were statistically analyzed using the SPSS 23.0 statistical package (IBM

SPSS Statistics for Windows, Armonk, NY, USA) and R software (version 4.0.3; http://www.Rproject.org). All statistical tests were bilateral. Age was the only continuous variable and all other variables were categorical variables. The maximum tumor diameter was obtained from preoperative CT. The best cut-off value for maximum tumor diameter was 36 mm, which was from the receiver operating characteristic (ROC) curve (Figure 1). The maximum tumor diameter in CT images was divided into two categories: <3.6 cm and ≥3.6 cm. The LNM status in CT images was classified as Yes or No. Tumor grade I and II differentiation were considered as well differentiation, tumor grade III was recorded as poor differentiation. Continuous variables were expressed using median and quartile range (QR). T-tests and chi-square tests were respectively used to compare the differences of continuous variables and categorical variables between the training cohort and the validation cohort. LNM recorded in postoperative pathological records was considered as the outcome event. All variables with P-values < 0.2 in the univariate analysis were selected for multivariate logistic regression analysis to further identify independent risk factors for neck LNM. In multivariate logistic regression analysis, variables with P-values < 0.2 were identified as independent risk factors and then used to construct the nomogram, while variables that were not statistically significant were excluded from nomogram development.

2.4. Construction and Validation of the Nomogram

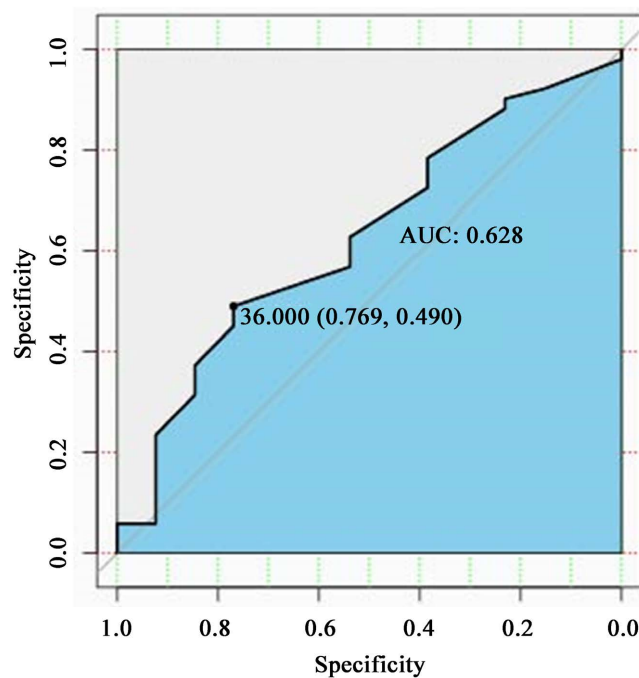A nomogram is a quantitative tool used to predict the probability of neck LNM

Figure 1. ROC curves for the cut-off value of tumor diameter. ROC curves were plotted with the maximum tumor diameter as the independent variable. The value corresponding to the largest area under the curve is the best cut-off value, 36 mm. AUC = 0.628.

before treatment in patients with HSCC. Independent risk factors identified in the multivariate logistic regression analysis were incorporated into the predictive model to construct the nomogram by using the rms package of R software. In the nomogram, the regression coefficients for each independent risk are scaled to a specific number in the 0 - 100 point range. To evaluate the discrimination of the nomogram, bootstrap validation (1000 bootstrap replicates) was performed based on the training cohort. The discrimination and accuracy of the column line graphs were assessed by plotting internal and external ROC curves and calibration curves, respectively. Clinical usefulness was assessed by plotting clinical decision analysis (DCA) curves.

3. Results

3.1. Clinicopathological and Biological Features of the Study Population

Table 1 shows basic information and pathological characteristics and biological information related to immunohistochemical staining results of HSCC patients in the training cohort and validation cohort. The pathological type of all patients was squamous cell carcinoma. The neck LNM rate was 79.7% (n = 51) in the training cohort and 73.6% (n = 39) in the validation cohort. The percentage of patients in the training cohort who had larger tumor diameters (≥3.60 cm) was 43.8% (n = 28). The percentage of those with well differentiation type was 45.3% (n = 29) and the percentage of those with poor differentiation type was 54.7% (n = 35). In the training cohort, the percentage of highly expressed stmn1 and Ki67 were 75% and 67.2%, respectively. 56.2% patients' lymphatic vessels and 40.6% patients' blood vessels stained by D2-40 and CD31 respectively were with tumor embolus. All the information apart from tumor cell differentiation, showed no statistically significant differences (P value >0.05) between the training cohort and the validation cohort.

3.2. Risk Factor Screening and Model Construction for LNM

The detailed results of the univariate analysis and multivariate logistic regression analysis are shown in Table 2. In univariate analysis, five variables including tumor cell differentiation, maximum tumor diameter in CT, LNM status in CT, lymphatic vessel invasion status, and STMN1 expression level showed P values less than 0.2 and were selected for multivariate logistic regression analysis. In multivariate logistic regression analysis, maximum tumor diameter ≥ 3.6 cm (P = 0.007), poor differentiation (P = 0.008), high expression of STMN1 (P = 0.052), lymphatic vessel invasion (P = 0.013), and occurrence of LNM in CT (P = 0.01) were confirmed as the associated risk factors for neck LNM of HSCC. Finally, the above independent factors were used to develop a nomogram (Figure 2) for predicting the neck LNM of patients with HSCC. No multicollinearity in the nomogram was found. Another prediction model that predicts LNM based only on LNM status in CT was also developed.

3.3. Internal Validation of Nomogram and Comparison with the CT-Based Prediction Model

While performing internal validation, the differences between the nomogram and the CT-based prediction model in terms of discrimination, accuracy and

Table 1. The clinicopathology and biological characteristics of the patients.

	All patients (N = 117)	Training cohort (N = 64)	Validation cohort (N = 53)	P value
Age (year) (Median, QR)	60.0[55.0; 64.0]	61.0 [55.0; 65.0]	60.0[56.0; 63.0]	0.331
Gender				0.138
Female	8 (6.84%)	2 (3.12%)	6 (11.3%)	
Male	109 (93.2%)	62 (96.9%)	47 (88.7%)	
Tumor cell differentiation				0.013
Well differentiation	66 (56.4%)	29 (45.3%)	37 (69.8%)	
Poor differentiation	51 (43.6%)	35 (54.7%)	16 (30.2%)	
Maximum tumor diameter in CT				0.498
<3.6 cm	70 (59.8%)	36 (56.2%)	36 (56.2%)	
≥3.6 cm	47 (40.2%)	28 (43.8%)	28 (43.8%)	
LNM status in CT				0.254
No	41 (35.0%)	19 (29.7%)	22 (41.5%)	
Yes	76 (65.0%)	45 (70.3%)	31 (58.5%)	
Ki67 expression level				0.436
Low	43 (36.8%)	21 (32.8%)	21 (32.8%)	
High	74 (63.2%)	43 (67.2%)	31 (58.5%)	
STMN1 expression level				0.135
Low	37 (31.6%)	16 (25.0%)	21 (39.6%)	
High	80 (68.4%)	48 (75.0%)	32 (60.4%)	
Vascular invasion status				1.000
No	69 (59.0%)	38 (59.4%)	31 (58.5%)	
Yes	48 (41.0%)	26 (40.6%)	22 (41.5%)	
Lymph vessels invasion status				1.000
No	51 (43.6%)	28 (43.8%)	23 (43.4%)	
Yes	66 (56.4%)	36 (56.2%)	30 (56.6%)	
LNM in pathology				0.576
No	27 (23.1%)	13 (20.3%)	14 (26.4%)	
Yes	90 (76.9%)	51 (79.7%)	39 (73.6%)	

QR: Quartile Range; CI: confidence interval CT: Computed tomography scan; STMN1: Stathmin1; LNM: Lymph node metastasis.

Table 2. Univariate and multivariate analyses for screening risk factor.

	Univariate analysis		Multivariate analysis	
	Odd Ratio (95%CI)	P value	Odd Ratio (95%CI)	P value
Age	0.99 (0.91, 1.07)	0.7		
Gender				
Female				
Male	$7 * 10^7$ (0.00, NA)	>0.9		
Tumor cell differentiation				
Well differentiation				
Poor differentiation	3.49 (0.99, 14.3)	0.061	31.9 (3.47, 672)	0.008
Maximum tumor diameter in CT				
<3.6 cm				
≥3.6 cm	3.21 (0.86, 15.6)	0.1	46.7 (4.24, 1272)	0.007
LNM status in CT				
No				
Yes	5.82 (1.63, 22.9)	0.008	21.5 (2.72, 327)	0.01
Ki67 expression level				
Low				
High	2.06 (0.58, 7.24)	0.3		
STMN1 expression level				
Low				
High	5.44 (1.49, 21.0)	0.011	7.11 (1.08, 66.1)	0.052
Vascular invasion status				
No				
Yes	2.74 (0.74, 13.3)	0.2		
Lymph vessels invasion status				
No				
Yes	6.11 (1.63, 29.9)	0.012	23.6 (2.69, 470)	0.013

CI: confidence interval; CT: Computed tomography scan; STMN1: Stathmin1; LNM: Lymph node metastasis.

clinical usefulness were compared. The results showed that the area under the ROC curve (AUC) (Figure 3(a)) of the nomogram was larger than that of the CT-based prediction model, which indicated the higher discrimination of the nomogram. The AUC = 0.916 (95% CI, 0.833 - 1.000), and the sensitivity and specificity for the diagnosis of neck LNM were 82% and 84%, respectively, which were significantly higher than those of the CT-based prediction model, which
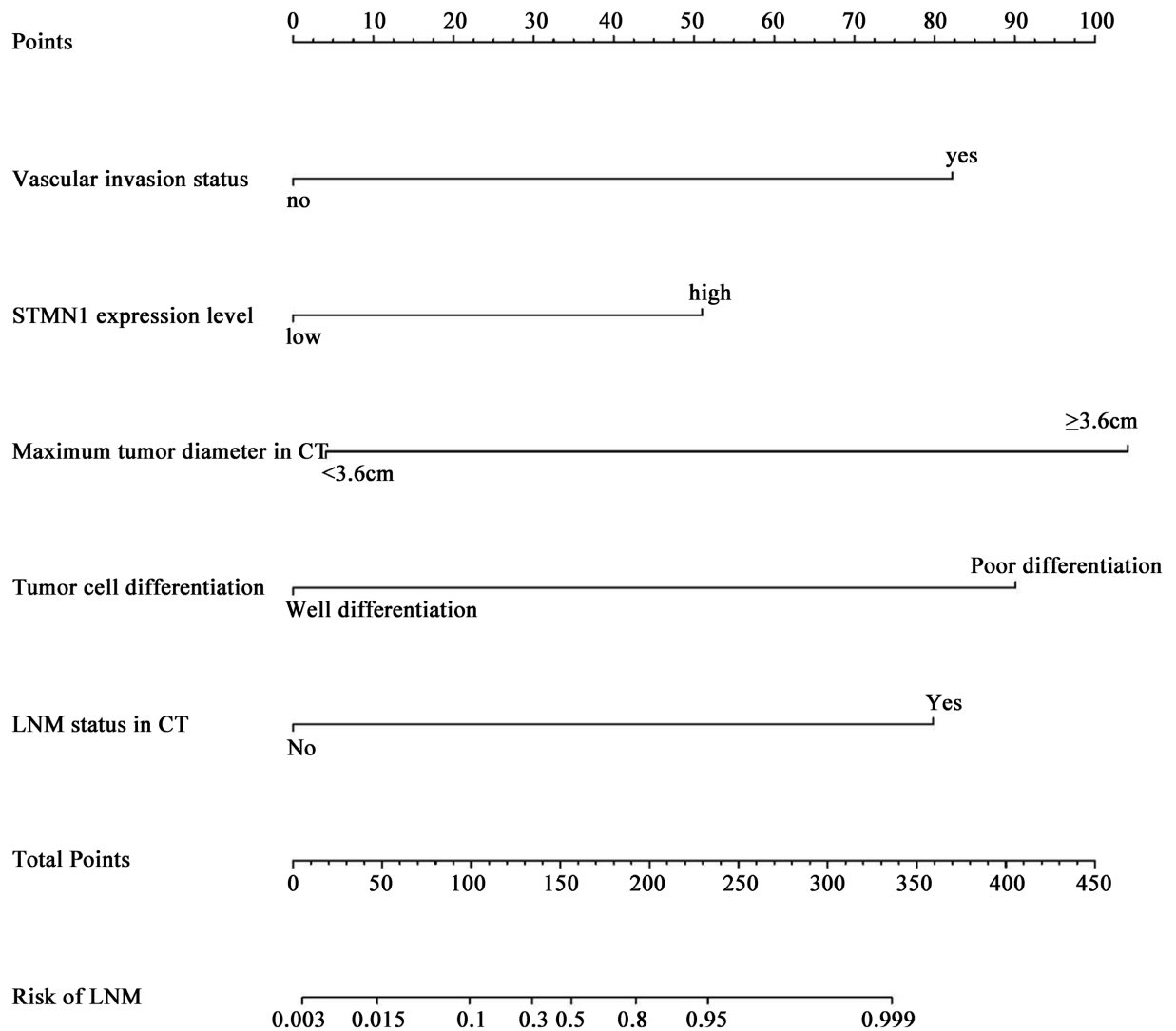
Figure 2. The nomogram based on clinicopathological and biological features. The nomogram for predicting the probability of neck LNM in patients with HSCC. The "total points" of a certain patient are calculated by adding all the points of each of the five predictors. Based on the total points, the risk of neck LNM is obtained.

had an AUC = 0.7 (95% CI, 0.551 - 0.849) and sensitivity and specificity of 78% and 61%, respectively. At the same time, the calibration curves (**Figure 3(b)**) reflected a high degree of consistency between model predictions and actual observations, indicating that the nomogram has a high degree of accuracy.

In addition, the potential clinical effects of the nomogram were tested using clinical DCA curves (**Figure 3(c)**), and the results showed that the nomogram had high positive net benefits in almost all threshold probabilities and much higher net benefits than the CT prediction model. Meanwhile, we also performed internal validation of the nomogram using 1000 bootstraps resamples to calculate the C-index. The value remained at 0.916 after bootstrap resampling, and the result was consistent with a high AUC, indicating that this nomogram could distinguish whether or not a patient would develop neck LNM in more than 90% of HSCC patients. Taken together, the results of the internal validation
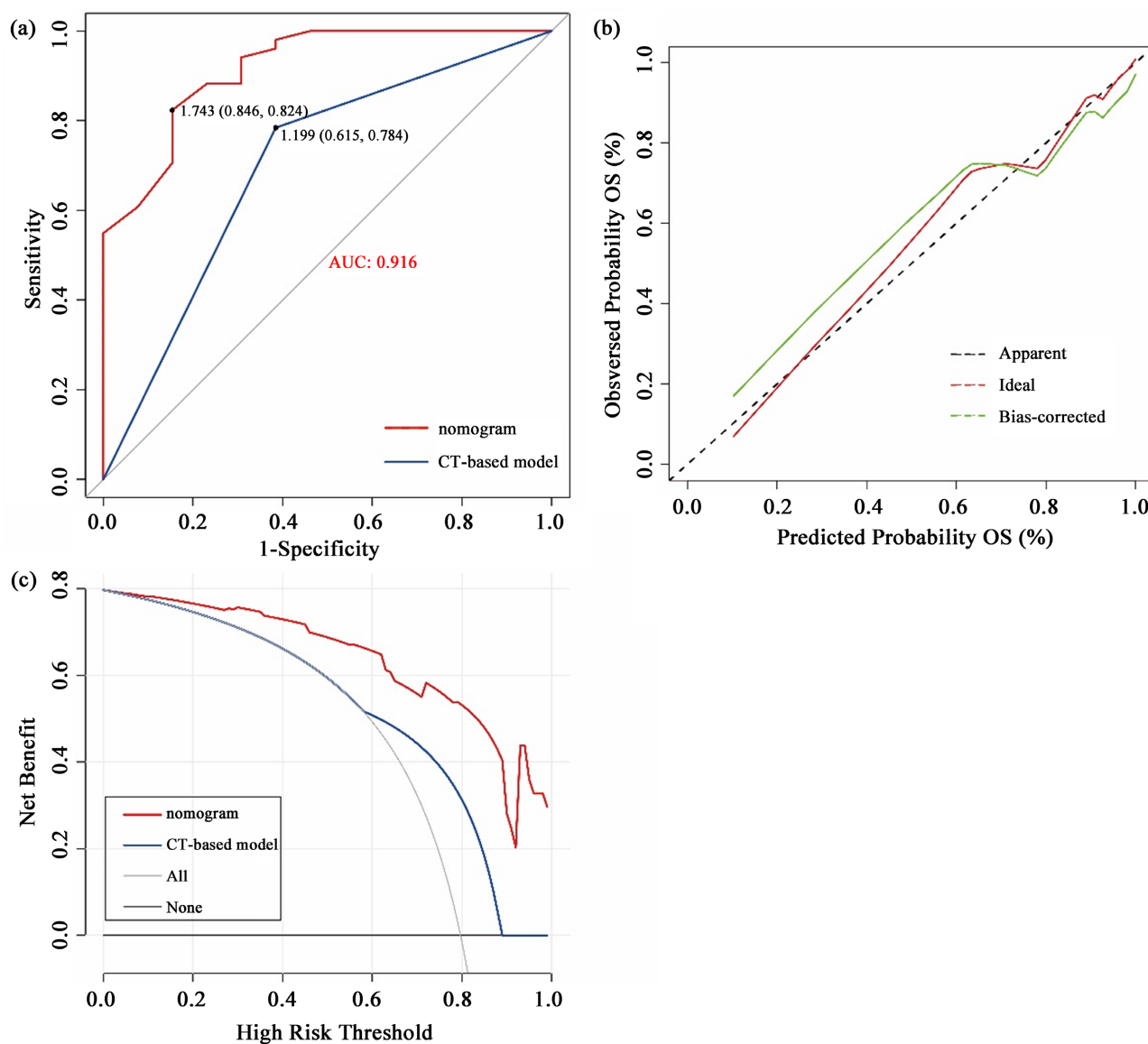
Figure 3. Internal validation including calibration curve, bias-corrected line, and DCA curves. (a) The red line and blue line represent the ROC curves of the nomogram and the CT-based prediction model in the training cohort, respectively. The value of the red line AUC = 0.916 (95% CI 0.833 - 1.000), which is higher than the green line AUC = 0.700 (95% CI 0.551 - 0.849). (b) The calibration curve of the nomogram; the logistic calibration curve was close to the ideal curve. (c) The red line and blue line represent DCA curves of the nomogram and CT-based prediction model in the training cohort, respectively. The curve labeled "all" assumes that all patients underwent neck lymph node dissection. The curve marked as "none" assume that no patients underwent neck lymph node dissection. The X-axis shows different thresholds, while the Y-axis represents the net benefit. Net benefit was calculated by summing the true positives and subtracting the false positives.

indicated good discrimination, accuracy, and clinical usefulness of the nomogram.

3.4. External Validation of the Nomogram

After the internal validation of the nomogram, external validation was performed in an independent validation cohort. The results showed that the AUC = 0.928 (95% CI, 0.864 - 1.000), and the sensitivity and specificity to diagnose neck

LNM of HSCC were 92% and 93%, respectively (**Figure 4(a)**). In addition, calibration curves (**Figure 4(b)**) displayed good consistency between the actual neck LNM status and the estimated probabilities of LNM. Its clinical usefulness is shown in the DCA curve (**Figure 4(c)**) with a high positive net benefit in almost all threshold probabilities. All of these results of the external validation further indicated the nomogram might be used for neck LNM prediction of HSCC to guide the lymph node dissection and irradiation.

4. Discussion

Currently, imaging examinations such as computed tomography are one of the
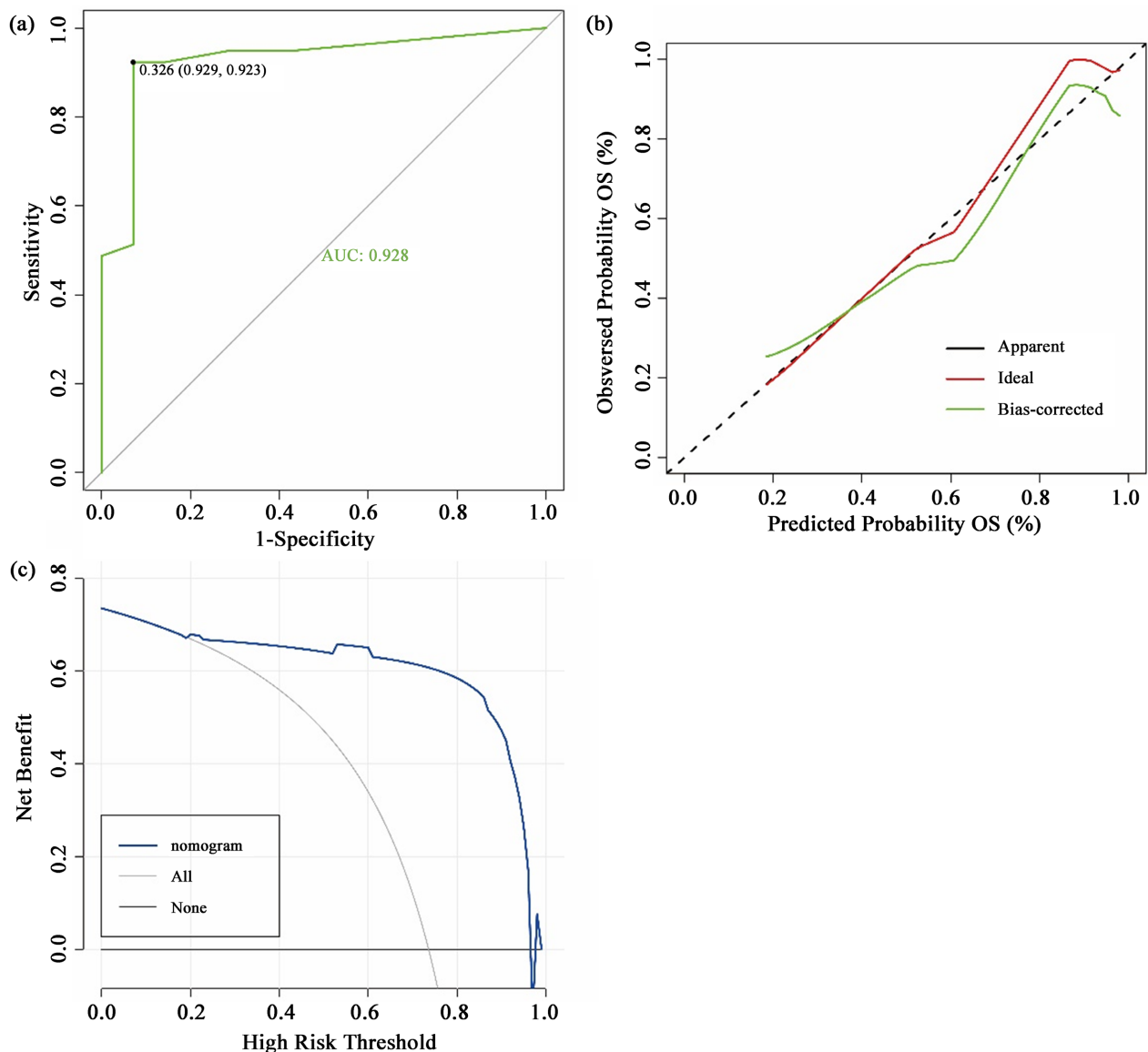

Figure 4. External validation including calibration curve, bias-corrected line, and DCA curves. (a) ROC curve of the external validation with AUC = 0.928 (95% CI 0.864 - 1.000). (b) The calibration curve showed that the apparent line and ideal line have good consistency, and are close to the bias-corrected line. (c) The DCA curves of the external validation and nomogram provided a good net benefit in the validation cohort.

main methods used by physicians to determine LNM in the neck before surgery, and all of the patients with HSCC included in this study underwent CT examinations of the head and neck before surgery. The results are noteworthy that 66% of imaging N0 cases were truly negative, Its sensitivity for diagnosing LNM is 78% and its specificity is 61%. The tendency to extensive neck LNM is the most important clinical feature of HSCC, which influences the prognosis and leads to poor survival of HSCC [1]. Neck lymph node dissection and irradiation make HSCC survivors suffer great pain. To effectively predict neck LNM and reduce unnecessary neck lymph node dissection and irradiation will improve the quality of life of HSCC survivors. As the neck LNM of HSCC is associated with many clinicopathological and biological molecular factors [8], no single factor could be used to predict neck LNM in clinical practice at present. Therefore, a combination of these factors to predict neck LNM of HSCC would be more effective. A nomogram provided us with a good option. Such an attempt had not been performed for the prediction of neck LNM of HSCC before and was worthy of being explored.

Univariate analysis and multivariate analysis were used to screen for independent risk factors of neck LNM in HSCC. Finally, maximum tumor diameter in CT, tumor cell differentiation, STMN1 expression level, lymphatic vessel invasion, and LNM status in CT were confirmed as the independent risk factors, which had been also widely reported to be associated with LNM [18] [19] [20] [21] [22]. Then, we developed and validated a clinicopathological and biological molecular features-based nomogram for the prediction of neck LNM in HSCC patients.

In these five independent risk factors screened, CT is the most commonly-used method to determine neck LNM of HSCC for physicians. All patients in this study underwent CT examinations of the head and neck before surgery. LNM status in CT was an important reference for guiding treatment decisions. Misa *et al.* found that the sensitivity of CT diagnosis of neck LNM was 68%, and the specificity was 79% in head and neck squamous cell carcinomas (HNSCC] [23]. This is consistent with our result that it is not accurate to determine LNM based only on CT examination. Maximum tumor diameter in CT was another risk factor for constructing our nomogram. The probability of neck LNM increased with the size of the primary tumor [18] [24], and in this study, patients with tumors with a maximum diameter ≥ 3.6 cm had a higher probability of neck LNM than those with tumors < 3.6 cm. STMN1, also known as oncoprotein 18 and included in the nomogram, is a cytoplasmic protein, controlling the dynamic balance of the microtubule system, influencing cell mitosis and regulating cell cycle progression, which in turn affects tumor cell proliferation and motility [25] [26] [27]. It has been widely reported that STMN1 is overexpressed in many different human cancers, and that it is closely associated with LNM [21] [22] [28] [29] [30]. In hepatocellular carcinoma, STMN1 induces HSC activation by triggering the HGF/MET signaling pathway, thereby promoting liver tumor migration and growth, distant metastasis, and recurrence [22]. In cholangiocarci-

noma, Watanabe, *et al.* found that knockdown of STMN1 significantly affected the proliferation and migration of tumor cells and increased the sensitivity of tumor cells to chemotherapeutic agents [31]. In 2020, Cao, S., Zhang, W. *et al.* retrospectively analyzed eight studies that included a total of 1240 patients with esophageal cancer for meta-analysis and found that patients with high STMN1 expression had a significantly higher risk of LNM than those with low STMN1 expression [32], further proving that high STMN1 expression is a risk factor for tumor susceptibility to LNM. In this study, we also found that HSCC patients with high STMN1 expression (Figure 5(a), Figure 5(b)) were more likely to develop neck LNM than those with low STMN1 expression. Tumor cell differentiation was a pathological risk factor for LNM [30] [33]. Poorer differentiation indicated a higher degree of tumor malignancy and a higher probability of LNM [19]. Lymphatic vessels constitute one of the most important pathways for the metastasis of solid tumors [20], and lymphatic vessel invasion indicates a high possibility of LNM. Ultimately, we applied CD31 for endothelial staining of blood vessels and showed that the presence or absence of tumor embolus in blood vessels was not statistically significant in relation to LNM in the neck. We compared the studies that have been used and found that quantitative assessment of CD31 staining results and calculation of MVD was more likely to lead to positive conclusions [14]. Instead, we used a qualitative assessment, which is the most common assessment method used by pathologists, thus raising a new question: clarifying MVD for CD31-stained sections may be more helpful for the treating physician to have a more accurate diagnosis of the patient. Ki67 is one
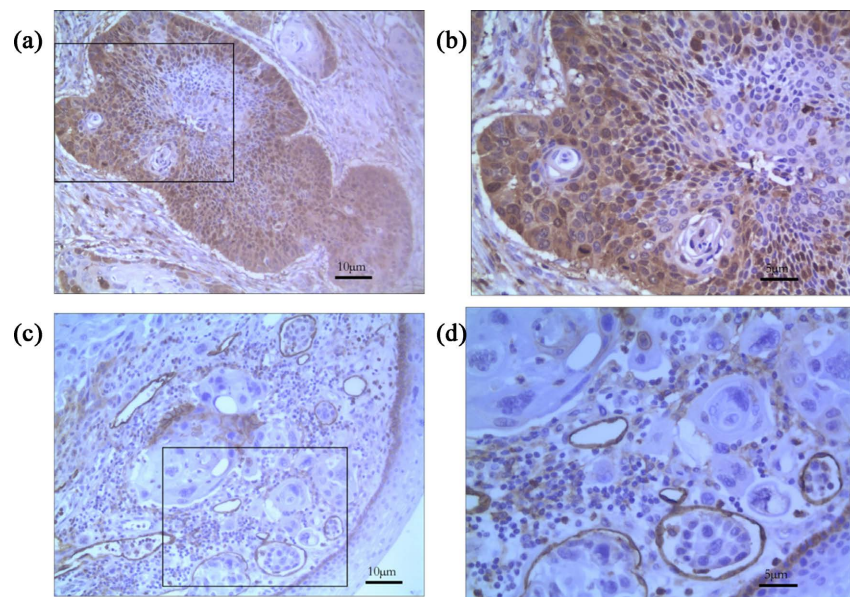

Figure 5. Representative immunohistochemical staining figures. (a) Under 200 times magnification, large areas of tumor cells are stained brown by the anti-STMN1 antibody. (b) Under 400 times magnification, the stmn1 protein can be seen in the cytoplasm of tumor cells. (c) Under 200 times magnification, brown-stained lymphatic vessels can be seen. (d) When magnified 400 times, tumor emboli can be seen in stained lymphatic vessels.

of the widely used cell proliferation indices, and the results of studies on its ability to predict LNM in head and neck tumors vary widely [11] [12] [13]. The analysis showed no obvious correlation with LNM in the neck of patients with HSCC, and it was ultimately not included in the nomogram. Finally, this study proved that patients with lymphatic vessel invasion (Figure 5(c), Figure 5(d)) had a significantly higher probability of LNM than patients without lymphatic vessel invasion.

While the five independent risk factors were screened and the nomogram was developed, a prediction model for LNM based only on CT was also constructed. During internal validation, the nomogram showed higher discrimination and better clinical usefulness than the CT-based prediction model. CT criteria for the assessment of neck LNM are limited by factors such as the size and shape of the lymph nodes, and the presence of central necrosis [34]. Among these, central necrosis is considered a biologically late-stage event in the evolution of tumors in lymph nodes [35], and is not easily visible in smaller diameter lymph nodes. In other words, early changes in tumor progression cannot be easily identified by CT, and may be manifested in ways including lymphovascular infiltration, EMT, and expression of some biomolecules. It has been confirmed that epithelial-mesenchymal transition plays a key role in tumor progression. The superior performance of this nomogram may be due to the inclusion of these clinicopathological characteristics and biological molecular features, which include the risk factors for early and late events of LNM to greatly improve the power of risk prediction for neck LNM in patients with HSCC.

Furthermore, an independent validation cohort was used for external validation and the results confirmed that the sensitivity and specificity of the nomogram in predicting neck LNM of HSCC reached 92% and 93%, respectively. In terms of clinical usefulness, the nomogram performed well in distinguishing LNM and could offer a net benefit over the "treat-all" or "treat-none" strategy within a range of threshold probabilities.

Of course, the nomogram has some limitations. First, this study is a retrospective design and a prospective study is needed to validate our results. Second, the sample numbers meet minimum sample requirements and we performed a covariance analysis and the results showed that there was no possibility of overfitting, however, data validation with multiple samples is still needed before clinical application.

5. Conclusion

In summary, the nomogram developed in this study is the first attempt to combine the clinicopathological and biological molecular features to predict neck LNM of HSCC, which overcomes the previous limitation of determining neck LNM of HSCC patients based on imaging examination alone. These gave the nomogram strong predictive power and could be used to predict neck LNM of HSCC and guide neck lymph node dissection and irradiation to improve the

treatment effect for HSCC patients. Moreover all risk factors including this nomogram are easily and conveniently available for HSCC patients through routine examinations and sample IHC staining, making the nomogram easy to apply in clinical practice.

Acknowledgements

Data Availability

The datasets analyzed in the current study are available from the corresponding authors on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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