

# High expression of GPNMB predicts poor prognosis in head and neck squamous cell carcinoma

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**Summary.** Glycoprotein non-metastatic protein B (GPNMB) is a transmembrane glycoprotein that is highly expressed in several malignancies compared with its expression in matched healthy tissues. The aim of this study was to investigate the clinical characteristics and prognostic value of GPNMB expression in tumor tissue derived from a cohort of patients with head and neck squamous cell carcinoma (HNSCC). GPNMB expression in human HNSCC, oral dysplasia and normal mucosal tissue was evaluated by immunohistochemistry (IHC). The correlations of GPNMB expression with the clinical characteristics of HNSCC were assessed by one-way ANOVA and *t* test analyses. Survival data were analyzed using Kaplan-Meier analysis and the Cox proportional hazards model. GPNMB was highly expressed in HNSCC tissue compared with dysplasia and normal mucosal tissue. Additionally, a high level of GPNMB expression in HNSCC was associated with poor prognosis ( $P < 0.01$ ). In the analysis of tumor-node-metastasis (TNM) staging, a high GPNMB expression level in HNSCC tissue, as well as metastatic lymph node tissue, correlated with an advanced N stage. In conclusion, GPNMB was overexpressed in human HNSCC tissue and predicted poor prognosis in human HNSCC tissue. In addition, GPNMB expression was closely correlated with N stage in patients with HNSCC.

**Key words:** GPNMB, Head and neck squamous cell carcinoma, Prognosis, TNM staging

## Introduction

Head and neck squamous cell carcinoma (HNSCC) is the cause of death for 600,000 people per year worldwide and its 5-year survival rate remains approximately 50% (Pulte and Brenner, 2010; Ferlay et al., 2015). The tumor develops in epithelial cells including the mucosal linings of the upper airway and food passages including the oral cavity, oropharynx, larynx and hypopharynx. HNSCC is characterized as one of the more aggressive human malignancies with a high incidence of regional lymph node metastasis in the head and neck (Iizuka et al., 2014). It results in a high mortality and poor prognosis. Moreover, the frequent recurrence of HNSCCs after treatment is closely associated with invasive and metastatic abilities (Iizuka et al., 2014). However, the mechanism of lymph node metastasis remains unclear (Leemans et al., 2011).

Glycoprotein non-metastatic gene B (GPNMB), also known as osteoactivin or dendritic cell-heparin integrin ligand, is a type I transmembrane glycoprotein. It was initially termed glycoprotein non-metastatic gene B (NMB) and was first cloned from a melanoma cell line with low metastatic potential that expressed GPNMB protein at high level (Weterman et al., 1995). It has been observed that GPNMB expression is elevated in various forms of cancer and correlated with patient's prognosis including ovarian cancer (Ma et al., 2018), melanoma (Tse et al., 2006), small cell lung cancer (Li et al., 2014),

breast cancer (Rose et al., 2010). *In vivo*, GPNMB glycoprotein is involved in many physiological processes, including mediating the transport of late melanosomes to keratinocytes, and regulating osteoblast and osteoclast differentiation and function (Maric et al., 2013). Several research studies have shown that upregulated GPNMB expression was associated with increases in invasion and metastasis (Rich et al., 2003; Maric et al., 2013; Fiorentini et al., 2014; Oyewumi et al., 2016).

Previous studies have shown that GPNMB is highly expressed in several HNSCC cell lines and confers invasion and migration potential (Arosarena et al., 2016). Additionally, it has been reported that GPNMB may promote HNSCC cell invasiveness by influencing the expression of matrix metalloproteinases (MMPs) (Arosarena et al., 2018). However, the correlation between GPNMB expression and metastasis in HNSCC and the prognostic value of GPNMB in patients with HNSCC are still unclear. In this study, we revealed the clinical characteristics and prognostic value of GPNMB expression in a cohort of HNSCC patients.

## Materials and methods

### *Human specimens*

Full ethical approval was granted by the School and Stomatology of Wuhan University Medical Ethics Committee (PI: Zhi-Jun Sun; 2014LUNSHENZI06) to use patient samples in this study. Human HNSCC specimens were derived from the Department of Oral and Maxillofacial Surgery, School and Hospital of Stomatology Wuhan University. The tissue microarrays (TMAs) (T12-412-TMA2, T15-411 and T17-790) were previously described (Wu et al., 2018). The TMAs were totally constructed from 42 normal oral mucosae, 69 oral epithelial dysplasia, 210 tumor tissues from primary HNSCC patients, 25 tumor tissues from recurrent HNSCC patients, 15 tumor tissues from HNSCC patients who received pre-surgical radiotherapy, 20 tumors tissue from HNSCC patients who received pre-surgical TPF (docetaxel, cisplatin and fluorouracil) inductive chemotherapy and 35 paired metastatic lymph node tissues from the primary HNSCC patients. All samples were fixed in formalin and embedded in paraffin. The clinical features, including tumor-node-metastasis (TNM) staging, histological grade and overall survival were available for all cases. The clinical stage of the HNSCC was classified according to the guidelines of the Union for International Cancer Control (8th edition). The histologic classification was diagnosed according to the World Health Organization Classification of Head and Neck Tumours. For all collected tissue samples, H&E slides were reviewed by two independent pathologists. The clinicopathologic characteristics and follow-up data for these patients were retrieved from the electronic medical records. Human papillomavirus (HPV) infection status was diagnosed by p16

immunostaining and the HPV DNA in situ hybridization technique.

### *TMA immunohistochemical staining*

The TMAs were baked at 60°C for 2 hours, deparaffinized in xylene, and rehydrated in decreasing concentrations of ethanol. Then, tissue sections were incubated in citrate buffer (pH 6) for 5 mins at high pressure for antigen retrieval. The sections were subsequently incubated in 3% hydrogen peroxide and in serum-free protein block solution. The primary antibody for GPNMB (1:500, Cell Signaling Technology, USA) was incubated with the samples at 4°C overnight. After signal amplification using a biotinylated-secondary antibody and streptavidin-horseradish peroxidase, chromogenic development was performed using 3,3'-diaminobenzidine (DAB) for 1-2 min. The slides were counterstained with hematoxylin, mounted with a glycerol-based mounting medium and scanned for digital imaging (Aperio ScanScope CS scanner). The data were processed with background subtraction and white balance.

### *Histological quantification and survival analyses*

The positive pixel immunohistochemical staining in membranes, nuclei or cytoplasm in HNSCC tumor cells was quantified by Aperio Quantification software (Positive Pixel Count v9). Using these data, the histoscore was subsequently generated by the following formula: [1×(the percentage of weakly positive staining)] + [2×(the percentage of moderately positive staining)] + [3×(the percentage of strongly positive staining)]. All data are presented as the mean values ± SEM. The statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA). Statistical significance was assessed by t-test or one-way ANOVA followed by Tukey's multiple comparison test. In univariate analysis, overall survival was estimated using Kaplan-Meier curves. The log-rank test was used to estimate the survival differences. The Cox proportional hazards model was used for multivariate analysis to assess the significance of overall differences. The statistical significance level for all comparisons was set at P<0.05.

## Results

*The GPNMB protein was highly expressed in human HNSCC tissue and was significantly correlated with reduced overall survival*

To explore the expression level of *GPNMB* in human HNSCC tissue, the OncoPrint™ Platform was applied as a tool to compute gene expression features and clusters. According to the analyses of the six datasets, *GPNMB* mRNA was expressed at higher levels in human HNSCC compared with matched normal mucosa (Fig. 1, P<0.001).

The prognostic value of GPNMB expression in HNSCC

Next, we stained HNSCC TMAs with GPNMB antibody to investigate whether GPNMB was expressed in human HNSCC tissue. As shown in Fig. 2A, representative tumor and mucosa tissue was stained with hematoxylin-eosin. Immunostaining revealed that GPNMB staining was mainly found in the cytoplasm of tumor cells (Fig. 2B). The immunostaining of GPNMB in HNSCC tumor cells was obviously stronger than the immunostaining in normal mucosa cells. However, the level of GPNMB immunostaining was variable in different HNSCC cells (Fig. 2B). Additionally, the statistical analysis showed that GPNMB was expressed at higher levels in primary HNSCC tissue (n=210) compared with dysplasia (n=69) and normal mucosa tissue (n=42) (Fig. 2C, P<0.001).

Then, to determine the value of GPNMB immunoexpression in prognosis, we analyzed the follow-up data of the patients; 201 of the 210 cases had follow-up data. The median histoscore was used as the cut-off. As shown in Fig. 2D, the Kaplan-Meier analysis demonstrated that the high expression levels of GPNMB were significantly correlated with poor overall survival in patients with HNSCC (P=0.0020). A multivariate Cox proportional hazard model used variables that had been determined to be prognostically significant by univariate analysis, including gender, age, pathological grade, tumor size, lymph node stage, smoking status, alcohol consumption and GPNMB expression level. The results indicated that a high expression level of GPNMB was a predictor of reduced survival (Table 1, P=0.028).

GPNMB was significantly associated with N stage in HNSCC patients

GPNMB immunoexpression was analyzed to identify correlations with the clinicopathologic

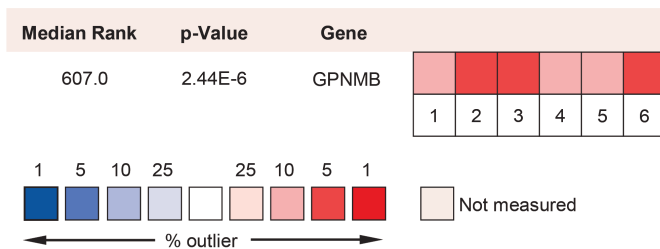
characteristics of the patients. Previous studies have demonstrated that GPNMB promoted invasive ability in HNSCC cell lines (Arosarena et al., 2016, 2018). In terms of TNM staging, we found that immunostaining of GPNMB in tumor cells of patients with N2 or N1 stage was obviously stronger than the immunostaining in tumor cells of patients with N0 stage (Fig. 3A). Statistical data also demonstrated that high GPNMB expression in the primary site was associated with an advanced N stage in HNSCC tissue (Fig. 3B, P<0.01). Interestingly, the analysis of the expression of GPNMB in metastatic lymph nodes indicated that a high

Table 1. Multivariate analysis for overall survival in primary HNSCC patients.

Parameters	HR (95%CI)	P value
Gender	0.931 (0.416-2.083)	0.826
Age	1.844 (0.986-3.451)	0.056
Smoking	1.402 (0.667-2.947)	0.373
Drinking	0.872 (0.433-1.758)	0.702
Pathological grade		
II vs. I	18.994 (2.554-141.240)	0.004*
III vs. I	13.987 (1.778-110.051)	0.012*
Tumor size		
T2 vs. T1	1.135 (0.452-2.855)	0.787
T3 vs. T1	1.709 (0.629-4.641)	0.293
T4 vs. T1	2.281 (0.745-6.988)	0.149
Node stage		
N1+N2 vs. N0	1.157 (0.647-2.069)	0.622
GPNMB expression	1.986 (1.078-3.657)	0.028*

Cox proportional hazards regression model. HR hazard ration, 95%CI 95% confidence interval. \* P<0.05.

Comparison of GPNMB across 6 dataset



Legend

- 1. Tongue Squamous Cell Carcinoma vs. Normal *Estilo Head-Neck, BMC Cancer, 2009*
- 2. Oral Cavity Carcinoma vs. Normal *Pyeon Multi-cancer, Cancer Res, 2007*
- 3. Tongue Cancer vs. Normal *Pyeon Multi-cancer, Cancer Res, 2007*
- 4. Tongue Squamous Cell Carcinoma vs. Normal *Talbot Lung, Cancer Res, 2005*
- 5. Oral Cavity Squamous Cell Carcinoma Epithelia vs. Normal *Toruner Head-Neck, Cancer Genet Cytogenet, 2004*
- 6. Tongue Squamous Cell Carcinoma vs. Normal *Ye Head-Neck, BMC Genomics, 2008*

Fig. 1. The mRNA expression feature of GPNMB in human head and neck cancer. The six mRNA databases were used to explore the expression features of GPNMB. GPNMB mRNA was found to be highly expressed in HNSCC tissue.

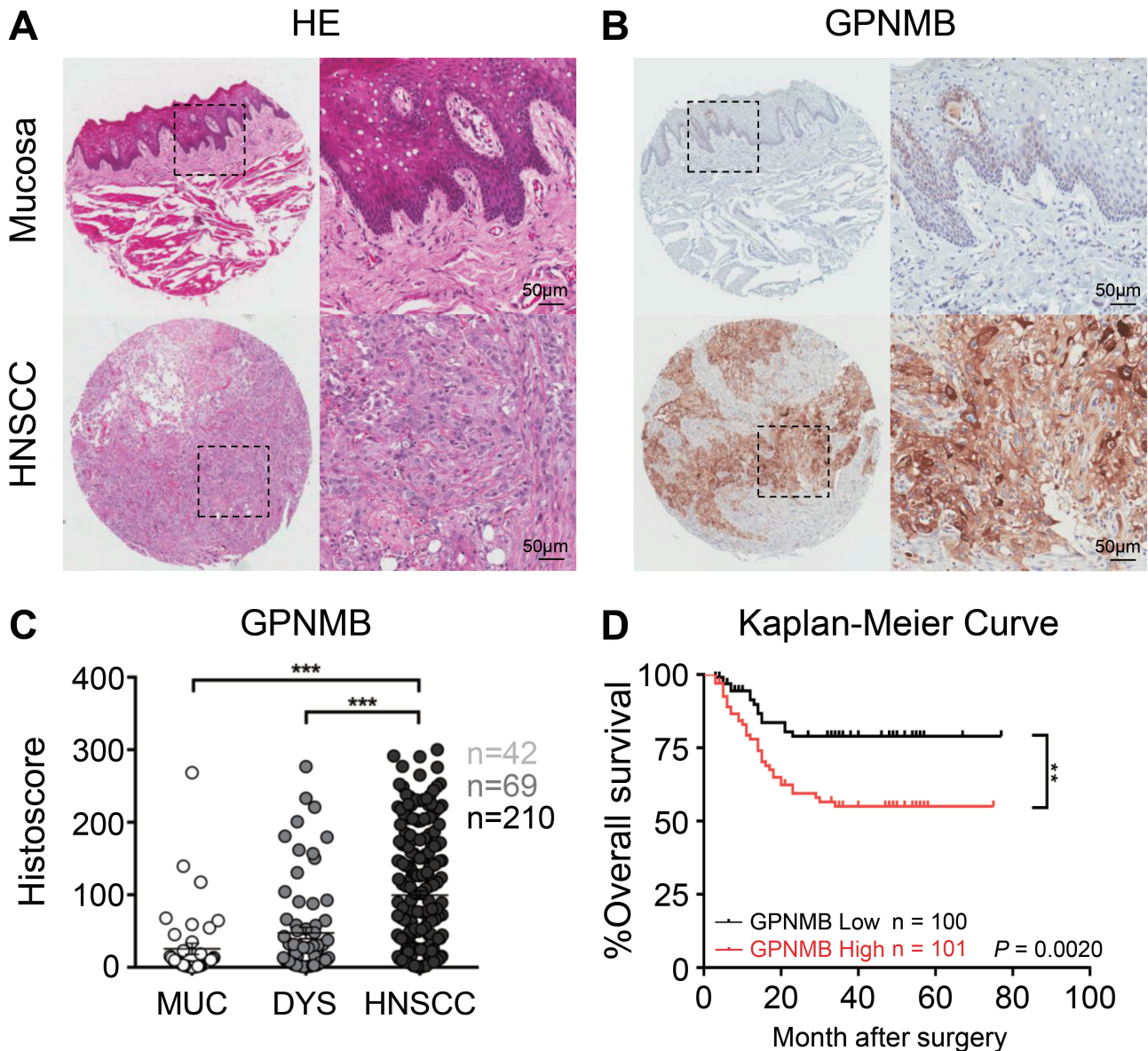
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expression of GPNMB also indicated an advanced N stage in the lymph nodes (Fig. 3C,  $P < 0.01$ ). Upon further analysis, there was no significant correlation between GPNMB expression level and pathologic grade (Fig. 3D,  $P > 0.05$ ) or tumor size (Fig. 3E,  $P > 0.05$ ).

Analyses of correlations between GPNMB expression and clinicopathologic characteristics

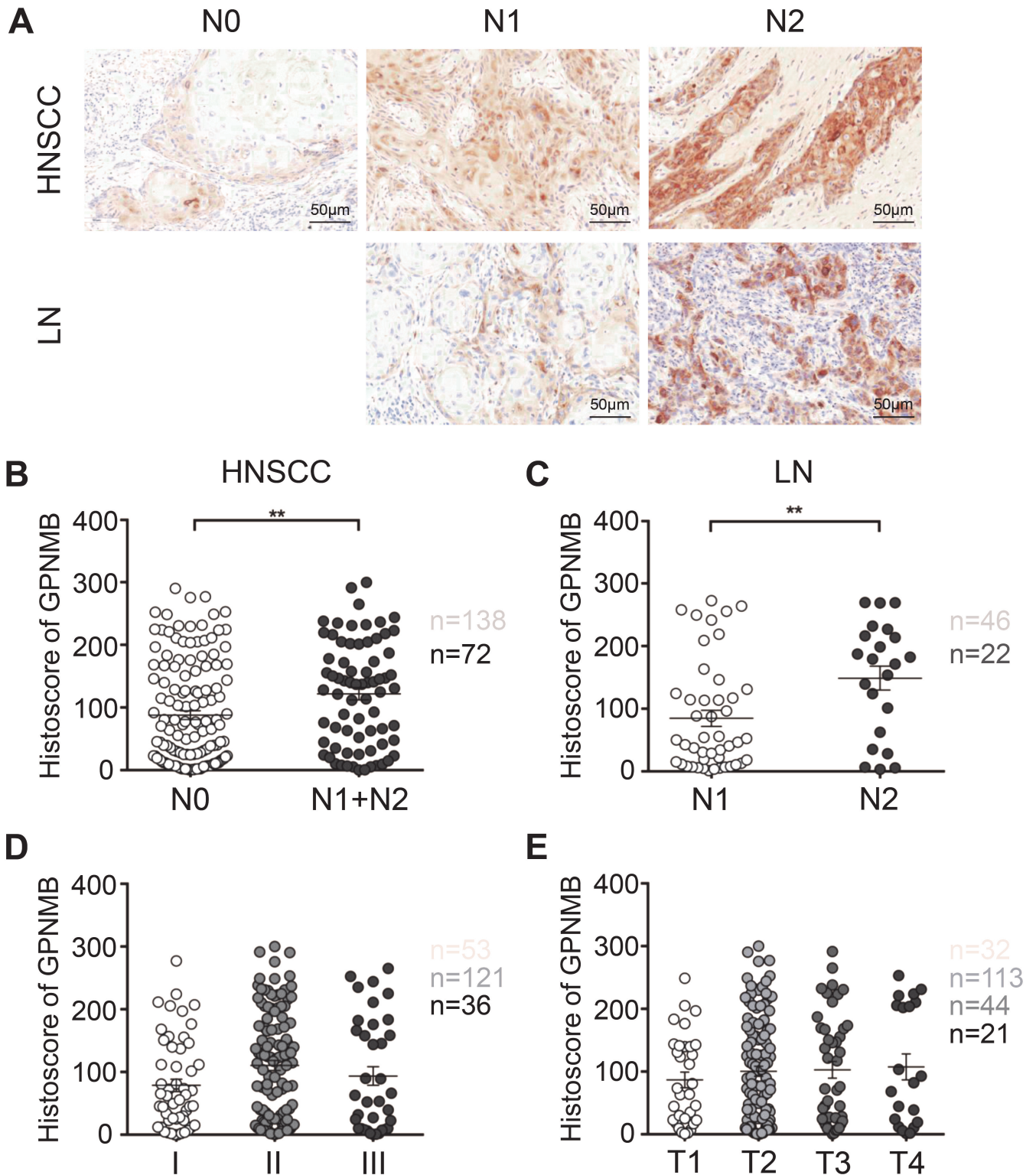
Further analysis revealed no significant difference in GPNMB expression, as detected by immunohisto-

chemistry, between patients' primary site and matched metastatic lymph node ( $n = 35$ ) (Fig. 4A,  $P > 0.05$ ). Additionally, there was no significant difference in GPNMB expression between primary HNSCC tissue ( $n = 210$ ) and recurrent HNSCC tissue ( $n = 25$ ) (Fig. 4B,  $P > 0.05$ ). In regard to a patient's pre-operative radiotherapy and TPF (docetaxel, cisplatin and fluorouracil) statuses, there was no difference in GPNMB expression between the untreated primary HNSCC ( $n = 210$ ) and the pre-operative radiotherapy ( $n = 15$ ) or pre-operative inductive TPF ( $n = 20$ ) groups



**Fig. 2.** High expression levels of GPNMB in human HNSCC correlated with poor prognosis. **A-B.** Representative hematoxylin and eosin (H&E) and immunohistochemical staining of GPNMB in human HNSCC tissue compared with staining of normal oral mucosa. **C.** Quantification of the immunohistochemical histoscores of GPNMB in HNSCC (HNSCC,  $n = 210$ ) compared with the histoscores in dysplasia (DYS,  $n = 69$ ) and normal mucosa (MUC,  $n = 42$ ); Tukey's multiple comparison test,  $***P < 0.001$ . **D.** Kaplan-Meier analysis of survival according to low and high immunoeexpression of GPNMB. The median was used as the cut-off value (cut-off=75.97);  $**P < 0.01$ . Scale bars: 50  $\mu$ m.

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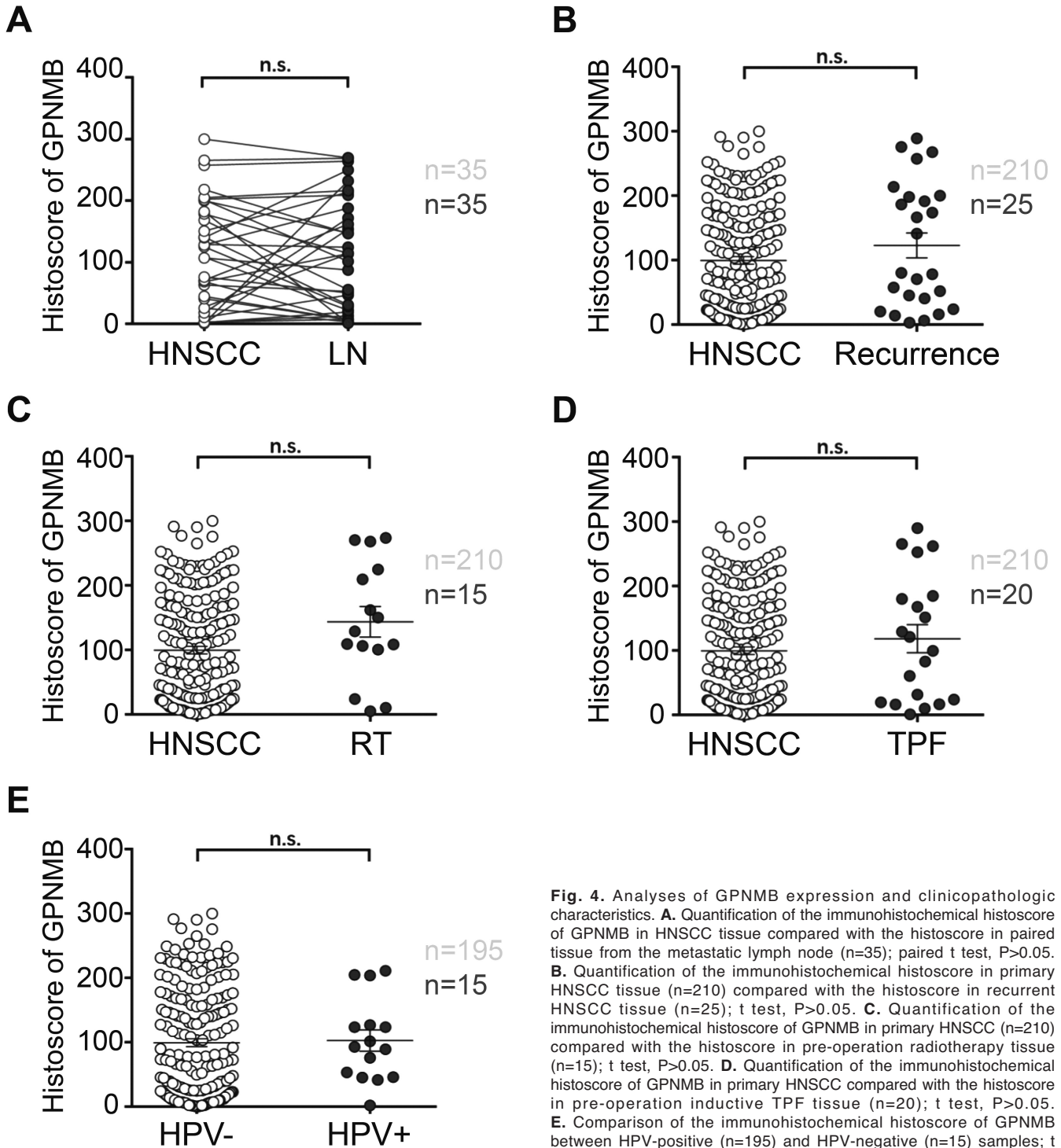


**Fig. 3.** The expression levels of GPNMB in HNSCC tissue and lymph nodes were both correlated with lymph node status. **A.** Representative immunohistochemical staining of GPNMB in human HNSCC tissue and metastatic lymph node tissue from patients in different N stages. **B.** Comparison of the immunohistochemical histoscores of GPNMB between N0 (n=138) and N1 + N2 (n=72) HNSCC tissue; t test, \*\* $P < 0.01$ . **C.** Comparison of the immunohistochemical histoscores of GPNMB between N1 (n=46) and N2 (n=22) lymph nodes; t test, \*\* $P < 0.01$ . **D.** Quantification of the immunohistochemical histoscores of GPNMB in I (n=53), II (n=121) and III (n=36) pathological grade; Tukey's multiple comparison test,  $P < 0.05$ . **E.** Quantification of immunohistochemical histoscores of GPNMB in T1, T2, T3 and T4 stages; Tukey's multiple comparison test,  $P > 0.05$ . Scale bars: 50  $\mu\text{m}$ .

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(Fig. 4C and D, both  $P > 0.05$ ). No significant differences were observed in GPNMB expression between HPV infection-positive ( $n=15$ ) and -negative patients ( $n=195$ )

(Fig. 4E,  $P > 0.05$ ). Furthermore, no significant differences in GPNMB expression were observed between patients with primary HNSCC who smoked



**Fig. 4.** Analyses of GPNMB expression and clinicopathologic characteristics. **A.** Quantification of the immunohistochemical histoscore of GPNMB in HNSCC tissue compared with the histoscore in paired tissue from the metastatic lymph node ( $n=35$ ); paired t test,  $P > 0.05$ . **B.** Quantification of the immunohistochemical histoscore in primary HNSCC tissue ( $n=210$ ) compared with the histoscore in recurrent HNSCC tissue ( $n=25$ ); t test,  $P > 0.05$ . **C.** Quantification of the immunohistochemical histoscore of GPNMB in primary HNSCC ( $n=210$ ) compared with the histoscore in pre-operation radiotherapy tissue ( $n=15$ ); t test,  $P > 0.05$ . **D.** Quantification of the immunohistochemical histoscore of GPNMB in primary HNSCC compared with the histoscore in pre-operation inductive TPF tissue ( $n=20$ ); t test,  $P > 0.05$ . **E.** Comparison of the immunohistochemical histoscore of GPNMB between HPV-positive ( $n=195$ ) and HPV-negative ( $n=15$ ) samples; t test,  $P > 0.05$ . All data are presented as the mean  $\pm$  SEM.

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and those who were nonsmokers (Fig. 5A,  $P>0.05$ ) nor were significant differences in GPNMB expression observed between patients with primary HNSCC who consumed alcohol and those who did not (Fig. 5B,  $P>0.05$ ).

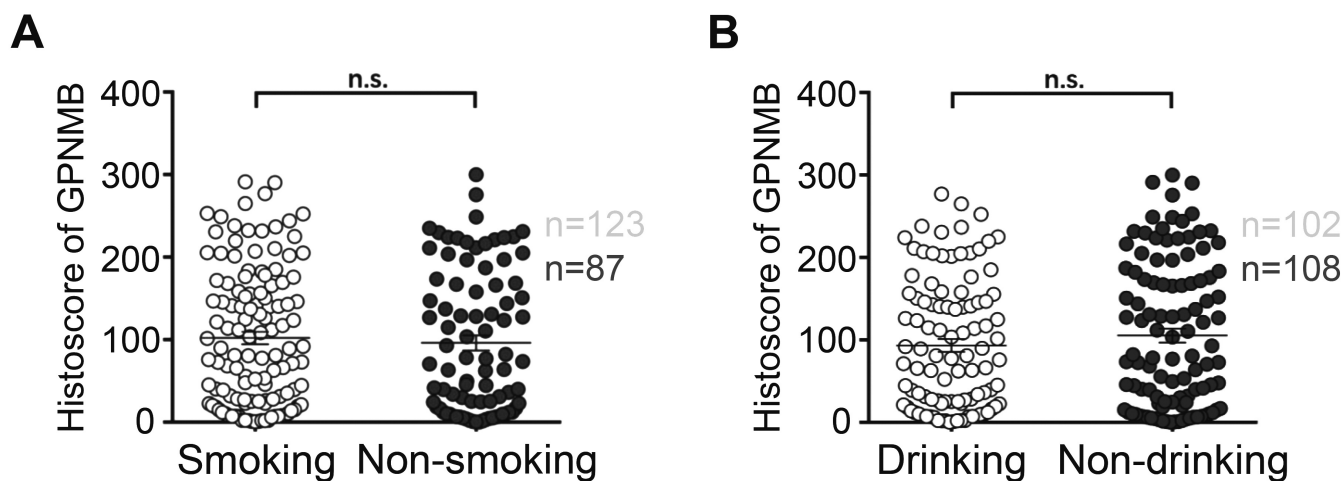
### Discussion

The present study identified that the GPNMB protein was highly expressed in HNSCC tissue compared with dysplasia and normal mucosa. Moreover, survival analysis revealed that the high level of GPNMB expression indicated an unfavorable prognosis for patients with HNSCC. This is the first study to verify that GPNMB protein was highly expressed in a HNSCC patient cohort. In addition, a close correlation between GPNMB expression and N stage (TNM staging system) was found in this study.

Metastatic dissemination of HNSCC initially occurs in the regional lymph nodes in the neck, and high mortality and poor prognosis of HNSCC are predicted by occurrence of lymph node metastasis (Iizuka et al., 2014). Unfortunately, the underlying molecular mechanisms contributing to tumor aggressiveness, recurrence, and metastasis are still not fully understood. Extracellular matrix degradation by MMPs plays a pivotal role in cancer progression by promoting motility, invasion and angiogenesis (Deryugina and Quigley, 2006; Morbini et al., 2015). Several MMPs are expressed in HNSCC and contribute to HNSCC migration and invasiveness (Iizuka et al., 2014; Morbini et al., 2015). Research studies have shown that GPNMB expression in several malignancies is positively associated with cancer invasion and metastasis (Maric et al., 2013; Fiorentini et al., 2014; Jin et al., 2018).

GPNMB expression was also associated with the EGFR/HER2 pathway that plays an important role in metastatic signaling in HNSCC (Carballeira et al., 2014; Leemans et al., 2018; Tajima et al., 2018). Moreover, in a study of HNSCC cell lines, the mRNA and protein expression of MMPs was assessed after GPNMB treatment or GPNMB gene silencing (Arosarena et al., 2018). The results showed that the effect of GPNMB treatment was variable. MMP-10 expression was upregulated in UMSCC14a and SCC15 cells after GPNMB treatment, while MMP-9 expression was decreased in UMSCC14a cells. GPNMB silencing decreased MMP expression widely in the study (Arosarena et al., 2018). In addition, GPNMB depletion negatively affected the invasive capacity of UMSCC12 cells (Arosarena et al., 2018). This evidence may influence the interpretation of our data that showed that the expression of GPNMB in HNSCC was associated with lymph node stage (of TNM staging) in both primary HNSCC tissue and metastatic lymph node tissue. Moreover, the results from the HNSCC cell line studies indicate poor prognoses for patients with HNSCC, which are consistent with the results from the present study.

In summary, these studies clearly indicate that GPNMB may be a prognostic marker. The high expression of GPNMB in primary HNSCC tissue and metastatic lymph node tissue can predict the high level of N stage in patients. Because this is a small HNSCC cohort, further study is still needed. Our data also provide evidence that GPNMB is a potential marker for estimating the patient's prognosis and may be a novel target for molecular-targeted therapy against HNSCC. Functional studies are needed to evaluate the mechanisms involved.



**Fig. 5.** The analyses of GPNMB expression relative to smoking and alcohol consumption status **A.** Comparison of the immunohistochemical histoscore of GPNMB between smokers ( $n=123$ ) and non-smokers ( $n=87$ );  $t$  test,  $P>0.05$ . **B.** Comparison of the immunohistochemical histoscore of GPNMB between drinkers ( $n=102$ ) and non-drinkers ( $n=108$ );  $t$  test,  $P>0.05$ .

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**Conflicts of interest.** The authors have no conflicts of interest to declare.

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