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ORIGINAL ARTICLE

Glycoprotein NMB promotes tumor formation and malignant progression of laryngeal squamous cell carcinoma

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Abstract

Laryngeal squamous cell carcinoma (LSCC), although one of the most common head and neck cancers, has a static or slightly decreased survival rate because of difficulties in early diagnosis, lack of effective molecular targeting therapy, and severe dysfunction after radical surgical treatments. Therefore, a novel therapeutic target is crucial to increase treatment efficacy and survival rates in these patients. Glycoprotein NMB (GPNMB), whose role in LSCC remains elusive, is a type 1 transmembrane protein involved in malignant progression of various cancers, and its high expression is thought to be a poor prognostic factor. In this study, we showed that GPNMB expression levels in LSCC samples are significantly higher than those in normal tissues, and GPNMB expression is observed mostly in growth-arrested cancer cells. Furthermore, knockdown of GPNMB reduces monolayer cellular proliferation, cellular migration, and tumorigenic growth, while GPNMB protein displays an inverse relationship with Ki-67 levels. Therefore, we conclude that GPNMB may be an attractive target for future LSCC therapy.

KEYWORDS
cell migration, dormancy, glycoprotein NMB, laryngeal squamous cell carcinoma, tumorigenesis

Abbreviations: bFGF, basic fibroblast growth factor; CSCs, cancer stem cells; CT, chemotherapy; DEGs, differentially expressed genes; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GPNMB, glycoprotein NMB; HNSCC, head and neck SCC; IHC, immunohistochemistry; LSCC, laryngeal SCC; LVI, lymphovascular invasion; OS, overall survival; OSCC, oral SCC; RT, radiation therapy; SCC, squamous cell carcinoma; SPF, specific pathogen free; TCGA, The Cancer Genome Atlas.

1 | INTRODUCTION

Laryngeal squamous cell carcinoma (LSCC) is one of the most common representatives of head and neck squamous cell carcinoma (HNSCC).[1] In 2020, the global incidence rates of LSCC worldwide in male and female patients were 160,265 and 24,350, respectively, in addition to 85,351 deaths among male patients and 14,489 deaths among female patients.[2–4] Although the survival rates for head and neck cancers have increased, LSCC survival has remained static[1,5] or slightly decreased.[6] The poor progression of LSCC is defined by several complications, including metastatic behavior through lymphovascular invasion (LVI)[7] or frequent relapse.[1]

Platinum-based chemotherapy (CT) is frequently used for LSCC[8–10]; however, acquired chemoresistance, relapse, and adverse side effects for patients persist as severe disadvantages.[10–13] High expression of the epidermal growth factor receptor (EGFR) is commonly observed in HNSCC, including LSCC, and targeting therapies, such as EGFR inhibitors (e.g., cetuximab), are frequently used in locally advanced, metastatic, and recurrent cases.[9,14–17] In combination with CT or radiation therapy (RT), these treatment outcomes result in enhanced overall survival (OS) over solitary CT or RT treatment scenarios.[18,19] However, monotherapy with cetuximab (or in combination with RT) results in limited positive response due to the cancer heterogeneity and various resistance mechanisms.[18,20–22] Therefore, an alternative novel target is needed to increase treatment efficacy and survival in LSCC patients.

Glycoprotein NMB (GPNMB), a type 1 transmembrane protein reported to be involved in malignant progression of various cancers, such as melanoma, glioma, and triple-negative breast cancer, may be such a target, as its high expression is thought to be a poor prognostic factor in those cancers.[23–26] We have previously demonstrated the importance of GPNMB in tumorigenic functions such as growth in vitro and in vivo, cellular migration and invasion, and induction of stem-like properties in breast cancer cells.[27–30] Although the oncogenic role of this protein was reported in some cancer types, little is known about GPNMB involvement in progression of HNSCC, especially LSCC. Previously, it was reported that GPNMB promotes migration of oral SCC (OSCC) and invasion of HNSCC,[31,32] while the contributory effects on tumorigenic function in LSCC remain unknown.

In the present study, we aimed to examine the roles of GPNMB in LSCC, finding that GPNMB expression levels in LSCC samples were significantly higher than those in normal tissues and, interestingly, GPNMB expression can be observed mostly in growth-arrested cancer cells. Furthermore, knockdown (KD) experiments indicate the importance of GPNMB in monolayer cellular proliferation, cellular migration, and tumorigenic growth such as sphere formation in vitro and tumor formation in vivo. Therefore, our results highlight GPNMB as a novel and unique target for the improvement of LSCC therapy.

2 | MATERIALS AND METHODS

2.1 | Cells and cell culture

Laryngeal squamous cell carcinoma cell lines UMSCC-10A, UMSCC-10B, UMSCC-11A, UMSCC-11B, UMSCC-12, UMSCC-13, and UMSCC-25 were obtained from the Head and Neck Cancer Biology Laboratory at the University of Michigan. Human embryonic kidney (HEK) 293T cells were obtained from the American Type Culture Collection (ATCC). For 2D monolayer culturing, cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate (Wako), and 100 μM MEM nonessential amino acids solution (Wako). For 3D sphere cultures, cells were maintained in DMEM/F-12 (1:1) medium (Invitrogen), 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate (Wako), 2% B-27 supplement (Invitrogen), 20 ng/mL epidermal growth factor (EGF) (Sigma), and 20 ng/mL basic fibroblast growth factor (bFGF) (Wako) in ultralow attachment culture dishes (Corning). All cells were maintained in a 5% CO_2-humidified atmosphere at 37°C.

2.2 | Short hairpin–mediated knockdown

To establish stable KD cell lines, two short hairpin RNAs (shRNAs) against GPNMB (shRNA#1 and shRNA#2) were inserted into a pLKO.1-puro vector, while an shRNA with a nontargeting sequence in the pLKO.1-puro vector was used as a control (shRNA#con) (Table S1). To produce lentivirus, plasmids psPAX2 and pMD2.G were transfected with pLKO.1-shRNAs into HEK293T cells using PEI Max (Polysciences Asia-Pacific). The filtered supernatant, containing lentivirus, was mixed with 8 μg/mL Polybrene (Sigma) and used to transduce UMSCC laryngeal cell lines. Stable cell lines were selected with puromycin (1 μg/mL for UMSCC-11A; 0.5 μg/mL for UMSCC-11B).

2.3 | Western blot (WB) analysis

Cell lysates were prepared as described previously[27] with protein concentrations quantified using a DC protein assay (Bio-Rad) according to the manufacturer's instructions. Total cell lysates were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), and WB analysis was performed as described previously[27] using the antibodies listed in Table S2.

2.4 | Reverse-transcription and quantitative PCR (qPCR) analysis

Total RNA was isolated by TRIzol (Life Technologies). Reverse transcription was performed with high-capacity RNA-to-cDNA Master Mix (Applied Biosystems). Quantitative PCR analysis was done

with SYBR Green I qPCR Master Mix (Applied Biosystems) and QuantStudio 5 (Thermo Fisher Scientific). Experiments were repeated three times, and data are represented as the mean of triplicate wells. The primer sequences used are listed in Table S3.

2.5 | Proliferation assay

Four thousand cells per well were seeded on 96-well plates, and cellular proliferation was measured by a CellTiter 96®AQueous One Solution Cell Proliferation Assay (MTS assay; Promega) according to the manufacturer's instructions. Experiments were repeated three times and are presented as the mean of triplicate wells.

2.6 | Sphere formation assay

Ten thousand cells per well were seeded on ultralow-attachment six-well plates (Corning) and maintained in 3D sphere culture medium. The numbers and sizes of spheres with diameters of more than 150 μm were counted on day 7.

2.7 | Wound-healing assay

Cells were seeded to confluence on 12-well plates (Falcon) and maintained in 2D monolayer culture medium. After 12 hours of adhesion, a cross-shape wound was scratched into the culture. Wound pictures were taken twice and relative closure/healing was determined by pixel percentages with ImageJ software (National Institutes of Health).

2.8 | Analysis of mRNA sequencing

After the isolation and extraction of total RNA, the RNA pellets were dissolved in 30 μL of Milli-Q water, and the integrity of the RNA was checked using an Agilent RNA 600 Nano Kit (Cat# 5067-1511; Agilent) on a Bioanalyzer (Agilent). Sample RNAs were subjected to library preparations for mRNA sequencing. After creation, libraries were adjusted to 1 nM and subjected to denaturation and neutralization. Subsequently, the libraries were diluted to 1.8 pM and then sequenced on a NextSeq 500 System (Illumina) using NextSeq500/550 v2.5 (75 Cycles) kits (Illumina, Cat#20024906). After sequencing, FASTQ files were exported, and basic information of the next-generation sequencing–run data was checked on CLC Genomics Workbench 20.0.3 software (CLC, QIAGEN) to identify changes in the mRNA expression profile as a result of GPNMB KD.

2.9 | Bioinformatics analysis

Differentially expressed genes (DEGs) were characterized with false discovery rates set at < 0.05 and a 1.5-fold-change cutoff. Filtered DEGs were classified into cellular proliferation, cellular migration (according to the GSEA database) (GO:0050673; GO:0016477), and LVI-related genes.[7] Heatmaps were made in Prism GraphPad version 8 software using standardized transcripts per kilobase million (TPM) expression values.

2.10 | Tumor formation in vivo

In vivo experiments were performed using 5-week-old female BALB/cAJcl-*nu*/*nu* mice kept under specific pathogen free (SPF) conditions (CLEA Japan). One million cells from each lentiviral transduction were injected subcutaneously to form three groups (shRNA#con, shRNA#1, and shRNA#2). After injection, mice were sacrificed at 6 (UMSCC-11B) and 10 (UMSCC-11A) weeks. Volumes of the extracted xenografts were calculated according to the formula: V(volume) = πL(length) W^2(width)/6. Animal experiments were performed with the approval of the Animal Ethics Committee of the University of Tsukuba in accordance with the university's animal experiment guidelines and the provisions of the 1995 Declaration of Helsinki.

2.11 | Patients and tissue specimens

Seventy-six FFPE tissue samples were acquired from 59 LSCC patients who had undergone a single or several surgeries (Table S4). Patients' consents and the approval from the Ethical Review Committee of University of Tsukuba Hospital were obtained to conduct experiments in this study. The project number is R02-147.

2.12 | Immunohistochemical and immunofluorescence staining

Paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated in ethanol, and autoclaved for 20 minutes in citrate-NaOH buffer at 121°C for antigenicity retrieval. After suppression of nonspecific antibody reactions in blocking solution (Perkin Elmer Life Science), samples were incubated with primary antibodies. For immunohistochemical (IHC) staining, reacted antibodies were detected by the Dako EnVision + System/HRP (DAB, DakoCytomation). For quantitative analysis we used NanoZoomer Digital Pathology system (2.0RS, Hamamatsu) and the QuPath.[33] For immunofluorescence (IF) staining, samples were then incubated with secondary fluorescently labeled antibodies and mounted with DAPI Fluoromount-G® (SouthernBiotech) for nuclei staining. We used a fluorescence microscope (Keyence BZ-X710) for detection and imaging. Antibody information is listed in Table S2.

2.13 | Statistical analysis

Quantitative data are represented as the means ± SD. Statistical analyses were performed using one-way or two-way analyses of variance (ANOVA) with the Tukey multiple comparison test using

Prism GraphPad version 8 software. A P value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | GPNMB is overexpressed in HNSCC Cancer Genome Atlas data and LSCC cell lines

Initially, we analyzed the mRNA expression levels of GPNMB in HNSCC and normal samples using the publicly available database from The Cancer Genome Atlas (TCGA) database by the UALCAN web resource.[34] In malignant tissues, GPNMB mRNA levels were significantly increased in all clinical stages and grades compared with normal tissues (Figure 1A–C; Table S5). On the other hand, according to data extracted from the Kaplan-Meier plotter database[35] of HNSCC patients, GPNMB expression was not significantly different between the two groups in OS analysis (Figure 1D). To elucidate the role of GPNMB in LSCC, we then examined GPNMB protein and mRNA expression levels in seven LSCC cell lines: UMSCC-10A, -10B, -11A, -11B, -12, -13, and -25 (Figure 1E, F; S1). All laryngeal cancer cell lines, except UMSCC-12, presented various expression patterns of GPNMB, while protein and mRNA levels were alike in each cell line. These experiments confirmed enhanced GPNMB expression in HNSCC patient tissues and LSCC cell lines.

3.2 | Knockdown of GPNMB impairs cellular proliferation, sphere formation, and cellular migration of LSCC cells

To determine the role of GPNMB in tumorigenesis, we established stable KD cells from UMSCC-11A and -11B by transduction with a lentivirus expression system using two independent shRNAs against human GPNMB (shRNA#1 and #2). After antibiotic selection, WB and qPCR analyses were used to examine GPNMB KD efficiency. As expected, GPNMB expression levels were significantly decreased (Figures 2A–D; S2A–C). Next, we used an MTS assay to define the 2D monolayer proliferative ability of UMSCC-11A and -11B, finding that the growth of GPNMB KD cells was significantly impaired compared with the control (shRNA#con) in both cell lines (Figure 2E, F). To confirm the role of GPNMB in 3D proliferation, we examined the sphere-forming ability of UMSCC-11A and -11B cell lines, with GPNMB KD cells showing significantly fewer spheres (diameter ≥150 μm) compared with controls in both cell lines (Figures 2G–J; S2D). We then assessed GPNMB involvement in cellular migration via wound-healing assays. Like previously reported results using OSCC cell lines,[31] the effect of GPNMB KD in our laryngeal cell lines significantly reduced migration ability compared with the control (Figures 2K, L; S2E). These results indicate that GPNMB is a key mediator of LSCC 2D monolayer proliferation, 3D sphere growth, and cellular migration.

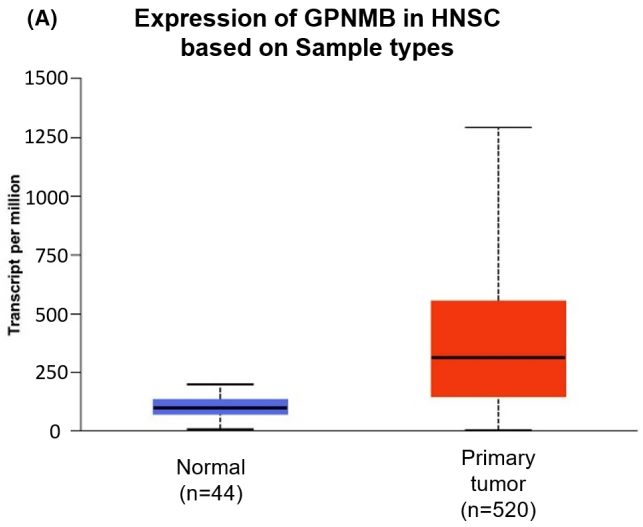
3.3 | Knockdown of GPNMB results in downregulation of cellular proliferation, migration, and LVI-related genes

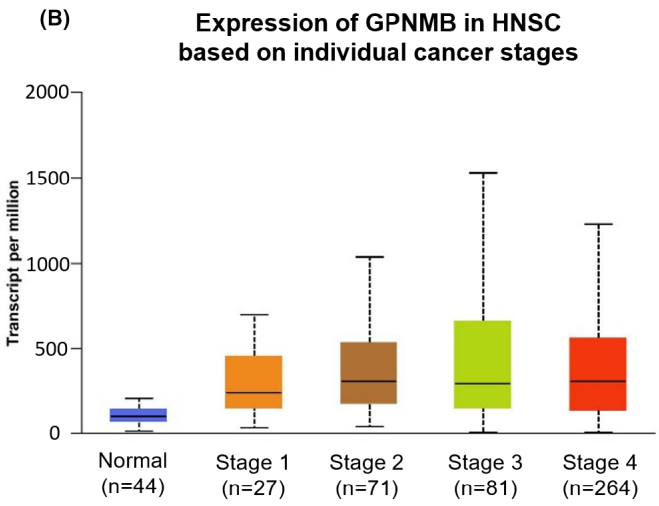
Next, we examined the effect of GPNMB KD in UMSCC-11A and -11B cells on other genes via RNA-sequencing analysis. After imposing cutoff criteria on the data, we assessed commonly affected genes (differentially expressed genes [DEGs]) after shRNA#1 and shRNA#2 treatment, finding that cellular proliferation and cellular migration genes were mostly downregulated, consistent with in vitro results (Figures 3A–D; S3A, B). Interestingly, several genes related to LVI[7] (*KIF18B*, *KIF23*, *PRC1*, *CCNA2*, *DEPDC1*, and *TTK*) were also downregulated in the case of UMSCC-11B (Figures 3E; S3C). These data suggest that GPNMB protein affects the expression of numerous cellular proliferation and cellular migration genes in both cell lines, while additionally affecting the expression of LVI-related genes in UMSCC-11B.

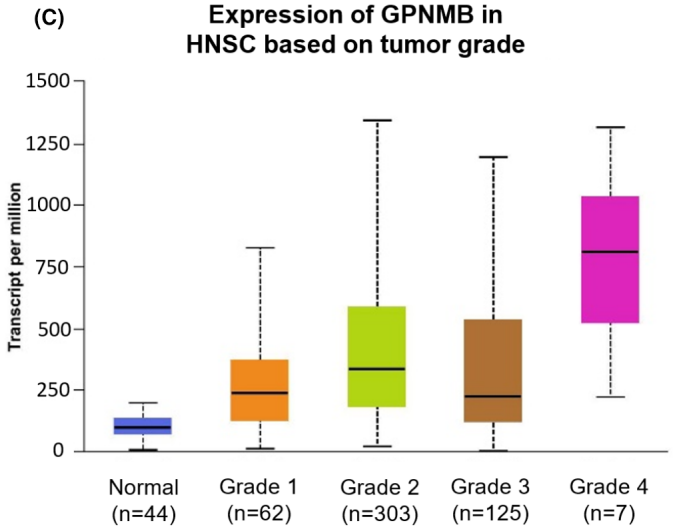
3.4 | Knockdown of GPNMB impairs tumor growth of LSCC cells in vivo

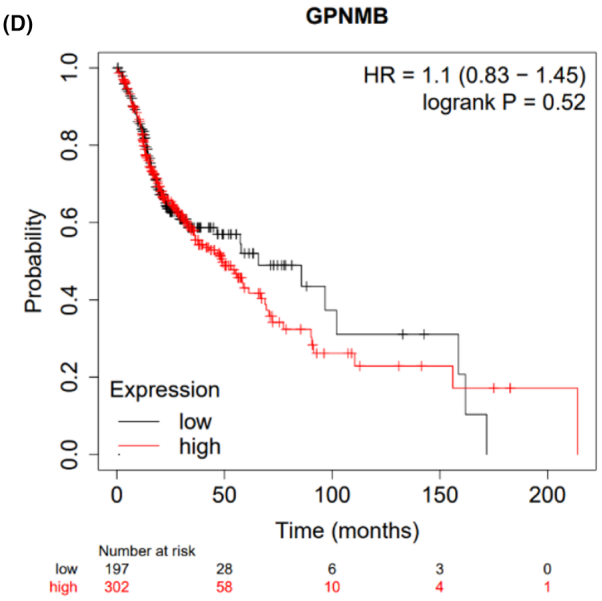
As GPNMB KD affects diverse genes in vitro, we further investigated the importance of GPNMB in laryngeal tumorigenesis in vivo by subcutaneous inoculation of UMSCC-11A and -11B cells with stable GPNMB KD into nude mice. Subsequently, GPNMB silencing resulted in significantly decreased tumor volume and weight in both cell lines (Figure 4A–F). Subsequent IHC analysis of the extracted tumors revealed that GPNMB protein expression levels seemed to diminish in the tumors of GPNMB KD compared with control, while the ratio of Ki-67-positive cells was not significantly different between GPNMB KD and control tumors (Figures 4G, H; S4A–D). In addition, IHC data showed the tendency of cells to differentially express GPNMB and Ki-67 (Figures 4G, H; S4E, F). To

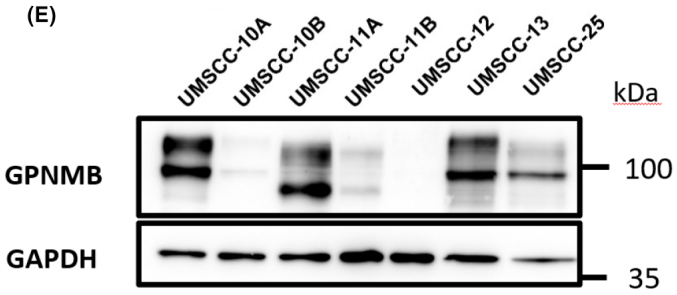
FIGURE 1 Enhanced GPNMB expression in head and neck squamous cell carcinoma (HNSCC) patients and laryngeal squamous cell carcinoma (LSCC) cells. A, GPNMB mRNA expression in primary HNSCC tissues and normal tissues from the UALCAN analysis (TCGA database). B, C, GPNMB mRNA expression in primary HNSCC tissues from patients with different clinical stages and tumor grades compared with normal tissues from the UALCAN analysis. Grades 1-4 are defined as well-, moderately, poorly differentiated, and undifferentiated lesions, respectively. D, Overall survival (OS) data of GPNMB$^{high/low}$ in HNSCC patients from Kaplan-Meier plotter analysis. E, Western blot analysis of differentially expressed GPNMB in LSCC cell lines. GAPDH was used as a loading control. Data are representative of three independent experiments. F, Quantitative PCR (qPCR) analysis for *GPNMB* in LSCC cell lines. Expression levels were normalized to *β-actin*. Data are presented as means ± SD, representative of three independent experiments

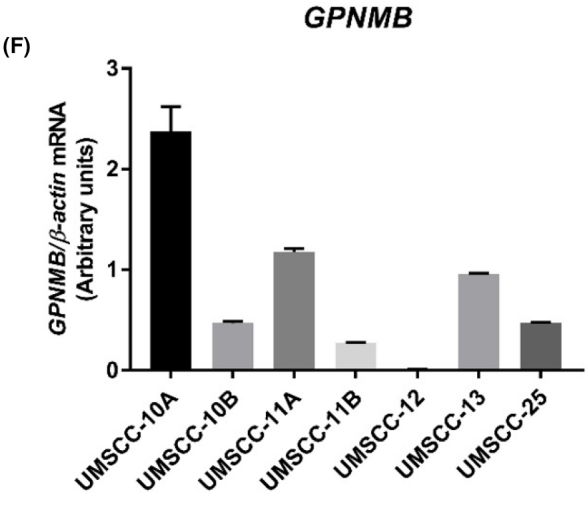












further elucidate this phenomenon, we performed IF staining of shRNA#con tumors (Figure 4I, J). We observed an inverse relationship between GPNMB and Ki-67, in which the majority of GPNMB-positive cells did not express Ki-67 and vice versa. These results indicate the importance of GPNMB in the tumorigenic growth of LSCC cells in vivo, as the inverse relationship between GPNMB and Ki-67 proteins suggest that GPNMB expression correlates with quiescent, nonproliferating cells.

3.5 | GPNMB is expressed in tumor tissues of LSCC patients

To understand the expression patterns of GPNMB in LSCC tumors, we analyzed 76 tissue samples from 59 LSCC patients (Table S4), comparing the expression of GPNMB between normal squamous epithelium (when available in the specimen) and tumorous lesions. The results of a representative case in Figure 5A–C indicated the high expression of GPNMB in malignant tissues, while the expression in normal epithelium was undetectable. However, the QuPath[33] analysis results from IHC staining did not show significant correlation between GPNMB expression and clinical development of LSCC

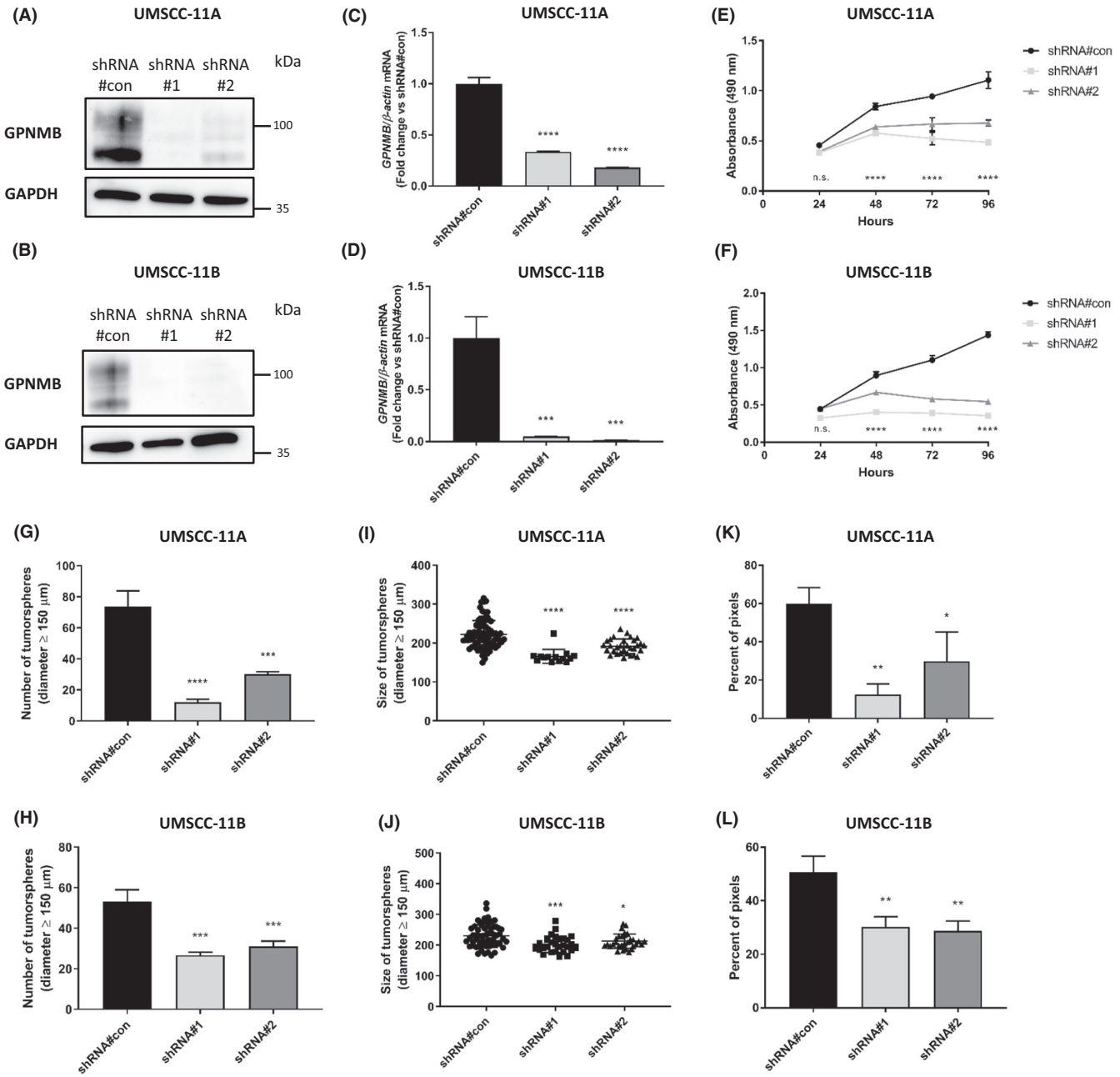


FIGURE 2 Role of GPNMB for laryngeal squamous cell carcinoma (LSCC) cell lines in 2D and 3D cellular proliferation and cellular migration in vitro. A, B, Western blot analyses for GPNMB in UMSCC-11A and -11B cells of control and GPNMB knockdown (KD) with shRNA#1 and shRNA#2. Data are representative of three independent experiments. C, D, Quantitative PCR (qPCR) analysis of *GPNMB* in UMSCC-11A and -11B with GPNMB KD. Expression levels were normalized to *β-actin*. E, F, 2D monolayer cell proliferation of UMSCC-11A and -11B with GPNMB KD was examined by MTS assay. G-J, Number and sizes of tumorspheres formed by UMSCC-11A and -11B with GPNMB KD. Only spheres ≥150 μm in diameter were counted. K, L, Wound-healing assay of UMSCC-11A and -11B with GPNMB KD. The area of the closed wound fracture was measured using ImageJ prior to the open area at 0 h. The results are presented at 16 h for UMSCC-11A and 23 h for UMSCC-11B after the scratch was performed. All data are presented as means ± SD, representative of three independent experiments. n.s., not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ one-way ANOVA (C-D, G-L) and two-way ANOVA (E-F) with Tukey's multiple comparison test

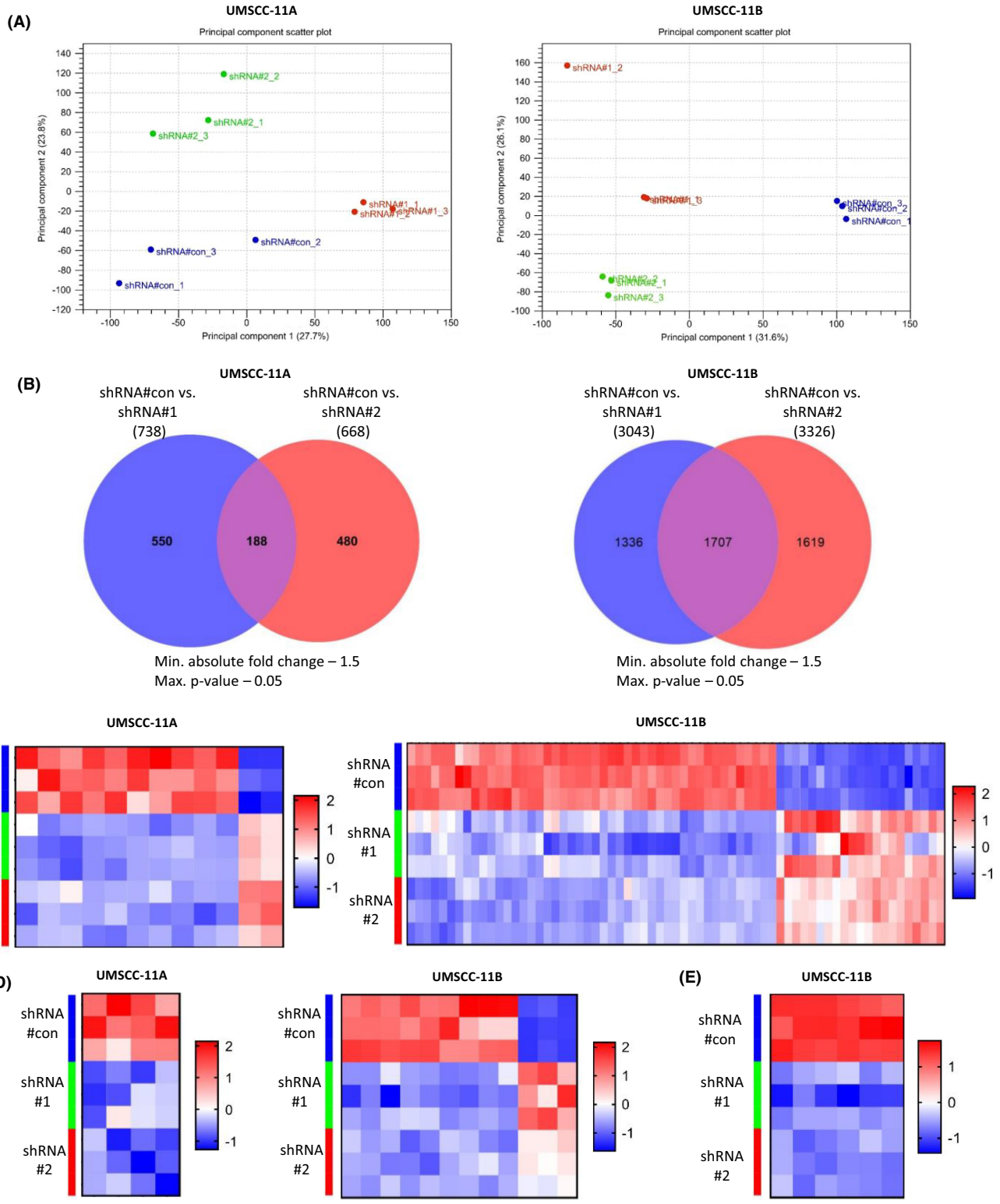


FIGURE 3 Downregulation of cellular proliferation, migration, and lymphovascular invasion–related genes in GPNMB knockdown (KD). A, Principal component analysis showing differences between control (red) and GPNMB KD (shRNA#1, green; shRNA#2, blue) clusters of samples. B, Venn diagram representing shared, differentially expressed genes (DEGs) of two KD conditions (shRNA#1 and shRNA#2) compared with control (shRNA#con). C, Heat map indicates the DEGs profile of cellular proliferation–related genes. Gene names are indicated in Figure S3A. D, Heat map indicates the DEGs profile of cellular migration–related genes. Gene names are indicated in Figure S3B. E, Heat map indicates the DEGs profile of lymphovascular invasion–related genes in UMSCC-11B. Gene names are indicated in Figure S3C

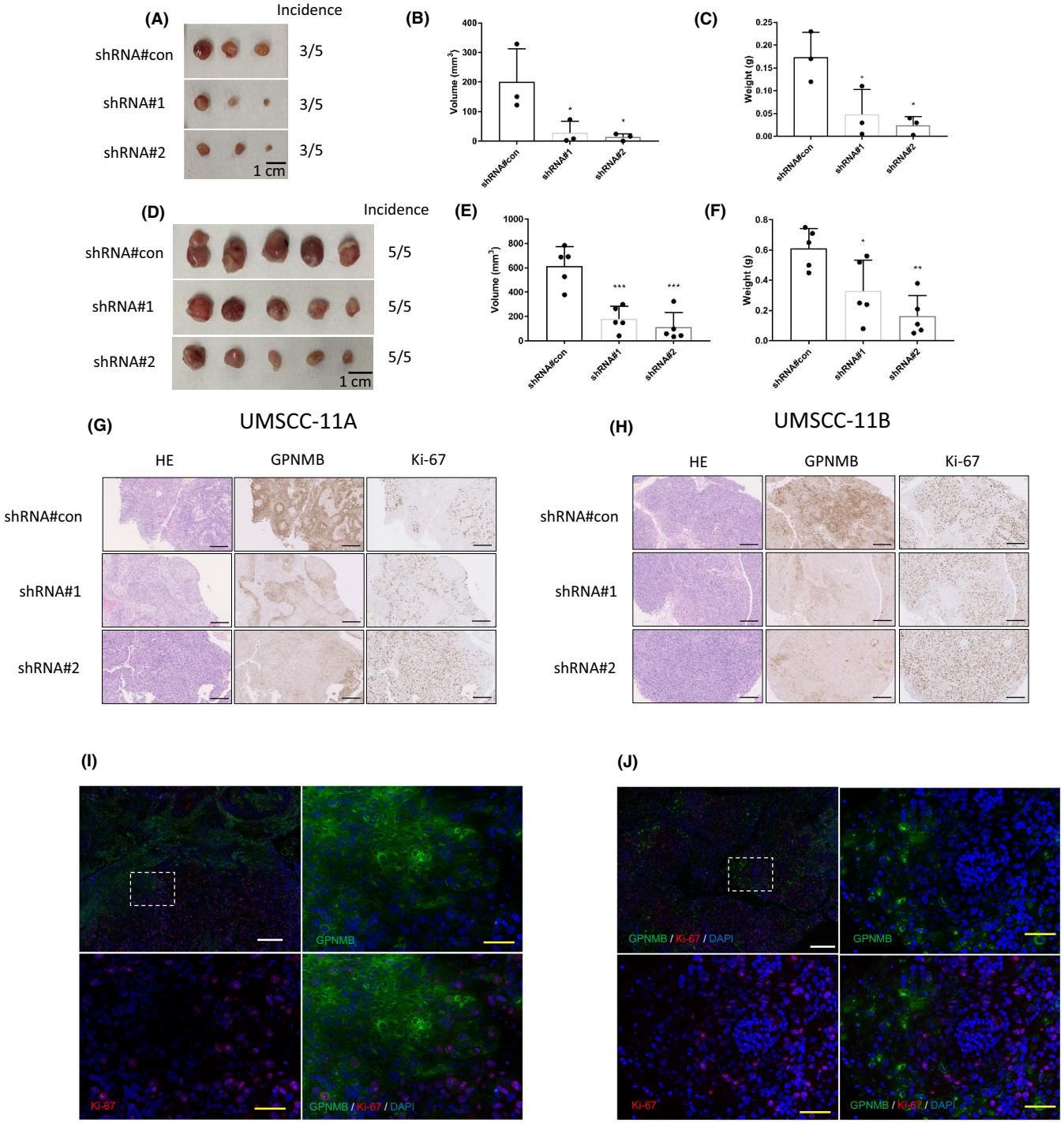


FIGURE 4 Importance of GPNMB for laryngeal squamous cell carcinoma (LSCC) cell lines in tumor formation in vivo. A-C, Tumor volumes and weights of UMSCC-11A with GPNMB KD subcutaneously injected cells into BALB/cAJcl-*nu/nu* mice. D-F, Tumor volumes and weights of UMSCC-11B with GPNMB KD subcutaneously injected cells into BALB/cAJcl-*nu/nu* mice. Incidence represents the rate of tumor occurrence versus the rate of subcutaneous injection (A, D). Data are presented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA with Tukey's multiple comparison test. G, H, Histology of the UMSCC-11A and -11B tumors with hematoxylin and eosin (HE) staining, and immunohistochemistry (IHC) of GPNMB and Ki-67. Scale bars: 250 μm. I, J, Immunofluorescence (IF) staining of UMSCCC-11A and -11B shRNA#con tumors with GPNMB (green), Ki-67 (red), and DAPI (blue). White scale bar: 200 μm; yellow scale bars: 50 μm

(Figure S5). To confirm the finding from two xenograft models of differential expression between GPNMB- and Ki-67-positive cells, we conducted IF staining of a representative specimen (Figure 5D). Similar to Figure 4I and J, most GPNMB-positive cells were Ki-67 negative and vice versa. These results confirm that GPNMB expression in tumor tissues is higher than in normal squamous epithelium as well as supporting the mutually exclusive relationship between GPNMB and Ki-67 proteins in primary, human LSCC cells.

4 | DISCUSSION

In this study, we present the role of GPNMB as a key mediator of LSCC tumorigenesis and progression. Silencing GPNMB from overexpressed malignant cell lines affected cellular proliferation, migration, sphere formation, and tumor growth in vivo. RNA-sequencing analyses revealed that GPNMB KD affects the expression profile of multiple genes and possibly leads to malignant progression of LSCC. Furthermore, our results showed that GPNMB expression patterns correlate with the quiescent state of LSCC cells, suggesting that it is involved in dormancy.

Our findings present similar outcomes of decreased cellular migration, 3D sphere formation in vitro, and tumor formation in vivo in LSCC during GPNMB depletion (Figures 2G-L; 4A–F) to those in breast cancer[27] or OSCC.[31] However, we previously showed that KD of GPNMB does not suppress 2D monolayer proliferation of breast cancer cell lines[27] but does impair proliferation in the case of LSCC cell lines (Figure 2E, F). Thus, the function of GPNMB might be

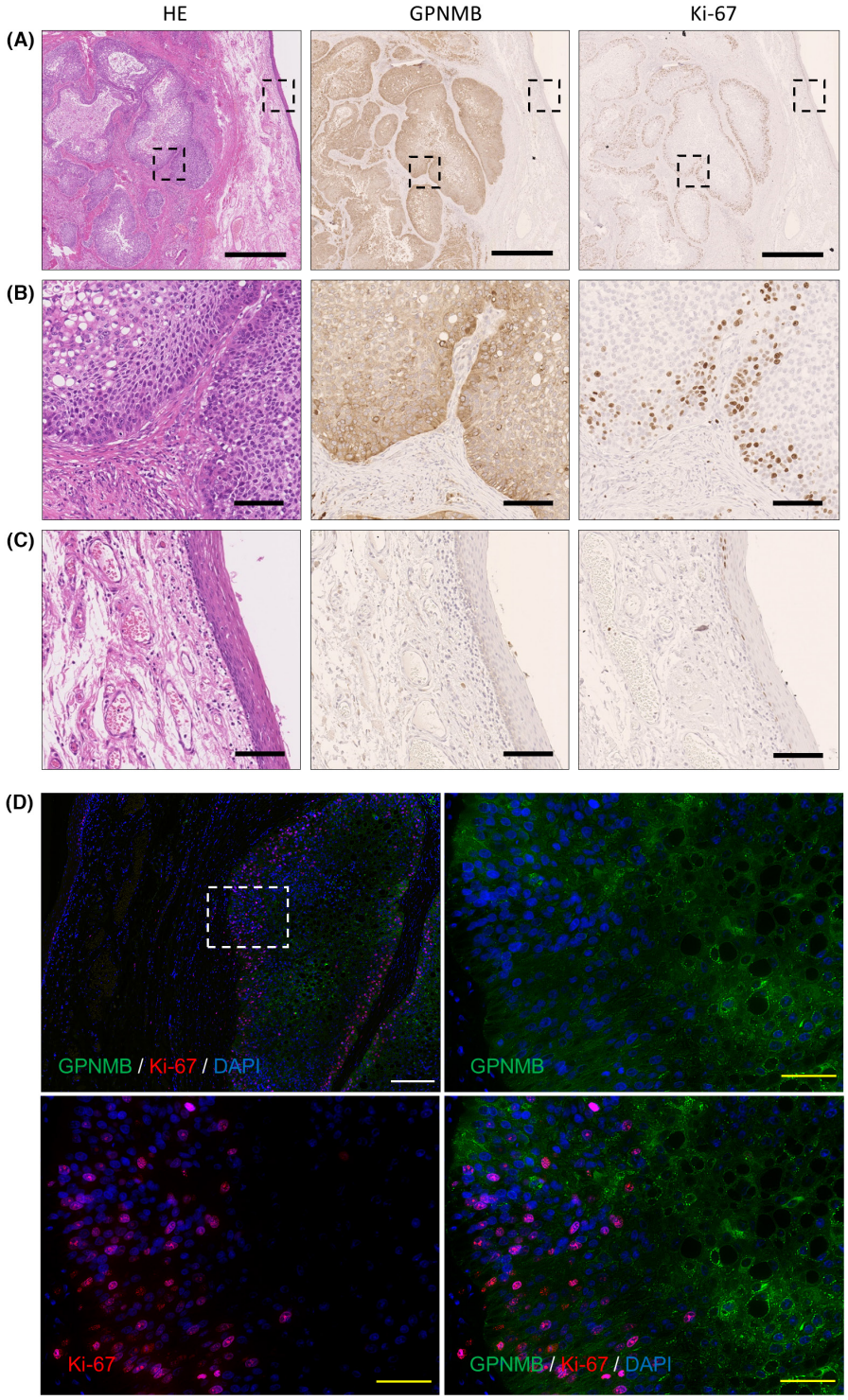


FIGURE 5 GPNMB and Ki-67 expression in tissue samples from laryngeal squamous cell carcinoma (LSCC) patients. A, HE and IHC staining for GPNMB and Ki-67 in a representative clinical sample. Scale bars: 1 mm. B, C, Tumor lesion and normal epithelium of the clinical sample from the annotated areas in A. Scale bars: 100 μm. D, IF staining of a representative clinical sample with GPNMB (green), Ki-67 (red), and DAPI (blue). White scale bar: 200 μm; yellow scale bars: 50 μm

versatile in different systems and organs, although it is required for tumorigenesis in multiple cancer types.

RNA sequencing results have confirmed the importance of GPNMB in numerous LSCC oncogenic processes. It was reported that simultaneously upregulated *TGFB2* and downregulated *DAB2* in HNSCC correlate with poor survival, cellular proliferation, and metastatic progression.[36] GPNMB KD downregulates *TGFB2* in both laryngeal cell lines and upregulates *DAB2* genes in UMSCC-11B, and these alterations might contribute to the cellular proliferation and migration in LSCC (Figures 3C, D; S3A, B). Another crucial mediator of carcinogenic cellular processes is IL-6, which facilitates HNSCC progression and metastasis via lymphangiogenesis[37]; however, silencing of GPNMB significantly suppressed *IL-6* expression in UMSCC-11B cell line, which was initially established from the post chemotherapeutic surgical site[38] (Figure 3C). Moreover, LVI was recognized as an underlying mechanism of lymph node metastasis in LSCC that worsens patient prognosis.[7] Zhang J et al. reported six LVI genes to be upregulated in HNSCC, and our GPNMB KD UMSCC-11B model exhibited reduced transcriptional levels of the same genes (Figure 3E), revealing the uniqueness of GPNMB and its broad regulatory role in laryngeal tumorigenesis.

Ki-67, on the other hand, is a marker which represents actively proliferating cells in G1, S, and G2 cell cycle phases.[39] While this protein can serve as a prognostic factor during treatment for some cancers (e.g., breast cancer),[40] its prognostic value was found to be inconclusive in HNSCC patients using conventional treatment options.[41–44] The assessment of tumors formed from GPNMB-depleted and control cells did not show significant differences in the ratio of Ki-67–expressing cells in the tumors (Figure S4A–D). Nevertheless, a remarkable impairment in tumor formation was achieved from GPNMB KD cells (Figure 4). Therefore, Ki-67 status cannot be a representative marker of tumorigenic activity changes with regard to GPNMB, at least within xenograft tumor models.

Furthermore, we have confirmed that GPNMB-positive cells do not express nuclear Ki-67 in most cases, an inverse relationship that seems robust (Figure 4I, J; 5D) and indicative that GPNMB-positive cells are nonproliferating in LSCC. Additionally, recent scientific studies have developed the idea that cancer stem cells (CSCs) are involved in cancer dormancy, drug resistance, relapse, and further tumorigenesis.[45–47] Our previous works have confirmed the direct involvement of GPNMB, exposed on the cell surface, in induction and maintenance of CSCs in breast cancer cell lines.[28] Hence, we suggest that the $GPNMB^{+}/Ki\text{-}67^{-}$ population of cells are cancer stem-like cells in LSCC. Although the role of GPNMB within CSC populations in LSCC has yet to be fully elucidated, it may serve as a novel target for future LSCC treatment.

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DISCLOSURE

The authors have no conflicts of interest to declare. Dr. Mitsuyasu Kato is the associate editor of the journal.

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