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LAMC2 acts as a novel therapeutic target of cetuximab in laryngeal cancer

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This study was set out to determine the function of LAMC2 in laryngeal cancer (LC). Initially, we identified the expression of LAMC2 in LC cells and tissues using TCGA datasets, GEO datasets (GSE143224), qRT-PCR, and western blot. Besides, we analyzed the correlations between LAMC2 and clinicopathologic features in LC patients. The CCK-8 assays were performed to detect cell viability and the half-maximal inhibitory concentration of cetuximab (IC50) in LC cells. We explored the correlations between LAMC2 and EGFR and further explored the regulation mechanism of cetuximab in LC. This study identified a high expression of LAMC2 in LC cells and tissues. The expression levels of LAMC2 were associated with TNM classification, lymph node (LN) metastasis, differentiation, and overall survival (OS). LAMC2 significantly promoted cell proliferation and cell viability. Besides, cetuximab significantly inhibited LAMC2 expression levels. LAMC2 significantly reversed the effect of cetuximab suppressing cell proliferation in LC cells. In conclusion, LAMC2 may act as a novel anti-cancer target in LC.

Key words: laryngeal cancer, LAMC2, EGFR, cetuximab, cell proliferation

Laryngeal cancer (LC) is the second most common malignancy of the head and neck [1]. Surgery, radiotherapy, and pharmacotherapy have prolonged the survival time of LC patients [2]. However, the 5-year survival rate for LC patients in advanced stages is less than 50% [1]. Studies on targeted therapy represent a growing field. Therefore, it is crucial to research potential targets for LC treatment.

LAMC2 is the γ2 subunit of laminin, which is located on chromosome 1q25-q31 [3]. Previous studies have reported that LAMC2 is amplified in many cancers, including non-small cell lung cancer [4], gastric cancer [5], colorectal cancer [6], hepatocellular carcinoma [7], cholangiocarcinoma [8], bladder cancer [9], anaplastic thyroid carcinoma [10]. Besides, the expression levels of LAMC2 are often correlated with survival time in human cancers [3]. Data from several studies suggest that LAMC2 regulates many phenotypes of cancer cells, such as cell migration, invasion [10], proliferation [11], angiogenesis [8]. In head and neck cancer, previous bioinformatic analyses suggested that LAMC2 significantly contributes to radioresistance [12]. However, no previous study has investigated the role of LAMC2 in LC.

Cetuximab is an anti-EGFR (epidermal growth factor receptor) monoclonal antibody, which is an effective approach for LC treatment [13]. Cetuximab combined with chemotherapy or radiotherapy improved the outcome of LC patients [14, 15]. Elevated EGFR contributed to the poor survival of patients with glottic laryngeal squamous cell carcinoma [16]. Evidence from some experimental studies has established that cetuximab suppresses the proliferation and invasion of LC [17]. Besides, several researches have reported the relationship between LAMC2 and EGFR in cancers. Grag et al. reported that LAMC2 regulates tumor progression, migration, and invasion by modulating the signaling of EGFR in anaplastic thyroid carcinoma [10]. LAMC2 promotes angiogenesis in cholangiocarcinoma by activating the EGFR signaling pathway [8]. However, no previous study has provided information on the relationship between LAMC2 and cetuximab or EGFR in LC.

In the pages that follow, the function of LAMC2 in LC will be discussed. This paper aims to explore the function of LAMC2 and the relationship between LAMC2 and cetuximab in LC. This study utilized the TCGA and GEO datasets to explore the expression levels of LAMC2 and the correlations between LAMC2 and EGFR in LC. The correlations between LAMC2 and clinicopathologic features were analyzed using forty-five patients' clinical data. Tu686 and AMC-HN-8 cells were used to further verify the results of bioinformatics analysis. This is the first study to explore the

relationship between LAMC2 and cetuximab in LC. LAMC2 may be a novel target for the treatment or screening of the advantaged population for cetuximab treatment in LC.

Patients and methods

Cell culture and clinical specimens. LC cells (Hep2, Tu212, Tu686, and AMC-HN-8) and HaCat cells were purchased from American Type Culture Collection (ATCC, USA). Forty-five clinical specimens (tumor and adjacent tissues) were provided by patients with LC in The First Affiliated Hospital, College of Medicine, Zhejiang University. All patients signed informed consent. All procedures involving human participants were performed following the ethical standards of the First Affiliated Hospital, College of Medicine, Zhejiang University, and with the 1964 declaration of Helsinki and its later amendments or comparable ethical standards. Ethical approval was obtained from The First Affiliated Hospital, College of Medicine, Zhejiang University (No. 2007A056). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 IU/mL penicillin, and 100 mg/ml streptomycin (Life Technologies, USA). Cells were cultured at 37 °C under 95% air, 5% CO_2.

Bioinformatics analysis. TCGA datasets were analyzed using GEPIA online tool (http://gepia.cancer-pku.cn/index.html). We analyzed LAMC2 expression levels and the correlation between LAMC2 and overall survival, EGFR in head and neck squamous cell carcinoma (HNSC). Besides, we searched the GEO datasets (GSE143224) [18] and analyzed LAMC2 and DMBT1 expression levels in LC.

Cell transfection. LAMC2 siRNA and overexpression vector were synthesized by GenePharm (Shanghai, China). The sequences of LAMC2 siRNA are listed in Table1. The sequence of LAMC2 was inserted into the GV492 vector. Lipofectamine 2000 reagent (Invitrogen, USA) was used to transfect Tu686 and AMC-HN-8 cells.

Quantitative real-time PCR (qRT-PCR). The mRNA expression level of LAMC2 was detected by qRT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen, USA). Complementary DNA (cDNA) was synthesized using the Primescript RT reagent kit (Takara, Japan). Relative expression levels were detected using SYBR Green PCR Kit (Takara, Japan) and calculated using the $2^{-\Delta\Delta CT}$ method. These parameters were used: 95 °C for 60 s, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 20 s. qRT-PCR primers are listed in Table 1.

Table 1. The primer sequences.

Gene	Sequences
LAMC2-Forward Primer	TGGCATTTGCTGAACGCATTT
LAMC2-Reverse Primer	TGCAGCCAGGTCTAATTGTTTT
GAPDH-Forward Primer	TGAAGGTCGGAGTCAACGGAT
GAPDH-Reverse Primer	GTCATGAGTCCTTCCACGATA
NC_siRNA	GCAAGCTGACCCTGAAGTTCAGT
LAMC2 siRNA1	CACAGACAAACTGGTAATGGATT
LAMC2 siRNA2	CAGACAAACTGGTAATGGATTCC
LAMC2 siRNA3	CAGAATACAGTGTCCATAAGATC

Western blot. To detect the protein expression levels of LAMC2, Ki67, and PCNA, we extracted total protein using trypsin EDTA digestive fluid (KeyGEN Biotech, China). BCA protein detection kit (Keygen Biotech, China) was used to detect protein concentrations. Equal amounts of protein lysates were run on SDS-PAGE gels. Next, proteins were transferred onto nitrocellulose membranes. The membranes were blocked for 1 h at room temperature and incubated with primary antibodies for LAMC2 (ab210959, Abcam), Ki67 (ab16667, Abcam), PCNA (ab29, Abcam), β-actin (ab115777, Abcam). Then, membranes were washed in PBST (PBS with 0.1% Tween) and incubated with the secondary antibody (#7074 and #7076, CST) for 1 h at room temperature. After washing in PBST three times, membranes were visualized by enhanced chemiluminescence (ECL) reagents (KeyGen, China).

Cell viability. Cell counting kit 8 (CCK-8) was performed to detect cell viability and the half-maximal inhibitory concentration of cetuximab (IC50) in LC cells. Cetuximab (2 mg/ml) was obtained from Merck Serono (Darmstadt, Germany). Two thousand cells were seeded on 96-well plates and further incubated for 24 h, 48 h, or 72 h, respectively. Next, the medium was replaced by a ten percent CCK-8 solution. After incubated for 1 h at 37 °C, cells were detected using a microplate reader at 450 nm.

Colony formation assay. To identify the effect of LAMC2 and cetuximab on cell proliferation, we performed colony formation and western blot (detecting proliferation-related biomarkers) assays. For colony formation, 200 LC cells (per well) were seeded on 12-wells plates and were incubated at 37 °C under 5% CO_2 after transfection. After two weeks, cells were fixed with 95% alcohol for 30 min and stained with crystal violet (0.1%) at room temperature. ImageJ software was used to calculate the number of clones.

Statistical analysis. All results were presented as the mean ± standard deviation (SD) for three independent experiments. Statistical analysis was performed with IBM SPSS 21.0 software. Significance levels of measurement data were analyzed by the Student t-test or one-way ANOVA. A p-value <0.05 was deemed statistically significant.

Results

LAMC2 is overexpressed in LC. To explore the expression levels of LAMC2 in LC, we compared LAMC2 mRNA expression levels in HNSC tissues (the number of tumors: 519) and normal tissues (the number of normal controls: 44) using the GEPIA online tool. We found that LAMC2 is overexpressed in HNSC (Figure 1A). Besides, we compared LAMC2 mRNA expression levels in LC tissues and normal tissues (normal control, NC) using the data in GEO datasets (GSE143224).

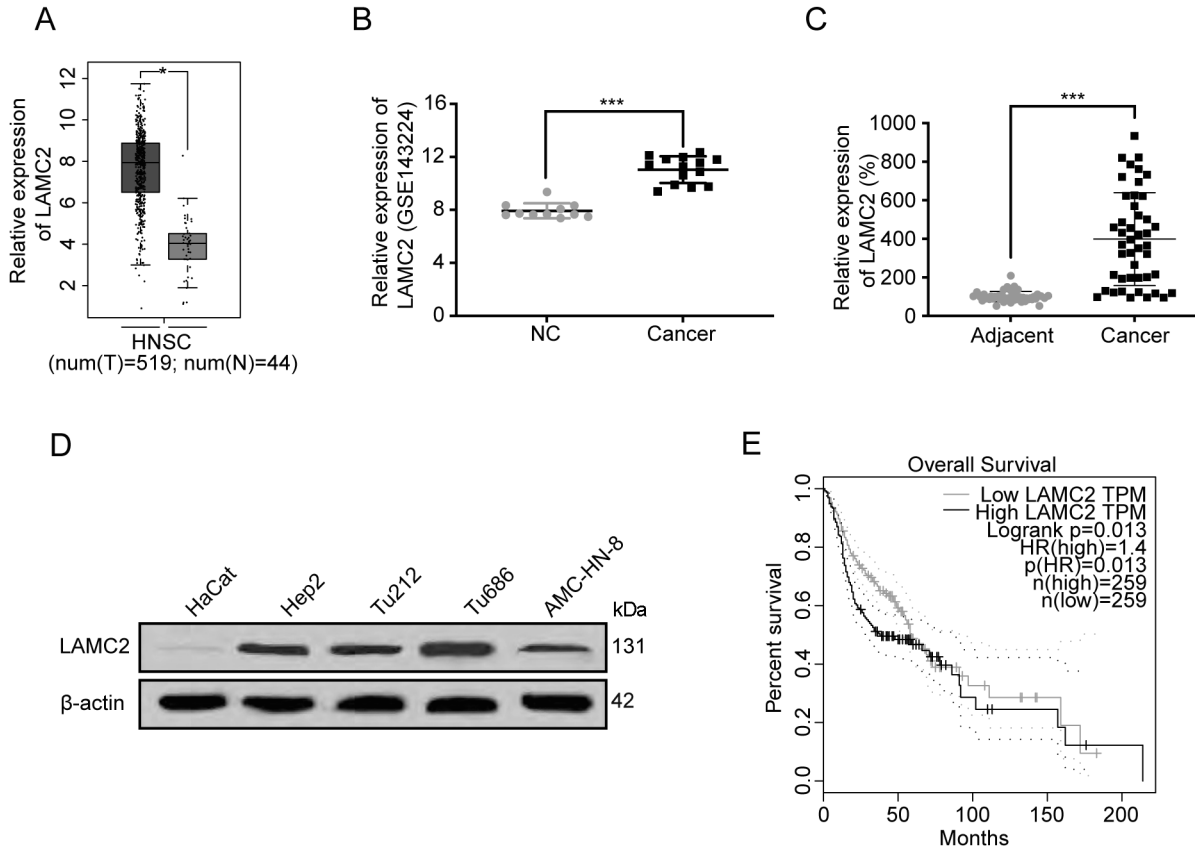

Figure 1. LAMC2 was overexpressed in LC. LAMC2 expression levels were analyzed in TCGA datasets (A), GEO datasets (B), patient tissues (C), and cell lines (D). E) Survival analysis of LAMC2 in LC. *p<0.05, ***p<0.001. Abbreviations: LC-laryngeal cancer; num(T)-numbers of tumor tissues; num(N)-numbers of normal tissues; NC-normal control

The results showed that LAMC2 is also upregulated in LC (Figure 1B). Next, we verified the results of bioinformatics analysis. qRT-PCR assays were performed to detect LAMC2 mRNA expression levels in LC tissues and adjacent cells (for control). Western blot assays were performed to detect LAMC2 protein expression levels in LC cell lines and control cell line (HaCat). We found that LAMC2 is indeed overexpressed in LC tissues (Figure 1C) and cell lines (Figure 1D). These results suggested that LAMC2 is overexpressed in LC.

LAMC2 expression levels are correlated with clinicopathologic features. As shown in Table 2, LAMC2 mRNA expression levels were correlated with TNM classification, lymph node (LN) metastasis, and differentiation. LAMC2 mRNA expression level was higher with later TNM classification, positive LN metastasis, and moderate and poor differentiation (Table 2). Besides, the results of survival analysis using TCGA datasets showed that LAMC2 decreases overall survival (OS) in HNSC (Figure 1E). Taken together, these results suggested that there is an association between LAMC2 and clinicopathologic features.

LAMC2 promoted cell proliferation in LC cells. To explore the effect of LAMC2 on cell proliferation, we

Table 2. Association of LAMC2 expression with clinicopathologic features in LC.

Clinicopathologic Features	Cases	LAMC2 expression (Mean ± SD)	p-value
Age (years)			
< 60	18	3.19±2.4	0.068
≥ 60	27	4.52±2.3	
Gender			
Male	33	4.20±2.44	0.330
Female	12	3.40±2.29	
TNM classification			
I+II	13	1.37±0.52	<0.001
III+IV	32	5.05±2.02	
LN metastasis			
negative	14	1.41±0.52	<0.001
positive	31	5.15±1.97	
Differentiation			
High differentiation	13	1.33±0.41	<0.001
Moderate and poor	32	5.07±2.00	

Abbreviations: LC-laryngeal cancer; LN-lymph node

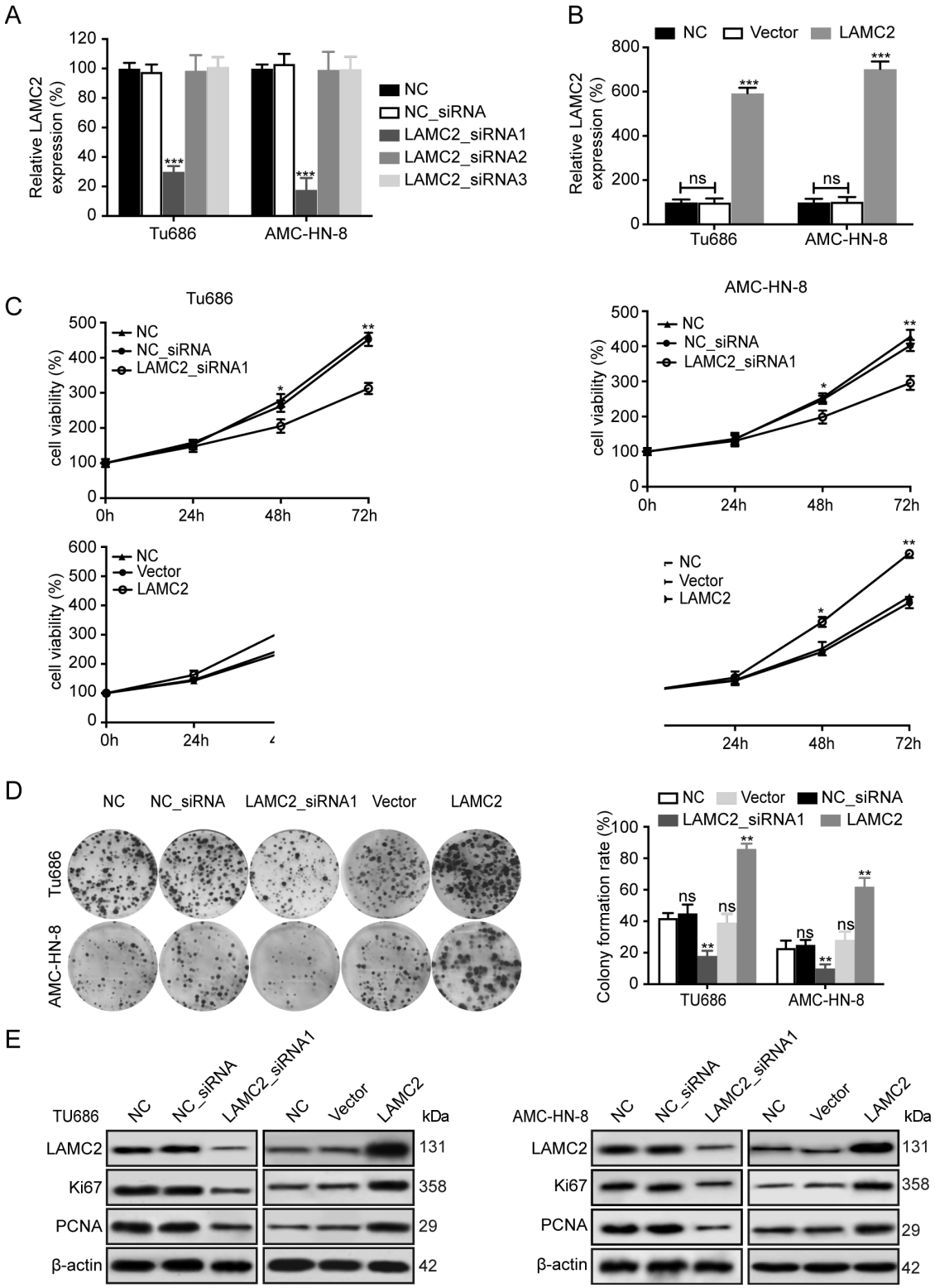

Figure 2. LAMC2 promoted cell proliferation in LC cells. A) LAMC2_siRNA1 significantly inhibited LAMC2 mRNA expression levels. B) LAMC2 overexpression vector significantly promoted LAMC2 mRNA expression levels. C) CCK-8 assays were used to detect the effect of LAMC2_siRNA or LAMC2 overexpression vector on cell viability. Colony formation assays (D) and western blot (E) were performed to detect the effect of LAMC2_siRNA or LAMC2 overexpression vector on cell proliferation. ns, not statistically significant, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: LC-laryngeal cancer; NC-normal control

knocked down and overexpressed LAMC2 expression in Tu686 and AMC-HN-8 cells, respectively. qRT-PCR assays were performed to screen siRNA with transfection efficiency and evaluate the overexpression efficiency of the LAMC2 plasmid. We found that LAMC2_siRNA1 significantly inhibits LAMC2 mRNA expression levels (Figure 2A), and LAMC2 vectors significantly promoted LAMC2 mRNA expression levels in Tu686 and AMC-HN-8 cells (Figure 2B). CCK-8 assays revealed that LAMC2 significantly promoted cell viability (Figure 2C). Besides, colony formation assays suggested that LAMC2 siRNA significantly inhibits cell proliferation, and LAMC2 significantly promoted cell proliferation in TU686 and AMC-HN-8 cells (Figure 2D). Next, western blot assays were used to detect the expression of proliferation-related biomarkers in Tu686 and AMC-HN-8 cells. We found that LAMC2 siRNA suppressed Ki67 and PCNA expression, and LAMC2 promoted Ki67 and PCNA expression (Figure 2E). In summary, these results indicated that LAMC2 promoted cell proliferation in LC cells.

The correlations between LAMC2 and EGFR. We also compared EGFR mRNA expression levels in LC tissues and normal tissues in GEO datasets (GSE143224). We found that EGFR is upregulated in LC (Figure 3A). TCGA and GEO datasets all presented the correlations between LAMC2 mRNA expression levels and EGFR mRNA expression levels in HNSC or LC (Figures 3B, 3C). As shown in Figure 3D, the half-maximal inhibitory concentration (IC50) of cetuximab was 100 mg/ml in Tu686 and AMC-HN-8 cells (Figure 3D).

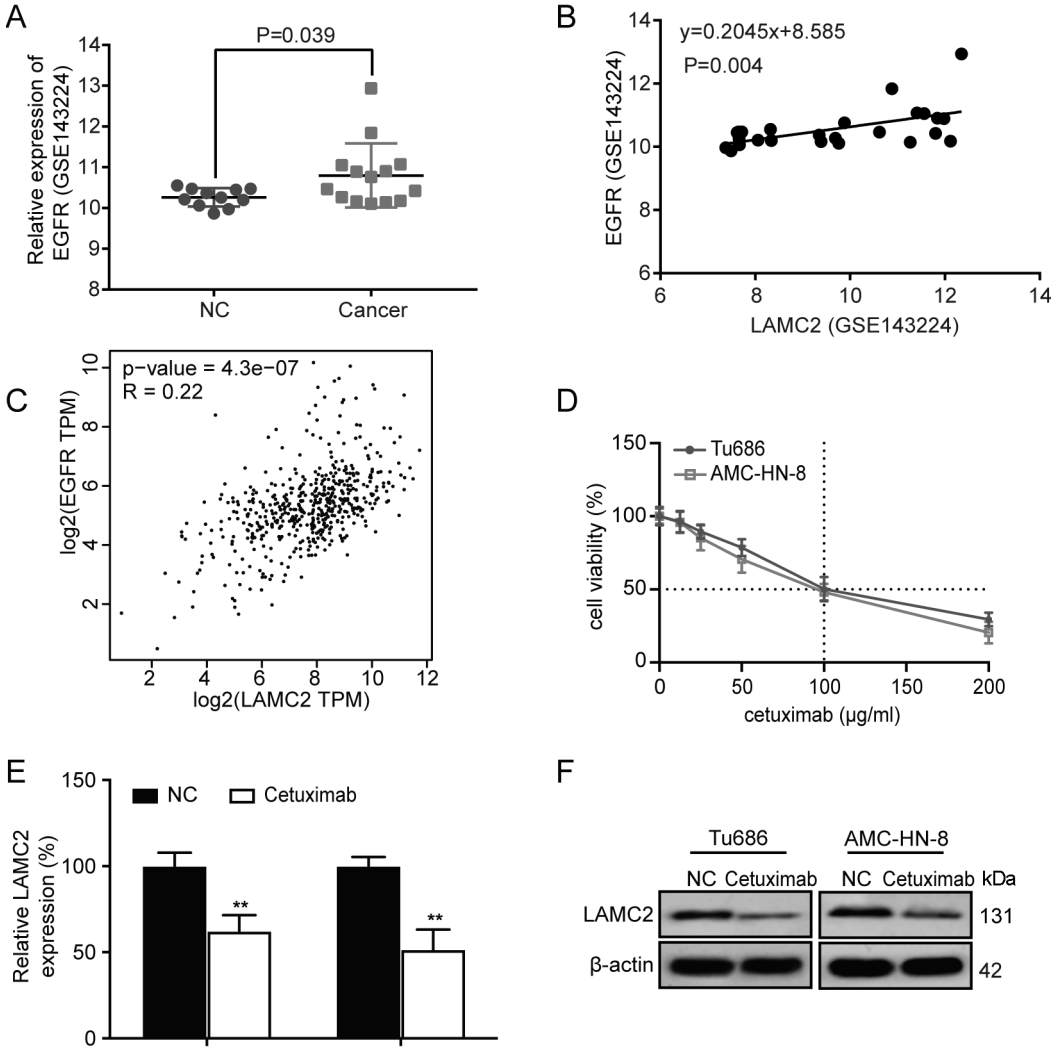

Figure 3. The correlations between LAMC2 and EGFR. A) The GEO datasets (GSE143224) revealed that EGFR is upregulated in LC. B, C) The correlations between LAMC2 and EGFR in mRNA expression levels in TCGA and GEO datasets. D) The IC50 of cetuximab in Tu686 and AMC-HN-8 cells. Cetuximab significantly suppressed the expression of LAMC2 mRNA (E) and protein (F). **p<0.01. Abbreviations: IC50-the half-maximal inhibitory concentration, NC-normal control

Further analysis showed that cetuximab significantly suppresses the expression of LAMC2 mRNA (Figure 3E) and protein (Figure 3F). In summary, these results suggested that there is an association between LAMC2 and EGFR.

Cetuximab inhibited cell proliferation through LAMC2. To identify the regulation mechanism of cetuximab, we divided the cells into four groups (including vector, cetuximab, LAMC2 overexpression, and cetuximab+LAMC2 overexpression). Next, we detected the effect of different interventions on cell proliferation. CCK-8 assays suggested that cetuximab significantly inhibited cell viability. Interestingly, LAMC2 significantly abolished the influence of cetuximab on cell viability (Figures 4A, 4B). Besides, colony formation and western blot assays all revealed that cetuximab significantly suppressed cell proliferation. LAMC2 also significantly reversed the effect of cetuximab on cell proliferation (Figures 4C, 4D). What emerges from the results reported here was that cetuximab suppressed cell proliferation by inhibiting the LAMC2 expression.

Discussion

As mentioned in the literature review, LAMC2 is the γ2 chain of laminin. A previous study demonstrated that the long arm of laminin-5 is formed by three chains (α3, β3, and γ2). Laminin-5 EGF-like fragments bind to the EGF

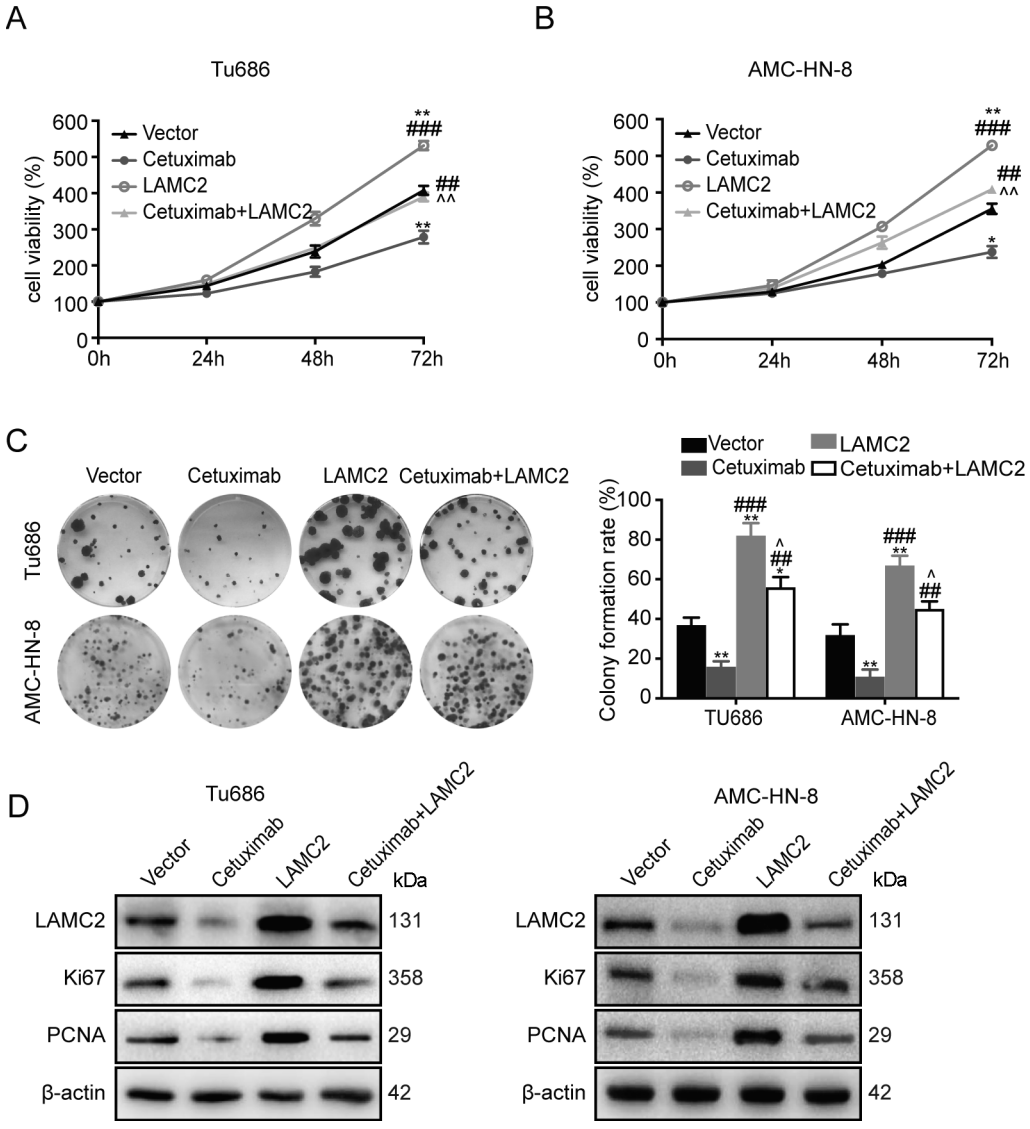

Figure 4. Cetuximab inhibited cell proliferation through LAMC2. A, B) CCK-8 assays were used to detect cell viability. C) Colony formation assays were performed to detect cell proliferation. D) Proliferation-related biomarkers were detected using western blot. *p<0.05 vs. vector group, #p<0.05 vs. cetuximab group, ^p<0.05 vs. LAMC2 group

receptor during gland involution [19]. Cetuximab is an anti-EGFR monoclonal antibody, which is often used to treat patients with LC. It has been observed that laryngeal cancer stem cells (LCSC) have more resistance to cetuximab when compared with non-LCSC [20]. One of the aims of this study was to explore a novel biomarker for the diagnosis or treatment of LC. The current study found that LAMC2 might be an oncogene in LC. One interesting finding is that cetuximab inhibits cell proliferation dependent on regulating the expression of LAMC2.

In reviewing the literature, no data was found on the role of LAMC2 in LC. The results of this study demonstrated that LAMC2 is upregulated in LC (Figures 1C, 1D). Tissues of advanced LC patients have higher expression levels of LAMC2 (Table 2). Besides, the silencing of LAMC2 significantly suppressed cell viability and cell proliferation (Figures 2C, 2D). These results indicate that LAMC2 plays an oncogenic role in LC. These results are consistent with data obtained in the TCGA and GEO datasets. Prior studies have noted the role of cetuximab in LC. Cetuximab suppressed Hep-2 cell proliferation and showed no pro-apoptotic effects. When administered with cisplatin, cetuximab synergistically suppressed proliferation and apoptosis [21]. Relationships between LAMC2 and EGFR have been reported in gastric cancer and anaplastic thyroid carcinoma. Xu et al. demonstrated that LAMC2 enhances cell invasion by promoting the phosphorylation of EGFR in gastric cancer [5]. Grag et al. found that LAMC2 promotes tumor progression, migration, and invasion via activation of EGFR. Surprisingly, cetuximab was found to enhance the antiproliferative activity of LAMC2 knockdown anaplastic thyroid carcinoma cells [10]. This study confirms that EGFR is correlated with LAMC2 (Figures 3B, 3C). Moreover, cetuximab markedly suppressed cell proliferation, and these effects were abolished by elevated LAMC2 (Figures 4C, 4D). These results suggest that cetuximab suppresses cell proliferation dependent on the inhibition of LAMC2. These results provide further support for the hypothesis that LAMC2 acts as a novel therapeutic target of cetuximab in LC.

In summary, we found that LAMC2 mRNA and protein expression is elevated in LC cells and tissues. Elevated LAMC2 significantly promoted cell proliferation in LC cells. This is the first study to explore the function of LAMC2 in LC. Besides, our results suggested that LAMC2 is a target of cetuximab in LC. Cetuximab inhibits cell proliferation dependent on the inhibition of LAMC2. These findings will help us to develop a novel target for the diagnosis or treatment of LC. Further studies are required to explore more effective drugs targeted LAMC2 in LC treatment.

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