Combination of β2-AR Blocker and COX-2 Inhibitor Suppresses Development of Oral Squamous Cell Carcinoma

Chong Zhang
Guangxi Medical University College of Stomatology
Zhen Ma
Guangxi Medical University College of Stomatology
Xianxiang Liao
Guangxi Medical University College of Stomatology
Shiqi Liu
Guangxi Medical University College of Stomatology
Fang Fang
Guangxi Medical University College of Stomatology
Shichen Tao
Guangxi Medical University College of Stomatology
Xiaotong Liu
Guangxi Medical University College of Stomatology
Xutao Wen
Guangxi Medical University College of Stomatology
Xingjin Chen
Guangxi Medical University College of Stomatology
Meiyu Chen
Guangxi Medical University College of Stomatology
Xuansheng Ning
Guangxi Medical University College of Stomatology
Huaming Mai (huamingmai@163.com)
Guangxi Medical University College of Stomatology https://orcid.org/0000-0003-1228-3952

Research

Keywords: oral squamous cell carcinoma, β2-adrenergic receptor, cyclooxygenase-2, invasion, metastasis

Posted Date: October 21st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-94033/v1

Abstract

Background To study the effect of inhibiting progression of oral squamous cell carcinoma (OSCC) through blocking β2-adrenergic receptor (β2-AR) and inhibiting Cyclooxygenase-2 (COX-2).

Methods Transwell invasion assay and wound-healing assay were used to investigate whether invasion and metastasis of OSCC cells (SCC9, Cal27 cell lines and primary OSCC cells) would be inhibited after treated by β2-AR blocker (ICI118,551), COX-2 inhibitor (Etodolac) or both. Real time quantitative polymerase chain reaction (RT-qPCR), Western blot and enzyme linked immunosorbent assay (ELISA) were used to detect expression change of genes related to invasion and metastasis in OSCC cells after drug treatment. In vivo, 6-8 weeks old female Balb/c nude mice were randomly divided into control group, ICI118,551 treatment group, etodolac treatment group and combined treatment group. Cal27 cells were used to establish OSCC xenograft models. The survival days, tumor sizes and lymph nodes metastasis were compared among these groups. Immunohistochemistry, Western blot and ELISA were carried out to detect expression change of invasion and metastasis relative genes.

Results ICI118,551 or etodolac could inhibit invasion and metastasis of OSCC cells, and the inhibition of combined treatment was more significant. Moreover, RT-qPCR and Western blot showed that epidermal growth factor receptor (EGFR), transforming growth factor-β1 (TGF-β1), interleukin-1β (IL-1β), matrix metalloproteinase 2 (MMP2) were downregulated after treatment, especially in combined treatment group. In vivo, the combined treatment group could significantly prolong survival days and inhibit tumor size. Immunohistochemistry, Western blot and ELISA showed the expression of above-mentioned genes were also downregulated.

Conclusions Invasion and metastasis of OSCC can be inhibited through blocking β2-AR and inhibiting COX-2, it can be a potential treatment of OSCC.

Background

Head and neck cancer is the seventh most common cancer worldwide, accounting for 3% of all cancers [1]. Oral squamous cell carcinoma (OSCC) is the most common cancer among head and neck cancer, accounting for about 90% of cases [2]. Therefore, the diagnosis and treatment of OSCC are very crucial. At present, the main treatment methods of OSCC include surgery, radiotherapy and chemotherapy. Surgery combined with radiotherapy and chemotherapy is still the standard combined and sequential treatment of OSCC [3–5]. However, despite the combination of multiple treatments, the overall 5-year survival rate of OSCC patients has not been significantly improved in the past few decades, OSCC still belongs to the malignant tumor with low survival rate, just about 50% [6, 7]. So, it is important to improve the overall survival rate of OSCC patients.

In past few years, different studies pointed out that β2-adrenoceptor (β2-AR) was overexpressed and played an important role in the occurrence and development of cancers [8–10]. It is closely related to the growth, angiogenesis, invasion and metastasis of cancers, including OSCC [11–14]. Meanwhile, our

previous study and other studies found that blocking β2-AR could inhibit the development of OSCC, propranolol combined with cisplatin had a synergistic effect and could weaken the resistance of OSCC cells after radiotherapy and chemotherapy treatment [15–17].

Cyclooxygenase-2 (COX-2) is overexpressed and closely related the poor prognosis and progression of malignant tumor, including OSCC [18–21]. Just like β2-AR, COX-2 is closely related to proliferation, apoptosis, angiogenesis, invasion, metastasis and immunosuppression of OSCC [22, 23]. Different studies reported that COX-2 inhibitor could significantly inhibit progression of OSCC [24–27]. Some clinical trials also reported that there was an obvious decrease of vascular endothelial growth factor-A (VEGF-A) serum level in patients with head and neck squamous cell carcinoma (HNSCC), after administered with celecoxib which is a COX-2 inhibitor. Moreover, celecoxib could improve progression-free survival time of these patients [28, 29].

Recently, some studies of colon and breast cancer found that the combination of β2-AR blocker and COX-2 inhibitor could significantly inhibit the occurrence and development of malignant tumors [30, 31]. They found that perioperative use of β-AR blockers and COX-2 inhibitors might improve immune competence and inhibit tumor metastasis [32]. However, there is not any study reports whether combination of β2-AR blocker and COX-2 inhibitor is effective on treatment of OSCC or not. We put forward the hypothesis that combined treatment of β2-AR blocker and COX-2 inhibitor could inhibit the development of OSCC. So, in this study, we explored the effect of combination of β2-AR blocker and COX-2 inhibitor on OSCC in *vitro* and *vivo*, as well as the related molecular mechanisms

Methods

All methods were performed in accordance with the relevant guidelines and regulations.

Cells and cell culture

OSCC cell lines. Cal27 cells and SCC9 cells both were obtained directly from BRJ Biological Technology Company, Ltd (Nanjing, China). Cal27 cells were cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (50 units/mL)/streptomycin (50 μg/mL) in a humidified atmosphere (5%CO_2) at 37 °C. SCC9 cells were cultured in dulbecco's modified eagle medium and ham's F-12 nutrient mixture (DMEM/F12) supplemented with 10% FBS and penicillin (50 units/mL)/streptomycin (50 μg/mL) in a humidified atmosphere (5%CO_2) at 37 °C. The two cell lines were both identified by short tandem repeat (STR) profiling (Supplementary Figure1,2).

The primary OSCC cells for the study were from tongue squamous cell carcinoma tissue of one patient. The patient, a 29-year-old man (T4N2M0); had left tongue low-differentiated squamous cell carcinoma with bilateral cervical lymph node metastases. Fresh specimens from tongue carcinoma were dissected promptly after operations carried out at the Department of Oral and Maxillofacial Surgery, Guangxi Medical University School and Hospital of Stomatology. Cells were cultured in DMEM supplemented with

14% FBS and penicillin (50 units/mL)/streptomycin (50 μg/mL) in a humidified atmosphere (5% CO_2) at 37 °C.

Reagents and their administration

ICI118,551

To block β2-AR activation, we used the selective β2-AR blocker ICI118,551 (MedChemExpress, USA). The drug was dissolved in dimethyl sulfoxide (DMSO) and added to a mixture of culture medium to 5μmol/L concentration for vitro assay. In vivo assay, it was dissolved in water and diluted to 0.5 mg/ml concentration.

Etodolac

The semi-selective COX-2 inhibitor Etodolac (MACKLIN, China) was used to inhibit COX-2 activation. It was dissolved in DMSO and added to a mixture of culture medium to 5μmol/L concentration for vitro assay. In vivo assay, it was dissolved in corn oil and diluted to 12.5 mg/ml concentration.

Animals and counterbalancing

Balb/c nude mice, purchased from the Animal Research Laboratory of Guangxi Medical University, were distributed randomly into a control group, ICI118,551 treatment group, etodolac treatment group and combined treatment group, 6 mice in each group. Cal27 cells (1×10^7/mL) in 50 μL phosphate buffer saline (PBS) were subcutaneously injected into in the flank of the tongue at day 0. Tumor growth was monitored in an assessor-blind trial, and tumor size was measured every 2 days. Tumor volume was estimated using the following equation: tumor volume = $((A \times B^2)/2)$ mm^3, where A is the largest dimension and B is the largest dimension perpendicular to A. ICI118,551 (5 mg/kg in a 0.5 mg/ml concentration) and Etodolac (50 mg/kg in a 12.5 mg/ml concentration) were given orally to the mice daily. PBS was given orally to the control group daily. Mice were euthanized 24 h after completion of treatment. The tumor and submandibular lymphatic node histology were analyzed. The Kaplan–Meier method was used to evaluate survival. All experimental procedures received approval by the Laboratory Animal Care and Use Committees of Guangxi Medical University.

Wound healing assay

OSCC cells were grown in 6-well plates to until reaching 100% confluence. A cell scratch-wound was established by a 200μl pipette tip. After this, the culture medium was changed to different drugs, which were diluted in DMEM or DMEM/F12. After 24h, the wound width was photographed, and the percentage of wound closure was determined based on the wound width at 0 h and the areas were measured using ImageJ software.

Transwell invasion assay

Cell invasion assays were performed using a modified 24-well chamber with a membrane which was pre-coated with Matrigel (Corning, USA). 100 μl of cells (1×10^5/ml) in FBS-free medium were seeded into the upper chamber, while different drugs were added 100μL into the upper chamber. In each lower chamber, 500μL medium containing 10% or 14% FBS was placed. After 24h, invasive cells on the undersurface of the membrane were stained with crystal violet (Thermo Fisher Scientific, USA) staining solution for 20 minutes. Quantification was measured by counting the invasive cells in five randomly selected fields.

Real time quantitative polymerase chain reaction

Total ribonucleic acid (RNA) was isolated using RNAiso Plus (Takara Bio, Japan) and according to the and reverse transcribed using PrimeScript™RT reagent Kit (Takara Bio, Japan) according to the instructions. We used SYBR®Premix Ex Taq™II Kit (Takara Bio, Japan) to detect expression of messenger ribonucleic acid (mRNA). The polymerase chain reaction (PCR) primer sequences used for each gene were showed in Table1. PCR reaction was carried out with Step One Plus TM Real-Time PCR System (Takara Bio, Japan): the first step is initial denaturation followed by 95°C for 30s, the second step is denaturation followed by 40 cycles of 95°C for 5s and 60°C for 30s. All the values were standardized with $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Total protein was extracted from cells or tissues using RIPA buffer in the presence of protease inhibitor (Fdbio science, China) and phosphatase inhibitor cocktail (Fdbio science, China). Proteins at 60μg per lane. Target proteins were separated based on their molecular weight on 10% SDS-PAGE gels (Fdbio science, China) and then transferred to poly vinylidene fluoride membranes (Millipore, USA). The membranes were incubated with the appropriate primary antibody overnight at 4°C. The primary antibody used for each gene were as follows: Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (FD0063, Fdbio science, China) at 1/5000 dilution, Anti-epidermal growth factor receptor (EGFR) (ab52894, abcam, USA) at 1/1000 dilution, Anti-transforming growth factor-β1 (TGF-β1) (ab179695, abcam, USA) at 1/1000 dilution, Anti-matrix metalloproteinase 2 (MMP2) (ab92536, abcam, USA) at 1/1000 dilution, Anti-VEGF-A (ab52917, abcam, USA) at 1/5000 dilution. Then membranes were incubated in corresponding species secondary antibodies for 1h. GAPDH was used as an internal control. Protein expression levels were detected by ChemiDocM universal imager system (Bio-Rad, USA) and gray value was analyzed by Image lab software. The relative expression of target protein (%) = gray value of target protein / gray value of GAPDH.

Enzyme-linked immunosorbent assay

Cytokine interleukin-1β (IL-1β) from culture supernatants of treated cells and serum of xenograft mice were determined by an enzyme-linked immunosorbent assay (ELISA) method using an ELISA kit (Solarbio, China) according to manufactures' introduction.

Immunohistochemical staining

Immunohistochemical staining was performed according to a previously described protocol, with minor modifications. Tissues were processed as formalin-fixed paraffin-embedded tissue blocks, the sections were then cut into 4 μm-thick sections and dewaxed in xylene, rehydrated using an ethanol gradient in water, and rinsed with PBS. Antigen retrieval was performed by boiling in sodium citrate buffer for 15 min. The cut sections were treated with 3% hydrogen peroxide and blocked with normal goat serum at 37℃ for 30 min. The sections were incubated overnight at 4°C with anti-EGFR (bsm-33050M, Bioss, China) at 1:400, anti-TGF-β1 (bs-0086R, Bioss, China) at 1:300, anti-IL-1β (bs-6319R, Bioss, China) at 1:300, anti-MMP2 (bs-0412R, Bioss, China) at 1:400, anti-VEGFA (bs-4572R, Bioss, China) at 1:400 and anti-cytokeratin (BH0149, Bioss, China) at 1:200, followed by a conjugated secondary antibodies (ZSGB-BIO, OriGene Technologies) for 20 min at 37 °C and DAB (ZSGB-BIO, OriGene Technologies) staining.

Statistical analysis

Statistical analysis was carried out using SPSS software (version 22.0, IBM, USA) and GraphPad Prism (version 7.00, GraphPad Software, Inc., La Jolla, USA). The data are presented as mean ± standard deviation. Analysis of Variance (ANOVA) was used to compare the means of multiple groups. P-values less than 0.05 were considered statistically significant.

Results

Combined application of ICI118,551 and etodolac inhibited the migration and invasion ability of OSCC cells

The results of wound-healing assay showed that ICI118,551 (5μmol/L) or etodolac (5μmol/L) alone could inhibit the migration ability of OSCC cells, while the migration ability of the cells treated with the combination of two drugs was more significantly inhibited than that treated alone. The statistical results of three OSCC cells (Cal27, SCC9 and the primary OSCC cells) showed that compared with the control group, the other three groups could significantly inhibit the migration ability of OSCC cells. Compared with ICI118,551 group (ICI) and etodolac group (Eto), the combination group (ICI+Eto) could substantially inhibit the migration ability of OSCC cells (Fig.1a-f). Same as the wound-healing assay, the results of transwell invasion assay showed that the invasion rate is the lowest in ICI+Eto group of each OSCC cell (Fig.1g-j).

In vivo assay, combined use of ICI118,551 and etodolac inhibited the development of OSCC and prolong the overall survival time of Balb/c nude mice with OSCC.

Balb/c tumor bearing mice were administered either with ICI118,551 (ICI), etodolac (Eto), both drugs (ICI+Eto), or PBS as control. 6 mice in each group. The average size of tongue tumors was 60.15±8.564 (mm^3) in control group, 24.16±7.302 (mm^3) in ICI group, 21.42±2.552 (mm^3) in Eto group and 14.18± 0.6627 (mm^3) in combination group (Fig.2a-b). And the average survival time was 13.33 ±1.022 days in control group, 20.833±1.327 days in the ICI group, 15±0.365 days in Eto group and 23.5 ±1.962 days in

combination group. The results showed that combined application of ICI118,551 and etodolac could inhibit the growth of tumor and prolong the overall survival time of Balb/c nude mice with OSCC (Fig.2c).

After mice were dead, the submandibular lymph nodes were resected to detect whether the combination of drugs could inhibit the metastasis of submandibular lymph nodes. H&E staining was combined with immunohistochemical staining, which was carried out to detect the expression of cytokeratin (CK), to analyze the metastasis of submandibular lymph nodes. The results showed that submandibular lymph node metastasis was found in 3 mice in control group, 2 in ICI group, 2 in Eto group, and only 1 in ICI+Eto group (Fig.2d-g).

Combined application of ICI118,551 and etodolac down-regulated genes relate to OSCC invasion and metastasis

After combined treatment of ICI118,551 and etodolac for 48 hours, the expression of some genes relate to invasion and metastasis in OSCC cells were detected by real time quantitative polymerase chain reaction (RT-qPCR), Western blot and ELISA. Results of RT-qPCR showed that EGFR, TGF-β1, IL-1β and MMP2 were substantially down-regulated after combined treatment. Among these, EGFR was up-regulated after treated with ICI118,551 in Cal27, but it was down-regulated after combined treatment and the relative expression was also lower than etodolac treatment. Moreover, the change of relative expression of MMP2 was not obvious after treated with ICI118,551 in two OSCC cell lines, but it was down-regulated in three OSCC cells after combined application. However, VEGF-A was not down-regulated in two OSCC cell lines after combined treatment, it was down-regulated after combined treatment in primary OSCC cell instead (Fig.3). Results of Western blot showed that EGFR, MMP2, VEGF-A were down-regulated after combined treatment in three OSCC cells, TGF-β1 was down-regulated in Cal27 and SCC9 cell lines (Fig.4a-o). ELISA showed that IL-1β was down-regulated after combined treatment, the concentration of IL-1β in combination group was the lowest. (Fig.4p)

Genes related to invasion and metastasis of OSCC in xenograft animal models were also downregulated after combined treatment.

In vivo assay, another four groups were set as above. On day 12, mice were sacrificed, tongue tumors and blood samples were collected for the analysis of genes relate to invasion and metastasis. Results of IHC showed that the mean optical density of EGFR, TGF-β1, MMP2 and VEGF-A in combination group were lower than other three groups, as same as results of Western blot (Fig.5). Interestingly, Western blot showed that the expression of VEGF-A in ICI or Eto group was either higher than control group. However, it was down-regulated in combination group (Fig.6a-e). In ELISA, the mean concentration of IL-1β in ICI or Eto group was either lower than control group. Same as vitro assay, in combination group, the concentration of IL-1β was the lowest (Fig.6f).

Discussion

OSCC is the most common cancer in oral and maxillofacial region. Surgery combined with chemotherapy and radiotherapy is still the standard treatment of OSCC, but the overall 5-year survival rate of OSCC patients has not been significantly improved in the past few decades. Recent years, β2-AR and COX-2 were found that overexpressed in OSCC, different studies also found that β2-AR blocker or COX-2 inhibitor could inhibit the progression of OSCC in *vitro* and *vivo*. However, a few clinical trials showed that post-operative use of β-AR blocker couldn't increase the survival rate of OSCC patients [33]. For COX-2 inhibitor, some clinical trials reported that celecoxib, a selective COX-2 inhibitor, could improve the prognosis of OSCC patients [34, 35]. Recent years, some studies of colon and breast cancer found that the combination of β2-AR blocker and COX-2 inhibitor could inhibit the occurrence and progression of malignant tumors and improve the prognosis of patients [30, 31]. So, we wanted to know whether the combined treatment could also inhibit the progression of OSCC.

To our knowledge, it is the first time to study the effect of combined use of β2-AR blocker and COX-2 inhibitor in OSCC. In *vitro*, we found that after combined treatment, the migration and invasion ability of OSCC cells were significantly inhibited. In *vivo*, the combined application of ICI118,551 and etodolac could inhibit the growth of tumor and prolong the overall survival time of Balb/c nude mice with OSCC. Moreover, to explore whether combined use of these two drugs could inhibit the progression of OSCC by regulating the expression of some genes related to invasion and metastasis further. We detected the expression of EGFR, TGF-β1, IL-1β, MMP2 and VEGF-A after combined treatment. The results of RT-qPCR showed that EGFR, TGF-β1, IL-1β and MMP2 were down-regulated in OSCC cells after combined treatment, except VEGF-A. The protein expression level of these above four genes were also the lowest in the four groups in *vitro* and *vivo*. EGFR, TGF-β1, IL-1β and MMP2 were all related to invasion and metastasis of malignant tumors, including OSCC [36–41]. So, we have verified that combined use of ICI118,551 and etodolac can inhibit the development of OSCC and the expression of EGFR, TGF-β1, IL-1β and MMP2 indeed.

Among these, we found that the expression change of EGFR was significant. EGFR, also known as ErbB1, belongs to EGF receptor family and is closely related to the growth and development of epidermal cells [42]. It has been found that EGFR is overexpressed in more than 80% invasive head and neck squamous cell carcinoma (HNSCC), which is closely related to the poor prognosis of the patients [43, 44]. EGFR can promote tumor cell proliferation, metastasis, anti-apoptosis and angiogenesis by activating its downstream signaling pathways Ras/Raf/MEK/ERK and phosphatidylinositide 3-kinases/protein kinase B (PI3K/Akt) [45, 46]. Some studies reported that EGFR also could promote the tumor immune escape mechanism [47, 48]. In recent years, EGFR-targeted therapy has been used to clinical treatment of OSCC [49–52]. It was also mentioned in the clinical guideline of national comprehensive cancer network (NCCN) in 2019 that cetuximab, a kind of EGFR-targeted drugs, could combine with radiotherapy and chemotherapy in the systematic treatment of OSCC [53]. However, many families with poor financial condition cannot afford the expensive price of cetuximab, and some studies reported that cetuximab had effective therapeutic effect on only 10–20% cancer patients [52, 54]. In our study, we found that the combination of common and inexpensive β2-AR blocker and COX-2 inhibitor could significantly down-regulate the expression of EGFR and inhibit the development of OSCC in *vitro* and *vivo*. Nasry et al.

reported that cetuximab could suppress the proliferation of cancer cells by inhibiting COX-2/PGE2/EGFR pathway [23]. But the molecular mechanism of β2-AR regulates the expression of EGFR has not been determined. Further studies need to explore the molecular mechanism of β2-AR combines with COX-2 to regulate EGFR. If we can clarify the mechanism clearly further, the combined use of β2-AR blocker and COX-2 inhibitor will be another treatment which can inhibit the expression of EGFR. And there will be one more choice of pharmaco-adjuvant therapy for the OSCC patients who cannot afford the price of cetuximab.

In this study, we also found that TGF-β1, IL-1β and MMP2 were down-regulated after combined treatment. TGF-β1 is a crucial inducer of epithelial-mesenchymal transition (EMT) [55]. EMT refers to the biological process in which cells transition from epithelial state to mesenchymal state. It is closely related to embryonic development, wound healing, and cancer metastasis especially [37]. In HNSCC, the expression of TGF-β1 is higher than normal tissues. Chen, et al. reported that the over-expression of TGF-β1 in OSCC was related to lymph node metastasis, recurrence and poor survival rate [56]. Moreover, IL-1β plays an important role in tumor microenvironment. IL-1β can increase the proliferation of dysplasia oral cells, stimulate oncogenic cytokines, and promote aggressiveness of OSCC [39, 57]. Similar to the above-mentioned genes, MMP2 was found to be overexpressed in HNSCC. Among MMPs, MMP2 is one of the most commonly identified in HNSCC and is associated with invasion and metastasis [58, 59]. Meanwhile, MMP2 was found that could be used for evaluating lymph node metastasis and tumor progression in OSCC [60]. So, the molecular mechanism still needs to be clarified. And, the clinical trials also need to proceed to determine the effect of this combined treatment in the future.

Conclusions

In conclusion, this study suggests that combined use of ICI118,551 and etodolac can inhibit the development of OSCC in *vitro* and *vivo*, especially the ability of invasion and metastasis. Meanwhile, the combination of these two drugs is more effective than ICI118,551 or etodolac treatment alone, the combined use of β2-AR blocker and COX-2 inhibitor will be a potential treatment for OSCC via downregulating expression of EGFR, TGF-β1, IL-1β and MMP2.

List Of Abbreviations

ANOVA=analysis of variance

β2-AR=β2-adrenergic receptor

COX-2=cyclooxygenase-2

DMEM=dulbecco's modified eagle medium

DMEM/F12=dulbecco's modified eagle medium and ham's F-12 nutrient mixture

DMSO=dimethyl sulfoxide

EGFR=epidermal growth factor receptor

EMT=epithelial-mesenchymal transition

Eto=etodolac

FBS=fetal bovine serum

GAPDH=glyceraldehyde-3-phosphate dehydrogenase

HNSCC=head and neck squamous cell carcinoma

ICI=ICI118,551

IL-1β=interleukin-1β

MMP2=matrix metalloproteinase 2

mRNA=messenger ribonucleic acid

NCCN=national comprehensive cancer network

OSCC=oral squamous cell carcinoma

PBS=phosphate buffer saline

PI3K/Akt=phosphatidylinositide 3-kinases/protein kinase B

RNA=ribonucleic acid

RT-qPCR=real time quantitative polymerase chain reaction

STR=short tandem repeat

TGF-1β=transforming growth factor-β1

VEGF-A=vascular endothelial growth factor-A

Declarations

Ethics approval and consent to participate

All animal experimental procedures received approval by the Laboratory Animal Care and Use Committees of Guangxi Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

The authors gratefully acknowledge the financial supported by National Natural Science Foundation of China (Research on beta-adrenergic receptor mediated signaling pathways regulating oral squamous cell carcinoma and its molecular mechanism 81460413).

Authors' contributions

HMM participated in formulating the study concepts and manuscript review. CZ carried out vitro and vivo assays, participated in formulating the study concepts, study design, data acquisition, manuscript preparation, editing and review. ZM carried out vitro and vivo assays, participated in study design, data acquisition, manuscript preparation and review. XXL participated in study design, vitro assay, data acquisition, manuscript preparation. SQL participated in study design, quality control of data and algorithms and statistical analysis. FF participated in data acquisition and data analysis and interpretation. SCT participated in vivo assay, quality control of data and algorithms and statistical analysis. XTL participated in quality control of data and algorithms, data analysis and interpretation. XTW participated in quality control of data and algorithms and data analysis and interpretation. XJC participated in data analysis and interpretation. MYC and XSN participated in statistical analysis. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References

1. Chow LQM. Head and Neck Cancer. N Engl J Med. 2020;382:60–72.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018;68:7–30.
3. Perri F, Muto P, Aversa C, Daponte A, Della Vittoria G, Pepe S, et al. Integrated therapeutic approaches in head and neck cancer: the importance of multidisciplinary team management. Anticancer Agents

Med Chem. 2013;13:834–43.

4. Quinlan-Davidson SR, Morrison WH, Myers JN, Gunn GB, William WN Jr, Beadle BM, et al. Recurrent oral cavity cancer: Patterns of failure after salvage multimodality therapy. Head Neck. 2017;39:633–8.
5. Allen C, Her S, Jaffray DA. Radiotherapy for Cancer: Present and Future. Adv Drug Deliv Rev. 2017;109:1–2.
6. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69–90.
7. Partecke LI, Speerforck S, Käding A, Seubert F, Kühn S, Lorenz E, et al. Chronic stress increases experimental pancreatic cancer growth, reduces survival and can be antagonised by beta-adrenergic receptor blockade. Pancreatology. 2016;16:423–33.
8. Aydiner A, Ciftci R, Karabulut S, Kilic L. Does beta-blocker therapy improve the survival of patients with metastatic non-small cell lung cancer? Asian Pac J Cancer Prev. 2013;14:6109–14.
9. Cole SW, Sood AK. Molecular pathways: beta-adrenergic signaling in cancer. Clin Cancer Res. 2012;18:1201–6.
10. Tang J, Li Z, Lu L, Cho CH. Beta-Adrenergic system, a backstage manipulator regulating tumour progression and drug target in cancer therapy. Semin Cancer Biol. 2013;23:533–42.
11. Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, et al. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nat Med. 2006;12:939–44.
12. Bravo-Calderón DM, Oliveira DT, Marana AN, Nonogaki S, Carvalho AL, Kowalski LP. Prognostic significance of beta-2 adrenergic receptor in oral squamous cell carcinoma. Cancer Biomarkers. 2012;10:51–9.
13. Zahalka AH, Arnal-Estapé A, Maryanovich M, Nakahara F, Cruz CD, Finley LWS, et al. Adrenergic nerves activate an angio-metabolic switch in prostate cancer. Science. 2017;358:321–6.
14. Le CP, Nowell CJ, Kim-Fuchs C, Botteri E, Hiller JG, Ismail H, et al. Chronic stress in mice remodels lymph vasculature to promote tumour cell dissemination. Nat Commun. 2016;7:10634.
15. Zhang C, Liao X, Ma Z, Liu S, Fang F, Mai H. Overexpression of β-Adrenergic Receptors and the Suppressive Effect of β_2-Adrenergic Receptor Blockade in Oral Squamous Cell Carcinoma. J Oral Maxillofac Surg. 2020; 78: 1871.e1-1871.e23.
16. Wolter NE, Wolter JK, Enepekides DJ, Irwin MS. Propranolol as a novel adjunctive treatment for head and neck squamous cell carcinoma. J Otolaryngol Head Neck Surg. 2012;41:334–44.
17. Lucido CT, Miskimins WK, Vermeer PD. Propranolol Promotes Glucose Dependence and Synergizes with Dichloroacetate for Anti-Cancer Activity in HNSCC. Cancers (Basel). 2018; 10.
18. Giaginis C, Alexandrou P, Tsoukalas N, Sfiniadakis I, Kavantzas N, Agapitos E, et al. Hu-antigen receptor (HuR) and cyclooxygenase-2 (COX-2) expression in human non-small-cell lung carcinoma: associations with clinicopathological parameters, tumor proliferative capacity and patients' survival. Tumour Biol. 2015;36:315–27.

19. Breinig M, Schirmacher P, Kern MA. Cyclooxygenase-2 (COX-2)--a therapeutic target in liver cancer? Curr Pharm Des. 2007;13:3305–15.
20. Erovic BM, Pelzmann M, Turhani D, Pammer J, Niederberger V, Neuchrist C, et al. Differential expression pattern of cyclooxygenase-1 and – 2 in head and neck squamous cell carcinoma. Acta Otolaryngol. 2003;123:950–3.
21. Pontes HA, Pontes FS, Fonseca FP, de Carvalho PL, Pereira EM, de Abreu MC, et al. Nuclear factor kappaB and cyclooxygenase-2 immunoexpression in oral dysplasia and oral squamous cell carcinoma. Ann Diagn Pathol. 2013;17:45–50.
22. Sano Y, Kogashiwa Y, Araki R, Enoki Y, Ikeda T, Yoda T, et al. Correlation of Inflammatory Markers, Survival, and COX2 Expression in Oral Cancer and Implications for Prognosis. Otolaryngol Head Neck Surg. 2018;158:667–76.
23. Nasry WHS, Rodriguez-Lecompte JC, Martin CK. Role of COX-2/PGE2 Mediated Inflammation in Oral Squamous Cell Carcinoma. Cancers (Basel). 2018; 10.
24. Wang Z, Fuentes CF, Shapshay SM. Antiangiogenic and chemopreventive activities of celecoxib in oral carcinoma cell. Laryngoscope. 2002;112:839–43.
25. Kurihara Y, Hatori M, Ando Y, Ito D, Toyoshima T, Tanaka M, et al. Inhibition of cyclooxygenase-2 suppresses the invasiveness of oral squamous cell carcinoma cell lines via down-regulation of matrix metalloproteinase-2 production and activation. Clin Exp Metastasis. 2009;26:425–32.
26. Lang S, Tiwari S, Andratschke M, Loehr I, Lauffer L, Bergmann C, et al. Immune restoration in head and neck cancer patients after in vivo COX-2 inhibition. Cancer Immunol Immunother. 2007;56:1645–52.
27. Lang S, Picu A, Hofmann T, Andratschke M, Mack B, Moosmann A, et al. COX-inhibitors relieve the immunosuppressive effect of tumor cells and improve functions of immune effectors. Int J Immunopathol Pharmacol. 2006;19:409–19.
28. Patil VM, Noronha V, Joshi A, Muddu VK, Dhumal S, Bhosale B, et al. A prospective randomized phase II study comparing metronomic chemotherapy with chemotherapy (single agent cisplatin), in patients with metastatic, relapsed or inoperable squamous cell carcinoma of head and neck. Oral Oncol. 2015;51:279–86.
29. Halamka M, Cvek J, Kubes J, Zavadova E, Kominek P, Horacek J, et al. Plasma levels of vascular endothelial growth factor during and after radiotherapy in combination with celecoxib in patients with advanced head and neck cancer. Oral Oncol. 2011;47:763–7.
30. Sorski L, Melamed R, Matzner P, Lavon H, Shaashua L, Rosenne E, et al. Reducing liver metastases of colon cancer in the context of extensive and minor surgeries through beta-adrenoceptors blockade and COX2 inhibition. Brain Behav Immun. 2016;58:91–8.
31. Shaashua L, Shabat-Simon M, Haldar R, Matzner P, Zmora O, Shabtai M, et al. Perioperative COX-2 and β-Adrenergic Blockade Improves Metastatic Biomarkers in Breast Cancer Patients in a Phase-II Randomized Trial. Clin Cancer Res. 2017;23:4651–61.

32. Benish M, Bartal I, Goldfarb Y, Levi B, Avraham R, Raz A, et al. Perioperative use of beta-blockers and COX-2 inhibitors may improve immune competence and reduce the risk of tumor metastasis. Ann Surg Oncol. 2008;15:2042–52.
33. Kim MS, Han KD, Kwon SY. Pre-diagnostic beta-blocker use and head- and neck cancer risk: A nationwide population-based case-control study. Medicine. 2019;98:e16047.
34. Lee DY, Lim JH, Kim YJ, Kim SD, Park SW, Kwon SK, et al. Effect of Celecoxib on Survival of Mobile Tongue Cancer. Anticancer Res. 2015;35:4235–41.
35. Lai V, George J, Richey L, Kim HJ, Cannon T, Shores C, et al. Results of a pilot study of the effects of celecoxib on cancer cachexia in patients with cancer of the head, neck, and gastrointestinal tract. Head Neck. 2008;30:67–74.
36. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, et al. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene. 2006;366:2–16.
37. Nieto MA, Huang RY, Jackson RA, Thiery JP. Emt: 2016. Cell. 2016; 166: 21–45.
38. Qiao B, Johnson NW, Gao J. Epithelial-mesenchymal transition in oral squamous cell carcinoma triggered by transforming growth factor-beta1 is Snail family-dependent and correlates with matrix metalloproteinase-2 and – 9 expressions. Int J Oncol. 2010;37:663–8.
39. Lee CH, Chang JS, Syu SH, Wong TS, Chan JY, Tang YC, et al. IL-1beta promotes malignant transformation and tumor aggressiveness in oral cancer. J Cell Physiol. 2015;230:875–84.
40. Wu T, Hong Y, Jia L, Wu J, Xia J, Wang J, et al. Modulation of IL-1beta reprogrammes the tumor microenvironment to interrupt oral carcinogenesis. Sci Rep. 2016;6:20208.
41. Celentano A, Yap T, Paolini R, Yiannis C, Mirams M, Koo K, et al. Inhibition of matrix metalloproteinase-2 modulates malignant behaviour of oral squamous cell carcinoma cells. J Oral Pathol Med. 2020.
42. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol. 1995;19:183–232.
43. Temam S, Kawaguchi H, El-Naggar AK, Jelinek J, Tang H, Liu DD, et al. Epidermal growth factor receptor copy number alterations correlate with poor clinical outcome in patients with head and neck squamous cancer. J Clin Oncol. 2007;25:2164–70.
44. Dai W, Li Y, Zhou Q, Xu Z, Sun C, Tan X, et al. Cetuximab inhibits oral squamous cell carcinoma invasion and metastasis via degradation of epidermal growth factor receptor. J Oral Pathol Med. 2014;43:250–7.
45. Zeng F, Harris RC. Epidermal growth factor, from gene organization to bedside. Semin Cell Dev Biol. 2014;28:2–11.
46. Forrester SJ, Kawai T, O'Brien S, Thomas W, Harris RC, Eguchi S, et al. Epidermal Growth Factor Receptor Transactivation: Mechanisms, Pathophysiology, and Potential Therapies in the Cardiovascular System. Annu Rev Pharmacol Toxicol. 2016;56:627–53.

47. Li X, Lian Z, Wang S, Xing L, Yu J. Interactions between EGFR and PD-1/PD-L1 pathway: Implications for treatment of NSCLC. Cancer Lett. 2018;418:1–9.
48. Ma W, Concha-Benavente F, Santegoets SJAM, Welters MJP, Ehsan I, Ferris RL, et al. EGFR signaling suppresses type 1 cytokine-induced T-cell attracting chemokine secretion in head and neck cancer. PLoS One. 2018;13:e0203402.
49. Ciardiello F. EGFR antagonists in cancer treatment. N Engl J Med. 2008;358:1160–74.
50. Cohen EEW, Licitra LF, Burtness B, Fayette J, Gauler T, Clement PM, et al. Biomarkers predict enhanced clinical outcomes with afatinib versus methotrexate in patients with second-line recurrent and/or metastatic head and neck cancer. Ann Oncol. 2017;28:2526–32.
51. Tahara M, Kiyota N, Yokota T, Hasegawa Y, Muro K, Takahashi S, et al. Phase II trial of combination treatment with paclitaxel, carboplatin and cetuximab (PCE) as first-line treatment in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck (CSPOR-HN02). Ann Oncol. 2018;29:1004–9.
52. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006;354:567–78.
53. The NCCN clinical. practice guidelines in oncology, head and neck cancers. National Comprehensive Cancer Network, lnc,2019.
54. Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, et al. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med. 2009;360:563–72.
55. Ahmadi A, Najafi M, Farhood B, Mortezaee K. Transforming growth factor-β signaling: Tumorigenesis and targeting for cancer therapy. J Cell Physiol. 2019;234:12173–87.
56. Chen MF, Wang WH, Lin PY, Lee KD, Chen WC. Significance of the TGF-β1/IL-6 axis in oral cancer. ClinSci (Lond). 2012;122:459–72.
57. Huang YH, Chang CY, Kuo YZ, Fang WY, Kao HY, Tsai ST, et al. Cancer-associated fibroblast-derived interleukin-1β activates protumor C-C motif chemokine ligand 22 signaling in head and neck cancer. Cancer Sci. 2019;110:2783–93.
58. Chien MH, Lin CW, Cheng CW, Wen YC, Yang SF. Matrix metalloproteinase-2 as a target for head and neck cancer therapy. Expert On Ther Targets. 2013;17:203–16.
59. Rosenthal EL, Matrisian LM. Matrix metalloproteases in head and neck cancer. Head Neck. 2006;28:639–48.
60. Lotfi A, Mohammadi G, Tavassoli A, Mousaviagdas M, Chavoshi H, Saniee L. Serum levels of MMP9 and MMP2 in patients with oral squamous cell carcinoma. Asian Pac J Cancer Prev. 2015;16:1327–30.

Tables

Table 1
The RT-qPCR primer sequences used for each gene we detected.

Genes	The primer sequences
GAPDH	Forward 5’-GAGTCAACGGATTTGGTCGT-3’
	Reverse 5’-TTGATTTTGGAGGGATCTCG-3’
VEGF-A	Forward 5’-CCTTGCTGCTCTACCTCCAC-3’
	Reverse 5’-ATGATTCTGCCCTCCTCCTT-3’
TGF-β1	Forward 5’-GCCGACTACTACGCCAAGGA-3’
	Reverse 5’-ATGCTGTGTGTACTCTGCTTGAAC-3’
IL-1β	Forward 5’-CCAGGGACAGGATATGGAGCA-3’
	Reverse 5’-TTCAACACGCAGGACAGGTACAG-3’
MMP2	Forward 5’-CTTCCAAGTCTGGAGCGATGTG-3’
	Reverse 5’-ATGAGCCAGGAGTCCGTCCTTA-3’
EGFR	Forward 5’-TGCATACAGTGCCACCCAGAG-3’
	Reverse 5’-GCACACTGGATACAGTTGTCTGGTC-3’

Figures

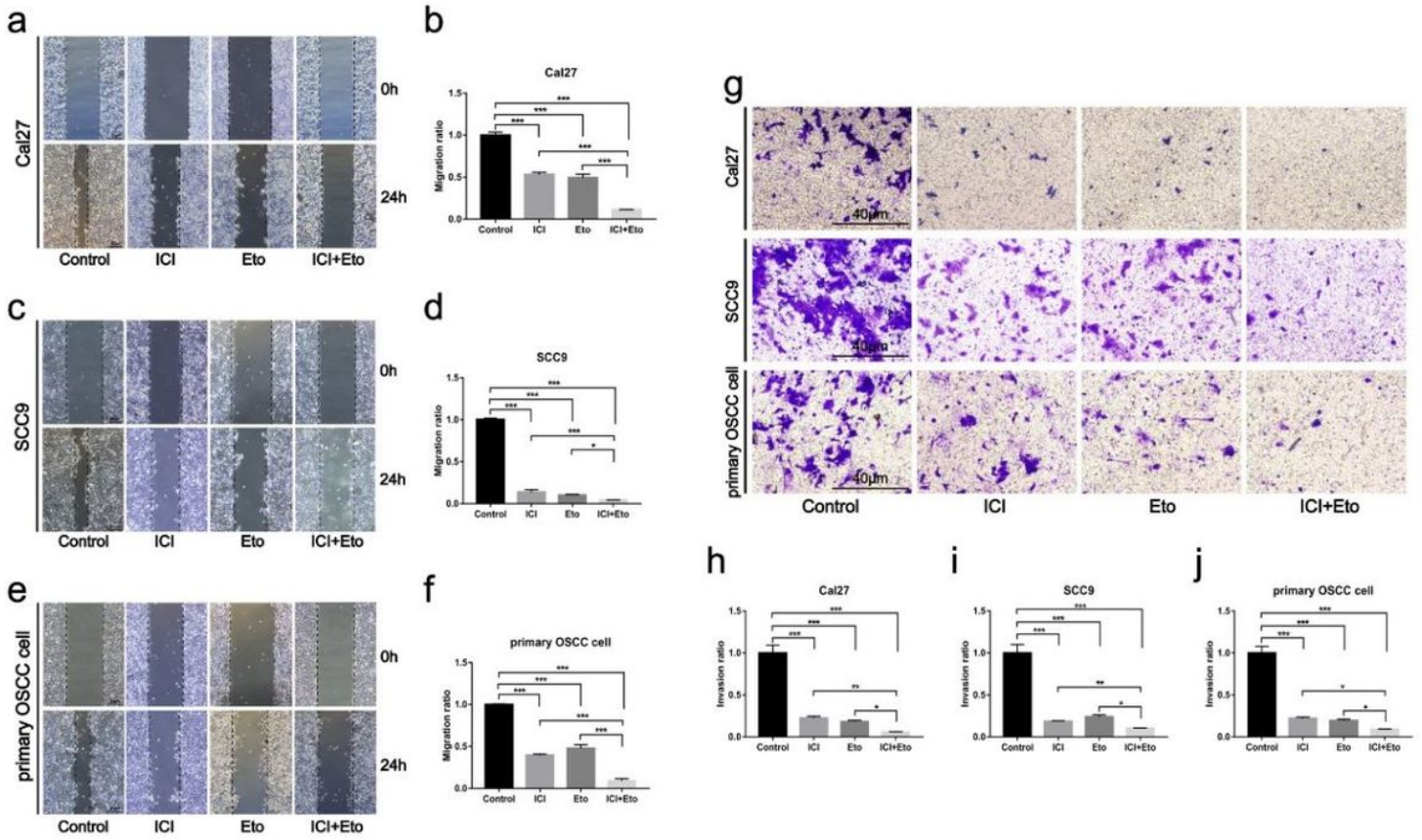

Figure 1

a. The combination of ICI118,551 and etodolac could inhibit the migration ability of Cal27 cell more significantly. b. The statistical analysis of migration ratio of Cal27 cell line. c. The combination of ICI118,551 and etodolac could inhibit the migration ability of SCC9 cell more significantly. d. The statistical analysis of migration ratio of SCC9 cell line. e. The combination of ICI118,551 and etodolac could inhibit the migration ability of primary OSCC cell more significantly. f. The statistical analysis of migration ratio of primary OSCC cell. g. The combination of ICI118,551 and etodolac could inhibit the invasion ability of OSCC cells more significantly. h. The statistical analysis of invasion ratio of Cal27 cell line. i. The statistical analysis of invasion ratio of SCC9 cell line. j. The statistical analysis of invasion ratio of primary OSCC cell. ICI=ICI118551, Eto= etodolac (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$).

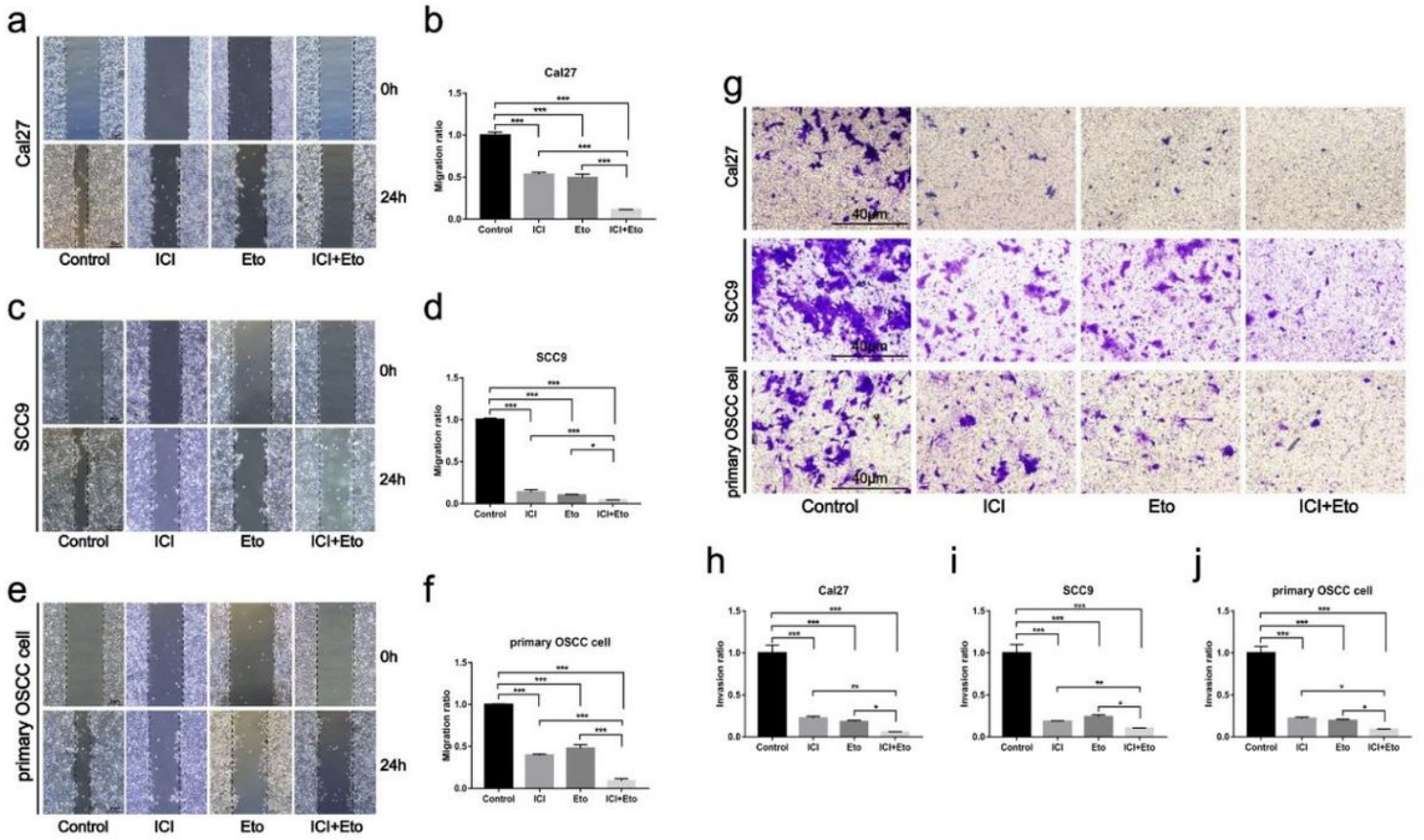

Figure 1

a. The combination of ICI118,551 and etodolac could inhibit the migration ability of Cal27 cell more significantly. b. The statistical analysis of migration ratio of Cal27 cell line. c. The combination of ICI118,551 and etodolac could inhibit the migration ability of SCC9 cell more significantly. d. The statistical analysis of migration ratio of SCC9 cell line. e. The combination of ICI118,551 and etodolac could inhibit the migration ability of primary OSCC cell more significantly. f. The statistical analysis of migration ratio of primary OSCC cell. g. The combination of ICI118,551 and etodolac could inhibit the invasion ability of OSCC cells more significantly. h. The statistical analysis of invasion ratio of Cal27 cell line. i. The statistical analysis of invasion ratio of SCC9 cell line. j. The statistical analysis of invasion ratio of primary OSCC cell. ICI=ICI118551, Eto= etodolac (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$).

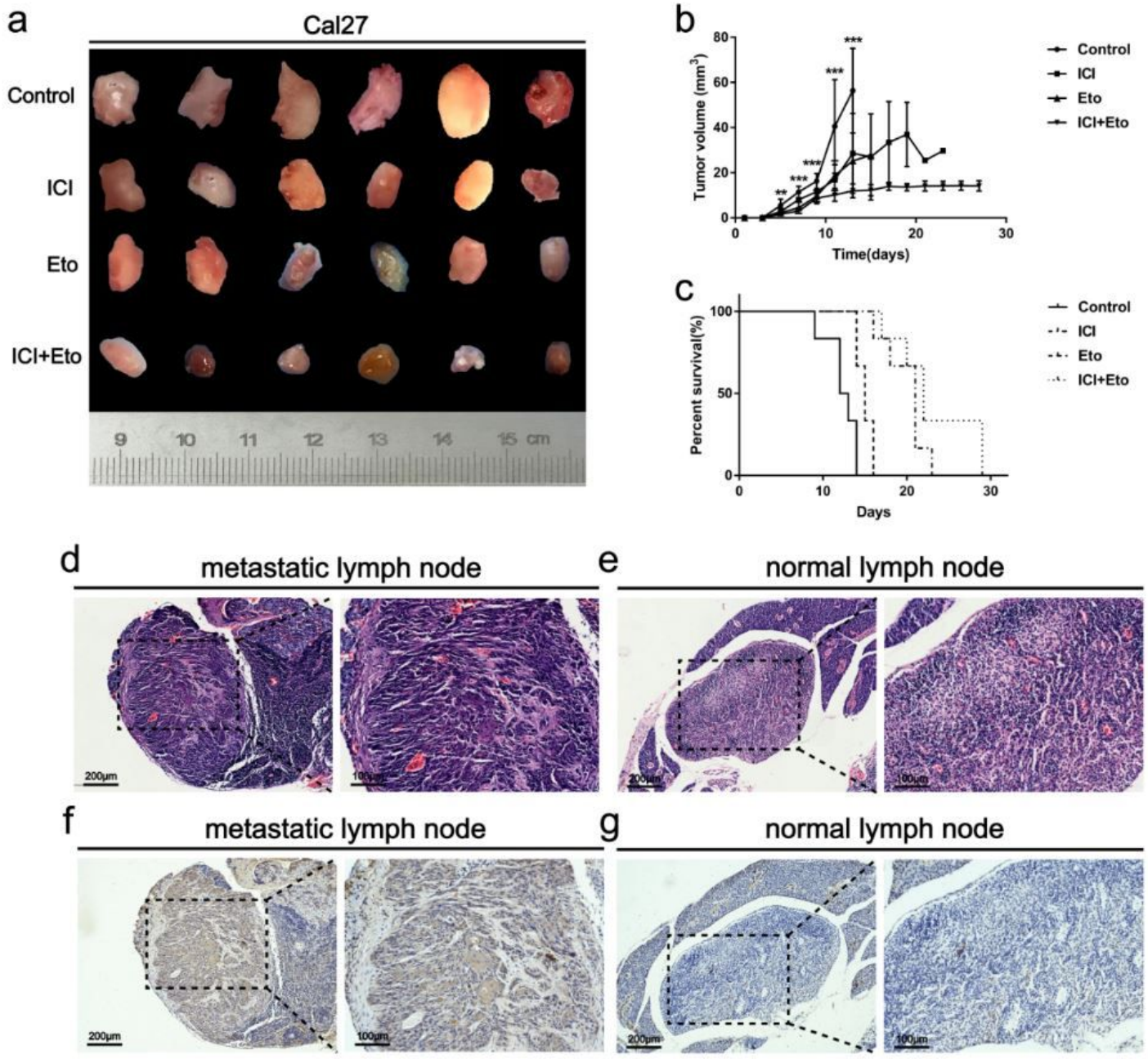

Figure 2

a. Compared with the size of tumors in four groups, the combination group could significantly inhibit the growth of the tumor compared with the other three groups. b. The statistical analysis of tumor size of four groups. c. Kaplan-Meier survival analysis showed that the combination group could significantly prolong the survival time of Balb/c nude mice with orthotopic implanted tumor. d, e. H&E staining of metastatic submandibular lymph nodes and normal submandibular lymph nodes. f, g. IHC was carried to detect the expression of cytokeratin in metastatic submandibular lymph nodes and normal submandibular lymph nodes.

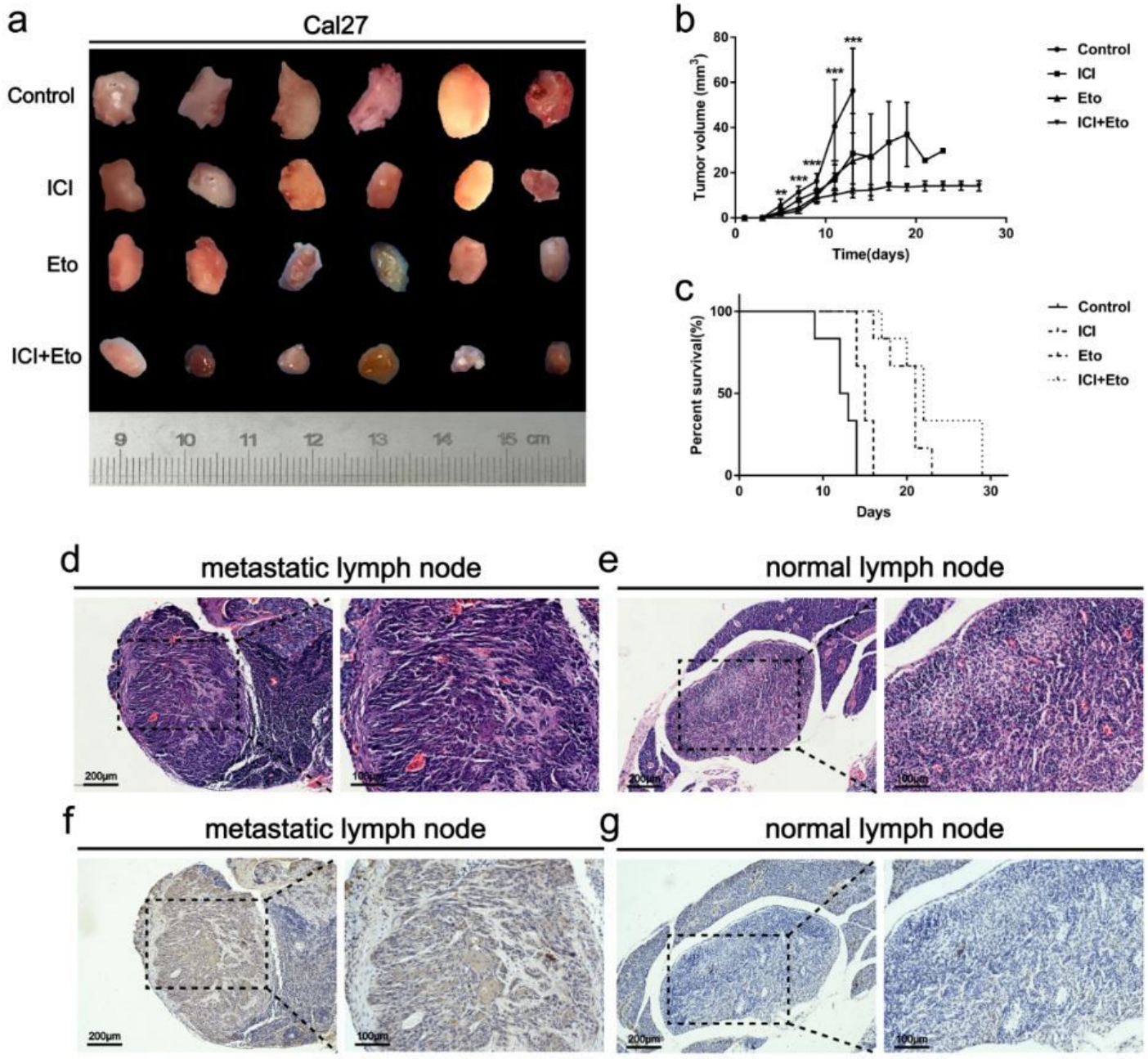

Figure 2

a. Compared with the size of tumors in four groups, the combination group could significantly inhibit the growth of the tumor compared with the other three groups. b. The statistical analysis of tumor size of four groups. c. Kaplan-Meier survival analysis showed that the combination group could significantly prolong the survival time of Balb/c nude mice with orthotopic implanted tumor. d, e. H&E staining of metastatic submandibular lymph nodes and normal submandibular lymph nodes. f, g. IHC was carried to detect the expression of cytokeratin in metastatic submandibular lymph nodes and normal submandibular lymph nodes.

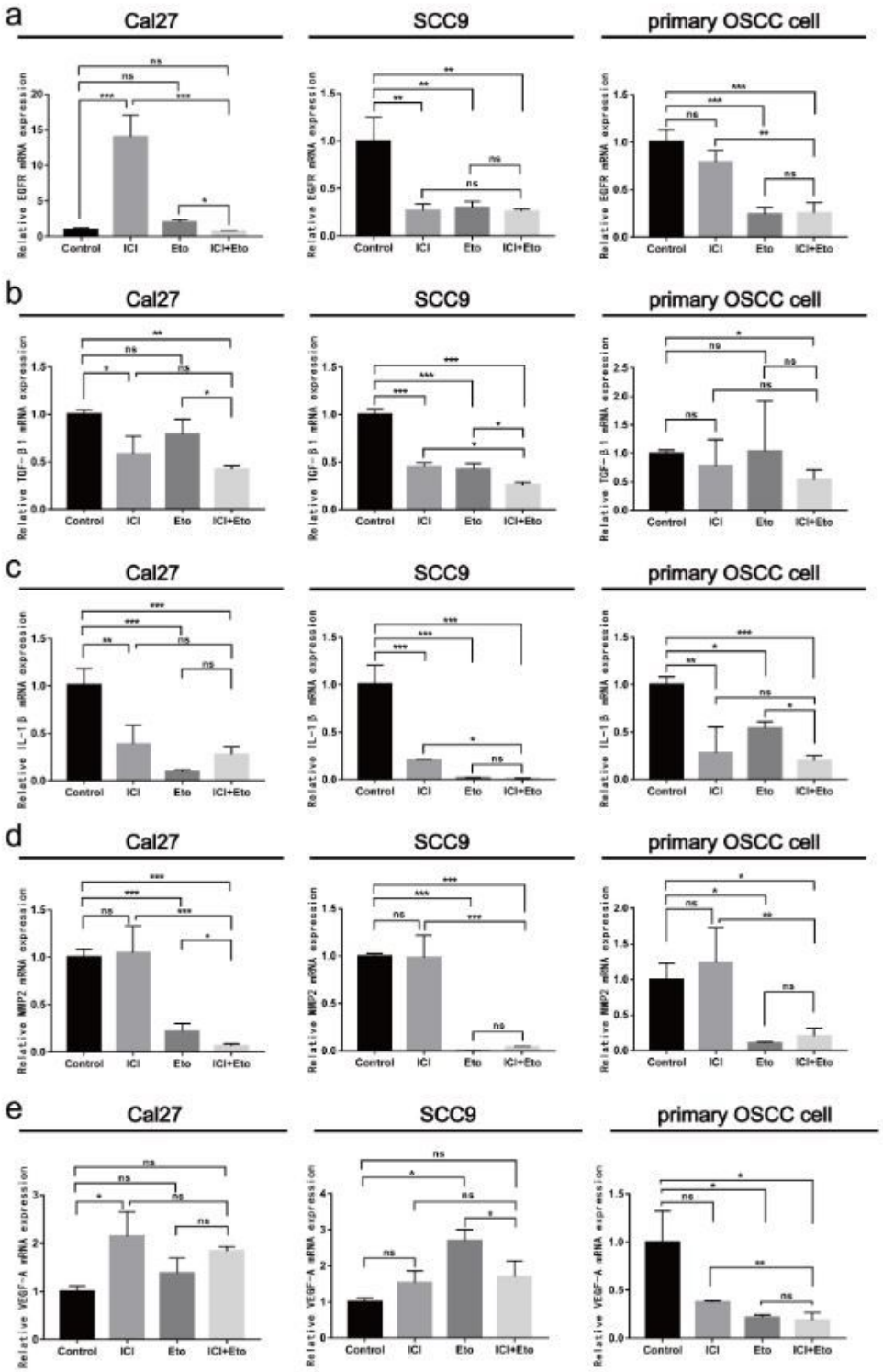

Figure 3

Comparison of the relevant mRNAs in three OSCC cells after treated by ICI118,551, etodolac alone and the combination of the two drugs. a. The relative mRNA expression of EGFR in four groups of three OSCC cells. b. The relative mRNA expression of TGF-β1 in four groups of three OSCC cells. c. The relative mRNA expression of IL-1β in four groups of three OSCC cells. d. The relative mRNA expression of MMP2 in four

groups of three OSCC cells. e. The relative mRNA expression of VEGF-A in four groups of three OSCC cells. ICI=ICI118551, Eto= etodolac. (ns P > 0.05, * P < 0.05, ** P < 0.001, *** P < 0.001).

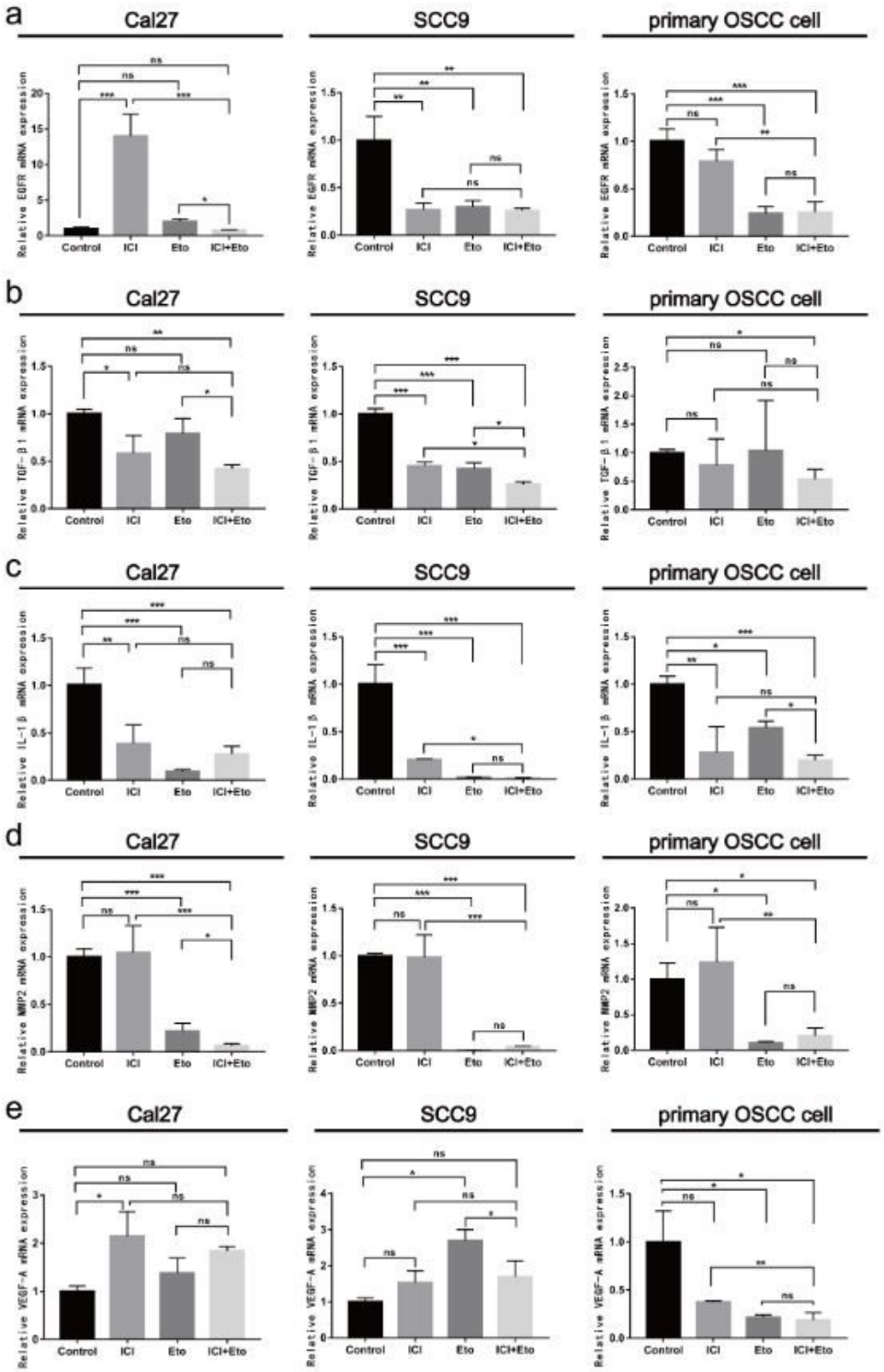

Figure 3

Comparison of the relevant mRNAs in three OSCC cells after treated by ICI118,551, etodolac alone and the combination of the two drugs. a. The relative mRNA expression of EGFR in four groups of three OSCC cells. b. The relative mRNA expression of TGF-β1 in four groups of three OSCC cells. c. The relative mRNA

expression of IL-1β in four groups of three OSCC cells. d. The relative mRNA expression of MMP2 in four groups of three OSCC cells. e. The relative mRNA expression of VEGF-A in four groups of three OSCC cells. ICI=ICI118551, Eto= etodolac. (ns P > 0.05, * P < 0.05, ** P < 0.001, *** P < 0.001).

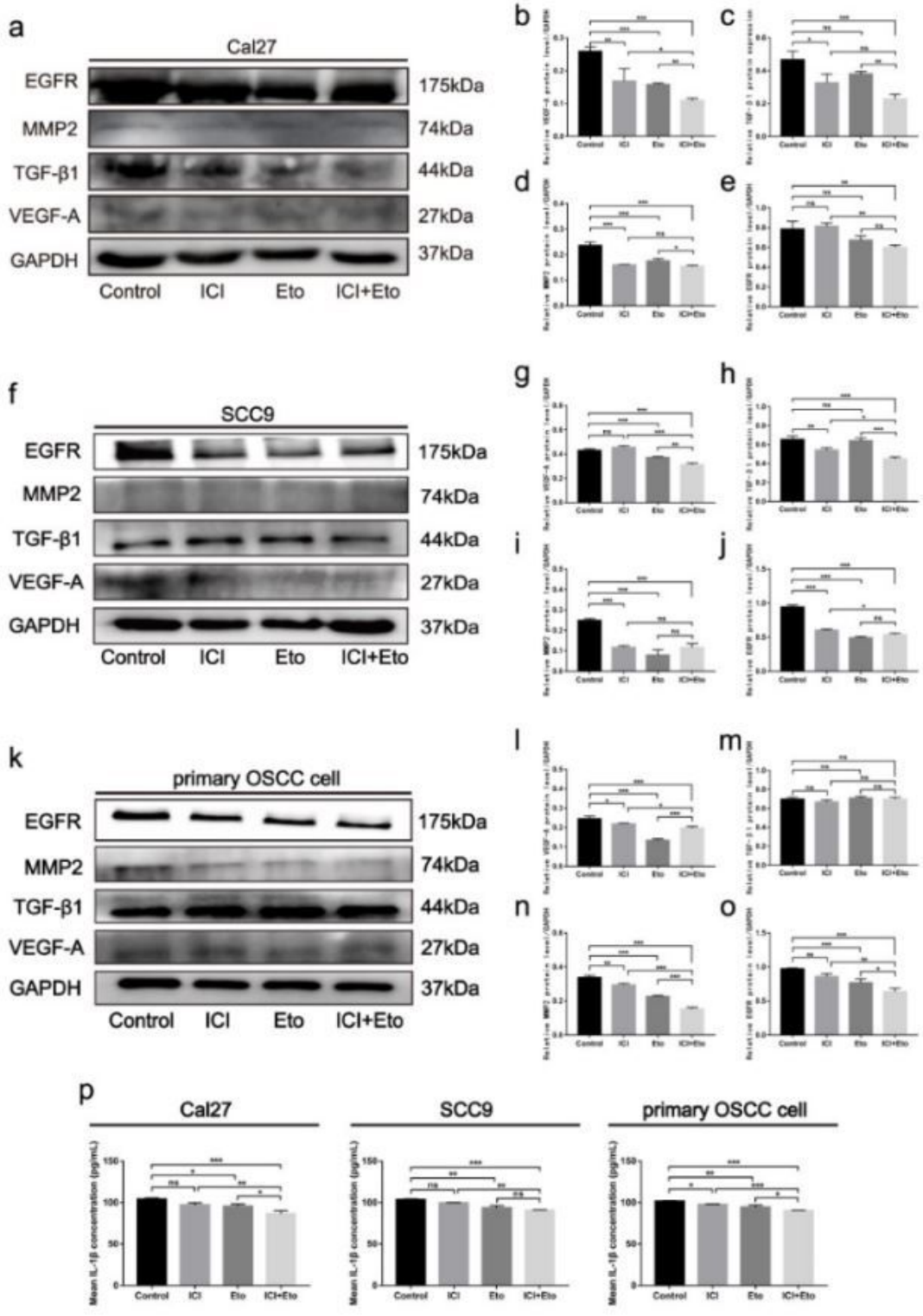

Figure 4

Comparison of the relevant protein expression levels of genes related to development of tumor in three OSCC cells treated with ICI118,551, etodolac alone and the combination of the two drugs. a. The relative

expression of VEGF-A, TGF-β1, MMP2 and EGFR in Cal27 cells treated with ICI118551, etodolac and the combination of the two drugs were compared by Western blot. b. The statistical analysis of relative protein expression of VEGF-A. c. The statistical analysis of relative protein expression of TGF-β1 protein. d. The statistical analysis of relative protein expression of MMP2 protein. e. The statistical analysis of relative protein expression of EFGR protein. f. The relative expression of VEGF-A, TGF-β1, MMP2 and EGFR in SCC9 cells treated with ICI118551, etodolac and the combination of the two drugs were compared by Western blot. g. The statistical analysis of relative protein expression of VEGF-A. h. The statistical analysis of relative protein expression of TGF-β1 protein. i. The statistical analysis of relative protein expression of MMP2 protein. j. The statistical analysis of relative protein expression of EFGR protein. k. The relative expression of VEGF-A, TGF-β1, MMP2 and EGFR in primary OSCC cells treated with ICI118551, etodolac and the combination of the two drugs were compared by Western blot. l. The statistical analysis of relative protein expression of VEGF-A. m. The statistical analysis of relative protein expression of TGF-β1. n. The statistical analysis of relative protein expression of MMP2 protein. o. The statistical analysis of relative protein expression of EFGR. p. Comparison of the concentration of IL-1β in cell supernatant after three OSCC cells treated with ICI118,551, etodolac alone and the combination of the two drugs. ICI=ICI118551, Eto= etodolac. (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$).

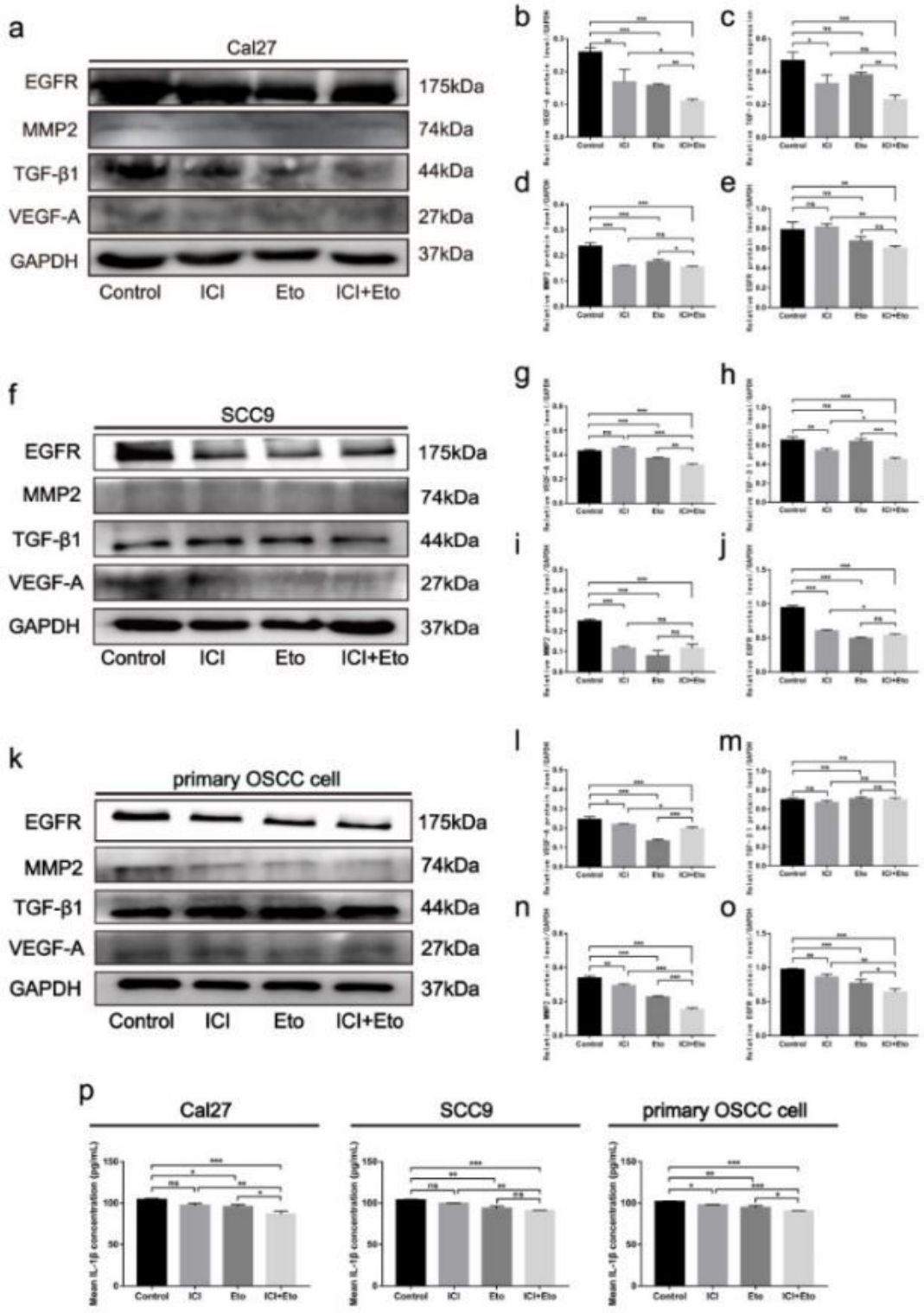

Figure 4

Comparison of the relevant protein expression levels of genes related to development of tumor in three OSCC cells treated with ICI118,551, etodolac alone and the combination of the two drugs. a. The relative expression of VEGF-A, TGF-β1, MMP2 and EGFR in Cal27 cells treated with ICI118551, etodolac and the combination of the two drugs were compared by Western blot. b. The statistical analysis of relative protein expression of VEGF-A. c. The statistical analysis of relative protein expression of TGF-β1 protein.

d. The statistical analysis of relative protein expression of MMP2 protein. e. The statistical analysis of relative protein expression of EFGR protein. f. The relative expression of VEGF-A, TGF-β1, MMP2 and EGFR in SCC9 cells treated with ICI118551, etodolac and the combination of the two drugs were compared by Western blot. g. The statistical analysis of relative protein expression of VEGF-A. h. The statistical analysis of relative protein expression of TGF-β1 protein. i. The statistical analysis of relative protein expression of MMP2 protein. j. The statistical analysis of relative protein expression of EFGR protein. k. The relative expression of VEGF-A, TGF-β1, MMP2 and EGFR in primary OSCC cells treated with ICI118551, etodolac and the combination of the two drugs were compared by Western blot. l. The statistical analysis of relative protein expression of VEGF-A. m. The statistical analysis of relative protein expression of TGF-β1. n. The statistical analysis of relative protein expression of MMP2 protein. o. The statistical analysis of relative protein expression of EFGR. p. Comparison of the concentration of IL-1β in cell supernatant after three OSCC cells treated with ICI118,551, etodolac alone and the combination of the two drugs. ICI=ICI118551, Eto= etodolac. (ns P > 0.05, * P < 0.05, ** P < 0.001, *** P < 0.001).

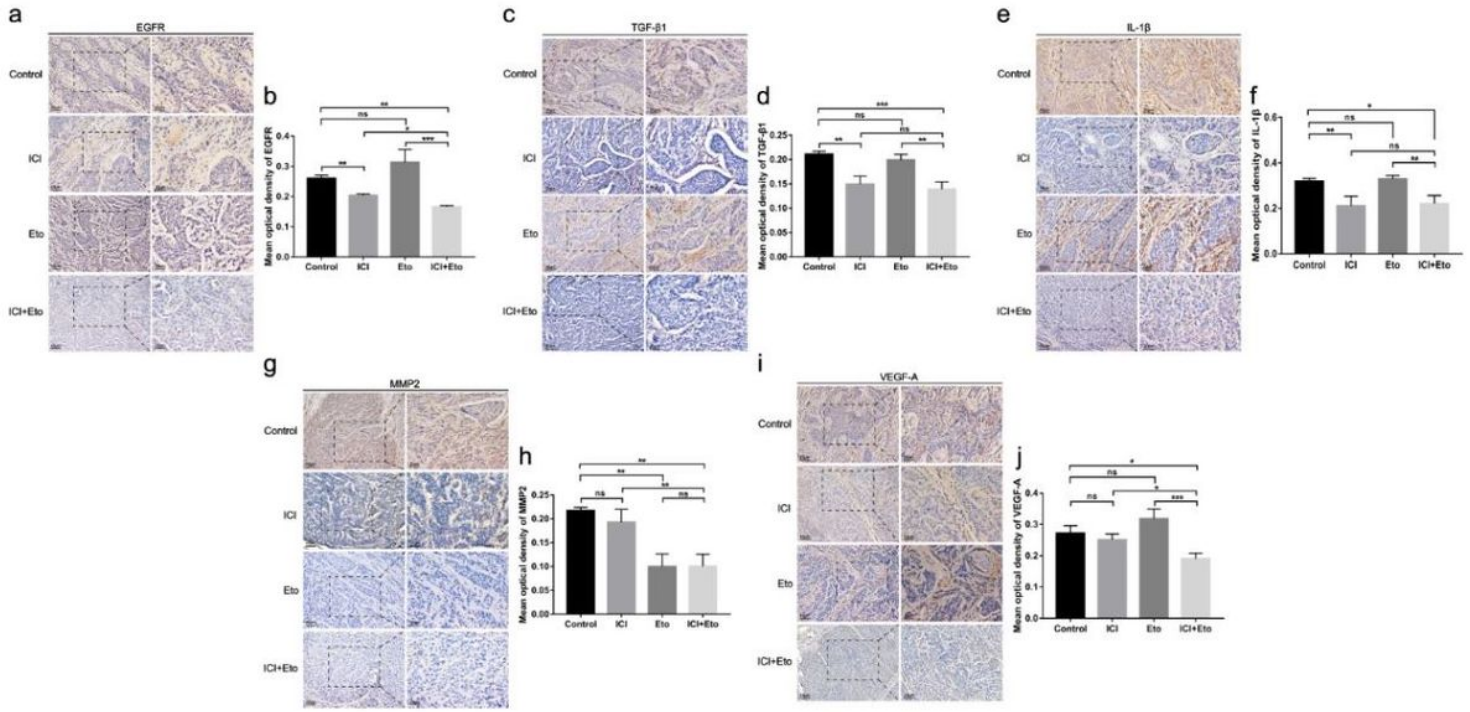

Figure 5

a. The difference of EGFR expression in tongue tumors of four groups was detected by IHC. b. The result of statistical analysis of EGFR. c. The difference of TGF-β1expression in tongue tumors of four groups was detected by IHC. d. The result of statistical analysis of TGF-β1. e. The difference of IL-1β expression in tongue tumors of four groups was detected by IHC. f. The result of statistical analysis of IL-1β. g. The difference of MMP2 expression in tongue tumors of four groups was detected by IHC. h. The result of statistical analysis of MMP2. i. The difference of VEGF-A expression in tongue tumors of four groups was

detected by IHC. j. The result of statistical analysis of VEGF-A. ICI=ICI118551, Eto= etodolac. (ns P > 0.05, * P < 0.05, ** P < 0.001, *** P < 0.001).

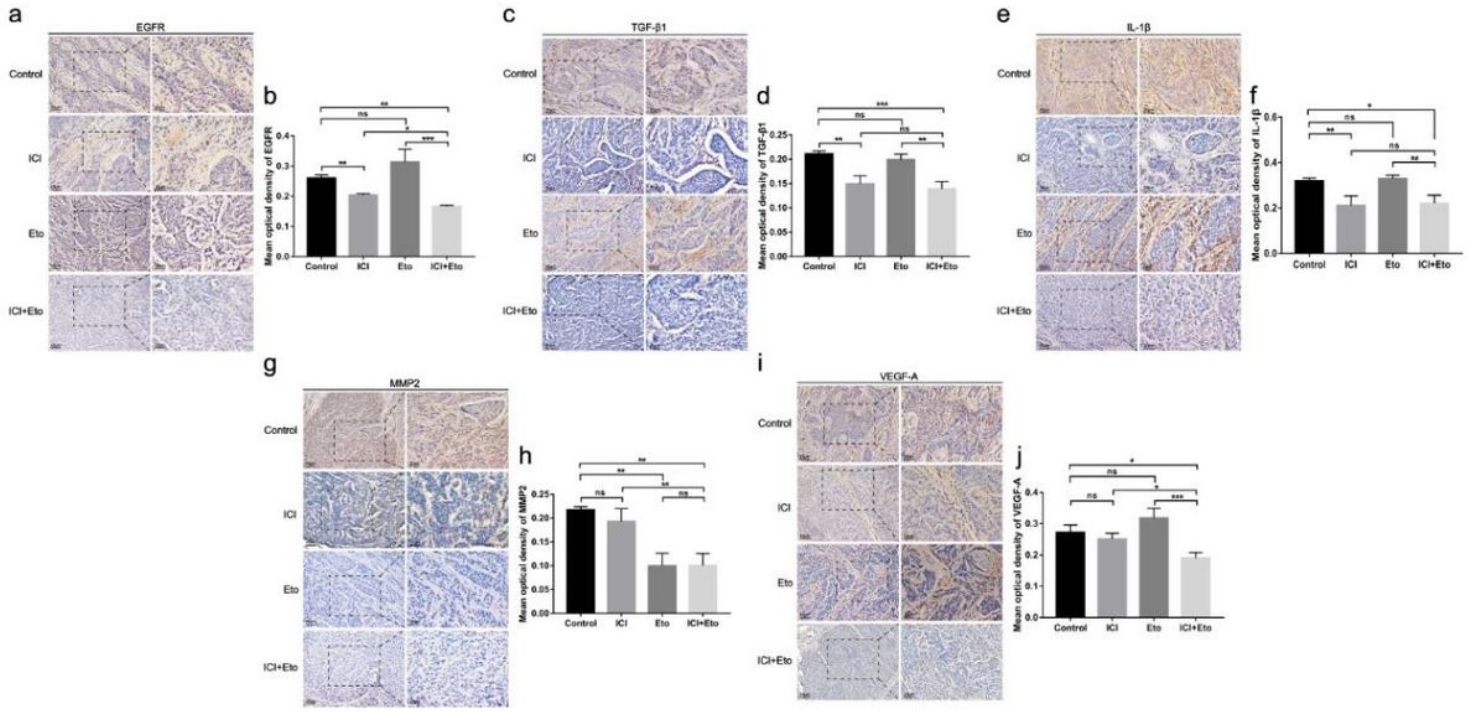

Figure 5

a. The difference of EGFR expression in tongue tumors of four groups was detected by IHC. b. The result of statistical analysis of EGFR. c. The difference of TGF-β1expression in tongue tumors of four groups was detected by IHC. d. The result of statistical analysis of TGF-β1. e. The difference of IL-1β expression in tongue tumors of four groups was detected by IHC. f. The result of statistical analysis of IL-1β. g. The difference of MMP2 expression in tongue tumors of four groups was detected by IHC. h. The result of statistical analysis of MMP2. i. The difference of VEGF-A expression in tongue tumors of four groups was detected by IHC. j. The result of statistical analysis of VEGF-A. ICI=ICI118551, Eto= etodolac. (ns P > 0.05, * P < 0.05, ** P < 0.001, *** P < 0.001).

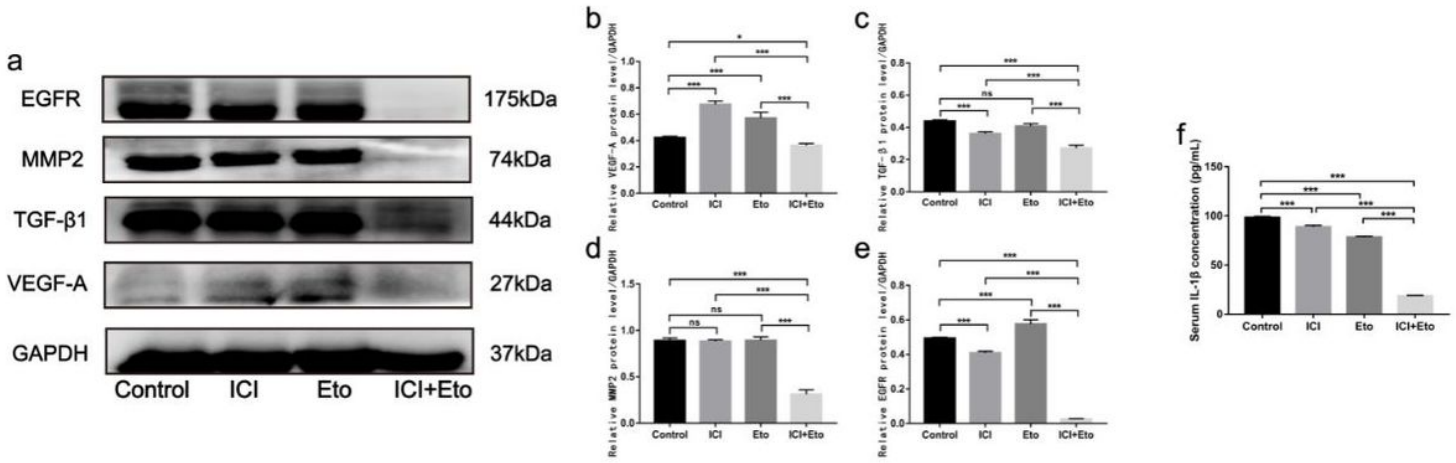

Figure 6

The relevant protein expression levels of genes related to the development of tumor. a. The relative expression of VEGF-A, TGF-β1, MMP2 and EGFR in tumor-bearing mice treated with ICI118551, etodolac and the combination of the two drugs were compared by Western blot. b. The statistical analysis of relative expression of VEGF-A protein. c. The statistical analysis of relative expression of TGF-β1 protein. d. The statistical analysis of relative expression of MMP2 protein. e. The statistical analysis of relative expression of EFGR protein. f. The concentration of serum IL-1β in tumor-bearing mice treated with ICI118551, etodolac and the combination of the two drugs were compared by ELISA. ICI=ICI118551, Eto= etodolac. (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$).

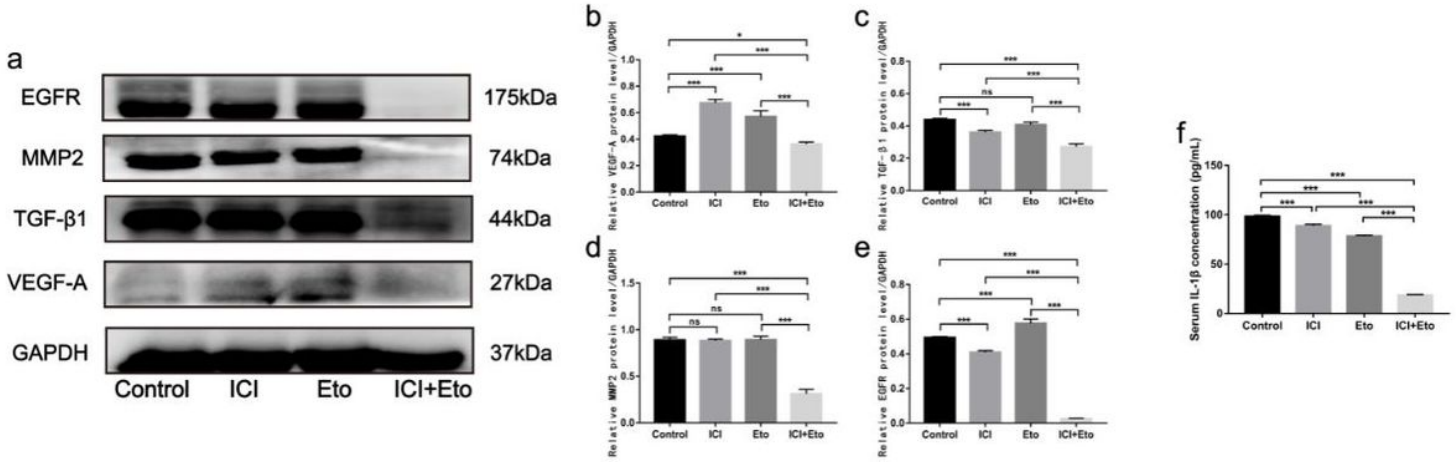

Figure 6

The relevant protein expression levels of genes related to the development of tumor. a. The relative expression of VEGF-A, TGF-β1, MMP2 and EGFR in tumor-bearing mice treated with ICI118551, etodolac and the combination of the two drugs were compared by Western blot. b. The statistical analysis of relative expression of VEGF-A protein. c. The statistical analysis of relative expression of TGF-β1 protein.

d. The statistical analysis of relative expression of MMP2 protein. e. The statistical analysis of relative expression of EFGR protein. f. The concentration of serum IL-1β in tumor-bearing mice treated with ICI118551, etodolac and the combination of the two drugs were compared by ELISA. ICI=ICI118551, Eto= etodolac. (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplymentarydata.docx
- supplymentarydata.docx