

Kv10.1 promotes the growth and progression of Oral Squamous Cell Carcinoma

Susanne Wolfer

`susanne.wolfer@med.uni-goettingen.de`

Universitätsmedizin Göttingen <https://orcid.org/0000-0002-4134-2301>

Yahya Al-Matary

Universitätsmedizin Göttingen

Julia Herzog

Universitätsmedizin Göttingen

Tammo Budde

Universitätsmedizin Göttingen

Nour Zeaiter

Universitätsmedizin Göttingen

Araceli Sánchez

Max Planck Institute for Multidisciplinary Sciences

Jialing Jiang

Max Planck Institute for Multidisciplinary Science

Thomas Asendorf

Universitätsmedizin Göttingen

Kai Antweiler

Universitätsmedizin Göttingen

Tobias Bürger

Universitätsmedizin Göttingen

Henning Schliephake

George Augusta University

Luis Pardo

Max Planck Institute for Multidisciplinary Science

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Abstract

Oral squamous cell carcinoma (OSCC) is one of the most prevalent malignancies of the head and neck, characterized by high rates of recurrence, metastasis, and, in some cases, resistance to conventional therapies. The overall survival rate remains unsatisfactory despite advances in treatment strategies. This underscores a need for novel targets and therapeutic approaches. The voltage-gated potassium channel Kv10.1 (ether-à-go-go 1; Eag1) is a promising candidate that is minimally expressed in normal somatic tissues but aberrantly upregulated across a wide range of tumor types. Recent studies have shown that Kv10.1 plays an important role in various oncogenic processes, including cellular proliferation and cell survival. The tumor-restricted expression and functional role of Kv10.1 in malignant progression make it a suitable target for therapeutic intervention in cancers. In this study, we investigated for the first time the role of Kv10.1 in OSCCs. We showed by immunofluorescence that OSCCs expressed high levels of Kv10.1. The OSCC cell line HN also expressed Kv10.1. The functional activity of Kv10.1 was validated electrophysiologically. Blocking of Kv10.1 in OSCC cell lines with astemizole and imipramine induced apoptosis. Furthermore, Kv10.1-positive tumors displayed a high level of proliferation marker (Ki67) compared to Kv10.1-negative tumors. Kv10.1 expression was associated with poor clinical outcomes in OSCC patients.

We conclude that Kv10.1 is aberrantly expressed in OSCC, enhancing cellular proliferation, suppressing apoptosis, and is associated with poor prognosis in OSCC patients. The tumor-specific expression of Kv10.1 makes it a potential target molecule for inhibition in the treatment of OSCCs.

Introduction

Head and neck cancers are among the most common malignant tumors worldwide. Ninety percent of oral malignancies are oral squamous cell carcinomas (OSCC) ¹. Locations of the OSCC are the floor of the mouth, the anterior two-thirds of the tongue, the gums of the upper and lower jaw, the hard and soft palate, and the buccal mucosa. OSCC are predominantly diagnosed between the fifth and seventh decade of life, where women are often older than men at the time of diagnosis ²⁻⁴. Men are affected more frequently than women, and the main risk factors are tobacco smoking and alcohol abuse ⁵⁻⁷. The development of metastases is mainly lymphogenic locoregional in the neck region. The presence of lymph node metastases is a well-known adverse prognostic factor ⁸⁻¹⁰. Distant metastases, most likely to the lung and liver, also affect patient survival, although they are less frequent than lymph node metastasis ¹¹.

The three main pillars of therapy are extensive surgical resection of the tumor, with neck dissection, and complex facial reconstruction, and radiation therapy and chemotherapy as adjuvant therapy options for advanced tumors or in the presence of lymph node metastases ¹²⁻¹⁴. Ablative surgical treatment affects important functions such as breathing, speaking, swallowing, and chewing, as well as the patient's aesthetic appearance and psychological health ¹⁵. Tumors that are no longer operable may undergo

primary radiochemotherapy¹⁵. Despite extensive resection, increasingly effective reconstruction, and improved adjuvant therapy, the 5-year survival rate remains only 50%-60%^{16,17}. Although the OSCC is easily detectable in the mouth, most patients only see the doctor when the tumors are well advanced. The prognosis gets worse as the tumor progresses¹⁸. Unfortunately, approximately 20% of patients with OSCC develop a recurrence; around 76% of these recurrences occur in the first two years after therapy, which importantly increases the mortality rate in these patients¹⁹. Immunotherapy using checkpoint inhibitors such as nivolumab and pembrolizumab has been available for several years as a palliative treatment option for patients with recurrence or metastatic disease who can no longer be treated curatively with surgery, radiation, or conventional chemotherapy²⁰. The toxicity of those therapeutics is less than that of chemotherapy with cisplatin, but only about 20% of patients respond to these immunotherapies^{21,22}. The KEYNOTE-689 study recently introduced the option of neoadjuvant therapy with pembrolizumab for advanced head and neck cancers²³; the outcome in routine clinical practice remains to be seen.

In search of more effective therapy options and less side effects, it is worth taking a deeper look into tumor biology. We have focused our attention on ion channels, specifically voltage-gated potassium channels, which are the most diverse channel family²⁴ and among those, on Kv10.1 (ether-a-go-go; EAG1), which was the first voltage-gated potassium channel unequivocally linked to tumorigenesis²⁵. Kv10.1 is the founding member of the EAG family, which also comprises ERG (Kv11.x) and ELK (Kv12.x) subfamilies^{26,27}. Physiologically, Kv10.1 expression in healthy tissues is abundant only in the brain and cerebellum; a few cells in the placenta, testes, or adrenal gland also express it, as do myoblasts at the onset of fusion²⁴. This restricted expression pattern is due to tightly controlled temporal expression during the cell cycle: Kv10.1 is expressed during a short window in the G2 and M phases, whereas expression disappears in G1²⁸. Interestingly, ectopic expression of Kv10.1 has been detected in a wide variety of human tumors, such as breast, cervix, lung, prostate or pancreatic carcinomas and malignant melanoma²⁹⁻³¹.

In tumors expressing Kv10.1, channel inhibition results in slower tumor progression. Various blockers of Kv10.1 have been described in the literature, with astemizole and imipramine being the best studied³²⁻³⁴. Neither blocker targets Kv10.1 primarily; astemizole is an antihistamine, and imipramine is a tricyclic antidepressant. In addition, these inhibitors cannot discriminate among channel family members; they block the closely related HERG (Kv11.1), a cardiac channel implicated in many cases of QT prolongation and whose blockade poses significant cardiac risk^{29,34}. Further investigations into specific binding to Kv10.1 led to the development of a monoclonal antibody (mAb56)^{35,36}. However, the required high concentrations and the observed partial inhibition speak against the clinical potential of this antibody. As an alternative, to exploit the specific expression of Kv10.1 in tumors, fusion proteins comprising anti-Kv10.1 antibody derivatives and the apoptosis-inducing cytokine TRAIL (TNF-related apoptosis-inducing ligand) have been proposed³⁷. Specific induction of apoptosis with an anti-Kv10.1 nanobody fused to

single-chain TRAIL could be shown to induce intense apoptosis in Kv10.1-positive prostate cancer cell lines³⁸.

To date, only a few studies have examined Kv10.1 expression in head and neck malignancies^{26,39}, and only larynx and pharynx carcinomas have been studied. We are not aware of any studies on Kv10.1 expression in OSCC.

In this study, we investigated Kv10.1 expression in OSCC using human tumor tissue and cell models, and examined its association with clinical and pathological characteristics and patient clinical outcomes. Furthermore, this study examines the potential of Kv10.1 inhibition in the management of OSCC.

Results

Clinical characterization of the study populations

Fifty-nine patients were included in this study, thirty-four male and twenty-five female. The mean age at diagnosis was 68 years, with a standard deviation (SD) of 12 years. The most frequent locations of OSCC were the gingiva (alveolar rim), tongue, and floor of the mouth (FOM). Most patients had a history of smoking, with 41 ± 28 (mean $\pm$ SD) pack-year (py). Patients' characteristics are provided in Table 1.

OSCC tumors and cell line express high levels of Kv10.1

To investigate the role of Kv10.1 in OSCC growth, we first assessed its expression in OSCC tumor tissue and normal mucosa derived from the same patients by using immunofluorescence (IF) staining. Eighty-five percent of tumor samples (50/59) showed areas of positive staining for Kv10.1, while 15% (9/59) were negative (Figure 1, Table 2A). Clear membrane-like staining was remarkably visible with a strong expression in 32% (16/50) of Kv10.1-positive tumor samples (Figure 1A). While we found approximately 85% positivity for Kv10.1 in tumor samples, only 24% of normal oral mucosa were positive for Kv10.1 in IF staining (Table 2A), further indicating tumor-specific expression, as the expression in the neighboring tissue was low ($p < 0.0001$ in paired t-test; Table 2B).

There were no differences in TNM status and differentiation between Kv10.1-positive and Kv10.1-negative tumors in our patient group. We did not detect higher-risk behavior in patients with Kv10.1-positive tumors. But we observed, in Kv10.1-positive patients, more tumors with no or poor keratinization compared with Kv10.1-negative tumors, which are moderately or strongly keratinized.

IF staining also revealed the presence of Kv10.1 channels in the OSCC cell line HN (Figure 1B). Following immunoprecipitation in HN cell line, Kv10.1 was detected at the protein level by Western Blot analysis (Figure 1C). The antibodies used for IP and immunoblot have different epitopes, further supporting the specific detection of Kv10.1 expression.

Functional Characterization of Kv10.1 channels in OSCC cell line

We further validated the presence of functional Kv10.1 channels in the OSCC cell line HN using whole-cell patch-clamp recordings⁴⁰. The marked dependence of Kv10.1 speed of activation on the potential prior to the stimulus, which identifies the channel almost unequivocally⁴¹, allowed us to isolate a K⁺ current compatible with Kv10.1 in HN cells (Figure 2A). The current showed pharmacological features compatible with Kv10.1, as it is reduced after application of astemizole and imipramine (Figure 2B); astemizole showed a higher potency with an IC₅₀ of 2.24 μM (95% CI: 1.34–4.93 μM), compared to imipramine with an IC₅₀ of 62.31 μM (95% CI: 34.28–153.2 μM) (Figure 2C).

Expression of Kv10.1 correlates with poor clinical outcome in OSCC patients

OSCC patients were observed for a maximum period of 191 weeks. Patients with Kv10.1-positive tumors exhibited a poorer overall survival (OS) compared to those with Kv10.1-negative tumors, with survival rates of 67% vs. 89% after one year, and 59% vs. 76% after two years, respectively. However, the difference did not reach statistical significance (HR 1.91; 95%-CI: 0.51-7.19; p=0.3; adjusted for age, T stage and N stage) (Figure 3A). Patients were censored at the most recent point for which survival status was known.

We validated the OS of OSCC patients in the TCGA database (n=276) by filtering by tumor location from the total HNSCC (n=563)⁴². OSCC patients with Kv10.1-positive tumors (n=256, 92.76%) had a shorter survival than patients with Kv10.1 negative tumors (n=20, 7.24%) (p=0.31) (Figure 3B).

We also found a trend towards shorter disease-free survival (DFS) in patients with Kv10.1-positive tumors compared to those with Kv10.1-negative tumors. However, these differences were not statistically significant. The DFS rates for Kv10.1-positive vs. Kv10.1-negative patients were 52% vs. 78% after one year, and 49% vs. 53% after two years (Figure 3C). The adjusted HR was 1.24 (95%-CI: 0.45-3.45; p=0.6). Events were defined as either death or recurrence. Patients were censored at the most recent point for which disease and survival status were known. When we focused on the initiation of palliative care, 13 patients transitioned to a palliative setting over time. Kv10.1-positive patients tended to start palliative care or die earlier than Kv10.1-negative patients. The rates for Kv10.1-positive versus Kv10.1-negative patients were 63% vs 89% after one year, and 53% vs 63% after two years (Figure 3D). The adjusted HR was 1.61 (95%-CI: 0.50-5.18; p=0.5). Events were defined as either death or the initiation of palliative care. Patients were censored at the most recent point with known survival and palliative care status.

Blocking of Kv10.1 channels reduces proliferation rate and produces apoptosis in OSCC

We further explored the potential effect of these functional Kv10.1 channels on the bio-oncological features of OSCC cells, in particular apoptosis and proliferation. To investigate the impact of Kv10.1 channels on the proliferation rate of OSCC cells, we performed IF staining of Ki67, a well-established marker of active cell cycling⁴³, on OSCC patient tumor samples (Figure 4A). Quantitative assessment showed that Kv10.1-positive OSCC tumors have significantly higher levels of Ki67 compared with Kv10.1-negative tumors (Figure 4B). (p = 0.035).

Blocking Kv10.1 channels has been shown to reduce proliferation and induce apoptosis in other tumor types. We thus tested if this is also the case for OSCC cells. We treated the Kv10.1-expressing OSCC cell line HN with Kv10.1 blockers imipramine or astemizole. Both drugs induced significant levels of apoptosis after 12 hours exposure (Figure 4C). Anti-Kv10.1-scTRAIL fusion constructs (scFv62-scTRAIL or VHH-D9-scTRAIL) have been reported to be specific and efficacious on several tumor types (prostate, breast, pancreas)^{38,44}. At the concentrations used in those experiments (150pM) and in the presence of subtoxic concentrations of cycloheximide to increase sensitivity to TRAIL, we could not observe apoptosis induction in HN over 12 and 24-hour treatments (Figure 4D). For this reason, we investigated the TRAIL receptor status in the HN cell line and found high expression of the decoy receptors TRAIL-R3 and TRAIL-R4 which inhibit the active receptors TRAIL-R1 and TRAIL-R2 (Figure 4E).

Discussion

Despite remarkable advances in our understanding of cancer biology as well as the continuous improvement of treatment strategies, the prognosis is still poor for patients with OSCC^{6,10,14}. Furthermore, the treatment outcomes of conventional cancer therapies, including surgery, chemotherapy, and radiotherapy, are not satisfactory because of side effects, toxicity, and morbidity⁸. In this study, we explored the role of Kv10.1 in the development and prognosis of OSCC. We also aimed to investigate the functional relevance and potential blockers of Kv10.1 in OSCC. Consistent with previously reported observations in other studies with different tumor types, we found, to our knowledge, for the first time a specific expression of Kv10.1 also in 85% of OSCC tissue samples, as well as in the OSCC cell line HN. In almost a third of the Kv10.1-positive tumor samples, we observed a Kv10.1-positive structure compatible with the plasma membrane, which has not been detected in any other type of malignant tumor before. In contrast, only one fourth of the samples of histologically normal mucosa surrounding the tumor (24%, Table 2) showed Kv10.1 expression, indicating tumor specificity. The small group of Kv10.1-positive normal oral mucosa may as well represent an early sign of tumor development that is not yet histologically detectable. It has already been shown that Kv10.1 is detectable by IHC in morphologically normal uterine cervix cells (27%), and that expression levels increase with higher dysplasia grades; hence, Kv10.1 was proposed as a marker for pre-cancerous cervical intraepithelial lesions⁴⁵. Thus, Kv10.1 was proposed as an early indicator of malignancy because its mRNA was present in different normal tissues with pre-cancerous conditions or in tumor-surrounding normal tissue⁴⁵. Although Kv10.1 expression has been demonstrated in a variety of human tumors, only a few studies on head and neck tumors have investigated Kv10.1 expression, exclusively in pharyngeal and laryngeal carcinomas⁴⁶. The results for pharyngeal and laryngeal carcinomas closely resemble our results for OSCC, with 83% positive tumor samples. In the study by Menendez et al., Kv10.1 was detected in 39% patient-related, tumor-free tissue, suggesting an early occurrence of aberrant Kv10.1 expression during tumorigenesis in HNSCC⁴⁶.

Kv10.1 is not only present but also functional in the plasma membrane of OSCC cells. Electrophysiologically, this channel exhibits characteristic properties that allow virtually unequivocal

identification³⁶. In the OSCC cell line HN, we detected electrophysiological behavior consistent with Kv10.1. Additionally, the known Kv10.1 blockers astemizole and imipramine significantly reduced the current flow across the cell membrane. The IC50 values for both drugs are larger than those reported for recombinant channels in HEK cells³⁴, and similar to the ones recorded in *Xenopus* oocytes⁴⁷.

The degree of keratinization is a significant prognostic factor of OS and DFS in OSCC; no or low keratinization is associated with a worse outcome^{48,49}. This correlation also holds true in HPV-negative HNSCC⁵⁰. Consistently, we found that Kv10.1-positive OSCC samples were more frequently non-keratinized or minimally keratinized than Kv10.1-negative samples (83% vs. 50%), and Kv10.1-positive tumors showed a worse prognosis than Kv10.1-negative tumors.

OSCC patients from our study and TCGA-OSCC-filtered patients with Kv10.1-negative tumors had a better prognosis than those with Kv10.1-positive tumors, although the difference was not statistically significant. The small sample size of Kv10.1-negative groups (both our series and TCGA database) might be the reason for the non-significant difference. However, other studies on head and neck tumors have demonstrated significantly reduced disease-specific survival in Kv10.1-positive tumors compared to Kv10.1-negative tumors²⁶. To assess our results, further studies with a larger cohort must be conducted.

Kv10.1 participates in several tumor-related biological processes, such as cell proliferation, apoptosis, cell cycle progression, and angiogenesis^{24,31,26}. In agreement with these findings, we also demonstrated that Kv10.1 expression in OSCC tumors was associated with a high proliferation rate, indicating its contributing role in the growth and progression of OSCC.

Block of Kv10.1 has been shown to induce apoptosis on cells derived from several tumor types^{33,51 52}. Likewise, both astemizole and imipramine induced apoptosis of HN cells. Together, the data indicates that Kv10.1 favors proliferation of OSCC cells, and that its inhibition promotes apoptosis.

Kv10.1 blockers slowed tumor growth but did not induce complete tumor regression in animal models^{24,53}. Therefore, attempts to use Kv10.1 as a target to achieve specific delivery of biologicals to the tumor have been undertaken. A combination of a single chain antibody (scFv62) or a nanobody (D9) with single-chain TRAIL has displayed its efficacy across several tumor models^{38,54,55}. However, scFv62-scTRAIL and VHH-D9-scTRAIL were not effective against the OSCC cell line. A possible explanation for this observation is that HN cells express high levels of the decoy receptors TRAIL R3 and R4 in OSCC cells (HN) (Fig. 4E) which lack functional death-signaling domains and may competitively inhibit TRAIL binding to the pro-apoptotic receptors TRAIL-R1 and TRAIL-R2, thereby reducing TRAIL-mediated apoptosis^{56,57}. Earlier Kv10.1-targeted TRAIL studies by Hartung and colleagues showed that TRAIL resistance mechanisms involve TRAIL receptor expression patterns and anti-apoptotic signaling^{55,58}.

Since no studies on the expression of Kv10.1 in OSCC have yet been conducted, a comparison of our study population with the literature is not possible. The most similar study is that by Menedez et al.

2012, which describes increased expression of Kv10.1 in head and neck tumors, particularly in advanced tumors with positive lymph node status. However, Menedez et al. did not examine OSCC, but rather laryngeal and pharyngeal carcinomas, which were similar in the distribution of the T stage, but differed in terms of age, sex and degree of differentiation⁴⁶. Therefore, a comparison of these studies is only possible to a limited extent. However, clinical and biological relevance of Kv10.1 has been described in early stages of tumorigenesis and for the progression of advanced head and neck tumors²⁶. Studies on the expression of Kv10.1 in gastric carcinoma also found no correlation with age, sex and differentiation grade, but a correlation with lymph node involvement and tumor stage⁵². In our study population, we found no correlation between Kv10.1 expression and age, sex, TNM stage, or differentiation grade. Further studies with a significantly larger population are needed. However, it should be noted that due to the highly specific expression of Kv10.1 in tumor tissue, obtaining enough Kv10.1-negative tumors for comparison is very difficult overall.

Conclusion

Kv10.1 is aberrantly expressed in OSCC tumors and the HN cell line, and is associated with high cell proliferation, reduced apoptosis, and an unfavorable clinical course. To date, no specific Kv10.1 blocker for OSCC has been identified; however, blockade has been successfully achieved using the best currently available agents—astemizole and imipramine. Future research must focus on identifying a highly specific blocker to enable targeted therapy with fewer side effects, to possibly reduce resistance to radiotherapy, chemotherapy, and immunotherapy, and ultimately improve the care of OSCC patients.

Materials and Methods

Sample collection and preparation

After written consent, 59 samples of tumor tissue were taken intraoperatively during regular tumor resection from August 1st, 2020, to February 28th, 2023. Twenty-nine samples of macroscopically normal tissue of oral mucosa were taken directly from the resection border and used as paired non-cancerous matched tissue. The samples were divided into two parts. One part was fixed with formalin and paraffin-embedded, and the other part was stored directly at -80°C. Two µm slices were cut from the formalin-fixed and paraffin-embedded samples. For HE staining, deparaffinized slices were incubated for 5 min in Mayer's Hematoxylin solution 1:2 (Merck, Weiterstadt, Germany) and subsequently in 1% Eosin (Merck). An experienced pathologist, who was blinded to the previous diagnosis (BT), confirmed the identification of OSCC and normal mucosa after HE-staining and assessed keratinization by determining the percentage of keratinized cells among all cells.

The clinical data and the outcome (overall survival (OS), palliative-free-survival, and disease-free-survival (DFS)) were retrieved from the regular medical records of the institution's certified tumor register and from the regular follow-up data. The following data were recorded: date of OSCC diagnosis, age at diagnosis, sex, tumor classification (TNM-status), tumor site, depth of invasion (DOI), tumor size,

extracapsular spread (ECS), grading, last contact with the patient, date of death, date of recurrence, date of deciding for palliative therapy, history of smoking and alcohol. Relapses of OSCC were defined as local recurrence or distant metastasis, confirmed by histology (biopsy) or radiological detection with clinical correlation.

Cell culture

HN cells (DSMZ ACC 417, derived from a lymph node metastasis of a palatal OSCC in a 60-year-old male), were purchased from DSMZ (Braunschweig, Germany). These cells were cultured in DMEM (Gibco, Thermo Fisher Scientific, Germany) supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich, Taufkirchen, Germany or anprotec, Bruckberg, Germany)[LP2.1], and 1% penicillin and streptomycin (PAN- Biotech, Aidenbach, Germany or Sigma-Aldrich) at 37°C in a humidified atmosphere with 5% CO₂.

Apoptosis and cytotoxicity measurement

We assessed the ability of specific and non-specific Kv10.1 inhibitors to induce apoptosis in HN cell lines as previously described³⁸. Briefly, HN cells cultured in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) were monitored using the IncuCyte live-cell imaging system (Sartorius, Goettingen, Germany). Cells were treated either with anti-KV10.1 antibody-TRAIL fusions (scFv62-scTRAIL or VHH-D9-scTRAIL, 150 pM)³⁸ in the presence of 8.9 µM cycloheximide (CHX, Fisher Scientific) or with 5 µM astemizole (Esteve Química, Barcelona, Spain) or 25 µM imipramine (Fisher Scientific) and Caspase 3/7 reagent (Sartorius; red or green, at a 1:1,000 dilution as recommended by the manufacturer). The Caspase 3/7 reagent substrate permeates the cell membrane, and upon cleavage by activated caspase-3/7, releases a DNA dye that renders the nucleus of the cell fluorescent. Cytotoxicity determinations were performed using the same approach in the presence of Incucyte Cytotox Green reagent (Sartorius); green fluorescence was normalized to cell confluence.

Immunofluorescence (IF)

Paraffin-embedded sections were deparaffinized in xylol and rehydrated through a series of ethanol/water solutions. Antigen retrieval was performed in a steamer for 20 minutes in citrate buffer pH 6. After blocking with SeaBlock solution (Dako, Wiesentheid, Germany) or with goat serum (BIOZOL, Hamburg, Germany) for 1h, the sections were incubated with anti-Kv10.1 antibody (Cat. Nr. 7453,1:500, Abcam, Muenchen, Germany), anti-p40 (mouse monoclonal, 1:50, Zytomed, Bargteheide, Germany) or anti-Ki67 (rabbit polyclonal, Cat. Nr 14-5699-82, 1:200, Abcam) overnight at 4 °C in a humidified atmosphere. We used the following secondary antibodies: Alexa Fluor 546 anti-mouse, Alexa Fluor 488 anti-rabbit [AA3.1], Alexa Fluor 488 anti-mouse IgG1 (Cat. Nr. A-21121, 1:2000, Thermo Fisher Scientific, Meerbusch, Germany) and Alexa Fluor 647 anti-rabbit (cat. Nr. Ab150079, 1:1000, Abcam). After washing, nuclei were counterstained either with Draq5 (1:1000) [AA4.1][AA4.2] or with DAPI (Cat. Nr. D9542, 1:1000, Sigma-Aldrich). Slides were mounted using ImmuMount (Thermo Fisher Scientific) and imaged in a Zeiss LSM 880 confocal microscope, a Nikon Eclipse Ti2, or a widefield fluorescence

microscope (BZ-X710, KEYENCE, Frankfurt am Main, Germany) and processed using Imaris v9.7 (Oxford Instruments) and cellprofiler (Broad Institute, Harvard). The assessment of the samples for categorizing Kv10.1 negative or Kv10.1 positive were performed by two blinded operators (PLA; JJ).

Protein extraction, immunoprecipitation and Western blot.

Adherent cells were washed twice with ice-cold PBS and scraped in lysis buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA and Complete Mini protease inhibitor cocktail (Roche)); the suspension was then incubated for 30 minutes on ice and centrifuged 15 min 14000 × g, 4 °C and protein concentration in the supernatant was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), following the protocol provided by the manufacturer. 1 mg of protein was then precleared by incubation with 25 µl of Protein G magnetic beads (New England Biolabs) and immunoprecipitated using 2 µg of mAb33³¹ or isotype mouse IgGκ2b as a negative control at 4 °C overnight. The beads were washed three times with lysis buffer, and once with Tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5), containing 10 ml of protease inhibitor. The protein-bound pellets were resuspended in TBS with Novex LDS sample buffer containing reducing agent (Thermo Fisher Scientific), heated at 70 °C for 10 min, and run in a 4–12% Bis-Tris polyacrylamide gradient gel using MOPS running buffer (Thermo Fisher Scientific). Separated proteins were transferred to a nitrocellulose membrane (0.2 µm pore size, Novex) in 10 mM NaHCO₃, 3 mM Na₂CO₃, 0.01% SDS, and 20% methanol using a voltage gradient (10–50V in 80 minutes). The membrane was then treated with Pierce Western blot signal enhancer according to the manufacturer's recommendations. The membrane was then blocked in 0.1% casein in TBST (TBS, 0.1% Tween-20) for 1 hour at room temperature, and overnight at 4 °C with anti-KV10.1 (9391 1:1,500)⁵⁹, and 1 hour with anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:10,000). The signal was developed using ECL substrate (Millipore) and visualized in a ChemoStar imaging system (iNTAS Science Imaging).

Quantitative RT-PCR

Total RNA was extracted from cells using RNeasy micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was carried out with iScript cDNA Synthesis Kit (Bio-Rad, Duesseldorf, Germany). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on an iCycler My iQ™ single-color Real-time PCR Detection System (Bio-Rad). β-Actin was used as an internal reference gene. The primer sequences used in this study were as follows: TRAIL-R1: 5′- GGG TCC ACA AGA CCT TCA AGT-3′ (forward) and 5′- TGC AGC TGA GCT AGG TAC GA-3′ (reverse), TRAIL-R2: 5′- AGA CCC TTG TGC TCG TTG TC-3′ (forward) and 5′-TTG TTG GGT GAT CAG AGC AG -3′ (reverse), TRAIL-R3: 5′- CCC TAA AGT TCG TCG TCG TC-3′ (forward) and 5′- TGG TGG CAG AGT AAG CTA GGA-3′ (reverse), TRAIL-R4: 5′- AAG TTC GTC GTC TTC ATC GTC-3′ (forward) and 5′- GAT GGT GGC AGA GTC AAC C-3′ (reverse), and Actin-B: 5′-AAAGACCTGTACGCCAACAC-3′ (forward) and 5′-GCTATACTCCTGCTTGCTGAT-3′ (reverse). RNA expression levels of TRAILs in HN cells were calculated by ΔCT.

Patch clamp

To investigate the functional activity of Kv10.1, electrophysiological recordings were performed in the whole-cell patch-clamp configuration as previously described^{34,40,41}. Briefly, cells were plated on glass coverslips coated with fibronectin. Patch clamp measurements were performed at room temperature using an EPC10 Plus amplifier (Heka Electronics, Reutlingen, Germany). We used pipettes made of borosilicate with a resistance of 1-3 M Ω when filled with the intracellular solution (in mM: 100 KCl, 45 NMDG, 10 1,1-bis(O-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA), 10 HEPES pH7.2). The external solution contained (mM): 160 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 8 Glucose, 10 HEPES, pH 7.4. Currents were activated by depolarization from a holding potential of -80 mV, filtered at 1-3 kHz and sampled at 5-10 kHz. Series resistance was compensated to at least 60%. Kv10.1 current was identified by taking advantage of the unique sensitivity of Kv10.1 current activation to the pre-pulse potential; the current activates slower the more hyperpolarized the membrane before the stimulus^{34,41}. Thus, currents elicited by an identical depolarization to +60 mV from a holding potential of -120 or -60 mV differ in their speed of activation. Therefore, subtraction of the resulting traces reveals the current whose activation is slowed down by hyperpolarization before the stimulus, which corresponds to Kv10.1. Pharmacological inhibition of the current was tested by superfusion with the indicated concentrations of imipramine or astemizole in the extracellular solution.

Transcriptomic study of OSCC patients from the Cancer Genome Atlas (TCGA)

RNA-sequencing data and clinical annotations of Head and Neck Squamous Cell Carcinoma (HNSC) were obtained from TCGA through the Genomic Data Commons portal⁴². Only samples corresponding to OSCC (276) were included in the analysis. OSCC samples were defined according to the site of tumor. Analysis was performed using R for windows 4.5.2 and Rstudio 2026.01.1 Build 403^{60,61}. Gene expression counts were normalized using a log2 transformation ($\log_2[\text{counts} + 1]$). Expression values for Kv10.1 (KCNH1) were extracted for all OSCC patients with available overall survival data. Overall survival time was defined as the number of days from diagnosis to death, or to last follow-up for censored cases. OSCC patients were classified into two groups according to Kv10.1 expression in their tumors. Tumors with detectable expression were classified as Kv10.1-expressing (Kv10.1 positive), whereas tumors without detectable expression were classified as Kv10.1-non-expressing (Kv10.1 negative). Kaplan–Meier survival curves were generated using the Kaplan–Meier estimator, and differences between groups were assessed using the log-rank test. Survival analyses were performed in R using the survival and survminer packages⁶¹.

Statistical methods

Continuous characteristics of the study population were described using mean, standard deviation and the five quartiles. Categorical variables were presented by their absolute counts and percentages. Group differences between Kv10.1-positive and Kv10.1-negative samples were tested using Mann-Whitney U-tests for continuous variables and Fisher's exact test for categorical variables. The asymmetry of the Kv10.1-classifications (positive/negative) between normal mucosa and tumor samples was assessed by McNemar's Test with continuity correction. Cox regression was employed to model the time from

diagnosis to the occurrence of an event. Events were defined differently for overall survival (death), disease-free survival (death or recurrence), and palliative-free survival (death or initiation of palliative care). All survival models were adjusted for T stage, N stage and age at surgery. Survival curves were generated based on Kaplan-Meier-estimates. Due to the explorative nature of this study, no adjustment for multiple testing was performed. Descriptive p-value (two-sided) of all numeric data such as RT-PCR and IF results, was reported and considered statistically significant if it was less than 0.05. All calculations were performed using R version 4.4.1 and GraphPad Prism.9.0.0.

Declarations

Data availability:

The datasets generated during and or analyzed during the current study are available from the corresponding author on reasonable request.

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Authorship and contribution

Y.A.-M., T.B., N.Z., J.J., L.A.P. and A.S. performed research.

S.W., L.A.P. and Y.A.-M. designed the study and wrote the manuscript.

S.W., L.A.P., Y.A.-M. T.A. and K.A. performed data analysis, interpretation and edited the manuscript.

T.B. critically evaluated the histological samples.

J.H. and K.A. collected and analyzed the clinical data of OSCC patients.

S.W. and H.S. provided funding and critically revised the manuscript.

S.W. and L.A.P. supervised the study

All authors reviewed and approved the content of the manuscript.

Conflict of interests:

Susanne Wolfer has no conflict of interest to declare.

Yahya Al-Matary has no conflict of interest to declare.

Julia Herzog has no conflict of interest to declare.

Tammo Budde has no conflict of interest to declare.

Nour Zeaiter has no conflict of interest to declare.

Araceli Sánchez has no conflict of interest to declare.

Jialing Jiang has no conflict of interest to declare.

Thomas Asendorf has no conflict of interest to declare.

Kai Antweiler has no conflict of interest to declare.

Tobias Bürger has no conflict of interest to declare.

Henning Schliephake has no conflict of interest to declare.

Luis Pardo has no conflict of interest to declare.

Ethic approval:

The study was approved by the local ethics committee (Reg.-Nr: 31/7/20).

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Tables

Table 1 and 2 are available in the Supplementary Files section.

Figures

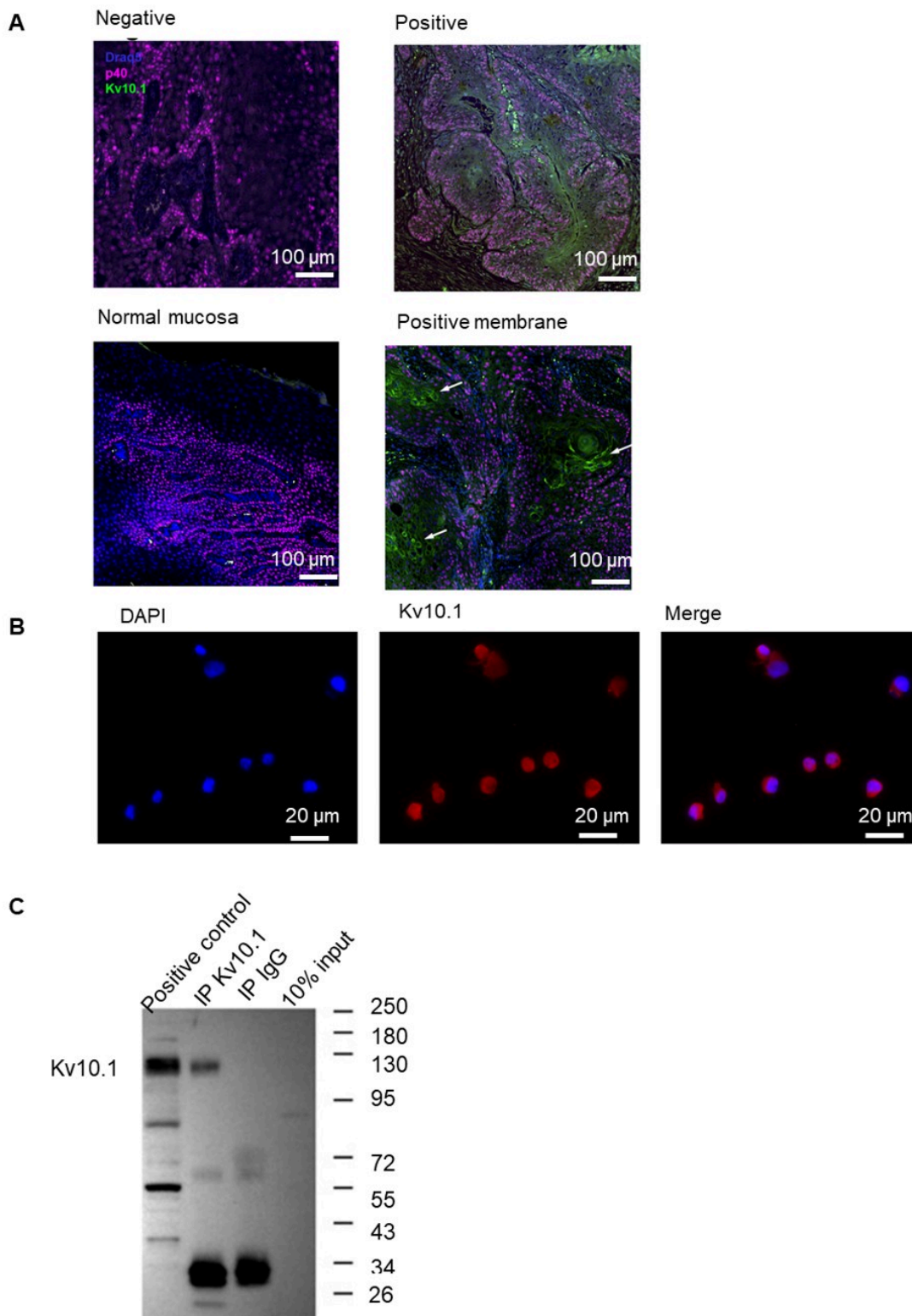


Figure 1

OSCC Tumors express high level of Kv10.1 compared to normal mucosa

A) Immunofluorescence (IF) staining of Kv10.1 in tumor samples from patients with OSCCs. p40 staining (magenta) was used as a tumor marker; nuclei were stained with Draq 5 (blue), and green represents Kv10.1 staining. The upper panels show examples of negative and positive staining. The lower panels

present a positive case with peripheral staining suggesting plasma membrane (white arrows), observed in a fraction of the positive cases (see text). The left panel corresponds to normal mucosa from the same patient. Scale bars: 100 μm .

B) IF staining of Kv10.1 in the OSCC cell line HN. Blue is DAPI, Red is Kv10.1. Scale bar: 20 μm

C) HN cells express Kv10.1 at the protein level. Immunoprecipitation from HN extracts using an anti-Kv10.1 monoclonal antibody (mAb33) and detected with an anti-Kv10.1 polyclonal antibody (9391).

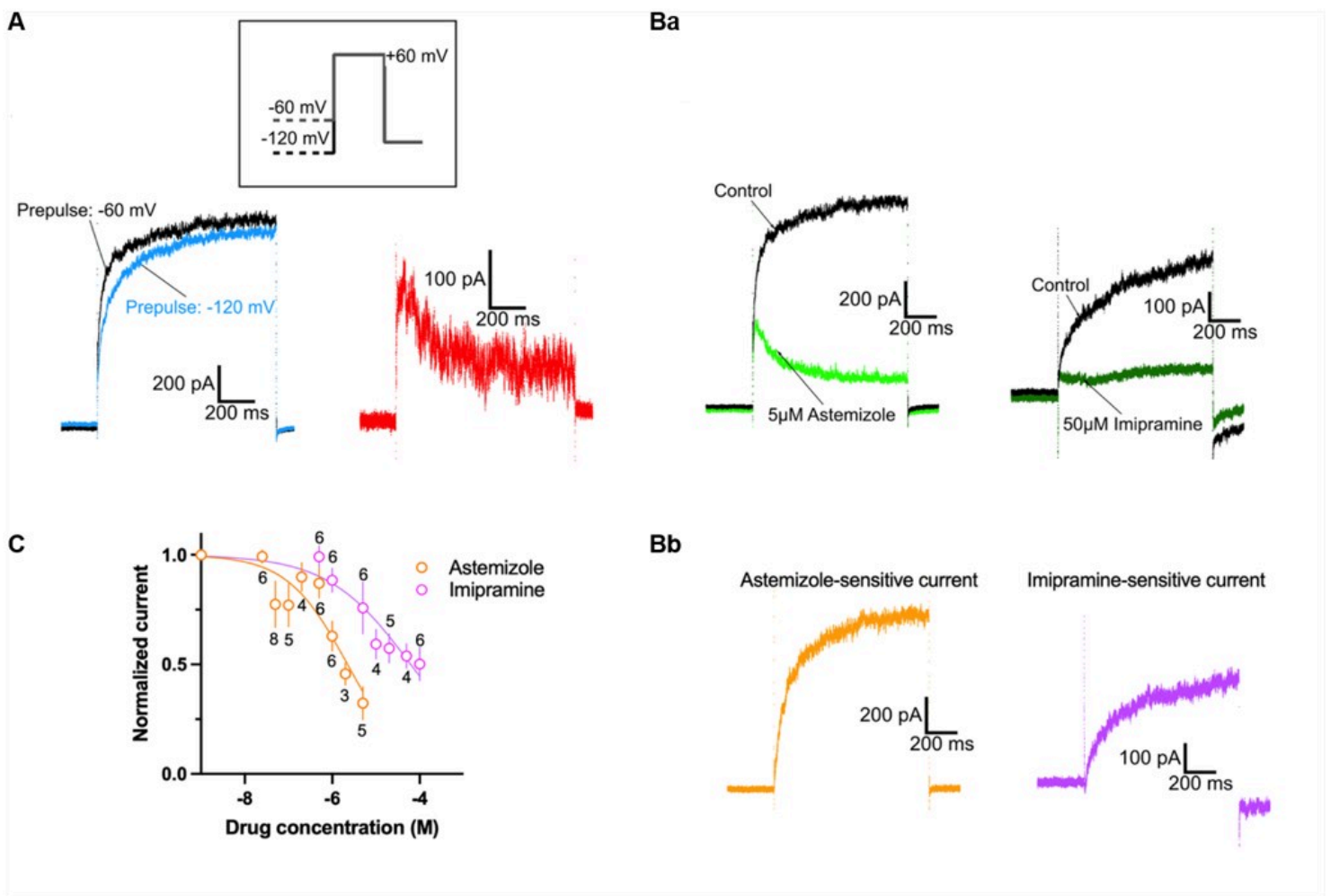


Figure 2

Functional characterization of Kv10.1 channels in HN cells

A) Whole-cell patch-clamp recordings from HN cells using the protocol described in the *inset* to detect the characteristic prepulse voltage-dependence of activation of Kv10.1. Subtraction of the traces at +60 mV from a prepulse potential of -120 mV (blue) from the current after a prepulse at -60 mV (black) reveals the current whose activation time constant depends on the prepulse potential (red trace), highly suggestive of Kv10.1.

B) Pharmacology of K⁺ currents in HN cells. Ba. Current traces at +60 mV before (black) or after (colored trace) the addition of astemizole (5 μ M, light green) or imipramine (50 μ M, dark green). Bb depicts the current sensitive to the drug (astemizole in orange, imipramine in purple) as the result of subtracting the traces in the presence of the drug from the control traces.

C) Dose-response of the inhibition of outward currents in HN cells by astemizole (orange) and imipramine (purple). Normalized current amplitudes are plotted against the drug concentration to determine the half-maximal inhibitory concentration (IC₅₀). Mean $\pm$ SEM for the indicated number of measurements.

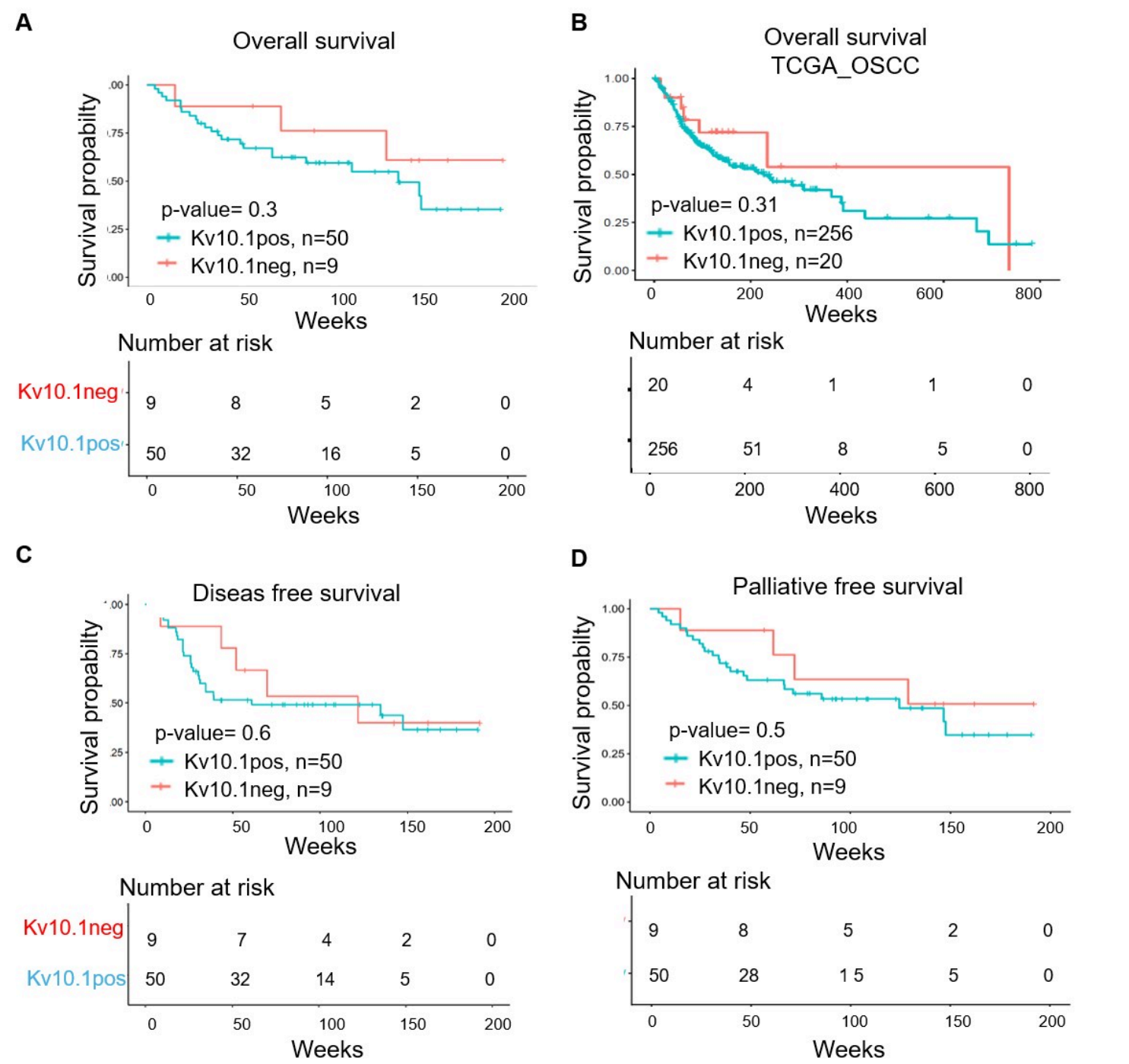


Figure 3

Kv10.1 expression in tumor correlates with poor survival of OSCC patients

- A) Correlation of *Kv10.1* expression with overall survival of patients with OSCC, p value= 0.3
- B) Correlation of *Kv10.1* expression with overall survival of patients with OSCC derived from TCGA_HNSC data, p value= 0.31
- C) Correlation of *Kv10.1* expression with disease free survival of patients with OSCC, p value=0.6
- D) Correlation of *Kv10.1* expression with palliative free survival of patients with OSCC, p value= 0.5

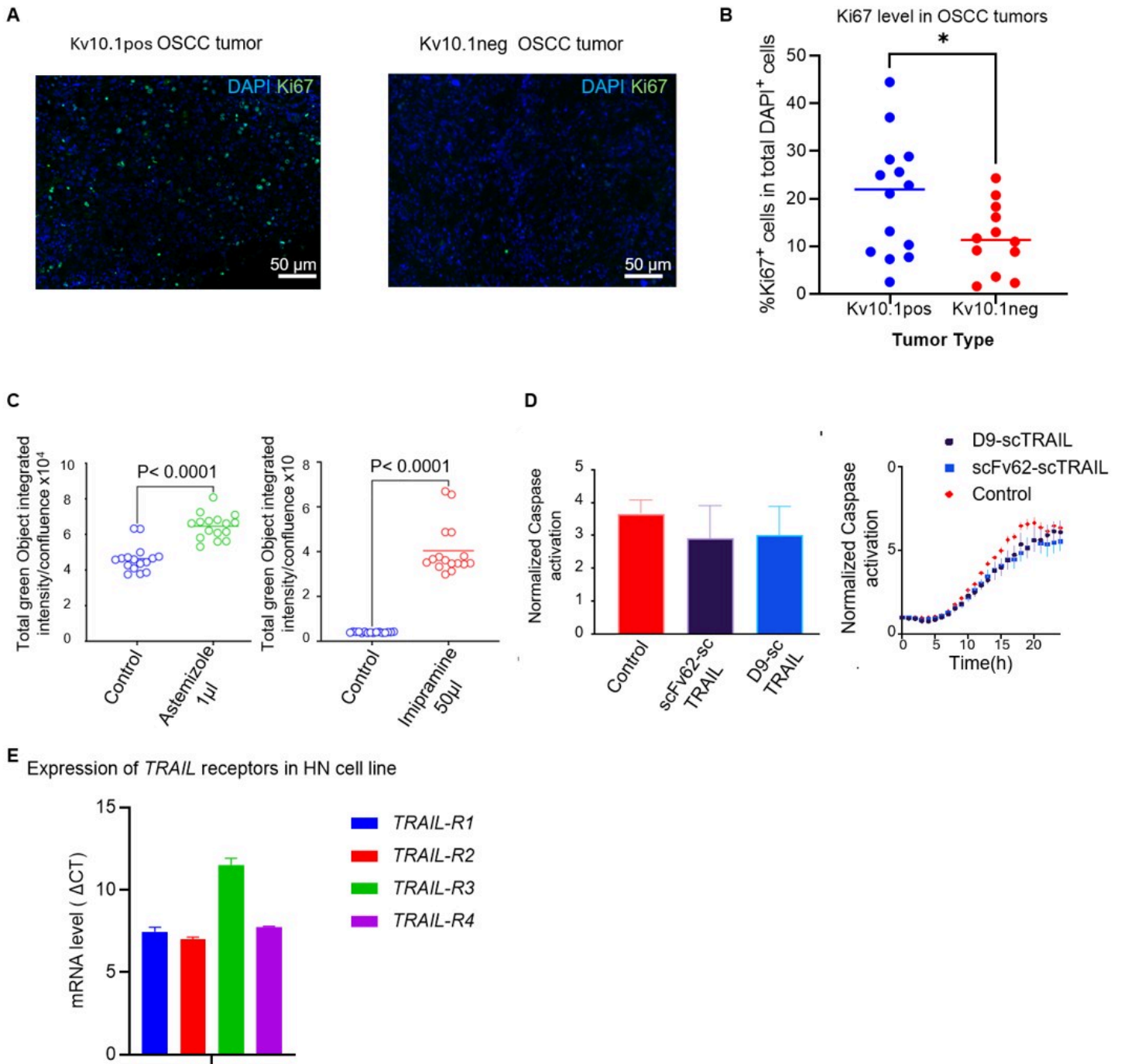


Figure 4

Effect of Kv10.1 channels on the proliferation and apoptosis in OSCC cells

A) IF staining of Ki67 in Kv10.1-positive (left) and -negative tumor samples (right) from OSCC patients. Blue is DAPI and green is Ki67.

B) The percentage of Ki67 in Kv10.1-positive and -negative tumors from patients with OSCCs, p-value= 0.035.

C) Cytotoxicity induction by astemizole (1 μ M) and imipramine (50 μ M) was determined by live-cell imaging over 38 hours (astemizole, left) or 40 hours (imipramine, right) using an Incucyte system. Cell death was determined by the increase in fluorescence of the Incucyte Cytotox Green reagent and quantified as total green integrated fluorescence (GCUx μ m²/image), normalized to cell count expressed as percent confluence.

D) Apoptosis rate measured by caspase activation after treatment of HN cells with scFv62-scTRAIL or VHH-D9-scTRAIL antibodies (150 nM).

E) RNA expression of tumor necrosis factor-related apoptosis-inducing ligand death receptors (TRAIL-Rs) in HN cells. Relative receptor expression was calculated using Δ CT values. β -Actin is used as a reference gene.

Supplementary Files

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- [Table1.docx](#)
- [Table2.docx](#)