INVESTIGATION OF THE EXPRESSION OF TRANZIENT RECEPTOR POTENTIAL ANKYRIN 1 AND VANILLOID 1 ION CHANNELS IN HUMAN ORAL SQUAMOUS CELL CARCINOMA

Ph. D. THESIS

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INTRODUCTION

1. Oral squamous cell carcinoma

The incidence of oral tumors in Hungary is the highest in Europe and 6th highest worldwide [1, 2]. The most common histological type of these malignant tumors, in 90% of cases is squamous cell carcinoma (SCC) [3].The mucosa of the oral cavity is exposed to several direct stimuli, including hot, cold and spicy foods [4, 5], and affected by other factors such as viral infections (e.g. human papilloma virus) [6, 7]. In the case of head and neck SCC (HNSCC), the recurrence of the disease is 70% despite the currently available multimodal treatment. Due to its high mortality rate, it is particularly important to introduce new targeted therapies in addition to standard cytostatic treatment [8]. The 3 main ways of curative therapy for locally limited HNSCC are surgical removal, radiation therapy, and systemic chemotherapy. In the case of extranodal expansion, affected surgical edges or perineural invasion, high doses of cisplatin chemotherapy and radiotherapy are able to improve disease-free survival (DFS) of the patients [9]. Cisplatin and the anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibody in combination with cetuximab, increase progression free survival (PFS) and overall survival (OS) in advanced HNSCC patients [10]. EGFR is a transmembrane receptor tyrosine kinase, a member of the erythroblastosis oncogenic B (ErbB) family. Both mutations and excessive expression of EGFR are prone to promote tumor formation: in HNSCC, excessive expression of the EGFR protein is observed in 80-100% of cases [11]. Anti-EGFR agents inhibit receptor activation, its downstream process and the resulting cell proliferation, which ultimately leads to a better prognosis for the disease [12]. Although many EGFR inhibitors have been shown to prolong PFS, they can often cause serious side effects such as skin rashes, fatigue and mucositis that can impair patient's quality of life and compliance [13]. In addition to their adverse side effects, it is also a proved that anti-EGFR therapy has no clinical advantage over immunotherapy, but the combination of currently approved immunotherapy and cetuximab may be a promising combination treatment strategy in the future. In patients with different mutations, such as p53, help tumor cells survive, elevate invasiveness, and develop therapeutic resistance [14, 15]. In the case of HNSCC, antibodies against programmed cell death protein 1 (PD1), pembrolizumab and nivolumab are the two approved drugs used in recurrent or metastatic HNSCC [16].

2. Tranzient Receptor Potential (TRP) ion channels

TRP ion channel family are non-selective Ca^{2+} and Na^{+} permeable polymodal ionchannels [17], which can be activated or sensitized directly by ligand binding or indirectly through other receptors or downstream intracellular pathways [18, 19]. TRP Ankyrin 1 (TRPA1) is activated by cold and mechanical stimuli, electrophilic compounds, hypertensive solutions and pharmacological agents [20, 21]. It is covalently modified and activated by allyl isothiocyanate (AITC), thiosulfinate compounds, which are pungent components of mustard oil and garlic, allicin in garlic, cinnamonaldehyde, polygodial in pepper and environmental irritants such as acrolein , tear gas and hypochlorite [22-26]. The compound of tobacco smoke acetaldehyde [27] and krontonaldehyde [28] can also activate the channel. Polymodal nociceptors of type C are significantly sensitive to capsaicin, an alkaloid isolated from the pungent red and chili peppers. Capsaicin acts on TRP Vanilloid 1 (TRPV1), selectively activates the heat-sensitive group of pain-sensitive nerve endings [29, 30]. Activation first occurs with increased sensitivity, hyperalgesia or allodynia, caused by the influx of Ca^{2+} into the cell, and then by the subsequent outflow of K^{+} . After a high dose of capsaicin, the nerve ending becomes relatively inoperable, due to cytoplasm and mitochondria swelling because of the high concentration of cations accumulating in the cell [31-33]. In addition to capsaicin and its ultrapotent analogue resiniferatoxin (RTX), TRPV1 channel can be activated by zingeron in ginger, eugenol in cloves and piperine in pepper [34]. TRPA1 is rarely found in neurons lacking TRPV1 [25] and plays an important role in vascularization by activating low and intermediate conductance Ca^{2+} -activated K^{+} channels, leading to endothelium-dependent smooth muscle cell hyperpolarization and vasodilation and acting as a molecular integrator of various stimuli in mediating sensation, pain and neurogenic inflammation [35]. In addition, TRPA1 is also expressed extraneuronally, for example in human airway epithelial cells [36], smooth muscle cells [35], fibroblast cells [37], epidermal keratinocytes [38], melanocytes [39] and immune cells [40].

Capsaicin-sensitive sensory nerve endings have a triple role: afferent and local and systemic efferent functions [41]. TRPV1 is also expressed in non-neuronal cell types of human skin, airways and bladder [42], mast cells [43], dendritic cells, T lymphocytes [44] vascular smooth muscle cells [45] and keratinocytes, the latter being involved in important functions such as growth, differentiation, cell survival and inflammation [34]. Capsaicin elevates intracellular Ca^{2+} levels in oral epithelial cells, demonstrating functional TRPV1 expression [46].

Literature data suggest that some proteins involved in Ca^{2+} homeostasis are associated with the progression of certain tumours. Ca^{2+}, as an intracellular secondary signal transducer, regulates

several processes in tumorigenesis, such as cell proliferation and cell motility. Altered ion channel expression has been demonstrated in a number of malignant tumours, actively promoting neoplastic transformation, malignant lesion progression, tumour microenvironment adaptation, metastasis formation and resistance to antitumour therapy. In contrast to oncogenes, ion channels are not responsible for initiating neoplastic transformation, but are rather regulators of oncogenic processes, for example through Ca^{2+} signalling and regulation of membrane tension. TRP channels can increase intracellular Ca^{2+} concentrations [19], thereby altering the transcription of genes that regulate cell proliferation, differentiation, migration, invasion, motility and apoptosis through Ca^{2+}-dependent signalling cascades [47]. The ultrapotent capsaicin analogue RTX did not promote tumour formation [48] and the TRPV1 antagonists AMG-980 and SB-705498 did not affect skin carcinogenesis [49]. In contrast, a putative tumor suppressor role of the TRPV1 channel has been described in melanoma [50] and colon cancer [51]. *TRPV1* was expressed in both non-tumour and oesophageal squamous cell carcinoma cells at both protein and mRNA levels and *TRPV1* was elevated in oesophageal squamous cell carcinoma cells than in healthy cells [52]. Increased *TRPV1* mRNA expression has also been detected in certain types of breast cancer [53], human breast adenocarcinoma of pleural metastasis origin MCF-7 cells, in addition to increased expression, were more sensitive to capsaicin-induced cell death [54]. *TRPV1* mRNA and protein levels are significantly reduced in human primary gastric cancer tissues compared to adjacent normal tissues, and reduced levels correlate with poor prognosis [55]. In renal cancer, TRPV1 is highly expressed in normal renal tubules, but their expression is significantly reduced or absent in carcinoma [56]. Some TRPA1-activators, such as AITC, promote cell survival and proliferation in small cell lung cancer [57]. However, TRPA1 has been shown to inhibit the migration of Panc-1 pancreatic ductal adenocarcinoma cell line cells through both channel-dependent and independent pathways [58]. In a rat model of OSCC, TRPV1 expression is increased in trigeminal ganglion neurons and TRPV1 regulates tumor-induced thermal hyperalgesia [59]. Increased TRPA1-specific immunopositivity has been described in human nasopharyngeal carcinoma (NPC) specimens and is a negative predictive factor for specific distant metastasis and local recurrence-free survival [60].

TRPV1-like immunopositivity has been observed in human tongue HNSCC cell lines, SCC4, C25 and HSC3. *TRPV1* mRNA expression in HSC3 cells is relatively similar to that in normal keratinocytes, but in SCC4 and SCC25 cells, its relative expression is significantly higher [61]. TRPV1 was also expressed on cultured CAL27 cells of human tongue squamous cell carcinoma

origin [62]. In human HNSCC, increased mRNA expression of *TRPV1-TRPV4* ion channels was found in several areas of the oral cavity by quantitative PCR (polymerase chain reaction) [63]. The expression level of TRPV1 is significantly higher in precancerous lesions of the tongue (leukoplakia) and in squamous cell carcinoma, but the expression level does not correlate with the degree of tumor malignancy [62].

AIMS

1. There are several results in the literature on the expression of TRPV1 in the oral mucosa under normal and pathological conditions, but no data are available on TRPA1 expression. For TRPA1 and TRPV1, most of the data on their expression are based on immunohistochemical studies, which, especially for TRPA1, need to be revised due to the questionable specificity of the antibodies. Our aim was therefore to determine the expression of TRPA1 and TRPV1 receptors in OSCC compared to healthy samples, map their expression and investigate their functionality in human OSCC cell lines.

2. In the second part of the reserach, we aimed to investigate the effects of additional anti-EGFR treatment to standard chemotherapy in patients with advanced HNSCC. We mainly focused on their effects on the overall survival (OS) and progression-free survival (PFS) of patients. In addition, we analysed the incidence and severity of anti-EGFR therapy-related side effects, such as skin rash, fatigue, mucosal inflammation of compared to patients receiving standard chemotherapy.

EXPERIMENTAL MODELS AND INVESTIGATIONAL TECHNIQUES

I. TRANSIENT RECEPTOR POTENTIAL ANKYRIN 1 AND VANILLOID 1 ION CHANNELS EXPRESSION STUDY IN HUMAN SQUAMOUS CELL CARCINOMA SAMPLES AND HUMAN OSCC CELL LINE

1. Study Participants and tissue collection and OSCC cell line

A total of 15 OSCC patients were included in the present study, aged between 57 and 92; all of them underwent surgical or experimental resection at the Department of Oral and Maxillofacial Surgery of Somogy County Kaposi Mór Teaching Hospital(license number: 7250-PTE 2018). Control samples of a similar size as the squamous cell carcinoma samples (n = 10) were taken from healthy volunteers by surgical excision. In addition, PE/CA-PJ41 (clone D2) OSCC cell line derived from the oral squamous cell of a 67-year-old female patient (Merck KGaA, Darmstadt, Germany) were also used.

2. RNAscope *in situ* hybridization in combination with immunohistochemistry

Human tissues were post-fixed for 24 h in 10% neutral buffered formalin solution (Merck, Darmstadt, Germany, Cat. No.: HT501128), rinsed in PBS, dehydrated and embedded in paraffin using standard procedures. Sections of 5 μm were cut using a sliding microtome (HM 430 Thermo Fisher Scientific, Waltham, MA, USA). The PE/CA-PJ41 cell line was collected in a subconfluent phase after 48 hours of breeding from a 6-hole cell breeding plate. The cells were treated for 5 minutes with a 1% trypsin-EDTA solution (Merck Kga. German), then the enzyme was stopped with 3 ml of RPMI solution. The cells were then collected in a 15 ml tube, and then quartzed with Luna-II automatic cell counter (Logos Biosystems, South Korea). The cells were diluted to 5,000,000/ml concentration, and then centrifuged for 5 minutes using Thermo Shandon Cytospin 3 centrifuge (Marshall Scientific, USA) centrifuge and citospin rotor at 500 rpm, during which 500,000 cells were transferred to Epredia™ Superfrost ultra -plus microscope for slides.The pre-treatment of PE/CA cell line was performed according to the RNAscope® Multiplex Fluorescent Reagent Kit v2 (ACD, Hayward, CA, USA) User Manual. In short, human sections were heat-treated, deparaffinized, H2O2-blocked, boiled and pre-treated with Protease Plus. In case of PE/CA-PJ41 cell line, Protease III treatment was performed. Samples were subsequently hybridized with probes specific to human *TRPA1* (ACD, Cat. No. 837411-C2), *TRPV1* (ACD, Cat. No.415381) mRNA and with 3-plex positive (ACD Cat. No. 320861) and negative (ACD, Cat.No. 320871) control probes. Sequential signal amplification and channel development was performed. Slides were subjected to an immunofluorescent labeling using monoclonal rabbit anti-cytokeratin-14 antiserum (Abcam, EPR17350, Cat. No. ab181595, diluted to 1:500) for 24 h at 24 ◦C. After 2 × 15 min washes in PBS, Alexa 647-conjugated donkey anti-rabbit (AB_2492288, Jackson Immunoresearch Europe Ltd., Cambridgeshire, UK; Cat. No. 711-605-152, diluted to 1:500,) serum was used for 3 h. Nuclear counterstaining with 40,6-diamidino-2-phenylindole (DAPI) was performed and sections were mounted with ProLong Diamond Antifade Mountant for confocal imaging. Human 3-plex positive (ACD; Cat. No.: 320881) control probes specific to POLR2A (Protein Coding RNA Polymerase II) mRNA (fluorescein), PPIB (Peptidylprolyl Isomerase B) mRNA (Cy3) and UBC (ubiquitin C) mRNA (cyanine 5, Cy5) and 3-plex negative (ACD; Cat. No.: 320871) control probes specific to bacterial dabP mRNA were tested in the human squamous cell carcinoma and PE/CA cell line. Fluorescent images were acquired using an Olympus Fluoview FV-1000 laser scanning confocal microscope (Olympus, Tokyo, Japan) and the Fluo-View FV-1000S-IX81 image

acquisition software system. The confocal aperture was set to 80 µm. The analogue sequential scanning was performed using a 40× objective lens (NA: 0.75). The optical thickness was set to 1 µm and the resolution was 1024 × 1024 pixel. The excitation time was set to 4 µs per pixel. Blue, green, red and white virtual colors were selected to depict fluorescent signals of DAPI (nuclear counterstain), Fluorescein (*TRPV1* mRNA), Cyanine 3 (*TRPA1* mRNA) and Alexa 647 (citokeratin-14 protein), respectively. DAPI was excited at 405 nm, Fluorescein at 488 nm, Cy3 at 550 nm and Alexa 647 at 650 nm.

3. RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

In the case of tissue samples, total RNA isolation was performed as follows: samples were placed into 1000 µL TRI-Reagent (Thermo Fischer Scientific, Waltham, MA, USA) and homogenized with stainless steel balls with the help off Tissue Lyser equipment (Qiagen, Hilden, Germany) and cordless pestle motor (VWR International Ltd., Debrecen, Hungary). PE/CA-PJ41 cell line was also homogenized in 1 mL TRI-reagent by vortexing. RNA contents of both sample types were isolated using Direct-zol™ RNA MiniPrep (for tissue lysates) and MicroPrep (for cell lysates, Zymo Research, Irvine, CA, USA), according toInt. J. Mol. Sci. 2022, 23, 1921 9 of 14
the manufacturer's instructions. The amount and purity of RNA were determined using a Nanodrop ND-1000 Spectrophotometer V3.5 (NanoDrop Technologies, Inc., Wilmington, DE, USA). Samples were treated with 1U DNase I enzyme to eliminate remaining genomic DNA. Tissue cDNA was synthesized from 500 ng RNA with a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), while for cell lysates, an Applied Biosystems™ High-Capacity Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used.

Relative gene expression ratios of the human *TRPA1* and *TRPV1* were determined with the QuantStudio™ 5 system (Life Technologies Magyarország Ltd., Budapest, Hungary) in a 96-well block using importin 8 (IPO8) as a reference gene [64]. Measurements were performed in triplicates, in a reaction volume of 10 µL, containing 1x SensiFAST™ Probe Lo-ROX mix (Meridiane Bioscience, Memphis, TN, USA), 400 nM probe primer mix (forward and reverse) and 20 ng cDNA. FAM conjugated TaqMan™ Gene Expression Assays (Thermo Scientific, Waltham, MA, USA), which were used to amplify the target loci: *IPO8*: Hs00914057_m1, *TRPA1*: Hs00175798_m1 and *TRPV1*: Hs00218912. Geometric means of the Cq values were calculated of both genes, and the gene expression was calculated using

the DDCt method [65]. The PE/CA-PJ41 PCR products were electrophoresed on a 2.5% agarose gel containing 0.01% GelRed (Biotium, Harward, CA, USA) at 70 V for 40 min, and visualized by a Safe View (Cleaver 182 Scientific Ltd., Warwickshire, UK) transilluminator.

4. Radioactive $_{45}Ca^{2+}$ uptake experiments on PE/CA-PJ41 cells and TRPV1 or TRPA1 receptor-expressing CHO(Chinese hamster ovarium) cell lines

For receptor selectivity experiments, PE/CA-PJ41 cells and CHO cells stably expressing TRPV1 or TRPA1 ion channels were investigated in response to capsaicin as well as AITC. CAPS and AITC responses were antagonized by capsazepine (10 μM) and HC-030031 (10 μM), respectively. Cells were plated in 15 μL cell culture medium onto Microwell Minitrays (Merck KGaA, Darmstadt, Germany) and incubated overnight at 37 ◦C in a humid atmosphere with 5% CO2. The following day, cells were washed with calcium free Hank's solution (pH 7.4), and then, they were incubated in 10 μL of the same buffer containing the desired amount of capsaicin (10 nM, 100 nM) or AITC (10 μM, 100 μM) and 200 μCi/mL $_{45}Ca^{2+}$ isotope (1.3 Ci/mmole, Ammersham) for 3 min at room temperature. After washing with ECS, the residual buffer was evaporated, the retained isotope was collected in 15 μL, 0.1% SDS and the radioactivity was measured in 2 mL scintillation liquid in a Packard Tri-Carb 2800 TR scintillation counter. $_{45}Ca^{2+}$ isotope-retention was presented in count per minute (CPM).

5. Cell viability assay

Cell were treated with capsaicin (Thermo Fisher Scientific) and AITC (Merck KGaA, Darmstadt, Germany) dissolved in dimethyl sulfoxide (DMSO) using the following concentrations.Capsaicin concentration ranged between 100 nM and 45 μM (100 nM, 1 μM, 2.5 μM, 5 μM, 10 μM, 15 μM, 25 μM and 45 μM) and AITC treatments were carried out at 100 nM, 1 μM, 5 μM, 7 μM, 10 μM, 15 μM and 45 μM (n = 6–6) [66]. DMSO concentration of the solvent treated control cells were adjusted to similar concentrations as the highest concentration of the treatments with capsaicin (0.45%, 0.25% and 0.15%) and AITC (0.45%,0.15% and 0.1%) [67]. Cells were seeded in 96-well tissue culture plates and cultured for 24 h. After the addition of AITC and capsaicin, cells were incubated for 24 h and cell viability was assessed using a CellTiter-Glo® Luminescent Cell Viability Assay (CTG, Promega, Madison, WI, USA) mixture, as recommended by the manufacturer. ATP-based luminometric measurement from the metabolically active cells in the culture were determined

by EnSpire® Multimode Plate Reader. Viability was calculated using the background-corrected luminescence as follows: Viability (%) = A of experiment well/A of control well × 100.

6. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software. The distribution of the data was examined with a Kolmogorov–Smirnov normality test, followed by one-way ANOVA and Dunnett's post hoc test (in the case of normal distribution) or Mann–Whitney U-test (if the data were not normally distributed). In all cases, $p < 0.05$ was considered as statistically significant.

II. THE USE OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) INHIBITORS IN THE TREATMENT OF PATIENTS WITH ADVANCED SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK REGION

1. Protocol, registration

We followed the Preferred Reporting Items for systematic reviews and meta-analyses (PRISMA) recommendation [68] for the preparation of reviews and meta-analyses of health interventions. The protocol and methods of our analysis were registered in the International Prospective Register of Systematic Reviews (PROSPERO, CRD42021233047), from which we did not deviate.

2. Search criteria

Literature searches were performed in MEDLINE, EMBASE and the Cochrane Central Register of Controlled Trials (CENTRAL) databases from 26 October 2020. The search was performed using the following search key: (HNSCC OR "head and neck squamous cell carcinoma" OR "squamous cell carcinoma of head and neck" OR "head and neck cancer" OR "head and neck neoplasm") AND (inoperable OR advanced OR recurrent OR metastatic OR metastasis OR unresectable) AND (random*). No restrictions were set based on the language and publication date of the literature. A reference search of the selected articles was also performed.

3. Selection of studies, eligibility criteria

The search results were organised using EndNote X9 3.3 (Clarivate Analytics), with first automatic and then manual duplicate removal. The study included articles with the following criteria: (1) randomized controlled trials (RCTs), (2) the population included adult patients (≥ 18 years) with histologically proven HNSCC (primary oropharyngeal, oral cavity,

hypopharyngeal, or laryngeal, recurrent and/or metastatic, and amenable to surgical resection or radiotherapy) (3) had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; with one or more measurable target lesions according to the Response Evaluation Criteria In Solid Tumors (RECIST) version 1.0, and (4) had an expected life expectancy of at least 3 months. Included studies had to include PFS and/or OS as endpoints, and an intervention group in which patients received EGFR-inhibitor treatment in addition to standard chemotherapy.

4. Extracting data

Primary outcomes were defined as PFS and/or OS, while secondary outcomes were defined as over level 3 and total fatigue, mucositis and skin rash. The following data were extracted from the included studies: first author, year of publication, DOI number, study design and duration, randomisation process, participating country and number of participating study centres. Participant data were also extracted, such as number of patients enrolled in the study, age, sex, inclusion and exclusion criteria. From both the intervention and comparator groups, the following data were collected: type of drug/placebo, doses, type and frequency of administration, duration of treatment, randomized and total number of patients. Data on the main outcomes (PFS, OS) were collected in the form of hazard ratios (HR) or odds ratios (OR) for dichotomous outcomes and their 95% confidence intervals (CI) were calculated.

5. Risk of bias in individual studies

To estimate the risk of bias for each study, we used the RoB 2 [69] software, a revised tool based on the current Cochrane Collaboration recommendation for assessing the risk of bias in randomised trials.

6. Statistical analysis

In the selection process, agreement and reliability between evaluators were determined using Cohen's kappa coefficient (κ) [70]. For dichotomous outcomes, ORs with 95% CI were calculated from the original raw data of the articles. Pooled HRs for PFS and OS were also estimated. The DerSimonian and Laird random-effects model [81] was used to determine heterogeneity in all cases. Heterogeneity was determined by calculating I^2 values. I^2 = 0% - 40% was considered as no significant heterogeneity, I^2 = 30% - 60% was considered as moderate heterogeneity, I^2 = 50% - 90% was considered as significant heterogeneity, I^2 = 75% - 100% was considered as heterogeneity worth considering. The results of each meta-analysis

(odds ratio and confidence intervals) were presented graphically using forest plots. Bias assessment was performed by examining funnel plots to detect non-symmetric distribution of standard errors and using Egger's test of one of the main outcomes (OS). In this case, $p < 0.05$ was considered statistically significant asymmetry. Subgroup analyses were also performed for each outcome, where subgroups were defined by different types of EGFR inhibitors (mAbs versus TKIs). As trials with low methodological quality, publication bias and small sample size may generate spurious p-values, we used a Trial Sequential Analysis tool (TSA, Copenhagen Trial Unit, Center for Clinical Intervention Research, Denmark) to calculate the required information size [71]. The TSA tool calculated the required sample size by taking into account an overall type I error of 5% and a type II error of 20%. This results in a two-sided graph where the red straight lines show the significance limits of conventional meta-analysis, the blue line shows the cumulative Z-score, and the inward sloping red lines show the limits of experimental sequential monitoring with adjusted p-values. All data and statistical analyses were performed using Stata (version 16.0, StataCorp) and TSA tools.

7. Assessment of the reliability of the results

The reliability of our results was assessed using the GRADE Approach (GRADEpro GDT: GRADEpro Guideline Development Tool McMaster University) [72].

RESULTS

I. TRANSIENT RECEPTOR POTENTIAL ANKYRIN 1 AND VANILLOID 1 ION CHANNELS EXPRESSION STUDY IN HUMAN SQUAMOUS CELL CARCINOMA SAMPLES AND HUMAN OSCC CELL LINE

1. *TRPA1* and *TRPV1* mRNAs are present in healthy human oral mucosa epithelial cells, OSCC and PE/CA-PJ41 cell line

Confocal laser scanning microscopy and qualitative morphological assessment revealed that both *TRPA1* and *TRPV1* mRNAs are expressed in the squamous epithelium of the healthy oral mucosa (n = 3) and OSCC (n = 6) samples. In agreement with this finding, both mRNAs were detected in PE/CA-PJ41 cells as well. Nearly all TRPA1- and TRPV1-positive cells contained the epithelium-specific keratinocyte marker citokeratin-14 in all samples. TRPA1-like immunopositivity was observed in the basal and prickle cell layers of thehealthy human oral mucosa, mainly in the cytoplasmic, but also in the nuclear regions. In most cancer samples, particularly in the poorly differentiated cases, TRPA1 immunopositivity was substantially stronger in the epithelial, vascular endothelial and some lymphoid

cells, but the staining was mainly located in the nuclei, clearly suggesting a non-specific reaction. The red and green refer to the computer-generated image analysis (ImageQuant, 3DHistech) artificial coloring, which are proportional to the intensity of the immunopositivity in a scale where red represents the strongest and blue no staining. These, of course, do not provide information about the specificity of the antibody. Furthermore, this antibody did not give any signal on CHO cells stably expressing the human TRPA1 receptor. Although several anti-TRPV1 antibodies are commercially available for human tissues (Biorbyt Ltd., Cat. no.: orb251483; Novus Biologicals, Cat. no.: NB100-98886; Abcam plc., Cat. no.: ab3487), none of them have been proven to be specific in our hands, on paraffin-embedded sections.

2. *TRPA1* and *TRPV1* mRNAs are significantly upregulated in OSCC samples

In agreement with the RNAscope results, both *TRPA1* and *TRPV1* mRNAs were stably expressed in the healthy human oral mucosa samples (n = 10). Significantly, approximately 4-fold and 2-fold *TRPA1* and *TRPV1* mRNA increases were detectable in OSCC (n = 15) samples, respectively, compared to the healthy control samples. Both *TRPA1* and *TRPV1* mRNAs were expressed in the OSCC PE/CA-PJ41 cell line, reaching the threshold cycle of Ct 29.4 and 25.1, respectively, during the qRT-PCR measurement.

3. Both TRPA1 and TRPV1 activations induce radioactive $_{45}Ca^{2+}$ uptake in PE/CA-PJ41 cells

In order to demonstrate that PE/CA-PJ41 cells express functionally active TRPA1 and TRPV1 ion channels, AITC- and capsaicin-induced 45Ca2+-influx was measured. The TRPA1 agonist AITC (10 and 100 μM) resulted in a concentration-dependent, 418.3 ± 120.2 and 1928 ± 315.8 CPM 45Ca2+-influx into PE/CA-PJ41 cells, respectively. For a positive control comparison, this value was 4874 ± 545.97 on stable TRPA1 receptor-expressing CHO cells in response to 100 μM AITC. The TRPV1 agonist capsaicin (10 and 100 nM) induced a concentration-dependent 1353.3 ± 315.4 and 5443.3 ± 1335.8 CPM 45Ca2+-retention, respectively, in PE/CA-PJ41 cells. This response was 12,324.7 ± 1168.1 CPM on the TRPV1 receptor-expressing CHO cell line for 100 nM capsaicin stimulation. The retention of 45Ca2+ was negligible; around 100–200 CPM in the Hank's solution alone and Hank's solution plus isotope controls. The TRPV1 antagonist capsazepine (CZP, 10 μM) significantly inhibited

the capsaicin-evoked, and the TRPA1 antagonist HC-030031 (10 μM) significantly reduced the AITC-induced $_{45}Ca^{2+}$- uptake.

4. Activation of both TRPA1 and TRPV1 reduces the viability of PE/CA-PJ41 cells

Incubation of PE/CA-PJ41 cells with 3 different concentrations of TRPA1 agonist AITC for 24 h resulted in a concentration-dependent reduction in cell viability. At 10 nM concentration, there was an 18% non-significant reduction in cell viability, at 100 nM, a 48% significant reduction, while at 5000 nM, there was a complete loss of cell viability (99%). Meanwhile, capsaicin induced a non-significant 19-32% reduction in cell viability in the 100-45000 nM concentration range. For solvent-treated control cells, DMSO did not affect cell viability at the highest concentrations.

II. THE USE OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) INHIBITORS IN THE TREATMENT OF PATIENTS WITH ADVANCED SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK REGION

The addition of epidermal growth factor receptor (EGFR) inhibitors in patients with advanced head and neck region squamous cell carcinoma increases progression free survival (PFS) and overall survival (OS), does not increase fatigue and mucosal inflammation, but increases skin rash. Ten studies were included in the analysis of OS and eight for PFS. The addition of EGFR inhibitors to standard chemotherapy significantly reduced the risk of death or disease progression (PFS HR: 0.68, 95% CI: 0.55-0.81) with significant heterogeneity (I^2 = 65.5%, p = 0.005) and reduced the risk of death (OS HR: 0.83, 95% CI: 0.72-0.94) with significantly moderate heterogeneity (I^2 = 42.3%, p = 0.076). No statistically significant differences were found between the EGFR inhibitor + chemotherapy and chemotherapy-only groups for all grade mucositis and fatigue. No significant difference was found for mucositis over 3 grade, but the TKI subgroup of EGFR inhibitors was significantly more likely to have mucositis over 3 grade. The EGFR inhibitor subgroup was significantly more likely to have mucositis over 3 grade compared to the conventional chemotherapy group (p = 0.017, OR: 0.39, 95% CI: 0.18-0.84). (p=0.008, OR: 4.86, 95% CI: 1.52-15.49), without significant heterogeneity (I^2=2.3%, p=0.407). In the mAb subgroup, a significant difference was detected between the control and EGFR inhibitor groups (p = 0.023, OR: 13.53, 95% CI: 1.44-127.58, I^2 = 0.0%), whereas this was not observed in the TKI subgroup (p = 0.120, OR: 3.42, 95% CI: 0.72-16.12, I^2 = 22.2%). In the analysis of all grade skin rashes, there was a significant difference between the EGFR-inhibitor and conventional chemotherapy groups (p < 0.001), with the EGFR-inhibitor group (OR: 18.32, 95%, CI: 8.07-41.60) more likely to have all grade skin rashes, with moderate

heterogeneity (I^2 = 56.6%, p = 0.032). There were significant differences between the two subgroups in both subgroups (mAB: $p < 0.001$, TKI: $p < 0.001$) TSA for skin rash adverse event testing could not be performed due to irrelevant risk reduction.

DISCUSSION AND CONCLUSIONS

In the first part of our work, we provided the first data on the expression and local mRNA upregulation of TRPA1 ion channels in OSCC and demonstrated the functionality of the TRPA1 and TRPV1 channels in human OSCC cell line. Furthermore, our results confirm the expression and up-regulation of *TRPV1* mRNA in human OSCC samples. Furthermore, with the help of our review article, we have shown that the expression levels of several TRP channels show significant differences between cancerous and normal tissues in HNSCC, with important implications for diagnosis, prognosis, and therapy [73]. TRPA1 protein enhancement has been described in several tumors in the literature, such as nasopharyngeal carcinoma [60]. Elevated TRPV1 protein expression levels have been found in cervical cancer [74], and in prostate cancer, *TRPV1* elevation has been described at both mRNA and protein levels [75]. *TRPV1* mRNA and immunopositivity were significantly elevated in all layers of the epidermis in HNSCC samples of the tongue, cheek, floor of the mouth and gingiva compared to normal oral mucosa [63]. Although there are many data available in the literature regarding receptor expression in the above-mentioned cell types, most of them rely on protein expression results detected by immunohistochemical methods. However, in 2019, Virk and his team highlighted a cornerstone methodological problem in TRPA1 immunohistochemistry in a paper published in Science Reports [76]. Our experiments support their conclusions, as we also found TRPA1 staining by immunohistochemistry to be aspecific. We have found that both TRPA1 and TRPV1 are functionally active in human OSCC cell lines, as both AITC and capsaicin induce Ca^{2+} influx into the cells. The effects induced by our measurements are consistent with other results obtained from HeLa cervical cancer cell lines [67, 77] and gastric cancer cells [66]. Treatment with 100 nM AITC for 24 h significantly reduced the viability of the OSCC line and at a concentration of 5 μM, reduced viability by 99%, i.e. almost completely. Liu et al. reported that high doses of AITC (≥20 μM) reduced the viability of HepG2 human hepatocellular carcinoma cells, increased DNA damage and inhibited cell migration [78], and in addition, in in vivo studies, AITC has antiangiogenic effects [79]. A number of studies have also shown that AITC negatively affects cell proliferation in breast [80], bladder [77] and cervical cancer cells [67, 77]. Data on the role of these receptors in carcinogenesis are contradictory in the literature. Some TRPA1- activators, such as AITC, promote cell survival and proliferation in small cell

lung cancer [57], but in contrast, TRPA1 agonists inhibit the migration of Panc-1 pancreatic ductal adenocarcinoma cell line cells [58]. In human breast cancer cells, AITC induced mitochondria-mediated apoptosis via cytochrome c, as well as apoptosis-inducing factor and endonuclease G release, and activation of caspase-9 and caspase-3 [80]. Our studies have shown that activation of TRPA1 and TRPV1 reduces OSCC cell viability, presumably by increasing intracellular Ca^{2+} levels and activating related signalling pathways. TRP channels may influence several aspects of malignant tumor progression by modulating Ca^{2+}-regulated cellular processes, including proliferation, survival and even tumorigenesis [73]. Clinically useful TRP modulators could be promising therapeutic agents, but the severe systemic side effects of TRPV1 antagonists developed for analgesic purposes, such as hyperthermia, have hampered the development of systemically applicable drugs. However, topically applied TRPA1 and/or TRPV1 agonists may be free of undesirable systemic side effects and therefore may have a beneficial treatment potential against the onset and progression of oral mucosal carcinoma.

In the second part of our research, we investigated the effect of additional EGFR inhibitor therapy on the patients PFS and OS in comparison with conventional chemotherapy. We found, that anti-EGFR therapy is able to increases patient survival with relatively good tolerability,. Our study has gathered up-to-date information on the efficacy and safety profile of EGFR inhibitors using material from three different databases. Only randomized clinical trials are included in our meta-analysis, as they allow for better comparability of experimental variables and thus make the results more reliable. Patients with advanced or unresectable HNSCC who progress within 6 months of combined radiotherapy with cisplatin or within 6 months of first-line platinum-based chemotherapy have a very poor prognosis and an overall median survival of only around 10 months [81]. In HNSCC, EGFR is highly expressed in most cases and its levels are negatively correlated with patient survival [82]. Cetuximab was approved as a major adjunctive treatment for HNSCC in combination with platinum-based and 5-fluorouracil therapy [83]. Although the beneficial effects of chemotherapy adjuvanted with anti-EGFR therapy have been established in several RCTs, survival rates for patients with advanced HNSCC remain low, due to, among other reasons, low patient compliance with treatment due to severe side effects. The most described side effect of mAbs and TKIs is a skin acneiform rash, which occurs in about 65-90% of patients and in severe cases may require dose reduction [84]. These lesions significantly impair the quality of life of patients and sometimes lead to discontinuation of anticancer treatment [85]. Our analysis showed that combining EGFR inhibitory mAbs or TKIs with standard chemotherapy increases both PFS and OS. In terms of

their safety profile and tolerability, we found that only the incidence of skin rash increased in the EGFR inhibitor group compared with standard chemotherapy alone. Nowadays, PD1 inhibitors play a crucial role in the treatment of advanced HNSCC cases and can control tumour progression by immune activation[86, 87]. Comparison of nivolumab, an anti-PD1 monoclonal antibody, with methotrexate, docetaxel or cetuximab showed that median overall survival was significantly improved in the nivolumab group (7.5 months) compared to the control group (5.1 months) [88]. However, despite the incorporation of PD1 inhibitors into the treatment protocol, the prognosis of patients with recurrent metastatic HNSCC remains poor and cetuximab-cisplatin remains the standard of care protocol for patients in whom PD-1 inhibitors are contraindicated and also in the group of patients with tumours not expressing PD-L1 [89]. Combination therapies and multi-targeted EGFR inhibitors that interfere with other signalling pathways may be potential tools against EGFR mutations leading to drug resistance. They have the advantage of reducing side effects and drug-drug interactions, facilitating dose optimisation and their clinical use [108]. In our study, the relatively small number of elements was a disadvantage due to the large patient population, the heterogeneity of EGFR inhibitors used in RCTs and the diversity of side effects. Median follow-up times were also different across RCTs, leading to more difficult comparisons of HR values. Our results demonstrate that a larger number of RCTs are needed to provide further evidence on the efficacy and safety of anti-EGFR therapy by investigating different types of this therapeutic option. Further research is needed to elucidate the precise molecular mechanisms of the therapeutic effect of EGFR inhibitors and the development of potential resistance. In conclusion, EGFR inhibitor treatment, despite the above-mentioned side effects, can be considered an effective second-line agent for the treatment of inoperable metastatic HNSCC patients who progress despite treatment with immune checkpoint inhibitors (ICIs).

SUMMARY OF THE FINDIGS PRESENTED IN THE THESIS

1) We provide the first evidence on the expression and local mRNA overexpression of the *TRPA1* ion channel in OSCC, as well as the functionality of the channels in the human OSCC cell line. In addition, our results confirm the expression and overexpression of *TRPV1* mRNA in this cancer. Based on these conclusions, clinically useful TRP modulators may be promising therapeutic agents, but severe systemic side effects of TRPV1 antagonists developed for analgesic purposes, such as hyperthermia, hinder the development of systemic drug targets. However, locally employed TRPA1 and/or TRPV1 agonists can avoid systemic undesirable

effects and thus provide beneficial treatment options on the oral mucosa, even against the development and progression of OSCC.

2) In our meta-analysis study, we found that in patients with advanced HNSCC, the use of anti-EGFR treatment added to standard chemotherapy improves the overall and progression-free survival of patients. Moreover, by examining their side effect profile and tolerability, we found that the incidence of skin rashes increased in the EGFR inhibitor group compared to the single use of standard chemotherapy.

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PUBLICATIONS RELATED TO THE THESIS

Kiss Fruzsina, Pohóczky Krisztina, Szállási Árpád, Helyes Zsuzsanna: Transient Receptor Potential (TRP) Channels in Head-and-Neck Squamous Cell Carcinomas: Diagnostic, Prognostic, and Therapeutic Potentials Review .Int J Mol Sci. 2020 Sep 2;21(17):6374. doi: 10.3390/ijms21176374 PMID: 32887395 Shared co-first authorship IF: 5, 923 Q1/D1

Kiss Fruzsina, Kormos Viktória, Szőke Éva, Kecskés Angéla, Tóth Norbert, Steib Anita, Szállási Árpád, Scheich Bálint, Gaszner Balázs, Kun József, Fülöp Gábor, Pohóczky Krisztina, Helyes Zsuzsanna: Functional Transient Receptor Potential Ankyrin 1 and Vanilloid 1 Ion Channels Are Overexpressed in Human Oral Squamous Cell Carcinoma.Int J Mol Sci. 2022 Feb 8;23(3):1921. doi: 10.3390/ijms23031921 PMID: 35163843 IF: 5, 923 Q1/ D1

Kiss Fruzsina, Pohóczky Krisztina, Görbe Anikó, Dembrovszky Fanni, Kiss Szabolcs, Hegyi Péter, Szakó Lajos, Tóth Lilla, Somogyiné Ezer Éva, Szalai Eszter, Helyes Zsuzsanna: Addition of EGFR inhibitors to standard chemotherapy increases survival of advanced head and neck squamous cell carcinoma patients: a systematic review and meta-analysis Oral Dis 2022 Apr 29. doi: 10.1111/odi.14228 PMID: 35485982 Shared co-first authorship IF: 3, 511 Q1

PUBLICATIONS NOT RELATED TO THE THESIS

Kiss Fruzsina, Fülöp Gábor, Oberna Ferenc, Battyáni Zita: Head and neck mucosal melanoma: clinicopathological analysis of 5 cases and review of the literature.Fogorv Sz. 2017 Mar;110(1):25-29.PMID: 29847065 Review. English, Hungarian. National journal with no impact factor that can be included in the publication criteria

Tóth Lilla, Juhász Márk F, Szabó László, Abada Alan, **Kiss Fruzsina**, Hegyi Péter, Farkas Nelli, Nagy György, Helyes Zsuzsanna: Janus Kinase Inhibitors Improve Disease Activity and Patient-Reported Outcomes in Rheumatoid Arthritis: A Systematic Review and Meta-Analysis of 24,135 Patients. Int J Mol Sci. 2022 Jan 23;23(3):1246. doi: 10.3390/ijms23031246.PMID: 35163173 IF: 5, 923 Q1/ D1

CUMULATIVE IMPACT FACTOR OF ALL PUBLICATIONS

Cumulative impact factor of publications related to the thesis:15,357

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ORAL PRESENTATION

2015: Pannon Section and the Hungarian Society of Maxillofacial and Oral Surgery Annual Meeting: Rare primary melanomas with oral manifestations

2016: Hungarian Society of Maxillofacial and Oral Surgery Annual Meeting: Treatment strategies of lip tumors in Somogy County (Károly Méhes Award)

2017: Hungarian Society of Maxillofacial and Oral Surgery Annual Meeting: Diagnostic possibilities and difficulties of neuropathic facial pains in everyday practice. Doctoral students in clinical research conference: Mass spectrometry study of tumors in head and neck area

2018: Pannon Section: Brachytherapy for head and neck tumors Hungarian Maxillofacial and Oral Surgery Annual Meeting: Brachytherapy for head and neck tumors

2019: The13th Congress of the Hungarian Dental Association, Szeged: Transient Receptor Potencial Ankyrin 1 ion channel expression and expression changes in squamous cell carcinoma and precancerosis in the head-neck region

2022: Pannon Section: Granulomatous polyangiitis and MRONJ case study

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