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Immunohistochemical expression of the cation channel TRPC6 in the submandibular and lacrimal gland and in salivary gland tumors

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ABSTRACT

Background: Canonical transient receptor potential channels play a crucial role in cancer cell proliferation. While TRPC6 subtype detection in submandibular glands and the relevance of some TRPC channels in this gland have been shown in animal models, its histological detection in human lacrimal and submandibular glands, as well as related tumors, lacks systematic study. Studying TRPC6 in humans could lead to new therapeutic options. This research aimed to immunohistochemically detect TRPC6 in human samples of physiological lacrimal and submandibular glands and of adenoid cystic carcinoma and mucoepidermoid carcinoma.
Methods: Seven fixed body donors and samples of six cancer patients were examined. The ten tissue samples collected from the submandibular and lacrimal glands were then processed into histological slides and stained with hematoxylin-eosin. Tumor samples were provided as sections. TRPC6 presence was determined by immunohistochemistry, which was performed by indirect detection with a primary TRPC6 antibody, a secondary HRP-conjugated antibody and the chromogen diaminobenzidine.
Results: Results confirm TRPC6 expression in all ten physiological gland samples: all samples showed a immunohistochemical signal with varying intensity. No significant gender-specific differences could be observed. TRPC6 was detected in four of six submandibular adenoid cystic carcinoma and the mucoepidermoid carcinoma samples, especially in tumor cells' cytoplasma and nuclei. Excretory ducts consistently showed TRPC6. Mucous tubules, their nuclei and the nuclei of adipocytes generally showed no signal while serous acini and their nuclei showed a weak TRPC6 signal.
Conclusion: The discovery of TRPC6 in glandular tissue indicates a role in salivary gland function and calcium homeostasis is a basis for further research into its significance for tumor development in adenoid cystic carcinoma and mucoepidermoid carcinoma of salivary glands. TRPC6 could be used as a target for treatment of these tumors. However, the correlation between TRPC6 and submandibular and lacrimal gland diseases requires further exploration.

1. Introduction

The transient receptor potential (TRP) ion channels are a superfamily of 28 membrane-bound, non-selective, calcium-permeable cation channels found in mammals. TRP channels play an increasingly important role in a variety of cellular functions, and dysfunction of these channels could potentially cause disease. As such, these channels are a major component in the intracellular Ca^{2+} homeostasis [34]. Similar to TRPC3 and TRPC7, TRPC6 is a diacylglycerol (DAG)-sensitive TRPC channel [25]. While existing data on the presence and function of the TRPC6 channel in the submandibular gland are based on animal models [9], there is no data regarding the existence of TRPC6 or even TRPC in the lacrimal gland. Similarly, there is no evidence of TRPC and subtype C6 expression in submandibular adenoid cystic carcinoma (ACC) and mucoepidermoid carcinoma (MEC).

It is suspected that certain channel subtypes, in particular TRPC6,

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may be present in human submandibular and lacrimal glands. The role of some TRPCs in salivary gland cells and salivary secretion has been described [2,3,6,7,9,15,16,18,26,28–30], but there are no systematic descriptions on the localization and role of TRPC6 in human exocrine glands such as the submandibular gland and lacrimal gland. This work is therefore important and contributes to future research on TRPC6. As TRPC6 channels are promising targets which can be pharmacologically manipulated [8], identifying their physiological expression may have clinical importance.

It has also been shown that TRPC channels such as subtype C6 are expressed in human tumors. Indeed, it was observed that TRPC6 shows a stronger expression in tumor tissues than in the corresponding normal tissues [20]. TRPC6 could play an important role in the development of human hepatocellular carcinoma (HCC) [21]. TRPC6 has also been shown to be overexpressed in breast cancer tumor tissues [24], as well as in benign prostatic space-occupying lesions and malignant human prostate cancer [40,42]. In squamous cell carcinoma (SCC) of the esophagus, the expression of TRPC6 is also significantly higher than in normal tissue. In addition, inhibition of TRPC6 suppressed the growth of SCC cells both in vitro and in rodents [20,38]. A similar phenomenon has been observed in renal cell carcinoma [39]. In glioblastoma (GBM), TRPC6 was found to affect various properties of GBM, including tumor growth, cellular survival, invasiveness and angiogenesis, thus inhibition of TRPC6 is associated with a reduction in glioma growth, invasion and angiogenesis [17]. In the lung, the TRPC6-mediated increase in intracellular Ca^{2+} by promoting cell cycle progression stimulates cell proliferation of non-small cell lung cancer (NSCLC) [41]. TRPC6 has been shown to be expressed in cervical cancer cell lines. TRPC6 could be a new target for the prevention and treatment of cervical cancer [8]. The influence of TRP channels on ACC and MEC tumors of the submandibular gland and whether they are present at all in these tumors has not been investigated. Whether TRPC6 is present in tumors of the salivary glands, in particular the submandibular gland, is not yet clear.

The main focus of this study was the immunohistochemical detection of TRPC6 channels in healthy human submandibular and lacrimal glands as well as in malignant tumors of the submandibular gland, ACC and MEC. The following objectives were studied: Is TRPC6 detectable in healthy human submandibular and lacrimal gland tissue by immunohistochemical staining? Are there differences in protein detection between histological structures (e.g. ducts, terminal glands)? Does gender influence the detection of TRPC6 in glandular tissue? Is TRPC6 detectable in human submandibular gland tumors such as ACC and MEC by immunohistochemical staining? Are there differences in protein detection between histological structures (e.g. ducts, terminals) and tumor lesions (e.g. tumor cell nuclei)? Are there differences in protein detection between healthy and tumorous glandular tissue? In the present study, human exocrine gland tissue samples of submandibular and lacrimal glands were obtained from cadavers from the Institute for Anatomy and Cell Biology of the Saarland University, as well as human submandibular tumor samples from the Institute of Pathology of the Saarland University Hospital. All samples were examined using immunohistochemistry (IHC) to determine the presence of TRPC6 in these tissues.

2. Methods

Seven nitrite pickling salt-ethanol-polyethylene glycol-400 (NEP) fixed body donors from the Anatomical Institute of Saarland University were examined (gender: 3 females 43 %; age [years]: median 84, range 16; postmortem interval to fixation [d]: median 1, range 3). Ten tissue samples were taken autonomously from submandibular and lacrimal glands using forceps and a scalpel. The collected samples were then processed in several steps into histological slides and first stained with hematoxylin-eosin, then using Immunohistochemistry (IHC). In addition, six tumor samples were included in this study (gender: 1 female 17 %; age [years]: median 63, range 24; Institute of Pathology of the Saarland University Hospital). This study has been approved by the Standing Ethics Committee of the Medical Association of Saarland, Saarbrücken, Germany (Approval no. 163/20 for samples from the Institute of Anatomy and approval no. 130/21 for samples obtained from the Institute of Pathology, Saarland University, Homburg, Germany).

Six submandibular and four lacrimal gland samples were excised in a standard procedure but obesity, atrophy glands, cachexia etc. were a challenge. Servello et al. have previously described the sample processing used in this study [37]. Briefly, the collected samples were fixed and then embedded in paraffin for microtome cutting, resulting in 7 μm thin sections. Samples underwent initial hematoxylin and eosin (H&E) staining to determine the morphology and integrity of the gland tissue, a prerequisite for IHC. The protocol for immunohistochemical staining has also already been described in detail in previous studies [1,37]. The first step was removal of paraffin. Initially sections required a long deparaffinization phase. This led to poorer results in terms of reduced accessibility to the TRPC6 channels, making it difficult for the primary antibody to bind to them. This phenomenon probably explains apartially reduced signal strength and intensity of the brown staining in some areas of the investigated samples. It could be microscopically detected when the slides were immersed in xylene 3 times for only 5 min according to an existing staining protocol for deparaffinization. To overcome this issue, the samples were subsequently immersed in each time freshly prepared xylene 3 times for 8 minutes for dewaxing. Next, the samples on histological slides underwent antigen retrieval using citrate buffer solution in a heated incubator, then an incubation with the primary 1:50 diluted TRPC6 antibody (Alomone Labs; Cat. No. ACC-017). Instead of TRPC6 antibody, negative controls were incubated with serum of untreated rabbits (Institute of Medical Biochemistry and Molecular Biology, Saarland University, Homburg, Germany) in each staining cycle. The samples were then subjected to another incubation using 1:500 diluted horseradish peroxidase-conjugated goat anti-rabbit antibodies (Invitrogen; Thermo Fisher Scientific, Inc.; Cat. No. A10547; 1:500) as secondary antibodies. Added chromogen, diaminobenzidine (Vector Laboratories, Inc.; Cat. No. SK-4103), appeared as a brown color following the HRP substrate catalyzed reaction. In this case, the incubation time with the DAB substrate was 11 minutes, which was determined under a light microscope. The samples were then counterstained with hematoxylin-eosin. A total of 16 tissue samples from 13 donors were immunohistochemically analyzed in this study. Results were assessed microscopically with samples graded according to staining intensity: Strong, Moderate, Weak and Negative. IHC-stained specimens were evaluated histologically by two blinded examiners using a light microscope (×10, ×20, or ×40 magnification).

3. Results

Immunohistochemical signals were detected in all 10 healthy samples tested and in four of six tumor samples tested (67 %), although with varying intensity. Of 16 total samples, specific staining was therefore observed in 14 samples, about 88 % of the cases. A grading scheme distinguished different histological gland structures such as serous acini, mucous tubules and excretory ducts. In tumors, in addition to the cell structures listed above, the tumor tissue as a whole and more specifically the tumor cells were examined.

3.1. Results of exocrine gland structures and gender

3.1.1. Submandibular gland

out of 6 samples, 3 samples showed a strong signal in IHC, especially in the excretory ducts, 3 samples (50 %) showed only a moderate signal, also in the excretory ducts and serous acini. Weak signals were found in all samples (100 %), in the excretory ducts and serous acini. The nuclei of the adipocytes, the mucous tubules and their nuclei, and the nuclei of the serous acini showed no signal. Regarding gender, among the female

samples, 1 (33 %) showed a moderate signal in IHC and 2 (67 %) showed a weak signal. In contrast, all 3 male samples showed only a weak signal (Fig. 1, submandibular gland).

3.1.2. Lacrimal gland

out of 4 samples, 1 sample (25 %) showed a strong signal in IHC, particularly in the nuclei of the serous acini. Weak signals were seen in all samples, in the excretory ducts and in the serous acini. The nuclei of the adipocytes, the serous acini and their nuclei mostly showed no signal. Both female samples were classified as having a medium signal by IHC, while a weak signal was observed in 1 of the male samples and a medium signal in the other (Fig. 2, lacrimal gland).

In conclusion, IHC analysis revealed a signal for TRPC6 in all exocrine glands examined, this confirms the detection of TRPC6 in human exocrine gland tissue.

3.2. Results of submandibular glands cancer

3.2.1. ACC

out of 5 samples, 1 sample (20 %) showed a strong signal in IHC, especially in the excretory ducts and a weak signal in the nuclei of the serous acini. There was no signal in the other glandular structures in this donor, as well as in adipocyte nuclei. However, the other donor samples

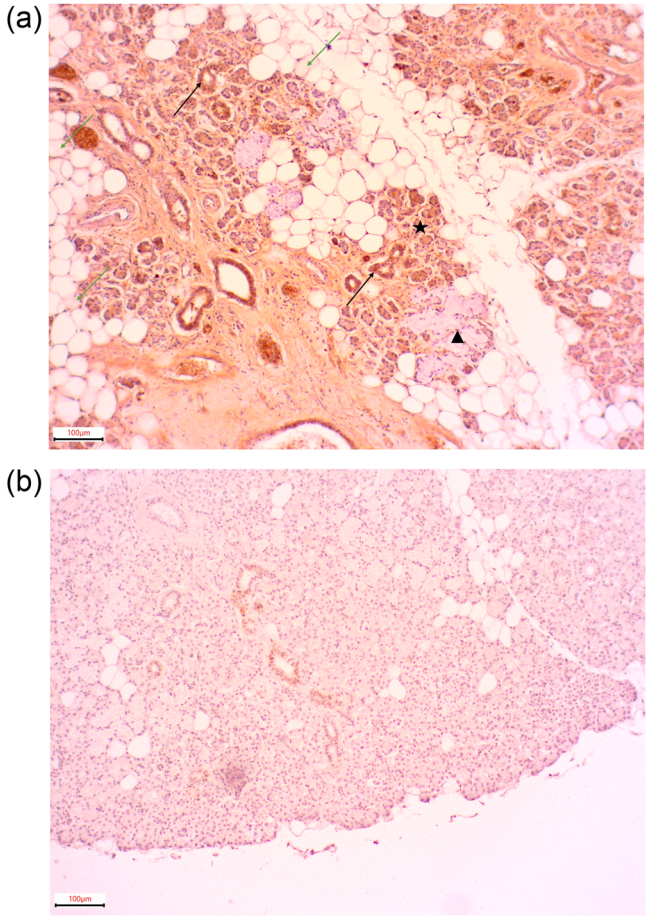

Fig. 1. Submandibular gland, IHC analysis with the primary antibody against TRPC6 and DAB staining at 10x magnification.(A) Staining: all excretory ducts show a brownish color (see arrows), as well as the serous acini, whereas the nuclei of the fat cells, the mucous tubules and their nuclei (see star), and the nuclei of the serous acini showed no signal. A background signal characterized by a slight brownish coloration in the lipid droplet of some fat cells (green arrows). (B) Corresponding negative control (serum from a non-immunized rabbit). The excretory ducts are not labelled with brown color (arrows).

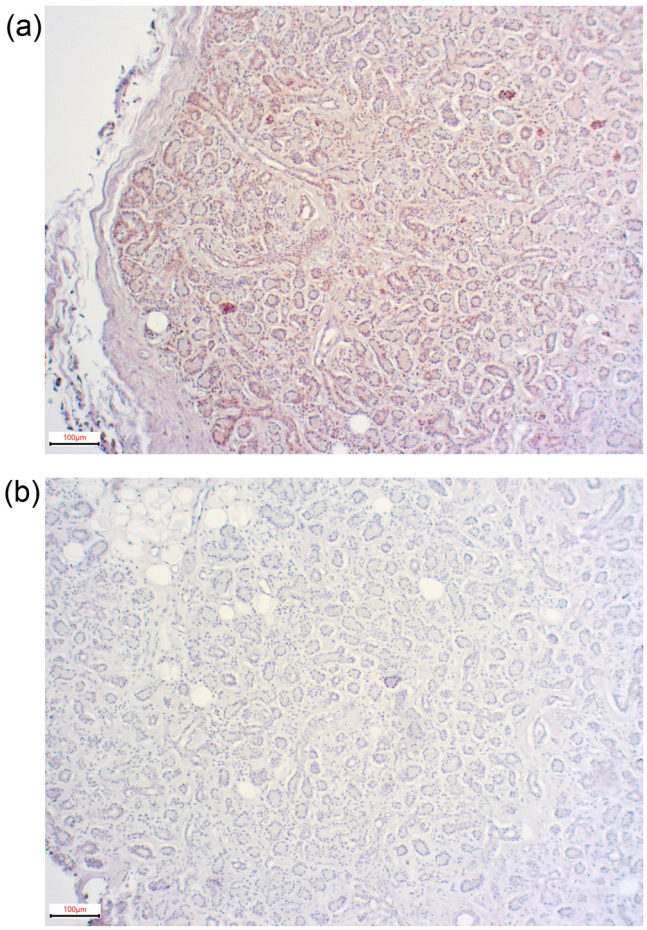

Fig. 2. Lacrimal gland, IHC analysis with the primary antibody against TRPC6 and DAB staining at 10x magnification.(A) Staining: all excretory ducts show a brownish color (see arrows), whereas the nuclei of the fat cells, the serous acini and their nuclei (see star) mostly showed no signal.(B) Corresponding negative control (serum from a non-immunized rabbit). The excretory ducts are not labelled with brown color (see arrows).

(4 out of 5) showed none of the typical glandular structures, i.e. no signal, because they were completely infiltrated with tumor tissue. Regarding the tumor tissue, IHC staining was found in 4 of the 5 samples (80 %), the signal was moderate in 3 samples and weak in 1 sample. The strongest signal intensity was found in the nuclei of the tumor cells in 3 samples (60 %). The cytoplasm of the tumor cells of the same samples showed a moderate signal. 1 sample generally showed no staining (Fig. 3A-C, ACC).

3.2.2. MEC

only 1 sample was provided and therefore investigated. Only the nuclei of the adipocytes shown as the only representatives of the typical parts of a submandibular gland and did not generate a signal. The tumor tissue in general was classified as having a strong signal, as were the nuclei of the tumor cells. The cytoplasm of the tumor cells was considered to have a moderate signal (Fig. 3D, MEC)

In conclusion, a signal for TRPC6 was detected by IHC in 4 of the 6 submandibular gland tumors (67 %) examined, both ACC and MEC. This confirms the detection of TRPC6 in human parotid gland tumors as described in literature. Based on these results, it can be assumed that the tumor tissue, in particular the cell nuclei of the tumor cells, show the strongest or best staining results. The reason for a lack of signal in one sample compared to another sample of the same tumor type may be due to tumor diversity.

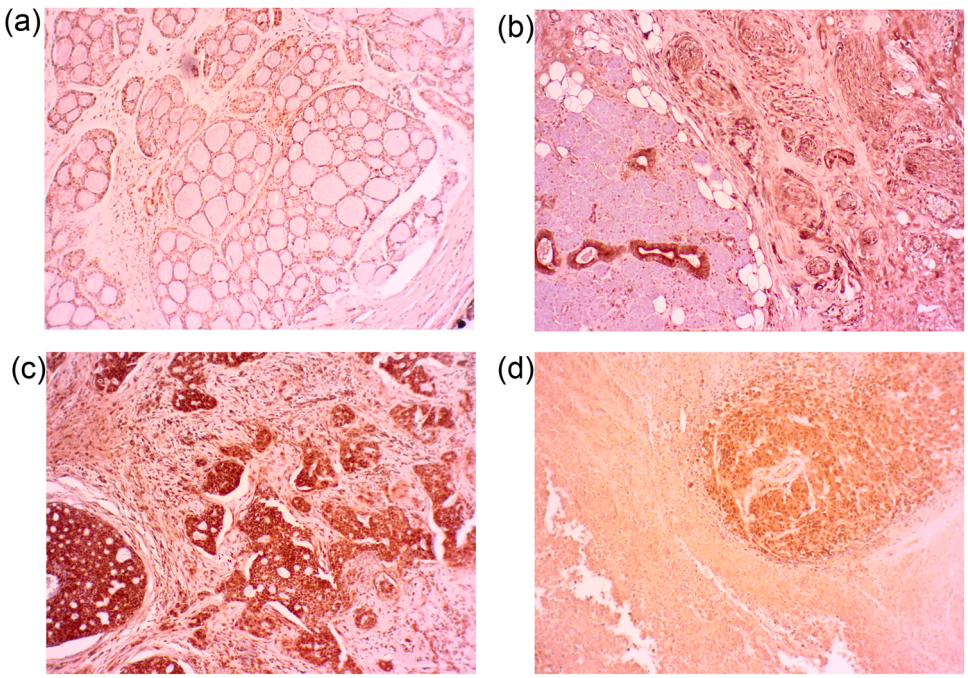

Fig. 3. ACC (A-C) and MEC (D), IHC analysis with the primary antibody against TRPC6 and DAB staining at 10x magnification.(A) Staining: tumor tissue shows a brownish color, especially the nuclei of the tumor cells (see arrows). Their cytoplasm also shows color. The nuclei of the fat cells show a slight coloring. All other structures are absent.(B) Staining: all excretory ducts show a brownish color, as well as the nuclei of the tumor cells (arrow). The nuclei of the serous acini show color. The serous acini and the fat cells and their nuclei showed no coloring. All other structures are absent.(C) Staining: the tumor tissue (see left-hand side arrow), especially the nuclei of the tumor cells (see right-hand side arrow) show a brownish color, as well as their cytoplasm. All other structures are absent.(D) Staining: the tumor tissue, especially the nuclei of the tumor cells (arrows) show a brownish color, as well as their cytoplasm. Fat cells showed no signal.

3.3. Healthy vs. tumorous

The structures with the strongest signal intensity in the IHC were the excretory ducts in the healthy glands and in the ACC (this structure is missing in the MEC). Next was the tumor tissue with the cytoplasm and nuclei of the tumor cells, which were also strongly stained, especially in the MEC. Weaker signals were found in the serous acini of healthy glands, especially in the submandibular gland, and in the nuclei of these cells, especially in the ACC. The mucous tubules and their nuclei generally showed no signal or were absent. Adipocyte nuclei generally showed no signal (Fig. 4).

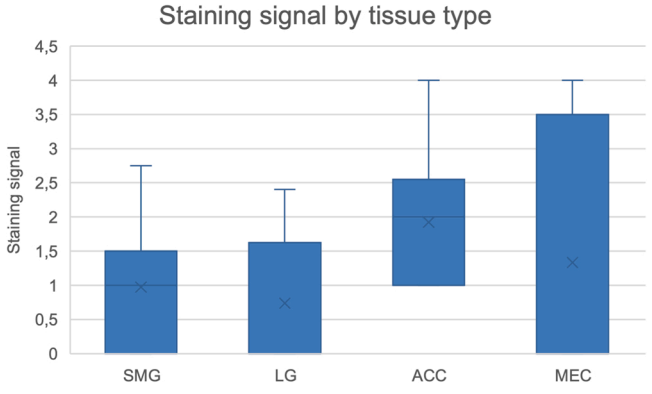

Fig. 4. Box plot illustrating the distribution of IHC staining signal intensities across four different tissue types: submandibular gland (SMG), lacrimal gland (LG), adenoid cystic carcinoma (ACC), and mucoepidermoid carcinoma (MEC). The y-axis represents the staining signal intensity by number: 0=missing structure, 1=no signal, 2=weak signal, 3=moderate signal and 4=strong signal. A Mann-Whitney U test was performed to determine whether there were differences in staining signal between these four groups. The distributions differed between the groups (with $p < 0.05$).

Again, the most intensely stained tissue structures overall, are the excretory ducts, especially in the tumors. In the tumors, this is followed by the nuclei of the tumor cells, the tumor tissue in general, and then the cytoplasm of the tumor cells. Finally, the nuclei of the serous acini were faintly stained. In healthy glands, the excretory ducts showed the strongest signal, followed by the serous acini and their nuclei. Differences in TRPC6 detection between samples with malignant changes of the submandibular gland and healthy submandibular tissue could therefore be demonstrated by IHC (Fig. 5).

4. Discussion

In this study, we were able to demonstrate for the first time the

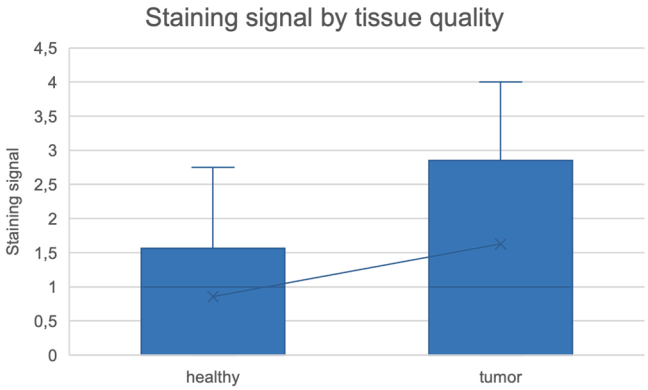

Fig. 5. Box plot illustrating the distribution of IHC staining signal intensities between healthy and tumor tissue samples. The y-axis represents the staining signal intensity by number: 0=missing structure, 1=no signal, 2=weak signal, 3=moderate signal and 4=strong signal. A Mann-Whitney U test was performed to compare staining signal in healthy and tumor samples. The ranks of the tumor groups were higher in comparison to healthy groups ($p < 0.05$).

presence of the TRPC6 protein in human healthy submandibular and lacrimal gland tissues as well as in submandibular adenoid cystic and mucoepidermoid carcinoma tissue on selected histologic structures, to the best of our knowledge. Differences in glandular distribution of TRPC6 expression were observed in the present study. The small number of samples is a weakness of this study. Ten tissue samples were taken from the submandibular and lacrimal glands of seven fixed body donors from the Institute of Anatomy. An important limitation of our study may be the limited age range, as the mean age of the examined cadavers at the time of death was 87 years. Therefore, no statement can be made about the occurrence of TRPC6 in physiological glandular tissue of the submandibular and lacrimal glands of young people. The distribution of TRPC6 could change with the aging process of the cells and thereby also of the organs. The donors all arrived at the institute on the day of their death, apart from donor 7, who arrived 3 days after his death. 4 of the 7 donors were fixed the next day, one the same day, one 3 days later and one even 4 days later. These postmortem differences also show a limitation of the method. Unfortunately, it was not possible to detect pre-existing diseases that potentially show a negative effect on the glandular tissue in any way. This further limits the significance of the results, even though no morphological abnormalities could be detected in the overview staining. Furthermore, only two tumors were represented: ACC and MEC. Therefore, no statement could be made about the occurrence of TRPC6 in other human salivary gland tumors. 6 tumor samples from 6 patients from the Department of Otorhinolaryngology, Head and Neck Surgery and the Institute of Pathology were examined. The mean donor age at biopsy was 64 years. This cohort is significantly younger than the body donor group at the Institute of Anatomy. The youngest patient was 53 years old, so no statement can be made about the occurrence of TRPC6 in ACC and MEC in younger people.

The antibody used is a rabbit polyclonal antibody. This antibody targets different binding sites of the target antigen and is therefore not specific for a particular binding site. This non-specific binding can lead to false results, such as non-specific background staining. An advantage of such polyclonal antibodies is that they are less sensitive to external influences such as pH changes. It was developed for the detection of TRPC6 from mouse, rat and human samples.

To create a standardized immunohistochemical staining protocol, it was necessary to find the correct antibody dilution for the experiment. Several antibody dilutions were tested. The 1:100 dilution of the primary antibody in the existing protocol proved to be too diluted for the selected tissue. This resulted in negative or questionable negative staining despite the presence of the desired TRPC6. After several trials, the 1:50 dilution proved to be suitable for human glands, in particular the submandibular salivary gland and the lacrimal gland.

When counterstaining immunohistochemical sections with distilled water, Ehrlich's hematoxylin, and rinsing with running tap water, a very short immersion time of 1 s in hematoxylin followed by rinsing with distilled water for 2–3 s before rinsing with tap water was chosen. In early attempts, longer hematoxylin staining without additional rinsing resulted in an overlapping of the brown staining, which could be misinterpreted as a false negative staining result in the evaluation. TRPC6 mRNA expression in humans was first determined by reverse transcription-polymerase chain reaction (RT-PCR): The protein was uniformly expressed throughout the CNS and peripheral tissues, with the highest levels measured in the placenta and lung. The only exocrine glands to be examined were the pancreas and the prostate gland [36]. The presence and role of TRPC6 channels in the human submandibular gland are still unclear. The TRPC1 subtype has been the subject of more extensive study. Trp1 is thought to be involved in the mechanism of calcium influx into human submandibular gland cells, as demonstrated in cell culture. Trp1 was also found in the plasma membrane of the acinar and ductal cells of the rat submandibular gland, where this mechanism of calcium influx is thought to take place [30]. For this purpose, TRPC1 forms a complex with caveolin-1 in the plasma membrane of human submandibular cells and MDCK cells to remain in the plasma membrane and allow calcium to enter the cells [14]. TRPC3 was localized in the apical parts of the ducts and acini in cell cultures of MDCK and rodent submandibular glands. On the other hand, TRPC1 was localized basolateral and TRPC6 was detected basolateral and apical [9]. TRPC1 plays a critical role in store-operated calcium entry (SOCE) and secretion in both salivary and pancreatic acinar cells. In addition, Orai1 and STIM1 have been identified as key components of SOCE that are required for TRPC1 function [3–5,7,12,30,33]. In rodents, immunohistochemistry has shown that TRPC3 plays a role in Ca2+ reabsorption from saliva by activating apical Ca2+-sensitive receptors in the submandibular gland. This reabsorption in the duct may contribute to the regulation of salivary flow, which is a critical factor in sialolithiasis [10]. So far, the influence of TRP channels on the human lacrimal gland remains unclear. The expression of TRPC, especially subtype C6, has not been extensively studied in the lacrimal gland. The expression of TRPV1 mRNA by quantitative RT-PCR (qRT-PCR) and TRPV1 protein by IHC was investigated in rabbit and human eyes: mRNA and protein were found in the secretory cells of the rabbit lacrimal gland. Thus, TRPV1 is expressed in cells that are particularly active in Ca2+ exchange and in cells with significant water transport activity. Since TRPV1 is a Ca2+ channel, it is likely to play a role in the regulation of water and Ca2+ balance in ocular tissues [31]. The TRPM3 channel is considered an important candidate for the development and function of the lacrimal gland, and its investigation was performed by qRT-PCR in rodents at different developmental stages and by IHC. The expression of TRPM3 in this gland is age-dependent and localized in the apical membrane of the glandular epithelium [27]. In 2018, researchers reported a functional interaction between the TRPV4 protein and ANO1 in acinar cells isolated from murine submandibular and lacrimal gland. TRPV4 interacts with IP3 receptors and ANO1 to regulate the muscarinic signaling pathway that mediates salivation and lacrimation [19]. Whether TRPC6 channels are also associated with human glandular cancer has been demonstrated by its involvement in the proliferation of prostate cancer cells, also on a molecular level through RT-PCR and Western blot [40]. A comparison of TRPC6 expression in benign versus malignant human glandular tissue has been performed in breast cancer [24], prostate cancer [42], oesophageal cancer [20], and kidney cancer [39], also using immunohistochemistry similar to our work. In addition to this method, Western Blot and RT-PCR were often performed. TRPC6 is highly expressed in SCC and plays an important role in cell proliferation and cell cycle, and inhibition of TRPC6 in human SCC cells suppresses their proliferation and leads to blockade of the G2/M phase [20]. The influence of TRP channels on ACC and MEC tumors of the submandibular gland, and whether they are present at all in these tumors, has not yet been investigated. In this study, we were able to show that the TRPC6 protein is mainly localized in the ducts of the submandibular gland, the lacrimal gland, and in the ACC and MEC of the submandibular gland. It has also been detected in the serous acini and their nuclei. TRPC6 is also highly expressed in tumor tissue, particularly in the nuclei and cytoplasm of tumor cells. Our immunohistochemical analysis of paraffin sections showed that TRPC6 expression was significantly associated with glandular tissue invasion in ACC and MEC. In each gland section of the submandibular and lacrimal gland and in most tumor sections, various cells showed moderate to strong brown staining, especially the excretory ducts, serous acinar cells, the nuclei of the serous acinar cells, the cytoplasm of the tumor cells as well as their nuclei. The mucous acini, their nuclei and the nuclei of the adipocytes were not stained in any specimen, i.e. no TRPC6 protein could be detected in these structures. However, two tumor sections showed no staining at all. The reason for the lack of signal in one sample compared to another sample of the same tumor type may be due to tumor diversity. This refers to the diversity of tumor cells within a tumor or between tumors of the same type in different patients [35]. There can also be great tumor diversity within a single tumor type, such as ACC in this case. This is referred to as intra-tumor heterogeneity [22]. This diversity can occur at different levels, for example at the cellular level, as different types of

cancer cells can be present within a tumor. Also at the genetic level: genetic diversity within a tumor can be attributed to small errors in DNA replication. These errors add up over the billions of cell divisions required to form a clinically detectable tumor and can lead to significant genetic diversity within the tumor [22]. A tumor can contain tumor stem cells, which have the ability to self-replicate and form a tumor, as well as differentiated cancer cells that perform specialized functions [32]. The tumor environment, which includes surrounding tissue, immune cells and blood vessels, can also be highly diverse. The composition and activity of this microenvironment can influence tumor diversity. Thus, biopsies of a small tumor area may not lead to a representative characterization of the genetic, epigenetic and/or phenotypic variations in the entire tumor [11,23]. Therefore, it seems interesting to investigate protein detection in ACC and MEC in a larger number of patients and to examine multiple sections of the same biopsy. In addition, only one MEC could be stained and examined. Since this is a rare tumor of the submandibular gland, only one specimen was available for this work. It would be useful to study protein detection in this tumor in a larger number of cases. The results of this study suggest that we were able to detect the TRPC6 protein immunohistochemically in human exocrine glands, such as the lacrimal and submandibular glands, as well as in two submandibular gland tumors, adenoid cystic carcinoma and mucoepidermoid carcinoma. The presence of TRPC6 in the submandibular gland has been demonstrated in animal models by other research groups [9]. Further studies on the presence of TRPC6 in these tissues with a larger number of cases are needed to deepen and confirm our results.

Nevertheless, the functional role of TRPC6 in the lacrimal gland and its diseases needs to be further investigated. It would also be useful to study and compare healthy and pathological lacrimal gland tissue, similar to our work on the submandibular gland. There may be differences in expression between the two groups. Diseases of the lacrimal gland that could be of interest are inflammation of the gland (dacryoadenitis) and tumors of both benign and malignant origin, although the latter are extremely rare. In addition to this work, it would be interesting to detect TRPC6 in other exocrine glands, especially in salivary glands. Examples would be the parotid gland or the sublingual gland. Moreover, the expression of other TRPC channels in the tissues examined should be sought in order to expand our knowledge of TRPC channels in lacrimal and salivary glands. TRPC6 is used as a therapeutic target in many diseases, via inhibitory, modulating or agonistic drugs [13]. Therefore, it is likely that TRPC6 could also be used as a target in the therapy of ACC and MEC. TRPC6 shows broad expression in both tumor and healthy tissues. Potential side effects must be carefully considered when using TRPC6 as a target for cancer therapy. Since TRPC6 is also expressed in neurons, smooth muscle cells, cardiac muscle cells and kidney cells, there is a potential risk that inhibiting TRPC6 activity in these cells could disrupt their normal function. A thorough investigation of the potential side effects of TRPC6 inhibitors on healthy tissues and organs is therefore of great importance [20]. A future challenge in the development of TRPC6 inhibitors is to achieve a targeted effect on ACC and MEC tumor cells without causing unwanted side effects on healthy tissues. Furthermore, TRPC6 in humans, similar to TRPC3 in rodents, might play a role in $Ca2^{+}$ reabsorption from saliva in the salivary glands, and thus in the regulation of salivary flow and the formation of salivary stones. It could also serve as a target molecule for the treatment of sialolithiasis. The salivary glands can be affected by a variety of diseases in addition to tumors and salivary stones, such as inflammation (sialo adenitis), hypertrophy (sialon adenosis) or hyposalivation (xerostomia) as a result of previous diseases or medication. Therefore, the function, significance and role of the TRPC6 protein in the salivary glands, in particular in the submandibular gland, in healthy physiological submandibular tissue and in pathological submandibular tissue should be investigated in more detail. This may lead to new therapies using TRPC6 as a target molecule.

Author statement

We have included one more of the clinicians as author, Bernhard Schick, Head of ENT-clinic.

All authors have read and approved the manuscript.

CRediT authorship contribution statement

Mathias Wagner: Supervision, Resources. **Céline Carl:** Writing – original draft, Methodology. **Thomas Tschernig:** Supervision, Conceptualization. **Maximilian Linxweiler:** Writing – review & editing, Resources.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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