CLIC1 plasma concentration is associated with lymph node metastases in oral squamous cell carcinoma

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Conflict of interest
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Abstract

Background. Previous studies have shown that the chloride intracellular channel 1 (CLIC1) protein is overexpressed in oral squamous cell carcinoma (OSCC) and nasopharyngeal carcinoma. Patients with these diseases had significantly higher CLIC1 plasma levels than healthy controls.

Objectives. To determine the plasma concentration of CLIC1 in patients with OSCC and laryngeal squamous cell carcinoma (LSCC).

Materials and methods. We collected blood samples from patients diagnosed with OSCC (n = 13) and LSCC (n = 7), as well as from healthy controls (n = 8). The blood samples were centrifuged to obtain plasma and stored at −80°C. The CLIC1 plasma concentration was determined using enzyme-linked immunosorbent assay (ELISA).

Results. The mean CLIC1 plasma concentration was higher in the OSCC group than in the LSCC and control groups. Patients with OSCC and nodal metastases had significantly higher CLIC1 plasma concentration levels than nonmetastatic patients ($p < 0.0001$; Tukey's multiple comparisons test) and controls ($p = 0.0004$). The CLIC1 concentration correlated significantly with the presence of nodal spread ($p = 0.0003$; Spearman's $r = 0.8613$) and overall TNM staging ($p = 0.0167$; Spearman's $r = 0.6620$). No differences in CLIC1 plasma levels were observed between the LSCC and control groups. The CLIC1 plasma concentration was not associated with age, sex, tumor stage, or tumor grade.

Conclusions. There were no differences in CLIC1 plasma concentration between healthy controls and patients with LSCC. However, our findings suggest that the presence of this protein in plasma may be associated with lymphatic metastasis in patients with OSCC. More research is needed to confirm this possible association.

Key words: HNSCC, oral squamous cell carcinoma, OSCC, CLIC1, cancer plasma marker

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Background

Head and neck squamous cell carcinoma (HNSCC) is a common malignancy with a poor prognosis. It is estimated that HNSCC accounts for more than 750,000 cases and 340,000 deaths annually worldwide.[1] Currently, there are only 2 widely accepted prognostic biomarkers for HNSCC, namely human papillomavirus (HPV) infection/p16 expression and programmed death-ligand 1 (PD-L1) expression.[2] Further research on the biological factors associated with HNSCC progression, recurrence and metastases is an important aim of contemporary oncology.

Numerous studies have evaluated biomarkers for the early diagnosis of cancer and to actively monitor treatment response. Recently, ion channels are being investigated as biomarkers of various diseases, including cancer.[3] Ion channels are integral membrane proteins present on the plasma membrane and within intracellular membranes. In the human genome, there are more than 400 genes encoding ion channel proteins.[3]

The chloride intracellular channel 1 (CLIC1) protein, which is representative of the chloride ion channel family, is one such biomarker. The CLIC1 is present in cells in both membrane and soluble forms.[4,5] It is widely distributed throughout the body and can be found in various epithelial tissues in apical domains.[6] This protein is involved in mitogen-activated protein kinase (MAPK) signaling pathways as well as in carcinogenic processes.[7–9] The CLIC1 is also considered to be a sensor and effector during oxidative stress in microglial cells.[10] The role of CLIC1 has been determined in several cancers, such as gallbladder cancer, glioblastoma multiforme, gastric cancer, colon cancer, ovarian cancer, breast cancer, liver cancer, pancreatic cancer, and others.[3,7]

Recent findings have shown that CLIC1 is overexpressed in patients with oral squamous cell carcinoma (OSCC) and is associated with a poor prognosis.[7] Cell culture studies have shown that *CLIC1* promotes cell viability, proliferation, migration, and invasion, as well as in vitro cell-mediated angiogenesis, in OSCC cells.[8] Although CLIC1 tissue activity has been identified in several cancer types,[7] elevated plasma levels have only been confirmed in 2 cancer types, namely nasopharyngeal carcinoma and OSCC.[11]

A cell culture study reported that the *CLIC1* gene is upregulated in laryngeal squamous cell carcinoma (LSCC).[12] Another cell culture study found that *CLIC1* suppression results in an increased radiosensitivity of laryngeal cancer cells.[13] However, CLIC1 plasma levels in laryngeal cancer have yet to be determined.

Objectives

This prospective study was performed to measure the concentration of CLIC1 in plasma obtained from patients diagnosed with LSCC and OSCC in order to determine whether this protein could serve as a potential biomarker in patients with HNSCC.

Materials and methods

Patients

The study group consisted of 20 patients (14 males and 6 females), with a mean age of 62.7 ±7.95 years (range: 48–75 years). All patients were histologically diagnosed with either oral or laryngeal HNSCC (Table 1). Pathological tumor staging was performed according to the 8th edition of the TNM classification published by the Union for International Cancer Control (UICC).[14] Patients were prospectively recruited between November 2019 and August 2020 at the Department of Head and Neck Surgery at Poznan University of Medical Sciences and the Greater

Table 1. Study group characteristics

Characteristics	Total (n = 28)
Cancer patients (n = 20)	
Age	
M ±SD	62.7 ±7.95
Median	63
Range	48–75
Sex	
Male	14
Female	6
T-stage	
T1	3
T2	6
T3	7
T4	4
N-stage	
N0	11
N1	1
N2	7
N3	1
Anatomical site	
Larynx	7
Oral cavity	13
Healthy individuals (n = 8)	
Age	
M ±SD	65.8 ±3.93
Median	67
Range	60–72
Sex	
Male	5
Female	3

M ±SD – mean ± standard deviation; T – tumor; N – lymph node.

Poland Cancer Centre in Poznań, Poland. Blood samples from patients with primary tumors were collected prior to the surgical treatment. The control group consisted of 8 healthy age- and sex-matched volunteers.

We measured and compared CLIC1 plasma concentrations in 4 groups: LSCC, OSCC, HNSCC (all patients from the LSCC and OSCC groups combined), and healthy controls.

The study protocol was in compliance with the Declaration of Helsinki and approved by the Ethics Committee of Poznan University of Medical Sciences (decision No. 598/19). Written informed consent was obtained from the participating individuals.

Exclusion criteria

Patients with any of the following were excluded from the study: second primary tumor, local recurrence, previous treatment with chemotherapy or radiotherapy, and positive HPV status.

Sample preparation

Blood samples were collected preoperatively following a standardized protocol. Plasma samples were prepared by collecting blood in ethylenediaminetetraacetic acid (EDTA) tubes and centrifuging them at 2000 g for 20 min at 4°C. After centrifugation, the plasma samples were apportioned into 0.5 mL aliquots and stored at –80°C for further analysis.

Enzyme-linked immunosorbent assay

The CLIC1 protein levels in plasma were measured with the Human CLIC1 enzyme-linked immunosorbent assay (ELISA) Kit (cat. No. orb438684; Biorbyt, Cambridge, UK), according to the manufacturer's instructions. Each sample was evaluated 3 times in order to confirm the consistency of the test. Briefly, 100 µL of samples and standards were added to the wells of microtiter plates pre-coated with anti-CLIC1 antibody and were incubated for 2 h at 37°C. The samples were removed, and 100 µL of biotin-conjugated detection antibody was added. The plates were incubated for 1 h at 37°C and washed 3 times with 1× Wash Solution (Biorbyt). Next, 100 µL of avidin conjugated to horseradish peroxidase (HRP) was added to each microplate well and incubated for 1 h at 37°C. The plate wells were washed 5 times using 1× Wash Solution, and 90 µL of tetramethylbenzidine peroxidase substrate was added. After 25 min of incubation at 37°C, the reaction was terminated with adding sulfuric acid solution. An automated plate reader (Multiskan™ FC Microplate Photometer; Thermo Fisher Scientific, Waltham, USA) was used to measure the absorbance at 450 nm. The CLIC1 levels were determined using a standard curve.

Statistical analyses

The GraphPad Prism v. 8 software program (GraphPad Software, San Diego, USA) was used to perform the statistical analyses. The value of $p < 0.05$ was considered statistically significant. The Kolmogorov–Smirnov normality test was performed to check for distribution normality. The Student's t-test, Kruskal–Wallis test, Dunn's multiple comparisons test, and Tukey's multiple comparisons test were used to calculate the differences in CLIC1 plasma levels between the groups. The Spearman's rank correlation coefficient was used to calculate the correlation between CLIC1 plasma concentration and TNM staging.

Results

Tumor

We found no significant differences in CLIC1 concentration levels between the HNSCC patients and the controls ($p = 0.6178$; unpaired t-test), nor between the OSCC ($p = 0.7023$), LSCC ($p = 0.7295$) and control groups ($p = 0.9973$; Tukey's multiple comparisons test) (Fig. 1A,B). The tumor stage was not correlated with the CLIC1 concentration ($p = 0.9749$; Kruskal–Wallis test) (Fig. 1C).

Lymph node metastases

The CLIC1 plasma concentration was significantly higher in OSCC patients with nodal metastases than in non-metastatic patients ($p < 0.0001$) and controls ($p = 0.0004$; Tukey's multiple comparisons test) (Fig. 1D). The CLIC1 plasma concentration was significantly correlated with the presence of nodal metastases in the HNSCC group ($p = 0.0043$; Spearman's $r = 0.6098$) and the OSCC group ($p = 0.0003$; Spearman's $r = 0.8613$)(Fig. 1D, Fig. 2A–C).

TNM staging

The TNM stage correlated significantly with the CLIC1 plasma concentration in the OSCC group ($p = 0.0167$; Spearman's $r = 0.6620$). In the HNSCC group, there was a nonsignificant trend toward the correlation ($p = 0.0860$; Spearman's $r = 0.3936$).

Grading

Tumor grade was not correlated with CLIC1 concentration in the HNSCC, OSCC or LSCC groups ($p = 0.1356$, $p = 0.2923$ and $p = 0.7597$, respectively; Kruskal–Wallis test) (Fig. 2D–F).

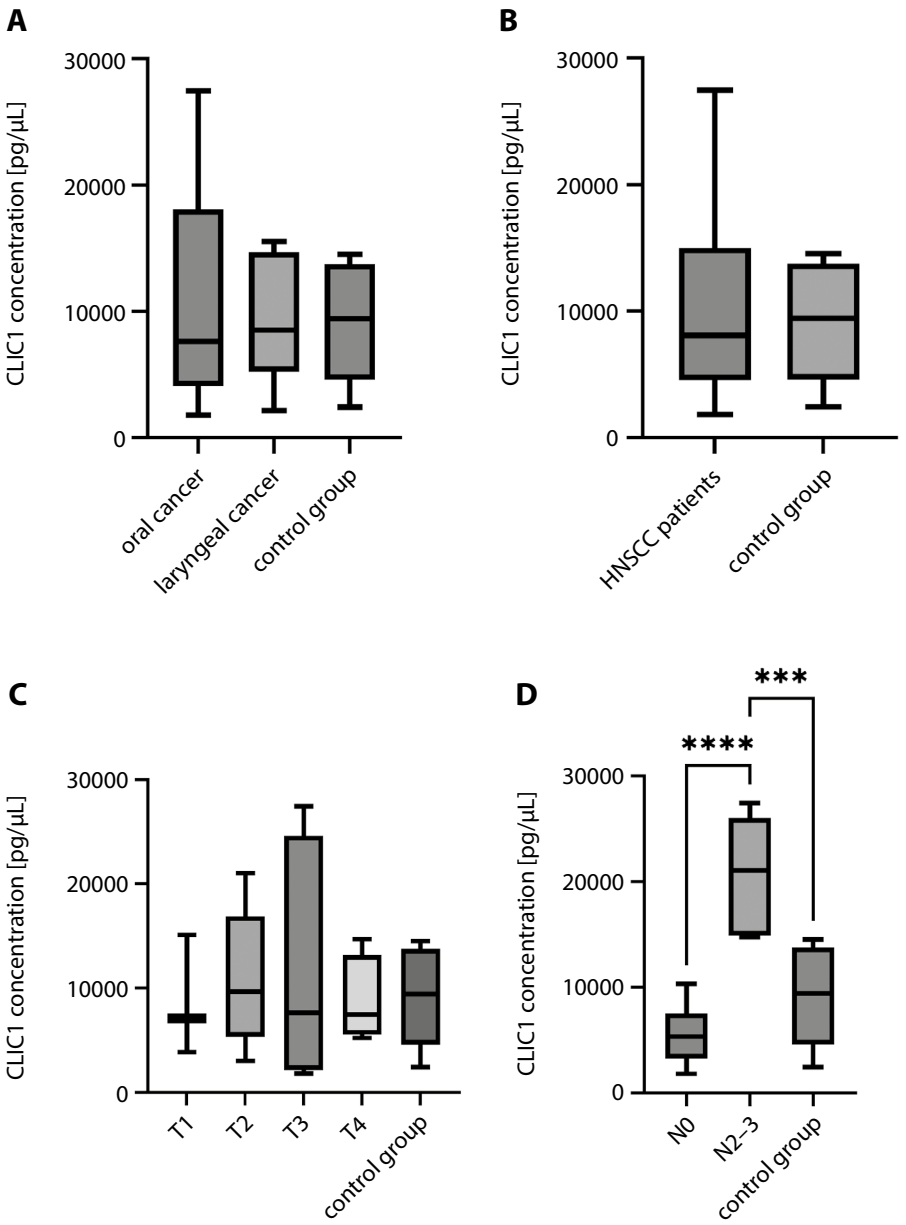

Fig. 1. A. CLIC1 plasma concentration in patients with oral cancer, laryngeal cancer and control group; B. CLIC1 plasma concentration in patients with head and neck squamous cell carcinoma (HNSCC) compared to controls; C. HNSCC patients compared to the control group); D. CLIC1 plasma concentration in patients with non-metastatic (N0) and metastatic (N2–N3) oral cancer compared to controls; *** p = 0.0004, **** p < 0.0001 (Tukey's multiple comparisons test)

Patient characteristics

The mean and median CLIC1 concentration levels were higher among women but the results were not statistically significant (p = 0.2761; unpaired t-test) (Fig. 2G,H). Age was not correlated with CLIC1 plasma concentration (p = 0.9349; Kruskal–Wallis test) (Fig. 2I).

Discussion

In this study, we measured CLIC1 plasma concentrations in patients with oral and laryngeal cancer and healthy controls. The mean CLIC1 plasma concentration was higher in the OSCC group than in the LSCC and control groups, but the results were not statistically significant. Patients with metastatic OSCC had significantly higher CLIC1 plasma concentrations than nonmetastatic patients (p < 0.0001). The CLIC1 concentration was significantly correlated with nodal metastases (p = 0.0003; Spearman's r = 0.8613) and overall TNM stage (p = 0.0167; Spearman's r = 0.6620). No differences in CLIC1 plasma levels were observed between the LSCC and control groups. The CLIC1 plasma concentration was not associated with age, sex, tumor stage, or tumor grade. These findings suggest that plasma CLIC1 concentration could be a useful biomarker in patients with OSCC but not in those with LSCC.

The CLIC family comprises 6 proteins (CLIC1–CLIC6).[3,15] Other members of the CLIC family have also been investigated as molecular targets in oncology.[16] Karsani et al. found an association between CLIC1 and the development and progression of OSCC.[17] Other studies have suggested that CLIC1 is involved in numerous cancers (nasopharyngeal, esophageal, stomach, liver, pancreatic, colorectal, lung, breast, gallbladder, prostate, ovarian, and brain cancers).[11,18–28] Two studies reported

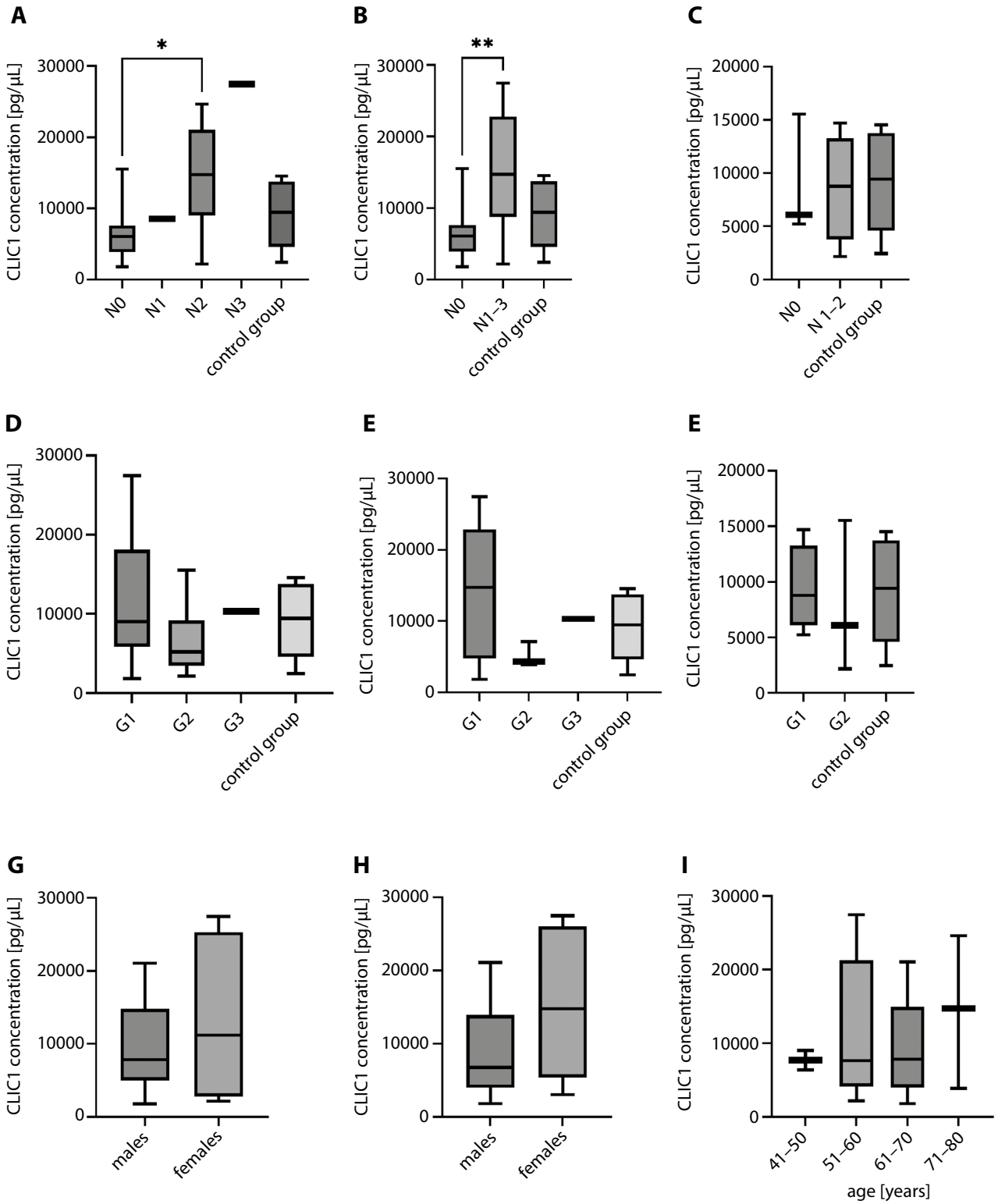

Fig. 2. A. CLIC1 plasma concentration by metastatic lymph node (N) stage in patients with HNSCC compared to controls; * p = 0.0463 (Dunn's multiple comparisons test); B. CLIC1 plasma concentration in patients with non-metastatic (N0) and metastatic (N1–N3) HNSCC compared to controls; ** p = 0.006 (Tukey's multiple comparisons test); C. CLIC1 plasma concentration in patients with non-metastatic (N0) and metastatic (N1–N2) laryngeal cancer compared to controls; D. CLIC1 plasma concentration by grading in HNSCC patients; E. CLIC1 plasma concentration by grading in oral cancer patients; F. CLIC1 plasma concentration by grading in laryngeal cancer patients; G. CLIC1 plasma concentration by gender in HNSCC patients; H. CLIC1 plasma concentration by gender in oral cancer patients; I. CLIC1 plasma concentration by age group

a possible association between *CLIC1* and laryngeal cancer.[12,13] In the present study, we investigated plasma CLIC1 concentration in patients with LSCC and found that the plasma CLIC1 concentration in these patients was similar to the healthy controls. These results suggest that further study of CLIC1 as a potential biomarker of LSCC

may not be beneficial. However, this biomarker may be useful in patients with OSCC, given the higher specificity of raised CLIC1 plasma levels in these patients. It is important to note that the plasma expression of this protein is also elevated in nasopharyngeal carcinoma, which was not investigated in this study.[11]

The lack of significant differences in CLIC1 plasma levels between the OSCC and control group may be due to the small sample size of the study (13 patients with OSCC and 8 healthy controls). However, the CLIC1 plasma concentration was significantly correlated with TNM staging, a finding that is in line with a previous report by Xu et al.[7] Nonetheless, this correlation was only significant for nodal staging (N), not tumor staging (T).

Xu et al. did not observe any correlation between the CLIC1 expression and the presence of metastatic lymph nodes in patients with OSCC.[7] By contrast, Feng et al. found that the upregulation of *CLIC1* was associated with the viability and proliferation of OSCC cells.[8] In that study, silencing of *CLIC1* inhibited these processes and promoted apoptosis. We found a strong correlation ($p = 0.0003$; Spearman's $r = 0.8613$) between CLIC1 plasma concentration and metastatic nodal staging, a finding that may have diagnostic and prognostic implications.

The role of *CLIC1* in metastatic lesions has been investigated in other cancer types. One study revealed that *CLIC1* knockdown inhibits gallbladder cancer metastasis by reducing migration and invasion of cells.[21] Other studies have demonstrated that the expression of *CLIC1* is correlated with metastatic spread in colon[9] and breast cancers,[28] as well as with nodal dissemination in gastric cancer.[19]

The CLIC1 plasma expression in patients with OSCC tends to change during the course of cancer treatment, which suggests that this protein could potentially play a valuable role in monitoring treatment response. In the study by Xu et al., CLIC1 concentration levels were lower in patients who underwent tumor resection than in those with ongoing disease.[7] Relevantly, tumor resection followed by adjuvant chemotherapy lowered plasma CLIC1 expression levels even further.[7] According to Feng et al., *CLIC1* knockdown increased the susceptibility of OSCC cells to cisplatin.[8]

Limitations

This study has several limitations, mainly the small patient population and the use of an ELISA test based on a single kit only, which could explain the lack of significant intergroup differences in some of the comparisons.

Conclusions

This study demonstrates that the CLIC1 plasma concentration is associated with metastatic nodal spread in patients with OSCC and, consequently, with overall TNM stage. These findings suggest that CLIC1 could be a feasible plasma biomarker to diagnose and monitor patients with oral cancer with nodal involvement. However, these findings need to be confirmed in larger studies.

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