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RESEARCH

The Prognostic Role of Claudins in Head and Neck Squamous Cell Carcinomas

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Abstract The expression of tight junction proteins is frequently altered in epithelial cancers. The loss of cell-cell adhesion associates with enhanced metastatic potential, which underlies the role of altered expression pattern of tight junction component claudins (CLDNs). Our study assessed the expression of CLDN 1, 2, 3, 4, 7, 8 and 10 in squamous cell carcinoma of the head and neck region (HNSCC) including oropahrynx, larynx, and hypopharynx in comparison to normal epithelial tissue of the same patient. The surgical samples were examined by tissue microarray and immunohistochemistry, the expression was calculated by H-score, which took account of intensity and percentage of positivity as well. Both normal and cancerous tissue proved negative for CLDN 3, 8 and 10. Normal epithelia showed mild expression of CLDN 4, but the minimal positivity disappeared in squamous cancer. In case of CLDN 1 and CLDN 7 we demonstrated significantly increased intensity in cancer, while CLDN 2 showed decreased expression compared with normal epithelium. The normal polarity and distribution of claudins were lost in HNSCC. Moreover, preserved expression of CLDN 2 (but not that of 1 and 7) was associated with better survival, which suggested a potential prognostic role of CLDN 2.

Keywords Claudin · Head and neck squamous cell carcinoma · Prognosis · Immunohistochemistry · Tight junction

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent tumor type with approximately 650,000 new cases each year and 350,000 deaths per year worldwide [1]. In Hungary HNSCC is the third most frequent malignant tumor type among males, and even though the incidence is usually much higher in southern Europe than in Central and Northern Europe, Hungary is ranked first in the world with respect to its incidence and mortality as well [2, 3]. Despite of improved tumor detection and advanced treatment modalities, 5-year overall survival did not change significantly in the past few years, usually more than 50% of HNSCC patients die [4, 5]. One of the most important factors of therapeutic decisions is clinical stage: early-stage (I–II) tumors are treated with surgery or irradiation as monotherapy, while the treatment of advanced tumors combines surgical tools and chemo-radiotherapy [6]. Based on the molecular characteristics of HNSCC, new treatment modalities were introduced, for instance anti-EGFR therapies [5]. Since the predictive value of traditional markers such as anatomical location (oral cavity, pharynx or larynx), extension, depth of invasion, grade, exophytic or endophytic spreading proved very limited, new parameters have been adopted, e.g. apoptosis index, Ki67 proliferation index, and expression of p53 [7–10]. Another determining factor is the etiology of cancer on its own: most of these malignancies link to extensive tobacco and alcohol consumption, however, HPV-positive squamous cancers showed better clinical outcome [8]. Nonetheless, the efficacy of these prognostic factors is limited, therefore, new markers are urgently needed.

Claudin proteins (CLDN) proved to be promising targets for diagnostics and therapeutics in many types of human epithelial malignancies [11]. The 28 members of human claudin family are closely related transmembrane proteins that are key components of tight junctions [11, 12]. Breast cancer was the first example in routine diagnostics where altered claudin expression was used as a prognostic marker: the claudin-low subgroup of triple negative tumors showed poor response to systemic therapy [13]. Furthermore, bioinformatics screening tools and immunohistochemical studies revealed the predictive role of CLDN 2, 3, 4 and 7 on overall survival of breast cancer patients [14]. CLDN 3 and 4 are frequently overexpressed in ovarian, pancreatic, prostate and urothelial cancers, and this overexpression is associated with poor prognosis [15–22]. Significantly increased expression of CLDN 1, 2, 4, and 7 was detected in the *in situ* and invasive lesions of the cervix [23]. Claudin molecules could serve not only as prognostic factors, but could also anchor rationally designed monoclonal antibodies, thereby opening new horizon for molecular targeted approaches, however, this drugs are not approved yet, only a few preclinical results are available [11, 24, 25].

Based on these data, question arose about the prognostic and therapeutic significance of claudins in HNSCC as well. Few studies have been previously focused on claudins in this region: when compared with normal epithelia, CLDN 1 was overexpressed in esophageal squamous cell carcinoma [26], and according to a small *in silico* study carried out on oral squamous cell carcinoma, CLDN 7 was down-regulated [27]. Another previous examination showed the alteration of CLDN 1 in various squamous cancers from different regions, including head-neck, skin, and genitourinary regions, and found that high CLDN 1 expression was prevalent in cancer in comparison to normal tissue [28]. The aim of the present study was to examine the expression pattern of claudins in oropharyngeal, hypopharyngeal and laryngeal cancers, to analyze its relation with clinicopathologic parameters and to identify its potential prognostic role.

Here we demonstrate that in comparison to normal epithelia, squamous cancers showed increased expression of CLDN 1 and 7, while CLDN 2 was significantly weaker. This distribution proved to be independent of primary location (oropharynx, hypopharynx or larynx). Moreover, high expression of CLDN 2 was associated with markedly better clinical outcome.

Materials and Methods

Patients

As we detailed in our previous work [29], we studied surgical specimens of 71 HNSCC patients who had been treated at Semmelweis University, Department of Otorhinolaryngology and Head and Neck Surgery, in the period of 2001–2006. Primary tumor samples were collected from the following regions: 20 from the hypopharynx, 16 from the oropharynx, 35 from the larynx (20 from the glottis and 15 from the supraglottic region). We also studied preserved mucosal regions in the surgical specimens. All investigations were performed under the permission of the Regional Ethical Committee of Semmelweis University.

Tissue Microarray

For the tissue microarray (TMA), hematoxylin and eosin-stained sections were used to define the tumor areas and preserved epithelial tissue from the same patient. One normal and one neoplastic representative 0.6 mm cores were obtained from each case and inserted in a grid pattern into a recipient paraffin block using a tissue arrayer (3D Histech, Budapest, Hungary). Sections (2 μm) were then cut from each TMA block and stained with antibodies.

Immunohistochemistry

Sections were deparaffinized in xylene for 2×20 min, then in ethanol for 2×15 min Endogenous peroxidase activity was blocked with methanol and hydrogen peroxide for 20 min, and continued with 3×5 min washing with distilled water. Microwave antigen retrieval (MFX-800-3 automatic microwave device, 750 W, Meditest, Budapest, Hungary) was performed at 95°C for 10+5 min in citrate buffer (0.05 mM, pH 6, DakoCytomation, Glostrup, Denmark). Sections were blocked for 20 min in 3% bovine serum albumin (Sigma–Aldrich, St. Louis, MO) at room temperature, and then washed for 5 min in TRIS buffer.

The CLDN-specific antibodies were as follows: mouse monoclonal anti-CLDN 2 and anti-CLDN 4 antibodies (Invitrogen, Camarillo, CA), rabbit polyclonal anti-CLDN 1 (Cell Marque San Francisco Rocklin, CA), rabbit polyclonal anti-CLDN 3, 7, 8 and 10 antibodies (Zymed, San Francisco, CA). Primary antibodies had been applied at a 1:80 dilution at room temperature for 1 h. For visualization, a standard avidin–biotin peroxidase technique (ABC system, DakoCytomation) was used with diaminobenzidine as chromogen. The sections were counterstained with hematoxylin. For each CLDN, a positive and negative control (with omission of the primary antibody) was included. The reactions were carried out in a Ventana ES automatic immunostainer (Ventana Medical Systems Inc., Tucson, AZ, USA) using the reagents provided by the manufacturer.

Semiquantitative Evaluation of Immunohistochemistry

Two independent examiners (BK, IK) evaluated the reactions to register staining intensity, tissue localization and

percentage of positive cells. According to the scoring system set up previously by our working group, intensity scores were 0 for negative, 1 for weak, 2 for moderate, and 3 for strong immunohistochemical reaction [30]. H-score was derived by summing percentages of cell staining at each intensity, multiplied by the weighted intensity of staining [31]. Determining between low and high level of CLDNs, medians were applied as thresholds.

Statistical Analysis

Categorical data were compared using Chi-square test. Asymmetrical numeric data (normal vs. cancer) were analyzed by matched Wilcoxon-test or Kruskal-Wallis test with *post hoc* analysis. Correlations were determined by Spearman rank order test. Overall survival analyses were done using the Kaplan-Meier method. Overall survival intervals were determined as the time period from initial diagnosis to the time of death. The comparison between survival functions for different strata was assessed with the log-rank statistics. Multivariate analysis of prognostic factors was done using Cox's regression model. Statistical significance was confirmed when P values were <0.05. Statistical analysis was performed using Statistica 9.0 software (StatSoft, Tulsa, OK).

Results

Expression of the CLDNs in Normal Squamous Epithelia in the Head and Neck Region

In the normal epithelia of the head and neck region the distribution of CLDNs showed individual variability, however, certain general features appeared. In the basal two third of the epithelia the majority of the samples expressed CLDN 1 (Fig. 1a), which showed moderate cell membrane positivity, while the reaction was absent from the apical layers (median: 10; minimum: 0; maximum: 200; lower quartile: 0; upper quartile: 100). CLDN 2 showed strong cytoplasmic positivity in all layers (Fig. 1c), but the reaction was explicit at the stratum germinativum and stratum spinosum and moderate in the stratum planocellulare (median: 120; minimum: 0; maximum: 300; lower quartile: 90; upper quartile: 200). In

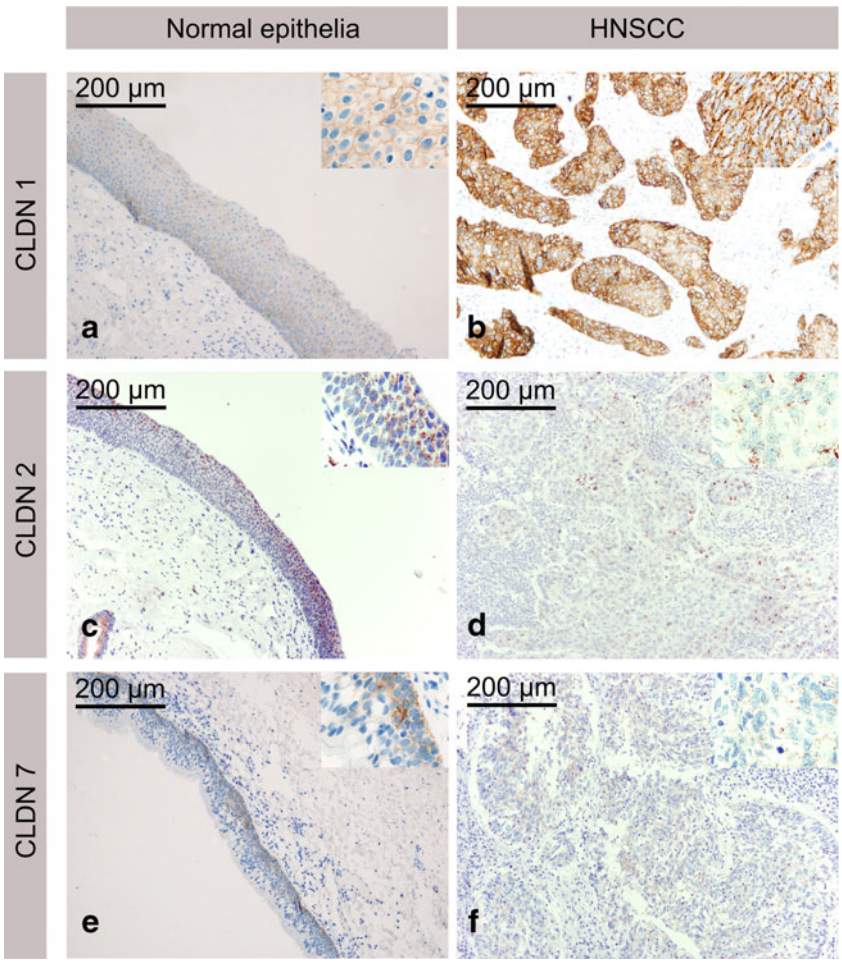

Fig. 1 Expression of CLDN 1, 2 and 7 in normal epithelia and head and neck squamous cell cancers. In normal epithelia the basal two third expressed CLDN 1, which showed moderate cell membrane positivity (**a**). In cancerous tissue CLDN 1 showed diffuse strong membrane positivity (**b**). In normal epithelia CLDN 2 showed strong cytoplasmic positivity in all layers (**c**). The anti-CLDN 2 reaction was significantly weaker in cancer (**d**). The expression of CLDN 7 showed the same pattern as CLDN 1 in normal epithelia (**e**) and in cancer (**f**) as well. In the right upper corners inserts are represented three-fold magnification of the large images

the case of CLDN 4, we found a pattern similar to that of CLDN 1, however, only 21 normal samples expressed the molecule, and the reaction was very weak (median: 0; minimum: 0; maximum: 180; lower quartile: 0; upper quartile: 15). In the studied slides, 28 samples expressed CLDN 7 (Fig. 1e), the intensity was weak, but the membrane positivity was more characteristic for the apical third of the epithelia (median: 0; minimum: 0; maximum: 100; lower quartile: 0; upper quartile: 20). The distribution between three different tumor sites (oropharynx, larynx and hypopharynx) did not differ significantly (all Ps>0.05, Kruskal-Wallis test). In all histology samples the normal epithelia of the head and neck region proved negative for CLDN 3, CLDN 8 and CLDN 10.

Expression of the CLDNs in Squamous Cancer of the Head and Neck Region

CLDN 1 was expressed in 90.1 percent of the studied HNSCCs (Fig. 1b). In comparison to the normal epithelia, CLDN 1 showed diffuse strong membrane positivity, which was significantly more intense (median: 170; minimum: 0; maximum: 300; lower quartile: 60; upper quartile: 300; $P<0.001$; matched Wilcoxon-test; Fig. 2a). Contrarily, the reaction against CLDN 2 was significantly weaker in cancer (median: 30; minimum: 0; maximum: 300; lower quartile: 6; upper quartile: 90) than in normal epithelia ($P<0.001$; matched Wilcoxon-test; Fig. 1d; Fig. 2b). In the studied HNSCCs CLDN 2 was expressed in 78.9 %. Expression of CLDN 7 showed the same pattern as that of CLDN 1, the H-scores showed modest correlation to each other ($P=0.024$, Chi-square test; $R=0.425$, $P<0.05$, Spearman rank order test), and compared to normal tissue, the intensity was increased in the neoplastic samples (median: 25; minimum: 0; maximum: 300; lower quartile: 0; upper quartile: 90; $P<0.001$; matched Wilcoxon-test; Fig. 1f; Fig. 2c), however, the reaction was weaker than CLDN 1, and only 64.8 percent of tumors was positive. If we stratified according to the tumor sites (oropharynx, larynx and hypopharynx) the differences of the expression between normal and squamous cell carcinoma were remained for all three CLDNs ($P<0.05$, matched Wilcoxon-test, data not shown). All studied neoplastic tissues of the head and neck region proved to be negative for CLDN 3, CLDN 4, CLDN 8 and CLDN 10.

Correlation between CLDNs and Clinicopathologic Parameters

A more detailed statistical analysis was performed on those CLDNs that are expressed in HNSCCs. As detailed in Table 1, the expression of CLDN 1, CLDN 2 and CLDN 7 was independent of most of the known clinicopathological features, e.g. age, gender, HPV-status, alcohol history, stage and grade. The expression of CLDN 1 in oropharyngeal cancers was significantly higher than in the case of the other two sites. The distribution of CLDN 2 and CLDN 7 proved to be independent of the location of the primary tumor (Kruskal-Wallis test, data not shown).

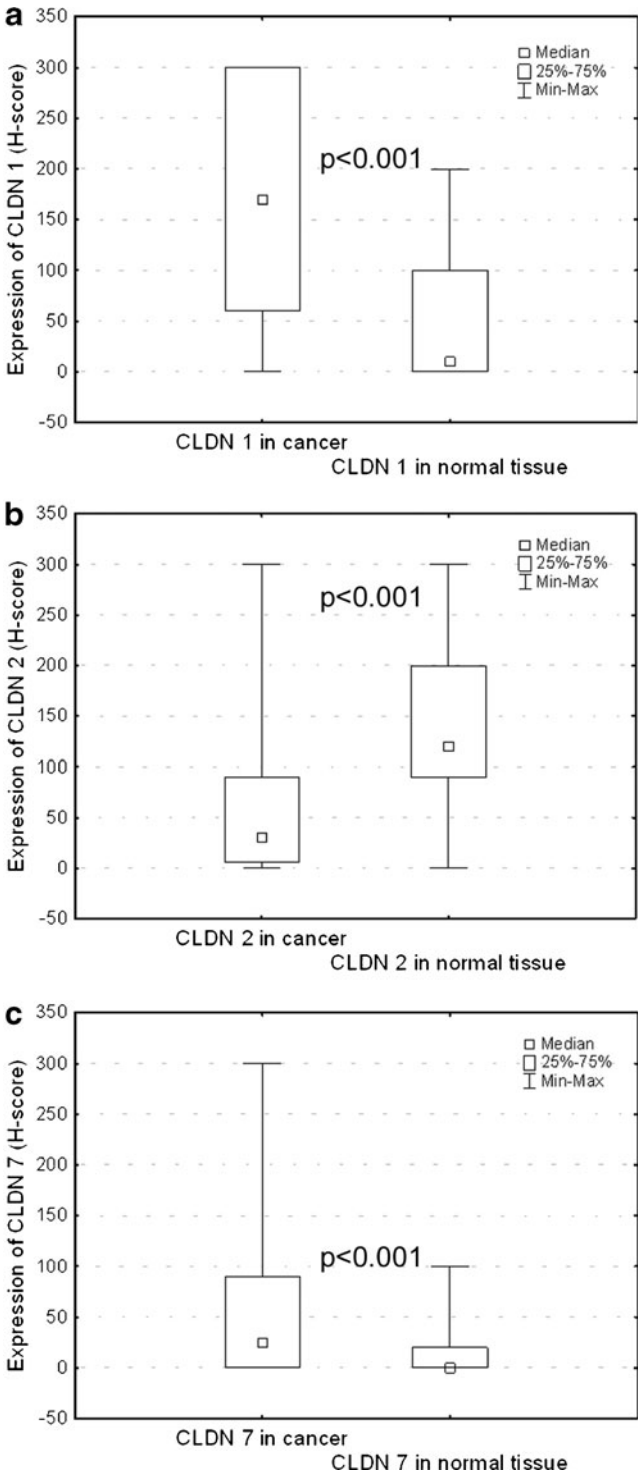

Fig. 2 Expression of CLDNs in head and neck squamous cell cancer compared to normal tissue. Expression of CLDN 1 (**a**) and 7 (**c**) was significantly elevated in cancer, while the level of CLDN 2 (**b**) was decreased (Wilcoxon's matched test)

Table 1 Correlation of clinicopathologic features and the expression of claudins in patients with HNSCC

	No. of patients (%)	CLDN 1 low[a] (%)	CLDN 1 high[a] (%)	*P value*	CLDN 2 low[a] (%)	CLDN 2 high[a] (%)	*P value*	CLDN 7 low[a] (%)	CLDN 7 high[a] (%)	*P value*
All patients	71 (100%)	36 (50.7%)	35 (49.3%)		33 (56.8%)	38 (42.2%)		36 (50.7%)	35 (49.3%)	
Age (years)[a]										
<54	34 (47.9%)	20 (55.6%)	14 (40%)		16 (48.5%)	18 (47.4%)		21 (58.3%)	13 (37.1%)	
≥54	37 (52.1%)	16 (44.4%)	21 (60%)	0.19 [b]	17 (51.5%)	20 (52.6%)	0.925 [b]	15 (41.7%)	22 (62.9%)	0.074 [b]
Gender										
Male	58 (81.7%)	29 (80.6%)	29 (80.6%		27 (81.2%)	31 (81.6%)		31 (86.1%)	27 (77.1%)	
Female	13 (18.3%	7 (19.4%)	6 (19.4%)	0.802 [b]	6 (18.8%)	7 (18.4%)	0.979 [b]	5 (13.9%)	8 (22.9%)	0.329 [b]
Tumor site										
Oropharynx	16 (22.5%)	2 (5.6%)	14 (40%)		4 (12.1%)	12 (31.6%)		6 (16.7%)	10 (28.6%)	
Larynx	35 (49.3%)	21 (58.3%)	14 (40%)		18 (54.5%)	17 (44.7%)		19 (52.8%)	16 (45.7%)	
Hypopharynx	20 (28.2%)	13 (36.1%)	7 (20%)	0.002 [b]	11 (33.3%)	9 (23.7%)	0.143 [b]	11 (30.6%)	9 (25.7%)	0.486 [b]
HPV										
Neg	57 (80.3%)	30 (83.3%	27 (77.1%)		27 (81.2%)	30 (79%)		29 (80.6%)	28 (80%)	
Pos	14 (19.7%)	6 (16.7%)	8 (22.9%)	0.512[b]	6 (18.8%)	8 (21%)	0.762[b]	7 (19.4%)	7 (20%)	0.953[b]
Alcohol consumption										
Never	19 (26.8%)	11 (30.6%)	8 (22.9%)		9 (27.3%)	10 (26.3%)		11 (30.6%)	8 (22.9%)	
Moderate	23 (32.4%)	10 (27.8%)	13 (37.1)		14 (42.4%)	15 (39.5%)		12 (33.3%)	11 (31.4%)	
Strong or ex-strong	29 (40.8%)	15 41.7%	14 (40%)	0.642[b]	10 (30.3%)	13 (34.2%)	0.939[b]	13 (36.1%	16 (45.7%)	0.666[b]
Clinical stage										
I–II	41 (57.7%)	19 (52.8%)	22 (62.9%)		18 (54.5%)	23 (60.5%)		17 (47.2%)	24 (68.6%)	
III–IV	30 (42.3%)	17 (47.2%)	13 (37.1%)	0.39[b]	15 (45.5%)	15 (39.5%)	0.611[b]	19 (52.8%)	11 (31.4%)	0.069[b]
Grade										
1	19 (26.8%)	13 (36.1%)	6 (17.1%)		8 (24.2%)	11 (29%)		10 (27.8%)	9 (25.7%)	
2	42 (59.2%	19 (52.8%)	23 (65.8%)		18 (54.5%)	24 (63.2%)		18 (50%)	24 (68.6%)	
3	10 (14.9%)	4 (11.1%)	6 (17.1%)	0.188[b]	7 (21.2%)	3 (7.9%)	0.274[b]	8 (22.2%)	2 (5.7%)	0.106[b]

[a] Using median as cut-off value; [b] Chi-square test

Data shown in parentheses are column percentages.

The Prognostic Role of CLDNs in Squamous Cancer of the Head and Neck Regions

Next we used Kaplan-Meier analysis to calculate the overall survival rates for patients with low and high expression of CLDNs with median values serving as thresholds (Fig. 3). We found that the expression of CLDN 1 and CLDN 7 did not affect overall survival, however, lower expression of CLDN 2 was associated with poorer outcome (P=0.02). Multivariate Cox regression analysis (including standard prognostic variables, such as age, gender, stage, histological grade, alcohol consumption, HPV-positivity) was performed to identify independent prognostic factors. The results showed that high level of CLDN 2 significantly decreased relative risk (P=0.04). Contrarily, CLDN 7 did not prove to be a determining factor (Table 2). Clinical stage was also found an independent prognostic factor, higher stages were related to poor survival, which is in accordance with previous studies [4]. Since Spearman rank order test showed modest, but significant correlation between CLDN 1 and CLDN 7 (R=0.425, P<0.05), Cox regression model could include only either of those.

Discussion

In case of HNSCCs the prognostic role of traditional markers is questionable, therefore, new markers are needed. Based on the lesson taught by other human tumors, components of the intercellular adhesion could be promising candidates. Tumor cells frequently show decreased differentiation and cell polarity, which accompany abnormal composition and function of tight junction proteins, such as claudins. Since, compared to the normal epithelia, altered expression was revealed in neoplastic tissue, claudins could open new horizon in the diagnostics as well as in therapeutics. Previous studies revealed the prognostic role of CLDNs in breast, ovarian, pancreatic, prostate, cervical and urothelial cancers [14–23,

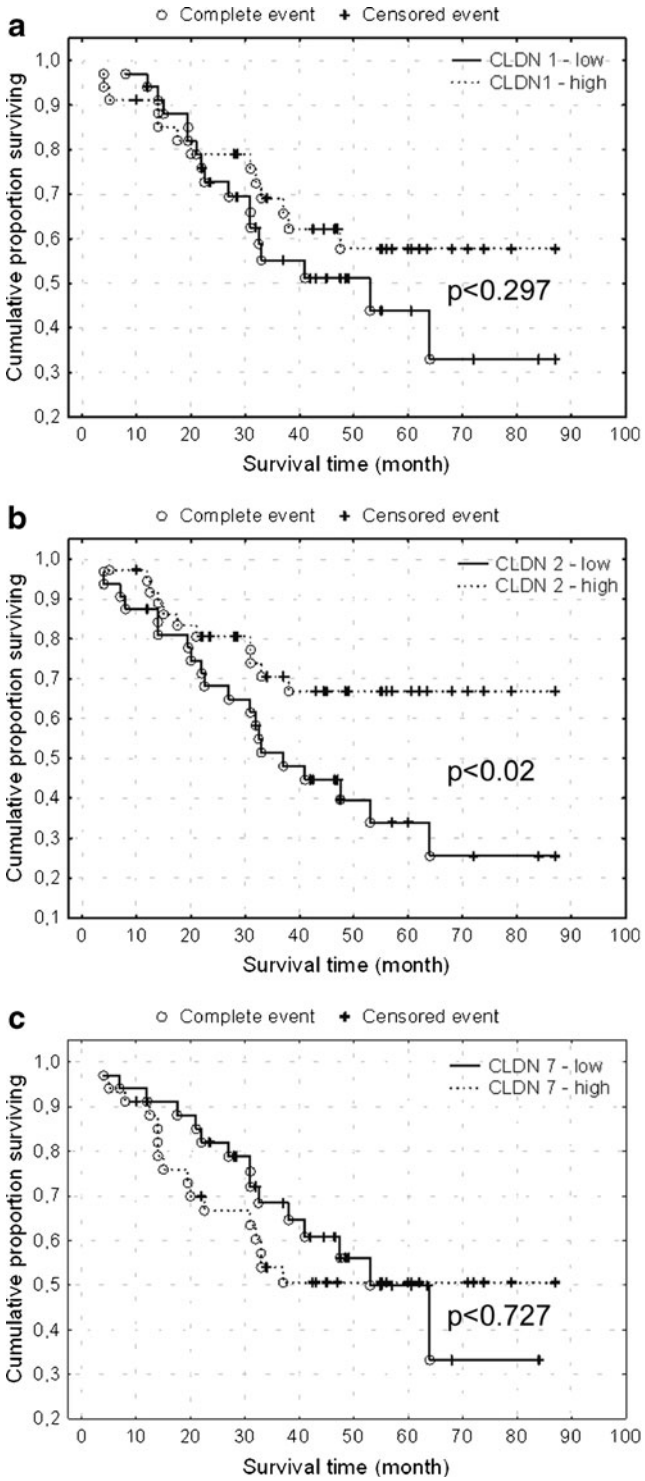

Fig. 3 Survival analysis of CLDN 1, 2 and 7 in head and neck squamous cancer. Significant survival difference was not found in the case of CLDN 1 (**a**) and 7 (**c**), while higher expression of CLDN 2 (**b**) was associated with better outcome

30], however, only few data are available in HNSCC. Our immunohistochemical examination systematically mapped the expression of claudin molecules in surgical samples of the head and neck region. We found thet the strength and distribution of claudin expression differed between normal epithelia and squamous cell carcinoma. We focused on CLDN 1, 2, 3, 4, 7, 8 and 10 expression, however, CLDN 3, 8, and 10 proved negative in the investigated squamous cell malignancies. In the normal epithelia CLDN 4 showed mild expression, while it completely disappeared in squamous cell cancer. Even though in other regions CLDN 4 is the most promising candidate as a target-based drug of human cancer [25], based on our study this approach does not seem to be effective in the case of HNSCC.

Table 2 Multivariate analysis of various independent prognostic factors in patients with HNSCC

Prognostic factor	RR	95% CI	*P*
Age in years (<54 vs. ≥54)	0.552	(0.189-1.619)	0.279
Gender (female vs. male)	1.299	(0.623-2.709)	0.485
Clinical stage (I-II vs. III-IV)	4.427	(2.004-9.781)	0.0002
Grade (1 vs. 2 vs. 3)	1.111	(0.693-1.78)	0.662
Alcohol consumption (never, moderate, strong or ex-strong)	1.03	(0.653-1.625)	0.897
HPV (negative vs. positive)	1.537	(0.613-3.849)	0.359
CLDN 2 (low vs. high)[a]	0.461	(0.22-0.967)	0.04
CLDN 7 (low vs. high)[a]	1.312	(0.619-2.814)	0.472

[a] Using median as cut-off value; RR: relative risk; CI: confidence interval

Protein expression of CLDN 1, 2 and 7 showed significant difference between normal and cancerous tissue. In the case of these three CLDNs, expression in both normal and neoplastic tissue was independent of the primary site, except for CLDN 1, which showed higher level of expression in the squamous cancers of oropharynx, compared to cancers of larynx and hypopharynx. Similarly to the esophageal, cervical and skin squamous malignancies [23, 26, 28, 32, 33], cancer cells showed higher expression of CLDN 1 than that of the normal epithelia, while in those cancers where the initiation of malignancy is the glandular component (breast, colon), the disappearance of CLDN 1 is more characteristic [34–36]. Of note, in our study, Spearman rank order test showed modest, but significant association between the expression of CLDN 1 and CLDN 7 (R=0.425, P<0.05). Ouban et al. found inverse correlation between CLDN 1 expression and tumor grade in squamous cell tumors of the genitourinary and gynecologic regions, but not that of the head and neck region which was further supported by our study [28].

Opposite to a previous finding, which showed the down-regulated of CLDN 7 [27], in HNSCC we measured higher expression of CLDN 7, however, the *in silico* examinations of Al Moustafa et al. were done on 12 tumor samples (all from the oral cavity). When compared our work to that of Yoshizawa et al., it appears that oral squamous cancer showed different

pattern of CLDN 7 expression than laryngeal, oropharyngeal and hypopharyngeal tumors [37]. Our examinations are rather in concordance with the complex analysis by Hewitt et al. which found elevated expression of CLDN 7 in pancreas, bladder, thyroid, fallopian tubes, ovary, stomach, colon, breast, uterus, and prostate cancer in comparison to normal tissues [38].

Our most interesting finding was CLDN 2 that unlike CLDN 1 or 7, had a significant effect on overall survival. Our group was the first to identify the prognostic role of CLDN 2 in any human cancer. We proved that CLDN 2 is more characteristic of normal tissue, and conservation of its expression (resembling that of normal structures) is associated with more favorable outcome.

Summarizing, the expression of CLDN 1, 2 and 7 show differences between normal and cancerous squamous epithelium in the head and neck region. Squamous cancers showed increased expression of CLDN 1 and 7, while the expression of CLDN 2 proved to be significantly weaker. Noteworthy, only CLDN 2 was found associated with the prognosis. These differences were mostly independent of the exact location of the tumors. Based on our results, CLDN 2 is a promising new diagnostic candidate for predicting the outcome of the disease in HNSCC patients, and CLDN 1 could be a good target or might serve as an anchor for rational therapy.

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