Membrane connexin 43 acts as an independent prognostic marker in oral squamous cell carcinoma

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Abstract. The aim of the present study was to evaluate the expression and localization of connexin (Cx) 26, -43 and -45 in a group of 35 patients with primary oral squamous cell carcinoma (OSCC) with the objective of making a more accurate disease prognosis. We analysed the expression of connexins in tissue samples of primary OSCC, matching oral mucosa free of dysplasia, and its associated lymph node metastases (LNM) by semi-quantitative immunohistochemistry of membrane, cytoplasmic and nuclear connexin expression. The levels of expression were correlated with the overall survival time (OS). Cx43 was overexpressed in tumour cells compared to epithelia in dysplasia-free mucosa. High membrane expression of Cx43 on tumour cells was the only statistically significant and independent prognostic factor of short OS (P=0.0088). Membrane expression of Cx43 in matching dysplasia-free mucosa acted similarly, but did not reach statistical significance (P=0.059). No correlation was found between the Cx26, Cx45 expression and OS. We conclude that Cx43 expression in dysplasia-free mucosa may indicate a very early stage of tumour promotion. Although overexpression of Cx43 is found in invasive tumours we only found membrane Cx43 expression to correlate with OS. This observation suggests that cytoplasmic Cx43 serves as storage and only membrane translocation may promote the formation of gap junctions and gap junctional intercellular communication (GJIC) with prognostic relevance.

Introduction

Each year approximately 650,000 new cases of head and neck carcinomas are registered and approximately 350,000 deaths

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are documented. These tumours of the sinuses, oral cavity, the pharynx and larynx (1) are almost exclusively squamous cell carcinomas (SCC). Alcohol and tobacco abuse are the most important risk factors (2). The 5-year survival rate, considering all tumour stages, is approximately 60%. Due to a high incidence of metastases hypopharyngeal carcinomas have a significantly poorer prognosis (3). To better predict the prognosis of cancer patients and thereby adjust the therapeutic concept, several molecular prognostic factors are discussed in the literature (4). Connexins are components of gap junction (GJ) hemi channels (connexons), which are formed by homomeric or heteromeric connexin hexamers in the plasma membrane. Two connexons of adjacent cells can form a GJ-channel and allow gap junctional intercellular communication (GJIC) by promoting the passage of small molecules like ions or second messengers (5). GJIC plays a central role in maintaining tissue homoeostasis, cell growth control and development (5). The association between GJIC and an increased contact inhibition was first described in cultured hepatoma cells (6). GJIC has a different function depending on the stage of tumour progression. Connexins possibly act on cell growth by controlling the expression of cell cycle regulatory genes such as cyclin A, D1, D2, and cyclin-dependent kinases (CDKs) through a variety of mechanisms (7). Cx43 transfected cells suppress degradation of p27, which inhibits the enzymatic activity of CDK. The hypophosphorylated retinoblastoma gene product (Rb) thereby accumulates in the cell, which characterizes the G1 phase (7). The absence of GJIC can lead to an accumulation of growth factors in the cell (6) and the suppression of contact inhibition, resulting in cell proliferation (8). King *et al* described a correlation between the endogenous Cx43 expression and an increased growth control, showing that the growth capacity was decreased in HeLa cells of cervical cancer (9). Cx43 knockout in mice leads to astrocytes with altered expression of genes associated with apoptosis, cell growth and transcription factors (10,11) and an increased susceptibility of mice to pulmonary neoplasia (12). Similar results have been published for Cx26 and Cx43 expression in mammary MDA-MB-231 cells (13). Cx26 is responsible for contact growth inhibition in HeLa and HepG2 cells (14,15). Because Cx45 can form altered heteromeric gap junctions in cooperation with Cx43, Cx45 overexpres-

Table I. The patient data.

N	Gender	Age (years)	Death	Survival (years)	pT	AJCC stage	Localisation
1	Woman	56	No	5.85	2	2	Right
2	Woman	62	No	2.32	Tis	0	Median
3	Men	77	No	4.98	2	2	Left
4	Men	60	No	4.64	2	2	Right
5	Men	61	Yes	1.01	4a	4	Median
6	Men	53	No	6.12	2	2	Right
7	Men	50	No	4.79	4	4	Right
8	Men	49	No	4.22	4	4	Left
9	Woman	79	No	4.20	4	4	Left
10	Men	67	No	5.72	4	4	Right
11	Men	39	Yes	0.10	4	4	Median
12	Woman	58	Yes	1.44	4	4	Median
13	Woman	62	Yes	1.62	4	4	Right
14	Men	31	No	5.73	2	2	Left
15	Men	45	Yes	0.97	4	4	Left
16	Woman	61	Yes	0.06	4a	4	Left
17	Woman	45	Yes	0.70	4a	4	Median
18	Men	47	Yes	1.75	4a	4	Median
19	Woman	66	Yes	0.64	4a	4	Left
20	Woman	64	Yes	1.93	4a	4	Right
21	Men	40	No	7.02	2	3	Right
22	Men	49	No	14.68	2	3	Right
23	Woman	64	No	8.94	2	4	Left
24	Men	71	Yes	1.56	4a	4	Left
25	Men	49	Yes	2.13	4a	4	Right
26	Men	70	Yes	3.56	1	1	Median
27	Men	46	Yes	4.94	1	1	Median
28	Men	52	Yes	1.19	3	3	Right
29	Woman	69	No	4.12	1	1	Left
30	Men	61	No	4.43	3	3	Right
31	Men	57	Yes	5.15	4a	4	Right
32	Men	65	Yes	2.38	4a	4	Median
33	Men	62	Yes	1.51	2	2	Right
34	Men	43	No	5.58	1	1	Right
35	Woman	49	No	6.56	1	1	Left

sion may affect intercellular contacts during carcinogenesis (16-18). Some connexin subtypes are also able to increase the attachment of tumour cells to the stroma during invasion and migration (7). Therefore, they can likely promote invasion by increasing communication with the endothelial barrier (19). Cx26 was detected in melanoma cells, surrounding small vessel endothelia (20), in squamous cell lung carcinoma (SCLC) and its associated lymph node metastases (21). Cx26 and Cx43 negative primary breast cancers developed Cx26 and Cx43 positive lymph node metastases (22). Cx43 enabled glioma cells to interact and establish functional GJ with astrocytes in the adult brain and facilitated direct paren-chymal invasion (23). Data on connexin expression in OSCC are rare and conflicting observations were reported by Ozawa *et al* and Villaret *et al*, who described Cx26 overexpression in tissue samples of OSCC and lymph node metastases (24,25).

In the present study we analysed in detail the expression and subcellular localization of Cx26, Cx43 and Cx45 in tumour epithelia of OSCC, matching oral mucosa free of dysplasia, and lymph node metastases using a subtle immunoreactive score. We found that the expression of Cx26 and Cx45 had no prognostic value. Cx43 was upregulated in tumour epithelia and membrane Cx43 expression was associated with short OS.

Table II. Staining protocol.

Antigen	Antibody (type, dilution)	Pre-treatment	Detection method	Source
Cx26	Rabbit, polyclonal, 1/200; 30 min	EDTA (pH 9)	Envision-peroxidase, Dako	Abcam, Cambridge
Cx43	Rabbit, polyclonal, 1/50; 30 min	EDTA (pH 9)	Streptavidin-alkaline phosphatase, Dako	Cell Signalling, Danvers
Cx45	Rabbit, polyclonal, 1/100; 30 min	Citrate (pH 6)	Streptavidin-alkaline phosphatase, Dako	Millipore, Temecula

Table III. Calculation of the immunoreactive scores.

Localisation (nucleus, membrane, cytoplasm)	Staining intensity (0, negative to 3, strong)	Percentage (%)	IRS
e.g. membrane	1	80	80+(2x15)+(3x5)=125
	2	15	
	3	5	

Materials and methods

Patients. Tissue samples of 35 patients were screened and connexin expression levels were evaluated using immuno-histochemistry. All patients suffered from a primary OSCC and were scheduled for surgical therapy in the Department of Oral and Maxillofacial Surgery of the University Medical Centre Goettingen without previous radiation or chemo-therapy. The initial histopathological diagnosis was made by a separate biopsy before tumour ablation. The extent of resection and lymph node metastases were diagnosed histo-logically. Metastases of lung, liver and bone marrow were evaluated for all patients by chest radiography, abdominal ultrasound examination and 99mTc-MDP-scintigraphy (Table I). Patients gave written informed consent before they were included in this study. The study was conducted according to the ethical standards approved by the local ethics committee of the University of Goettingen (vote number 07/06/09).

Tissue samples. Tissue samples of primary OSCC, matching oral mucosa free of dysplasia, and associated lymph node metastasis were obtained immediately after tumour resec-tion, fixed in neutrally buffered 4% formalin and embedded in paraffin. Immunohistochemical reactions were performed on 2-μm tissue sections. The staining procedures are summa-rized in Table II. Sections were counterstained with Meyer's haematoxylin. Two independent investigators evaluated all tissue sections by light microscopy (Olympus, Tokyo), considering nuclear, membrane and cytoplasmic connexin staining by using an immunoreactive staining score (IRS). The intensity of staining was evaluated on a graded scale (0, negative; 1, weak; 2, intermediate; 3, strong). A total of 100% of positively stained cells was distributed on every density. For the final IRS, the scores of intensity and staining were multiplied and the mean value was calculated by forming the sum of the individual products. Total values ranged between 0 and 300. The application of the score is demonstrated in Table III.

Statistical analysis. The influence of general clinical para-meters, such as gender, age at first diagnosis, nicotine and alcohol abuse, limiting systemic diseases, AJCC criteria and -stages, radio- and chemotherapy, localization of the primary tumour, extent of neck dissection and type of reconstruc-tion, on the overall survival time was analysed using Cox proportional hazard regressions. The strength of the influence was described by the hazard ratio (with an additional 95% confidence interval). Connexin expression was correlated with survival time using the Kruskal-Wallis test and the associ-ated baseline characteristics described above. The influence of the type of tissue (oral mucosa, primary cancer, lymph node metastasis and the subcellular localization (nucleus, cytoplasm, plasma membrane) and their interaction with the expression of each protein was analysed by non-parametric analysis of variance for data with repeated measurements (26). If significant interactions were observed, all tissue types and subcellular localization were also compared pairwise with each other using non-parametric analysis of variance. All tests were performed at a significance level of α=5% using the statistical software R (version 2.15, www.r-project.org).

Results

Connexin 26. No Cx26 expression was detected in dysplasia-free oral mucosa in any of the cellular compartments (Fig. 1A). Cx26 expression was significantly detected in the cytoplasm and membrane of primary OSCC (P<0.01). However, no nuclear expression was observed in primary OSCC tissue (Fig. 1B). Nevertheless, the signal strength was often at the lower limit of detection. Compared to primary OSCC, cyto-plasmic (P<0.01) and membrane (P<0.01) Cx26 expression was
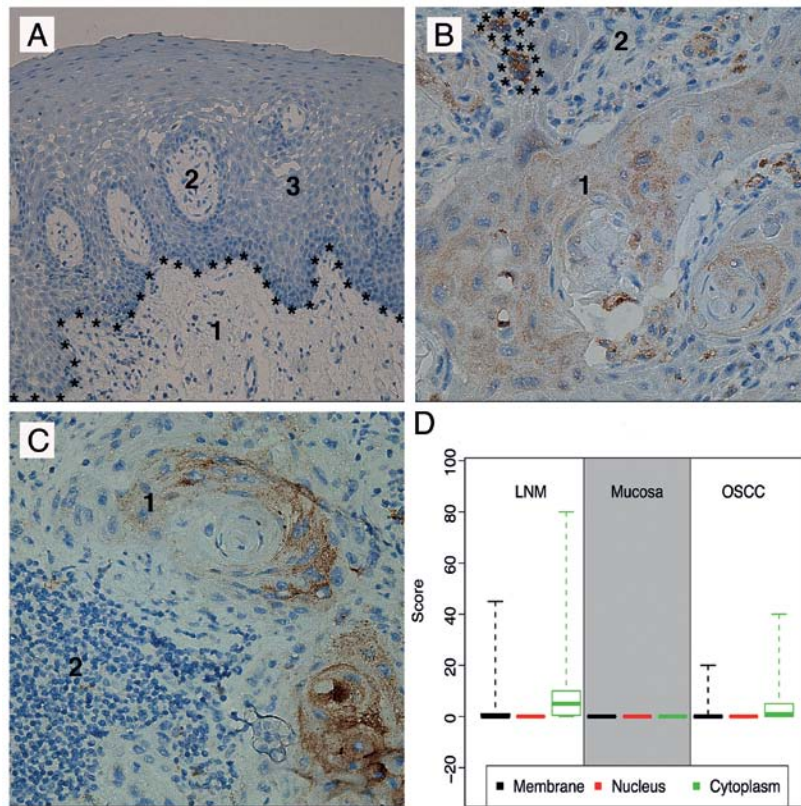
Figure 1. Illustration of Cx26 immunohistochemistry; (A) Oral mucosa (magnification factor x20) (1, submucosa; *, basal membrane; 2, papillary submucosa; 3, mucosa); (B) OSCC (magnification factor x40) (1, tumour cells; 2, stroma; *, polynuclear giant cells); (C) LNM (magnification factor x40) (1, tumour cells; 2, stroma); (D) Morphological distribution pattern.
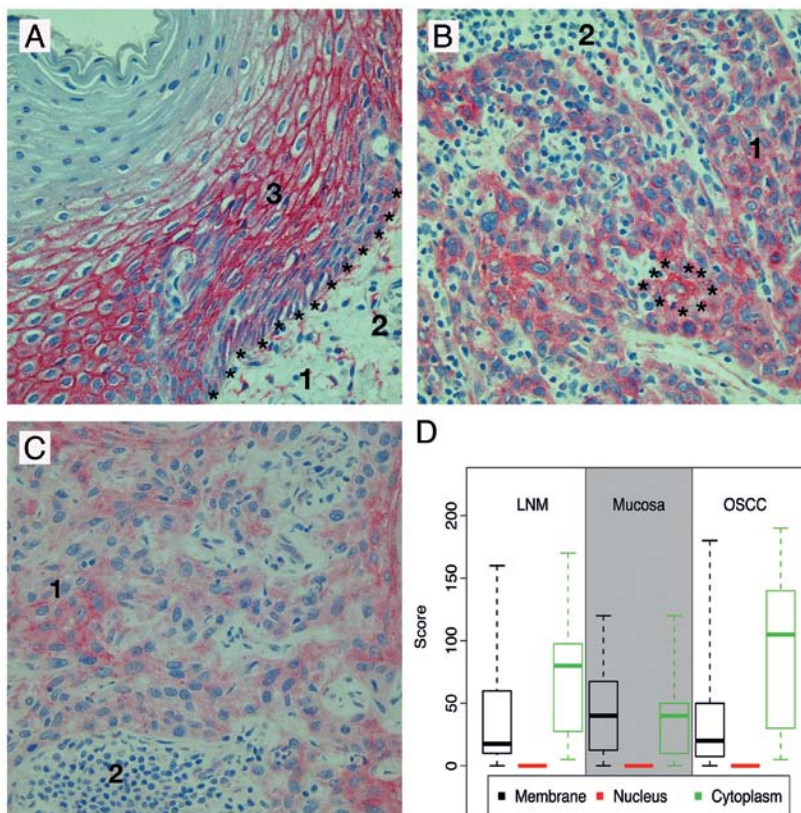

Figure 2. Illustration of Cx43 immunohistochemistry; (A) Oral mucosa (magnification factor x20) (1, submucosa; 2, blood vessel; *, basal membrane; 3, mucosa); (B) OSCC, original magnification x40 (1, tumour cells; 2, stroma; *, perinuclear Cx43 enrichment); (C) LNM, original magnification x40 (1, tumour cells; 2, stroma); (D) Morphological distribution pattern.

Table IV. Pairwise comparisons of connexin expression between the different localizations, separated by tissue type.

Antigen	Tissue	Cell location comparison	P-value
Cx26	LNM	Membrane vs. nucleus	0.13
		Membrane vs. cytoplasm	<0.01
		Nucleus vs. cytoplasm	<0.01
	Oral mucosa	Membrane vs. nucleus	NA
		Membrane vs. cytoplasm	NA
		Nucleus vs. cytoplasm	NA
	OSCC	Membrane vs. nucleus	<0.01
		Membrane vs. cytoplasm	<0.01
		Nucleus vs. cytoplasm	<0.01
Cx43	LNM	Membrane vs. nucleus	0.47
		Membrane vs. cytoplasm	<0.01
		Nucleus vs. cytoplasm	<0.01
	Oral mucosa	Membrane vs. nucleus	<0.01
		Membrane vs. cytoplasm	0.80
		Nucleus vs. cytoplasm	<0.01
	OSCC	Membrane vs. nucleus	<0.01
		Membrane vs. cytoplasm	<0.01
		Nucleus vs. cytoplasm	<0.01
Cx45	LNM	Membrane vs. nucleus	0.04
		Membrane vs. cytoplasm	<0.01
		Nucleus vs. cytoplasm	<0.01
	Oral mucosa	Membrane vs. nucleus	0.31
		Membrane vs. cytoplasm	<0.01
		Nucleus vs. cytoplasm	<0.01
	OSCC	Membrane vs. nucleus	0.67
		Membrane vs. cytoplasm	<0.01
		Nucleus vs. cytoplasm	<0.01

Table V. Pairwise comparisons of connexin expression between the different tissue types, separated by localizations.

Antigen	Cell location	Tissue comparison	P-value
Cx26	Membrane	LNM vs. oral mucosa	<0.01
		LNM vs. OSCC	<0.01
		Oral mucosa vs. OSCC	<0.01
	Nucleus	LNM vs. oral mucosa	<0.01
		LNM vs. OSCC	<0.01
		Oral mucosa vs. OSCC	NA
	Cytoplasm	LNM vs. oral mucosa	<0.01
		LNM vs. OSCC	<0.01
		Oral mucosa vs. OSCC	<0.01
Cx43	Membrane	LNM vs. oral mucosa	<0.01
		LNM vs. OSCC	<0.01
		Oral mucosa vs. OSCC	0.24
	Nucleus	LNM vs. oral mucosa	<0.01
		LNM vs. OSCC	<0.01
		Oral mucosa vs. OSCC	NA
	Cytoplasm	LNM vs. oral mucosa	<0.01
		LNM vs. OSCC	<0.01
		Oral mucosa vs. OSCC	<0.01
Cx45	Membrane	LNM vs. oral mucosa	<0.01
		LNM vs. OSCC	<0.01
		Oral mucosa vs. OSCC	0.53
	Nucleus	LNM vs. oral mucosa	<0.01
		LNM vs. OSCC	<0.01
		Oral mucosa vs. OSCC	0.07
	Cytoplasm	LNM vs. oral mucosa	<0.01
		LNM vs. OSCC	<0.01
		Oral mucosa vs. OSCC	0.26

significantly increased in lymph node metastases (Fig. 1C). No nuclear expression was detected in local metastases. The morphological distributions of Cx26 in the analysed tissues are shown in Fig. 1D. The data from the pairwise comparisons are summarized in Tables IV and V.

Connexin 43. In dysplasia-free oral mucosa the strongest Cx43-signals were detected within the plasma membrane and at a lower level nearly exclusively in the lower half of the stratum spinosum (Fig. 2A). Compared to the matching oral mucosa tumour cells showed an upregulation of cytoplasmic Cx43 expression within tumour cells of the primary tumour and to a lower extent in lymph node metastases (P<0.01), whereas membrane expression was not significantly altered (P=0.24) (Fig. 2B-D). Pairwise comparisons are summarized in Tables IV and V.

Connexin 45. In contrast to Cx43, Cx45 expression increased from the basal to the superficial layers of the squamous oral mucosa. Parakeratosis was Cx45-negative. Cx45 was almost exclusively expressed in the cytoplasm and weak signals were seen in the membrane (Fig. 3A). The Cx45 distribution pattern did not change between dysplasia-free oral mucosa, OSCC and associated lymph node metastases. In OSCC, the cytoplasmic Cx45 expression was not significantly different from the healthy oral mucosa (P=0.26) (Fig. 3B). In lymph node metastases, intracellular Cx45 expression increased significantly compared to primary OSCC (P<0.01) (Fig. 3C). The strongest signals in both types of tissue could be seen in the perinuclear region. The morphological distributions of Cx45 in the examined tissue types are shown in Fig. 3D. Pairwise comparisons are summarized in Tables IV and V.

Connexins and survival of patients. Cx26- and Cx45 expressions did not influence survival. High membrane expression of Cx43 was significantly associated with a shortened overall survival time (P=0.0088) (Fig. 4). Additionally, a high expression level in the cell membrane of matching dysplasia-free oral
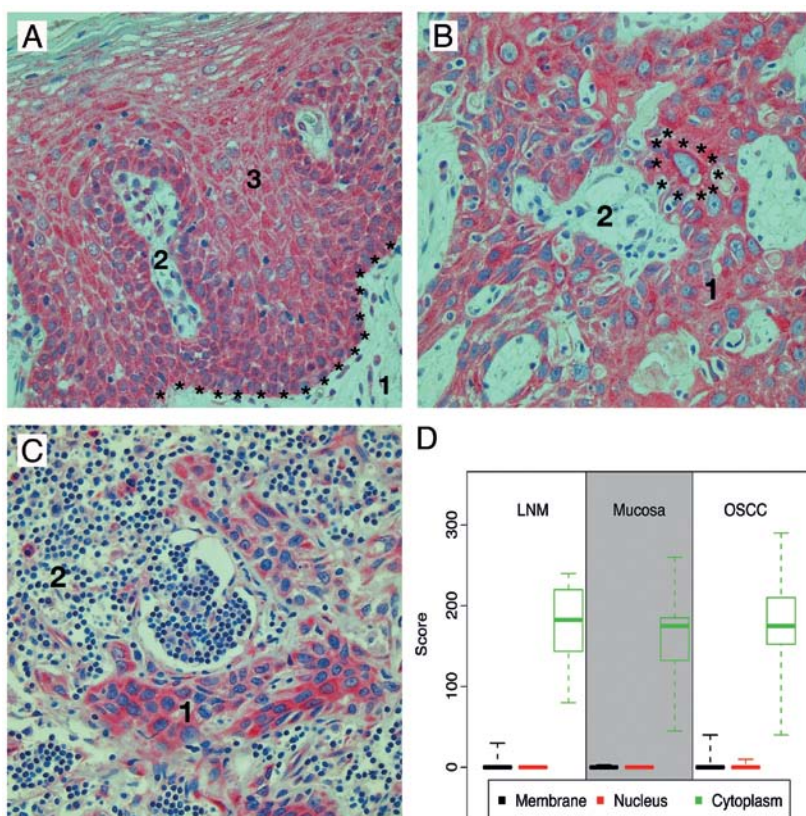
Figure 3. Exemplary illustration of Cx45 immunohistochemistry; (A) Oral mucosa (magnification factor x20) (1, submucosa; *, basal membrane; 2, papillary submucosa; 3, mucosa); (B) OSCC (magnification factor x40) (1, tumour cells; 2, stroma; *, perinuclear Cx45 enrichment); (C) LNM (magnification factor x40) (1, tumour cells; 2, stroma); (D) Morphological distribution pattern.
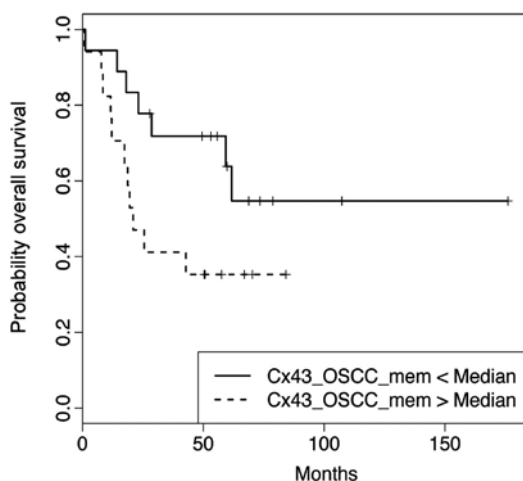

Figure 4. Correlation of overall survival time with the Cx43 expression in OSCC membrane (P=0.0088); Median of IRS-values = 20; IRS-values above the median (dashed curve); IRS values below the median (solid curve).
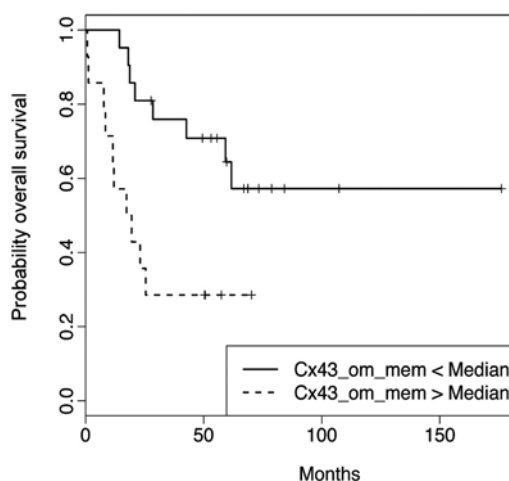
Figure 5. Correlation of overall survival time with the Cx43 expression in the membrane of oral mucosa (P=0.0596); Median of IRS-values = 40; IRS-values above the median (dashed curve); IRS values below the median (solid curve).

mucosa showed a nearly significant association (P=0.0596) (Fig. 5). Correlation analysis is shown in Table VI.

To investigate the association of general clinical parameters with Cx43 expression levels, we performed correlation tests. Expression of Cx43 correlated with clinical parameters, which correlated with the overall survival time, such as the T stage, the AJCC stage and the localisation of the primary tumour.

Membranous Cx43 expression in dysplasia-free oral mucosa showed a significant association with the T stage (P<0.01) and AJCC stage (P<0.01), but not with the localisation of the primary OSCC (P=0.89). Cx43 expression within the tumour cell membrane was independent of the T stage (P=0.06), the AJCC stage (P=0.07) and the localisation of the primary tumour (P=0.79). Correlation analysis is summarized in Table VII.

Table VI. Influence of connexin expression on overall survival.

Expression	HR	HR.lower	HR.upper	P-value
Cx26_OSCC_nuc	1.00	1.00	1.00	NA
Cx26_OM_nuc	1.00	1.00	1.00	NA
Cx26_LNM_nuc	1.00	1.00	1.00	NA
Cx43_OSCC_nuc	1.00	1.00	1.00	NA
Cx43_OM_nuc	1.00	1.00	1.00	NA
Cx43_LNM_nuc	1.00	1.00	1.00	NA
Cx45_OSCC_nuc	0.96	0.79	1.18	0.6986
Cx45_OM_nuc	1.00	1.00	1.00	NA
Cx45_LNM_nuc	1.00	1.00	1.00	NA
Cx26_OSCC_mem	1.04	0.94	1.15	0.4648
Cx26_OM_mem	1.00	1.00	1.00	NA
Cx26_LNM_mem	0.99	0.94	1.04	0.7483
Cx43_OSCC_mem	1.02	1.00	1.03	0.0088
Cx43_OM_mem	1.01	1.00	1.02	0.0596
Cx43_LNM_mem	0.99	0.97	1.01	0.1984
Cx45_OSCC_mem	0.98	0.88	1.09	0.6877
Cx45_OM_mem	1.48	0.74	2.95	0.2637
Cx45_LNM_mem	0.91	0.71	1.16	0.4298
Cx26_OSCC_cyto	0.98	0.92	1.04	0.5562
Cx26_OM_cyto	1.00	1.00	1.00	NA
Cx26_LNM_cyto	0.99	0.95	1.03	0.5186
Cx43_OSCC_cyto	1.00	1.00	1.01	0.4277
Cx43_OM_cyto	1.01	0.99	1.02	0.3379
Cx43_LNM_cyto	1.02	1.00	1.03	0.1195
Cx45_OSCC_cyto	1.00	0.99	1.00	0.2803
Cx45_OM_cyto	1.00	0.98	1.01	0.6068
Cx45_LNM_cyto	1.01	0.99	1.03	0.2083

HR.lower, HR.upper and mark the boundaries of a 95% confidence interval.

Discussion

Connexin 26. Although Cx26 was not expressed in dysplasia-free oral mucosa, expression was increased in primary OSCC. Because of its almost exclusive intracellular expression there is no morphologic evidence for the involvement of Cx26 in the formation of gap junctions. Compared to oral mucosa and primary OSCC, an increased cytoplasmic and membrane Cx26 expression was measured in lymph node metastases. Similar results have been reported by Kanczuga-Koda *et al*, who studied the expression of Cx26 in the tumour cells of mammary carcinoma and associated lymph node metastases and detected overexpression of Cx26 in lymph node metastases compared to the primary carcinoma (22). Saito-Katsuragi *et al* investigated the involvement of Cx26 during the metastasis of human malignant melanoma and observed a distinct Cx26 expression in the tumour cells and in tumour-associated microvessel endothelia. Cx26 expression was not detected in control tissue from healthy dermis nor nevus cell nevi (20). Therefore, the tumour cells are likely to induce Cx26 in tumour-associated microvessel endothelia and form homomeric gap junctions between the two cell types furthering perivascular accumulation of tumour cells and extravasation. This hypothesis is supported by the recent results of membrane Cx26 expression in lymph node metastases, which was not detectable at the primary tumour site.

Connexin 43. In dysplasia-free oral mucosa Cx43 was predominantly expressed at the membrane in the stratum spinosum. The expression decreased with increasing keratinization. The course of keratinization leads to a reduction of the cell membrane (27), which likely leads to the internalization and degradation of Cx43 containing connexons. In primary OSCCs a significant increase of cytoplasmic Cx43 expression was detected compared to matching oral mucosa. Membrane Cx43 expression was not significantly different between these two types of tissue. Tada and Hashimoto (27), who examined

Table VII. Correlation between Cx34_OSCC_mem and Cx43_OM_mem to parameters, which are also correlated with overall survival.

Parameter		Cx43_OSCC_mem	P-value	Cx43_OM_mem	P-value
Tis		20.0 (20.0, 20.0)	0.06	10.0 (10.0, 10.0)	<0.01
T1		10.0 (0.0, 60.0)		15.0 (0.0, 40.0)	
T2		10.0 (0.0, 40.0)		30.0 (0.0, 110.0)	
T3		6.0 (2.0, 10.0)		0.0 (0.0, 0.0)	
T4		47.5 (0.0, 180.0)		62.5 (0.0, 120.0)	
Stage	0	20.0 (20.0, 20.0)	0.07	10.0 (10.0, 10.0)	<0.01
	1	10.0 (0.0, 60.0)		15.0 (0.0, 40.0)	
	2	10.0 (0.0, 30.0)		30.0 (10.0, 110.0)	
	3	7.5 (2.0, 40.0)		0.0 (0.0, 20.0)	
	4	45.0 (0.0, 180.0)		60.0 (0.0, 120.0)	
Side	Left	25.0 (0.0, 180.0)	0.79	40.0 (0.0, 120.0)	0.89
	Right	15.0 (2.0, 100.0)		30.0 (0.0, 110.0)	
	Median	35.0 (0.0, 90.0)		40.0 (0.0, 120.0)	

the localisation of Cx43 by immunofluorescence and immune electron microscopy in normal human skin, basal cell carcinoma and squamous cell carcinoma, described similar results. In these two cancer types, Cx43 was mainly intracellular. Also Dubina *et al* (28) investigated the involvement of Cx43 in advanced stages of colorectal carcinoma and identified several mutations that led to a frame shift and were localized in the carboxyl group of the protein. The expression of these mutant proteins was detected only in the invasive components of the tumours.

These results suggest that gap junctions, composed of Cx43, are lost during carcinogenesis and thereby cause a loss of GJIC. Thus far, however, is not entirely clear whether this loss is due to an increased degradation of intercellular channels or faulty transcription and post-transcriptional modifications within the connexins. An aberrant cytoplasmic localization and connexin related disruption of GJIC might be important events during tumourigenesis, invasion and metastasis. In the recent study we found a relatively high expression of Cx43 in dysplasia-free matched mucosa and, unexpectedly, an association of a significantly shortened post-operative survival time of patients with a high Cx43 expression in the plasma membrane. We speculate that Cx43 expression is upregulated at a very early stage of promotion in morphologically dysplasia-free mucosa because of its association with an advanced T stage and AJCC stage and the trend to a shortened OS. However, this cannot be proved, due to the fact that it is almost impossible to obtain unaffected oral mucosa. The association of increased membranous Cx43 expression on tumour cells with a worse prognosis indicates that Cx43 acts as a tumour promoter in OSCC in contrast to other cancer types. It should also be kept in mind that there are no standardised quantitative immunohistochemical Cx expression procedures developed so far regarding the antibodies, the detection systems and the immunoreactive scores. The particular characteristic of the recent study is an immunoreactive staining score that includes every level of staining intensity for each subcellular localisation that allows a very detailed quantification. This is an important difference from many other studies in which the scores were calculated on the basis of an average staining intensity.

Connexin 45. Cx45 has been extensively studied with regards to its co-expression with Cx43 resulting in altered GJIC. In heart failure, Cx45 is upregulated compared to Cx43 (29). The diffusion capacity of cationic fluorescent dyes over gap junction function is reduced when Cx45 is overexpressed and forms heteromeric gap junctions in co-localization with Cx43 (18), leading to altered intercellular voltage gating mechanisms (17). The relative upregulation of Cx45 compared to Cx43 was shown to cause an increased susceptibility for cardiac arrhythmias *in vivo* (16). There is only slight evidence on the relevance of Cx45 expression in malignant tumours. It has been shown, that Cx45 is variably expressed in human lung fibroblasts and human lung carcinoma cells (30). Cx45 is also expressed in normal lung tissue and advanced stages of mouse lung carcinomas (31). GJIC-dependent contact inhibition can block cell proliferation (8). Decreased GJIC may therefore enhance cell growth of an initiated cell clone.

In the present study, Cx45 was almost exclusively expressed intracellularly in dysplasia-free oral mucosa and primary OSCC,

without any difference between these two tissues. The strongest Cx45 expression was measured in the lymph node metastases. In addition to the cytoplasmic expression, membrane Cx45 expression increased significantly in lymph node metastasis to a very low level in a few tumour cells. Therefore, the formation of homo- or heteromeric gap junctions is unlikely.

High membrane Cx43 expression is an independent marker of poor prognosis of OSCC. Membrane expression of Cx43 in matching tumour-free mucosa is also associated with poor prognosis. This provides evidence for a relevant expression of Cx43 at very early stage of tumour promotion in damaged mucosa. The recent data are suggestive of homodimeric Cx43 containing GJs. The mechanisms of the turnover of high cytoplasmic Cx43 levels without prognostic relevance and lower membrane Cx43 levels with prognostic relevance remain to be elucidated. Due to their expression levels and dominant cytoplasmic expression pattern Cx26 and Cx45 seem to be of minor relevance for tumour progression.

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