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LOXL4 is a selectively expressed candidate diagnostic antigen in head and neck cancer

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ABSTRACT

Selective up-regulation of the mRNA of LOXL4, a member of the LOX matrix amine oxidase family, significantly correlated with lymph node metastases and higher tumour stages in head and neck squamous cell carcinomas (HNSCC). To evaluate the diagnostic and prognostic value of the protein we produced an antibody specific for LOXL4 and assessed the expression in 317 human HNSCC specimens. The LOXL4 protein was detected in 92.7% of primary tumours, in 97.8% of lymph node metastases and in affected oral mucosa with high-grade dysplasia, but was absent in various non-neoplastic tissues of the head and neck. TNM categories and overall survival did not link to grades of immunoreactivity. Studies in cultured primary hypopharyngeal HTB-43 carcinoma cells detected perinuclear and cell surface expression of LOXL4, but no nuclear localisation. Therefore, its interactive SRCR-domains and catalytic activity combined with tumour cell specific expression and cell surface associated location indicate multiple functions in tumour cell adhesion and interactions with the extracellular matrix. Our data suggest that LOXL4 is useful both as tumour marker and target in the treatment of HNSCC.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Unfortunately, local or regional disease recurs in one third of patients with advanced tumour stage, and distant metastases appear in 25% with a 5-year survival rate of around 40% despite aggressive bi-or trimodality standard treatments.[1] The availability of prognostic parameters or tumour antigens would allow the selection of patients for adjuvant treatment regimes as target therapies. Yet over the past decades, only limited success has been achieved in identifying unique targets or prognostic markers for TNM stage and behaviour of tumour growth such as invasion depth and lymphangiosis carcinomatosa along with patients' characteristics (e.g. age, co-morbidity) in HNSCC.

Lysyl oxidase (LOX) and the four lysyl oxidase-like proteins (LOXL, 2, 3 and 4) are a family of copper and lysyl-tyrosine

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quinone cofactors containing amine oxidases active in the assembly and homeostasis of the extracellular matrix (ECM). Elevated levels of LOX were reported to promote tumour cell invasion[2] and expression of the active LOX enzyme correlated with metastatic disease in head and neck cancer (HNC) and breast cancer[3] suggesting its suitability as a therapeutic target for the prevention and treatment of metastases. In differential display experiments we have noted overexpression of the latest member, LOXL4, in HNSCC cell lines.[4] Furthermore, we demonstrated significant correlation between increased LOXL4 mRNA levels and lymph node metastases and higher tumour stages in HNSCC. Our results also revealed that chromosome band 10q24.2, which contains the LOXL4 gene, was present in HNSCC cells in supernumerary copies of isochromosomes that may have contributed to LOXL4 overexpression in these cells.[5]

The significant role of ECM during tumour progression is increasingly recognised, yet only a few studies have addressed the function of ECM components in HNSCC. The matrix protein SPARC/osteonectin was identified as an independent prognostic marker for short disease-free interval in HNC.[6] Matrix metalloproteinases including MMP-2, 9, 13, and 7 have been linked to digestion of the basement membranes and infiltration of tumour cells into the surrounding tissue in HNSCC.[7] A role has been also proposed for invadopodia in HNSCC, branched actin-rich structures associated with the ECM, and the protein contactin within the invadopodia was found to be important in the regulation of MMP secretion and coupling of the dynamic actin assembly and the secretory machinery to enhance ECM degradation and invasiveness.[8] While the MMP dependent ECM degrading processes have been recognised for their roles in dissolving the matrix and promoting free cell movement, migration and invasion, results from our and other laboratories demonstrated that the LOX amine oxidases active in the assembly and maintenance of the ECM also play important roles in tumour cell invasion.

We have demonstrated that increased LOX expression and activity promoted migratory and invasive potential of breast tumour cells. Furthermore, this LOX induced mechanism, via the activation of FAK/Scr/paxillin signalling, was effective not only in invasive breast tumour cells, but in other tumour cell types including uveal melanoma[2] and invasive astrocytes.[9] In spite of these results, there is very little and contradictory information concerning the expression and functional significance of LOX family members in the upper aerodigestive tract. LOX up-regulation has been reported previously in oral submucosal fibrosis and SCC.[10] However, reduced expression of LOX– and LOXL2–mRNA expression in HNSCC was also noted and proposed as playing a role in tumour suppressive processes.[11]

In this study, we have examined the reactivity of our new anti-LOXL4 antibody[5] in subcellular compartments of cultured HNSCC cells and used this antibody to evaluate LOXL4 protein expression in a series of primary and metastatic tumour specimens, with different histopathological features and clinical parameters, to determine its prognostic value in HNSCC, and attempted to correlate the degree of LOXL4 immunoreactivity and various tumour parameters in these HNSCC samples.

2. Patients and methods

2.1. Cell lines

HTB-43 SCC cells, originally derived from the pharynx, were maintained in RPMI 1640 medium supplemented with 1% nonessential amino acids, 10% foetal calf serum (Invitrogen Corp, Grand Island, NY, USA), and 1% streptomycin-penicillin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Head and neck tumours

A specimen collection of 233 primary tumours and 45 nodal metastases of a total of 257 different patients with invasive head and neck carcinoma obtained during primary treatment were investigated. In 30 of these patients both primary tumour and metastasis were comparatively analysed. Furthermore, nine biopsies of oral mucosa and tongue with different degrees of dysplasia (three of each) were assessed for LOXL4 expression. Histopathological features and clinical parameters were obtained from pathology reports, clinical databases and patient's files. Nearly all patients exhibited alcohol drinking (94%) and smoking (97%) behaviour. Tumour samples were collected between 1988 and 2005. In addition, normal oral mucosa samples of 30 healthy patients were also collected to serve as controls. Control samples were taken from the tonsils and palate during regular surgical treatment of tonsillar hyperplasia or snoring without additional excisions. Other non-neoplastic tissue samples including normal keratinising and non-keratinising squamous epithelium,

Table 1 – Patient data with TNM categories and histological grading of primary tumours and regional lymphnode metastases

	Primary tumour	Nodal metastasis
Age years (mean ± stdv)	59.6 (±10.7)	63.3 (±8.9)
Sex male/female (%)	195/38 (83.3% / 16.7%)	32/13 (71.1% / 28.9%)
T stage		
Tx (%)	–	6 (13.3%)
T1 (%)	41 (17.6%)	12 (26.7%)
T2 (%)	61 (26.2%)	10 (22.2%)
T3 (%)	63 (27.0%)	11 (24.4%)
T4 (%)	68 (29.2%)	6 (13.3%)
N stage		
N0 (%)	76 (32.6%)	–
N1 (%)	50 (21.5%)	13 (28.9%)
N2 (%)	100 (42.9%)	27 (60.0%)
N3 (%)	7 (3.0%)	5 (11.1%)
M stage		
M0 (%)	220 (94.4%)	41 (91.1%)
M1 (%)	13 (5.6%)	4 (8.9%)
Grading		
G1 (%)	2 (0.9%)	–
G2 (%)	151 (64.8%)	29 (72.5%)
G3 (%)	80 (34.3%)	11 (27.5%)
n.a.	–	5

Table 2 – LOXL4 expression in primary tumours of different localisations and in regional nodal metastases

Localisation	LOXL4				
	– (negative)	+	++	+++	Total
Oral cavity	1	2	6	5	14 (6.0%)
Oropharynx	10	38	56	54	158 (67.8%)
Hypopharynx	6	9	10	24	49 (21.0%)
Larynx	0	1	5	6	12 (5.2%)
Primary tumour	17 (7.3%)	50 (21.5%)	77 (33.0%)	89 (38.2%)	233
Nodal metastasis	1 (2.2%)	9 (20.0%)	22 (48.9%)	13 (28.9%)	45

glandular epithelium, and mesenchymal tissue components were obtained from adjacent sites of tumour resection during laryngectomy. All 317 samples were retrieved following informed consent approved by the local ethical committee. Patient data, TNM categories and histological grading of primary tumours and nodal metastases are summarised in Table 1, distribution of localisation is itemised in Table 2.

2.3. *Immunohistochemistry*

Formalin fixed paraffin embedded samples were deparaffinised and blocked by incubation for 10 min with a 3% goat serum in TBS for 20 min. Primary affinity purified polyclonal rabbit anti-LOXL4 antibody (own production 5) was added to the slides for 60 min at room temperature followed by incubation with a biotin-conjugated swine anti-rabbit IgG secondary antibody (Dako, Hamburg, Germany) at room temperature for 1 h. A labelled peroxidase complex system (ABC-Vectorstatin, Dako, Hamburg, Germany) was used to visualise all immune reactions. Sections were counterstained with Mayer's haematoxylin. The optimum concentration of the primary antibody was determined using serial dilutions of highly purified synthetic peptide (Eurogentec, Seraing, Belgium). Negative controls were obtained by omission of the primary antibody and incubating the primary antibody with specific blocking peptide in 5 to 10-fold molar excess before staining. The slides were reviewed by two of the authors (T.G. and J.B.W.) who confirmed the diagnosis and tumour grading. To assess LOXL4 protein levels, 300 cells were examined in at least five areas at ×400 magnification and a mean percentage of positive tumour cells was determined assigning cases to one of the four following categories: (–) <5%; (I) 5–30%; (II) 31–75%; (III) >75%. Cases with score of (–) were considered negative, cases with scores of (I–III) positive. To consider bias, the immunohistochemical and histopathological analyses were additionally reviewed by our senior pathologist (P.R.) independently from the working hypothesis of the project. For survival correlation analyses, low LOXL4 expression was categorised as below 30%, and high expression as over 30% mean percentage of LOXL4 immunopositive cells within tumour samples.

2.4. *Confocal laser scanning microscopy*

Cells were grown on sterile slides in minimum essential medium in 5% CO_2 at 37 °C in a humidified atmosphere, washed with PBS, fixed with 2.5% paraformaldehyde for 10 min at room temperature and post-fixed with ice cold methanol for 10 min. The cells were then washed in PBS and blocked with 10% goat serum in PBS for 1 h and then incubated with affinity purified primary polyclonal rabbit anti-LOXL4 antibody at 4 °C overnight. After washing the cells with PBS they were incubated with Alexa Fluor 488-conjugated secondary goat anti-rabbit antibody (Molecular Probes, Eugene, Oregon USA) for 1 h at room temperature. Subsequently, the cells were incubated with DAPI (Sigma, Taufkirchen, Germany) for 2 min at room temperature for nuclear counterstaining (final dilution 1:300 with PBS). Confocal analysis was carried out using a Zeiss ISM 510 UV confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

2.5. *Flow cytometric (FACS) analyses*

HTB-43 SCC cells were harvested, counted, and resuspended in PBS at a concentration of 3×10^6 cells/ml. 100 μl of reagent A or B (FIX&PERM, Caltag Lab., Burlingame, CA, USA) were incubated with 100 μl of tumour cell suspension according to the manufacturer's instruction. After centrifugation, cells were resuspended in 100 μl of either PBS (for cell surface staining) or permeabilisation reagent (for cytoplasmatic staining). Subsequently, cells were incubated (20 min) with each 10 μl PBS, rabbit serum (Serotec, Dusseldorf, Germany) or polyclonal rabbit ant-human LOXL4 antibody at a dilution of 1:5 (in-house antibody) at room temperature. Goat-anti-rabbit:FITC (Dianova, Hamburg, Germany) was used as secondary antibody. FACS analyses were performed by flow cytometry (FACSCalibur, Becton Dickinson) and CellQuest using WinMDI software.

2.6. *Statistical analysis*

Statistical evaluations were performed using the statistical analysis system 'R'.[12] Overall survival, defined as the time between initial treatment (mostly surgery) and time of death, was evaluated with the log-rank statistics for individual potential prognostic factors including age, sex, T stage, N stage, histological grading, and LOXL4 expression. Survival curves were obtained using the method of Kaplan–Meier.

Fisher's exact test was used to assess significant differences between HNSCC samples and their benign counterparts with respect to LOXL4 expression. The chi-square test was used to evaluate the relationship between LOXL4 expression and clinical and pathological features. Frequency distribution of LOXL4 expression between primary tumours and lymph node metastases to prove agreement or disagreement was

tested with a special symmetry test.[13] All normally distributed data are displayed as mean ± standard deviation, the non-normally distributed as median with 1st and 3rd quartile. Data were considered statistically significant if P values were $\leqslant 0.05$.

3. Results

3.1. *LOXL4 is expressed in primary tumours of the head and neck cancer region*

Immunohistochemistry was performed to evaluate the LOXL4 expression in 317 tissue samples derived from 257 patients with histologically confirmed HNSCC and controls. In 233 primary tumour samples from different localisations of the head and neck region and with various T stages, we have found overall LOXL4 expression in 92.7% of primary tumours (Fig. 3A). The 95% confidence interval ranged from 88% to 96%. The distribution of LOXL4 with respect to localisation of cancer showed only slight differences of expression frequencies. Oral cavity amounted to 92.9%, oropharynx to 93.7%, hypopharynx to 87.8%, and larynx to 100% respectively (Table 2). In LOXL4 positive tumours, the staining reaction appeared cytoplasmic, in a dot-like perinuclear distribution, with occasional accentuation at the cell membrane. Nuclear labelling was not present. The staining intensity was variable between tumours, but within an individual tumour, the majority of the tumour cells were evenly labelled (Fig. 1D–F). No significant correlation was noted between LOXL4 expression in primary tumours / lymph node metastases and T-stage ($P = 0.226$ / 0.638), N-stage ($P = 0.19$ / 0.084), M-stage ($P = 0.62$ / 0.719), histological grading ($P = 0.194$ / 0.557), localisation ($P = 0.265$ / 0.58), age ($P = 0.629$ / 0.58) and sex ($P = 0.435$ / 0.351).

3.2. *LOXL4 expression in nodal metastases is higher than in primary tumours*

We next investigated LOXL4 expression in 45 samples of regional nodal metastases. Among these neck metastases, 44 samples stained positive with an overall expression rate of 97.8% (Fig. 3A and Table 2). The 95% confidence interval ranged from 88% to 100%. The intensity of the immunostaining was similar to the positive primary tumours. To assess

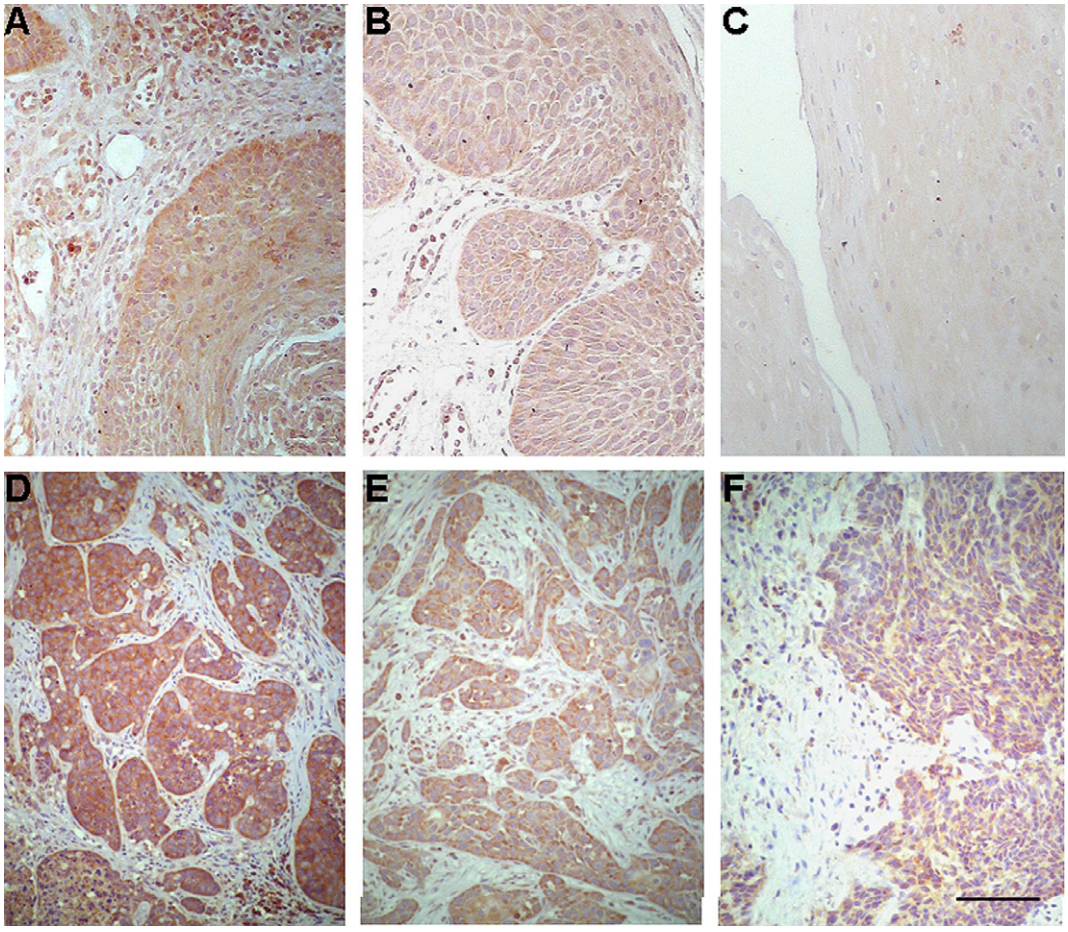

Fig. 1 – Immunohistochemistry of LOXL4 in different grades of dysplasia and in HNSCC. A, **Medium intensity of LOXL4 immunoreaction in high-grade dysplasia, invasive SCC.** B, **Weak immunoreactivity in moderate dysplasia.** C, **No immunopositivity in a specimen with slight dysplasia.** D–F, **Invasive squamous cell carcinomas with** D, **strong,** E, **moderate, and** F, **weak LOXL4 expression. Scale bare = 100 μm.**

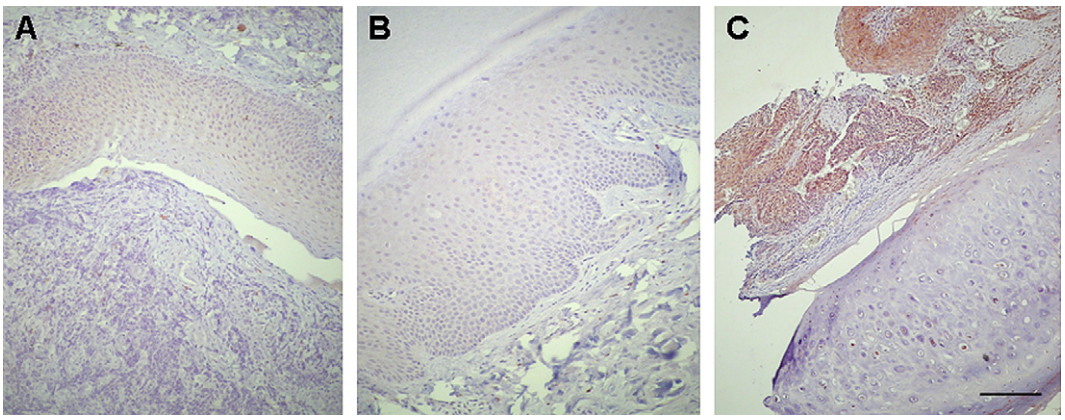

Fig. 2 – Immunohistochemistry in normal and in a neoplastic tissue containing masses of cartilage. A, Normal squamous epithelium of the tonsil (non-keratinising), and B skin (keratinising) do not express LOXL4. C, In contrast, a poorly differentiated SCC of the larynx shows a strong LOXL4 reactivity in the tumourous part. The surrounding tissues, notably the adjacent cartilage, are negative. Scale bar = 100 μm.

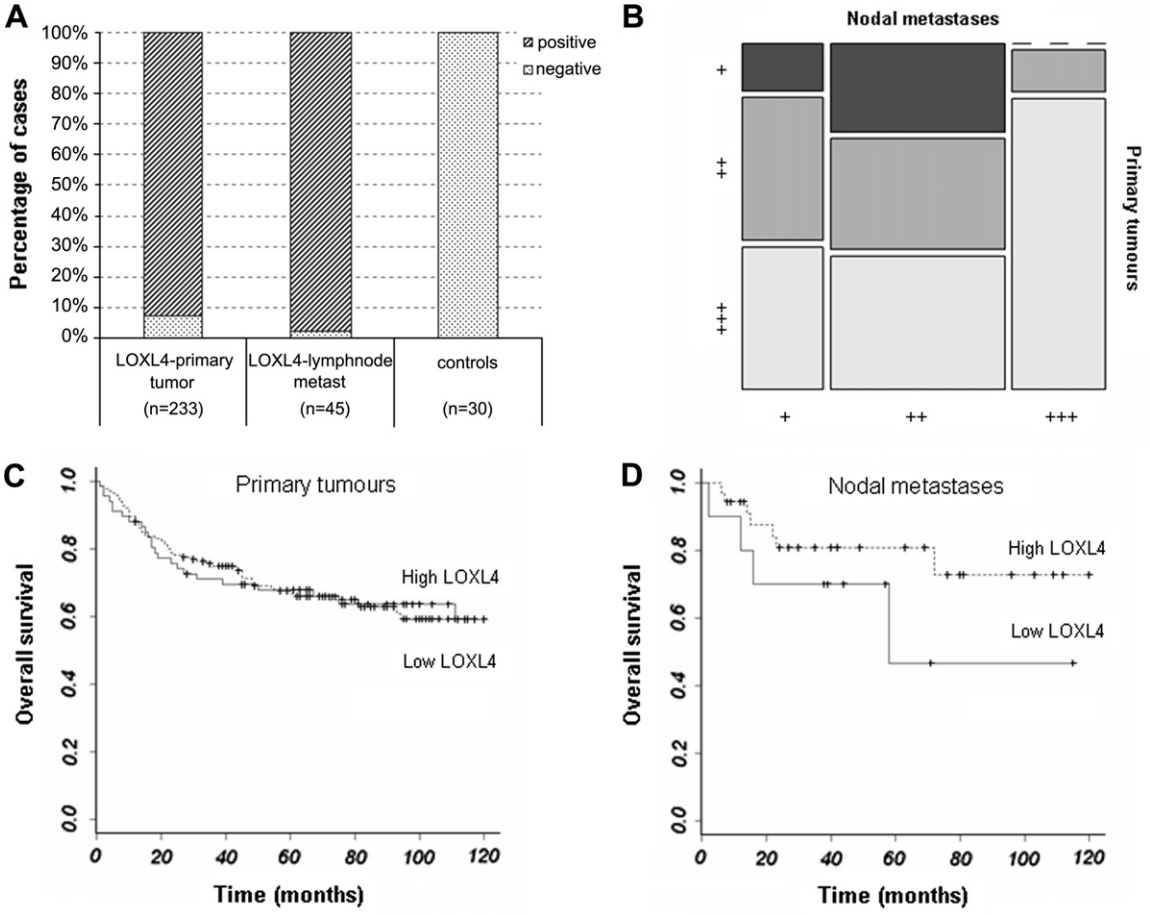

Fig. 3 – Comparative clinicopathological data with respect to LOXL4 expression. A, Comparison of LOXL4 expression levels with those of primary tumour, nodal metastasis and control of normal oral mucosa. Filled columns with strips represent LOXL4 positive; filled column with dots represents LOXL4 negative data. B, Mosaic plots showing agreement of the intensity of LOXL4 immunoreactivity with primary tumours and lymph node metastases in 13 (43.3%) patients. LOXL4 expression was comparatively analysed in a total of 30 patients. $\chi^2 = 6.7$ ($P = 0.0821$). C, D, Kaplan–Meier plots of patients with primary head and neck cancer expressing high or low level of LOXL4 revealed no difference in overall survival (C; $P = 0.983$). However, plots of those patients whose nodal metastases stained high for LOXL4 revealed a prolonged overall survival even though this difference was not significant (D; $P = 0.193$).

potential individual variations in LOXL4 protein levels we examined expression differences in both primary tumours and nodal metastases of the same individuals in a cohort of 30 patients. We have found the same LOXL4 immunostaining intensity in both primary tumours and lymph node metastases in 13 patients, a higher intensity in primary tumours in 12 patients (Fig. 3B) and a more intense LOXL4 immunostaining in nodal metastases in five patients ($\chi^2 = 6.7$ with $P = 0.082$) with no statistically significant diversity of LOXL4 expression in favour of either primary tumour or metastasis.

3.3. *LOXL4. is absent in normal oral mucosa and non-neoplastic tissues, but expressed in pre-malignant lesions including leukoplakia*

To confirm the value of LOXL4 as a novel marker for detecting HNSCC, we first performed LOXL4 immunostaining in 30 samples of normal oral mucosa as control for HNSCC. In contrast to the positive tumour samples, all 30 normal control samples were negative for LOXL4 immunostaining (Fig. 2A). We further analysed LOXL4 expression in other non-neoplastic tissues of the head and neck region including normal keratinising and

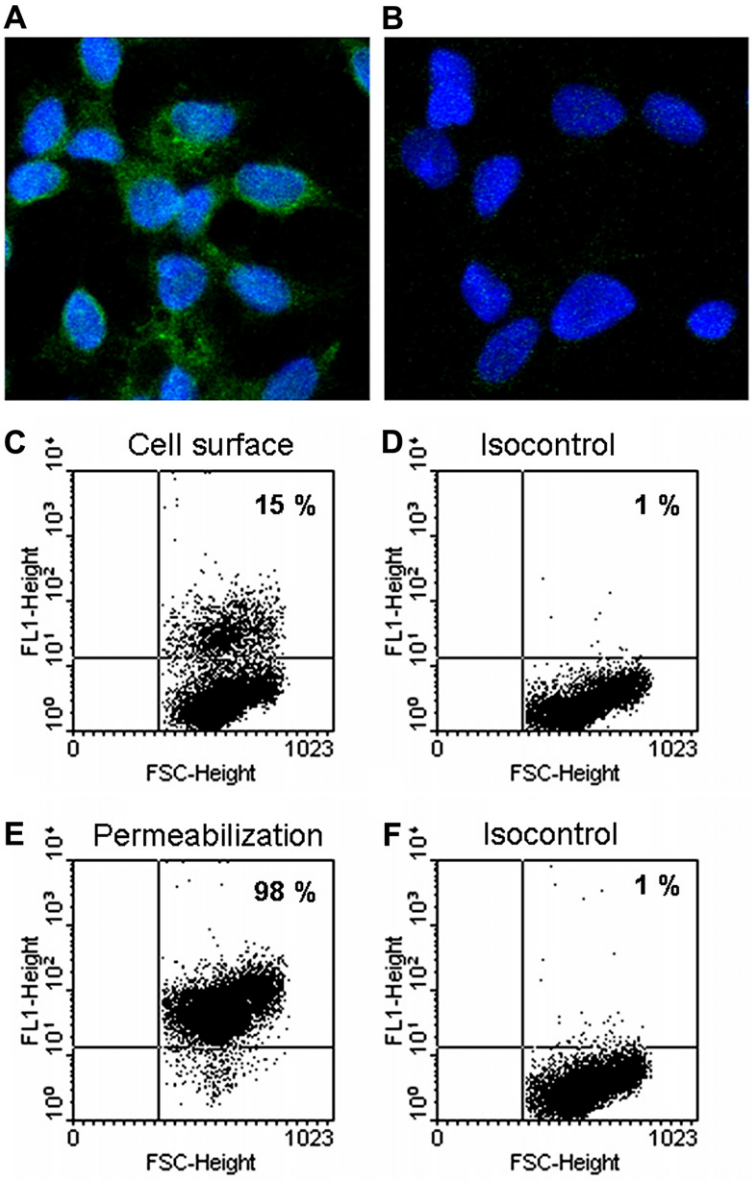

Fig. 4 – LOXL4 cell location analyses in HTB-43 cells. A, Confocal laser scanning microscopy of LOXL4 staining (green) is located throughout the cells. Cisternal patterns are visible surrounding the nucleus (blue), punctuate, vesicular staining is also found towards the periphery of the cell. B, Specificity of the reaction is shown with the primary antibody omitted (Original magnification 40×). C–F, LOXL4 expression on the cell surface and in cytoplasm of HTB-43 cells analysed by flow cytometry. C, Labelling of cells with primary polyclonal rabbit anti-human LOXL4-Abs and with FITC-conjugated second goat-anti-rabbit Abs indicates a LOXL4 surface staining rate of an average of 15%. E, After permeabilisation, both intracellular and cell surface localisation of LOXL4 were detected. D and F, Representative reaction patterns are shown compared with control.

non-keratinising squamous epithelium, glandular epithelium, and mesenchymal tissue samples, which were all consistently negative (Fig. 2A–C). Some staining was seen in polymorph nuclear granulocytes, possibly due to either an endogeneous peroxidase activity or specific expression of LOXL4 in these cells.[14]

In nine samples of pathologically confirmed leukoplakia of the tongue and oral mucosa, we observed LOXL4 expression exclusively in intraepithelial neoplasia with moderate to high-grade dysplasia. A representative sample is shown in Fig. 1A,B. In three samples of slight dysplasia there was no detectable LOXL4 immunoreactivity, and a representative specimen is shown in Fig. 1C.

3.4. *HNSCC patients with either high or low level of LOXL4 immunoreactivity in their primary tumours or lymph node metastases showed no difference in overall survival*

To further evaluate whether there is a prognostic value for the level of LOXL4 protein immunoreactivity, univariate analysis comparing low and high LOXL4 detection levels in both primary tumours ($n = 233$) and nodal metastases ($n = 45$) was carried out. The median observation time after initial diagnosis was 98 months (ranging from 2 to 209). In patients whose primary tumours showed low immunostaining for LOXL4, the two-year overall survival rate was 75.7% (SDF 5.3) while it was 78.1% (SDF3.2) for patients whose tumours demonstrated a high level of LOXL4 staining ($P = 0.98$). This small difference disappeared when we compared five- and ten-year overall survival rates (Fig. 3C). Correlation of LOXL4 protein immunoreactivity levels in nodal metastases with low LOXL4 showed a two-year overall survival rate of 70.0% (SDF 14.5), while it was 80.8% (SDF 7.1) in those with high LOXL4 reactivity (Fig. 3D). The median overall survival time was 70.2 months in patients with low LOXL4 and 95.9 months in patients with high LOXL4, but without significance ($P = 0.19$) due to the low number of cases and events (four versus seven death events).

3.5. *The LOXL4 antigen is localised in the cytoplasm and cell surface in HNSCC cells*

As reported previously, members of the LOX family, LOX and LOXL, were localised not only in the ECM, but also in the nuclei of cultured cells and certain cell types within tissues.[15] We have investigated subcellular localisation of LOXL4 in cultured HTB-43 cells derived from SCC of the pharynx using confocal laser scanning microscopy. LOXL4 immunostaining revealed a punctuate pattern within these cells, and an enrichment in cisternal structures close to the nuclei, but not within the nuclei (Fig. 4A). This pattern argues for containment of LOXL4 in the endomembrane system of the cells, where protein synthesis and vesicular transport towards secretion at the cell surface takes place consistent with the presence of a predicted signal peptide on LOXL4.

Flow cytometry results of cell surface and intracellular LOXL4 detection localised the antigen in both cell membrane and intracellular compartments. The LOXL4 antigen was increased from 15% (Fig. 4C) on the surface of non-permeabilised cells to 98% (Fig. 4E) after permeabilisation of the cell membrane and counting cytoplasmatic and surface antigen signals together. Isotype controls of non-specific antibody binding assessed less than 1% reactivity (Fig. 4D and 4F). These data further supported the immunohistochemical observation that the main sites for LOXL4 expression are both associated with the cell membrane and within the cytoplasm, but not the nucleus.

4. Discussion

We have previously demonstrated up-regulation of the LOXL4 mRNA in HNSCC, confirmed the up-regulation of LOXL4 mRNA with immunoreactivity of the LOXL4 protein in various tumour cell lines, and importantly, found a significant correlation with lymph node metastases and higher tumour stages.[5] In this study, we have further evaluated LOXL4 expression and the immunoreactivity of the LOXL4 protein in a large cohort of patients with HNSCC. LOXL4 positive immunostaining was detected in almost all tissue samples obtained from primary tumours and lymph node metastases. Among the samples of pathologically confirmed pre-malignant leukoplakia of the tongue and oral mucosa, only those with a moderate to high degree of dysplasia stained positive for LOXL4. In all non-neoplastic tissue components obtained from adjacent sites of tumour samples and in those derived from 30 healthy controls containing mesenchymal tissues, keratinising, non-keratinising squamous and glandular epithelia, no LOXL4 immunoreactivity was detected.

LOXL4 immunostaining showed only slight variations relative to tumour localisation with the hypopharynx being the lowest (87.8%), the oral cavity (92.9%) and the oropharynx (93.7%) being intermediate and the larynx being the highest (100%) frequency. Additionally, we have found that while 92.7% of primary tumours were LOXL4 positive, an even higher percent (97.8%) of lymph node metastases stained positive. Collectively, these results, obtained in over 300 samples, indicate that LOXL4 can serve as a selective molecular marker in primary and metastatic HNSCC.

We have not detected statistically significant differences in overall survival rates between these groups when compared based on the grades of LOXL4 immunostaining intensities. The antibody used in these experiments was raised against the KVWDLKMR epitope within the second of four N-terminal SRCR domains of LOXL4. While there is no experimental evidence for processing of LOXL4, in other LOX family members N-terminal proteolytic processing does occur and in the case of LOX and LOXL was proven to be necessary for catalytic activation. If LOXL4 is similarly processed/activated and the N-terminal proteolytic processing affects the first and second SRCR domains[14,16], our immunodetection may have missed some forms of LOXL4 in HNSCC tissues. Additionally, LOXL4 may undergo post-translational modifications such as glycosylation and/or phosphorylation noted for other members of the LOX family, (17 and unpublished observation) and these modifications can, in a tissue and/or cell type dependent fashion, influence the availability of the epitope and limit immunodetection of the LOXL4 protein under certain conditions. Subsequent functional studies of LOXL4 will determine if this is the case and advance the development of more

specific and/or multiple antibodies to allow us improved LOXL4 detection and refine the correlation between immunostaining intensities and diverse tumour parameters.

Numerous antibodies to tumour-associated antigens have been reported in recent years and only a few have been identified against head and neck squamous epithelia and HNSCC. The antibodies specific for squamous epithelia are useful in confirming an epithelial origin of the tissue being stained, e.g. antibodies against cytokeratins[18,19], the basal layer of squamous epithelium[20], involucrin[21], EGFR[22] or survivin.[23,24] Although these antibodies have been found to be useful for immunohistochemistry, only a few of them are reactive on frozen tissue section, or react with normal epithelial cells (including normal squamous epithelia). Our results confirm that the anti-LOXL4 antibody has a high specificity for SCC and that well-defined staining patterns result when the antibody is applied to SCC specimens of different grading and staging. In this study, all statistical variables were considered in their original and grouped form and multiple regression modelling of these variables supported the histological and immunostaining observations with tumours of different locations and nodal metastases.

Results with SCC and squamoproliferative lesions i.e. leukoplakia with different grade of dysplasia suggest that the LOXL4 immunostaining pattern closely reflects functional activity of LOXL4 associated with disordered squamous cell behaviour. While all SCC biopsies were derived from the upper aerodigestive tract, squamous epithelial cells examined in healthy skin did not exhibit LOXL4 reactivity. An absence of LOXL4 expression was also observed in both normal oral mucosa and normal mucosa of the tonsil. Because of its restrictive and highly sensitive SCC cell type specific reactivity, the anti-LOXL4 antibody may prove particularly valuable for the histopathological recognition of SCC. In addition to the diagnostic value of LOXL4, our recent feasibility study[25] demonstrated the potential for LOXL4-transfected dendritic cells to serve as an efficient tumour vaccine and support their suitability as a vaccination strategy applicable to cancer patients with tumour specific upregulation of LOXL4.

While without statistical significance, it is of interest that we have observed a trend towards a better overall survival in patients with head and neck cancer with high LOXL4 protein expression in nodal metastases and a slightly better two-year overall survival rate with high LOXL4 expression in primary tumours. As our analyses have considerable practical importance in the clinical management of patients, we believe that this observation needs to be further investigated. Most recently, LOXL4 was reported to exert its functions through the inhibition of the Ras/ERK signalling pathway in human bladder cancer and was proposed to act as a tumour suppressor gene.[26] Our clinical survival results, if confirmed, may support a similar function for LOXL4 in certain stages of HNSCC.

These results appear to be somewhat in contradiction with our previous observations on the essential role of LOX in breast tumour cell invasion, where additional evidence indicated that the active LOX induced FAK/Scr mediated migratory/invasive behaviour in breast[27] and also in other tumour cell types.[3,9] While in breast cancer LOX and LOXL2 demonstrated the strongest association with invasive and metastatic phenotype[2,28], we have also found such association for LOXL4 mRNA in HNSCC.[5] This paradox between our data and those reported by Wu and colleagues[26] may result from the lack of full understanding of the cellular function of the LOXL4 protein in normal epithelial and tumour cell types, and limitations inherent in conclusions derived from different experimental systems (promoter methylation in the Wu study, versus our immunostaining) and different tumour types (bladder cancer cells in the Wu, versus HNSCC in our study).

In order to begin to address ECM and/or cellular LOXL4 functions, we determined its location in HTB-43 cells. The LOXL4 protein immunoreactivity was found predominantly in the cytoplasm and on the cell surface, but not in the nuclei of these cells. While LOX and LOXL have been well described as secreted proteins in the ECM associated with fibrillar collagen and elastic fibres and also in the nuclei of certain cell types[17], there is no detailed information about the tissue and cellular localisation of the SRCR-domain containing LOX family members, LOXL2, LOXL3 and LOXL4, though the signal peptide present in these proteins suggests that these are also secreted.[29] Results from this study support that, similar to LOXL2 (unpublished observation), LOXL4 is not likely to be localised within the nuclei in epithelial cells. However, it is found in close association with the cell membrane, a location that has been noted for other SRCR domain containing proteins in which the SRCR domains serve as protein interactive sites.[29,30] While currently there is no in depth information regarding the function of LOXL4 in squamous epithelial cells, its tumour cell specific expression and cell surface associated location demonstrated by our results, its catalytic activity determined by its conserved C-terminal domains[31] and its four N-terminal, potentially interactive SRCR domains[32] indicate multiple functions that may play important roles in HNSC tumour cell adhesion and interactions with the ECM.

In summary, results from this study provide evidence that LOXL4 is selectively expressed in a very high percentage of both primary tumours and lymph node metastases in HNSCC, is likely to play a significant role(s) in HNSCC and that the antibody raised against LOXL4 is suitable to detect primary and metastatic HNSCC with high sensitivity and specificity. Finally, it's selective expression and absence in non-neoplastic tissues of head and neck produces interest in LOXL4 as a target for various treatment strategies in HNSCC.

Conflict of interest statement

None declared.

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