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Lysyl oxidase expression is an independent marker of prognosis and a predictor of lymph node metastasis in oral and oropharyngeal squamous cell carcinoma (OSCC)

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Proteins of the lysyl oxidase (LOX) family are important modulators of the extracellular matrix. However, they have an important role in the tumour development as well as in tumour progression. To evaluate the diagnostic and prognostic value of the LOX protein in oral and oropharyngeal squamous cell carcinoma (OSCC) we performed QRT-PCR and immunohistochemical analysis on two tissue microarrays (622 tissue samples in total). Significantly higher LOX expression was detected in high grade dysplastic oral mucosa as well as in OSCC when compared to normal oral mucosa ($P < 0.001$). High LOX expression was correlated with clinical TNM stage ($P = 0.020$), lymph node metastases for the entire cohort ($P < 0.001$), as well as in the subgroup of small primary tumours (T1/T2, $P < 0.001$). Moreover, high LOX expression was correlated with poor overall survival ($P = 0.004$) and disease specific survival ($P = 0.037$). In a multivariate analysis, high LOX expression was an independent prognostic factor, predicting unfavourable overall survival. In summary, LOX expression is an independent prognostic biomarker and a predictor of lymph node metastasis in OSCC. Moreover, LOX overexpression may be an early phenomenon in the pathogenesis of OSCC and thus an attractive novel target for chemopreventive and therapeutic strategies.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common human cancer in the western world and is associated with high morbidity and low survival[1,2] HNSCC comprises a heterogeneous group of tumours located at different anatomic sites, including the oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx, each of which is characterized by different clinical behaviour. Local or regional disease recurrence occurs in one third of patients with advanced tumour stages (stage III and IV) and distant metastases appear in 25% of patients despite bi- or trimodality standard treatments.[3] Novel molecular predictive biomarkers of regional and distant metastatic potential at the time of diagnosis are urgently needed to guide clinical therapy decisions.

Identification and evaluation of new molecular parameters are of outmost importance in OSCC. Using tissue microarrays, it is now possible to perform large-scale expression analysis to identify candidate biomarkers. Previously, we have demonstrated that the combination of tumour tissue microarrays and cDNA arrays is a powerful approach to rapidly identify and further evaluate genes that play a role in tumour biology.[4] Here, we used the tissue microarray approach to identify candidates in OSCC. Since we focus our research on hypoxia-related proteins,[5] we have selected for this thorough analysis lysyl oxidase (LOX), an extracellular matrix protein, which was identified to be overexpressed in human hypoxic cells.[6]

Lysyl oxidase, a copper-dependent metalloenzyme that belongs to the lysyl oxidase gene family, acts as an extracellular modulating enzyme and initiates the covalent cross-linking of collagens and elastin in extracellular matrice (ECM)[7–9]

Key words: Oral and oropharyngeal squamous cell carcinoma, dysplastic oral mucosa, lysyl oxidase, tumour biomarker, tissue microarray, cDNA microarray
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and is essential for normal connective tissue function, embryonic development, and wound healing.[10,11] However, LOX can function also intracellularly, regulating cell signalling and gene expression.[12] Interestingly, LOX was originally identified as a tumour suppressor in non-tumorigenic revertants of *ras*-transformed fibroblasts.[13,14] Concerning human cancer there are several reports describing reduced LOX expression in cancer patients, supporting the idea that *LOX* acts as a tumour suppressor.[7,15–17] In contrast, recent publications have demonstrated up-regulation of the LOX mRNA as well as of the LOX protein in various cell lines and tumour tissues (including head and neck cancer, breast cancer and lung cancer etc.).[18,19] Several studies evaluated the relationship between tumour behaviour and LOX protein expression *in vitro* and *in vivo* with inconsistent and inconclusive results.[18,19]

So far, the significance of LOX proteins in head and neck squamous cell carcinoma is controversial. While in some studies LOX was shown to be upregulated in oral squamous cell carcinoma[20] and to be essential for hypoxia-induced metastasis in HNSCC,[19] reports on LOX and LOXL2 down-regulation in head and neck cancer patients also exist.[15]

In order to investigate the predictive and prognostic significance of the lysyl oxidase in malignant tumour tissue samples derived from OSCC patients we have implemented a comprehensive analysis of the LOX protein on tissue microarrays.

Material and Methods

Patients

Consecutive patients who presented at the Ear Nose and Throat Department of the University Hospital Zurich between 1993 and 2000 with histologically proven previously untreated oral or oropharyngeal squamous cell carcinoma were included.

A review of the records identified 267 patients fulfilling the entry criteria. Fifteen of these were subsequently excluded because of the small size of the biopsy ($n = 8$), previous or synchronous malignancies ($n = 4$), intercurrent death ($n = 1$), lack of follow-up ($n = 2$). Finally, primary tumour tissues of 252 patients were enrolled into this study. Furthermore, 93 biopsies of oral mucosa with different degrees of dysplasia (14 samples of low grade and 79 samples of high grade dysplasia), as well as 20 samples from hyperplastic mucosa, 20 samples from non-neoplastic ulceration derived from oral cavity and oropharynx were assessed for LOX expression. For the evaluation of dysplastic changes a simplified WHO classification was used. Mild dysplasia (SIN 1) was considered low grade dysplasia, whereas moderate dysplasia (SIN 2) and severe dysplasia/carcinoma in situ (SIN 3) were considered high grade dysplasia.

In addition, normal mucosa samples of 60 patients not suffering from tumour disease derived from palate, tongue and buccal mucosa were collected to serve as controls. With the approval of the regional board of the Medical Ethics Commission, paraffin-embedded tissue samples were obtained from the archives of the Institute of Surgical Pathology. The histopathological diagnosis and grading of biopsies were reviewed by an experienced pathologist (I.H.). Clinicopathological co-variables were derived from patient charts. The principal clinical characteristics of the patient cohort are summarized in Table 1.

Table 1. Clinicopathological characteristics of the patients in the OSCC tissue microarray

Variable	N	%
Eligible patients	252	
Age (years)		
Median	55	
Range	36-81	
Sex		
Female	83	33
Male	169	67
Localisation		
Oral Cavity	98	38,9
Oropharynx	154	61,1
Histological grade		
G1	18	7
G2	159	63
G3	75	30
Tumour stage		
T1	66	26
T2	112	45
T3	46	18
T4	28	11
Nodal stage		
N0	92	36
N1	45	18
N2	111	44
N3	4	2
Follow-up		
Median length of follow-up (mo)	81	
Median follow-up 95% CI	71,2-82,8	
End state		
Dead	127	50,4
Alive	125	49,6

Patients in this study were treated according to recommendation of the Swiss Head and Neck Cancer Society. Basically, the treatment decisions were made based on the clinical and pathological TNM diagnosis. The treatment decision did not account for the oxygenation status. 115 (45,6%) out of 252 patients were treated with surgery alone, 22 (8,7%) with radiation only, 86 (34,1%) with surgery and radiation, 16 (6,4%) with surgery, radiation and chemotherapy, as well as

13 (5,2%) with radiation and chemotherapy. In the surgery group (surgery without or with radiotherapy and/or chemotherapy) in 178 (70,6%) patients additional neck dissection was performed. No neck dissection was performed in 39 (15,5%) patients of the surgery group and in 35 (13,9%) patients treated with radiotherapy alone or with chemotherapy.

RNA extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction

In addition to paraffin-embedded tissue samples in 7 patients with OSCC also fresh frozen tissue samples with corresponding normal tissue from distant region were available. The localization of normal mucosa samples was adjacent, but not in close vicinity to the patient primary tumor. The samples were used as normal controls only in cases where macroscopically and histological examination revealed normal epithelial tissue without any signs of abnormal tissue architecture or tumor disease. These samples were also obtained at time of surgery for OSCC at the Ear Nose and Throat Department of the University Hospital Zurich, after approval of the regional board of the Medical Ethics Commission. The histopathological diagnosis and grading of biopsies were reviewed by an experienced pathologist (I.H.). Clinicopathological co-variables were derived from patient charts. Tissue samples were fresh frozen in liquid nitrogen and stored at −80°C before RNA extraction.

For RNA extraction 15–20 of 30 μm thick frozen sections were prepared with a cryomicrotome and transferred to a chilled 1.5 ml eppendorf tube. RNA isolation was performed using the RNeasy RNA mini kit (Qiagen, Valencia, CA) employing the tissue homogenizer Ultra-Turrax T8 (IKA-Werke) according to supplier's instructions. Following isolation procedure, RNA concentration and RNA degradation were checked using the Agilent 2100 Bioanalyzer (Agilent Technologies). Tissue quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Delaware, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Only those samples with a 260 nm/280 nm ratio between 1.8–2.1 and a 28S/18S ratio within 1.5–2 were further processed.

The LOX forward and reverse primer were "CAGAGGA GAGTGGCTGAAGG", and "CCAGGTAGCTGGGGTT TACA" respectively, synthesized by Microsynth Company (Balgach, Switzerland). The RT-PCR reaction was performed using LightCycler®FastStart DNA Master SYBR Green I (Roche). PCR conditions were as follows: 42 cycles, 95°C for10 min, 95°C for 10 sec, 57°C for 6 sec, 72°C for 3 sec, 72°C for 1 min.

Tissue microarray preparation

Two tissue microarrays were constructed as described previously.[4] Briefly, suitable areas for tissue retrieval were marked on routine haematoxylin and eosin (H&E) sections, punched out of the paraffin block and inserted into recipient block. The tissue arrayer was purchased from Beecher Instruments (Woodland, USA). The punch diameter was 0,6 mm. The OSCC-TMA was constructed to represent 429 samples (252 primary tumour samples, 128 samples of lymph node metastasis, 29 samples of recurrent tumours and 20 normal mucosa samples). The whole OSCC-TMA was accomplished on six paraffin blocks using cores from different areas of each tumour in order to account for tumour heterogeneity. A second premalignant tissue PMT-TMA with 193 samples was constructed using 93 tissue samples from dysplastic mucosa (14 samples of low grade and 79 samples of high grade dysplasia), 20 samples from hyperplastic mucosa, 20 samples from non-neoplastic ulceration and 60 samples from normal oral mucosa. Also this TMA was accomplished on six paraffin blocks. After the block construction was completed, 4.0μm sections of the resulting tissue microarray block were cut for further analysis.

Immunohistochemistry

LOX immunohistochemistry was performed on paraffin sections of formalin-fixed tissue on TMA, using a Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, Arizona) as described elsewhere.[21] The samples were deparaffinised and blocked by incubation for 10 min with a 3% goat serum in TBS for 20 min. Antigen retrieval was achieved by cooking in 0,01 M citrate buffer for 5 min. Primary polyclonal rabbit anti-LOX antibody (a gift from Dr. D. Kirschmann, University of Iowa) was added to the slides for 60 min at room temperature and revealed with the iVIEW DAB detection kit, yielding a brown reaction product. The signal was enhanced with the Ventana amplification kit. Slides were counterstained with haematoxylin prior to glass coverslipping. As a negative control we included sections where primary antibody was replaced by nonhuman reactive rabbit IgG (DAKO).

Evaluation of LOX immunostaining

All immunohistochemical specimens were analyzed independently by 2 investigators (AA, IH), who were blinded to the clinical information pertaining to the subject. Only cytoplasmic immunoreactivity of epithelial cells was regarded as LOX expression. Staining localized in the stromal component was not considered. An immunoreactivity score (IRS), modified from the German IRS[22] was calculated multiplicating percentage of positive cells (PP) with staining intensity (SI). To calculate the IRS, we assigned the following points for PP: 0 = 0%; 1 = 1% to 20%; 2 = 21% to 40%; 3 = 41% to 60%; 4 = 61% to 80%; 5 = 81% to 100%. The staining intensity was rated on a scale 0 to 3, with 0 = negative; 1 = weak; 2 = moderate; 3 = strong staining intensity. On the basis of PP and SI the IRS could range from 0 to 15. The median IRS of all samples, which was 8, was used to categorize the cases as high LOX expression (IRS $\geq$ 8) and low LOX expression (IRS $<$ 8). The consensus opinions were used to assign final IRS scores to the disputed cases before data analysis. To

evaluate intratumoural heterogeneity of LOX expression we additionally stained 10 conventional tissue slides with OSCC.

Statistical analyses

The statistical analyses were carried out using the SPSS for Windows 15.0.1 for windows (SPSS Inc, Chicago, IL). Overall survival (OS) was defined as the time between diagnosis and time of death of the patient or until the last follow-up visit and disease specific survival (DSS), was defined as time between diagnosis and death of cancer or until the last follow-up visit. Survival curves were calculated using the Kaplan-Meier method, with significance evaluated using 2-sided log-rank statistics. The chi-square test was used to evaluate the relationship between LOX expression and clinical and pathological features. Continuous baseline variables were dichotomized as follows; T-category (T1/2/ Vs T3/4), N-category (N0/ Vs N1/2/3). Wilcoxon tests were used to examine the relationships of LOX expression between primary tumours and lymph node metastases. Cox regression analysis was performed to determine if LOX status is an independent risk factor for overall survival (OS) in both the univariable and multivariable level. Inclusion criteria for covariates and confounders for entry into the multivariate analysis was a p-value < 0.1 in the univariable analysis. A backward elimination procedure was then performed to eliminate nonsignificant variables ($P > 0.1$). Probability values less than 0.05 were regarded as significant.

Results

Analysis of the LOX expression by Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-PCR)

To demonstrate that LOX is up-regulated in squamous cell carcinoma in comparison with normal oral mucosa we applied QRT-PCR analysis on paired tumour samples with corresponding normal oral mucosa. Seven human OSCC tumour and normal mucosa samples from patients included in the tissue microarray analysis were used for QRT-PCR. Expression of LOX in each sample was normalized by the expression of 18s rRNA. The ratio of LOX expression in tumour tissue to normal tissue was regarded as the relative quantitative mRNA expression of LOX. As shown in Figure 1, levels of LOX mRNA were increased in 5 out of 7 patients when compared with corresponding normal tissue samples.

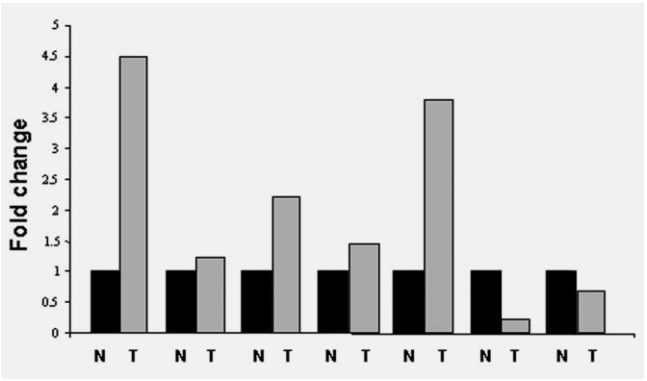

Figure 1. *LOX* mRNA expression in OSCC tumour tissue samples (T, grey bars) and normal tissues samples (N, black bars) examined by quantitative reverse transcription-polymerase chain reaction (QRT-PCR). The ratio of *LOX* expression in tumour tissue to normal tissue was regarded as the relative quantitative mRNA expression of *LOX*. Levels of *LOX* mRNA were increased in 5 out of 7 patients tested when compared with normal tissue samples.

Expression pattern of the LOX protein in normal oral mucosa, premalignant lesions and in squamous cell carcinoma of the oral cavity and oropharynx

Immunohistochemistry of LOX was performed to evaluate the expression of this marker in 429 tissue samples derived from 252 patients with histologically confirmed OSCC. Moreover, LOX expression in premalignant lesions and control samples was evaluated on additional 193 samples on TMA (Fig. 2). LOX immunoreactivity in normal oral mucosa (NOM) and hyperplastic oral mucosa (HYP) was mainly weak and localized suprabasal in the lower half of the oral mucosa (Fig. 2*a*; NOM, HYP). The median of the immunoreactivity score (IRS) was 3 and 3.5 for NOM and HYP respectively (Fig. 2*b*; NOM, HYP). Heterogeneous LOX immunoreactivity was detected in low grade dysplastic epithelia with a median IRS of 3 (Fig. 2*a*; LGD). Interestingly a strong and diffusely distributed LOX immunoreactivity was detected in high grade dysplastic epithelia (Fig. 2*a*; HGD) and a mainly strong but heterogeneously distributed LOX positivity was observed in OSCC tissue (Fig. 2*a*; OSCC), with median value of IRS of 10 and 8 respectively (Fig. 2*b*, HGD and OSCC). The distribution of LOX is depicted with boxplot, where each plot represents the median, upper and lower quartiles as well as minimum and maximum data values and outliers (Fig. 2*b*). The differences of LOX immunoreactivity between each tissue samples were significant as showed by Kruskal and Wallis H-test ($P < 0.001$). The median value of immunoreactivity score for LOX in all OSCC samples (median $= 8$) was used as a cutoff to differentiate tumour positivity. Low LOX expression was defined as negative or weak cytoplasmic staining (IRS < 8), whereas high LOX expression was defined as moderate or high cytoplasmic staining with IRS ≥ 8. Table 2 summarizes the immunohistochemical results for non-, preund malignant oral mucosa on the TMA. The cytoplasmic LOX expression was increased in high grade dysplastic mucosa and OSCC with positive lymph node status.

LOX expression and clinical and pathological characteristics

Using Person's chi-square test the LOX expression in OSCC samples was analysed for relationship with clinical and pathological parameters. No relation was identified between age, histological grade, T-stage and expression of LOX (Table 3). There was also no difference in LOX protein expression between different anatomic localisations (tongue, base of the

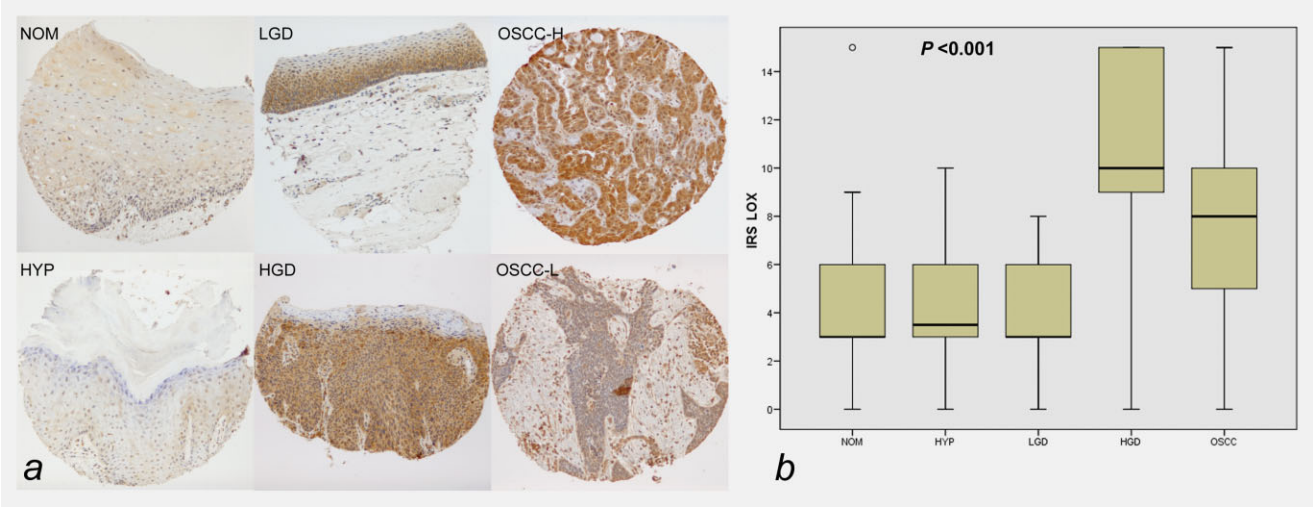

Figure 2. (*a*) typical staining patterns observed with LOX immunohistochemistry in normal mucosa (NOM), low grade (LGD) and high grade dysplastic mucosa (HGD) as well as in the squamous cell carcinoma of the oral cavity and oropharynx (OSCC) on high-density tissue microarray. Weak LOX immunoreactivity in NOM, HYP and heterogeneous reactivity in LGD. Strong homogeneous LOX immunoreactivity in HGD and weak to strong heterogeneous immunoreactivity in OSCC (×80). (*b*) box plot analysis of immunoreactivity scores of LOX showing a significant increase of IRS in high grade dysplasia (HGD) and OSCC compared to normal (NOM), hyperplastic oral mucosa (HYP) and low grade dysplastic epithelia (LGD) ($P < 0.001$, Kruskal and Wallis H-test).

Table 2. Analysis of LOX expression in non-, premalignant and malignant oral mucosa on TMA

Samples	Total	LOX low No (%)	Lox high No (%)
Normal oral mucosa	80	73 (91)	7 (9)
Oral Ulcus	20	16 (80)	4 (20)
Hyperplastic oral mucosa	20	17 (85)	3 (15)
Low grade dysplastic mucosa	14	13 (93)	1 (7)
High grade dysplastic mucosa	79	9 (11)	70 (89)
Non-metastasized OSCC	92	51 (55)	41 (45)
Metastasized OSCC	160	36 (23)	124 (78)

tongue, floor of the mouth; data not shown). However, significant relation was observed between the lymph node status and the LOX expression ($P < 0.001$) when evaluating the entire patient population. High LOX expression was seen in regionally lymph node metastasized tumours (N1 to N3, UICC) compared with regionally non-metastatic tumours (N0) (Table 3). Moreover, significant relation was observed between clinical stage and the LOX expression ($P = 0.020$). High LOX expression was detected in advanced-stage tumours (Table 3).

In our group of patients, 178 (70,6%) had small tumours (T1 and T2; < 4cm). In this group of small primary OSCC high LOX expression was significantly associated with lymph node status ($P < 0.001$). Furthermore, for patients with T1 or T2 tumours, only 33 out of 81 (40,7%) patients with low LOX expression had evidence of regional lymph node metastases, whereas 70 out of 97 (72%) patients with high LOX expression developed lymph node metastases. For all OSCC tumours, when using high LOX level as a predictor of pathologically proven lymph node metastases, the sensitivity was 75% and the specificity was 58%. For the small primary OSCC tumours (T1 and T2) the sensitivity was 72% and the specificity was 59%.

LOX expression and patient survival

To determine whether the level of LOX protein expression is associated with overall survival (OS) and disease specific survival (DSS) of OSCC patients, we performed univariate survival analysis. This analysis was performed for the entire patient cohort (Fig 3*c*, 3*d*) as well as for patient suffering from oropharyngeal (Fig 3B) and oral cavity carcinoma (Fig 3*a*), separately. As shown in Figure 3 high LOX expression correlated with poor overall survival independently whether the primary tumour localisation was oral cavity ($P = 0.023$, log rank), oropharynx ($P = 0.050$, log rank), or the whole patient cohort was analysed ($P = 0.004$ for OS and 0.037 for DSS, log rank).

In univariate analysis, several factors were statistically significantly associated with overall survival, including sex, T stage, N stage, clinical stage and LOX expression (Table 4). We then performed a Cox proportional hazards regression analysis to determine whether the effect of LOX expression on overall survival is dependent on other known risk factors. In the subsequent multivariate analysis both T stage and LOX expression was associated with worse overall survival ($P < 0.001$ and $P = 0.042$, respectively; Table 4).

Table 3. LOX and its clinical and pathological correlations

Parameters	LOX low No (%)	LOX high No (%)	P-value
Age at diagnosis (≤55/>55)			
≤55	43 (17.1)	87 (34.5)	0.691
>55	44 (17.5)	78 (31.0)	
Histological grade			
Well	10 (4.0)	8 (3.2)	0.149
Moderate	52 (20.6)	107 (42.5)	
Poor	25 (9.9)	50 (19.8)	
T-stage			
T1	22 (8.7)	43 (17.1)	0.456
T2	42 (16.7)	71 (28.2)	
T3	17 (6.7)	29 (11.5)	
T4	6 (2.4)	22 (8.7)	
N-stage for all tumours (n=252)			
N0	51 (20.2)	41 (16.3)	<0.001
N1	4 (1.6)	36 (14.3)	
N2	30 (11.9)	86 (34.1)	
N3	2 (0.8)	2 (0.8)	
N-stage for T1/T2 tumours (n=178)			
N0	48 (27.0)	27 (15.2)	<0.001
N1	5 (2.8)	23 (12.9)	
N2	27 (15.2)	47 (26.4)	
N3	1 (0.6)	0 (0.0)	
M-stage			
M0	95 (37.7)	156 (61.9)	
M1	0 (0.0)	1 (0.4)	
Clinical TNM stage			
I	19 (7.5)	17 (6.7)	0.02
II	20 (7.9)	19 (7.5)	
III	12 (4.8)	39 (15.5)	
IVA	42 (16.7)	80 (31.7)	
IVB	2 (0.8)	2 (0.8)	

Discussion

In the present study we performed analysis of the LOX protein on a large cohort of patients suffering from oral and oropharyngeal squamous cell carcinoma. This is the first report of LOX protein expression analysis including samples from normal mucosa, precancerous lesions and OSCC tumour samples.

In previous reports, differences in chromosomal pattern, carcinogenic progression and disease course in patients with squamous cell carcinoma of the head and neck were observed, but there was a relationship to the primary tumour localisation.[23] In order to analyze a homogeneous patient cohort, only OSCC patients with primary manifestation in the oral cavity and oropharynx were included in this study and analysed together as well as separately. Moreover, only patients who underwent standard therapy protocols and who were accessible for follow up for a minimum of 5 years were included.

In this project we have focused on the role of the lysyl oxidase protein in OSCC. With the implementation of the TMA technology we analysed the association between clinicopathological data and expression levels of the LOX protein. Different LOX expression levels were observed in non-, pre- and malignant oral mucosa. Concerning LOX protein our data demonstrate that increasing levels of LOX expression are associated with high grade dysplasia and with advanced-stage tumours.

High LOX expression in the primary tumour was highly indicative for the simultaneous presence of lymph node disease ($P < 0.001$), independently of the primary tumour stadium. Furthermore, the LOX protein expression correlated not only with advanced tumour stage, and lymph node metastasis, but also a clear correlation with overall survival and disease specific survival was observed ($P < 0.004$ and $P < 0.037$).

Heavy smoking, alone and in combination with high level of alcohol consumption are well established risk factor for HNSCC.[24] Regardless of tobacco and alcohol use, also exposure to HPV shows increased association especially with oropharyngeal carcinoma.[25–27] Therefore two distinct pathways were proposed for the oropharyngeal cancer development: one driven mostly by the carcinogenic effects of alcohol and tobacco use (or combination of these) and the other one by HPV induced genomic instability.[25] In our patient cohort high LOX expression was associated with impaired overall survival independently whether the primary tumour was localised in the oral cavity or oropharynx. This could implicate that the LOX expression is not essential for the HPV associated cancer development. In order to clarify the role of the LOX protein in HPV related cancerogenesis, we have initiated an independent risk analysis of the HPV infection in regard to disease outcome as well as in regard to LOX expression in oropharyngeal squamous cell carcinoma.

Outcome indicators in cancer patients are in general derived from clinical and pathological features.[28] However, currently it is impossible to reliably predict treatment outcomes, even in patients within the same TNM category.[29] Other clinical and pathological variables, not included in the TNM system, have been identified as prognostic predictor variables.[30–33]

Surprisingly, the nodal status was in our cohort significant negative prognostic factor only in the univariate analysis. Similar results were observed by Pedruzzi et al.[34] This could be partially explained by the analysis of a cohort of consecutive patient at our institute regardless of the applied therapy modality. This very interesting observation requires further examination.

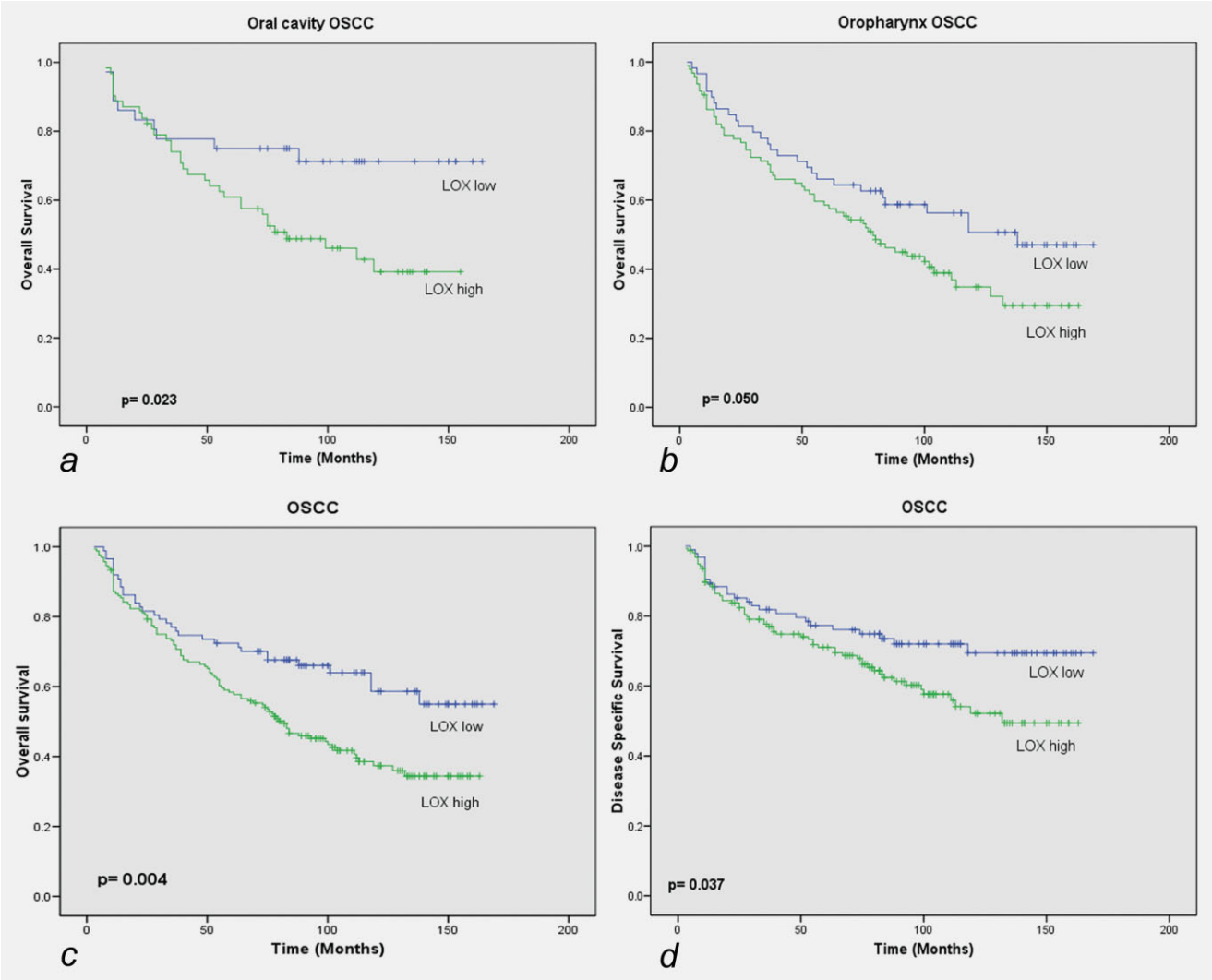

Figure 3. Kaplan-Meier survival analysis of oral cavity OSCC (*a*), oropharynx OSCC (*b*) as well as oral cavity and oropharynx OSCC together (*c, d*) according to LOX expression. Patients with high LOX expression had a significantly inferior overall survival time (*a, b, c*) and disease specific survival (*d*) than those with low LOX expression in all investigated anatomical localisation ($P = 0.023$ for oral cavity OSCC, $P = 0.050$ for oropharyngeal OSCC and $P = 0.004$ for both group together, log rank test). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 4. Univariate and multivariate survival analysis

	All patients ($n = 252$) death events ($n = 127$, 50,4 %)					
	Univariate Analysis			Multivariate Analysis		
Overall survival	**RR**	**CI (95%)**	***P***	**RR**	**CI (95%)**	***P***
Age at diagnosis (≤55/>55)	1.01	0.71–1.42	0.972			
Gender (male/female)	1.46	0.99–2.15	0.052	1.32	0.89–1.95	0.169
Tumour grade (G1-2/G3-4)	0.73	0.48–1.08	0.114			
Tumour stage (T1-2/T3-4)	2.66	1.88–3.77	<0.001	2.27	1.51–3.42	<0.001
Lympn node metastasis (N0/ pN1-3)	1.615	1.11–2.35	0.012	1.01	0.53–1.93	0.981
Clinical TNM stage (I-II/III-IV)	2.13	1.38–3.27	0.001	1.28	0.58–2.85	0.538
LOX (low/high)	1.77	1.19–2.63	0.004	1.53	1.01–2.32	0.042

In head and neck carcinoma *LOX* down as well up regulation was described: Initially, *LOX* was shown to be up regulated in oral squamous cell carcinoma as well as in oral submucous fibrosis.[20] Rost *et al.*[15] have observed the reduction of *LOX* as well as of *LOXL-2* mRNA expression in cell lines as well as tissue samples originating from HNSCC. Erler *et al.*[19] observed association between *LOX* overexpression and decreased overall survival in head and neck cancer patients. *LOX* was also included among the endogenous molecular markers indicative of hypoxia in HNSCC and indicative for shorter overall survival.[35]

Recently, Shieh *et al.*[36] have demonstrated up regulation of LOX mRNA as well as increased LOX protein expression in OSCC associated with areca nut. Interestingly, in their experimental setting precancerous lesions displayed the highest LOX mRNA expression. These recent report support our observations of dynamic LOX protein up regulation in oral squamous carcinoma, with low LOX protein expression in normal oral mucosa, hyperplastic oral mucosa and in low grade dysplastic tissue. In our experimental setting tumour tissue samples of OSCC displayed up regulation of the LOX protein expression when compared with normal mucosa and low grade dysplasia, however in agreement with results observed on RNA level by Shieh *et al.*, we have observed highest LOX protein expression levels in high grade dysplastic tissue. At this point it is tempting to speculate about the role of the LOX protein in cancer progression in OSCC. The here observed up regulation of the LOX protein in precancerous lesions may represent a crucial step allowing for modification and modulation of the ECM environment and allow the further progression and metastatic spread of the neoplastic process. However, for clearer understanding of the metastatic progression in OSCC, further functional studies are needed.

In a recent study a similar pattern of expression of the LOX4 protein in premalignant lesions and HNSCC was observed.[37]

There is some controversy concerning the biologic relevance of LOX protein in molecular pathogenesis of invasive carcinomas. Interestingly, initially *LOX* was first identified as a "ras recision gene" in normal cells transformed by LTR-*c-H-ras*. A putative *ras*-related tumour suppressor function had been attributed to *LOX* in transformed fibroblasts.[38–44] A decrease in *LOX* activity was noted in different malignant cell lines including fibrosarcoma, rhabdomyosarcoma, and choriocarcinoma.[45] Consistent with these reports, poorly invasive/non-metastatic breast cancer, uveal and cutaneous melanoma, and rat prostate cancer cell lines were shown to express little to no *LOX*.[18] Csiszar *et al.*[46] demonstrated a decrease in *LOX* expression in colon tumours and Ren *et al.*[47] described a progressive reduction of *LOX* expression with the transition from normal prostate epithelium to malignant prostate epithelium.

Subsequently however, both up- and down-regulation of *LOX* was noted not only in cell lines but also in tumour tissue, e.g., in squamous oral, head and neck, gastric, prostate, colon, invasive breast, lung, and renal carcinoma. Up-regulation in *LOX* expression was observed in some osteosarcoma cell lines[48] and certain malignant tumours.[49,50] In addition, Stassar *et al.* demonstrate that *LOX* expression is significantly associated with a higher staging in clear cell renal carcinoma tissues.[51]

Systematic studies have been directed mostly at the role of *LOX* in invasive breast carcinoma cells. These studies revealed, that *LOX* activity was essential for promotion of the invasive phenotype of breast carcinoma cells[18,52] and for metastatic behaviour in mice.[19]

The reasons for contradictory results of LOX expression in various cancers may be multifactorial. Concerning the *in vitro* experimentally transformed fibroblasts it was repeatedly shown that *ras* oncogene expression results in the establishment of a new equilibrium of gene expression patterns which includes gene activation as well as gene repression. When using this experimental system one has to keep in mind that the molecular effects that result from transient and sustained oncogene expression might be of quite different nature. Our own investigations showed that transient *ras* oncogene expression results in activation of different set of genes when compared with sustained *ras* oncogene expression.[53] Therefore, changes in gene expression patterns observed *in vitro* must be interpreted with caution when applied *in vivo*.

Another possible explanation may lie in differences in fluctuations in LOX expression at different stages of transformation, in differences in sample size and also in variation in the molecular environment in different tissue types. The different modulation of LOX activity in different tumours can partially be explained by global genetic differences in particular tumours, by different availability of LOX downstream targets or substrates in different cell types.[54] It is possible that tumorigenic and/or invasive properties can be gained either by LOX up or down regulation, in reliance of the intrinsic cellular environment, as well as in dependence of natural presence or absence of the LOX protein under normal conditions in a particular cell or tissue type.

In conclusion, our study is the first in examining the expression of the LOX protein in a panel of tissue samples representing the particular steps of the carcinogenesis including normal mucosa samples, precancerous tissue, as well as tumour tissue samples of OSCC. Our results demonstrate that LOX is a specific marker of lymph node metastases in patients with this challenging form of carcinoma. Importantly, LOX levels seem to identify a subset of patients with early tumour stage primary tumour and high metastatic potential that might benefit from more aggressive therapy. However, the appropriate treatment still has to be determined for this group of high risk patients. The emerging role of LOX in the metastatic process strongly suggests that attenuation of LOX activity may be a potential target for novel strategies in the treatment of head and neck as well as other solid carcinomas. As LOX inhibition had minimal effects on the growth of the primary tumour,[36] it is conceivable, that combination of anti-LOX therapy with other agents, such as radio-chemotherapy, would act supportive to target both primary and metastatic growth.

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