# LOXL4 as a Selective Molecular Marker in Primary and Metastatic Head/Neck Carcinoma

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**Abstract.** *Background: Overexpression of the lysyl oxidase-like 4 (LOXL4) gene is associated with a variety of human malignancies. The purpose of this study was to compare the gene expression of LOXL4 to the expression of epidermal growth factor receptor (EGFR) in head and neck squamous cell carcinomas. The overexpression of EGFR has been well examined and already serves as a therapeutic target. The expression of both genes was compared in head and neck squamous cell carcinomas and their diagnostic and prognostic value was evaluated. Materials and Methods: Messenger RNA from 58 head and neck carcinomas and 11 healthy upper aerodigestive tract mucosa samples was extracted and subjected to electrophoresis. Northern hybridisation was carried out using digoxigenin-labelled gene-specific probes, and the level of gene expression was measured by densitometry. Results: High expression of LOXL4 gene was detected in 71% of all carcinomas and only in 9% of the healthy mucosa samples (p=0.0002). In comparison, a high level of expression was detected for the EGFR gene in 78% of the carcinomas and in 36% of normal mucosa (p=0.01). Conclusion: Although both genes revealed a similar level of overexpression in the carcinoma samples, it was found that the a notably higher percentage of healthy mucosa tested positively for EGFR than LOXL4, indicating that LOXL4 may serve as a selective molecular marker in primary and metastatic head and neck carcinoma.*

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Unfortunately, local or regional disease recurs in one third of the patients with advanced tumour stage, and distant metastases appear in 25% of cases, with a 5-year survival rate of around 40% despite aggressive bi- or tri-modality standard treatments (1).

To date, the underlying molecular processes involved in HNSCC are not well understood, although knowledge of the overexpression of certain genes has allowed the development of new forms of therapy. The blockade of the epidermal growth factor receptor (*EGFR*) by the use of anti-*EGFR* monoclonal antibody cetuximab has been introduced as a potential anticancer therapy (2).

Preliminary studies examined the expression of genes in a series of primary and metastatic tumour specimens, and found that an isoform of lysyl oxidase, namely lysyl oxidase-like 4 (LOXL4), a copper-dependant amine oxidase that is important for the assembly and maintenance of components of the extracellular matrix, was overexpressed in 74% (46 of 62) of invasive HNSCC tumours and 90% of both primary and metastatic HNSCC cell lines (3). Moreover, a significant correlation was found between LOXL4 expression and local lymph node metastasis *versus* primary tumour types (3, 4). To date, the expression of *LOXL4* has not been compared to the expression of well-established genes in HNSCC in order to investigate the potential use of the *LOXL4* gene as a new target for the development of new cancer therapies.

In this study, the expression of *EGFR* and *LOXL4* was analysed in 58 HNSCC samples. The gene expression level was related to clinical and histopathological parameters from each patient such as TNM stage, gender and age at time of biopsy excision and the degree of gene expression was correlated with these parameters in the HNSCC samples studied.

## Materials and Methods

*Histology samples.* A total of 58 tissue samples (44 primary HNSCC, eight regional lymph node metastases and six distant metastases of HNSCC) underwent mRNA expression analysis for *EGFR* and *LOXL4*. A glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probe was used to enable comparison between *EGFR* and *LOXL4* expression levels. Four biopsies of benign tumours of the head and neck (two papillomatoses and two parotid adenomas) and

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*Key Words: EGFR*, LOXL4, gene expression, head and neck squamous cell carcinoma, metastasis.

Table I. *Differential expression of GAPDH, EGFR and LOXL4 mRNA.*

| No. | Location | Type | Designation | Age (years)/Gender | TNM | *GAPDH* | *EGFR* | *LOXL4* |
|---|---|---|---|---|---|---|---|---|
| 1 | Larynx | P | Malignant | 81/m | T2N2M0 | +++ | – | – |
| 2 | Larynx | P | Malignant | 81/m | T3N0M0 | +++ | +++ | + |
| 3 | Larynx | P | Malignant | 52/m | T4N2M0 | +++ | +++ | +++ |
| 4 | Larynx | P | Malignant | 81/f | T3N0M0 | +++ | +++ | – |
| 5 | Larynx | P | Malignant | 71/m | T3N0M0 | ++ | ++ | – |
| 6 | Hypopharynx | P | Malignant | 74/m | T4N2M0 | +++ | + | +++ |
| 7 | Hypopharynx | P | Malignant | 73/m | T4N0M0 | + | – | + |
| 8 | Hypopharynx | P | Malignant | 65/m | T4N2M1 | + | + | ++ |
| 9 | Hypopharynx | P | Malignant | 54/m | T4N3M0 | +++ | + | + |
| 10 | Hypopharynx | P | Malignant | 66/f | T4N2M0 | ++ | +++ | – |
| 11 | Hypopharynx | P | Malignant | 56/m | T4N2M0 | ++ | – | +++ |
| 12 | Oropharynx | P | Malignant | 67/m | T4N2M0 | ++ | + | +++ |
| 13 | Oropharynx | P | Malignant | 66/m | T2N2M0 | ++ | + | +++ |
| 14a | Tonsils | P | Malignant | 80/f | T2N2M0 | +++ | + | – |
| 15b | Oropharynx | / | Benign | / | / | ++ | – | – |
| 16a | Base of tongue | P | Malignant | 55/m | T3N1M0 | ++ | + | +++ |
| 17 | Oropharynx | P | Malignant | 65/m | T4N2M0 | + | – | – |
| 18a | Base of tongue | P | Malignant | 62/m | T4N2M0 | + | – | +++ |
| 19a | Base of tongue | P | Malignant | 55/m | T3N1M0 | + | – | +++ |
| 20a | Base of tongue | P | Malignant | 59/m | T4N2M0 | + | – | – |
| 21c | Thyroid gland | M | Malignant | 68/m | TxN2M1 | + | ++ | + |
| 22d | Parotid gland | / | Benign | / | / | + | + | – |
| 23d | Parotid gland | / | Benign | / | / | + | + | – |
| 24a | Base of tongue | P | Malignant | 55/m | T3N1M0 | ++ | +++ | ++ |
| 25a | Tonsils | P | Malignant | 44/f | T2N1M0 | +++ | +++ | ++ |
| 26 | Larynx | P | Malignant | 51/m | T2N0M0 | +++ | +++ | ++ |
| 27 | Larynx | P | Malignant | 58/m | T3N2M0 | +++ | +++ | +++ |
| 28a | Base of tongue | P | Malignant | 66/f | T3N0M0 | ++ | +++ | +++ |
| 29e | Hypopharynx | M | Malignant | 50/m | T2N2M1 | +++ | +++ | +++ |
| 30 | Larynx | P | Malignant | 56/m | T2N2M0 | +++ | – | – |
| 31 | Larynx | P | Malignant | 64/m | T4N0M0 | +++ | +++ | +++ |
| 32 | Larynx | P | Malignant | 61/f | T4N1M0 | +++ | +++ | +++ |
| 33 | Hypopharynx | P | Malignant | 64/m | T4N2M0 | +++ | + | + |
| 34 | Larynx | P | Malignant | 66/m | T4N1M0 | +++ | – | ++ |
| 35 | Oropharynx | P | Malignant | 56/m | T4N1M0 | +++ | +++ | ++ |
| 36 | Larynx | P | Malignant | 68/m | T3N0M0 | +++ | +++ | +++ |
| 37 | Larynx | P | Malignant | 77/f | T3N2M0 | +++ | ++ | +++ |
| 38 | Hypopharynx | P | Malignant | 45/m | T3N2M0 | ++ | – | + |
| 39 | Hypopharynx | P | Malignant | 67/m | T4N3M0 | +++ | + | + |
| 40 | Larynx | P | Malignant | 70/m | T3N0M0 | ++ | +++ | +++ |
| 41b | Larynx | / | Benign | 11/m | / | ++ | – | – |
| 42 | Larynx | P | Malignant | 58/m | T3N2M0 | +++ | + | +++ |
| 43 | Hypopharynx | P | Malignant | 64/m | T2N1M0 | ++ | + | – |
| 44 | Hypopharynx | P | Malignant | 69/f | T2N0M0 | ++ | + | – |
| 45 | Larynx | P | Malignant | 73/m | T2N0M0 | +++ | ++ | ++ |
| 46c | Hypopharynx | M | Malignant | 53/m | TxN2M1 | ++ | – | – |
| 47f | Lymph node | M | Malignant | 49/m | T4N2M0 | +++ | – | + |
| 48f | Lymph node | M | Malignant | 55/m | T3N2M0 | +++ | + | – |
| 49e | Lymph node | M | Malignant | 63/m | T3N2M0 | +++ | ++ | +++ |
| 50e | Lymph node | M | Malignant | 67/m | T4N3M0 | +++ | ++ | +++ |
| 51e | Lymph node | M | Malignant | 79/m | T4N2M0 | +++ | + | – |
| 52c | Lymph node | M | Malignant | 53/f | TxN2M0 | +++ | – | ++ |
| 53e | Lymph node | M | Malignant | 80/f | T3N2M0 | + | + | – |
| 54e | Lymph node | M | Malignant | 46/m | T4N2M0 | +++ | +++ | +++ |
| 55a | Tonsils | P | Malignant | 46/f | T3N3M0 | +++ | +++ | + |
| 56a | Tonsils | P | Malignant | 51/f | T3N2M0 | +++ | + | +++ |
| 57c | Hypopharynx | M | Malignant | 90/f | TxN3M1 | +++ | +++ | +++ |
| 58e | Lymph node | M | Malignant | 45/m | T3N2M0 | +++ | + | +++ |

Table I. *continued overleaf*

Table I. *continued*

| No. | Location | Type | Designation | Age (years)/Gender | TNM | GAPDH | EGFR | LOXL4 |
|---|---|---|---|---|---|---|---|---|
| 59g | Parotid gland | M | Malignant | 68/m | T1N1M1 | +++ | +++ | – |
| 60h | Paranasal sinuses | P | Malignant | 82/f | T4N0M0 | ++ | +++ | +++ |
| 61h | Paranasal sinuses | P | Malignant | 65/f | T4N0M0 | ++ | + | – |
| 62a | Base of tongue | P | Malignant | 55/f | T3N0M0 | +++ | +++ | – |
| 63 | Mucosa | / | Benign | 66/f | / | ++ | – | – |
| 64 | Mucosa | / | Benign | 36/f | / | ++ | – | – |
| 65 | Mucosa | / | Benign | 42/m | / | +++ | – | – |
| 66 | Mucosa | / | Benign | 59/m | / | ++ | – | + |
| 67 | Mucosa | / | Benign | 57/m | / | ++ | ++ | – |
| 68 | Mucosa | / | Benign | 78/m | / | ++ | – | – |
| 69 | Mucosa | / | Benign | 73/m | / | ++ | ++ | – |

a: These tumours formally belong to oropharyngeal cancer; b: biopsy of papillomatosis; c: metastasis from cancer of unknown primary; d: biopsies of parotid adenoma; e: primary in hypopharynx; f: primary in larynx; g:primary in nasopharynx; h: exact location: maxillary sinus. P: Primary HNSCC; M: metastasis; m: male; f: female; (–) no expression; (+) weak expression; (++) medium expression; (+++) strong expression; /, not applicable.

seven epithelial cell cultures established from the oral mucosa of healthy individuals were used as control samples. All samples were retrieved during surgery at the Department of Otorhinolaryngology (University Kiel, Kiel, Germany), following informed consent approved by the local Ethics Committee (AZ:D413/07) and the study was, therefore, performed in accordance with the ethical standards of the 1964 Declaration of Helsinki. The control samples were taken during regular surgical treatment without additional excision. Patient characteristics, specimen characterization and histopathological parameters are summarised in Table I.

*Isolation of RNA*. For rapid and reproducible RNA preparation, Trizol total RNA kit (Fermentas, St. Leon-Rot, Germany) was used according to the manufacturer's instructions. Measurement of the RNA concentration was carried out photometrically at 260 nm.

*Northern hybridisation*. For the Northern hybridisation analysis, 20 μg heat-denatured RNA derived from HNSCC and healthy mucosa samples were used. Agarose electrophoresis and subsequent transfer on nitrocellulose were carried out according to the method described by Sambrook *et al*. (5).

For the construction of the *EGFR* probe, RT-PCR with *EGFR* gene specific oligonucleotides was conducted using 5'-ctctggatccacaggaactg-3' as the sense and 5'-gtggcactgtatgcactcag-3' as the antisense oligonucleotide. The probe was labelled with digoxigenin (Roche Diagnostics, Mannheim, Germany). LOXL4 and GAPDH probes were prepared as described previously (6).

Quantification of the Northern hybridisation results was performed using the gel documentation system EASY Win-32 (Herolab, Wiesloch, Germany). Densitometric values were therefore defined from the sum of the pixel intensities of weak (+), moderate (++), and strong bands (+++) to create a calibration curve, where the GAPDH band intensity was equated with moderate expression.

*Statistical analysis*. Data analysis was performed using the two-tailed Fisher's exact test. The following parameters were considered in the analysis: TNM stage, primary tumour *vs*. metastasis, tumour localisation, age and gender. Probability *p*-values lower than 0.05 were considered as statistically significant.

## Results

*Comparison of malignant and benign samples*. In 71% of the HNSCC samples, the *LOXL4* gene was found to be significantly overexpressed in contrast to the benign mucosa specimens (9%) used ($p$=0.0002). Regarding the *EGFR* gene, the overexpression amounted to 78% in HNSCC *vs*. 36% in benign mucosa without significant differences ($p$=0.01). Further analysis revealed no *LOXL4* gene expression in the benign tumour specimens (2 papillomatoses, 2 parotid adenomas) ($p$=0.01), whereas 50% of these samples were positive for *EGFR* gene expression.

*Gene expression with regard to TNM stage*. The gene expression in T1-T3 tumours (T1=2%, T2=17%, T3=35%) was compared to the expression in stage T4 tumours (40%). Expectedly, *EGFR* and *LOXL4* were overexpressed in both groups, however, no significant difference was found between stage T1-T3 and stage T4 tumours (*EGFR*: 87% *vs*. 70%, $p$=0.2; LOXL4 65% *vs*. 78%, $p$=0.4).

The analysis of gene expression in tumours with local lymph node metastasis (stage N1-N3, n=38) and without metastasis (stage N0, n=14) revealed no significant differences between metastatic and non-metastatic tumours (*EGFR*: 93% *vs*. 71%, $p$=0.1; LOXL4: 64% *vs*. 74%, $p$=0.5). Interestingly, all tumours in stage N3 expressed both *LOXL4* and *EGFR* (n=4). Similarly, analysis of stage M0 tumours (n=52) *vs*. stage M1 (n=6) showed no significant differences in the expression pattern of either gene (*EGFR*: $p$=1; *LOXL4*: $p$=1).

A comparison between primary tumours (n=44) and metastases (eight local lymph node metastases and six distant metastases) revealed an overexpression of *LOXL4* and *EGFR* in both metastases and primary tumours (*EGFR*: metastases 79%, primary tumours 77%; *LOXL4*: metastases 64%, primary tumours 73%).

*Gene expression data in terms of tumour location, age and gender.* In addition to TNM, the *EGFR* and the *LOXL4* gene expressions were analysed in 44 primary tumours with regard to tumour location (base of tongue: n=7, tonsils: n=4, other location in the oropharynx: n=4, larynx: n=16, hypopharynx: n=11 and paranasal sinuses: n=2). Of the tumour biopsies representing different locations, the analysis showed gene overexpression in all tumour locations. Finally, gene expression data were analysed statistically to determine possible correlations with age and gender. No significant correlations were found between gene expression and either age (age range: 44-64 years, n=30, *EGFR*: 70%, *LOXL4*: 80%; age 65-90, n=28, *EGFR*: 86%, *LOXL4*: 61%) or gender (*LOXL4*: male 76%, female 59%, $p$=0.2). However, a more frequent expression of *EGFR* in tumours of female patients was noticeable compared to male patients (female 94%, male 71%, $p$=0.08).

## Discussion

In recent years, there have been many studies evaluating molecular markers of HNSCC aiming to obtain a reliable indicator of tumour differentiation or prognosis.

Although the body of literature continues to grow at a high rate, there is still little knowledge on the prognostic and therapeutic value of the markers in the diagnosis and treatment of HNSCC. The early detection of recurrences after surgical or radiation treatment of squamous cell cancers of the head and neck is often difficult. Molecular markers such as the squamous cell carcinoma antigen (SCCA) and the carcinoembryonic antigen (CEA) give an insight into the biology of HNSCC. Thus, the combined analysis of these markers can facilitate the early detection of local relapse or distant recurrence and can therefore accelerate specific diagnostic and/or therapeutic procedures (7). Another tumour-associated antigen is the epithelial cell adhesion molecule (EpCAM) which is differentially glycosylated in all head and neck carcinomas in which its expression is detectable (8). The accumulation of this protein in disseminated tumour cells is of particular importance as such cells are considered as the origin of metastases.

Chaubal *et al*. (9) investigated the suitability of EpCAM to serve as a specific marker for disseminated tumour cells and found that this adhesion molecule can be used as a tumour marker for the diagnosis of single tumour cells in patients suffering from HNSCC.

An increasing number of publications highlight the importance of the tumour marker Cyfra 21-1 (10-12). Patients with Cyfra 21-1 <1.5 ng/ml had a higher survival rate compared to patients with Cyfra 21-1 1.5 ng/ml (13). The authors suggested that the elevated levels of Cyfra 21-1 without clinical evidence of disease can be attributed to the marker's mean lead-time as compared to the clinical appearance of the disease.

*EGFR* is a member of the ErbB family of receptors and its stimulation by endogenous ligands, EGF or transforming growth factor-alpha (TGF-α), results in the activation of an intracellular tyrosine kinase. High levels of *EGFR* expression are correlated with poor prognosis and resistance to radiation therapy in a variety of cancers, mostly in HNSCC (14). Most preclinical and clinical studies demonstrated a lower local control after radiation therapy in tumours overexpressing *EGFR* (15). A study in 140 patients with primary laryngeal squamous cell carcinoma showed that the 5-year survival rate was 81% for patients with *EGFR*-non-expressing tumours, compared with 25% for patients with *EGFR*-expressing tumours ($p$<0.0001) (16). These results were also confirmed by other studies (17).

In a quantification study, Nestor *et al*. (18) analysed molecular targets in HNSCC and found that *CD44v6* is generally expressed at a higher level than *EGFR*, suggesting that CD44v6 could be more readily targeted by the specific monoclonal antibodies cetuximab and U63.

Comparative analysis of clinical relevance of the HNSCC markers CEA, carbohydrate antigen (CA19-9), SCCA, thymidine kinase and deoxythymidine-5'-triphosphatase revealed no significant correlation between serum levels and tumour location, staging, or grading, suggesting that the routine assessment of this markers is of limited practical value (19). In an analysis of twenty-nine molecular markers which are associated with tumour grading and different degrees of dysplasia in premalignant lesions, only the nucleolar organizer regions, which are considered to be required for RNA transcription, were of prognostic significance in oral SCC (20).

Despite the increasing information on tumour associated molecules, as in most human carcinomas, the findings are often disparate to determine the chronology of the molecular events leading up to the malignant transformation of the cells in the head and neck region (21). Furthermore, it should be noted that there is often insufficient information due to the small sample size used in individual studies. Schliephake noted that the studies which were able to show a significant association between the occurrence of a marker and the prognosis of the disease have considerably larger patient populations on average than those which failed to do so (20). Another shortcoming for the low predictive value of the HNSCC markers is the fact that most markers are detectable not solely in HNSCC but also in their non-neoplastic benign counterpart.

In HNSCC, *EGFR* and its ligand, TGF-α, are overexpressed in 80-90% of cases; the corresponding magnitudes of increase are 1.7-fold ($p$=0.005) and 1.9-fold ($p$=0.006), respectively, when compared to healthy controls (22).

As shown in the present study, high frequency *EGFR* gene expression was detected in the tumour biopsies (78%); however, 36% of the benign mucosa samples were also

positive for this gene. This finding was not observed for the *LOXL4* gene where only 9% of the benign mucosa revealed *LOXL4* positivity. In a previous study (6) and recently (4), using a large cohort of patients with HNSCC, it was shown that LOXL4 expression is detectable in 92.7% of primary tumours, in 97.8% of lymph node metastases and in affected oral mucosa with high-grade dysplasia; however, it was absent from various non-neoplastic tissues of the head and neck (4).

In conclusion, the present study showed that *LOXL4* is a suitable tumour marker due to its high percentage expression in histopathologically varied HNSCC, suggesting its functional significance in carcinogenesis of HNSCC.

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