EGFR and Cortactin: Markers for potential double target therapy in oral squamous cell carcinoma

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Abstract. Survival periods of patients following surgical therapy of oral squamous cell carcinoma (OSCC) have previously been demonstrated to decrease over recent decades. Epidermal growth factor receptor (EGFR) and Cortactin are molecular markers that are important in tumour progression and development, and interact within the EGF pathway. Although EGFR antibody therapy exists, sufficient efforts for increased survival are still lacking due to the present limited response rates. The aim of the present study was to examine the association between EGFR and Cortactin expression on survival rates of OSCC patients and to determine whether EGFR and Cortactin expression levels are associated with advanced tumor sizes and lymphnode-metastases. In total, 222 OSCC patients were included in the study. EGFR and Cortactin expression in tumor tissue was evaluated by immunohistochemistry. Cox regression was used for survival analysis. Categories were tested for associations by using cross tabs (Chi-square test). Groups were compared by the non-parametric Mann Whitney U-test. Probabilities of less than 0.05 were considered significant and significant expression of Cortactin was observed in Advanced Union Internationale Contre le Cancer stage (P=0.032), including advanced tumour stage (P=0.021) and lymph node metastasis (P=0.049). High Cortactin expression was significantly associated with poorer survival rates (P=0.037). Further Cortactin expression was not associated with extracapsular spread, however EGFR exhibited a significant association (P=0.034). Neither EGFR nor Cortactin expression was correlated to grading. EGFR and Cortactin co-expression was demonstrated to be significantly associated with poorer survival rates in OSCC patients, suggesting that identification of predictive biomarkers for adjuvant therapies are of primary concern in OSCC. In particular, efficient dual-target therapy may act as an appropriate therapy to improve survival time for patients at advanced OSCC tumor stages.

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Introduction

Oral cancer is one of the most common cancers. Male patients between the fifth and sixth decade are mainly affected by oral squamous cell carcinoma (OSCC) (1). Advanced stages are often present at primary diagnosis. Unfortunately, new insights into the understanding and further treatment options of OSCC are still lacking (1). Surgery has advanced opportunities for good functional and reconstructive results (2). Nevertheless, recurrence and second tumours are dominating and influencing survival rates (3). TNM classification and grading were discussed as having a high impact on patients outcomes. However, even recent studies indicate that this is not a sufficient explanation system for predicting recurrence (4). Poor survival rates reflect the status quo of OSCC and further therapy is frustrating in many cases. The need to identify molecular markers with a therapeutical impact on metastasis and recurrence is urgent. The correlation between tumour biology and survival rates is becoming more relevant. Further, tumour biology might lead to suitable therapeutic strategies. The creation of a variable patient-specific key target therapy with acceptable side effects is a main goal of today's research (5). Various molecular markers have emerged and have provided a new understanding of pathogenesis in OSCC. Epidermal growth factor receptor (EGFR) is one well studied target. The EGFR tyrosin kinase and its signal transduction pathway is a key route for distinct molecular interactions. Intracellular signalling chains, e.g., Ras/mitogen-activated protein kinase (MAPK) and the activation of transcription and extracellular chains, e.g., extracellular signal-regulated kinase (ERK) are activated through the EGFR. Endpoints of the signalling chain are supporting tumour growth, invasion, angiogenesis, metastases and interactions with lymph nodes. Essential research was carried out on the EGFR pathways with the emergence of EGFR antibody therapies (6).

Recently, three EGFR antibodies were developed. Cetuximab is a monoclonal immunglobulin G1 antibody inhibiting the receptor, whereas Erlotinib and Afatinib are

blocking proteins of the ErbB family (7) and inhibit the tyrosin kinase activity of the receptor. To date, Cetuximab is the only EGFR antibody used in OSCC. EGFR overexpression was associated with poor prognosis and decreased survival rates in several OSCC studies. The first clinical study use of Cetuximab therapy in combination with radiotherapy in advanced OSCC was assessed in the often-cited study of Bonner *et al* (8). Effects of EGFR antibody therapy on survival rates were reported within this trial, but with a limited time benefit for the patients.

Despite these milestones at the beginning of the clinical use, the treatment response to EGFR inhibitors is not always sufficient resulting in a low or lack of impact on survival rates and is also dependent on the amount of EGFR expression (6). Further, up to 12 potential ligands next to EGF exist that could interact with the receptor and mutations of the receptor are not rare and could negatively interact with the response (9). Therefore improvements in therapy influencing survival rates are necessary. One option to maximise therapeutical effects is to block more targets than one in a single signal transduction pathway. Dual-blocking or dual-targeting was also considered as a method to enhance the anti-tumour response and is a topic of high clinical impact (10). The combination of antibodies targeting two patterns offers the chance to strengthen effiacacy without increasing side-effects. One substrate of the EGFR cascade is Cortactin (11). Cortactin is located within the cytoplasm and around the nucleus. It also co-localises with actin in the plasma membrane and at peripheral adhesion sites (12,13). Currently, the activity of Cortactin presents many unsolved questions because of its complexity (14). Important actions of Cortactin include cell spreading and adhesion (15). Hence, Cortactin is clearly also essential in tumour progress. An interpretation of the intensity of staining, of the proportion of stained cells, of the score of positivity and of the use of recommended scores is possible and has to be carried out carefully and independently (16). Therefore, our aim was, to use immunohistochemistry (IHC) indexing to create subgroups with meaningful numbers of patient samples in order to avoid overlaps, any interference of subgroups with insufficient numbers and any lack of clarity. Our objective was further to investigate the clinicopathological and prognostic significance of the co-expression of EGFR and Cortactin via immunohistochemical staining and to determine whether a collective of OSCC patients had sufficient numbers for evaluation.

Patients and methods

Patients. In total, 222 patients were included in the current study. They were treated between 2009 and 2011 at our maxillofacial surgery department. Relevant data (Table I) from patients diagnosed with OSCC for statistical evaluation and formalin fixed and paraffin embedded tissue (FFPE) for laboratory use were available in every single case. Regular follow up examinations of every included patient were held at our department according to the German guidelines of oral cancer (17). All included patients received regular follow up. In the first 2 years after the diagnosis the follow up was done every 3 months, after 2 years the follow up was done every 6 months until the fifth year. After the fifth year our follow up was completed.

Table I. Clinical Parameters of the cohort-not subdivided.

Clinical Parameters	Total (n=222)
Median age in years (range)	60.1 (49.2-69.7)
Gender	
Male/female	175/47
UICC stage	
I	25
II	40
III	42
IVa	118
Tumour size	
T1	46
T2	92
T3	34
T4a/b	50
N Stage	
N0	97
N1	37
N2	88
Extracapsular spread	24
Grading	
G1	12
G2	113
G3	97

The therapy regimes of the included patients were primary surgery, with intra-operative margin control via the help of frozen sections and with neck dissection with the intention of curative treatment. All tumour tissues were collected at the main tumour operation, which also included neck dissection. The tumour was operated by excisional biopsy of the whole tumour. Postoperative adjuvant cisplatin-based chemoradiation was performed in cases of pN1, pN2 or tumour infiltration of the jaw or locally infiltrating tumour growth of the oral cavity (T4a/b) and of positive microscopic resection margins and/or extracapsular spread, also according to the German guidelines for oral cancer as previously described (18).

Exclusion criteria were death resulting from a cause other than OSCC, distant metastasis at primary diagnosis and the use of primary radiochemotherapy before operation. The methods were approved by the ethics committee of the Technische Universität München (no. 212108) and are in accordance with the Declaration of Helsinki.

Tissue microarray (TMA) construction. Two independent pathologists defined the centre of the tumour and the invasion front of every study patient. The tissue was formalin-fixed and paraffin-embedded in blocks. The pathologists then marked the areas to be represented in the TMA. A minimum of two tumour cores from the centre of the tumour, the invasion front and the corresponding lymph nodes with a 6-mm core size were assembled into the TMA by using a Tissue Microarrayer (Beecher Instruments, Inc., Sun Prairie, WI, USA) as previously described (18,19). All lymph nodes, used for the TMA,

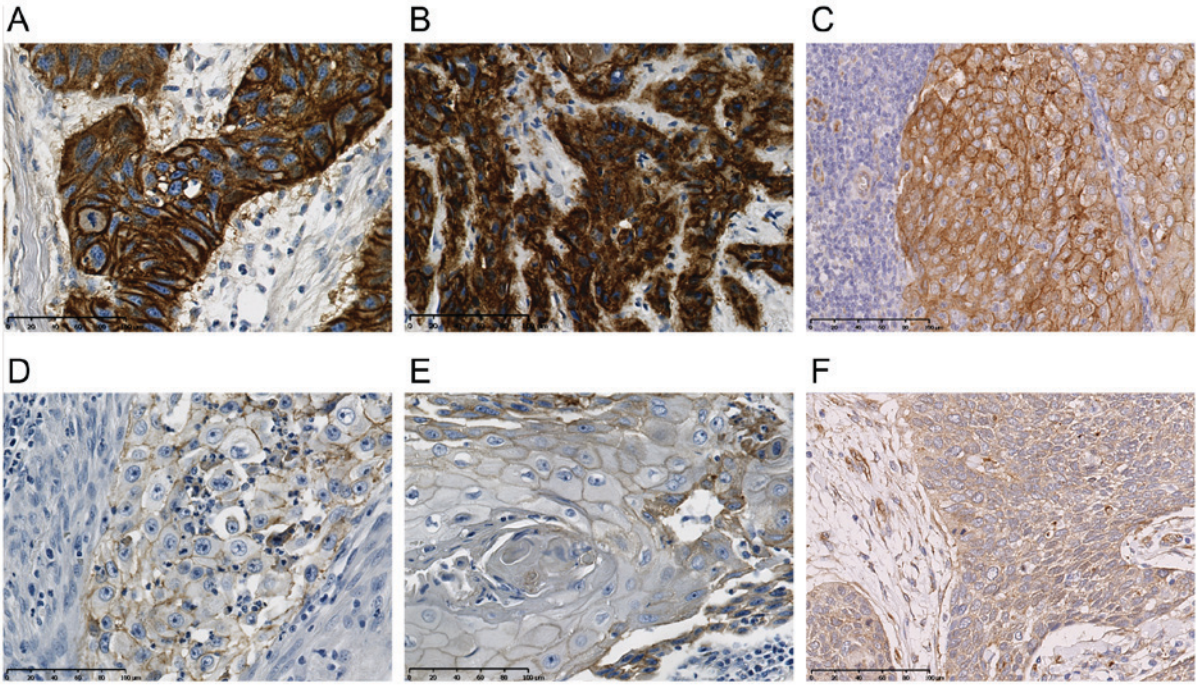

Figure 1. Immunohistochemistry results. (A) membrane EGFR II, (B) cytoplasmic EGFR II, (C) Cortactin II, (D) membrane EGFR I, (E) cytoplasmic EGFR I, and (F) Cortactin I. EGFR, epidermal growth factor receptor.

were positive lymph nodes if the patient had positive lymph nodes. If the patient had no positive lymph nodes, negative lymph nodes were taken. Therefore lymph nodes of every patient were presented in the TMA.

Immunohistochemistry. Immunohistochemical staining was performed as described previously (20) by using 4-μm-thick sections of the TMA. The sections were incubated with primary antibodies against EGFR (1:50; Dako, Hamburg, Germany); and Cortactin (1:100; BD Bioscience, Heidelberg; Germany) overnight according to the manufacturers' recommendations.

Scoring. Immunohistochemical samples were blind-scored by two investigators and checked by one pathologist. EGFR and Cortactin staining was evaluated under a light microscope (magnification, x200). The immunostaining intensity and positive cell proportion were assessed for both markers. Further, the staining was evaluated via an immunoreactive score (IRS) (21). We also evaluated the EGFR expression of both the cell cytoplasm and the cell membrane independently, since EGFR has two cellular loci of expression.

The staining intensity score was adjusted on a scale of 0-1-2-3: no staining was scored as 0; weak staining as 1; intermediate staining as 2; and strong staining as 3. Positive cell proportion was also assigned (0<25%; 1 if 25-50%; 2 if 50-75%; and 3 if >75%) as previously described (22). For a combination of quality and quantity, scores of intensity and quantity were multiplied (IRS results: 0-1-2-4-6-9). For the evaluated markers of the current cohort a final cut off score was determined as I (low expression: 0-4) and II (high expression: 6-9).

Statistical analysis. Data was analyzed with the SPSS for Windows, release 24.0.0, 2016 (SPSS, Inc., Chicago, IL, USA) and results were presented as figures. Cox regression and Kaplan Meier curves were used for survival analysis. Categories were tested for associations by using cross tabs (Chi-square test). To compare groups, the non-parametric Mann Whitney U-test was used. $P<0.05$ was considered to indicate a statistically significant difference.

Results

IHC scoring system of EGFR and Cortactin. We analysed staining as described in the methods section for cytoplasmic EGFR, membrane EGFR and Cortactin. The staining results of the cut off value groups I and II for the evaluated markers of the current cohort are shown in Fig. 1 (cytoplasmic EGFR, membrane EGFR and Cortactin).

Association of Cortactin expression to the survival rates. We used the Chi-square test to compare the clinicopathological parameters between the Cortactin low expression (I) and high expression (II) group. We did these tests for every TMA localisation. All the following results are only valid for the expression of the central tumour area. The invasion front and lymph nodes had no impact on the evaluation of expression. The analysis showed that overall survival was significantly poorer (P=0.037) in the case of Cortactin II: 50.3 months [SD 3.59; 95% confidence interval (CI): 43.28-57.36] compared with Cortactin I: 63.7 months (SD 4.82; 95% CI: 54.22-73.13: Kaplan Meier curves of Cortactin). Remarkably, during the analysis, Cortactin with a high expression score had an influence on clinicopathological data (Table II). Cortactin II was significantly associated with advanced UICC stages, especially III and IV (P=0.032). T1 stages were rare in Cortactin II (P=0.021). The incidence of lymphatic invasion (P=0.049) also dominated in Cortactin II and showed

Table II. Clinical parameters of the cohort-subdivided to EGFR and Cortactin expression.

Clinical parameters	EGFR	Cortactin
Median age in years (range)	57.6 (49.5-64.8)	57.4 (44.3-65.8)
Gender		
Male/female	84/15	91/32
UICC stage		
I	15	10
II	14	26
III	19	23
IVa	49	66
Tumour size		
T1	28	18
T2	33	59
T3	12	22
T4a/b	25	25
N Stage		
N0	44	53
N1	21	16
N2	30	58
Extracapsular spread	8	16
Grading		
G1	7	5
G2	47	45
G3	66	52

EGFR, epidermal growth factor receptor.

significantly more N2 stages in this cohort. Grading (P=0.057) and extracapsular spread (P=0.15) had no influence.

Association of EGFR expression to the survival rates. The Chi-square test was also used to compare the clinicopathological parameters between the EGFR low expression (I) and high expression (II) group. We performed these tests for every TMA localisation as for the Cortactin cohort. We evaluated cytoplasmic EGFR and membrane EGFR. All the following results are only valid for the expression of the central tumour area. The invasion front and lymph nodes had no impact on the evaluation of expression. The analysis showed that overall survival did not differ in dependence on EGFR expression (cytoplasmic EGFR, P=0.636; membrane EGFR, P=0.978). The average survival of the cytoplasmic EGFR cohort for I was: 84.5 months (SD 7.98; 95% CI: 68.92-100.18) and for II was 89.6 months [SD 7.49; 95% CI: 74.92-104.30, Fig. 2A (Kaplan Meier curves of cytoplasmic EGFR)]. The membrane EGFR cohort had an average survival for I of 99.5 months (SD 13.29; 95% CI: 73.43-125.50) and for II of 99.5 months [SD 13.29; 95% CI: 73.43-125.50, Fig. 2B (Kaplan Meier curves of membrane EGFR)]. Furthermore, cytoplasmic EGFR and membrane EGFR in an high expression score did not have an influence on clinicopathological data (Table II). EGFR was not significantly associated with advanced UICC stages

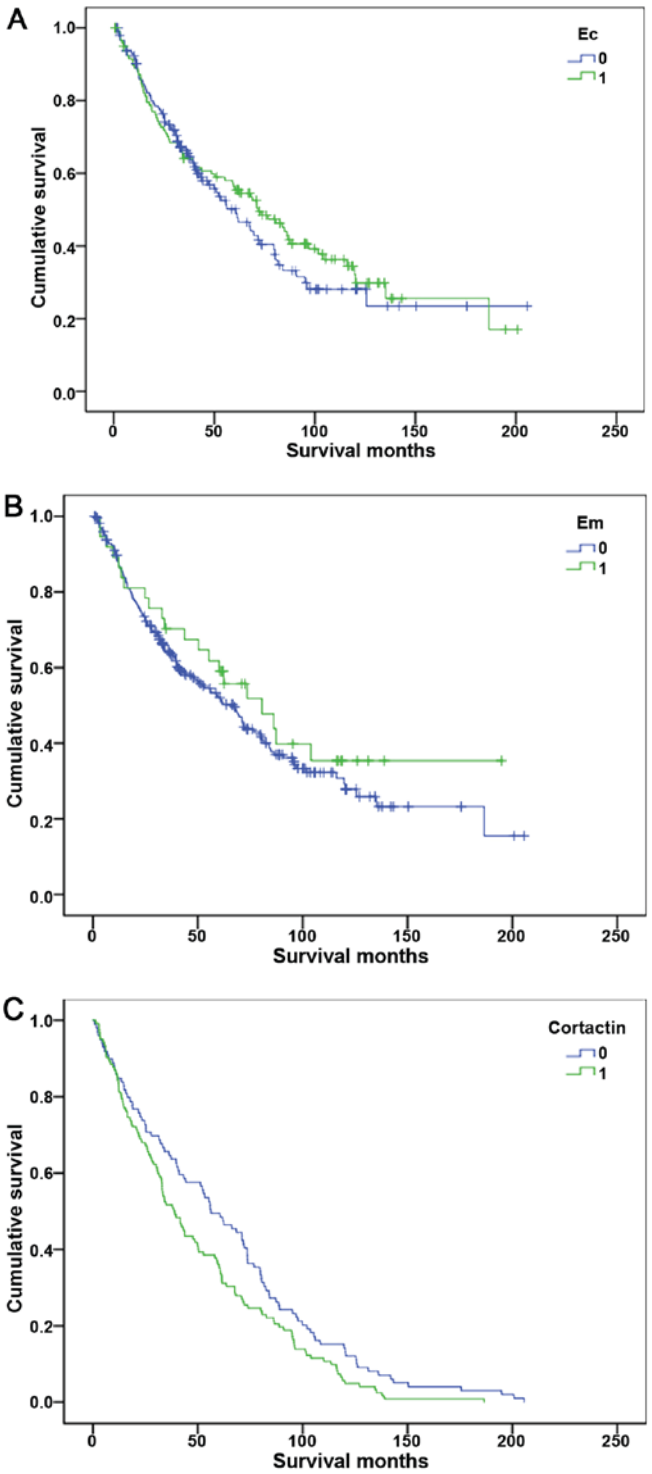

Figure 2. Graphical survival analysis. (A) Kaplan Meier curves of Cortactin subgroup I (marked as '0' curve) and II (marked as '1' curve). (B) Kaplan Meier curves of cytoplasmic EGFR (Ec) I (marked as '0' curve) and II (marked as '1' curve). (C) Kaplan Meier curves of membrane EGFR (Em) I (marked as '0' curve) and II (marked as '1' curve). EGFR, epidermal growth factor receptor.

(cytoplasmic EGFR, P=0.094; membrane EGFR, P=0.113) nor T stage (cytoplasmic EGFR, P=0.670; membrane EGFR, P=0.439) or N stage (cytoplasmic EGFR, P=0.473; membrane EGFR, P=0.113). Moreover, grading (P=0.33) had no influence. Remarkably, extracapsular spread was significantly associated with high cytoplasmic EGFR expression (P=0.034).

Association of EGFR expression to Cortactin expression and the survival rates. Interestingly, a strong co-expression in the tumour centre of EGFR II and Cortactin II led significantly to reduced survival rates (P=0.04) with a median of a reduction in survival by 8 months.

Clinical data. Relevant clinical data from the 222 included patients with an OSCC diagnosis are listed in Table I. The average survival of the cohort was 88.4 months (SD 4.8; 95% CI: 78.84-90.04). Risk factors such as smoking and alcohol consumption were evaluated, with approximately 50% of the patients having a positive anamnesis. Expression of EGFR II and Cortactin II in combination of smoking and regular alcohol consumption was observed in 10% of all patients.

Lymph node recurrence played a major role in survival (P=0.028), whereas local recurrence did not (P=0.128). Lymph node recurrence occurred in 21 patients, which means that recurrence of lymph nodes metastasis occured after surgical removal and neck dissection. Local recurrence was evaluated in 35 patients.

The UICC stage (P=0.031), age of patients (P=0.012) and lymph node metastasis (P=0.003) at the time of primary diagnosis had a significant influence on overall survival rates.

In contrast, patient gender, T category, extra capsular spread and tumour grading were not significantly associated with overall tumour-related survival (P>0.05) and were independent of the marker expression status.

Discussion

The EGF cascade is an important pathway that is upregulated in a high percentage of human tumours (23). EGF and its receptor have complex influences on cell signalling and are key targets in oncology. EGFR expression has been studied in various malignomas (24) and EGFR interaction was often correlated with survival rates (25). Several studies emphasised that expression level of EGFR is proportional to recurrence, therapy failure and worse overall survival in OSCC (26). However, on the other hand, various authors have argued that this does not reflect reality and, to date, many trials are questioning the statement of the proportionality of EGFR to worse survival rates (27). Therefore, one of our aims was to do further research in the field of EGFR expression in a cohort of OSCC with a large number of patients. Our results from the current study do not confirm the unlimited correlation of high EGFR expression to lower survival rates. Monoclonal antibody therapy is linked to specific targets such as glycoproteins, vascular targets, growth factors, stromal antigens and the cluster of differentiation antigens (28). These therapies are applied only in well-defined special clinical cases. Currently, these individual target therapies are rescue therapies in OSCC and other malignancies and are applied after the first- or second-line therapy failed or in the case of recurrence after the first line therapy protocol has been administered (29). Further, these antibody therapies depend on the expression of the molecular target in order to be started. In the case of OSCC, EGFR antibody therapy is selected if cisplatin-based radiotherapy was unable to lead the tumour into remission (6). Cetuximab is the antibody of choice in the therapy of OSCC. The tyrosin kinase inhibitors Erlotinib and Afatinib are developed e.g., for use in lung cancer and gastric cancer (30,31). Yet these tyrosin kinase inhibiting antibodies are not authorised for the clinical use in OSCC. Due to our results showing the lack of influence of EGFR expression on survival rates, as previously suggested by other studies, we emphasise hereby the importance of double-target blocking with additional key targets as EGFR monotherapy might not be sufficient to eliminate EGFR-positive tumours (32). Another important issue is that resistance of EGFR to the antibody therapy are emerging, and could cause an altered therapy response. In particular, associations to the expression of multi-drug resistance proteins are newly being discussed (33,34). Molecular cross-talk offers options for identifying targets for future therapies (35). Further, a strong clinical correlation of every target is necessary. Several co-targets are considered in the literature. Cortactin plays a major role in cell interactions and Cortactin influences survival in OSCC in a significant way according to our current results. We could set a proof-of-principle in our cohort with regard to the influence of Cortactin in OSCC. To the best of our knowledge, the expression of both EGFR and Cortactin was not evaluated previously in OSCC. Our results show, that these interactions should not be disregarded. Discordance to previous published results regarding Cortactin expression can be explained on the basis of the use od smaller cohorts (36). Evaluation methods such as IHC scoring must be extremely detailed and well thought out to provide safe prognostic values of potential biomarkers (16,37). Therefore, we conducted IRS scoring and further divided the collective into the score cohorts I and II to avoid any interferences of subgroups with insufficient numbers. Moreover, we evaluated distinct localisations of the tumour, as also heed in the TMA: the centre of the tumour, the invasion front and the corresponding lymph nodes because of the potential differential expression of the biomarkers (38). Hence, we evaluated the mentioned tumour regions independently. Our results showed that significant interactions of Cortactin occur in the central tumour area. The area of tumour invasion and the lymph nodes play no significant role in Cortactin expression and have no influence on clinical features. In previous studies, Cortactin expression was reported in advanced stages of OSCC (39). Nevertheless, none of these few studies evaluated distinct tumour areas separately for Cortactin (40) as it was conducted successfully in the present study. EGFR staining is very common in clinical routine and is the basis of several studies. However, to our knowledge, studies having the topic EGFR and OSCC did not differ between the two expression sites of EGFR as we have for cytoplasmic EGFR and membrane EGFR (41). In the present study we were able to evaluate differences in these localisations. We found a significant correlation of high cytoplasmic EGFR expression and extracapsular spread in the central tumour area. In the literature, this interesting fact was not reported before. Only the general presence of EGFR expression, rather than its detailed cellular localisation and increased extracapsular spread were reported (42). According to the present understanding of molecular oncology, the differential results regarding the localisations of EGFR expressions might lead to significantly different outcomes and should be taken into consideration. The results of our current study based on a cohort with a large size and the complete availability of after-care data, suggest that the staining and evaluation of Cortactin expression have translational clinical impact and the results of our study

are of high relevance. In particular, the majority of included patients were primarily diagnosed with advanced UICC stages (III and IV). Curative surgical treatment is often not possible for these stages and further therapy strategies are all the more important for these cohorts. Our results indicate for the first time that Cortactin is a protein having a concomitant and not a compensatory pathway next to EGFR. This result is essential, since cross-talk therapy is based on molecules that are independent of each other in expression. In summary, we showed that the dual-antibody-therapy targeting EGFR and Cortactin is superior to EGFR targeting alone in OSCC (43). Cortactin might represent an important molecule for the therapeutic approaches urgently needed to solve the problems of mutations and therapy resistances (44) and of recurrence. Regarding other malignancies, for example lymphocytic leukaemia, Cortactin plays also an important role as a checkpoint molecule (45). In colon cancer, Cortactin promotes cell migration and invasion (46). These findings are showing the importance of further studies with the subject Cortactin.

Immunohistochemical evaluations have their limitations. Because only protein expression can be evaluated by IHC, genetic profiles and further cellular interactions remain unknown. Further studies are needed to answer these open molecular questions.

Our results indicate that Cortactin could be a prognostic marker for OSCC and also that the co-expression of EGFR and Cortactin could have a clinical impact on survival rates. The development of a Cortactin antibody to improve the stagnated survival rates of OSCC patients is worthy of further studies. Mainly in advanced UICC stages (III and IV) this cross link antibody therapy could be the future therapy of choice, since conventional therapies have only a limited range. The genetic regulations of these markers should now be evaluated to substantiate the findings of the current study.

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