Characterisation of Prognosis and Invasion of Cutaneous Squamous Cell Carcinoma by Podoplanin and E-Cadherin Expression

Kristina Hesse a Imke Satzger a Vivien Schacht a Brigitta Köther a Uwe Hillen b Joachim Klode b Katrin Schaper a Ralf Gutzmer a

a Skin Cancer Center Hannover, Department for Dermatology and Allergy, Hannover Medical School, Hannover, and b Department for Dermatology, University Medical Center Essen, Essen, Germany

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Abstract
Background: Around 5% of all cutaneous squamous cell carcinoma (cSCC) metastasise. Metastases usually locate in regional skin and lymph nodes, suggesting collective cancer invasion. The cellular level of tumour invasion and prognostic parameters remain to be characterised. Methods: We performed immunohistochemical analyses of E-cadherin (marker for collective cancer invasion) and podoplanin (marker for epithelial-mesenchymal transition [EMT], single-cell invasion) expression in 102 samples of metastatic and non-metastatic cSCC and 18 corresponding skin and lymph node metastases to characterise the invasion of cSCC. Immunohistochemical results were retrospectively correlated with clinical data. Results: E-cadherin was highly expressed in metastatic and non-metastatic cSCC and skin metastases. This suggests collective cancer invasion. However, E-cadherin was downregulated in poorly differentiated cSCC and lymph node metastases, suggesting partial EMT. Podoplanin was significantly upregulated in metastatic (p = 0.002) and poorly differentiated (p = 0.003) cSCC. Overexpression of podoplanin represented a statistically independent prognostic factor for disease-free survival (p = 0.014). Conclusion: Collective cancer invasion is likely in cSCC. In lymph node metastases and poorly differentiated cSCC, partial EMT is possible. Podoplanin is an independent prognostic parameter for metastasis.

Introduction

Cutaneous squamous cell carcinoma (cSCC) is the second most common skin cancer [1]. It arises predominantly in sun-exposed skin and is derived from the keratinocytes in the epidermis [2]. The incidence is rising due to an ageing population and augmented ultraviolet exposure, however showing geographic variation [3, 4]. In North America, an increase between 50 and 200% during the last 20–30 years was registered [5]. It mainly affects elderly men [6, 7]. The mortality rate varies between 1.5 and 2.1% [8, 9]. cSCC has an excellent prognosis since a histologically controlled complete surgical excision can achieve local control with a cure rate of about 95% [2, 10, 11]. However, a subset of approximately 5% of cSCC metastasise [2, 5]. Metastases are usually located in regional skin or lymph nodes and mainly arise within the first 2
years after diagnosis [11]. If metastases occur, prognosis decreases drastically [5]. Prognostic parameters, being associated with an elevated metastatic risk, are immunosuppression, moderate/poor histological differentiation, perineural and lymphovascular invasion, local recurrence, median tumour diameter $>2$ cm, tumour thickness $>2$ mm and location on the ear [10, 12]. The identification of further reliable parameters, indicating the metastatic risk, is important for the implementation of additional diagnostic and therapeutic procedures such as sentinel lymph node biopsies or intensified follow-up care including imaging procedures.

Malignant transformation is triggered by cancer cells that are able to remove themselves from the primary tumour in order to form distant metastases. Recent studies indicate that this mechanism is either based on collective cancer invasion of adherent cell groups or single-cell invasion in the setting of epithelial-mesenchymal transition (EMT). The latter is declared to be a premise of tumour invasion, dedifferentiation and metastasis. This process is characterised by the loss of epithelial differentiation and gain of mesenchymal phenotype. Single tumour cells detach from the primary tumour through loss of adhesion and attenuation of epithelial phenotype. This is enabled by the downregulation or functional loss of adhesion molecules like E-cadherin, a 120-kDa calcium-dependent transmembrane glycoprotein [13, 14]. Intravasating blood and lymphatic vessels, cancer cells reach remote regions of the body, where they regain their epithelial features, enabling them to form distant metastases. This is called EMT [15, 16]. EMT is known to play a role in metastasis of constitutional isolated tumours (e.g. leukaemia, lymphoma) and tissue repair [17].

In contrast, the mechanism of collective cancer invasion involves adherent cell groups that detach from the primary tumour, featuring the development of malignant transformation and metastasis. Adhesion proteins such as E-cadherin are maintained to ensure cellular integrity. At the leading edge, molecules like podoplanin, a mucin-type transmembrane glycoprotein, mediating cellular contractile properties and cytoskeletal reorganization, are upregulated, and it is assumed that a small number of cells undergo a partial EMT in order to guide the adherent cell complex. Collective cancer invasion is involved in tumour progression of various malignancies such as carcinoma of the mamma, prostate, pancreas and epithelial wound healing [18, 19].

In a retrospective study we performed immunohistochemistry for E-cadherin und podoplanin in 102 samples of metastatic and nonmetastatic cSCC and 18 corresponding skin and lymph node metastases. We intended to characterise the invasion of cSCC by correlating the expression of the potential biomarker with metastatic risk and prognosis and investigated if there are prognostic parameters for metastasis.

### Material und Methods

For further details, see the supplementary materials (for all online suppl. material, see www.karger.com/doi/10.1159/000450920) (Fig. 1).

### Results

#### Analysis of Clinicopathological Parameters and Immunohistochemical Expression

We analysed 80 non-metastatic and 22 metastatic cSCC of 98 patients. The two groups were compared regarding clinicopathological parameters and immunohistochemical expression of E-cadherin and podoplanin (online suppl. Tables 1, 2). Metastasis occurred after a mean of 22.8 months (95% CI: 11.868–35.208, median: 10.8 months). The mean follow-up time was 39.2 months (median: 29.6 months). Primary metastatic cSCC displayed a significantly deeper invasion depth compared to non-metastatic cSCC ($p = 0.034$, online suppl. Table 1).

The immunohistochemical expression of E-cadherin in 102 investigated metastatic and non-metastatic cSCC displayed no significant differences. E-cadherin was strongly expressed in both groups with mainly a membranous pattern. Podoplanin was significantly more strongly expressed in metastatic cSCC ($p = 0.002$, online suppl. Table 2).

#### In Poorly Differentiated cSCC E-Cadherin Was Attenuated and Podoplanin Was Upregulated

Clinicopathological parameters of cSCC samples (invasion depth, localisation and histological grading) were registered. Concerning the percentage of positively stained tumour cells, E-cadherin was significantly downregulated ($p < 0.001$) whereas podoplanin was significantly upregulated in poorly differentiated cSCC ($p = 0.003$, Table 1).

In G1 and G2 tumours, the staining pattern of E-cadherin was predominantly membranous, whereas it changed significantly to membranous, cytoplasmic and no staining in G3 tumours ($p = 0.002$). The parameters
invasion depth and localisation displayed no significant correlations with immunohistochemical staining parameters (data not shown).

**E-Cadherin Was Attenuated in Metastases, Particularly in Lymph Node Metastases**

The Student t test was applied to compare immunohistochemical results of 14 pairs of cSCC and corresponding skin and lymph node metastases. They were analysed together and also divided into subgroups (skin metastases, lymph node metastases; 10 and 4, respectively). The majority of all metastases displayed significantly attenuated E-cadherin compared to the corresponding primary cSCC ($p = 0.031$). The subgroup primary cSCC/lymph node metastases revealed that all lymph node metastases displayed downregulated E-cadherin ($p = 0.069$). In contrast, podoplanin was highly expressed in lymph node metastases, thus showing no significant differences to the corresponding primary cSCC ($p = 0.391$, Fig. 2, online suppl. Table 4).

**Podoplanin Might Serve as Independent Prognostic Parameter for Metastasis**

In order to perceive a correlation of immunohistochemical staining characteristics and tumour parameters with metastasis, we performed a univariate (Kaplan-Meier) and multivariate (Cox regression) analysis. An upregulation of podoplanin was significantly associated with metastasis ($p = 0.014$, Fig. 3). Tumour invasion depth revealed no significant correlation with metastasis. However, poor differentiation correlated with metastasis ($p = 0.036$). The multivariate analysis to evaluate which parameters may offer an independent prognostic value for metastatic risk considered invasion depth, histopathological grading ($<3$/$\geq 3$) and immunohistochemical expression of podoplanin/E-cadherin (quantity $\leq 1$/$>1$, respectively). Podoplanin expression correlated significantly with metastasis (95% CI: 1.076–8.738, $p = 0.036$) and might serve as independent prognostic parameter (Table 2).
Characterisation of Prognosis and Invasion: Podoplanin and E-Cadherin in cSCC

**Fig. 2.** Comparison of immunohistochemical expression of E-cadherin and podoplanin in primary cSCC and corresponding lymph node metastasis. The figure shows the comparison of the immunohistochemical expression of E-cadherin (a, c) and podoplanin (b, d) of a primary cSCC (a, b) and the corresponding lymph node metastasis (c, d). The primary cSCC displays >75% positively stained tumour cells. E-cadherin is attenuated in lymph node metastases (<25% positively stained tumour cells), whereas podoplanin remains upregulated in lymph node metastases (>75% positively stained tumour cells).

**Table 1.** Podoplanin is upregulated and E-cadherin is downregulated in poorly differentiated cSCC

| Parameter                     | G1   | G2   | G3   |
|-------------------------------|------|------|------|
| Podoplanin-positively stained |       |      |      |
| tumour cells*, %              | <25% | 65.7 | 43.2 | 44.4 |
| 25–75%                        | 34.3 | 43.1 | 22.2 |
| >75%                          | 0    | 13.7 | 33.3 |
| E-cadherin-positively stained |       |      |      |
| tumour cells**, %             | <25% | 11.5 | 17.6 | 77.7 |
| 25–75%                        | 34.2 | 56.9 | 11.1 |
| >75%                          | 54.3 | 25.5 | 11.1 |

* p = 0.003, ** p < 0.001.

**Table 2.** Multivariate analysis (Cox regression) of potential prognostic markers in primary cSCC

| Parameter                     | CI – 95% | CI + 95% | p value | Hazard ratio |
|-------------------------------|----------|----------|---------|--------------|
| Invasion depth                | 0.918    | 1.178    | 0.540   | 1.040        |
| Grading (<3/≥3)               | 0.958    | 13.801   | 0.058   | 3.636        |
| E-cadherin (quantity ≤1/>1)   | 0.601    | 9.813    | 0.213   | 2.429        |
| Podoplanin (quantity ≤1/>1)   | 1.076    | 8.738    | 0.036   | 3.066        |

CI, confidence interval.
Previous studies have identified E-cadherin and podoplanin being involved in tumour invasion processes as single-cell (EMT) and collective cancer invasion. The cellular level of invasion in cSCC and the contributing role of E-cadherin and podoplanin as potential biomarkers of malignant transformation as well as prognostic parameters remain to be characterised.

The immunohistochemical expression of E-cadherin displayed no significant differences in metastatic and non-metastatic cSCC. In primary cSCC we detected a high and predominantly membranous expression that was retained in skin metastases. Our results emphasize the hypothesis of collective cancer invasion. In contrast, Toll et al. [20] registered a loss of membranous E-cadherin at the leading edge in the primary tumour in 77%, with no difference between metastatic and non-metastatic cSCC. They conclude that partial EMT may be involved in malignant transformation of cSCC [20].

Comparing primary cSCC with corresponding metastases, we demonstrated a significant decrease in E-cadherin particularly in lymph node metastases. This corresponds to Koseki et al. [21], who registered a loss or reduced E-cadherin in 91.3% of lymph node metastases. Additionally, we noted the membranous staining being attenuated in favour of a cytoplasmic E-cadherin expression. This is in concordance with Vinicius et al. [22], who detected a cytoplasmic staining of E-cadherin in 27.3% in lymph node metastases. Membranous E-cadherin was decreased in lymph node metastases so that augmenting cytoplasmic staining might be related to tumour progression in cSCC.

Skin metastases displayed a similar staining pattern to the corresponding primary tumours with high and primarily membranous E-cadherin expression. Together with the frequency of regional metastases (>60%), it presumes a united cell structure, leading to the hypothesis of collective cancer invasion in cSCC.

E-cadherin was significantly attenuated in poorly differentiated cSCC \((p < 0.001)\). Similar results were described by Lan et al. [23], who detected a high E-cadherin expression in well-differentiated and reduced or missing staining in poorly differentiated tumours. In contrast, Jensen et al. [24] revealed no significant changes of E-cadherin expression with respect to the histopathological grading of primary cSCC. This observation might arise from the fact that they assigned all \(\geq G2\) tumours to the group of poorly differentiated cSCC. In our analysis, however, immunohistochemical staining of G2 tumours rather resembled well-differentiated (G1) tumours. We detected that G1 and G2 tumours predominantly exposed membranous staining which was significantly replaced by a cytoplasmic expression in G3 tumours \((p = 0.002)\), presuming an E-cadherin translocation in poorly differentiated cSCC. The translocation might indicate proteolysis or lysosomal degradation of E-cadherin [25]. This outcome corresponds to recent studies where cytoplasmic staining of E-cadherin was interpreted as functional loss, promoting an attenuation of cellular integrity and featuring the development of malignant transformation and metastasis in the setting of EMT [20, 22, 25, 26].

The Kaplan-Meier analysis in order to investigate the influence of these staining characteristics to metastasis achieved no significant results.

Podoplanin was significantly upregulated in metastatic cSCC \((p = 0.002)\). Toll et al. [27] had already detected high podoplanin expression in metastatic cSCC. However, skin and lymph node metastases did not diverge in podoplanin expression, displaying similar staining properties to the corresponding primary cSCC. This observation confirms prior analysis of Vinicius et al. [22], who detected podoplanin-staining in 41.8% of cSCC and in
41.7% of metastases, thus revealing no significant differences.

Referring to the univariate analysis, podoplanin overexpression in primary tumours correlated significantly with metastasis \( (p = 0.014, \text{Fig. 3}) \). The multivariate analysis confirmed podoplanin as independent prognostic marker for metastasis \( (p = 0.036, \text{Table 2}) \). These results correspond to recent studies, whereby the podoplanin expression was associated with poor prognosis referring to local recurrence, reduced overall survival and elevated risk for metastasis, predominantly to regional lymph nodes \([27–29]\). In contrast, a Brazilian study demonstrated increased podoplanin expression in the context of locally aggressive cSCC, but without any association with lymph node metastasis \([22]\). Due to the small number of patients with tumour-related deaths, we did not conduct an analysis of overall survival. Prior studies confirmed that life expectancy significantly drops through high podoplanin expression in cSCC \([22]\). Kreppel et al. \([29]\) performed an analysis of 63 patients, including 20% of cSCC displaying high podoplanin expression. In this group, the 5-year-survival rate was 0% compared to a distinctly advanced survival rate of 91.3% in the group with podoplanin-negative tumours. Thus, podoplanin can serve as prognostic marker for lymph node metastasis and overall survival \([22, 29]\). Moreover, podoplanin was significantly overexpressed in poorly differentiated cSCC in our study \( (p = 0.003, \text{Table 1}) \), corresponding to recent studies that correlate reduced histopathological differentiation of cSCC to upregulation of podoplanin expression \([30, 31]\).

Our results indicate that E-cadherin and podoplanin influence tumour invasion in the context of collective cancer invasion as well as the process of EMT. The immunohistochemical results of highly expressed E-cadherin presume a united cell structure, leading to the hypothesis of collective cancer invasion. By emerging filopodia at the leading edge and thus shaping the actin cytoskeleton, podoplanin is also discussed in the context of collective cancer invasion by Wicki et al. \([19]\). However, the expression of podoplanin-inducing tumour progression is not dependent on attenuated E-cadherin. Instead, E-cadherin is preserved in invasive cSCC \([19]\). Consistently, we detected a highly expressed adhesion protein in metastatic and non-metastatic cSCC.

In contrast to collective cancer invasion in cSCC, Toll et al. \([27]\) describe podoplanin being involved in the process of EMT. They observed podoplanin overexpression in metastatic cSCC, and immunohistochemical staining correlated significantly with an increased risk of lymph node metastasis \([27]\). Correspondingly, we detected an overexpression of podoplanin in metastatic cSCC, being positively associated with metastasis \( (p = 0.014, \text{Fig. 3}) \).

According to Toll et al. \([27]\), podoplanin contributes to the downregulation of epithelial proteins by initiating a cellular translocation of E-cadherin so that the decrease in membranous expression results in functional loss and weakens intercellular integrity, referring to the mechanism of EMT. This corresponds to our observation of E-cadherin being attenuated and the fact that membranous expression decreases in favour of a cytoplasmic staining in poorly differentiated cSCC. In conclusion, we can affirm the hypotheses outlined above that collective cancer invasion seems to be involved in the majority of cSCC whereas in case of lymph-node metastases and poorly differentiated cSCC partial EMT is possible.

In addition, we analysed whether tumour characteristics such as invasion depth and histopathological grading might serve as potential prognostic parameters for metastasis. The invasion depth of > 5 mm is associated with a metastatic risk of 20–45.5% \([12, 22, 32–35]\). This analysis verified a significantly deeper invasion depth in metastatic cSCC \( (p = 0.034, \text{online suppl. Table 1}) \). Poor differentiation is correlated with an increased metastatic risk and part of the “high-risk-features” in the AJCC classification of cSCC \([8, 12, 24, 32, 34, 36–38]\). The univariate analysis revealed that G3 tumours are significantly associated with metastasis \( (p = 0.036) \). According to Cox regression, however, histopathological grading (≥ G3) only tends to be an independent prognostic parameter for metastasis \( (p = 0.058, \text{Table 2}) \). Recent analyses displayed a metastatic risk of G3 tumours between 17.9 and >30% \([24, 32, 34, 37, 39, 40]\).

Metastasis of cSCC is infrequent but associated with poor prognosis \([8, 42–44]\). Thus, early identification of unfavourable courses is necessary but insufficiently possible with respect to previous classifications. Therefore further biomarkers and prognostic parameters are needed to estimate the metastatic risk. We analysed E-cadherin and podoplanin and correlated their immunohistochemical expression with clinical and tumour characteristics of cSCC patients. The detection of E-cadherin in the majority of metastatic and non-metastatic cSCC and the frequency of regional skin and lymph node metastases, and no distant metastasis suggest collective cancer invasion in the majority of cSCC. Subsequently, in the setting of diagnosis, clinical and ultrasound surveillance of regional lymph nodes and cutaneous in-transit regions are reasonable. Beyond that podoplanin can serve as prognostic marker, since its expression correlates significantly with metastasis. Two thirds of sentinel-positive cSCC
expressed >75% podoplanin-positive tumour cells. Particularly in case of high podoplanin expression, sentinel lymph node biopsies might be recommended in order to detect and treat metastasis at an early stage.

Study Limitations
The small sample size and the focus on immunohistochemistry limit the significant evidence for the hypothesis of EMT in lymph node metastases and poorly differentiated cSCC, where E-cadherin was significantly down-regulated. Further markers like vimentin or ß-catenin and EMT transcription factors such as Snail, ZEB1, ZEB2 or TWIST using additional molecular and genetic methods are necessary to verify EMT in cSCC.

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Author Contributions
K.H. wrote the manuscript, performed the clinical research of patients’ files and conducted the statistical and immunohistochemical analysis and scoring. R.G. designed the research study and conducted the immunohistochemical analysis and scoring and drafted the article. I.S. designed the research study, organized clinical and pathological data and assisted with data analysis and interpretation. B.K. helped to establish and perform the immunohistochemical staining. V.S. evaluated tumour characteristics in tumour samples of cSCC. K.S. assisted with analysis and interpretation of the data. U.H. helped to analyse the immunohistochemical staining and reviewed the manuscript. J.K. helped to design the research study. All authors reviewed and approved the final version of the paper.

Statement of Ethics
The approval to conduct this study was obtained from the Ethics Committee of the Hannover Medical School (vote No. 2071-2013).

Disclosure Statement
The authors have no conflict of interest to declare.
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