

Expression patterns of CD168 correlate with the stage and grade of squamous cell carcinoma of head and neck

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Abstract. The receptor for hyaluronan-mediated motility CD168 is associated with the processes of oncogenesis and metastasis. The objective of the present study was to determine the possible association between the expression and distribution of CD168 and the tumor stage of head-and-neck squamous cell carcinoma (SCC). Formalin-fixed and paraffin-embedded tumor samples obtained from 100 patients during primary resection of SCC from the oral cavity, oropharynx, hypopharynx or larynx were included in the present study. The patients were divided into two risk groups: Low risk, representing the early stage of completely resected SCCs with good-to-moderate differentiation, and the high-risk group, representing the advanced stage SCCs with positive resection margins, vascular invasion or locoregional metastasis. All specimens were stained with a monoclonal antibody against CD168. Percentage and staining intensity of CD168-positive cells were scored, and their spatial distribution within the tumor nests was noted. The results obtained were correlated with the tumor stage. The quantification of CD168 expression revealed significant differences between the two risk groups (t-test, $P=0.002$), with higher scores in tumors resected from the high-risk SCC group compared with those from the low-risk group. In addition, in the high-risk group, the CD168-positive cells were present predominantly in the periphery (70.4%) of tumor nests, whereas in the low-risk group, only 56.6% were located there; however, this trend did not reach the level of statistical significance. Taken together, the results from the present study suggested that CD168 expression patterns could potentially be

used as a predictor of tumor aggressiveness, and therefore they may be a prognostic factor in head-and-neck SCC.

Introduction

Head-and-neck cancers are among the six most common malignant tumors, and their incidence is still increasing (1). Squamous cell carcinomas (SCCs) comprise 90% of head-and-neck cancers (2). High consumption of alcohol and/or tobacco (3,4) and viral infections [e.g., human papilloma virus (HPV)] (5,6) represent two major risk factors for the development of head-and-neck SCC. Although the appropriate primary prevention measures, such as change of lifestyle or vaccination against HPV, already exist, not all cases of SCC can be prevented (7,8). Therefore, an early detection of primary cancer and its recurrence remain the most important pillars of successful curative treatment.

To date, no immunohistochemical biomarkers predicting the clinical behavior of head-and-neck SCC have been described. Several reports (9,10) have demonstrated that the overexpression of hypoxia-associated proteins, such as hypoxia-inducible factor- α and glucose transporter 1, is associated with poor prognosis and resistance to radiotherapy in the oral SCCs (OSCCs). Similarly, the expression of transketolase-like protein 1 and the Apo10 protein epitope of the endonuclease, DNaseX, in OSCC in tissue or in peripheral blood has been successfully used for monitoring these tumors.

CD168, also known as a receptor for hyaluronic acid-mediated motility (RHAMM), is a member of the hyaladherin family of hyaluronan-binding proteins. It is involved in cell signaling, migration, and adhesion via interactions with hyaluronan, microtubules, actin, calmodulin, and components of the extracellular regulated kinase (ERK) signaling pathway (11). Hyaluronan accumulates during high tissue turnover (e.g. in regeneration, wound healing, etc.), and is essential in cell migration and proliferation during embryogenesis (12). CD168 has been extensively studied in different tumor cell lines and a variety of tumor types, including lymphoid and myeloid malignancies, and colorectal, breast, ovarian and urothelial carcinoma (13-16), although only a few studies have dealt with its expression and significance in the head-and-neck SCCs (17-19). It has been demonstrated that CD168 is required

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to facilitate proliferation of head-and-neck cancer cells (18). In addition, CD168 was found to contribute to cell cycle abnormalities by co-expression with microtubule-associated protein homolog (TPX2), resulting in an increased proliferative activity of head-and-neck SCC. CD168 was found to be highly expressed in the clinical specimens of oropharynx, hypopharynx and laryngeal cancer (18). Notably, CD168 was recognized by cytotoxic CD8⁺ effector T cells, therefore having possible relevance in the development of an anti-SSC vaccine.

CD168 binds hyaluronan (20,21) and together with another hyaluronan receptor, CD44, forms complexes that coordinately activate the mitogen-activated protein (MAP)/ERK1,2 pathway (22). CD168 is involved in the epithelial-to-mesenchymal transition (EMT) during the process of metastasis, and in the generation of breast cancer stem cells with enhanced resistance to therapy (19).

The present study aimed to characterize the CD168 expression pattern in primary SCC tissues resected from the oropharynx, hypopharynx, larynx and oral cavity. The total number of CD168-positive cells in tumor tissues were scored, and the spatial distribution pattern of CD168-positive tumor cells within the tumor cell nests (peripheral or central) were characterized, as well as the proportion of the positive tumor cells. The results obtained were analyzed in the context of clinical and pathological classification of the tumor-node-malignant (TNM) tumors system for each patient, and within the risk groups.

Patients and methods

Patients and tissue specimens. The specimens were obtained from 100 patients diagnosed with the primary SCC of the oral cavity, oropharynx, hypopharynx or larynx. All patients included in the present study were admitted to the Charité University Hospital in Berlin. The study was approved by the local Ethics Committee (approval no. EA1/312/14).

In addition to the tumor localization, patients' gender, age and the TNM stage at diagnosis were evaluated. According to the Munich Tumor Registry, the clinical and pathological TNM stage represents significant prognostic factors independent of gender, age and HPV infection status (23). Therefore, our study sample was divided into two risk groups: Patients with early stage carcinomas (clinical/pathological T1 or clinical/pathological T2, N0, R0, L0, V0 stage) and a high-to-moderate histological differentiation (G1 and G2) were designated as the "low-risk" group, whereas patients with advanced stage cancer [clinical/pathological T3 or clinical/pathological T4, R0/1, L0/1, V0/1, N1-3, extracapsular spread (ESC)] and usually moderately to poorly differentiated carcinoma were assigned to the 'high-risk' group. ESC was defined according to the criteria of Woolgar and Triantafyllou (24). The lymph node specimens were separately screened by two pathologists for capsular destruction with or without tumor nests with desmoplastic reaction, as well as for focal ESC in the lymph node hilus. In difficult cases, serial sections were prepared and carefully examined.

The low-risk group consisted of 46 patients. In 5 of these patients (1 female and 4 males), the primary carcinoma was located in the oropharynx; in 2 (both males), the primary

Table I. Correlation between CD168 expression and the clinicopathological features (n=100).

Feature	n	CD168 expression	
		Positive	Negative
Age (years)			
20-39	1		1
40-59	40	18	22
60-79	55	16	39
80-99	4		4
Gender			
Female	16	6	10
Male	84	28	56
Primary tumor site			
Oral cavity	25	3	22
Oral pharynx	22	12	10
Hypopharynx	13	9	4
Larynx	40	10	30
Low-risk group	46	8	38
High-risk group	54	26	28

carcinoma was located in the hypopharynx; in 17 patients (3 females and 14 males), it was located in the larynx carcinoma; and in the remaining 22 patients (7 females and 15 males), the primary carcinoma was located in the oral cavity. All the SCCs in these cases were highly or moderately differentiated (G1/G2). The high-risk group consisted of 54 patients. In 17 patients (2 females and 15 males), the tumor was located in the oropharynx; in 11 patients (1 female and 10 males), it was located in the hypopharynx; in 23 patients (2 females and 21 males), it was located in the larynx; and in the remaining 3 patients (1 female and 2 males), it was located in the oral cavity. The carcinomas had a variable differentiation grade (grade 1-3), but were predominantly G2.

Histology and immunohistochemistry. Formalin-fixed and paraffin-embedded tumor tissues were retrieved from the archives of the Pathology Institute at the Charité University Hospital in Berlin. Representative paraffin blocks encompassing vital tumor areas were selected. Each specimen was stained with hematoxylin and eosin for the histological tumor evaluation. Immunohistological analyses were performed using 4 μ m sections obtained from the selected paraffin blocks. To detect the CD168 protein (RHAMM), a mouse monoclonal antibody raised against human CD168 (clone 2D6, NCL-CD168; Leica Biosystems, Ltd., Newcastle, UK) was used at a dilution of 1:50. All immunostainings were performed with an automated immunostainer (BondMaxTM; Leica Biosystems, Ltd.) according to the appropriate protocols and reagents for antigen retrieval and visualization, using a Bond Polymer Refine DAB Detection kit (Leica Biosystems, Ltd.). Depending on the size of specimens, 100-250 tumor cells were scored. The CD168-positive cells were noted as a percentage of all, positive and negative cells, and the staining intensities were evaluated.

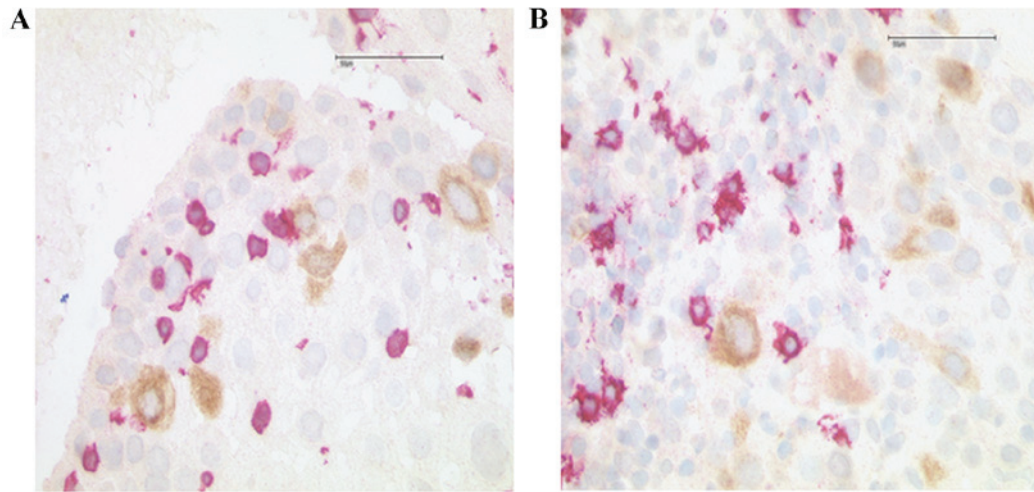


Figure 1. Verification of CD168-expressing cells. Double immunohistochemical staining experiments were performed for (A) CD3 and CD168 and (B) CD20 and CD168 proteins. The tumor-infiltrating T-cells (labeled with a monoclonal antibody against CD3; red immunostain) or B-cells (labeled with a monoclonal antibody against CD20; red immunostain) did not co-express CD168 (shown by the brown immunostain). The CD168-positive cells corresponded to neoplastic epithelial cells.

In order to verify CD168 expression by carcinoma cells, and not by tumor-infiltrating immune cells, a double immunostaining protocol was used, according to the protocol provided with the BondMax™ immunostainer (Leica Biosystems, Ltd.). CD168 expression was visualized using the Bond Polymer Refine DAB Detection kit (Leica Biosystems), resulting in a brown stain, whereas CD20 or CD3 were visualized with the Bond Polymer alkaline phosphatase kit, resulting in a red stain.

Statistical analysis. To quantitatively assess the immunohistochemical staining of CD168, the percentage of positive tumor cells and the staining intensity on a scale of 0, 1.0, 1.5, 2.0, 2.5 and 3.0, corresponding to negative, weak, weak-to-moderate, moderate, moderate-to-strong and strong staining, respectively, were evaluated. The CD168 staining score was subsequently calculated as the product of positive tumor cells (as a percentage) and the intensity. The statistical significance of differences in the CD168 staining score between the low- and high-risk groups was calculated using the two-sided Student's t-test.

In addition to the continuous score described above, a cut-off of 20% CD168-positive tumor cells was applied to dichotomize the cohort into CD168-positive and -negative tumors, and the results were correlated with the patients' age and localization of the primary tumor. The association between age and CD168 expression was analyzed by performing Pearson's correlation analysis. To compare the results regarding the spatial distribution of CD168-positive cells in the two risk groups, Pearson's Chi-square test was applied. Statistical calculations were performed using R software (version 3.2.0; The R Foundation for Statistical Computing; see <https://www.r-project.org/>). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Verification of CD168-expressing cells. Double immunohistochemical labeling revealed that the tumor-infiltrating

T- or B-cells did not express CD168, whereas all the CD168-positive cells corresponded to neoplastic epithelial cells (Fig. 1).

Quantification of CD168 expression. There was a significant difference in the CD168 staining scores for the low-risk [mean, 15.48; standard deviation (SD), 23.87] and high-risk (mean, 33.54; SD 31.72) groups [$t(95) = -3.141$, $P = 0.002$]. It was also observed that, in the high-risk group, more CD168-positive cells were localized to the periphery (70.4%) compared with the low-risk group (56.6%), although this trend was not statistically significant.

Using anti-CD168 staining with a positivity threshold arbitrarily set at 20%, 36% ($n = 36$) of all 100 samples were identified as being CD168-positive. In patients with laryngeal cancer, there was a significant negative correlation between the age and CD168-positivity ($r = -0.350$; $P = 0.027$). Carcinomas located in the oropharynx and hypopharynx had a higher proportion of CD168-positive cases compared with the negative cases (ratio of 1.2 and 2.25, respectively). The positivity of CD168 in the oral cavity carcinomas was low (ratio of 0.09) (Table I).

Localization of CD168 positive tumor cells in the low-risk group: Peripheral/central. In 20 of the total of 46 low-risk group cases (43.4%), the CD168-positive tumor cells were localized either in the central tumor areas (11 cases), or no CD168 cells were detected (9 cases). In the remaining 26 cases (56.6%), the CD168-positive tumor cells were detected at the tumor edge, with some cells located in the central areas (Fig. 2A).

Localization of CD168 positive tumor cells in the high-risk group: Peripheral/central. In 8 of the total of 54 high-risk group cases (14.8%), the CD168-expressing tumor cells were localized in the central tumor areas, whereas 8 cases were CD168-negative. In the remaining 38 (70.4%) cases, the CD168-positive cells were localized exclusively at the

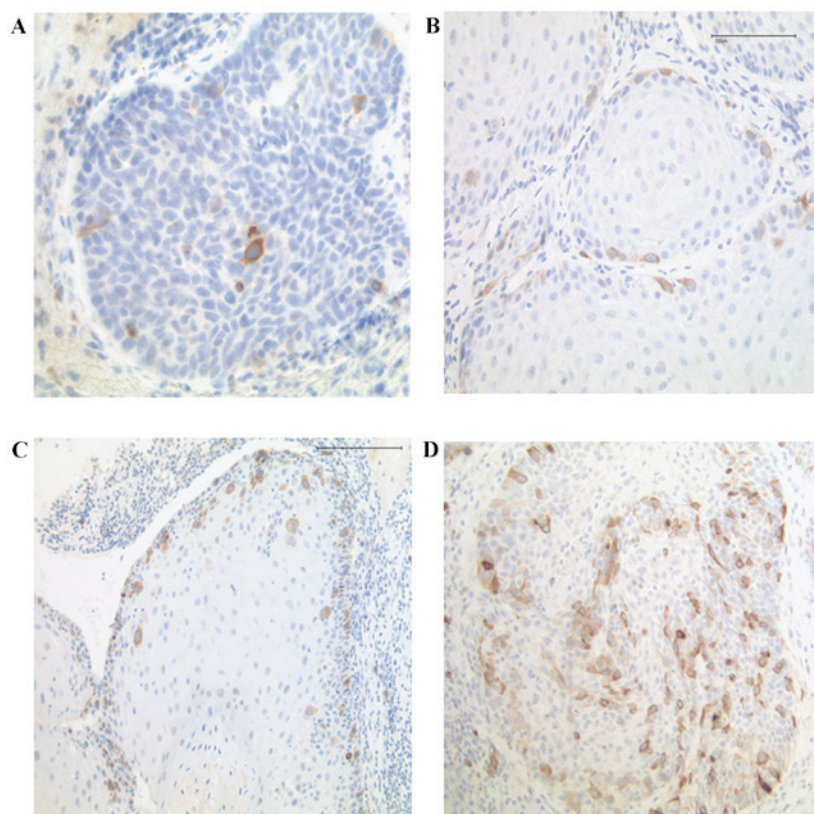


Figure 2. Representative examples of CD168 staining results. (A) Only a few tumor cells were CD168-positive, mainly in the central part of the tumor nest (low-risk tumors). (B and C) The CD168-positive cells accumulated at the edge of the tumor nest (high-risk tumors). (D) The CD168-positive cells were present at the edge and in the central parts of the tumor nest (high-risk tumors).

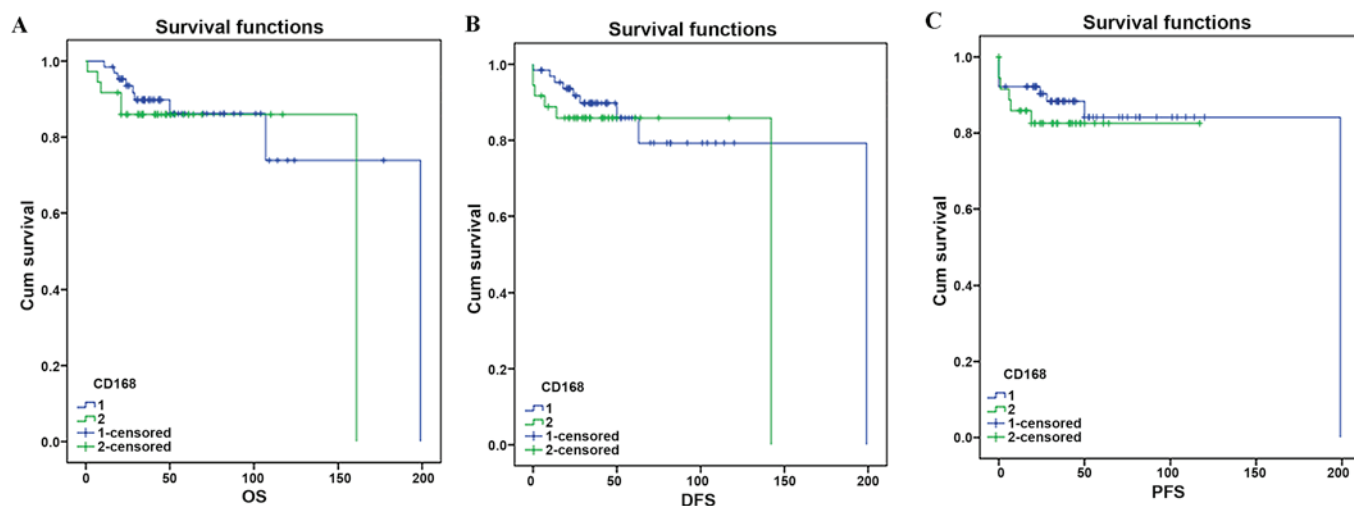


Figure 3. Kaplan-Meier-analysis for (A) OS, (B) DFS and (C) PFS, which did not reveal any significant differences for CD168 positive vs. negative cases (dichotomized at 20% CD168 positivity). OS, overall survival; DFS, disease-free survival; PFS, progression-free survival; Cum, cumulative.

tumor edge, or both at the edge and in the central tumor areas (Fig. 2B-D).

Kaplan-Meier analysis. Kaplan-Meier analysis was performed to determine overall survival, disease-free survival and progression-free survival. This analysis, however, did not reveal any significant differences for CD168-positive vs. -negative cases (dichotomized at 20% CD168 positivity; Fig. 3).

Discussion

CD168, a RHAMM, has a dual function as an extracellular and an intracellular hyaluronan-binding-protein (25,26). Hyaluronan serves an important role in tissue organization and in cellular migration, growth and differentiation (27). Several studies have highlighted a correlation between the expression of CD168 and cancer progression or a worse prognosis, particularly in the

breast, stomach or colorectal cancers, and in multiple myelomas. A previous CD168 knockdown study demonstrated growth reduction of transplanted tumors (28). Increased CD168 expression was revealed to be indicative of an elevated, or abnormal, level of cell proliferation in human OSCC (17).

Several research groups subsequently turned their attention to the special subcellular localization of CD168. At the cell surface, CD168 mediates cell motility and growth, whereas within the cell, it is involved in the organization of the cytoskeletal network. Finally, when localized to the nucleus, CD168 functions as a regulator of cell cycle progression. In the present study, CD168 expression was observed predominantly in the cytoplasm, and a nuclear staining pattern was not detectable. The results of the present study corroborate those of Yamano *et al* (29), who investigated CD168 expression in oral SCC and determined a correlation not only with the TNM stage, but also with mRNA and protein expression, and also reported an exclusively cytoplasmic CD168 expression pattern. The novelty of the present study concerned an investigation of whether the topographic distribution of CD168-positive cells within the carcinoma nests correlated with the tumor stage.

The patients in the study were divided into two risk groups, according to the Munich tumor registry (23). This registry has evaluated the survival curves of head-and-neck cancer over the time period between 1998 and 2011, and the TNM stage was identified as a significant predictive factor (23). In the low-risk group, patients in the early stage of SCC (clinically/pathologically T1 or T2) were included, with total tumor resection (R0), without lymph node metastases, without vascular invasion and with good-to-moderate histological differentiation. The high-risk group included patients in the advanced stage of SSC (clinically/pathologically T3 or T4), with the infiltration of blood vessels or margins and with a variable differentiation grade. A highly significant statistical correlation was identified between the high-risk group and the presence of CD168-positive tumor cells in the present study, using the CD168 staining score. The data in the present study support the results of Yamano *et al* (29), who determined statistically significant higher expression levels of CD168 in the advanced stage of SCC (T3 and T4).

Since the expression of CD168 correlates positively with the migratory tumor potential (30), a further aim of the present study was to investigate the localization of CD168-positive tumor cells within the tumor tissues. It was identified that, indeed, there was a positive correlation between the presence of CD168-positive tumor cells at the edge of the tumor nests and the stage of cancer; however, this correlation did not reach the level of statistical significance.

Additionally, a significant negative correlation was identified between the patient's age and CD168 expression in the larynx carcinoma group. Studies revealing a similar correlation in other tumor types have delivered conflicting results: For example, in a breast cancer study, a positive correlation between the age and visceral metastases was identified (31). In another study on melanoma, the prognosis worsened with age, whereas the number of CD168-positive sentinel nodes decreased (32). The authors of that study suggested that hematogenous tumor spread may account for this paradox (32).

Kaplan-Meier analysis for overall survival, disease-free survival and progression free-survival was performed, which

did not reveal any significant differences for CD168 positive vs. negative cases. It is worth noting that our survival analysis has (at present) very limited utility due to the short observation time. The clinical course of the patients will continue to be recorded and assessed, and the survival analysis will be performed again at a certain time in the future.

In conclusion, using a representative group of head-and-neck SCC specimens, it has been demonstrated, to the best of our knowledge for the first time, that an increased number of CD168-positive cells correlates with tumor grade and the TNM stage. Consequently, CD168 may be used as a possible prognosis marker. The present authors suggest that a routine evaluation of the CD168 expression pattern in cases of head-and-neck SCC may be helpful in evaluating the tumor aggressiveness, and in predicting individual patient prognosis.

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