CD44 as a stem cell marker in head and neck squamous cell carcinoma

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Abstract. In the recent past, evidence is increasing indicating the existence of a subpopulation of resistant tumor cells in head and neck squamous cell carcinoma (HNSCC) that cannot be eradicated by established antineoplastic treatments. These cancer stem cells (CSCs) have features of somatic stem cells such as selfrenewal, proliferation and differentiation. $CD44^+$ cells in tumors of the head and neck are referred to as CSCs of HNSCC. Expression profiling of CD44 in 29 HNSCC tumors was performed by fluorescence microscopy. ELISA analysis was performed to detect concentration of soluble CD44 in the peripheral blood of 29 HNSCC patients and 11 healthy controls. Expression of CD44 was determined in all HNSCC tissue samples (n=29). In all samples a surface staining pattern was found. The concentration of CD44 in the peripheral blood of HNSCC patients was significantly higher compared to a healthy control group (m_{HNSCC}=13.5±0.5 ng/ ml; m_{Cont}=9.3±0.6 ng/ml; P=0.6x10^{-12}). The role of CD44 as a marker for CSCs in HNSCC remains to be ascertained. Further experiments might reveal its role as a diagnostic and prognostic factor, and possibly as a therapeutic target.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is an aggressive and recurrent malignancy, largely because it is usually diagnosed at a late stage. Tobacco and alcohol exposure are the main risk factors and account for ~85% of HNSCC (1). Despite advances in surgical and non-surgical therapy mortality from this disease remains high because of the development of distant metastases and the emergence of therapy-resistant local and regional recurrences. Antineoplastic treatments such as chemotherapy or radiation can efficiently eradicate a majority of proliferating malignant cells within malignant tumors. However, there is increasing evidence that there is a subpopulation of resistant tumor cells that can not be reached by these regimens. These cancer stem cells (CSCs) have distinct features of somatic stem cells such as selfrenewal, extensive proliferation and differentiation. Therefore, these cells are required and responsible for initiation, but also maintenance and recurrence of disease. In recent years, the CSC hypothesis has been coined for HNSCC as well (2,3). Prince *at al* showed that $CD44^+$ cancer cells, which typically comprise <10% of the cells in an HNSCC tumor, but not the $CD44^-$ cancer cells, gave rise to new tumors *in vivo* (2). Since then, $CD44^+$ cells in tumors of the head and neck are referred to as CSCs of HNSCC.

CD44 is an integral cell membrane glycoprotein and it comprises different isoforms that arise from alternative splicing of a region of variable exons. They differ in primary amino acid sequence as well as amount of N- and O-glycosylation (4), thereby its apparent molecular mass ranges from 85 to 250 kD (5). At least 20 variants of CD44 have been reported due to the alternative splicing of 10 exons that encode the membrane's proximal portion of the extracellular domain (6-8). Originally, it was described as a receptor on circulating lymphocytes involved in homing, cell adhesion and migration (9,10).

In 1991 Günthert *et al* showed that the expression of CD44 gave metastatic potential to a non-metastatic line of cells in a rat carcinoma model (11,12). Since then, several analysis have indicated that there is a correlation between the expression of CD44 variants and progression, metastasis and prognosis of malignant disease. This has also been shown in different types of epithelial carcinoma in addition to HNSCC such as colorectal carcinoma (13,14), breast carcinoma (15) and certain types of gastric carcinoma (5,16).

The in-depth analysis of expression markers such as CD44 in tissue samples of HNSCC patients may reveal their role as potential prognostic biomarkers or therapeutic targets, e.g. for antigen directed immunotherapy. The analysis of soluble CD44 in peripheral blood of HNSCC patients may reveal important findings concerning diagnosis and possible pathways of metastasis in head and neck cancer.

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Table I. Tumor tissue and blood sample collection.

Patient	Gender	Age (yrs.)	Tumor location	TNM	Smoking/ ETOH
1	M	54	Larynx	T4N2xMx	+/+
2	F	55	Larynx	T1N0	+/-
3	M	62	Larynx	T4N0	+/-
4	M	70	Larynx	T4N0	1
5	M	68	Larynx	T3N2b	1
6	M	68	Larynx	1	1
7	M	55	Larynx	T4N2b	1
8	M	74	Larynx	T4N0	1
9	M	59	Larynx	1	1
10	M	57	Larynx	T4N1	+/+
11	M	55	Oral Cavity	T4N1	+/+
12	M	69	Oral Cavity	T1N2b	1
13	F	54	Oral Cavity	T1N2b	1
14	M	48	Oral Cavity	1	1
15	M	49	Oral Cavity	T3N1	1
16	M	51	Oral Cavity	1	1
17	M	66	Oral Cavity	T2N0	+/+
18	F	50	Oropharynx	T3N2c	1
19	M	64	Oropharynx	1	1
20	M	60	Oropharynx	T4N2c	1
21	M	64	Oropharynx	T3N2	1
22	F	55	Oropharynx	T4N3	1
23	M	61	Hypophyarnx	T4N2b	+/+
24	M	66	Hypopharynx	T2N0	1
25	M	76	Hypopharynx	1	1
26	M	59	Hypopharynx	T4N2b	1
27	F	62	Hypopharynx	T2N2b	1
28	M	51	Hypopharynx	1	1
29	M	57	Hypopharynx	T3N2c	+/-

1, information not available.

Materials and methods

Tissue and peripheral blood sample collection. A total of 29 HNSCC tissue samples from tumor patients were selected out of a tissue data base collected from 1997 to 2010 at the Department of Otorhinolaryngology, Head and Neck Surgery at the University of Mannheim. Samples were fixated immediately after excision by freezing in liquid nitrogen. All samples were confirmed by pathology after H&E staining. In addition to the tissue collection, peripheral blood samples were taken from HNSCC patients and healthy donors. Peripheral blood was collected before, during, but never after tumor surgery. A group of 11 healty blood donors served as a control. Blood samples were centrifuged at 2500 rpm for 10 min. Afterwards serum samples were harvested and fixated by freezing at -80˚C. The histological and clinical characteristics of all tumor samples are summarized in Table I.

Immunofluorescence labeling. To detect the expression of CD44 in HNSCC tissue samples, tumors underwent fixation by freezing in liquid nitrogen as mentioned above. Specimens were prepared in slices of 5-8 μm, air-dried and fixed in acetone for 10 min. Afterwards, slices were treated with 4% paraformaldehyde (PFA) for 10 min at room temperature. After 3 washing steps with PBS tumor samples were treated with 1% serum (goat) for another 10 min. Then slices were incubated with CD44 antibody (mouse monoclonal, 1:100, Abcam, Cambridge, UK) for 1 h at 37˚C followed by incubation with a second biotinylated antibody (anti-mouse, 1:100) for 30 min. After further washing steps with PBS, slices were treated with Streptavidin-Cy3 (1:1000) for 30 min at room temperature. Subsequently, slices were stained with DAPI after washing with PBS. Finally, slices were covered in fluorsave and dried to be evaluated by fluorescence microscopy.

Enzyme-linked immunosorbent assay. Serum levels of CD44 were measured with a human CD44 ELISA Kit (Abcam). A monoclonal antibody against soluble CD44 was adsorbed to microwells in 96-well microtiter plates. Samples, including standards of known CD44 concentrations and samples were pipetted into these wells. During the first incubation, the CD44 antigen was added to wells. After washing, a biotinylated monoclonal antibody specific for CD44 was incubated and the enzyme (streptavidin-peroxidase) was added. After incubation and washing to remove all unbound enzyme, a substrate solution was added, which catalyzed a reaction on the bound enzyme and so induced a coloured reaction product. The intensity of this product is directly proportional to the concentration of CD44 present in the samples.

Statistical analysis. All results were plotted as the mean ± standard deviation. To estimate the probability of differences, we employed the Student's t-test. $P<0.05$ denoted statistical significance.

Results

Tissue and peripheral blood sample collection. A total of 29 HNSCC tissue samples and the according blood serum samples were selected out of a data base collected from 1997 to 2010 at the Department of Otorhinolaryngology Head and Neck Surgery at the Faculty of Medicine Mannheim, University of Heidelberg, Germany. Samples entering the study were derived from 5 female and 24 male patients aged 48-76 (mean age: 60) years. Locations of the primary tumor were differentiated as larynx, oropharynx, hypopharynx and oral cavity (10 larynx, 5 oropharynx, 7 hypopharynx, 7 oral cavity). Patient characteristics are summarized in Table I. A group of 11 healthy blood donors served as a control for experiments concerning CD44 concentration in peripheral blood of HNSCC patients. All studies were approved by the Ethics Committee of the Faculty of Medicine Mannheim, University of Heidelberg.

Expression of CD44 in HNSCC tissue samples. Immunofluorescence labeling of 29 tissue samples was performed to detect the expression of CD44 in HNSCC. In all samples,

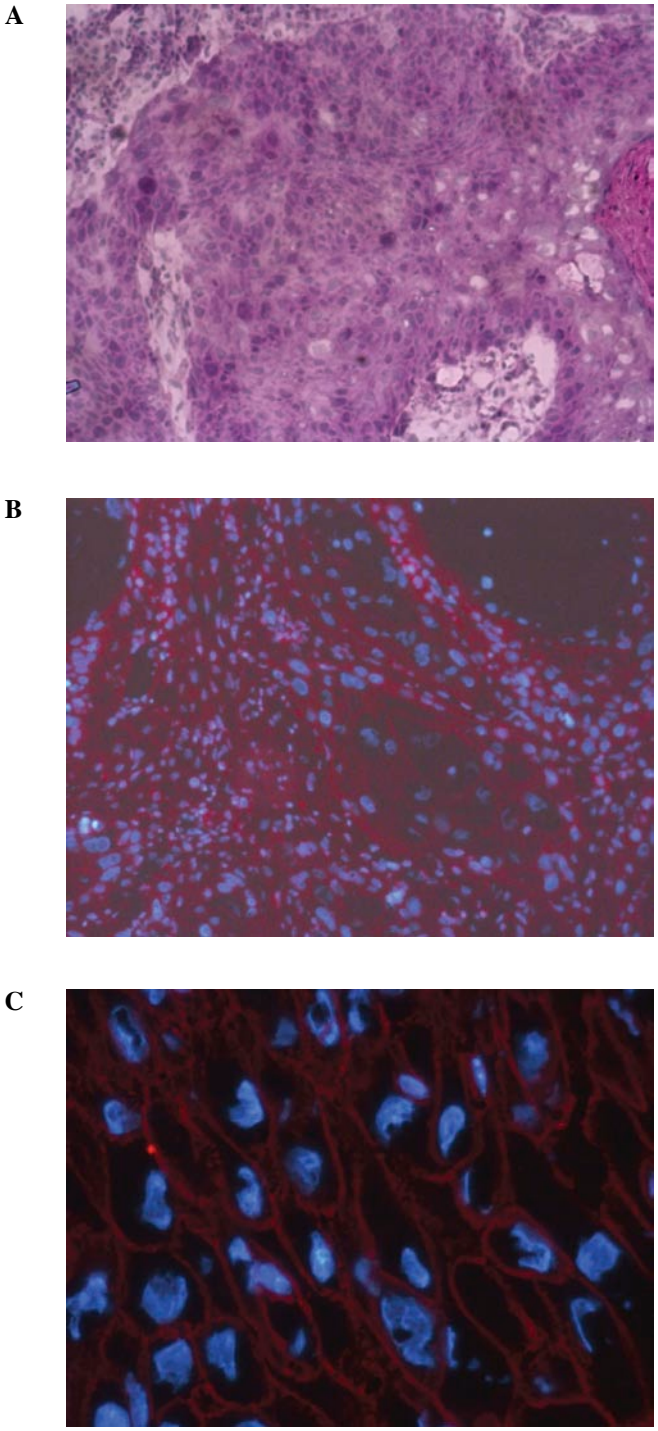

Figure 1. Expression of CD44 in HNSCC samples. Pathology of HNSCC was established for each sample by H&E staining during routine diagnostics (A). CD44 was visualised in red color by immunofluorescence labeling via Cy3 (B and C). Cell nuclei were stained in blue by DAPI. High fluorescence intensity for CD44 was found in each of 29 HNSCC samples stained (B and C). Each sample showed a surface staining pattern of CD44 (C). Stromal cells did not show any fluorescence by staining of CD44 via Cy3 (B).

pathology of HNSCC was confirmed via H&E staining by pathology during routine diagnostics (Fig. 1A). CD44 was visualised in red color by immunofluorescence labeling via Cy3. In all 29 HNSCC tissue samples an intense fluorescence signal could be detected (Fig. 1B and C). In HNSCC tissues, CD44 was mainly expressed on cell surface in all samples

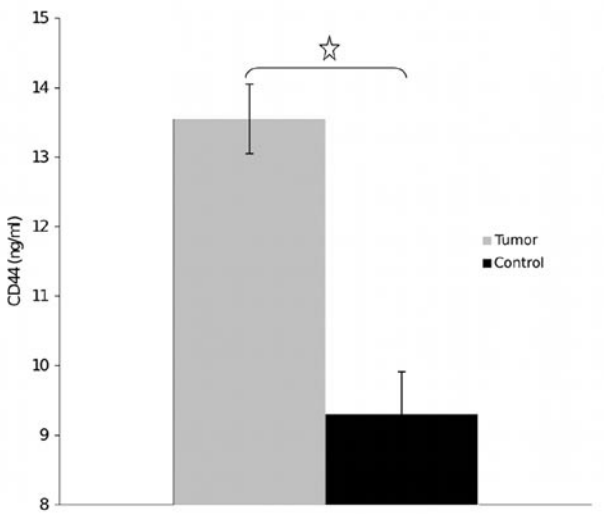

Figure 2. CD44 in peripheral blood of HNSCC patients. Concentration of soluble CD44 in peripheral blood of HNSCC patients was evaluated by ELISA and compared to healthy controls. There was a significantly higher concentration of CD44 in peripheral blood of HNSCC patients in comparison to healthy controls (☆$P=0.6x10^{-12}$).

stained (Fig. 1C). Stromal cells did not show any fluorescence by staining of CD44 (Fig. 1B).

Concentration of CD44 in peripheral blood of HNSCC patients. ELISA experiments were performed to measure the concentration of soluble CD44 in the peripheral blood of HNSCC patients compared to healthy controls. The expression of soluble CD44 in serum of HNSCC and healthy controls is shown in Fig. 2. There was a significantly higher concentration of CD44 in peripheral blood of HNSCC patients compared to the healthy control group (m_{HNSCC} = 13.5±0.5 ng/ml, m_{Cont}=9.3±0.6 ng/ml, $P=0.6x10^{-12}$). Primary tumor location (Fig. 3A), T-stadium (B) and N-stadium (C) did not significantly influence the concentration of CD44 in peripheral blood of HNSCC patients. The values determined and showed in Fig. 3 are: m_{larynx}=13.9±0.4 ng/ml, m_{oroph}=13.6±0.5 ng/ml, m_{hypoph}=13.1±0.3 ng/ml, $m_{oralcav}$ =13.5±0.3 ng/ ml (A); m_{T1}=13.8±0.7 ng/ml, m_{T2}=13.0±0.3 ng/ml, m_{T3}=13.5±0.5 ng/ ml, m_{T4}=13.8±0.5 ng/ml (B); m_{N0}=13.8±0.5 ng/ml, m_{N1}=13.6±0.3 ng/ml, m_{N2}=13.5±0.6 ng/ml, m_{N3}=14.1 (C); m_{Cont} 9.3±0.6 ng/ml (A-C).

Discussion

Expression of CD44 in HNSCC tissue samples. In this study, we showed that CD44, which is referred to as a cancer stem cell (CSC) marker of the head and neck (2), can be detected in HNSCC tissue samples by immunofluorescence labeling. Others have used flow cytometry analysis (2,17), immunohistochemistry (18-20), or microarray technology (18) to verify $CD44^+$ cells in HNSCC tissue samples. Results consistently indicate the presence of CD44 in HNSCC tumors on both protein and gene level. In our experiments CD44 was abundantly expressed in all HNSCC samples tested (n=29) and therefore conform to data collected by Han *et al* (18), who performed immunohistochemical analysis of 16 HNSCC tumor samples.

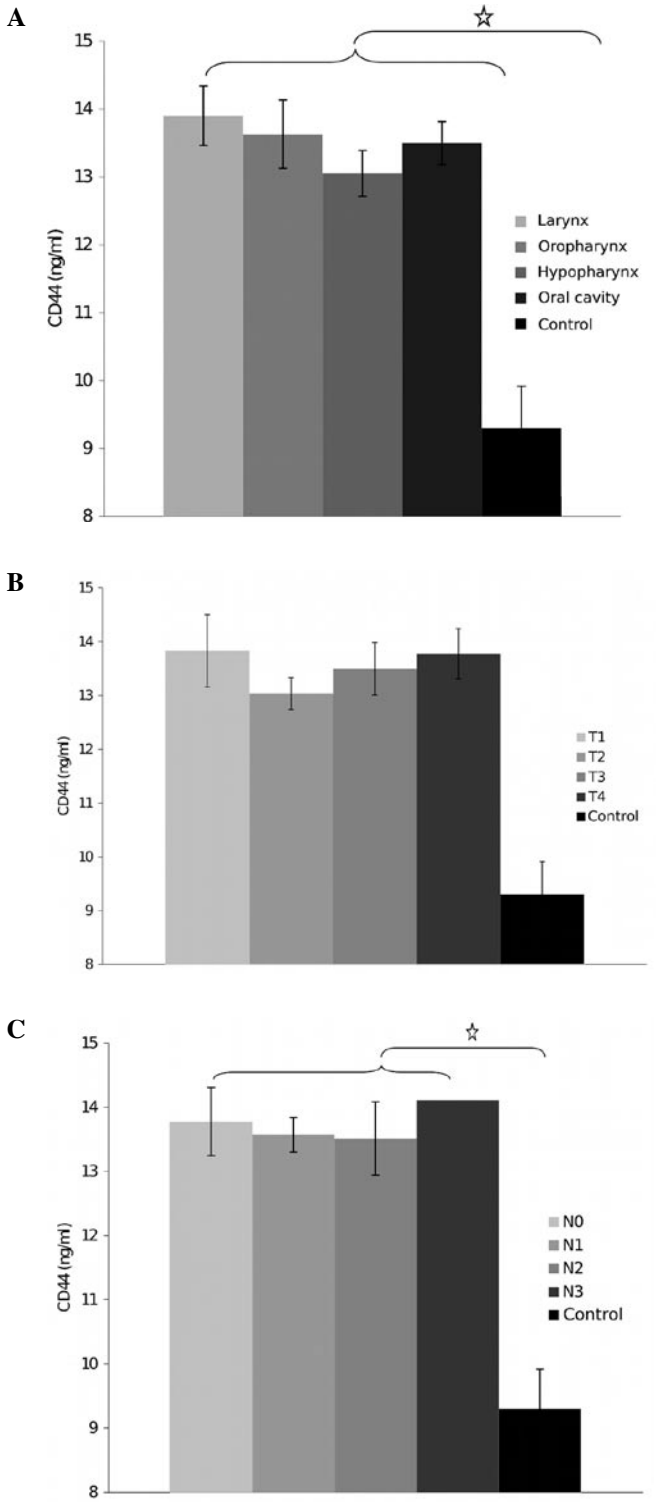

Figure 3. CD44 in peripheral blood of HNSCC patients depending on location of primary tumor, T-stage and N-stage. There are no significant differences in the concentration of soluble CD44 in HNSCC patients according to primary tumor location (A), T-stage (B) or N-stage (C). Note the significantly higher concentration of CD44 in the peripheral blood of HNSCC patients compared to healthy controls (A) ☆P=$0.6x10^{-12}$, (B) ☆P=$0,7x10^{-13}$, (C) ☆P=$0.7x10^{-13}$.

The staining pattern shown in immunofluorescence (Fig. 1B and C) and immunohistochemistry (18) shows a monotonic staining of almost all cells except stromal and vascular cells. This stands in contrast to previous work e.g. by Prince *et al*, who first postulated $CD44^+$ cells as CSCs of the head and neck (2,21). Prince *et al* reported, that $CD44^+$ cancer cells typically comprise <10% of the cells in an HNSCC tumor (2). It is obvious that the percentage of cells of the tumor samples stained as $CD44^+$ in our experiments is much higher than 10%. A possible reason might be the different experimental methods of CD44 detection, e.g. flow cytometry versus immunofluorescence labeling. It is even more reasonable that the percentage of <10% considered by Prince *et al* comes off because of the exclusion of Lin- cells. Lin- was defined as negative for the surface markers CD2, CD3, CD10, CD18, CD31, CD64 and CD140b (2). The flow cytometric analysis done by Pries *et al* showed, that CD44 expression in HNSCC tumors differs inter-individually. In their study, the percentage of cells in HNSCC tumors varied from ~4 up to over 90% (17). It is possible that this is correct because Pries *et al* had a look at the whole of the tumor and not just at a small specimen cut out of the bulk of an HNSCC. It has been shown in previous work, that CD44 is a ubiquitary marker e.g. in the hematopoietic or mesenchymal system (22).

Consistent with the work of others (18,19,23) CD44 showed a surface staining in immunofluorescence labeling in our study. The location of CD44 on the cell surface of HNSCC might represent its role as an adhesion molecule in tumor survival and progression. There is evidence that CD44 proteins anchor the cells to the extracellular matrix (ECM) and that adhesive properties of malignant cells have to be altered to detach from the primary tumor in order to build metastases (24). It has been shown that the extracellular portion of CD44 serves as a substrate for proteolytic cleavage by matrix metalloproteinases (MMPs) on several cancer cell lines and in human tumors. This indicates that CD44 proteins can serve as 'platforms' for enzymes such as MMPs, which are required for their effective functions. The inhibition of the reactions catalyzed by these MMPs can block tumor cell migration (25,26). As tumor cells get in contact with the ECM via several cell surface adhesion molecules such as CD44, integrins and cadherins it is likely that these proteins are also needed for metastatic processes (27). As CD44 obviously gets in contact with the ECM it is imaginable that there is a $CD44^-$ dependent assembly and organization of the ECM, which might be useful to protect tumor cells from immune defence (28,29). Pre-treatment of chondrocytes with an anti-CD44 antibody blocked their interaction with the ECM (30).

Concentration of CD44 in peripheral blood of HNSCC patients. Soluble CD44 has been evaluated in blood serum of patients with different primary cancers (5,21,31) as well as in oral rinses of patients with HNSCC (4,32). Elevated CD44 serum levels have been found for several tumor types using ELISA analysis (31,33,34).

In this study, we showed that the concentration of soluble CD44 is significantly higher in peripheral blood of HNSCC patients compared to healthy controls. This is in contrast to the findings reported by Van Hal *et al* (21), who did not find significant differences between the CD44v6 plasma levels of HNSCC patients, healthy controls and non-cancer patients. A reason might be the measurement of different isoforms of CD44. Van Hal *et al* focussed on the detection of CD44v6, the total quantity of CD44 including all isoforms was measured

in our experiments. An explanation for the findings of Van Hal *et al* might be given by Herold-Mende *et al* (19). They found that the variant exons of CD44 v5, v6, v7, v7-8 and v10 are expressed in epithelia of healthy donors and that variant exons v7, v8 and v10 were significantly downregulated in primary squamous cell carcinoma and were not detected at all in the majority of metastasis-derived specimens (19). Expression of CD44v5 and CD44v6, on the other hand, was mainly unaltered. However, this might give an explanation for the findings of Van Hal *et al* (21). In contrast to this Mack *et al* found a slight increase of CD44s and CD44v6 levels in oral leukoplakia and in moderately differentiated carcinomas (20). For other types of cancer CD44v6 has already been shown to be a potential marker of prognosis. Saito *et al* postulated that the serum concentration of sCD44v6 and its expression in tumors were associated significantly with the depth of invasion of the tumor, lymph node metastasis and clinical stage in patients with diffuse type gastric carcinoma (5).

In our experiments, a highly significant difference between concentration of CD44 in serum of HNSCC patients was found in comparison to healthy controls. In contrast to Saito *et al* (5), there was no correlation to the stage of primary tumor expansion, lymph node metastasis or distant metastasis in our results. It is not the stage of disease, but rather the mass of the tumor or the specific number of tumor cells that should be seen in congruence with the concentration of CD44 in peripheral blood in HNSCC patients. In any case, the tissue samples available for experimental analysis are small specimens out of the bulk of a tumor. This is to guarantee benefit of surgery to patients and optimal terms and conditions of pathological assessment to ensure the best possible treatment and follow-up for the patient.

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