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Soluble CD44v6 is Not a Sensitive Tumor Marker in Patients with Head and Neck Squamous Cell Cancer

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Abstract. *Background: In some epithelial tumors, isoforms of CD44 are overexpressed and soluble isoforms detectable in serum samples are elevated. In squamous cell cancer of the head and neck (SCCHN) the alteration of CD44 isoforms could be associated with poor prognosis. A comprehensive study was undertaken to examine the value of CD44v6 as a tumor marker for SCCHN. Patients and Methods: Serum samples of SCCHN and healthy smokers were analyzed for soluble CD44v6 by ELISA. The expression of CD44 isoforms was determined by immunohistochemical staining of healthy and dysplastic tissue. Results: There was no significant difference between the serum levels of sCD44v6 in SCCHN and healthy smokers. Nor was there a correlation between the serum level of sCD44v6 and UICC stage, TNM stage or histological grading. In tissue of primary SCCHN, expression of CD44v6 was found as a strong, specific staining of the lower epithelial layers. Similar amounts of CD44v6-positive-labelled tumor cells were found in invasive carcinoma. Conclusion: Soluble CD44v6 is not a valuable tumor marker for SCCHN since the soluble form appears to be present in healthy smokers and does not reflect the stage of the disease.*

The expression of CD44, a cell adhesion protein, and its isoforms has been associated with the presence of distant metastases, a shortened overall and disease-free survival of many human cancer types and has proven to be a useful tumor marker, *e.g.* in gastric cancer (18, 33).

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CD44 is a glycoprotein located on the cell surface, which is expressed by a variety of tissues and which is essential for numerous physiological events, plus being involved in cell-cell and cell-matrix interactions (11, 19, 31). It is composed of four main parts: (a) a distal extracellular domain, (b) a membrane-proximal region, (c) a transmembrane spanning domain and (d) a cytoplasmic tail. The CD44 protein is encoded by one single gene located on chromosome 11 and is partly subjected to alternative splicing encoding parts of the extracellular domain (14). Therefore, there are two different groups of the CD44 protein. The standard form, also called CD44st, and the various isoforms of CD44, known as CD44v1-10. CD44st, a molecule of 80 to 90 kDa without variant domains, is expressed on the surface of many normal cells. In contrast, the CD44 variants are expressed in a restricted manner: on T-lymphocytes and other leukocytes following activation (v6, v9), on epithelial cells as epithelial CD44 (v8, v9, v10) and on keratinocytes (CD44v3-v10) (19). Different functions have been assigned to CD44, apparently restricted to subsets of the different isoforms (31). The intracellular part of CD44 linked to the cytoskeleton is responsible for two main functions. Cell adhesion is regulated by changes in the binding affinity of CD44 ("inside-out signaling"). Secondly, it contributes to cell activation and regulation of cell death and growth, influencing the signal transduction ("outside-in signaling") (1). It is included in the development and functioning of the normal immune system, with regard to the activation of leukocytes, hematopoesis and the homing of lymphocytes (11). Malignant cells may imitate activated lymphocytes and, therefore, may be able to interfere with the cell-matrix and cell-cell interactions, resolving in metastatic spread. Additionally, CD44 molecules function as receptors for the extracellular matrix components laminin, fibronectin, types I and VI collagen, proteoglycan sulfate and hyaluronate (19). No specific ligands for the CD44 isoforms have been identified to date (17). Binding to hyaluronate, an

Table I. *Characteristics of the patients for the ELISA and the immunohistochemical study.*

	UICC		TNM		Histological differentiation		Tissue for immunohistochemical study
SCCHN (n=29)	I	4	T1	5	G1	2	1
	II	2	T2	7	G2	10	4
	III	6	T3	5	G3	17	5
	IV	17	T4	12			
Dysplastic tissue							5
Normal tissue							
smokers (n=5)							5
non smokers (n=5)							5

ubiquitous extracellular polysaccharide, enables CD44+ lymphocytes and malignant cells to migrate, invade and enter lymphatic vessels (12, 19).

Because CD44 proteins are released from different cells, especially lymphocytes, soluble CD44 molecules can be detected in the human blood circulation (6). Increased serum levels of CD44 have been correlated with alterations of the cell surface, tumor size, lymph node status and the presence of distant metastases, as already shown for gastric cancer (9). Antibodies against the different soluble isoforms of CD44 allow the detection of CD44 variants in the serum. *In vivo* experiments have already shown that the isoform containing the v6 domain plays an important role in terms of the frequency of metastases (22). The CD44v6 domain is highly expressed by squamous cell carcinoma of the head and neck (SCCHN). Thus, the present study was performed to evaluate the clinical significance of CD44v6 serum levels as a potential tumor marker in patients with SCCHN.

Patients and Methods

ELISA. The blood samples of 29 patients with primary SCCHN were collected over a period of 6 months. The primary tumors were located in the oral cavity, the oropharynx, the larynx and the hypopharynx in equal shares. Tumor stage was evaluated according to the UICC and the TNM classifications. Histological differentiation was determined during the routine pathological work-up. The sera were obtained from patients with different tumor stages: T 1 (n=5), T 2 (n=7), T 3 (n=5), T 4 (n=12), UICC 1 (n=4), UICC 2 (n=2), UICC 3 (n=6) and UICC 4 (n=17). The histological grading was as follows: Grade 1 (n= 2), Grade 2 (n=10) and Grade 3 (n=17). The reference group was represented by 12 healthy smokers suffering from benign non-inflammatory diseases of the head and neck region (Table I).

The serum concentration of sCD44v6 was determined by ELISA. The principle of this assay is based on the so-called "sandwich principle": Two different specific murine monoclonal antibodies are used to verify the solid phase and the carrier peroxidase, enabling verification of corresponding CD44-molecules in a specific manner. There is no detectable cross-reactivity with circulating immune factors such as TNF alpha. The sCD44v6 levels were evaluated in bar charts, with calculation of the mean and the standard deviation.

Immunohistochemistry. Patients: The present study was conducted on a consecutive series of 10 SCCHN of different origin that had been treated in our department. The material was obtained from a prospectively recruited study population. In addition, for each patient detailed clinical data were recorded, particularly the tumor stage and the presence of lymph node metastases according to the UICC classification. Further histopathological data, such as the degree of tumor cell differentiation, were also collected.

Additionally, normal mucosa of 5 nonsmokers and 5 smokers, which showed no evidence of epithelial dysplasia or malignant growth, as well as 5 cases of dysplastic tissue, were examined for the expression of CD44v6. All patient data are presented in Table I. All tissue samples were embedded in paraffin according to routine protocols.

Immunohistochemical analysis of CD44v6. For immunolocalization of CD44v6, a specific antibody against the surface receptor of CD44v6 was used (DAKO, Glostrup, Denmark). All immunostaining techniques applied have been described in detail previously (20). Briefly, appropriate paraffin sections were enzymatically pre-treated (0.4% pepsin) and the antibody-antigen-detection was performed by the ABC-peroxidase method.

Morphometric data evaluation. All immunostaining was initially qualitatively evaluated by light microscopy by two independent observers for the quality and extent of the staining results and their distribution in the different tissue compartments. Discrepancies in the observations were resolved in conference. In addition, a semi-quantitative morphometric analysis of the amount of positive marked cells was performed (normal epithelium, dysplastic or squamous carcinoma cells). This analysis covered 10 randomly selected areas from each case.

For better evaluation, the mean values from these data were classified into 3 different groups of positively-labelled cells. These groups comprised almost completely positively-marked cells (90-100% of all cells; group 1), a strong staining pattern (50-89 % positive cells; group 2) and a slight expression of CD44v6 positively-labelled cells (0-49 % marked cells; group 3).

Results

ELISA. Serum levels of sCD44v6 in primary SCCHN and healthy controls. The serum levels of the soluble CD44 isoform v6, obtained from patients with primary SCCHN and healthy smokers, are shown in Figure 1. The mean±SD value in the reference group of healthy smokers of 137.2±34.5 ng/ml (range: 69.4 to 187.0 ng/ml) was comparable to the sCD44v6 serum levels of patients with SCCHN at 146.7±52.2 ng/ml (range: 71.0 to 280.0 ng/ml).

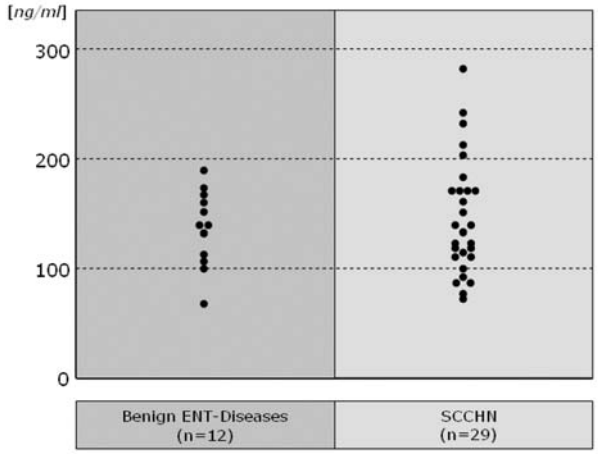

Figure 1. *Dot plot and bar chart of sCD44v6 serum levels of patients with primary SCCHN and healthy smokers with benign ENT diseases.*

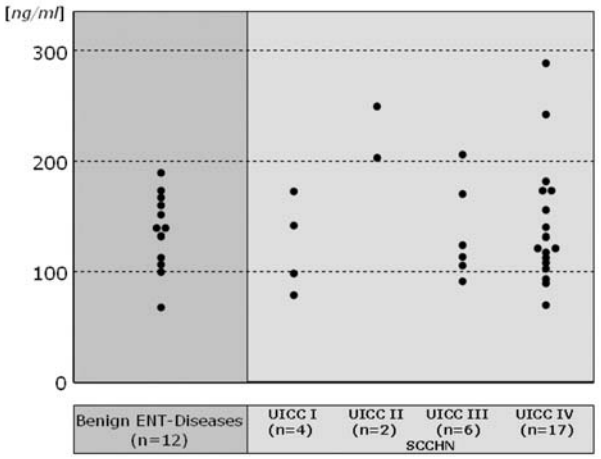

Figure 3. *Dot plot and bar chart of sCD44v6 serum levels correlated to the UICC stages.*

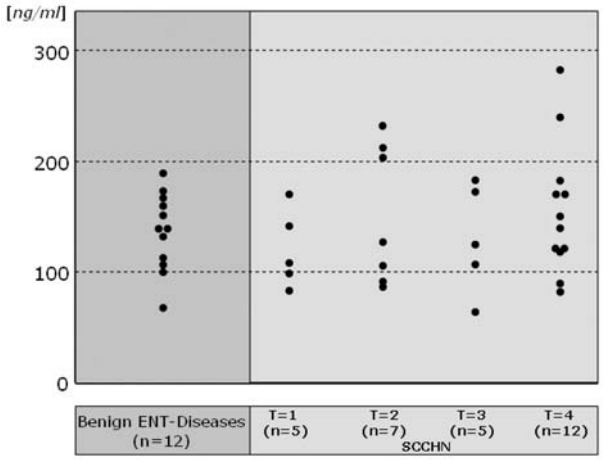

Figure 2. *Dot plot and bar chart of sCD44v6 serum levels correlated to the tumor size (TNM classification).*

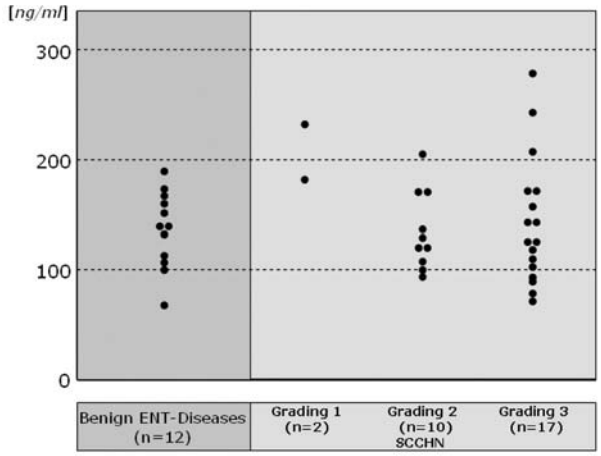

Figure 4. *Dot plot and bar chart of sCD44v6 serum levels correlated to the histological differentiation of the tumor.*

Serum levels of sCD44v6 correlated to the T stages according to the TNM classification. The serum levels of sCD44v6 from patients with primary SCCHN and healthy smokers were related to the size of the tumor according to the TNM classification, as illustrated in Figure 2. The mean±SD serum levels of sCD44v6 were distributed as follows: T1=121.3±37.0 ng/ml (n=5), T2=140.9±55.4 ng/ml (n=7), T3=121.0±42.5 ng/ml (n=5) and T4=146.6±44.4 ng/ml (n=12). Compared to the control group of healthy smokers, there was no significant difference.

Serum levels of sCD44v6 correlated to the UICC stages. The serum levels of sCD44v6 from patients with primary SCCHN and healthy smokers in different UICC stages are shown in Figure 3. The mean±SD serum levels of sCD44v6 were distributed as follows: UICC 1=124.2±42.0 ng/ml (n=4), UICC 2=229.4±32.2 ng/ml (n=2), UICC 3=137.4±44.4 ng/ml (n=6) and UICC 4=143.6±54.7 ng/ml (n=17). According to the T stages, there was no significant difference between SCCHN and healthy smokers.

Serum levels of sCD44v6 correlated to the degree of histological differentiation. The serum levels of sCD44v6 from patients with primary SCCHN and healthy smokers of different degrees of histological differentiation are demonstrated in Figure 4. The mean±SD serum levels of sCD44v6 were distributed as follows: Grade 1= 204.1±42.3 ng/ml (n=2), Grade 2=121.5±57.8 ng/ml (n=10) and Grade 3=133.0±57.8 ng/ml (n=17). There was also no significant difference between the sCD44v6 serum levels of healthy smokers and SCCHN regarding the histological grading.

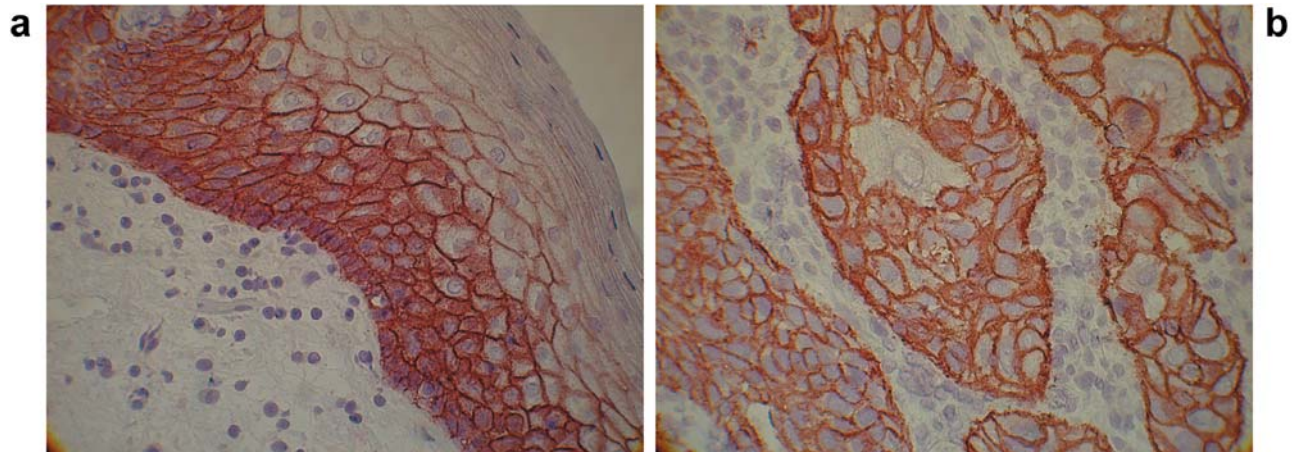

Figure 5. *(a) Expression of CD44v6 in the normal laryngeal epithelium: strong specific staining of the lower epithelial layers. (b) Similar amount of CD44v6-positive-labelled tumor cells in invasive carcinoma.*

Immunohistochemistry. Expression of CD44v6 in normal tissue. The immunolocalization of CD44v6 in all samples of normal squamous mucosa showed a positive expression of the lower epithelium layers, where all epithelium cells expressed CD44v6 (Figure 5 a). As expected, adjacent blood vessels, small nerve fibers and adipocytes and smooth muscle cells revealed no positive staining for CD44v6. There was no difference between smokers and non smokers.

Immunohistochemical distribution of CD44v6 in dysplastic tissue and invasive carcinoma. The immunolocalization of CD44v6 revealed in all dysplastic lesions a specific labelling of the dysplastic cells. Nearly all cases provided a group 1 expression of CD44v6; only one case was classified as group 2.

In invasive carcinomas, we again observed a strong staining of the squamous carcinoma cells; all cases demonstrated a specific localization of the CD44v6 at the surface of the tumor cells (Figure 5 b). The amount of positively-labelled tumor cells were classified as following: none of the cases showed a slight expression (group 3), in 3 of the analyzed cases we observed a strong CD44v6 expression (group 2). In the remaining 7 cases, almost all tumor cells were positively-marked (group 1).

With regard to the degree of tumor cell differentiation, no difference could be observed between tumors which were poorly- or well-differentiated.

Discussion

CD44 may be a candidate serum tumor marker due to overexpression of certain CD44 isoforms and the presence of soluble CD44 isoforms in the blood circulation in different human tumors (9, 16). In order to test the value of soluble isoforms of CD44 in the peripheral blood of patients with SCCHN, it is important to take a closer look at the expression of the molecules and its splice variants in solid SCCHN. Many others have focused on the expression of CD44v6.

Squamous cell tissue is the only healthy human epithelium which physiologically expresses CD44v6. Stoll *et al.* postulated that down-regulation and loss of special isoforms can be regarded as pathological alterations in SCCHN (28). This loss of one or more of the splice variants of CD44 has been proven to be an independent prognostic risk factor. Stoll *et al.* argued that there are three different groups regarding the expression of CD44: the first group shows a significant correlation between the expression of CD44 and survival, like gastric cancer, colorectal cancer, breast cancer, cervical cancer and malignant lymphoma (13); the second group shows expression of CD44 without a direct influence on the survival rate, like ovarian cancer (26), bronchial carcinoma (7), renal cell carcinoma (30) and malignant melanoma (15); the third group shows a correlation between the loss of CD44 expression and a bad prognosis, like skin cancer and laryngeal cancer (24). Investigations of squamous cell carcinoma of the tongue showed a down-regulation of CD44v3, CD44v4/5 and CD44v6 and a correlation between cell differentiation, tumor grade and presence of lymph node metastases (3).

Kanke *et al.* found a significant correlation between reduced CD44v6 expression and poorer differentiation, as well as an increased number of lymph node metastases in SCCHN. Larger tumors and the ability to invade were concomitant with decreasing CD44v6 expression (14). According to this, Soukka *et al.* assumed that cancerous changes are strongly associated with the down-regulation and loss of CD44v6 (27). Carinci *et al.* described decreased survival due to decreased expression of CD44 in

oropharyngeal cancer. Accordingly, they assumed that a loss of cell adhesion due to decreased CD44 expression is determinant of prognosis (2).

In contrast, an increased expression of CD44 isoforms by different malignoma, especially CD44v6, has been postulated by other investigators (4). Salles *et al.* assumed that the overexpression of CD44 isoforms, like v5, v6, v7 and v8, enhance the metastatic potential of these tumors (23). Güler *et al.* described that CD44v6 expression in normal squamous epithelium is restricted to the lower one-third of the layers. They showed a correlation between a significant decrease in disease-free survival and extensive staining for CD44v6. Therefore, they assumed CD44v6 to be a good prognostic factor in laryngeal cancer (8). We found similar results on immunohistochemical staining; positively-labelled cells were mainly located in the lower layers of the normal epithelium. Furthermore, we did not observe any differences between the amount of positively-labelled cells in terms of dysplastic or malignant tumor tissue. In line with our results, Sikorska *et al.* demonstrated that there is no significant difference regarding the expression of CD44v6 in laryngeal squamous cell carcinoma comparing patients with and without lymph node metastases. High or low CD44v6 expression showed no influence on survival time (25). Also, other groups have described a decreased expression of CD44v5 and CD44v6 isoforms without having influence on the frequency of metastases (21, 27).

Soluble CD44 (sCD44) is present in human serum, detectable by an enzyme-linked immunosorbent assay (ELISA). The presence of CD44 in the serum and the tumor was investigated as a possible prognostic factor of the overall survival and the metastatic potential (5, 29, 31). Generally, the concentrations of sCD44v6 are not as sensitive as routinely established tumor markers, since the levels of sCD44v6 markers do not reflect the true expression of CD44 on the tumor surface. Patients suffering from chronic inflammatory intestinal disease also showed increased serum levels of CD44, probably due to the activation of the immune system (32). Accordingly, there are detectable levels of CD44 and CD44v6 in the sera of healthy donors and patients with lymphoma, benign or malignant lung and breast cancer (10, 29). This phenomenon was further investigated in pancreatic carcinoma with a direct correlation between sCD44v6 and tumor size, lymph node status and survival rate, but not for sCD44. The presence of isoforms like sCD44v6 in patients with pancreatic carcinoma were not attributed to malignant cells, but as being released from activated lymphocytes. The decrease of the serum level on increasing tumor stage is concomitant with the lower number of activated lymphocytes representing the suppression of the immune system (6). Confirming these findings, Matsumura *et al.* verified measurable serum levels of CD44v6 in the control group of healthy donors. This lowers the significance of sCD44v6 as a potential tumor marker.

Our investigations, including patients with primary SCCHN and patients with benign non-inflammatory ENT diseases, made clear that there is no significant difference between the CD44v6 serum concentrations of the two groups. Additionally, there is no direct correlation between the serum level of CD44v6 and tumor size, histological differentiation or tumor stage. These results are new, but underline the data of Van Hal *et al.,* who could not demonstrate a correlation between the soluble levels of CD44v6 of patients with SCCHN and healthy donors. Their observation that resection of the tumor does not alter the level of soluble CD44v6 is in accord with our observation that the level of sCD44v6 in SCCHN is not tumor-dependent, since none of the classic clinical parameters predicting clinical prognosis can be related.

In conclusion, the serum level of CD44v6 is of no clinical value in initial screening and follow-up of patients with SCCHN.

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