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# Increased soluble CD44 concentrations are associated with larger tumor size and lymph node metastasis in breast cancer patients

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**Abstract**

*Purpose*   CD44 is a cell surface glycoprotein involved in cell–cell and cell–substrate interactions, which may be shed or released into circulation by proteolytic enzymatic mechanisms. Alternative splicing of CD44 and aberrant levels of soluble CD44 variants in the serum of cancer patients have been correlated to tumor progression and metastasis in different tumors including breast cancer. In this study we evaluated the clinical value of CD44 serum levels (sCD44) in patients with primary breast cancer.

*Methods*   Concentrations of soluble isoforms sCD44std, sCD44v5 and sCD44v6 were determined with a sensitive ELISA and normalized against the total protein concentration (TP). Pre-operative serum samples from 82 patients and 67 age-matched healthy blood donors were analyzed. The results were correlated to clinico-pathological parameters (tumor size, grading, lymph node metastasis, etc.).

*Results*   In sera of breast cancer patients, we detected elevated concentrations of sCD44v6 ($P = 0.0001$) and total protein TP ($P = 0.0001$) in comparison to healthy controls, whereas overall sCD44 (sCD44std) and sCD44v5 did not differ. Patients with sCD44v6-concentrations above the 75%-percentile showed an increased T stage (2.9 cm vs. 1.8 cm) as well as a higher risk for lymph node metastasis (55% vs. 35%). In breast cancer patients with lymph node metastasis the median value of sCD44v6 was significantly higher ($P = 0.025$) in comparison to patients without lymph node metastasis and healthy controls.

*Conclusions*   Our data suggest an upregulated expression of alternatively spliced soluble CD44 isoforms in breast cancer patients. The specific alterations of certain CD44 isoform concentrations (especially sCD44v6) may reflect disturbances of the nuclear splicing machinery in tumor cells. The clinical significance of our findings are underlined by the positive correlation of elevated sCD44v6 concentrations and lymph node metastases ($r_s = 0.25$).

**Keywords**   Soluble CD44 · Primary breast cancer · Tumor size · Lymph node metastasis

**Abbreviations**

| | |
|---|---|
| C I | Control group I |
| CD | Cluster of differentiation |
| CD44s | CD44 standard |
| CD44v | CD44 variant |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Estrogen receptor |
| FSH | Follicle stimulating hormone |
| PR | Progesterone receptor |
| $r_s$ | Spearman correlation coefficient |
| sCD44 | Soluble CD44 |
| TP | Total protein concentration |

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## Introduction

CD44 designates a heterogeneous family of cell surface glycoproteins. The diversity is due to alternative RNA splicing

of an internal cassette of variant exons (v1–v10). Different isoforms of CD44 are generated by the insertion of different combinations of alternative exons (v1–v10) at a single site within the membrane-proximal portion of the extracellular domain (Cichy and Pure 2003; Ponta et al. 2003).

CD44 is involved in cell–cell and cell–substrate interactions, such as lymphocyte homing, cell-migration and -adhesion. As a cell surface glycoprotein, CD44 may be shed or released into circulation by proteolytic enzymatic mechanisms (Cichy and Pure 2003).

Most cells express the standard isoform of CD44 in which no alternative exon is included, while expression of membrane-bound CD44 variable isoforms has been found to be associated with cancer progression and metastasis in different human tumors including breast cancer (Ponta et al. 2003; Sanchez Lockhart et al. 2001). In addition, altered levels of soluble CD44 proteins in sera of cancer patients have been correlated to tumor progression and metastasis in different carcinomas. In non-Hodgkin's lymphoma as well as B-cell chronic lymphocytic leukaemia elevated sCD44 levels are associated with poor outcome and high risk of disease progression (Molica et al. 2001; Niitsu and Iijima 2002). In head and neck cancer elevated serum levels were also detected (Kawano et al. 2005a, b).

In breast cancer CD44 splice variants v5 and v6 have been reported to be expressed in tumor tissue. Correlations between elevated CD44 expression and common diagnostic and prognostic factors are still under investigation (Sanchez Lockhart et al. 2001; Guriec et al. 1996, 1997; Lockhart et al. 1999). Especially CD44v6 seems to play an important role in breast cancer (Lackner et al. 1998; Martin et al. 1997; Sheen-Chen et al. 1999). Previous studies on soluble CD44 isoforms in breast cancer patients have shown significant elevation of sCD44v6 concentrations and correlations to clinico-pathological parameters like steroid-receptor status, grading (Sheen-Chen et al. 1999), lymph node metastasis (Martin et al. 1997) and the presence of distant metastasis (Lackner et al. 1998; Sheen-Chen et al. 1999). For other soluble CD44 isoforms the situation remains unclear.

In this study we evaluated the clinical value of CD44 serum levels (sCD44std, sCD44v5, sCD44v6) in patients with primary breast cancer.

## Materials and methods

### Cases and controls

Pre-operative sera were available from 82 consecutive patients with histological proven invasive primary breast cancer without distant metastasis, who received adjuvant treatment in the Department of Obstetrics and Gynaecology at the University of Freiburg, Germany, in the period

between April 2000 and September 2001. Approval by the local ethics committee (No. 313/2000) and written informed consent of each patient was obtained. The blood samples were initially taken before surgery in women diagnosed with breast cancer without prior primary systemic therapy. An experienced pathologist performed in all cases the diagnosis of invasive tumor according to TNM-guidelines in the Institute of Pathology, University Hospital Freiburg. The median age of patients was 58.5 years (range 33–87 years), 27 patients were premenopausal or received HRT and 55 patients were postmenopausal at the time of diagnosis. The characteristics of the study population are summarized in Table 1. A group of 107 healthy female blood donors served as a control for serum analysis of soluble CD44 isoforms. The control group was divided into two subgroups, C I: age $\leq$ 30 years ($n = 40$, median age 24 years, range 19–30 years) and C II: > 30 years ($n = 67$, median age 58 years, range 35–67 years) in order to create an adequate age-matched control group.

### Enzyme-linked immunosorbent assay (ELISA)

Pre-operative sera were stored at −20°C until analysis. Pre-treatment serum concentrations of the soluble isoforms sCD44std (overall sCD44 serum level) and the specific isoforms sCD44v5 and sCD44v6 were assessed with a quantitative ELISA for sCD44std, sCD44v5 and sCD44v6, respectively, using the CD44 antibody clones SFF-2 (for human CD44std), VFF-8 (for human CD44v5) and VFF-18 (for human CD44v6). The antibodies were either part of ELISA-Kits or were bought separately (all Bender MedSystems, Vienna, Austria). All measurements were performed in triplicates using a modified manufacturer's protocol.

Intra- and inter-assay controls were performed parallel to each ELISA. The panel of 107 sera collected from female blood donors was also examined for serum concentrations of sCD44std, sCD44v5 and sCD44v6.

The detection level of the system was 0.07 ng/ml for sCD44std, 0.22 ng/ml for sCD44v5 and 0.09 ng/ml for sCD44v6, respectively. The intra-assay and inter-assay variability ranged from 5.26 to 10.89%.

### Serum concentration of total protein

The sCD44 concentrations were normalized against the total protein concentration (TP) assessed by a modified Bradford assay.

### Steroid receptor status

Estrogen and progesterone receptor expression was routinely analyzed by immunohistochemistry. An immunoreactive (IRS) score was determined by a trained pathologist.

**Table 1** Characteristics of breast cancer patients evaluated in the study ($n = 82$)

| | No. of patients | (%) |
|---|---|---|
| Age (years) | | |
| <50 | 17 | 20.7 |
| >50 | 65 | 79.3 |
| Menopausal status | | |
| Premenopausal | 27 | 32.9 |
| Postmenopausal | 55 | 67.1 |
| Tumour cell type | | |
| Invasive ductal | 47 | 57.3 |
| Invasive lobular | 17 | 20.7 |
| Other | 18 | 22.0 |
| Tumour stage | | |
| T1 | 37 | 45.1 |
| T2 | 29 | 35.4 |
| T3 | 4 | 4.9 |
| T4 | 12 | 14.6 |
| Lymph node metastases | | |
| Yes | 48 | 60.0 |
| No | 32 | 40.0 |
| Grading | | |
| G1 | 4 | 4.9 |
| G2 | 39 | 47.6 |
| G3 | 39 | 47.6 |
| Estrogen receptor | | |
| Negative | 23 | 28.0 |
| Positive | 59 | 72.0 |
| Progesterone receptor | | |
| Negative | 31 | 37.8 |
| Positive | 51 | 62.2 |
| HER2/neu status | | |
| Score 0 | 13 | 20.0 |
| Score 1 | 14 | 21.5 |
| Score 2 | 10 | 15.4 |
| Score 3 | 28 | 43.1 |

A score higher than 3 for estrogen receptor or progesterone receptor, respectively was considered positive (Remmele and Schicketanz 1993).

Statistical analysis

The statistical analysis focussed on differences in the distribution of serum levels of soluble CD44 isoforms in breast cancer patients and healthy controls. In cancer patients, the correlations between soluble CD44 isoforms and clinico-pathological parameters were analyzed. The Kolmogorov-Smirnov test demonstrated a non normal distribution of the results, which was not improved by logarithmic transformation. Therefore, the Wilcoxon test for paired samples was used. Different soluble CD44 isoforms were tested for correlation with each other by Spearman's correlation coefficient ($r_s$). Furthermore, serum levels of CD44 were correlated to the following clinico-pathological parameters: age, menopausal status (premenopausal vs. postmenopausal), FSH, hormone receptor status (estrogene and progesterone, IRS = 3 vs. IRS $\geq$ 4), tumor size, tumor grading (G1 vs. G2 vs. G3), tumor cell type (invasive-ductal vs. invasive-lobular vs. others), lymph node metastasis, HER2/neu-Score (score 1 and 2 vs. score 3), CA 15–3 using Spearman's correlation coefficient ($r_s$). The SPSS Software Version 13.0.1 was used for statistical analysis.

## Results

### Serum levels of soluble CD44 in healthy controls

Serum samples obtained from 107 healthy female blood donors were analyzed for soluble CD44std, v5 and v6 concentrations as well as TP concentration. Mean values range and *P*-values were determined. Healthy blood donors were divided into two groups according to age (C I < 30 years, C II $\geq$ 30 years).

While sCD44std and sCD44v5 concentrations did not show differences, serum concentrations of sCD44v6 were significantly higher in older blood donors than in younger blood donors (152.06 ng/ml vs. 142.05 ng/ml; $P = 0.0449$). However, the TP concentration was significantly lower in older compared to younger healthy controls (47.0 g/l vs. 49.77 g/l; $P = 0.0002$). These findings were supported by the highly statistical significant differences of the sCD44v6/TP ratio between the two groups ($P = 0.0037$).

### Serum levels of soluble CD44 isoforms in breast cancer patients

In a second step, serum samples of the 82 breast cancer patients and 67 age matched healthy controls were analyzed. Serum concentrations of sCD44v6 were significantly higher in breast cancer patients than in controls (194.41 ng/ml vs. 152.06 ng/ml; $P = 0.0001$) (Fig. 1). Furthermore TP concentrations were significantly elevated in breast cancer patients compared to age matched controls (55.41 g/l vs. 47.00 g/l; $P = 0.0001$) (Fig. 2). However, there were no significant differences for overall sCD44 (sCD44std) and sCD44v5, although higher sCD44v5 concentrations were more often seen in breast cancer patients (75-percentile: 51.4 ng/ml vs. 41.5 ng/ml) (Table 2). Additionally, in breast cancer patients the ratio of sCD44v6 to overall CD44 (sCD44v6/sCD44std) as well as the ratio of sCD44v6 to total protein (sCD44v6/TP) changed significantly in favor for sCD44v6 compared to controls (sCD44v6/sCD44std:
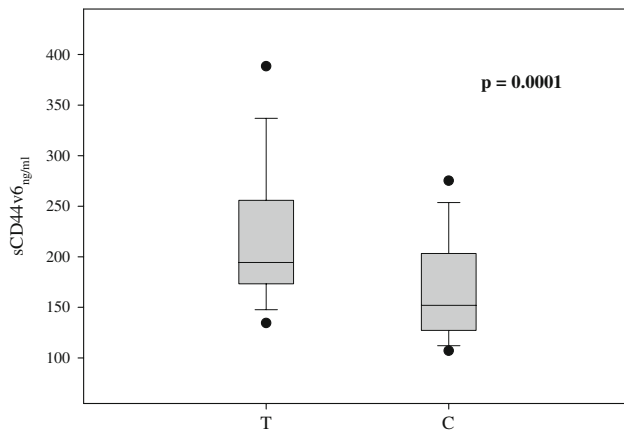
**Fig. 1** Serum levels of soluble CD44v6 in breast cancer patients (T) and healthy controls (C). sCD44v6 serum levels are significantly elevated in breast cancer patients ($P = 0.0001$). *Box-plots* show 5/95-percentile
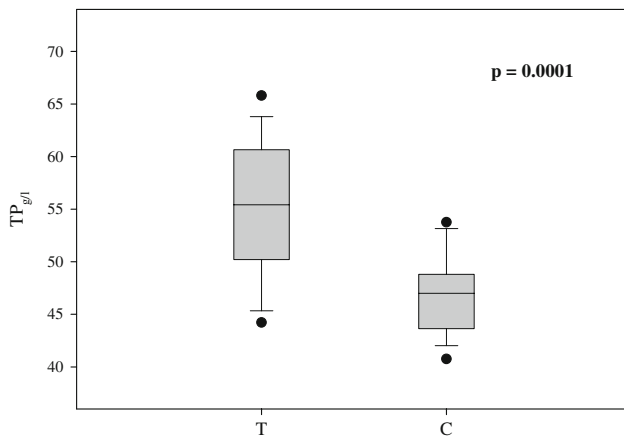


**Fig. 2** Total protein concentration in breast cancer patients (T) and healthy controls (C). The total protein concentration is significantly elevated in breast cancer patients ($P = 0.0001$). *Box-plots* show 5/95-percentile

**Table 2** sCD44-variants in the serum of breast cancer patients

|  | sCD44std | sCD44v5 | sCD44v6 | TP |
|---|---|---|---|---|
| Patients | 504.88 | 37.89 | 194.41 | 55.41 |
|  | 264.89–1634.41 | 18.68–119.94 | 98.78–506.32 | 42.53–68.47 |
| Controls | 507.71 | 33.80 | 152.06 | 47.00 |
|  | 222.52–1079.74 | 17.31–81.32 | 95.36–349.31 | 38.79–62.01 |
| P-value[a] | 0.5055 | 0.0950 | 0.0001 | 0.0001 |

Serum levels of soluble CD44 (median value and range in ng/ml), TP (median value and range in g/l) in breast cancer patients and healthy controls

[a] Wilcoxon rank-sum test

0.42 vs. 0.36; $P = 0.0025$; and sCD44v6/TP: 3.64 vs. 3.08; $P = 0.0289$) (Table 3).

Further detailed analyses revealed that patients with sCD44v6 concentrations above the 75%-percentile presented with an increased T stage as well as a higher proba-

**Table 3** sCD44v-ratios in breast cancer patients

|  | sCD44v6/TP | sCD44v6/ sCD44std | sCD44v5/ sCD44v6 |
|---|---|---|---|
| Patients | 3.64 | 0.42 | 0.19 |
|  | 1.88–9.13 | 0.16–0.88 | 0.09–0.40 |
| Controls | 3.08 | 0.36 | 0.20 |
|  | 2.07–7.48 | 0.12–0.60 | 0.11–0.44 |
| P-value[a] | 0.0289 | 0.0025 | 0.0052 |

sCD44v6/TP-, sCD44v6/sCD44std- and sCD44v5/sCD44v6-ratio in breast cancer patients and healthy controls

[a] Wilcoxon rank-sum test

bility for lymph node metastasis in comparison to patients with concentrations lower than the 75%-percentile. The median tumor size was 2.9 cm versus 1.8 cm and the risk of lymph node metastasis 55% versus 35%. On the other hand patients with sCD44v6 concentrations below the 25%-percentile showed a smaller tumor size and a lower risk for lymph node metastasis. In this patient group, the median tumor size was 1.7 cm versus 2.2 cm and the risk of lymph node metastasis was 19.1% versus 47.5% (Table 4). In a subset of patients ($n = 31$) we monitored sCD44std, sCD44v6 and TP concentrations up to day 10 postoperatively. After an initial drop of 15–17% (day 3–5) sCD44std and TP concentrations showed an increase to almost preoperative levels at day 10 ($-6\%$). In contrast, sCD44v6 concentrations stayed at a decreased level ($-13\%$) even at day 10 postoperatively (data not shown).

## Serum levels of soluble CD44 in breast cancer patients with lymph node metastases

A subgroup analysis of patients with lymph node metastases ($n = 32$) showed higher sCD44v6 concentrations compared to patients without lymph node involvement

**Table 4** Correlation of sCD44v6 with lymph node involvement and tumour size

| Parameter | Lymph node involvement % (n) | Tumor size (cm) |
|---|---|---|
| sCD44v6 > 75% (255.15 ng/ml) | 55.0 (11/20) | 2.90 |
| sCD44v6 < 75% (255.15 ng/ml) | 35.0 (21/60) | 1.80 |
| sCD44v6 > 25% (173.70 ng/ml) | 47.5 (28/59) | 2.20 |
| sCD44v6 < 25% (173.70 ng/ml) | 19.1 (4/21) | 1.70 |

Correlation of high (>75%-percentile) and low (<25%-percentile) sCD44v6-concentrations in the serum of breast cancer patients with respect to lymph node involvement and tumour size. Patients with high (>75%-percentile) sCD44v6-concentrations have an increased risk for lymph node involvement and reveal a larger tumour size

($n = 48$) (217.36 ng/ml vs. 184.12 ng/ml) (Fig. 3) respectively. In addition the ratios of sCD44v6 to overall CD44 and of sCD44v6 to TP changed in favor of sCD44v6 (sCD44v6/sCD44std: 0.47 vs. 0.37; sCD44v6/TP: 4.05 ng/mg vs. 3.45 ng/mg), respectively (Fig. 4). These alterations were statistically significant (sCD44v6: $P = 0.025$; sCD44v6/TP: $P = 0.016$) (Table 5).

### Correlation of soluble CD44 isoforms and clinico-pathological parameters

In breast cancer patients the Spearman test revealed a positive correlation for sCD44v6 and lymph node metastases
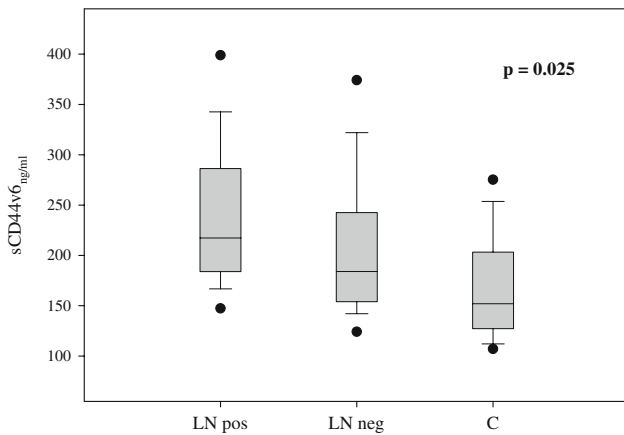


**Fig. 3** Serum levels of soluble CD44v6 in breast cancer patients with lymph node metastases (LN pos), without lymph node metastases (LN neg) and healthy controls (C). The sCD44v6 serum levels are significantly elevated in patients with lymph node metastasis compared to patients without lymph node metastases ($P = 0.025$). *Box plots* show 5/95-percentile
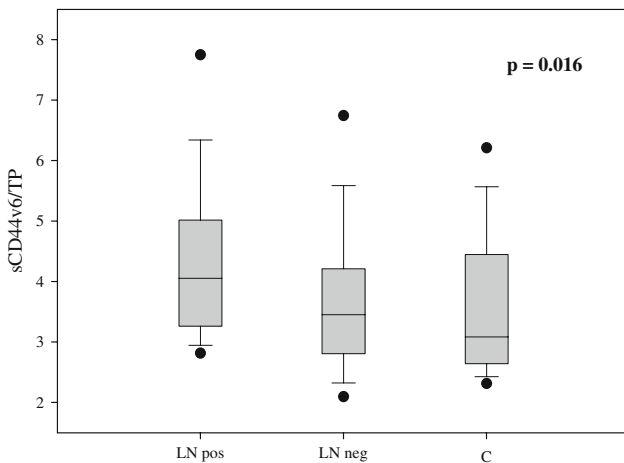


**Fig. 4** The ratio of sCD44v6 to total protein concentration (sCD44v6/TP) in breast cancer patients with lymph node metastases (LN pos), without lymph node metastases (LN neg) and healthy controls (C). The sCD44v6/TP ratio is significantly elevated in patients with lymph node metastasis compared to patients without lymph node metastases ($P = 0.016$). *Box plots* show 5/95-percentile

(0.25) as well as a negative correlation for sCD44v6 and TP (−0.31). In addition, strong correlations were found for the coexpression of sCD44v5 and sCD44v6 in breast cancer patients (0.63) as well as in healthy controls (0.51). Furthermore, in healthy blood donors sCD44std (0.29) and sCD44v6 (0.27) were associated with menopausal status. Additional correlations of soluble CD44 isoforms with other clinical parameters were not seen. In a subset of breast cancer patients ($n = 66$) we determined the CA 15–3 concentration and correlated the results to sCD44-levels. There was no correlation seen for any of the investigated sCD44-variants (sCD44std, sCD44v5, sCD44v6) and CA 15–3 (data not shown).

## Discussion

CD44 splice variants v5 and v6 are expressed in breast cancer. Previous studies described an overexpression of different CD44 isoforms not only for breast cancer but also for other human tumors. However, the diagnostic relevance of CD44 in breast cancer is still controversially discussed. While the CD44 epitope encoded by exon v6 has been described as an independent marker for prognosis by several groups (Guriec et al. 1996; Kaufmann et al. 1995), others could not confirm these data (Tempfer et al. 1996; Friedrichs et al. 1995).

Previous studies of soluble CD44 isoforms in breast cancer patients have shown a significant elevation of sCD44v6 concentrations and correlation to clinico-pathological parameters like steroid-receptor status, grading, lymph node metastases or presence of distant metastases (Lackner et al. 1998; Martin et al. 1997; Sheen-Chen et al. 1999). For other soluble CD44 isoforms the situation remains unclear.

In our study we evaluated soluble CD44 isoforms (sCD44std, sCD44v5 and sCD44v6) in patients with primary breast cancer without distant metastases.

In healthy controls we found an age dependent increase in sCD44v6 concentrations, whereas no difference was detected for sCD44std and sCD44v5 concentrations. These findings suggest that age influences sCD44v6 concentrations which might be due to altered hormonal regulation of CD44 splicing (by TPA, PDGF, IGF-1) (Fichter et al. 1997).

In breast cancer patients, the sCD44v6 concentration was significantly higher compared to controls whereas no difference was observed for overall sCD44 (sCD44std). These findings are consistent with previous published studies. There was no significant difference for sCD44v5 concentrations between breast cancer patients and controls, although high sCD44v5 concentrations were more often seen in breast cancer patients. In addition, we detected a positive correlation of sCD44v5 and sCD44v6 (breast

**Table 5** sCD44-variants in patients with lymph node involvement

|  | sCD44std | sCD44v5 | sCD44v6 | sCD44v6/TP |
|---|---|---|---|---|
| Lymph node positive | 494.61 | 38.98 | 217.36 | 4.05 |
|  | 342.12–984.65 | 21.62–119.94 | 145.63–424.17 | 2.75–8.10 |
| Lymph node negative | 504.88 | 36.35 | 184.12 | 3.45 |
|  | 264.89–1634.41 | 18.68–91.87 | 98.78–506.32 | 1.88–9.13 |
| $P$-value[a] | 0.930 | 0.191 | 0.025 | 0.016 |

Serum levels of soluble CD44 (median value and range in ng/ml), TP (median value and range in g/l) and sCD44v6/TP-ratio in breast cancer patients with and without lymph node metastases

[a] Mann–Whitney test

cancer patients: $r_s = 0.63$; healthy controls: $r_s = 0.51$) indicating en bloc splicing of both variant exons, which was recently functionally described (Stickeler et al. 2001). In patients with breast cancer the ratio of sCD44v6 to sCD44std (sCD44v6/sCD44std) and of sCD44v6 to total protein concentration (sCD44v6/TP) was altered in favor of sCD44v6. Since the total protein concentration in breast cancer patients was also significantly elevated, these results show that in breast cancer cells expression of alternatively spliced CD44 isoforms is not only shifted towards sCD44v6 but also that this shift is highly specific. The range of sCD44v6 levels in breast cancer patients and controls showed an overlap. Therefore sCD44v6 might not be suitable as screening marker for the detection of breast cancer. However, it might serve as a marker for patients with a higher risk for lymph node involvement and advanced T stages. In our study, patients with high sCD44v6 concentrations (>75-pecentile) had an increased tumour size and a higher risk of lymph node metastasis whereas patients with low sCD44v6 concentrations (<25-percentile) revealed smaller tumour sizes and a decreased risk of lymph node metastasis compared to other breast cancer patients. Patients with lymph node metastasis had significantly higher sCD44v6 concentrations than patients without lymph node metastasis or healthy controls. Unlike other previously published studies we did not find other correlations to clinico-pathological parameters for any of the investigated sCD44 isoforms. This possibly could be due to the inclusion criteria of patients with primary breast cancer without distant metastases.

In summary, our study showed a direct correlation of CD44 serum concentrations and the occurrence of breast cancer. Specific alterations of certain CD44 isoform concentrations (especially sCD44v6) may reflect disturbances of the nuclear splicing machinery in tumor cells. Our finding of an involvement of sCD44v6 in lymphatic tumour spread renders the sCD44v6 isoform an interesting target for further investigation and possibly points towards new approaches of breast cancer therapy.

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