

Tenascin-C serum levels and its prognostic power in non-small cell lung cancer

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ABSTRACT

Background: Tenascin-C is overexpressed in the stroma of most solid malignancies and may function as a diagnostic tumor marker. This study was conducted to evaluate the potential significance of Tenascin-C as a predictive marker for tumor progression in the sera of non-small cell lung cancer (NSCLC) patients.

Results: Serum concentration of Tenascin-C is significantly elevated in NSCLC patients compared to healthy controls ($p=0.013$). The sensitivity of Tenascin-C in detecting NSCLC was 74% at a specificity of 57%. Elevated Tenascin-C serum values are associated with larger tumor size and lymph node involvement ($p=0.022$ and $p=0.036$, respectively). The Kaplan-Meier-curves showed a significant association of Tenascin-C with the patient's overall survival ($p=0.004$), but not with the recurrence-free survival ($p=0.328$).

Methods: We quantified Tenascin-C in the sera of 103 NSCLC patients and 76 healthy blood donors by enzyme-linked immune-absorbance assay tests. Prognostic significance was determined by area under the curve analysis and Youden-index. The results were correlated with clinical, histopathological, and patient survival data (Chi-square test, Kaplan-Meier analysis, log-rank test, multivariate Cox-regression analysis).

Conclusion: Although significantly elevated in patients with NSCLC, the sensitivity and specificity of the Tenascin-C serum quantification test was low. However, although failing to be an independent prognosticator in multivariate analysis, the results implicate Tenascin-C as a predictive prognostic marker for NSCLC patients. The data must be further validated in future prospective trials with larger patient cohorts.

INTRODUCTION

Due to its aggressive biology, lung cancer causes the most cancer-related deaths worldwide [1]. Non-small-cell-lung cancer (NSCLC) accounts for 85% of all lung cancers with a high metastatic potential and a high rate of tumor recurrence, even after curative intended tumor resection. The prognosis for patients with NSCLC depends mainly on classical clinical aspects such as tumor size, grading and the existence of metastases; clinical decision-making is still based on the classical UICC staging system [2]. Although significant improvements of the individualized therapy for NSCLC patients have been made during the

last few years with introduction of several molecular markers (such as EGFR and EML4-Alk) in the clinical routine, the search for a clinically relevant serum marker as diagnostic and prognostic markers is still ongoing [3]. Tumor biomarkers with prognostic power regarding treatment success or prognostic impact on survival might significantly help to stratify the therapy of patients and improve the diagnosis and therapy.

In the case of NSCLC, known biomarkers such as SCCA, CYFRA and CEA show low sensitivities, bounded diagnostics and low aftercare value [4]. Extracellular matrix proteins play a significant role in the survival and metastasis of cancer cells. Thus,

a group of extracellular matrix proteins, the Tenascins, recently came into the focus of cancer research [5–7]. Tenascins are a group of extracellular matrix glycoproteins that are expressed during the development of multicellular organisms and in pathological processes such as inflammation and tissue injury as well as tumor angiogenesis and metastasis [8]. Tenascin-C is a multi-domain disulfide-linked homohexamer consisting of 15 EGF-like domains followed by 15 fibronectin-type-III domains and a C-terminal fibrinogen domain [9]. The Tenascin protein family consists of four members (-X, -R, -C, -W), of which Tenascin-X and -R are also present in differentiated, healthy tissues. However, in adults Tenascin-C and -W are expressed only in pathophysiological conditions such as cancer development [5]. A significant expression of Tenascin-C can be measured in embryogenesis, at sites with a high cell turnover, such as stem cell niches and wound healing and inflammatory diseases, i.e. rheumatoid arthritis [6]. Several isoforms of Tenascin-C are formed by alternative splicing and it was reported that especially its large isoform plays a decisive role in the regulation of angiogenesis and in tumorigenesis of cancer [9–11]. The prognostic significance of Tenascin-C expression was investigated in several malignant diseases, such as squamous cell carcinoma of the head and neck, breast, colorectal and prostatic cancer [12–16]. Little is known regarding the expression and functional role of the large isoform of Tenascin-C in NSCLC, it is overexpressed in specimens of NSCLC and Parekh and colleagues described increased expression in patients with an early recurrence of disease [17–20]. In an analysis of endobronchial epithelial-lining fluid of NSCLC patients,

Tenascin-C was significantly elevated and the authors suggested that it might be a potential diagnostic tool for early cancer detection [21]. Ishiwata and colleagues described a significant correlation of overall survival and elevated Tenascin-C serum values in a cohort of 63 NSCLC patients, but there was no association of clinical and pathological data and the molecule [22]. Moreover, Tenascin-C expression was investigated in COPD lung specimen and patients' serum with divergent results [23, 24].

We conducted the present study to determine the association between preoperatively sampled serum values of the large isoform of Tenascin-C and clinical and histopathological data and to evaluate its significance as a diagnostic and prognostic marker of resectable NSCLC patients.

RESULTS

Serum Tenascin-C levels in NSCLC patients and healthy controls

Tenascin-C concentration in NSCLC-patients was significantly elevated (n=103, mean 5.5 ng/ml, standard deviation (SD) \pm 3.8 ng/ml) compared to healthy blood donors (n=76, mean 6.3/ml, SD \pm 6.6ng/ml, p=0.013; Figure 1A). Receiver operating characteristic curves were used to establish the sensitivity-specificity relationship for Tenascin-C (1B). The AUC was 0.690. The cut-off level determined by the Youden index was 35.6 ng/ml. The sensitivity of Tenascin-C in detecting NSCLC was 74.4% at a specificity of 57.4% compared to the control group.

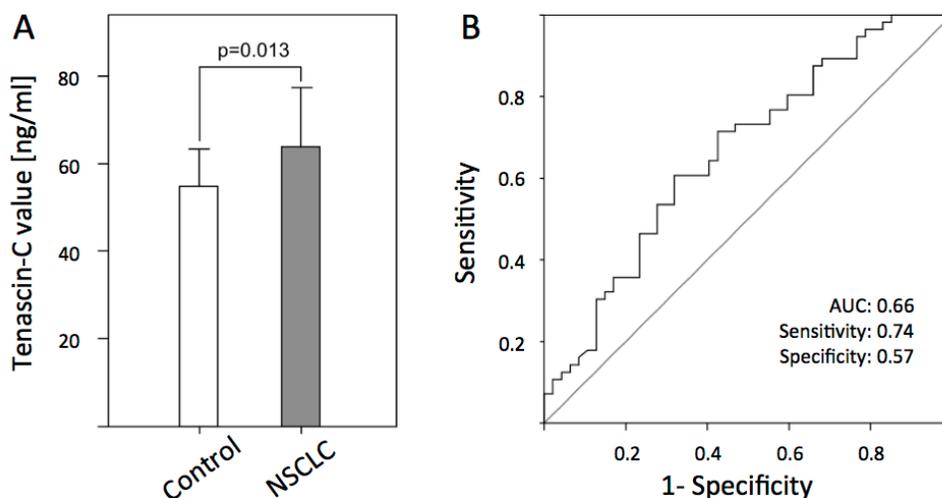


Figure 1: Serum concentration of Tenascin-C in healthy controls and non-small-cell bronchogenic carcinoma (NSCSC) patients. A. Bars represent the mean of n= 76 healthy controls and n = 116 NSCLC patients as determined by ELISA (p<0.013). B. Receiver operating characteristic (ROC) curves of Tenascin-C for the diagnosis of NSCLC patients versus healthy control samples.

Table 1: Association of clinicopathological characteristics of NSCLC patients and Tenascin serum levels

		All (n)	Tenascin-C high (n and %)	Tenascin-C low (n and %)	Significance (p)
Age (cut65)	<65	43	18 (42%)	25 (58%)	1.000
	≥ 65	60	25 (42%)	35 (58%)	
Sex	Male	70	31 (44%)	39 (58%)	0.523
	Female	33	12 (36%)	21 (64%)	
Asbestos exposition[†]	Yes	5	2 (40%)	3 (60%)	1.000
	No	85	35 (41%)	50 (59%)	
Nicotine abuse[†]	Yes	15	5 (33 %)	10 (67%)	0.579
	No	76	32 (42%)	44 (58%)	
Neoadjuvant Therapy[†]	Yes	82	32 (39%)	50 (61%)	0.347
	No	11	6 (55%)	5 (45%)	
Tumor size[†]	pT1+2	74	26 (35%)	48 (65%)	0.022
	pT3+4	20	13 (65%)	7 (35%)	
Lymph nodes[†]	pN0+N1	45	13 (29%)	32 (71%)	0.036
	pN2+N3	50	26 (52%)	24 (48%)	
Distant metastases[†]	M0	94	38 (40%)	56 (60%)	1.000
	M1	8	4 (50%)	4 (50%)	
Grading[†]	G1 and G2	41	16 (39%)	25 (61%)	1.000
	G3	51	21 (41%)	30 (59%)	
Cell type	Adeno	39	13	26	0.115
	Sqamous Cell	37	21	16	
	Large Cell	12	6	6	
Resection[†]	R0	68	25 (37%)	43 (63%)	0.419
	R1	7	4 (57%)	3 (43%)	
30-day Mortality[†]	Yes	10	3 (30%)	7 (70%)	0.516
	No	91	39 (43%)	52 (57%)	
Adjuvant Therapy[†]	Yes	42	17 (41%)	25 (59%)	1.000
	No	51	21 (41%)	30 (59%)	

[†]Due to the retrospective character of the study, numbers do not always add up to 103 patients.

Association of Tenascin-C serum concentrations and clinical and pathological parameter

Patients were divided into low level (<8.33 ng/ml) and high-level Tenascin-C groups (≥8.33 ng/ml). The correlation of clinico-pathological data with Tenascin-C using cross tabulation showed a significant association of increased Tenascin-C and tumor sizes (pT3 and pT4 compared to pT1 and pT2 p = 0.022) and the status of the lymph nodes (pN2 and pN3 versus pN1; p = 0.036), respectively. Clinical parameters such as age, sex, perioperative mortality (30 days), resection, nicotine

abuse, asbestos exposure, neoadjuvant therapy and adjuvant therapy did not show any significant differences compared to controls. We found no significant correlation of Tenascin-C with the development of distant metastases; however, we only assessed a very small number (n=3) of metastasized patients (Table 1).

Kaplan-Meier survival curve for recurrence-free survival in NSCLC patients

Survival curves plotted by the Kaplan-Meier analysis (log-rank test) did not show a significant

correlation of Tenascin-C-level and RFS (median calculated RFS 41.3 months and 24 months, respectively; $p=0.328$, Figure 2A). In contrast, prolonged OS was significantly associated with a lower Tenascin-C serum value ($p=0.004$, Figure 2B). Patients with concentrations under 8.33 ng/ml showed a median OS of 48.1 months, whereas those with concentrations over 8.33 ng/ml only survived for 15.4 months as a median.

Multivariate analysis

In univariate survival analysis performed by cox-regression, a significant impact of clinic-pathological data such as tumor size, lymph node status, resection, grading and distant metastasis was shown (Table 2). While a significant factor in univariate analysis, Tenascin-C failed to be an independent prognosticator for survival in multivariate analysis. The analysis identified the resection status (R) and the tumor cell grading (G) as independent characteristics.

DISCUSSION

The aim of the current study was to evaluate the potential of Tenascin-C serum quantification (large isoform) as a diagnostic and prognostic marker in the peripheral blood of NSCLC patients. Until now, only few groups have analyzed the functional role of Tenascin-C in NSCLC. The results showed that Tenascin-C serum levels were significantly elevated compared to the sera of healthy controls ($p<0.001$). The AUC showed an acceptable discriminatory power of Tenascin-C (AUC = 0.66; Figure 1B). The sensitivity (74%) and specificity (57%) of Tenascin-C was clearly inferior to the most frequently used tumor markers for NSCLC, SCCA, NES

or CEA [25]. These data, which are similar to those of Ishiwata and colleagues, who did not find a significant difference between NSCLC patient sera and healthy controls, enabled us to conclude that Tenascin-C might not be a proper screening tool for the diagnosis of NSCLC [22]. Another aspect underlying this hypothesis is the potential overexpression of Tenascin-C in COPD patients and smoking, which are frequent comorbidities of NSCLC patients [23, 24, 26].

We furthermore investigated the correlation of Tenascin-C serum values and histopathological factors, which revealed a significant association of elevated Tenascin-C values with larger tumor sizes and lymph node metastasis ($p=0.022$ and $p=0.036$, respectively), indicating a relatively elevated Tenascin-C expression and release in higher tumor stages with a larger tumor burden. This is contradictory to the results of Ishiwata and colleagues, who revealed no association with tumor stage [22].

According to our findings, patients with higher Tenascin-C serum values have a shortened OS compared to the low Tenascin-C group. These correlations were already indicated by Parekh and colleagues, who had a small cohort of ten patients and found elevated Tenascin-C mRNA and protein expression in patients with early recurrence of the disease and later confirmed by Ishiwata and colleagues, who described a significant correlation in a group of 63 patients [19]. However, as the multivariate Cox regression analysis in the present study failed to reveal Tenascin-C as an independent prognostic parameter for overall survival ($p=0.354$), the role as a potential prognostic tumor marker for NSCLC patients remains inconclusive. However, data on serum levels were published in few studies only. Depending on the tumor type, the findings of Tenascin-C serum levels and linkage to clinical parameter are variable. For example, analysis

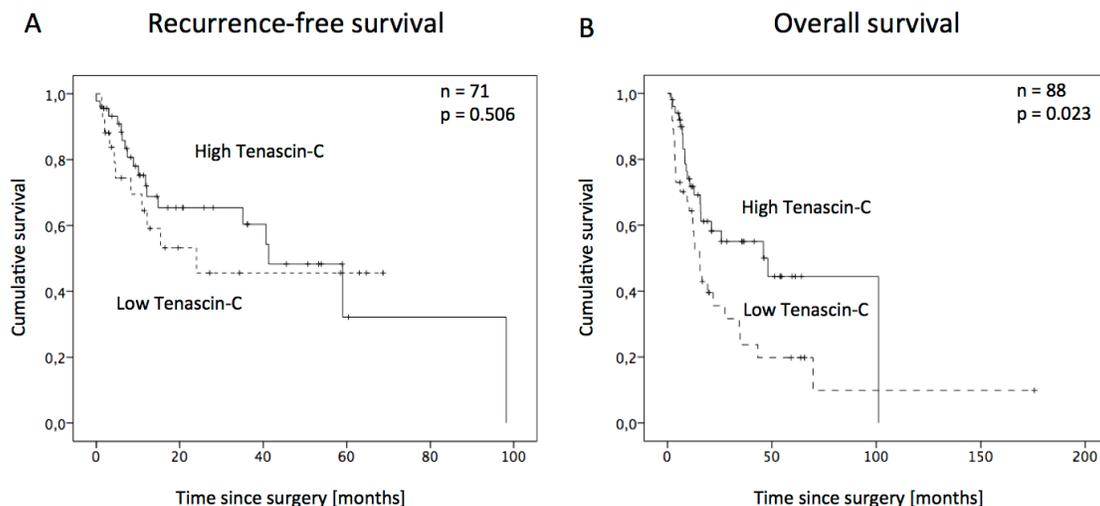


Figure 2: Kaplan-Meier survival curve for recurrence-free survival. A. and overall survival B. in NSCLC patients after surgery for Tenascin-C low- and high-level group.

Table 2: Prognostic value of Tenascin-C expression for overall survival in univariate and multivariate analyses by Cox regression

	Univariate analysis			Multivariate analysis		
	HR	95%CI	P-value	HR	95%CI	P-value
Age	1.325	0.940/1.868	0.108	-	-	-
Sex	0.882	0.614/1.268	0.498	-	-	-
Tumor size (pT3/4 vs 1/2)	2.135	1.434/3.233	0.001	1.606	0.706/3.652	0.259
Lymph node metastases (pN2/3 vs 1/2)	1.850	1.301/2.630	0.001	2.098	0.901/4.885	0.086
Resection margin (R1/2 vs 0)	2.360	1.411/3.948	0.001	3.470	1.163/10.355	0.026
Grading (G3 vs 1/2)	1.600	1.129/2.269	0.008	2.853	1.310/6.213	0.008
Distant metastases (M1 vs M0)	2.806	1.220/6.457	0.015	1.105	0.218/5.600	0.904
Tenascin-C (low vs high)	2.374	1.301/4.321	0.005	1.434	0.669/3.074	0.354

(HR- hazard ratio; CI- confidence interval)

of Tenascin-C serum levels in breast and ovarian cancer did not reveal any correlations to the overall-survival or clinical data [27, 28].

Several studies have investigated the expression of Tenascin-C in various solid tumors and tumor microenvironments and supposed a functional role in tumor progression, migration and the formation of metastases [6]. Interestingly, some researchers identified low Tenascin-C as a marker for a better prognosis for some diseases [29–31], but others described a contrary effect, a correlation of high Tenascin-C and a better prognosis [32, 33]. These results are not surprising, as Tenascin-C mediates complex remodeling of the tumor microarchitecture by close interactions between tumor cells and surrounding stromal cells. Expression of Tenascin-C is associated with intra-tumoral angiogenesis i.e. micro-vessel density and a pro-migratory influence on endothelial cell migration [22]. Tenascin-C expression was found in both, stromal and cancer cells and has a direct influence on epithelia-mesenchymal transition.

Another functional role of Tenascin-C is immunomodulatory; it inhibits the proliferation of blood lymphocytes *in vitro* and interferon-gamma production by tumor-infiltrating lymphocytes isolated from NSCLC specimens [19]. The results of these studies might reveal an association between Tenascin-C and a malignant biology, which results in an elevated shedding of

Tenascin-C by matrix metalloproteinases (MMPs) such as MMP-13 [34]. Additionally, the shedding of the molecule might be caused by a transformation of the surrounding tumor environment. Both mechanisms, the regulation of the molecule's shedding, the migration into the tumor environment and even the access into the bloodstream through the endothelial barrier are understood only in part. However, the elevated expression of Tenascin C in tumor tissue and corresponding serum levels is in line with the well-established idea of a relationship between inflammation and cancer. But, as previously shown, the immune response during malignant progression and treatment resistance is a dynamic process and can even initiate pathways that are pro-tumorigenic [35]. The interactions of the immune system with tumor cells are of high complexity since several different types of cells and, in addition, sub-differentiations in dependency of the surrounding cytokine environment, are reported. The effects, how inflammation promotes tumor initiation, progression and treatment resistance are still poorly understood.

In summary, the detected Tenascin-C might be a useful prognostic parameter, which power might be increased by combination with others. It could reveal additional discriminatory or prognostic power additional to innovative staging parameter, such as circulating disseminated tumor cells status or other molecular

markers together and to established systems, such as the TNM staging. The goal is to identify those patients, which might best profit from the available treatments to tailor an individual therapy for each patient.

Further prospective studies with larger patient cohorts are needed to evaluate the predictive potential of Tenascin-C serum levels. Moreover, the functional role of Tenascin-C in NSCLC is poorly understood and needs to be investigated in *in vitro* and *in vivo* analyses; to increase the knowledge of Tenascin-C associated tumor proliferation and metastatic invasion.

MATERIALS AND METHODS

Characteristics of patients and healthy controls

Blood sera of Caucasian 103 patients, aged between 31 and 82 years (median 65.5 years) with the diagnosis of NSCLC and Caucasian 76 healthy blood donors, aged between 31 and 79 (median 51 years), were included in this study. All patients were treated surgically between 1993 and 2010 in the Department of General, Visceral and Thoracic Surgery of the University Medical Center Hamburg-Eppendorf, Germany. All blood samples were obtained directly before surgery. All sera were processed at the latest after four hours [36]. Operation methods were oncological lobectomies, bilobectomies and pneumectomies with hilar and mediastinal lymphadenectomy.

All data including sex, histology, tumor size, lymph node metastasis, disease stage (UICC 7th edition) and follow-up data were obtained from a combination of clinical and pathological record reviews, from outpatient clinic medical records and communication with patients and their attending physicians, and from the cancer registry. Reliable follow-up data were available for 88 patients. Written consent for using the samples for research purposes was obtained from all patients prior to surgery or blood drawing.

The Ethics Committee of the Chamber of Physicians in Hamburg, Germany approved the study. The study was performed in accordance with the principles of the declaration of Helsinki (as revised in Seoul 2008) and REMARK criteria [37].

Disease-free survival (DFS) was calculated from the date of operation to the first detection of tumor recurrence, while overall survival (OS) was accordingly from the date of operation to the date of death or last follow-up. Patients who did not survive the first 30 days after surgery were excluded from the survival analysis. Median follow-up time of the patients included in the survival analysis was 12.2 months (range 0-176 months); median calculated OS was 27.6 months (95% CI 9.7-45.4 months). Four (6.8%) patients died within the first 30 days after surgery.

Enzyme-linked immunosorbent assay for human Tenascin-C

The serum concentration of human Tenascin-C was quantified using a TNC-ELISA (ELISA (Human Tenascin-C Large Assay Kit - Immuno-Biological Laboratories Co., Ltd., Japan) according to the manufacturer's instructions.

In brief, a microtiter plate pre-coated with monoclonal mouse anti-human Tenascin-C antibody (4C8MS) was incubated with the serum of NSCLC patients and serum of healthy controls, respectively for 1h at 37°C. After washing, a HRP conjugated anti-Tenascin-C mouse antibody (4F10TT) specific for the EGF-like domain was added and incubated for 30 min at 4°C. Another washing step followed before the addition of Tetra-Methyl-Benzidine as the coloring agent (Chromogen) for 30 min at room temperature. The reaction was stopped by adding 1N H₂SO₄ solution. The absorbance was measured at 450 nm on an ELISA reader (Dynatech MR5000; Pegasus Scientific, Rockville, MD, USA).

In order to ensure that the immunoassay was suitable for measuring clinical serum samples, reproducibility and linearity were examined. The assay showed excellent linearity with serial dilutions and showed <10% coefficient of variation (CV) for the intra- and inter-assay variability studies.

Statistical analysis

The statistical analysis was performed using SPSS Statistics for Windows (Version 20, SPSS Inc., Chicago Ill, USA). Interdependence between ELISA results and clinical data was calculated using Chi-square and Fisher's exact tests and displayed in cross tables. The cut-off level for Tenascin-C quantification was determined using the Youden-index. Group differences were calculated by the t-test, ANOVA; Mann-Whitney or Kruskal-Wallis test. Survival curves were plotted using the Kaplan-Meier method and analyzed using the log-rank test. All tests were two-sided and p-values less than 0.05 were considered statistically significant. All variables achieving a P value ≤ 0.05 were included in a multivariate Cox regression model for determining independent prognosticators of DFS and OS.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

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