

Correlation of Expression of Tenascin C and Blood Vessel Density in Non-small Cell Lung Cancers

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Abstract. *Background/Aim: Non-small cell lung cancers are cancer diseases that rank second in terms of incidence and first in terms of mortality, worldwide. Stromal cells of these cancers express tenascin C (TNC) – hexameric glycoprotein, which is also expressed during foetal life. TNC is also observed in stromal cells of most human cancers. In some cancers, TNC was shown to influence proliferation and migration of cancer cells and angiogenesis. The aim of this work was to analyze the correlation of expression of TNC with the markers of vascular endothelial cells, CD31 and CD34, and clinicopathological data in NSCLC. Materials and Methods: Archival paraffin blocks from 101 cases of NSCLC were used for the studies. Immunohistochemical reactions were carried out on paraffin sections using mouse monoclonal antibodies anti-TNC, anti-CD31 and anti-CD34, with the use of Autostainer Link-48. Results: Statistical analysis of the results showed positive correlation between TNC expression and CD31(+) and CD34(+) microvessel density (MVD) ($r=0.456$, $p<0.0001$; $r=0.296$, $p<0.01$, respectively). Conclusion: Based on the obtained results it can be concluded, that TNC may be involved in angiogenesis in NSCLC.*

Lung cancer is the most common cause of death and the second cancer disease in terms of incidence in the world. Despite gradual, small improvements in lung cancer therapy over the last decades (from 14.3% to 18.4% 5-year survival), it is still a very important clinical problem (1-3).

Taking into account histological structure, course of the disease and the type of therapy used, lung cancers are classified

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as small-cell and non-small cell lung cancers (NSCLC). Among the latter, one can distinguish 3 basic histological subtypes: squamous cell carcinoma, adenocarcinoma and large cell carcinoma, which account for over 90% of all diagnosed cases of non-small cell cancers (4). The most common subtype of lung cancer among women is adenocarcinoma, whereas in men – squamous cell carcinoma (5-7).

Tenascin C (TNC) is an extracellular matrix hexameric glycoprotein. In the human body, it is expressed during foetal life, and plays a role in the development of cardiac muscle, valves and coronary vasculature. During foetal life, tenascin is found in large amounts in extensively migrating cells, *inter alia* in neural tube cells, as well as in tendinosis, bone and chondroid tissue (8, 9). In the adult human body, tenascin is not present and can be found in small amounts only in inflammations and during wound healing. It can also be found in pathological conditions in various types of cancers (10, 11). Tenascin structure contains domains that interact *e.g.* with elements of Wnt and RhoA pathways or with components of the extracellular matrix (*e.g.* fibronectin, integrin) (10-11). In some tumours, TNC may stimulate proliferation and migration of cancer cells. Additionally, tenascin can affect angiogenesis process. Angiogenesis is a process of new blood vessels formation by expansion of already existing vasculature (12). CD31 is a marker of endothelial cells from existing blood vessels, whereas CD34 expression is characteristic of newly formed vasculature (12).

Increased expression of TNC was observed in a large number of malignant neoplasms, such as *e.g.* glioblastoma multiforme, osteosarcoma, chondrosarcoma, bladder cancer, pancreatic carcinoma, NSCLC and ductal breast carcinoma (13-20). In case of bladder cancer, it was shown, that high TNC expression in extracellular matrix was associated with significantly shorter patients' survival time, whereas expression of TNC in the cytoplasm of cancer cells was associated with longer survival (21). In glioma, higher TNC expression was correlated with an increased ability for migration and

proliferation of neoplastic cells (22). In pancreatic cancer, TNC can reduce cellular adhesion and thus increase the ability of cancer cells for migration and metastasis (16, 19). In ductal breast cancer, increased TNC expression was correlated with the expression of fibroblast growth factor (FGF) and vimentin. TNC overexpression in the same types of cancer was associated *e.g.* with the presence of metastases into lymph nodes, shorter survival time, increased expression of Ki-67 as well as lack of expression of estrogen receptor (18).

It is also suggested, that TNC affects angiogenesis. In an *in vitro* model, subjecting glioblastoma multiforme cells to TNC resulted in an increased synthesis of proangiogenic factors (23). On the other hand, lung cancer and clear cell renal cell carcinoma showed high TNC expression adjacent to vascular endothelial cells CD31(+) (24, 25). In NSCLC, increased TNC was correlated with tumour size and the presence of lymph node metastases (26). Moreover, intensified TNC expression in this type of cancer was shown in patients with recurrent disease (26).

The aim of this study was to analyze TNC expression in correlation with microvascular density (MVD) CD31(+) and CD34(+), and clinicopathological data.

Materials and Methods

An immunohistochemical (IHC) study was performed on 101 paraffin blocks obtained from patients diagnosed with NSCLC and treated between years 1998-2010 in the Department and Clinic of Thoracic Surgery of Wroclaw Medical University, Poland. All patients were treated surgically, and tissue specimens were prepared prior to chemotherapy. Patients from the IHC group were followed up for 31.53±41.18 (median: 14.0; range=1-145 months) months. In this period 73 patients died. The experiment was performed in accordance to ethical standards and was approved by the Ethics Committee of Wroclaw Medical University (decision no. KB 83/2011). Patients' demographic and clinicopathological characteristics are presented in Table I.

Immunohistochemistry (IHC). All reactions were performed on 4-µm-thick paraffin sections. In order to examine the expression of TNC, CD31 and CD34, the protocol using Dako Autostainer Link48 (Dako, Glostrup, Denmark) was introduced. Deparaffinization, rehydration and antigen retrieval were conducted by boiling in EnVision FLEX Target Retrieval Solution (pH=9, 20 min, 97°C; Dako, Glostrup, Denmark) using the Pre-Treatment Link platform (Dako, Glostrup, Denmark). Activity of endogenous peroxidase was blocked by 5 min incubation in EnVision FLEX Peroxidase-Blocking Reagent (Dako, Glostrup, Denmark). Sections were incubated with the primary mouse monoclonal antibody for anti-human TNC (Leica Novocastra, Nussloch, Germany), diluted 1:50, CD31 antibody (ready-to-use, IR610, Dako, Glostrup, Denmark), CD34 (ready-to-use, IR632, Dako, Glostrup, Denmark) for 20 min at room temperature (RT). Following this EnVision FLEX/HRP – secondary antibodies were applied (20 min at RT; Dako, Glostrup, Denmark). Diaminobenzidine (DAB, Dako, Glostrup, Denmark) was used as the peroxidase substrate and the sections were incubated for 10 min at RT. All slides were counterstained with EnVision FLEX Hematoxylin (Dako, Glostrup,

Table I. Characteristics of patients and tumours.

IHC (n=101)		
Mean age (range)	62.86±8.862 (39-87)	
Parameters	N	%
Gender		
Male	80	79.21
Female	21	20.79
Tumour size		
T1	25	24.75
T2	52	50.50
T3	10	8.91
T4	16	15.84
Lymph nodes		
N0	48	47.52
N1, N2, N3	53	52.48
Grade		
G1	8	7.92
G2	58	57.43
G3	26	25.74
No data	9	8.91
pTNM		
I	41	40.59
II	16	15.84
III	41	40.59
IV	3	2.97
Stage		
Early	57	56.44
Advanced	45	43.56
Tenascin C		
0	18	17.82
1-12	83	82.18
CD31		
≤median (8.33)	52	51.49
>median (8.33)	49	48.51
CD34		
≤median (9.33)	52	51.49
>median (9.33)	49	48.51

IHC: Immunohistochemistry; n: number of cases.

Denmark). Subsequently, the preparations were mounted in Mounting Medium (Dako, Glostrup, Denmark).

Evaluation of IHC reactions. All sections were evaluated using a BX-41 light microscope (Olympus, Tokyo, Japan) by two pathologists who were blinded to the patients' clinical data. In doubtful cases, a re-evaluation with a double-headed microscope was performed until a consensus was achieved. CD31 expression was observed in a cytoplasm of endothelial cells. Initially, whole slide was analysed as follows: the sections were first scanned at low power (×40 and ×100 magnification) to identify the hot-spots (areas of potentially highest microvessel density (MVD) count). Subsequently, these areas were examined under ×200 magnification using a Chalkley Point Array graticule (Pyser Sgi., Edenbridge, UK). The Chalkley count was regarded as the number of grid points that hit stained vessels. An average score was determined for three

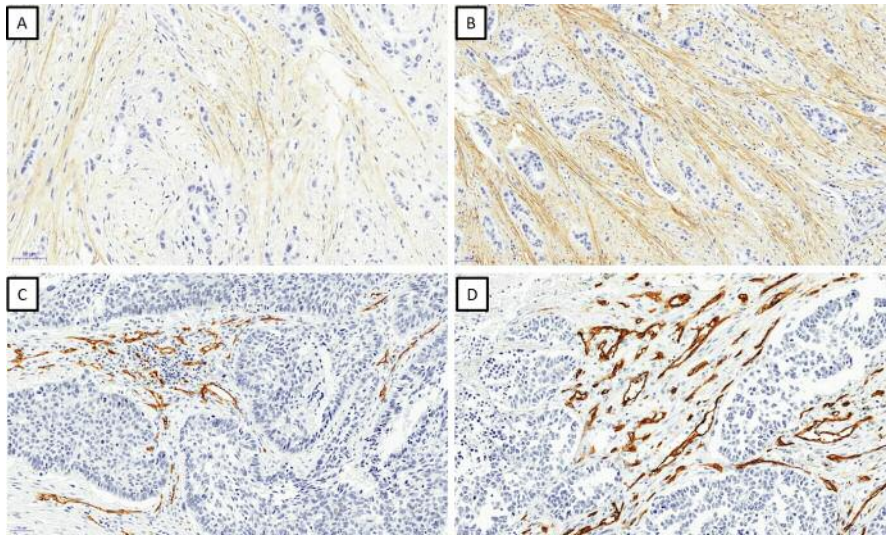


Figure 1. Low (A) and high (B) expression of tenascin C in stromal cells of non-small cell lung cancer (NSCLC) and expression of CD31 (C) and CD34 (D) in vascular endothelium in NSCLC (Magnification $\times 200$).

hot-spots. The evaluation of tenascin-C reaction intensities was appraised using the 12-point immunoreactive score (IRS) of Remmele and Stegner, in which the percentage of positive cells and the intensity of the reaction were taken into account (27). The final score assigned was the multiplicative product of these two parameters (Table II).

Statistical analysis. Shapiro-Wilk test was used for evaluating the normality assumption of the examined groups. For comparing the differences in the expression of examined markers in all groups of patients' pairs and clinical-pathological data, the Mann-Whitney test was used. Additionally, the Spearman correlation test was used to analyse the existing correlations. The Kaplan-Meier method was used to construct survival curves. To evaluate the analysis of survival, Mantel-Cox test was performed. All statistical analyses were performed using Prism 5.0 (GraphPad, La Jolla, CA, USA). The results were considered as statistically significant when $p < 0.05$.

Results

Based on the conducted experiments, cytoplasmic TNC expression in stromal cells was shown in 82.17% of analyzed cases (Figure 1A and B). Statistical analysis of the obtained results did not show any differences between histological subtypes of NSCLC: squamous cell carcinoma, adenocarcinoma and large cell carcinoma. Evaluation of TNC expression and clinical-pathological data of NSCLC patients revealed a statistically significantly higher expression in G2 tumours in comparison to G1, and TNC expression higher in G3 in comparison to G1 (Figure 2A).

Density analysis of CD31-positive (CD31(+)) blood vessels showed that they were present in 93.06%, and CD34-positive

Table II. The immunoreactive (IRS) score was used to evaluate cytoplasmic expression of tenascin C (TNC). The final score is multiplication of percentage of positive cells (A) and the intensity of colour reaction (B).

Percentage of positive cells	Intensity of colour reaction
0 pts – 0% cells	0 pts – no staining
1 pt – 1%-10% cells	1 pt – low
2 pts – 11%-50% cells	2 pts – moderate
3 pts – 51%-80% cells	3 pts – intense
4 pts – >80% cells	

pt: Point; pts: points.

(CD34(+)) were present in 95.04% of cases (Figure 1C and 1D). No statistically significant differences were observed in the case of CD34(+) blood vessel density analysis and the following features: G, pT, stage, sex or patient survival.

The expression correlation analysis showed a medium positive correlation between TNC expression and the density of CD31(+) vasculature ($r=0.456$, $p < 0.0001$) (Figure 2B), as well as a weak positive correlation between TNC expression and the density of CD34(+) blood vessels ($r=0.296$, $p < 0.01$) (Figure 2C).

Discussion

The increased TNC expression in cancer and stromal cells of various cancer types has been described on many occasions (11, 13-20). In our studies, we observed an increased TNC expression

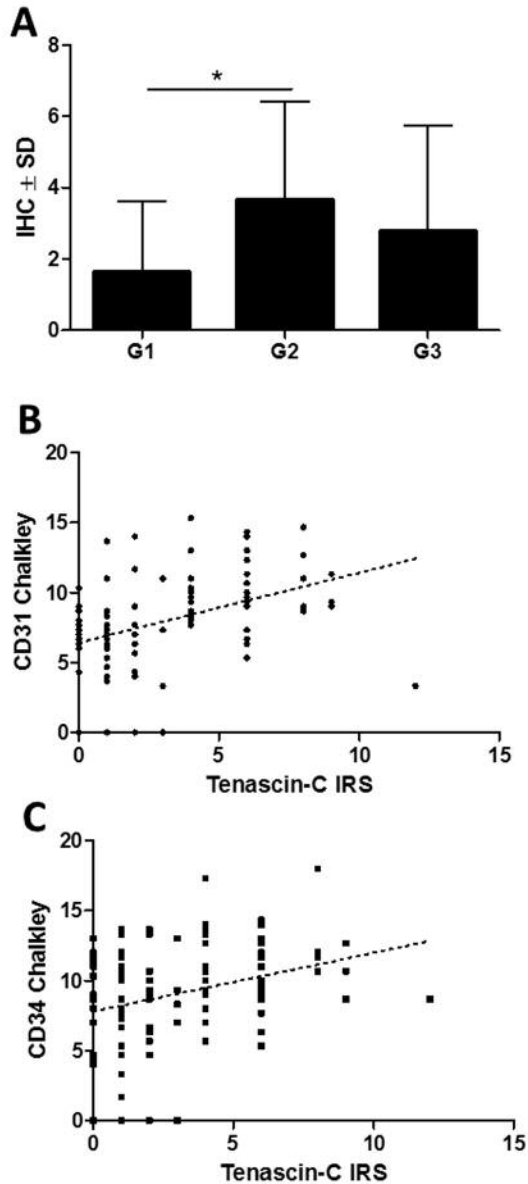


Figure 2. There is a higher expression of tenascin C in cases with a G2 grade in comparison to G1, and an upward trend in G3 in comparison to G1 (A). Positive correlation between tenascin C expression and microvessel density (MVD) CD31(+) (B) and MVD CD34(+) (C) ($r=0.456$; $p<0.0001$ and $r=0.296$; $p<0.01$, respectively). * $p<0.05$.

in stromal cells in cases of higher histological malignancy, which may prove involvement of TNC in cancer progression.

We observed positive correlation between TNC expression and the density of CD31(+) and CD34(+) blood vessels. So far, correlation between TNC expression and angiogenesis in NSCLC has been poorly understood (24). In the analysed literature, an attempt was made to evaluate only correlation between angiogenesis in NSCLC and plasma TNC

concentration, but not TNC expression in stromal cells of NSCLC (17, 24). TNC involvement in angiogenesis is supported also by the synthesis of proangiogenic factors by glioblastoma multiforme cells treated with TNC, as well as TNC expression in the stroma of NSCLC and clear cell renal cell carcinoma adjacent to CD31-expressing blood vessels (23, 25, 28). On the other hand, other authors observed also positive correlation between TNC expression and vasculature density in prostate cancer (29).

The above data show that TNC expression may play different roles in the progression of various human cancer types. In lung cancer, TNC degradation may be an additional factor affecting tenascin expression in stromal cells, but this will require further confirmatory analysis (30). Based on the obtained results, it seems that TNC is probably engaged in the mechanism of cancer angiogenesis in NSCLC.

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