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**MYOFIBROBLASTIC STROMAL REACTION AND EXPRESSION OF
TENASCIN-C AND LAMININ IN PROSTATE ADENOCARCINOMA**

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ABSTRACT

The aim of this study was to analyse relationship between changes of the stroma and expression of tenascin-C and laminin in prostate carcinoma. Tenascin-C immunostaining was increased, and laminin decreased in carcinomas compared with peritumoral tissue and benign prostate hyperplasia ($p<0.05$). Statistical analysis confirmed connection between stromal changes and tenascin-C expression in prostate carcinoma ($p<0.05$). Gleason pattern 3 carcinomas showed more pronounced stromal reaction and tenascin-C expression compared with Gleason pattern 4 carcinomas ($p<0.05$). The main cells in prostate cancer stroma are myofibroblasts that are also responsible for tenascin production. Degradation of laminin was not connected with myofibroblastic stromal changes.

Key words: myofibroblasts, tenascin-C, laminin, prostate adenocarcinoma

INTRODUCTION

It is well established that reciprocal interactions between prostate stromal cells and prostate epithelial cells are central to mechanisms of prostate gland development and differentiation.¹ Prostate adenocarcinoma has been shown to induce a stromal reaction that is associated with changes of stromal cells phenotype as a component of tumour progression and spread. Prostate cancer-reactive stroma is composed of a myofibroblast/fibroblast mix with a significant decrease of fully differentiated smooth muscle, whereas in normal prostate stroma is predominantly consisting of smooth muscle.^{2,3} Myofibroblasts typically appear at sites of pathologic tissue remodelling and their phenotype is considered to be intermediate between fibroblasts and smooth muscle. Hence, myofibroblasts could be immunohistochemically distinguished by coexpression of vimentin and α -smooth muscle actin (α -SMA) without expression of desmin or calponin.⁴⁻⁶ Recent investigations have shown that the stromal reaction was more pronounced in Gleason pattern 3 compared to other Gleason pattern tumors.^{2,3}

To become invasive, prostate cancer cell must first penetrate acinar basement membrane (BM) proteins and extracellular matrix adhesive glycoproteins.⁵ Structural components of the BM are laminins, type IV and type VII collagens, nidogens, and proteoglycans.⁶

Laminins are heterotrimeric molecules made up by one α , one β and one γ chain. Until today we know of five α -chains, three β -chains and three γ -chains. These chains combine into at least 14 different laminins. The distribution of these laminin isoforms varies between tissues, but in most BMs more than one laminin is present.⁷

Laminins are associated with a variety of biological activities such as cell differentiation, cell shape and movement, maintenance of tissue phenotypes, and

promotion of tissue survival and their functions in tumour invasion is under extensive research today.^{7,8}

Tumour cell invasion is also accompanied by remodelling of extracellular matrix (ECM) structures, especially at the invasion front, which is marked by proteolytic digestion of pre-existing components and synthesis of new components to form a different, tumour-associated ECM that promotes the proliferation, survival and migration of tumour cells.^{9,10}

It was shown in numerous studies that tenascin-C (TN-C) is an extracellular matrix protein highly up-regulated in many different cancers.¹¹

Tenascin-C is a large (180–300 kDa), hexameric multidomain glycoprotein located mainly in the ECM that is involved in tissue interactions during embryogenesis, wound healing, inflammation and oncogenesis.¹² The most prominent function of TN-C includes anti-adhesion effects, favouring cell motility and growth promotion. These proposed activities suggest a potential role for TN-C in regulation of tumour cells proliferation, invasion and metastasis.^{13,14}

The aim of this study was to analyse the relationship between changes of the stroma and expression of tenascin-C and laminin in prostate carcinoma.

MATERIALS AND METHODS

Our study group included randomly chosen radical prostatectomy specimens of 52 patients with prostate adenocarcinoma and 21 patients with benign prostate hyperplasia (BPH) as a control group. All slides with tumour that were analyzed contained also areas of nonneoplastic prostate tissue. The age range of the patients with carcinoma was between 52 and 76 years (median age 65.2), and of the patients with BPH between 68 and 87 years (median age 74.4 years).

Specimens were fixed in 10% buffered formalin, embedded in paraffin, cut at 5 μm thickness, and routinely stained with hematoxylin and eosin. The diagnosis of adenocarcinoma and benign prostate hyperplasia was histologically confirmed in all cases.

Immunohistochemical staining for vimentin, desmin, α -SMA and laminin was performed following Microwave Streptavidin ImmunoPeroxidase protocol on DAKO Tech-MateTM Horizon automated immunostainer (DAKO, Copenhagen, Denmark). We used primary monoclonal antibodies to vimentin (clone V9; code M 0725; dilution 1:50), α -SMA (clone 1A4; code M 0851; dilution 1:50), desmin (clone D33; code M 0760; dilution 1:100), and laminin (clone 4C7; code M0638; dilution 1:25) (all purchased from DAKO, Copenhagen, Denmark). Immunohistochemical staining for tenascin-C was performed on NexES Ventana automated immunostainer (Ventana, Tucson, United States of America). Primary monoclonal antibody to tenascin-C (clone 49; dilution 1:100; Novocastra Laboratories, Newcastle, United Kingdom) was used. Slides stained with laminin were pretreated with pepsin (DAKO, Copenhagen, Denmark) in water bath on 30°C during 25 minutes, and slides stained with tenascin-C were pretreated with protease 1 (Ventana, Tucson, United States of America) during 8 minutes. Pepsin and protease 1 were “ready to use”.

As positive controls we used: myometrium for vimentin (cytoplasmic reaction); leiomyoma for α -SMA (cytoplasmic reaction); leiomyoma for desmin (cytoplasmic reaction); tonsil for tenascin-C (extracellular reaction) and tonsil for laminin (reaction in basal membrane). Removals of the primary antibodies were used as negative control.

To evaluate the intensity of vimentin, α -SMA, desmin, laminin, and tenascin-C expression in prostate carcinoma, in adjacent peritumourous tissue in same prostate and in prostate with BPH, the percentage of positively stained stromal cells was examined in ten fields for each antibody under high magnification (400x). Peritumourous tissue was defined as stromal tissue immediately adjacent to carcinoma but without microscopically visible signs of infiltration with malignant glands. Analyzed regions were previously selected on low power magnification and marked on slides by different colours. The staining intensity was graded on a scale of 0-3, and expressed as 0, 0% positive stromal cells; 1, up to 33% positive stromal cells; 2, 33-66% positive stromal cells; 3, more than 66% positive stromal cells.^{2,3} To compare expression of each investigated markers immunohistochemical analysis was performed in previously selected areas as stated above, thus all immunohistochemical markers were analyzed in same area. The immunohistochemistry results were evaluated by three independent observers (DT, MU and BK).

Statistical analysis was performed using χ^2 test, Fisher's exact test and Spearman's rank correlation test. Fisher's exact test was used to compare 0/1 grading to 2/3 grading for each immunohistochemical marker (vimentin, α -SMA, desmin, tenascin-C and laminin) in prostate carcinoma, adjacent peritumourous tissue, and BPH. The level of significance was set at $p < 0.05$.

RESULTS

The Gleason distribution with Gleason patterns is shown in Table 1. The most common Gleason pattern was 3. In 48 (92.3%) tumours, one or both pattern was 3. Twenty cases (38.5%) had both Gleason patterns 3. The second most common Gleason pattern was 4, which was found in 22 (42.3%) cases.

In analyzed areas 32 (61.5%) adenocarcinomas were Gleason pattern 3, and 20 (38.5%) were pattern 4. The age range of the patients with carcinoma Gleason pattern 3 was between 52 and 73 years (median age 65.4), and age range of the patients with Gleason pattern 4 was between 54 and 76 years (median age 66.4 years). Statistical analysis revealed no significant difference between age of patients with Gleason pattern 3 and 4 ($p=0.62882$).

In 41 (78.9%) patients tumours were confined to prostate (pT2) while 11 (21.1%) patients had tumour, which extended through prostate capsule (pT3). At the time of the diagnosis all patients were without lymph node or distant metastases.

Immunohistochemical expression of vimentin, α -SMA, desmin, tenascin-C and laminin in prostate adenocarcinoma, adjacent peritumourous tissue and BPH is shown in Table 2.

Compared to adjacent peritumourous tissue and BPH, stroma of prostate adenocarcinoma showed statistically significant increased vimentin ($p=0.00000$, $p=0.00000$) and decreased desmin ($p=0.00001$, $p=0.01179$) expression whereas expression of α -SMA showed no statistically significant difference between all three analyzed groups ($p>0.05$). Peritumourous tissue and BPH showed no statistically significant difference in immunohistochemical expression of vimentin and desmin ($p=0.36196$, $p=0.62149$).

Tenascin-C was predominantly expressed in stroma around neoplastic glands while laminin was the most increased around benign glands (Fig. 1A-F). Both markers were also expressed in wall of medium sized blood vessels, which served as internal positive control. However, in only few cases we also noted weak positive tenascin reaction in cytoplasm of neoplastic epithelial cells.

Tenascin-C was significantly increased while laminin was significantly decreased in carcinoma compared to adjacent peritumourous tissue and BPH ($p < 0.05$). Differences in expression of tenascin-C and laminin in adjacent peritumourous tissue and BPH were statistically insignificant ($p = 0.68194$, $p = 0.73385$).

Spearman's rank correlation test revealed statistically significant correlation between increased vimentin and decreased desmin expression in prostate carcinoma ($p < 0.05$) but not in adjacent peritumourous tissue and BPH ($p > 0.05$). In all three analyzed groups expression of tenascin-C showed no significant correlation with α -SMA expression ($p > 0.05$). Expression of laminin did not correlate with expression of vimentin, α -SMA and desmin in prostate carcinoma, peritumourous tissue and BPH ($p > 0.05$).

Immunohistochemical expression of vimentin, α -SMA, desmin, tenascin-C and laminin in prostate adenocarcinoma Gleason pattern 3 and 4 is shown in Table 3.

Fisher's exact test showed significantly increased vimentin and decreased desmin expression in adenocarcinoma Gleason pattern 3 compared to Gleason pattern 4 ($p = 0.00452$, $p = 0.03818$). The expression of tenascin-C was statistically significantly stronger in Gleason pattern 3 ($p = 0.00003$). Expression of α -SMA and laminin was similar in both analyzed Gleason pattern ($p = 0.99999$).

DISSCUSSION

Prostate cancer is the most frequent cancer in elderly men, with an increasing incidence in industrial countries but exact mechanisms of its appearance and progression is not yet entirely known.¹⁵ Newest concepts which point out the importance of tumourous stroma in tumour development and progression intensified investigation of stroma in many tumours, including prostate adenocarcinoma.^{2,3,16-20}

Reactive stroma in breast and colon carcinoma has been described and in these cancers reactive stroma is composed of mixture of myofibroblasts, fibroblasts, endothelial cells and immune cells. Although all of these cells could be potentially involved in cancerogenesis, myofibroblasts were of special interest.¹⁶⁻¹⁸

Using a co-implantation tumour xenograft model, Orimo et al.¹⁸ demonstrated that carcinoma-associated fibroblasts (CAFs) extracted from human breast carcinomas promoted the growth of admixed breast carcinoma cells significantly more than do normal mammary fibroblasts derived from the same patients. The CAFs, which exhibited the traits of myofibroblasts, played a central role in promoting the growth of tumour cells through their ability to secrete stromal cell-derived factor 1 (SDF-1), which stimulated tumour growth directly, acting through the cognate receptor, CXCR4 that is expressed by carcinoma cells. CAFs also promoted angiogenesis by recruiting endothelial progenitor cells into carcinomas.¹⁸

Martin et al.¹⁶ showed that myofibroblasts were accumulated at the invasive front of the colorectal carcinomas. Myofibroblasts produced lytic enzymes able to degrade the basement membrane surrounding tumour glands, and also participated in the synthesis of the ECM components of the tumour stroma, which subsequently altered the adhesive and migratory properties of the epithelial colon cancer cells.¹⁶

In prostate cancer reactive stroma is mainly composed of myofibroblast and fibroblast what has been clearly shown in some previous investigation.^{2,3} Our current study confirmed appearance of myofibroblasts in prostate cancer reactive stroma, especially in Gleason pattern 3 tumours, but not in adjacent peritumourous tissue and stroma in BPH. Immunohistochemical markers of myofibroblasts were more expressed in Gleason pattern 3 compared to Gleason pattern 4 carcinomas. **Stromal density in Gleason pattern 4 carcinomas was significantly reduced compared to Gleason pattern 3 carcinomas, but residual stromal cells clearly showed immunohistochemical profile toward smooth muscle cells.** Tuxhorn et al.³ in their study noted similar results and suggested the most pronounced myofibroblastic stromal reaction in Gleason pattern 3 prostate carcinoma by double-labelled fluorescent immunohistochemistry.

Appearance of myofibroblasts in cancer stroma precedes invasive stage of cancer; therefore one of main proposed roles of myofibroblasts in cancer progression is transition from noninvasive to invasive phenotype. This effect may be mediated directly through cell-cell contacts or through diffusible molecules which are produced and secreted by myofibroblasts.^{21,22} They synthesize collagen I and III, fibronectin, versican, vascular endothelial growth factors (VEGFs) (especially type VEGF-D), SDF 1 and tenascin and also express proteases, fibroblasts activation protein, and matrix metalloproteinase's (MMPs).^{23,24}

The role of tenascin in tumours growth and progression is still controversial. Some authors have proposed that tenascin was involved in the maintenance of normal prostate stromal–epithelial homeostasis and protected against the effects of neoplasia.²⁵ This opinion is supported by studies which showed that tenascin was secreted by stromal cells (fibroblasts/myofibroblasts), but not by prostate cancer cells.²⁵⁻²⁷ Another view of tenascin expression in prostate carcinoma is that as a part of a reactive stroma

compartment, it could enhance tumour progression by stimulating angiogenesis, and by promoting cancer cell survival, proliferation, and invasion.³

In our current study tenascin-C was mainly expressed in stroma of carcinoma compared to adjacent peritumourous tissue and BPH. When we compared two analyzed Gleason patterns, stromal components in Gleason pattern 3 carcinomas showed significantly more intense tenascin expression than in Gleason pattern 4 carcinomas.

Xue et al.²⁵ in their study showed similar results and confirmed increased tenascin-C expression in the ECM, mainly at the periphery of the glands, in tumour foci and blood vessels. Low- and moderate-grade tumours were characterized by strong tenascin expression while high-grade tumours had sparse tenascin staining. In normal glands and hyperplastic lesions tenascin was also weakly expressed.²⁵

The same author in another study noted increase of tenascin expression from low-grade prostate intraepithelial neoplasia (PIN), where tenascin expression was similar to normal glands and BPH to high-grade PIN where tenascin expression was high and partly overlapped that of well to moderately differentiated prostate carcinoma.²⁸

Statistical analysis of our results showed that increased tenascin-C expression positively correlated with expression of myofibroblastic and negatively correlated with expression of smooth muscle immunohistochemical markers. Tenascin-C was also more increased in carcinomas with more prominent stromal reaction (Gleason pattern 3) compared to carcinomas with less prominent (Gleason pattern 4) stromal changes. These findings directly and indirectly confirmed the role of myofibroblasts in tenascin-C expression and proposed myofibroblasts as primary origin of tenascin-C in prostate carcinoma reactive stroma.

Our results are consistent with results reported by Tuxhorn and co-workers.³ In their study myofibroblasts were also more numbered in Gleason pattern 3 prostate carcinomas and in all cases strong periglandular tenascin expression was shown. However, they studied tenascin expression on 7 cases of Gleason pattern 3 prostate carcinomas only. The same authors in the same study confirmed that overexpressed transforming growth factor-1 in PIN epithelial cells was capable of inducing HPS-TZ1A human prostate fibroblasts to transdifferentiate in the myofibroblasts and express tenascin *in vitro*.

Recent investigations indicated that one of the most important processes during prostate cancer cells migration is laminin degradation by MMP, especially membrane type-1 MMP (MT1-MMP). MT1-MMP is a transmembrane member of the MMP family that has been demonstrated to be upregulated in prostate cancer cells as cancer progresses from normal to prostate intraepithelial neoplasia and to invasive cancer, suggesting their potential role in the invasion of prostate cancer.^{29,30} However, it is also well known that some types of proteases and MMPs are synthesized and secreted by myofibroblasts.³¹⁻³³

Expression of laminin in tumourous stroma was investigated in many tumours and loss of laminin has been connected with poor prognosis in colon, urinary bladder and pancreas cancers.³⁴⁻³⁶

In prostate, laminin types in BM were changing during prostate development and different types were observed in prepubertal, adult benign and malignant prostate glands.³⁷

Few other previous investigations showed decreased and disordered laminin expression in prostate carcinoma compared to benign glands.^{25,29,30,37-40} Laminin was

also poorly expressed around glands with PIN, and especially accentuated loss of laminin was noted in cases with increased tenascin expression.²⁵

Current study confirmed results of previous investigations of laminin expression in prostate carcinoma.^{25,29,30,37-40} Laminin was decreased in carcinoma compared to adjacent peritumourous tissue and BPH while peritumourous tissue and BPH showed similar pattern of laminin expression. We found no differences in laminin expression between Gleason pattern 3 and 4 carcinomas. Unlike tenascin-C, expression of laminin showed no significant correlation with expression of myofibroblastic immunohistochemical markers thus it seems that degradation of laminin is not dependent to myofibroblastic stromal changes and could be probably due to MMP produced by cancer cells.

In conclusion, we confirmed that the reactive stroma that occurs in prostate carcinomas is different from stroma in adjacent peritumourous tissue and BPH. Gleason pattern 3 prostate carcinomas showed more pronounced stromal changes compared to Gleason pattern 4 carcinomas. The main cells in this reactive stroma are myofibroblasts that are also responsible for tenascin production and secretion.

The value of these changes in different Gleason patterns of prostate carcinoma for the routine diagnostics should be further analysed, especially in needle core biopsies.

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TABLE 1. Distribution of Gleason patterns and scores in 52 patients with prostate adenocarcinoma

Gleason patterns with scores	Number of cases	%
5 (3+2)	10	19.2
6 (2+4)	1	1.9
6 (3+3)	20	38.5
6 (4+2)	3	5.8
7 (3+4)	10	19.2
7 (4+3)	8	15.4
Total	52	100

TABLE 2. Immunohistochemical expression of stromal markers, tenascin-C and laminin in prostate adenocarcinoma, peritumourous tissue and benign prostate hyperplasia

		Vimentin	SMA	Desmin	Tenascin-C	Laminin
Prostate adenocarcinoma (n=52)	0/1	14 (26.9%)	2 (3.8%)	22 (42.3%)	18 (34.6%)	51 (98.1%)
	2/3	38 (73.1%)	50 (96.2%)	30 (57.7%)	34 (65.4%)	1 (1.9%)
Peritumourous tissue (n=52)	0/1	38 (73.1%)	4 (7.7%)	3 (5.8%)	47 (90.4%)	8 (15.4%)
	2/3	14 (26.9%)	48 (92.3%)	49 (94.2%)	5 (9.6%)	44 (84.6%)
Benign prostate hyperplasia (n=21)	0/1	18 (85.7%)	0 (0%)	2 (9.5%)	18 (85.7%)	4 (19.0%)
	2/3	3 (14.3%)	21(100%)	19 (90.5%)	3 (14.3%)	17 (81.0%)

Fisher's exact test was used to compare 0/1 grading to 2/3 grading in prostate adenocarcinoma, peritumourous tissue and benign prostate hyperplasia.

Legend:

0 – 0% stromal cells

1 – up to 33% positive stromal cells

2 – 33% to 66% positive stromal cells

3 – more than 66% positive stromal cells

TABLE 3. Immunohistochemical expression of stromal markers, tenascin-C and laminin in prostate adenocarcinoma Gleason pattern 3 and 4

		Vimentin	SMA	Desmin	Tenascin-C	Laminin
Gleason pattern 3 (n=32)	0/1	4 (12.5%)	1 (3.1%)	18 (56.2%)	4 (12.5%)	31 (96.9%)
	2/3	28 (87.5%)	31 (96.9%)	14 (43.8%)	28 (87.5%)	1 (3.1%)
Gleason pattern 4 (n=20)	0/1	10 (50.0%)	1 (5.0%)	4 (20.0%)	14 (70.0%)	20 (100%)
	2/3	10 (50.0%)	19 (95.0%)	16 (80.0%)	6 (30.0%)	0 (0%)

Fisher's exact test was used to compare 0/1 grading to 2/3 grading in prostatic adenocarcinoma Gleason pattern 3 and 4.

Legend:

0 – 0% stromal cells

1 – up to 33% positive stromal cells

2 – 33% to 66% positive stromal cells

3 – more than 66% positive stromal cells

TITLES AND LEGENDS TO FIGURE

FIGURE 1. Immunohistochemical expression of tenascin-C and laminin in prostate carcinoma, adjacent peritumourous tissue and benign prostate hyperplasia.

Tenascin-C was significantly elevated in prostate carcinoma (A) compared to adjacent peritumourous tissue (B) and benign prostate hyperplasia (BPH) (C). Expression of laminin was low in prostate carcinoma (D) while adjacent peritumourous tissue (E) and BPH (F) showed high laminin expression. (All microphotographs were made under high magnification, 400X.)

