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High stromal hyaluronan level is associated with poor differentiation and metastasis in prostate cancer

P. Lipponen [a,*], S. Aaltomaa [b], R. Tammi [c], M. Tammi [c], U. Ågren [c], V.-M. Kosma [a,d]

[a] *Department of Pathology and Forensic Medicine, University of Kuopio, POB1627, FIN-70211, Kuopio, Finland*
[b] *Department of Urology, Kuopio University Hospital, Kuopio, Finland*
[c] *Department of Anatomy, University of Kuopio, Kuopio, Finland*
[d] *Department of Pathology, Kuopio University Hospital, Kuopio, Finland*

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Abstract

Several epithelial tumours accumulate hyaluronan (HA) which promotes cancer cell invasion and metastasis. We analysed the expression of HA and its receptor CD44 and their prognostic value in 166 prostate cancer patients followed up for a mean of 13 years; standard deviation (S.D.) 2.7; range 8.7–21.4 years. HA was detected with a specific biotinylated probe prepared from cartilage aggrecan and link protein, and CD44 with an antibody recognising all forms of CD44. The peri- and intratumoral stroma from half of the patients strongly expressed immunohistochemically detectable HA in $\leqslant 15\%$ of the stromal area; the tumours in the remaining half expressed HA in $>15\%$ of the area. The staining of cancer cells for HA was scored positive or negative, and for CD44 the median value of 80% of positive tumour cells was used as a cut-off point. The expression of HA in cancer cells was weakly associated with perineural infiltration of the tumour ($P=0.03$) and high Gleason score ($P=0.002$). There was also a significant inverse relationship between the expression of HA and CD44 in cancer cells ($P<0.001$). The high level of HA in the peri- and intratumoral stroma was related to metastasis, high T-category, high Gleason score, perineural infiltration and high mitotic activity of the tumour (for all $P<0.001$). There was a significant inverse relationship between the expression of CD44 in cancer cells and high level of strong expression of HA in the tumour stroma ($P<0.001$). A low fraction of CD44-positive cells was related to a high TM-category, high Gleason score and rapid cell proliferation (for all $P<0.0001$; M/V P value $=0.0013$). In the univariate survival analysis, the high level of strong expression of HA in tumour stroma predicted an unfavourable outcome in the entire series ($P=0.003$) and also in the M0 tumours ($P=0.07$), while in T1–2 M0 tumours the prognostic value did not reach the level of statistical significance ($P=0.1$). A low fraction of CD44-positive cells predicted a poor outcome in the entire series ($P<0.001$) and also in M0 tumours ($P=0.003$). Cancer cell-associated HA expression had no prognostic value in any tumour categories. In the multivariate analysis of prognostic factors, HA expression in the cancer cells or in the tumour stroma had no additional value to the standard prognostic factors TM-classification, Gleason score and CD44 expression. Our results show that stromal HA accumulation is related to several malignant features and adverse clinical outcome in prostate cancer. However, further studies based on uniformly treated patient cohorts are needed to establish the clinical significance of these findings in current clinical practice.

Keywords: Hyaluronan; CD44; Prostate neoplasm; Prognosis

1. Introduction

Prostate cancer is the most common malignancy in men in the western world. The prognostic evaluation and the decisions on treatment strategy are mainly based on the histological differentiation [1] and the extent of the tumour [2] at diagnosis. These prognostic parameters perform in an acceptable way in tumours which have progressed beyond the prostate capsule, while in local tumours these factors can not predict accurately the clinical outcome in all cases. The role of tumour supressor genes [3], cell proliferation [4], angiogenesis [5] and cell adhesion molecules [6] has been extensively studied in prostate cancer to find out new accurate prognostic tools.

Studies from several epithelial neoplasms show that the extracellular matrix polysaccharide hyaluronan

* Corresponding author. Fax: +358-17-162753.
E-mail address: pertti.lipponen@kuopio.fi (P. Lipponen).

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(HA) [7] has a significant role in tumour progression and metastasis [8–11]. HA controls cell migration, differentiation and cell proliferation, which all are important in tumour growth [5,12–15]. Increased HA concentrations may help invasion by providing a less dense matrix for cancer cells, stimulating cancer cell motility and forming an immunoprotective coat for cancer cells [16–18]. In addition, the cell surface receptor of HA, cell determinant (CD)44, has been shown to be important in cancer cell adhesion, cell migration and tumour neovascularisation [19,20]. Alterations in CD44 expression with prognostic significance have also been shown in prostate cancer [21,22].

Although there are many previous studies of CD44 in prostate cancer [21,22], the content of HA, its major ligand, has received no attention. Therefore, we evaluated the expression of HA in a series of 166 cases of prostate cancer. The results of HA histochemistry were compared with standard prognostic factors, CD44 expression and prognosis during a long-term follow-up.

2. Patients and methods

2.1. Patients

The current study comprised 166 patients with prostate cancer diagnosed and treated at the Department of Urology, Kuopio University Hospital, between 1973 and 1992. The mean observation period was 13 years standard deviation (S.D.) 2.7, range 8.7–21.4 years) and the mean age of patients at presentation was 71 years (S.D. 7, range 40–89 years). The cohort was not entirely consecutive, since sufficient tumour specimens for histochemistry were not available in all cases. Tumour-metastasis-classification was done according to the International Union against cancer (UICC) standards [2]. Radical prostatectomy was rarely done during the study period which explains why the node (N) classification was not available. The patients were treated by orchiectomy in 67 cases (40%), other endocrine therapy was used in 17 cases (10%), radical prostatectomy or radiation therapy was done in 7 cases (4%), and careful follow-up only was used in 75 cases (45%). The follow-up reviews were done at 3 monthly intervals during the first 2 years and thereafter at 6 monthly intervals. At the time of diagnosis, 42/166 (25%) of cases had distant metastasis. The causes of death were verified from the patient files, autopsy reports and from the files of Finnish Cancer Registry.

2.2. Histological methods

The histological samples were core needle biopsies or transurethral resection (TURP) specimens fixed in buffered formalin (pH 7.0), embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin. The histological differentiation of tumours was evaluated as described by Gleason [1]. The perineural growth was categorised into two groups; present or absent. The mitotic figures were identified and calculated from the most actively proliferating area in the section. The volume corrected mitotic index method (M/V index) was used, which expresses the number of mitotic figures/square millimetre of tumour tissue in the section [4].

2.3. Hyaluronan histochemistry

The visualisation of HA in sections was based on the specific HA binding region of the articular cartilage proteoglycan, aggrecan and link protein. Both of these molecules have HA-binding sites, homologous with each other and also with CD44 [23]. The biotinylated complex of the HA binding region and link protein (bHABC) was prepared from bovine articular cartilage as previously described [24,25]. Briefly, proteoglycans were extracted from the bovine cartilage with 4 mmol/l guanide chloride. The extract was dialysed against distilled water in the presence of high molecular weight HA. The C-terminus of the proteoglycan molecule was cleaved off with trypsin, and the protease-resistant complex of the aggrecan HA binding region, link protein, and HA was purified using hydroxyapatite chromatography and gel filtration. The complex was biotinylated, and the bHABC was separated from HA using gel filtration under dissociative conditions. The purity of the preparation was tested by polyacrylamide gel electrophoresis and Western blotting.

The 5 micrometre thick sections from the primary tumours were deparaffinised in xylene, rehydrated with graded alcohols, and washed with sodium phosphate buffer (PB, 0.1 mol/l, pH 7.4). Endogenous peroxidase activity was blocked with 1% H_2O_2 for 5 min and non-specific binding was blocked with 1% bovine serum albumin (BSA) in PB for 30 min. The sections were incubated in bHABC (2.5 ug/ml, diluted in 1% BSA) overnight at 4°C. The slides were washed with PB and treated with avidin-biotin-peroxidase (ABC, Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA, 1:200 dilution) for 1 h at room temperature. Following washes with PB, the colour was developed with 0.05% diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis. MO, USA) and 0.03% hydrogen peroxide in PB at room temperature. The slides were counterstained with Mayer's haematoxylin for 2 min, washed, dehydrated, and mounted in Depex (BDH, Poole, UK). The specificity of the staining was controlled by digesting some of the sections (Fig. 1) with Streptomyces hyaluronidase in the presence of protease inhibitors prior to the staining or blocking the bHABC-probe by pre-incubation with HA oligosaccharides [26].

2.4. CD44 immunohistochemistry

CD44 expression was demonstrated by using a mouse monoclonal anti-human CD44 antibody (clone 2C5) (R&D Systems, Abingdon, UK) at a dilution of 1:2500. The slides were incubated with 1% BSA for 30 min, and the primary antibodies diluted with 1% BSA were incubated on the slides overnight at +4°C. Endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide in anhydrous methanol for 3 min at room temperature. The protocol thereafter included sequential incubations for 1 h at room temperature with the biotinylated secondary antibody and an avidin-biotin-peroxidase detection kit (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Between each step, the slides were washed three times with phosphate buffer. Finally the DAP colour was developed. As a positive control, the previously studied prostate cancer biopsy specimen known to be positive was used. The same biopsy specimen processed without the primary antibody was used as a negative control. The positive and negative controls were positive or negative, respectively, in all the batches.

2.5. Scoring of HA expression

The intensity of stromal HA expression varied within each section, but the strongest intensity could always be identified and was comparable between different sections. The percentage of stroma with the strongest HA intensity of the total peri- and intratumoral stromal area was analysed. The peri- and intratumoral stroma from half of the patients strongly expressed immunohistochemically detectable HA in $\leqslant 15\%$ of the stromal area; the tumours in the remaining half expressed HA strongly in $>15\%$ of the area. The percentage of HA-positive tumour cells of all neoplastic cells in the section was also evaluated and later categorised into two groups: HA-positive or -negative. A tumour was considered positive if any cancer cell-associated HA signal was observed. Sections predigested with Streptomyces hyaluronidase and those stained with a probe pretreated with HA oligosaccharides gave no signal, an indication of the specificity of the method (Fig 1).

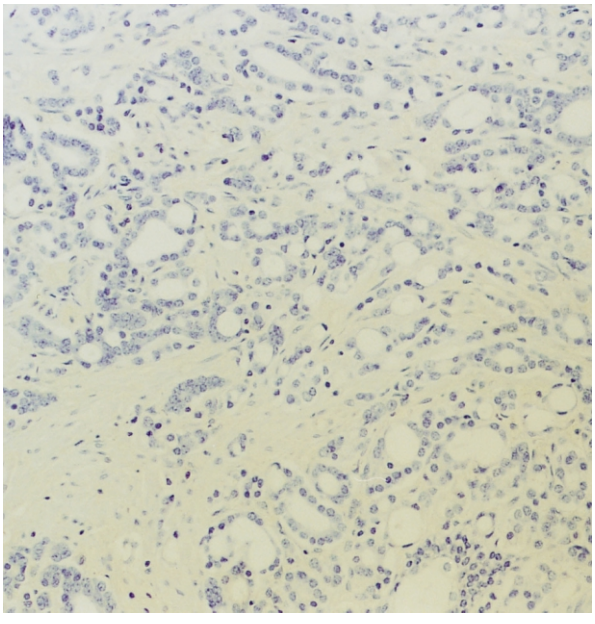

Fig. 1. A negative control section indicating the specificity of hyaluronan (HA) staining (magnification 250×).

2.6. Scoring of CD44 expression

The expression of CD44 was evaluated in the entire section. The cells were determined positive when a visible staining signal was detected on the cell membrane. The fraction of positively stained cancer cells was analysed in the entire section using a continuous scale. Later, the fractions of positive tumour cells were categorised into two groups using the median value (80% of positive cells) as a group limit. In normal or hyperplastic prostate tissue, the basal cells were often positive, but the staining signal of the basal cells of malignant glands (when relevant) was not included in the scoring process.

2.7. Statistical analysis

In the basic statistical calculations, the Statistical Package for the Social Sciences (SPSS)-X program package was used in an IBM computer, and the statistical tests used are indicated in connection with the results when appropriate. Univariate survival analysis (logrank analysis) was based on the life-table method with statistics by Gehan (SPSS-X). Multivariate survival analysis (Cox's analysis) used deaths from prostate cancer as events. A P value of <0.05 was considered significant.

3. Results

The expression of HA in the cancer cell epithelium was weakly associated with the perineural infiltration of the tumour and a high Gleason score, but inversely related to CD44 expression (Table 1). The high level of strong expression of HA in the tumour stroma was significantly related to the TM-classification, Gleason score, perineural infiltration of the tumours and high proliferation rate of the cancer cells (Table 2). There was an inverse relationship between stromal HA expression and the expression of CD44 in the cancer cells (Table 2).

A low fraction of CD44-positive cells was associated with a high TM-classification, high Gleason score, no perineural infiltration and a high mitotic index (Table 3).

Table 1
The relationship between the expression of hyaluronan (HA) in cancer cells and other clinicopathological features

	HA-negative cancer cells ($n=102$)	HA-positive cancer cells ($n=64$)	P value[a]
T1	57	33	
T2	17	11	0.9
T3	21	15	
T4	7	5	
M0	75	49	
M1	27	15	0.6
PNI−[b]	50	23	
PNI+	18	20	0.03
Gleason score			
2–4	6	7	
5–7	65	20	0.002
8–10	31	37	
M/V $\leqslant 4/mm^2$[b]	53	26	
M/V $>4/mm^2$	46	38	0.1
CD44 % $\leqslant 80$[b]	50	36	
CD44 % >80	50	27	<0.001

PNI, perineural infiltration; M/V, mitotic index; CD44, cell determinant 44. The group limit of M/V is based on previous studies [4].
[a] Chi-square test.
[b] Not available in all cases.

Table 2
The relationship between the strong expression of HA in the peri- and intratumoral stroma and other clinicopathological features

	Strong HA expression $\leqslant 15\%$ (weak level) ($n=83$)	Strong HA expression $>15\%$ (high level) ($n=83$)	P value[a]
T1	62	28	
T2	11	17	
T3	8	28	<0.001
T4	2	10	
M0	75	49	
M1	8	34	<0.001
PNI−[b]	53	20	
PNI+	7	31	<0.001
Gleason score			
2–4	9	4	
5–7	61	20	<0.001
8–10	13	59	
M/V $\leqslant 4/mm^2$[b]	53	26	
M/V $<4/mm^2$	27	57	<0.001
CD44 % $\leqslant 80$[b]	29	57	
CD44 % >80	52	25	<0.001

PNI, perineural infiltration; M/V, mitotic index; CD44, cell determinant 44. The group limit of M/V is based on previous studies [4].
[a] Chi-square test.
[b] Not available in all cases.

In the univariate survival analysis, the high level of strong expression of HA in the tumour stroma predicted unfavourable outcome in the entire series (Fig. 2) and also in the M0 tumours (Fig. 3). However, in the T1–2 MO tumours, the prognostic value did not reach the level of statistical significance ($P=0.1$).

The expression of HA in the cancer cells was not related to prognosis. A low fraction of CD44-positive cells was associated with a low survival probability in the entire series (Fig. 4) and also in the M0 tumours (data not shown; $P=0.003$). In the multivariate analysis of the prognostic factors, HA had no independent prognostic value over the standard prognostic factors and CD44 expression (Table 4).

Normal or hyperplastic prostate epithelium adjacent to the prostate cancer showed no HA expression in the prostate epithelial cells. The stromal expression of HA in the areas of morphologically normal or hyperplastic prostate epithelium was weak. HA was expressed intra- and extracellularly.

In prostate cancer, 64 out of 166 (39%) specimens showed expression of HA in the epithelial cells (cytoplasm and cell membranes were equally stained) (Fig. 5a) but the expression was usually focal. The mean (S.D.) fraction of the positive cancer cells was 8 (17)% (range 0–90%). HA was invariably expressed in the peri- and intratumoral stroma of the specimens. The intensity of expression showed marked intratumoral variation ranging from weak (Fig. 5b) to strongly positive (Fig. 5c). The staining pattern for CD44 was membranous and it was seen in nearly all acinar and ductal cells in normal prostate tissue. In the areas of benign prostatic hyperplasia, the cell membranes were positively stained as well. In carcinomas, the staining intensity and the fraction of positive cells was variable (Fig. 5d).

Table 3
The relationship between the strong expression of CD44 and other clinicopathological features

	CD44 expression $\leqslant 80\%$ ($n=86$)	CD44 expression $>80\%$ ($n=77$)	P value[a]
T1	30	57	
T2	19	9	
T3	30	6	<0.001
T4	7	5	
M0	53	69	
M1	33	8	<0.001
PNI−[b]	53	20	
PNI+	7	31	<0.001
Gleason score			
2–4	7	5	
5–7	30	53	<0.001
8–10	49	19	
M/V $\leqslant 4/mm^2$[b]	31	46	
M/V $>4/mm^2$	55	29	0.0013

PNI, perineural infiltration; M/V, mitotic index; CD44 immunohistochemistry was available in 163 cases. The group limit of M/V is based on previous studies [4].
[a] Chi-square test.
[b] Not available in all cases.

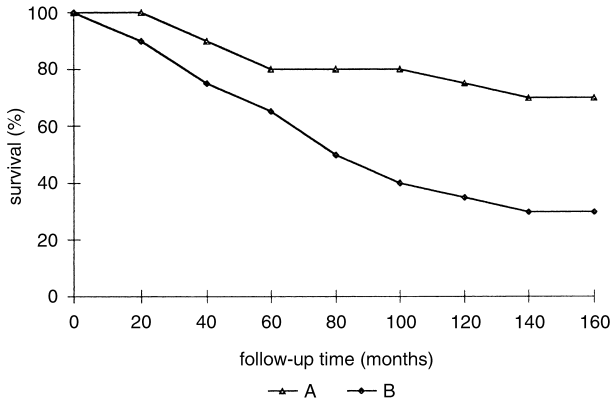

Fig. 2. The survival of patients categorised according to the fraction of strongly hyaluronan (HA) stained stroma ($P=0.003$, $X2=13.2$). Curve A: fraction of low level of strongly stained stroma ($\leqslant 15\%$), $n=83$; curve B: fraction of high level of strongly stained stroma ($>15\%$), $n=83$.

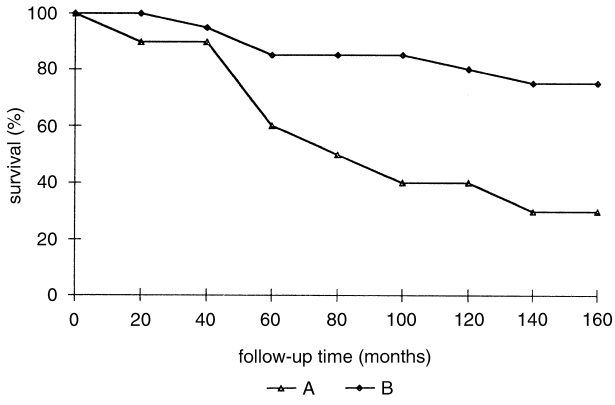

Fig. 4. The survival of patients categorised according to the fraction of CD44-positive cancer cells ($P<0.001$, $X2=23.1$). Curve A: fraction of CD44-positive cells $\leqslant 80\%$, $n=86$; curve B: fraction of CD44-positive cells $>80\%$, $n=77$.

4. Discussion

Several malignant tumours contain elevated levels of HA [8–11], which may be caused by increased HA production by the tumour cells themselves or by interactions between the tumour cells and surrounding stromal cells that induce increased HA production by the latter.

Experimental analyses show that HA and HA receptors are important in tumour growth and metastasis [20,27]. The intracellular HA receptor RHAMM/IHABP interacts with microtubules and actin filaments in interphase and in dividing cells possibly interfering with cell proliferation [16]. Overproduction of HA promotes tumour growth by promoting cell proliferation [14], and it also stimulates tumour cell migration by modulating the fibrin fibre architecture [18]. Moreover, HA facilitates cell migration by creating hydrated pathways that allow cellular or fibrous barriers to be penetrated by cells [17]. The interactions between HA and its major cell surface receptor CD44 [27,28] and cytoskeletal proteins [16] may play a pivotal role in regulating tumour cell behaviour during cancer development. Experimental [27] and clinical findings [21,22] suggest that downregulation of CD44 plays an important role in the development of human prostate cancer, possibly through the reduction of the ability to bind HA. Earlier experimental studies have documented the important value of HA in prostate cancer development [20,27], but there are no previous studies available on the role of HA in clinical prostate cancer.

The expression of HA seems to be tissue-specific since in colon [10] and stomach cancer [11] most of the cancer cells express HA, whereas prostate cancer cells were usually HA-negative. In line with the present results lung adenocarcinomas [29] seem to be HA-negative. The rare expression of HA in cancer cells was associated with poor tumour differentiation and perineural invasion, which is in accordance with the results in gastric, breast and colon cancers [10,11,30]. In these cancers, cellular HA expression is also related to the TNM-classification, while in prostate cancer no such a relationship existed. A similar association between malignant tumour features and strong stromal expression of HA

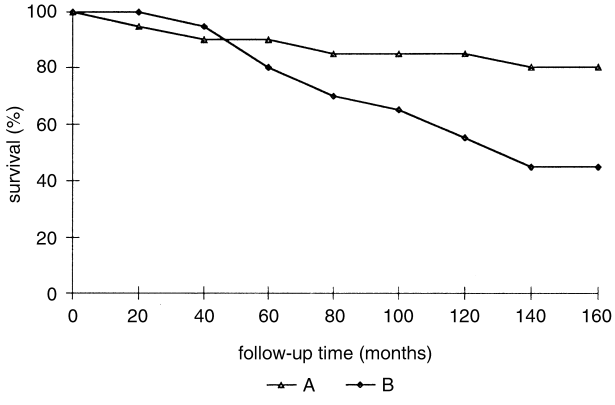

Fig. 3. The survival of M0 patients categorised according to the fraction of strongly hyaluronan (HA) positive stroma ($P=0.07$, $X2=3.0$). Curve A: fraction of low level of strongly stained stroma ($\leqslant 15\%$), $n=75$; curve B: fraction of high level of strongly stained stroma ($>15\%$), $n=49$.

Table 4
The independent prognostic factors in multivariate analysis

	RR (95% CI)
All cases	
T-category	1.50 (1.08–2.09)
Gleason score	2.45 (1.42–4.21)
M-category	2.92 (1.51–5.63)
CD44%	0.38 (0.20–0.73)
M0 tumours	
T-category	2.32 (1.65–3.24)
CD44 %	0.43 (0.19–0.97)

RR, risk ratio; CI, confidence interval.

has been documented in gastric [11], ovarian [8] and in breast [9] cancers, but not in colon [10] cancer. Experimental analyses show that HA has a direct effect on cell proliferation [14] which is in line with the close interrelationship between high mitotic index and strong HA expression in the present series.

In spite of the interaction between HA and CD44 [27], the results indicate that the ability of prostate cancer cells to induce strong peri- or intratumoral HA accumulation is independent of the expression of CD44 on tumour cell surface. However, none of the cancer specimens were HA-negative in the stroma of the tumour, which means that HA and CD44 have important interactions at the cellular microenvironment. In addition, in ovarian cancer, strong stromal expression of HA was inversely related to CD44 expression [8]. In the present series, the reduced expression of CD44 was related to other unfavourable prognostic factors and poor outcome, which is in full agreement with previous results [21,22].

There are no previous prognostic studies available on HA expression in prostate cancer, while the role of the conventional prognostic factors and CD44 has been previously widely discussed in the literature [3,4,6]. The

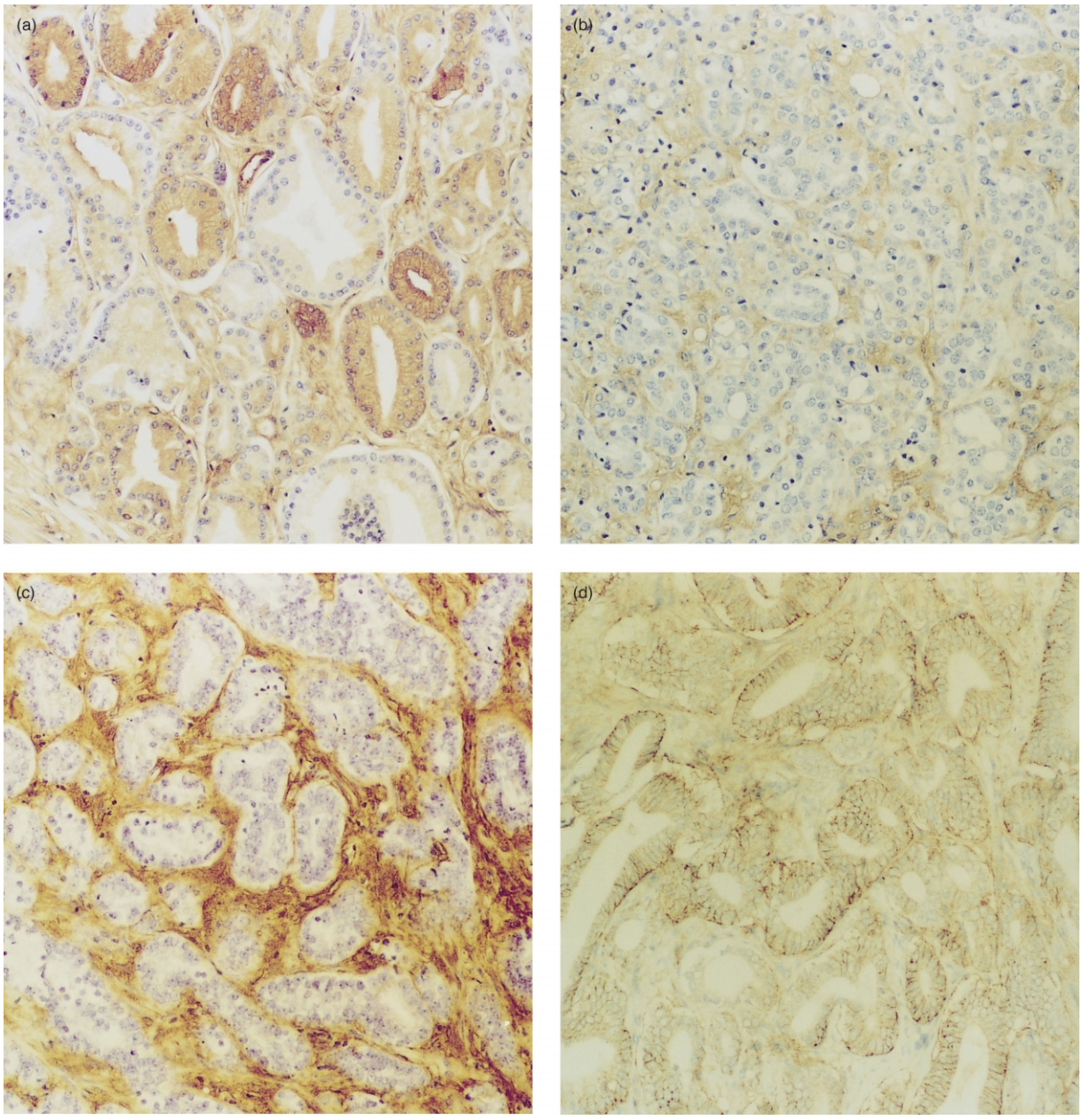

Fig. 5. (a) Focal expression of hyaluronan (HA) in prostate cancer epithelium (magnification 250×); (b) weak stromal expression of HA in prostate cancer (magnification 250×); (c) strong stromal expression of HA in a poorly differentiated prostate cancer (magnification 250×); (d) strong heterogenous expression of CD44 in a moderately differentiated prostate cancer (magnification 250×).

prognostic results are in line with the results in breast [9] and ovarian [8] cancers. In the tumours of the gastrointestinal tract cancer, cell-related HA seems to be important as regards prognosis [10,11], and stromal expression seems to have no prognostic value. Although univariate analysis showed significant prognostic value for stromal HA expression in prostate cancers, in multivariate analysis, the prognostic power of the TM-classification and Gleason score and CD44 % were better than that of HA. This may be due to the close interrelationship between HA expression and the other important prognostic factors. An advantage of our patient cohort is the long follow-up, but the patient cohort was treated by several different methods which may be a confounding factor. The number of cases is, however, too low to do survival analyses within the different therapy groups. Moreover, a high proportion of patients' tumours had metastases at diagnosis which does not correspond to currently detected new cancer cases. Therefore, the clinical significance and the prognostic value of HA expression in prostate cancer in the current clinical setting should be confirmed in uniformly treated patients, possibly by radical prostatectomy. From the methodological point of view, the prostatectomy specimens provide also better starting material for stainings than the TURP specimens or core needle biopsies in the current study. In addition, since tumours seemed to be heterogenous in their HA expression, TURP specimens may not thus be completely representative of the entire tumour.

We conclude that HA is rarely expressed in prostate cancer cells, but it is expressed in the tumour stroma. Increased stromal HA expression is related to all the important unfavourable prognostic factors and also to unfavourable prognosis. Accordingly, HA seems to be a new promising prognostic factor in prostate cancer. However, additional studies based on uniformly treated cancer cases are needed to establish the role of HA in current clinical settings.

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