The Prognostic Value of CD44 Isoforms in Prostate Cancer Patients Treated by Radical Prostatectomy

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ABSTRACT

CD44 forms a group of transmembranous glycoproteins formed by alternative splicing of a single mRNA. The expression of v6 exon-containing variants correlates with metastasis and poor prognosis in a number of malignancies. The distribution and prognostic value of CD44s, CD44v5, and CD44v6 were studied immunohistochemically in the radical prostatectomy specimens of 97 patients with prostate cancer and in 12 lymph node metastases. The mean follow-up period was 84 months. The percentage of CD44-immunoreactive cells was scored semiquantitatively. CD44 mRNA expression was checked by RT-PCR. In 86, 23, and 69% of the adenocarcinomas, respectively. Gleason sum score (GSS) and pT stage were correlated inversely. Eighty-nine percent of the tumors and BPH samples, respectively. Loss of CD44s and CD44v6 was present in 44 and 75% of the tumors and BPH samples, respectively. Loss of CD44s and CD44v6 predicted an adverse prognosis at univariate analysis. The independent prognosticators identified by multivariate analysis were: GSS, pT stage, and CD44s for clinical progression; GSS and CD44s for prostate-specific antigen progression; and GSS for tumor-specific survival. The prognostic value of CD44s in prostate adenocarcinoma predicts a poor prognosis, independent of stage and grade.

INTRODUCTION

Prostate cancer is the most prevalent non-skin tumor in Western males, and its mortality is second only to that of lung cancer (1). The incidence and prevalence of the disease have been increasing dramatically over the last decade, which can partially be explained by the introduction of PSA2 as a serum tumor marker (2, 3). On average, patients are now diagnosed at an earlier stage of the disease and often are candidates for curative treatment by means of a radical prostatectomy (4). At present, there are no tumor markers that are sufficiently able to divide patient groups between those who will and those who will not benefit from radical surgery. A lot of prostate cancer research aims at the development and application of new prognostic tissue markers, such as proliferation-associated molecules (e.g., Ki-67; Ref. 5) and adhesion molecules (e.g., E-cadherin; Ref. 6).

CD44 is a transmembranous glycoprotein that was discovered in lymphocytes as a lymphocyte homing molecule (reviewed by Matsumura and Tarin; Ref. 7). Subsequently, it was found that numerous normal and tumor tissues express CD44 as well (8, 9). The human CD44 gene is composed of 19 exons, 9 of which are variably expressed due to alternative splicing of the mRNA (10). Standard CD44 (CD44s) is composed of exons 1–5 and 15–19 (s1–s10). Variant forms contain one or more of the exons 6–14 (v2–v10); v1 is not expressed in humans) that are positioned between exons 5 and 15. The structure of the gene is depicted in Fig. 1. Variant CD44 molecules containing exon v6 appear to play a causal role in tumor metastasis (11). The presence of CD44 molecules has been found to have prognostic value in several tumors (12–15).

As yet, the expression of CD44 molecules in prostate cancer has only been investigated in cell lines. Preliminary immunohistochemical data from clinical prostate cancer indicated that tumor glands show heterogenous expression of CD44s and no CD44v6 expression (16). In the present study, the immunohistochemical expression pattern of CD44 was studied in prostate cancer tissues, and the prognostic value was determined in patients treated by radical prostatectomy. In addition, CD44 mRNA expression was checked by RT-PCR.

MATERIALS AND METHODS

Patients. In the period 1980–1988, 159 patients were operated on at the University Hospital (Rotterdam, the Netherlands) for prostate cancer (T1-3N0M0, TNM classification 1992; Ref. 17). In 17 of these, the tumor was diagnosed in a transurethral resection specimen performed for BPH, i.e., stage T1a–b. In

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2 The abbreviations used are: PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia; GSS, Gleason sum score; PIN, prostatic intraepithelial neoplasia; RT-PCR, reverse transcriptase-PCR; TSA, tyramide signal amplification.
49 patients, the pelvic lymph node dissection specimen con-
tained metastatic tumor on examination of frozen sections. 
These patients did not undergo subsequent radical prostate-
tomy, except for two younger patients with micrometastasis, 
leaving 112 patients treated by radical prostatectomy. The 
patients were followed regularly, and all data concerning diag-
nosis, treatment, and follow-up were stored prospectively in a 
comprehensive database. Clinical progression was defined as 
cytologically or histologically proven local recurrence or the 
appearance of distant metastasis. Tumor death was defined as 
death due to direct tumor effects, metastasis, or tumor therapy. 
PSA progression was defined as a PSA level of $\geq$1.0 at two 
subsequent measurements, in which case the first elevated PSA 
level was considered the date of failure. PSA progression was 
only considered in patients with a complete PSA history, i.e., 
patients operated on in 1987–1988 ($n = 29$). If necessary, 
patients were treated during follow-up for local recurrence or 
metastatic disease. One patient was lost to follow-up. Two 
patients died within 1 month following radical prostatectomy, 
one from myocardial infarction and one from pulmonary embo-
lism.

**Tissues.** All radical prostatectomy specimens were fixed 
in 10% buffered formalin and totally embedded in paraffin. The 
H&E-stained slides were reviewed by a single experienced 
genitourinary pathologist (T. H. v. d. K.) to obtain the GSS and 
to stage the tumors according to the 1992 TNM classification 
system. The material from 10 patients could not be retrieved, 
and one tumor was a metastasis of a large bowel carcinoma. The 
previously diagnosed tumor was not found in the radical pros-
tatectomy specimen of three patients with a T$_{max}$ tumor (final 
stage, pT$_{max}$). All 14 of these patients were excluded from 
analysis, leaving 97 patients. Of each radical prostatectomy speci-
men, two or three tissue blocks were selected that contained at 
least all the Gleason growth patterns observed in that tumor and, 
if applicable, also contained normal prostatic glands, BPH, 
and/or PIN. PIN lesions were graded 

**Table 1** Relationship between CD44 expression and GSSs

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<td>12 (29)</td>
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<td>22 (23)</td>
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<tr>
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<td>&gt;50%</td>
<td>4 (100)</td>
<td>3 (60)</td>
<td>2 (12)</td>
<td>8 (19)</td>
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<td>18 (19)</td>
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<td>3 (18)</td>
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<td>24 (57)</td>
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<td>10 (71)</td>
<td>2 (100)</td>
<td>58 (60)</td>
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<td>10–25%</td>
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<td>11 (26)</td>
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<td>13</td>
<td>14</td>
<td>2</td>
<td>97</td>
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$^a$ Numbers of tumors; percentages in parentheses.

$^b$ Spearman’s rank correlation.
Fig. 2 CD44 immunohistochemistry of nonmalignant prostatic tissues; counterstaining with hematoxylin. A, CD44s, benign glands (×241). B, CD44v5, benign glands (×241). C, CD44v6, benign glands (×241). D, CD44s, BPH (×117). E, CD44s, high-grade PIN (×117).

have only been collected as of 1989. Directly after removal of the prostate, macroscopic tumor lesions (or BPH tissue) were identified and a piece of tumor tissue was removed in a sterile fashion, snap-frozen in liquid nitrogen-chilled isopentane (Merck, Darmstadt, Germany) and stored at −80°C until use. A H&E-stained frozen section was prepared from these tissue fragments to confirm the presence of prostate cancer and BPH.

**Immunohistochemistry.** The selected tissue blocks of the radical prostatectomy specimens and the lymph nodes were immunostained with monoclonal antibodies against CD44s (clone SFF-2), CD44v5 (clone VFF-8), and CD44v6 (clone VFF-7), all from Bender MedSystems (Vienna, Austria; Refs. 12, 13, and 19-21). CD44v5 has not been related to malignant processes, but it was included for comparison with CD44s and CD44v6. For visualization of antigen-antibody binding, an ultrasensitive method (TSA system; DuPont NEN Research Products, Boston, MA) was used (22). This method uses the activity of second antibody-coupled horseradish peroxidase to catalyze the deposition of a large number of biotin molecules near the peroxidase molecule. The biotin molecules can be made visible...
by fluorochrome- or enzyme-labeled streptavidin. This procedure was used because preliminary results showed that the more well-known avidin-biotin complex method (23) yielded false-negative results in a substantial number of cases.

Tissue sections were cut at 5 μm and mounted on 3-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO)-coated glass slides (24). After deparaffinization through xylene and ethanol, endogenous peroxidase activity was blocked by rinsing the slides for 10 min in 3% hydrogen peroxide in ethanol. The slides were rinsed with distilled water and placed in a 10 mmol/liter citrate buffer at a pH of 6.0. Antigen retrieval was performed in a microwave oven at 700 W for 3 min (25). After cooling and rinsing with PBS, the slides were placed in a Sequenza immunostaining system (Shandon, Uncorn, United Kingdom). Following a 15-min preincubation with 10% normal goat serum (DAKO A/S, Glostrup, Denmark) in PBS, the slides were incubated for 90 min with either the primary antibody or PBS (negative control). The antibodies were diluted in PBS at 1:200 (SFF-2 and VFF-7) or 1:100 (VFF-8). Subsequently, the slides were incubated for 30 min with a blocking buffer consisting of 0.15 mol/liter Tris buffer (Life Technologies, Inc., Breda, the Netherlands) at pH 7.5, 0.15 mol/liter NaCl (Merck), and 0.5% blocking reagent (DuPont, TSA kit). After a 30-min incubation with a 1:50-diluted horseradish peroxidase-labeled goat antimouse antibody (DAKO), an amplification solution consisting of biotinyl tyramide (DuPont) diluted 1:50 in 50% distilled water and 50% amplification diluent (DuPont) was applied. Finally, the slides were incubated with 1:400-diluted horseradish peroxidase-labeled streptavidin. All the components of the TSA system were diluted in the blocking buffer. In between the incubations, the slides were rinsed three times in a buffer consisting of 0.1 mol/liter Tris, 0.15 mol/liter NaCl, and 0.05% Tween 20 (Merck). The antigen-antibody binding was visualized with 0.75 mg/ml PBS of diaminobenzidine hydrochloride (Fluka, Neu-Ulm, Germany) with 0.08% hydrogen peroxide as substrate. The slides were counterstained with Mayer’s hematoxylin, dehydrated, and covered.

Quantification. The different Gleason growth patterns as well as areas of normal prostatic glands, BPH, and PIN were identified and marked on the immunohistochemically stained tissue slides. The slides were studied at ×25 magnification without knowledge of the clinical outcome of the patients. The percentage of CD44-positive cells (CD44s, CD44v5, and CD44v6) in a particular area was scored semiquantitatively as:

### Table 2 Relationship between CD44 expression and primary Gleason growth patterns

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<td>CD44s</td>
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<td>9 (15)</td>
<td>20 (29)</td>
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<td>13 (7)</td>
<td>6 (4)</td>
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<tr>
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<td>&gt;50%</td>
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<tr>
<td>CD44v6</td>
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<td>26 (43)</td>
<td>34 (50)</td>
<td>18 (75)</td>
<td>82 (48)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10-25%</td>
<td>2 (13)</td>
<td>15 (25)</td>
<td>15 (22)</td>
<td>3 (13)</td>
<td>35 (21)</td>
<td></td>
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<tr>
<td></td>
<td>&gt;50%</td>
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<td>10 (15)</td>
<td>3 (13)</td>
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<td>P = 0.25b</td>
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<td>68</td>
<td>24</td>
<td>170</td>
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</table>

* Numbers of primary Gleason growth patterns; percentages in parentheses. If a tumor contained more than one area of the same Gleason growth pattern, the lowest score was taken.

b Sign test.

### Table 3 Relationship between CD44 expression and pT stage

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<th>Score</th>
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<th>pT2-4</th>
<th>pT2</th>
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<tbody>
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<td>18 (72)</td>
<td>62 (94)</td>
<td>9 (36)</td>
<td>49 (68)</td>
</tr>
<tr>
<td>10-25%</td>
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<td>18 (25)</td>
<td>5 (20)</td>
<td>2 (3)</td>
<td>7 (28)</td>
<td>14 (19)</td>
</tr>
<tr>
<td>25-50%</td>
<td>9 (36)</td>
<td>19 (26)</td>
<td>1 (4)</td>
<td>2 (3)</td>
<td>4 (16)</td>
<td>9 (13)</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>11 (44)</td>
<td>7 (10)</td>
<td>1 (4)</td>
<td>5 (20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P &lt; 0.001b</td>
<td>P &lt; 0.001b</td>
<td>P &lt; 0.001b</td>
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<tr>
<td>Total</td>
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<td>72</td>
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<td>72</td>
<td>25</td>
<td>72</td>
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</table>

* Numbers of tumors; percentages in parentheses.

b X² test for trend.
CD44 immunohistochemistry of prostatic cancer, counterstaining with hematoxylin. A-C, Gleason growth pattern 3 adenocarcinoma with strong labeling of CD44s (A), CD44v5 (B), and CD44v6 (C). D, Gleason growth pattern 3 with reduced CD44s labeling. E, Gleason growth pattern 4 with reduced CD44v6 labeling. Note basal localization in immunoreactive cells. F, prostate cancer lymph node metastasis. Lymphocytes strongly immunoreactive with CD44s, metastatic tumor cells negative. A–E, magnification ×117; F, magnification ×341.

<10%, 10–25%, 25–50%, and >50%. Scores were obtained independently for all marked areas in the tissue slides of one prostate. In nonmalignant glands, only luminal cells were scored. A tumor CD44 score was obtained by taking the lowest score given to a tumor area present in that tumor.

The cellular localization of CD44 immunostaining was examined in detail in 12 sections that were chosen randomly from the tissue sections of all 97 patients using a confocal laser scan microscope system (Carl Zeiss, Jena, Germany). A fluorescent TSA method, which uses FITC-labeled streptavidin during the final incubation, was applied to these sections. The nuclei were counterstained with propidium iodide, and the slides were covered with Vectashield (Vector Laboratories, Burlingame, CA).

**CD44 mRNA Expression.** Total RNA of the snap-frozen BPH and cancer samples was prepared by the guanidine isothiocyanate/cesium chloride centrifugation method using the...
RESULTS

Clinical Data. After exclusion of the patients that were not appropriate (1 patient was lost to follow-up, 10 radical prostatectomy specimens could not be retrieved, 3 tumors were of final stage pT3b, and 1 tumor was not of prostatic origin), 97 patients were left for analysis. After clinical evaluation all patients were staged T1a, T1b, T2, T3a, T3b, and T4. The T-stage distribution was as follows: T1a in 17, T2 in 50, and T3 in 30. The mean age at operation was 63 years (range, 45–76). The following combinations were used for PCR: P1+ with P1−, P1+ with V10−, and V6+ with P1−. PCR was performed with the total volume of cDNA using super Taq DNA polymerase (Sphaero, HT Biotechnology, United Kingdom) in a thermal cycler (Perkin-Elmer Corp.). The PCR mix was heated to 94°C for 4 min followed by 40 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. After the last cycle, the tubes were kept at 72°C for 10 min. The PCR products were separated on a 1% agarose gel in the presence of ethidium bromide and analyzed under UV light. RNA isolated from PC-3 and DU 145 cells served as positive control (16, 27–29). Sequence analysis of the PCR fragments was performed by the dideoxy chain termination method (30) according to the protocol of the T7 kit (Pharmacia, Uppsala, Sweden).

Statistics. Statistical analysis was performed using the SPSS (statistical package for social sciences) and STATA statistical computer packages. The sign test, χ² test for trend, and Spearman’s rank correlation test were used to analyze the relation between several pathological variables and CD44 expression. For the analysis of survival data, Kaplan-Meier curves were constructed and the log-rank test for trend was performed. Multivariate survival analysis was performed using Cox’s proportional hazards model.

CD44 Expression in Prostatic Tissues. Only a weak cytoplasmic nonspecific background staining was observed in some of the negative control slides. This could easily be distinguished from the specific immunostaining. CD44s and CD44v6 immunohistochemistry of benign prostatic glands showed a very intense membranous staining of almost all basal cells (Fig. 2). In addition, most luminal cells (>75%) also expressed both antigens, although in general weaker. Identical staining patterns were found in BPH tissues (Fig. 2). Because benign glands were present in almost all slides, these could serve as an internal control of the immunostaining procedure. Areas with basal cell hyperplasia showed a very intense labeling of all basal cell layers. Detailed analysis by confocal laser scan microscopy in a subset of tissue sections showed that immunostaining was only present at the intercellular membranes; thus, the basal membranes of the basal cells and the apical membranes of the luminal cells lacked immunoreactivity. In PIN lesions, the majority of basal cells was labeled but with decreased intensity as compared to normal glands (Fig. 2). The luminal cell layer of PIN lesions showed reduced immunostaining of both CD44s and CD44v6. The scores of CD44s and CD44v6 were lower in high-grade PIN lesions than in low-grade PIN lesions (P < 0.05, χ² test for trend). The patterns of CD44v5 labeling were comparable but with a much lower intensity. In all areas, the percentage of CD44v5-immunoreactive cells was smaller than that found with the other two antibodies (P < 0.001, sign test). The lymphocytes that were present in most tissue sections showed strong immunoreactivity for CD44s. Occasionally, CD44v5 and CD44v6-immunoreactive lymphocytes were seen as well, especially in areas with prostatitis. Prostatic urothelium that was present in a number of tissue slides showed a strong immunolabeling of CD44s in all cell layers except for the luminal cell layer and a comparable but weaker labeling of CD44v5 and CD44v6. A more cytoplasmic staining pattern was present in seminal vesicles.

CD44-immunoreactive tumor cells were found in 83 adenocarcinomas (86%) for CD44s and in 22 (23%) and 67 (69%) low-up period, 21 patients had died of other causes, and 63 patients were still alive. Of the 29 patients who were evaluable for PSA progression (complete PSA history available), 12 (41%) showed progression. The mean overall follow-up period for PSA progression was 81 months (range, 21–109).

CD44 mRNA Expression in Prostatic Tissues (RT-PCR with primer set P1+ with P1−; Lanes 1–9, prostate cancer (PC); Lanes 10–17, BPH. Size indication in bp.}

Fig. 4 Expression of CD44 mRNA in prostatic tissues (RT-PCR with primer set P1+ with P1−). Lanes 1–9, prostate cancer (PC); Lanes 10–17, BPH. Size indication in bp.
for CD44v5 and CD44v6, respectively. Immunostaining was membranous and in most cases limited to the intercellular membranes. This was identical to the pattern found in benign tissues. CD44v6 tumor scores were lower than CD44s tumor scores (P < 0.001, sign test). CD44v5 tumor scores were considerably lower than CD44s and CD44v6 tumor scores (P < 0.0001, sign test). It appeared that the heterogeneity of immunostaining within one Gleason growth pattern was limited. The relationship between tumor CD44 scores and primary Gleason growth pattern and GSSs is shown in Tables 1 and 2. The relationship between tumor CD44 scores and pT stage is depicted in Table 3. The expression of CD44 molecules was associated with favorable pathological factors such as a low GSS (Table 1), pT2 stage (Table 3), and the absence of perineural invasion (data not shown). Twelve lymph nodes containing metastatic prostatic tumor cells were studied for the expression of CD44 molecules. Immunoreactive tumor cells could not be identified, whereas lymphocytes showed strong CD44s immunostaining and occasionally weak CD44v5 and CD44v6 immunostaining (Fig. 3).

Fig. 4 shows the results of RT-PCR with a CD44-specific primer set (P1 + with P1 -) of BPH and tumor mRNA. In eight of nine tumors (89%) and in all eight BPH samples, a ~500-bp CD44 mRNA was found. In four of the tumors (44%) and six of the BPH samples (75%), an additional mRNA of ~900 bp was present. Both fragments were also present in the RNA of PC-3 and DU 145 cells (not shown). Sequence analysis showed that the ~500-bp fragment (actually 481 bp) was identical to CD44s. RT-PCR with exon-specific primers identified the ~900-bp fragment as CD44v8-10 (epithelial form, 877 bp). In addition, all the samples that expressed CD44v8-10 also expressed CD44v6v10 (1138 bp). The expression level of the latter mRNA was lower in all samples and therefore probably not visible in Fig. 4.

**Prognostic Value of CD44 Molecules.** For prognostic evaluation, GSSs were grouped into three categories: 4–5, 6–7, and 8–10. pT stage and pN stage were grouped into two categories: pT2 versus pT3–4, and pN0 versus pN1–2. The results of the univariate analysis of prognostic markers by means of a log-rank test for trend are shown in Table 4. In addition to the well-known prognostic value of the GSS and pT stage, CD44s and, to a lesser extent, CD44v6 were also found to have prognostic value. For both CD44s and CD44v6, the prognosis was better if the percentage of immunoreactive cells was higher. pN stage, age at operation (P > 0.25 in both cases), and CD44v5 expression (Table 4) did not show prognostic value. The relationships between CD44 expression and the several outcome parameters are shown in the Kaplan-Meier curves of Figs. 5–7. To investigate whether or not CD44 molecules also showed independent prognostic value, the variables were stepwise entered into Cox’s proportional hazards model. Table 5 shows the models that best fitted the data with all individual variables at P < 0.05. The expression of CD44s was of independent value in predicting clinical and PSA progression. In both cases, the hazard ratio was <1, indicating a favorable prognosticator. The inclusion of CD44v6 expression did not improve the models. The GSS was the only independent variable to predict tumor-specific survival. If only patients with pT2 tumors were considered, identical results were found for the prediction of clinical progression and tumor-related death (data not shown). Because CD44 expression has been shown to be associated with the metastatic process, the predictive value of CD44 was also investigated for the risk of local recurrence and metastasis separately. Both CD44s and CD44v6 expression showed a significant relationship, but at multivariate analysis only CD44s expression was of independent value, together with GSS.

**DISCUSSION**

A number of functions attributed to CD44 may play a role in tumor biology (31). For this reason, studying the prognostic value of CD44 molecules in malignant processes is warranted. The availability of a group of prostate cancer patients treated by radical prostatectomy and with long-term postoperative follow–up allowed us to study the distribution of CD44-positive cells in benign and malignant prostatic tissues and to investigate the prognostic value of these cells in prostate cancer. It is important to recognize two factors that may hamper the interpretation of the results of the present study. First, the distributions of pT stages and GSSs show a large proportion of locally extensive and/or poorly differentiated tumors. This can be explained by the fact that 30% (31%) of the patients were operated on with a clinical T2 tumor and that more than half of the clinical T2 tumors were upstaged to pT3, which is a well-known fact (32). The distributions of pT stages and GSSs have changed over the past decade (4), which makes the patient sample from the present study difficult to compare with contemporary patients. Subgroup analysis of patients with pT2 tumors did not change the results significantly, however. Secondly, the PSA progression data are based on a small sample of patients and should therefore be interpreted with care.

The immunohistochemical data were scored semiquan-
titatively. On the one hand, this might lead to impaired

<table>
<thead>
<tr>
<th>Variable</th>
<th>Clinical progression</th>
<th>PSA progression</th>
<th>Tumor-specific survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>χ²</td>
<td>P</td>
<td>χ²</td>
</tr>
<tr>
<td>GSS</td>
<td>17.6</td>
<td>&lt;0.0001</td>
<td>5.5</td>
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<tr>
<td>pT stage</td>
<td>18.7</td>
<td>&lt;0.0001</td>
<td>9.0</td>
</tr>
<tr>
<td>CD44s</td>
<td>19.6</td>
<td>&lt;0.0001</td>
<td>9.6</td>
</tr>
<tr>
<td>CD44v5</td>
<td>0.96</td>
<td>0.33</td>
<td>2.2</td>
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<tr>
<td>CD44v6</td>
<td>6.7</td>
<td>0.0097</td>
<td>2.2</td>
</tr>
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</table>
Fig. 5 Relationship between clinical progression and tumor scores of CD44s (A), CD44v5 (B), and CD44v6 (C). Kaplan-Meier curves show fractions of nonprogressing tumors. Curves are truncated at 120 months. Groups: 1, <10%; 2, 10–25%; 3, 25–50%; and 4, >50%. Censored patients are indicated by a number along their line. Numbers of patients per group: A, group 1, n = 29; group 2, n = 22; group 3, n = 28; and group 4, n = 18; B, group 1, n = 86; group 2, n = 7; group 3, n = 3; and group 4, n = 1; C, group 1, n = 58; group 2, n = 21; group 3, n = 13; group 4, n = 5.

Fig. 6 Relationship between PSA progression and tumor scores of CD44s (A), CD44v5 (B), and CD44v6 (C). Kaplan-Meier curves show fractions of nonprogressing tumors. Curves are truncated at 75 months. Groups: 1, <10%; 2, 10–25%; 3, 25–50%; and 4, >50%. Censored patients are indicated by a number along their line. Numbers of patients per group: A, group 1, n = 8; group 2, n = 8; group 3, n = 9; and group 4, n = 4; B, group 1, n = 27, and group 2, n = 2; C, group 1, n = 17; group 2, n = 7; and group 3, n = 5.

accuracy and the risk of missing subtle changes. Also, interobserver variability might be a problem. On the other hand, CD44 immunohistochemistry is an easily applicable and relatively fast procedure. If a marker is going to be used clinically, the results should be quantitated in a convenient way. Finally, because the range of positivity varied from 0 to almost 100%, a semiquantitative score probably does not impair accuracy.
Patients are A, per group: Groups: I, CD44s indicated by a number along their line. Numbers of patients (B), group 1, n = 58; group 2, n = 21; group 3, n = 29; group 4, n = 18; group 4, n = 11; C, group 1, n = 58; group 2, n = 21; group 3, n = 13; and group 4, n = 5.

For tissue markers, the presence of which indicates a poor prognosis, the most intensely stained area of the tumor is generally used for grading or scoring based on the assumption that this part will determine the prognosis. It was initially thought that this also would apply to the expression of CD44 in prostate cancer. When the results were analyzed, it became clear that the opposite was true. For this reason, the tumor score was defined as the lowest score obtained in an area of a tumor (compare, e.g., the expression of E-cadherin in prostate cancer; Ref. 6).

Immunohistochemistry of CD44s and CD44v6 in benign prostatic tissues was very much alike. Intense immunolabeling was found in almost all basal cells and most luminal cells (Fig. 2). It was believed that variant CD44 molecules were expressed preferentially by malignant cells (7, 31), but several studies demonstrated high expression of these molecules in many normal organs, among which is the prostate (8, 9). Staining of benign prostatic glands could be used as internal control for the immunohistochemical procedure. The staining pattern was membranous and appeared to be limited to the intercellular membranes. This suggests that CD44 plays a role in the attachment of benign prostatic cells to each other rather than to the extracellular matrix. Because the benign glands of the present study were from prostates containing a malignant tumor, it cannot definitively be concluded yet whether these results hold true for normal prostates as well.

PIN lesions showed reduced immunolabeling of CD44 (Fig. 3). In high-grade PIN, the semiquantitative CD44 scores were lower than in low-grade PIN, suggesting that CD44 molecules are related to the maturation of prostatic cells. Because high-grade PIN is the putative precursor of prostate cancer, one would expect even lower CD44 scores in tumors. Indeed, if high-grade PIN and adenocarcinoma were present in one tissue slide, CD44s and CD44v6 scores tended to be lower in the tumor (P < 0.01, sign test).

Most tumors expressed CD44s and CD44v6 (86 and 69%, respectively). In general, tumor CD44 scores were correlated inversely with GSSs (Table 1), Gleason growth patterns (Table 2), pT stage (Table 3), and the presence of perineural invasion. Prostatic tumor cells metastatic to a lymph node did not show immunolabeling of CD44 (Fig. 3). Although CD44v5 was present in only 23% of the tumors, the patterns were comparable with CD44s and CD44v6. These results suggest that prostatic CD44 expression decreases gradually from benign to premalignant to low-grade tumor to high-grade tumor and finally disappears in metastatic cancer. The staining heterogeneity within one tumor area appeared to be limited. In ongoing studies this will be quantitated.

CD44 was also detected at the mRNA level (Fig. 4), strongly indicating that prostatic tissues indeed produce CD44.

### Table 5: Multivariate analysis of prognostic markers

<table>
<thead>
<tr>
<th>Outcome parameter</th>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical progression</td>
<td>GSS</td>
<td>2.20</td>
<td>1.20-4.04</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>pT stage</td>
<td>4.89</td>
<td>1.11-21.5</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>CD44s</td>
<td>0.674</td>
<td>0.467-0.974</td>
<td>0.036</td>
</tr>
<tr>
<td>PSA progression</td>
<td>GSS</td>
<td>2.90</td>
<td>1.09-7.73</td>
<td>0.042</td>
</tr>
<tr>
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<td>CD44s</td>
<td>0.381</td>
<td>0.179-0.974</td>
<td>0.012</td>
</tr>
<tr>
<td>Tumor-specific survival</td>
<td>GSS</td>
<td>4.43</td>
<td>1.55-12.7</td>
<td>0.006</td>
</tr>
</tbody>
</table>
molecules. The percentage of tumors with CD44s mRNA (eight of nine, 89%) and CD44v6−10 (four of nine, 44%) was comparable to the immunohistochemical findings. Because normal prostatic tissues express these molecules as well, it cannot be excluded that the mRNA present in cancerous tissues was derived from contaminating benign cells.

The first experimental studies with prostate cancer cell lines showed expression of CD44s and splice variants, including v6-containing molecules at the mRNA and protein levels (16, 27–29). Lokeshwar et al. (27) showed that a neutralizing CD44 antibody inhibited the cell proliferation and basement membrane invasion activity of PC-3 and TSU-P14 cells. Furthermore, it was shown that prostatic CD44 molecules interact with hyaluronic acid and the cytoskeleton (29). These results indicate an active role of CD44 in the malignant behavior of prostate cancer. This is apparently at variance with the results from the present study. Because in vitro growing cell lines may have changed considerably during long-term culture, these experimental results may therefore not hold true for the tissue of origin.

CD44s and to a lesser extent CD44v6 were found to be of prognostic value in predicting clinical progression and PSA progression (Table 4; Figs. 5–7), with loss of expression being associated with an increased risk. As described above, CD44 expression was correlated with several pathological factors. This might explain part or all of the prognostic value of CD44. However, multivariate analysis showed that decreased expression of CD44s is an independent predictor for clinical progression and PSA progression (Table 5). Most studies on CD44 expression in clinical tumors have, however, found a positive correlation with pathological variables and an adverse prognosis (12–14, 21, 33–35). In other studies, such correlations were not found (36), or CD44 expression was even correlated with favorable prognostic markers or a better prognosis (15, 26, 37). It appears, therefore, that the biological role of CD44 molecules is not identical in all organs and tumors. It could very well be that in tumors arising in epithelia that normally do not express CD44 (e.g., gastrointestinal epithelium; Ref. 9), acquired expression of CD44 is correlated with an adverse outcome (11, 12, 33), with CD44 acting as a growth- and metastasis-promoting molecule. Prostatic epithelium normally expresses CD44, and the presence of CD44 in prostatic tumors could be related to a less malignant tumor, with CD44 acting as an intercellular adhesion molecule that conserves tissue architecture and inhibits local spread and metastatic behavior.

In conclusion, CD44 immunohistochemistry can be performed reliably on formalin-fixed, paraffin-embedded prostatic tissues with an internal control present but requires an ultrasensitive immunostaining protocol. CD44 molecules are expressed in normal prostatic epithelium as well as in prostatic adenocarcinoma. Loss of CD44s expression is correlated with pathological variables and is an independent prognostic marker for clinical and PSA progression. Additional studies with more patients with locally confined tumors are needed. Experimental studies on CD44 expression in prostate cancer have focused on the adverse role of CD44; given the results of the present study, the opposite should be considered as well.

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