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Prognostic Value of Cancer Stem Cell Markers (CD44 and CD24) In Combination with Clinical and Morphological Characteristics of Prostate Cancer

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ABSTRACT

AIM: Determine the number of cells with cancer stem cells (CSC) markers (CD44 and CD24) in prostate cancer (PC) tissue and evaluate their prognostic significance. Materials and Methods: We performed retrospective analysis of the results of examination and treatment of 102 stage II and III PC patients, who were treated in SI "Institute of Urology, NAMS of Ukraine" and in Kyiv City Clinical Cancer Center in 2014-2015. Morphological and immunohistochemical studies of CD44 and CD24 expression were performed on paraffin-embedded sections of surgical material. STATISTICA 6.0 software was used for processing of the results. Results: CD24 and CD44 expression were identified in 51.0% and 75.5% of tumor samples of patients with PC, respectively. Number of tumors with positive expression of CSC markers (CD44+CD24-/low) was 32.3%. We observed significant correlation between the expression of CD44 and CD44+CD24-/low and the stage of disease ($r = 0.45$, $p \leq 0.05$, and $r = 0.65$, $p \leq 0.05$, respectively). It was shown that high serum PSA levels in patients with PC (>15 ng/ml) were associated with increased levels of CD44 and CD44+CD24-/low expression. In patients with CD24- and CD44+CD24-/low tumor cell phenotype incidence of recurrence was 3 times higher compared to patients with CD44+ phenotype. A correlation between the level of CD44+CD24-/low expression and the development of recurrent disease was significant ($r = 0,4$; $p \leq 0,05$). Conclusions: Detection of the tumor cells with CD44+CD24-/low phenotype in PC tissue may be an additional criterion for prognosis of PC course, particularly for predicting the risk of recurrence.

INTRODUCTION

Prostate cancer (PC) is one of the most common tumors in men of old and senile age [1-3]. However, its etiology remains unclear, which may be partly due to genetic and epigenetic mechanisms that cause the heterogeneous nature of the disease. Despite significant advances in the treatment of PC, about a third of patients faces the development of resistance [4]. Mortality from common PC forms usually results of their insensitivity to androgenic deprivation and development castration-resistant prostate cancer [4].

Recent studies are aimed at finding markers of inter- and intra-tumor heterogeneity of PC, as well as markers that are important in its molecular pathogenesis. Attention of scientists is focused on molecular markers of PC heterogeneity that would have clinical and biological significance. In modern literature, there is actively discussed intratumor heterogeneity, in which cancer stem cells (CSC) may be of particular importance, but their relationship with clinical characteristics of different histological types of PC with metastasis and patient's survival is not fully elucidated [5]. Therefore, the study of heterogeneity of PC and the relation between clinical features of these tumors with markers of CSC is an urgent problem whose solution can help to improve knowledge of the biology of the tumor growth.

Up-to-date CSC was found in patients with cancer of the brain, breast, lung, skin, pancreas, rectum [1]. To identify CSC of PC, several markers were studied. The most promising and significant among them are CD24, CD44, CD49f, CD133, CD166, and α2β1-integrin [3, 6, 7]. These markers were investigated separately and in combinations, but optimal set is not found still. The reason for this is the large variety of histological types of tumors and their genetic heterogeneity [8].

In 2008, Hurt and colleagues identified $CD44^{+}CD24^{-/low}$ cells subpopulation of human prostate as tumor stem cells that have the ability to grow as nonadherent spheres in serum replacement medium. Only $CD44^{+}CD24^{-/low}$ cell population has the potential to form tumors in NOD / SCID mice [1, 8].

The aim of the work was to determine the frequency of cells expressing markers of cancer stem cells (CD44 and CD24) in prostate cancer tissue and to evaluate their prognostic significance.

MATERIALS AND METHODS

The work is based on a retrospective analysis of the results of examination and treatment of 102 patients with prostate cancer of stages II and III, which were treated in SI "Institute of Urology NAMS of Ukraine" and the Kyiv City Clinical Cancer Center during 2014-2015.

Clinical diagnosis is established based on an assessment of prostate-specific antigen (PSA) in the blood serum, and digital rectal examination, pelvic CT and/or transrectal ultrasound of the prostate, osteoscintigraphy, radiography of the chest cavity, ultrasound of the abdomen. In all patients, the diagnosis was verified after transrectal multifocal biopsy of the prostate under ultrasound guidance.

Tumor stage was determined according to the international classification of tumors (TNM, 7 edition, 2009). The histological type of the removed tumors was verified by morphological study of histological sections of paraffin blocks of tumors (staining of sections with hematoxylin and eosin) according to the International Histological Classification of WHO.

Immunohistochemical study of the CD24 and CD44 expression of in tumor cells was performed on parallel 5 micron thick paraffin sections. As primary antibodies, monoclonal antibodies specific for CD24 (clone SN3b, Thermo Scientific, USA) and CD44/HCAM (clone 156-3C11, Diagnostic BioSystems, USA) were used in dilutions recommended by the manufacturers. The number of CD24 and CD44 immunopositive tumor cells > 10% was considered as positive expression. Immunohistochemical reactions were visualized using EnVision system (Dako LSAB2 system, Denmark) according to the manufacturer's recommendations, histological sections were stained with Mayer’s hematoxylin. The results of immunohistochemical reactions were analyzed using optical microscopy XSP-137-BP (JNOEC) at magnification x200-x400.

After surgery, all patients were scheduled for examination to detect the possible development of biochemical recurrence. Observation period was 24 months. The presence of biochemical recurrence was established if PSA level was elevated > 0.2 ng/mL in 2 consecutive surveys.

Statistical analysis of the results was performed using the methods of variation statistics and the program STATISTICA 6.0. The relation between expression of the studied markers with clinical and pathological characteristics of PC was assessed using Pearson's coefficient of mutual

contingency (s). The differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Clinical characteristics of 102 patients with prostate cancer of stages II-III are given in Table 1. Number of patients with stage II PC was 68.6%, stage III - 31.4%. The age of patients ranged from 51 to 81 years, mean age — 58.0 ± 3.1 years. The largest number of cases were in the age range of 61 years or more.

Table 1 General clinical characteristics of the patients with PC of stages II-III

Index	**Number of patients**	
	n	**%**
Total number of patients	102	100
Age (years)		
Average age	58.0±3.1	
Range of age	51 – 81	
PCA level		
<4 ng/ml	13	12.7
4-10 ng/ml	59	57.8
>10 ng/ml	30	29.5
PC stage by TNM		
Stage II	70	68.6
Stage III	32	31.4
Gleason grading		
Score 6	26	25.5
Score 7	58	56.8
Score 8	13	12.7
Score 9	5	5.0
Surgical margins		
Positive	36	35.2
Negative	66	64.8
Biochemical recurrence		
Established	37	36.3
Not found	65	63.7

The results of a comprehensive survey of patients (radiological, ultrasound, laboratory) showed no metastases in regional lymph nodes and distant metastases of PC.

Patients with PC were distributed into four subgroups depending on the Gleason grading: patients with moderately differentiated adenocarcinoma (Gleason score 6 and 7 - 25.5% and 56.8% respectively) and patients with low differentiated adenocarcinoma (Gleason score 8 and 9 - 12.7% and 5.0% respectively).

Positive surgical margins were in 35.2% patients, and negative in the remaining 64.8% cases.

By the level of PSA, patients were distributed into 3 groups: group I - PSA below 4 ng/ml (12.7%), group II - 4-10 ng/ml (57.8%), and group III - more than 10 ng/ml (29.6%).

In 36.3% of patients, biochemical recurrence of PC was detected. The highest recurrence rate was observed in the first year after surgery, significant reduction was noted in the second year of observation.

Immunohistochemical study showed a significant heterogeneity of the studied tumors by expression of CSC markers (Fig. 1). For example, the presence of CD24 and CD44 was identified in 51.0% and 75.5% of PC samples respectively of all PC patients (Fig. 2).

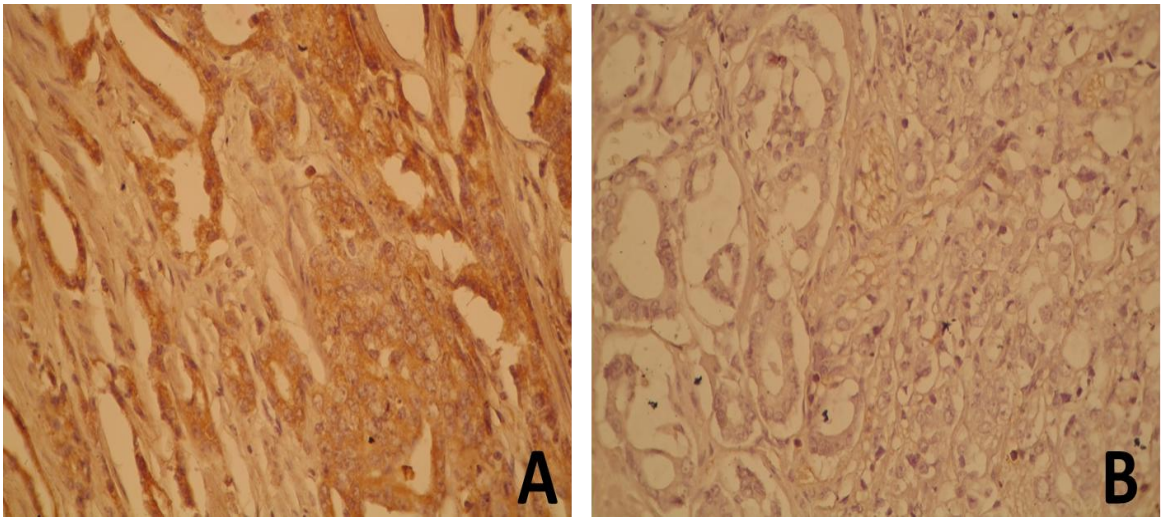

Fig.1. Expression of CD44 (A) and CD24 (B) in prostate cancer cells. Immunohistochemical method, staining with Mayer's hematoxylin, x400

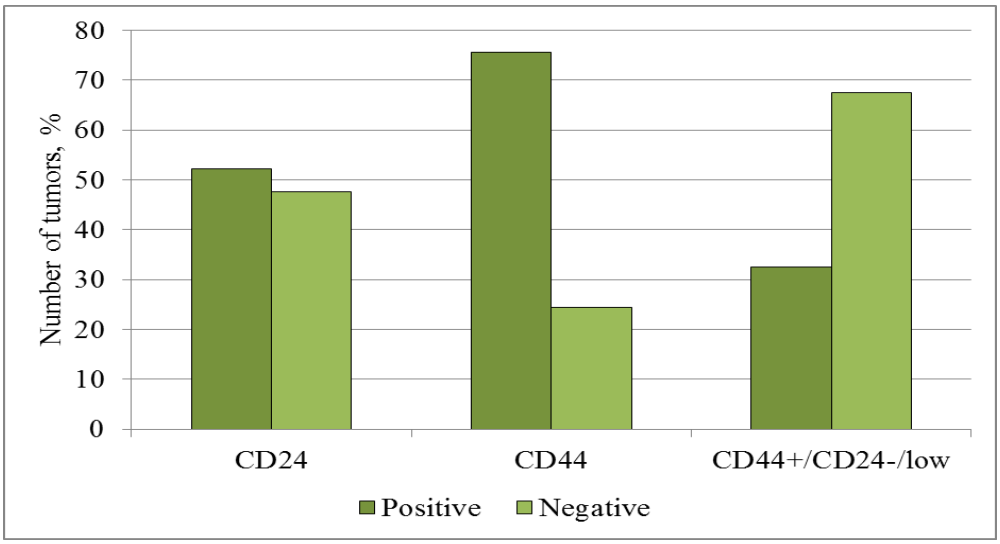

Fig. 2. Distribution of the studied tumors by expression of CSC markers

Number of tumors positive for expression of CSC markers (CD44+CD24-/low) was 32.3% (Table 2).

Table 2 Expression of CSC markers in tumor tissue of PC patients

CSC markers	Number of tumors with positive expression N (%)	Number of tumors without expression N (%)
CD24	52 (51.0)	50 (49.0)
CD44	77 (75.5)	25 (24.5)
CD44+CD24-/low	33 (32.4)	69 (67.6)

The criteria, which have significant clinical implications for prognosis and indications for adequate therapy for PC patients, is the stage of the disease. The largest number of CD44 and CD44+CD24-/low positive tumors was identified in patients with stage III (77% and 53% respectively) compared to stage II PC (65% and 28% respectively). Number of tumors expressing CD24 in stages II and III was practically equal and accounted for 40.5% and 40% respectively.

A correlation between expression of CD44 and CD44+CD24-/low and the stage of the disease ($r = 0.45$, $p \leq 0.05$ and $r = 0.65$, $p \leq 0.05$ respectively) has been revealed (Fig. 3).

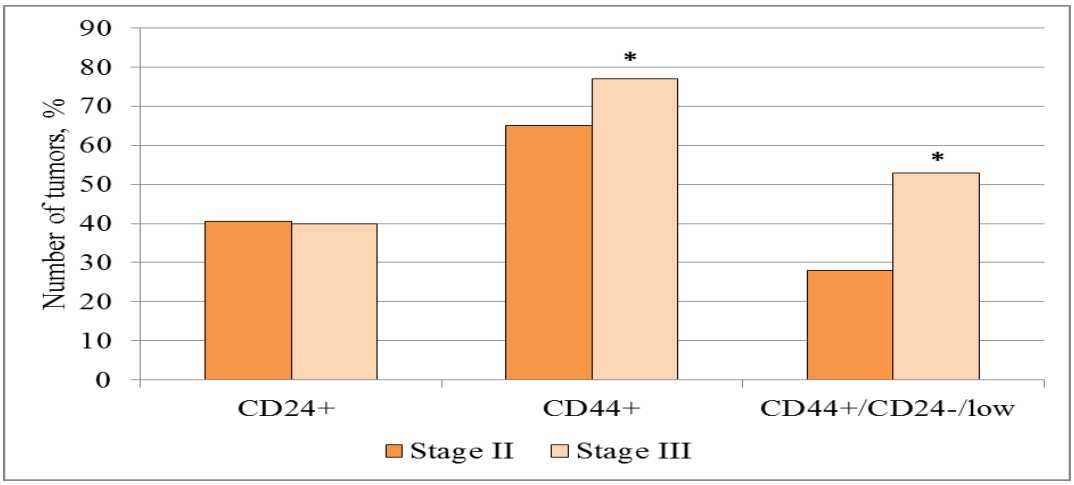

Fig. 3. Expression of CSC markers dependent on disease stage.

Note: *the difference is significant compared to the group of 42-50 year-old PC patients ($p < 0.05$).

Investigation of CSC markers revealed a positive trend to an increase in the relative amount of tumors with CD44+CD24-/low phenotype along with increasing Gleason score. In particular, moderately differentiated adenocarcinoma (Gleason score 6 (15.4%)) had tumor phenotype CD44+CD24-/low, with Gleason score 7 and 8 - 44% and 75% respectively, while all low differentiated tumors (9 points by Gleason) were with this phenotype ($r \geq 0.05$). Among tumors with different Gleason score, significant differences in the expression of CD44 were not found (Fig. 4).

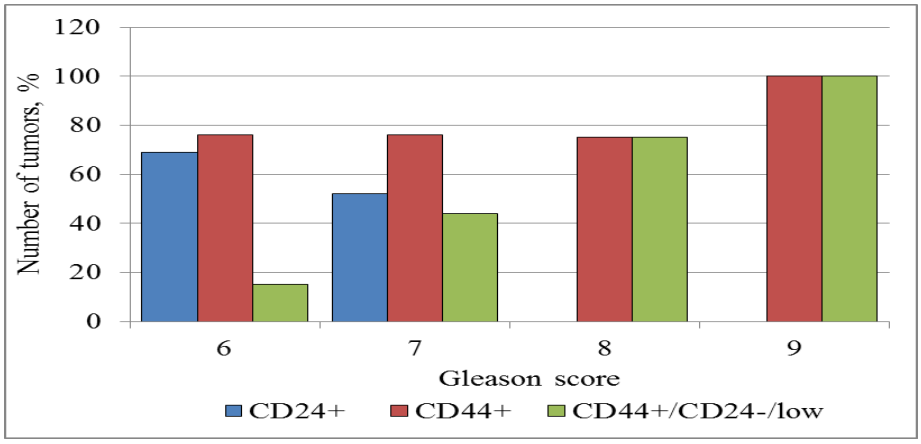

Fig. 4. Expression of CSC markers dependent on Gleason score

These data are consistent with the results already known in the literature. In particular, it was shown that overexpression of CD44v7-v8 increased proportionally to the degree of differentiation of PC by Gleason [9, 10]. However, there are also studies [11, 12, 13], which showed a statistically significant negative correlation between the expression of CD44 in tumor cells and Gleason score of PC. Other authors found no correlation between CD44 expression and Gleason grading [14, 15].

The next stage of our work was to determine the presence of a relation between expression of CSC markers and PSA level (Table 3). It is shown that in 50.0% of patients with positive expression of CD24 PSA level was higher than 15 ng/ml. It was found that high PSA level in blood serum of PC patients (> 15 ng/ml) is associated with high levels of CD44+CD24-/low expression. For example, in patients with expression of CD44 and CD44+CD24-/low, PSA levels in blood serum higher than 15 ng/ml were detected in 59.7% and 64.3% cases respectively.

Table 3 Expression of CSC markers dependent on PSA level

PSA levels	Number of tumors with expression of CD24 % /(N)	Number of tumors without expression of CD24 % /(N)	Number of tumors with expression of CD44 % /(N)	Number of tumors without expression of CD44 % /(N)	Number of tumors with expression of CD44+CD24-/low %/(N)	Number of tumors without expression of CD44+CD24-/low % /(N)
PSA <15 ng/ml	50.0 (26)	47.0 (23)	40.7 (31)	72.0 (18)	36.4 (12)	53.6 (37)
PSA >15 ng/ml	50.0 (26)	53.0 (27)	59.7 (46)*	28.0 (7)	64.3 (21) *	46.4 (32)

Note: * the difference is significant compared to the group with the index PSA <15 ng/ml ($p < 0.05$).

A study of the frequency of tumor phenotype CD44+CD24-/low in patients with recurrent prostate cancer has been performed. The dependence between CD24 expression and recurrence has been revealed (Table. 4). In 76.0% of patients with positive CD24 expression in tumor cells,

the development of relapse has been detected. There was no significant relationship between CD44 expression and recurrence.

Table 4 Relation between expression of CSC markers and PC recurrence

Biochemical recurrence	Number of tumors with expression of CD24 % (N)	Number of tumors without expression of CD24 % (N)	Number of tumors with expression of CD44 % (N)	Number of tumors without expression of CD44 % (N)	Number of tumors with expression of CD44+CD24-/low % (N)	Number of tumors without expression of CD44+CD24-/low % (N)
Established	48.1 (25)	76.0 (38)	44.0 (34)	12.0 (3)	75.8 (25)	17.4 (12)
Not found	51.9 (27)	24.0 (12)*	56.0 (43)	88.0 (22)	24.2 (8)*	82.6 (57)

Note: *the difference is significant compared to the group with established biochemical recurrence ($p < 0.05$).

It has been shown that in patients with tumor cell phenotype CD24- and CD44+CD24-/low incidence of relapse was 3 times higher compared with patients with other tumor cell phenotype (Fig. 5). In particular, in 75.8% of patients with recurrent PC, the primary tumor was of CSC phenotype. Correlation between the level of expression of CSC markers and development of the disease recurrence has been established ($r = 0.4$; $p \leq 0.05$).

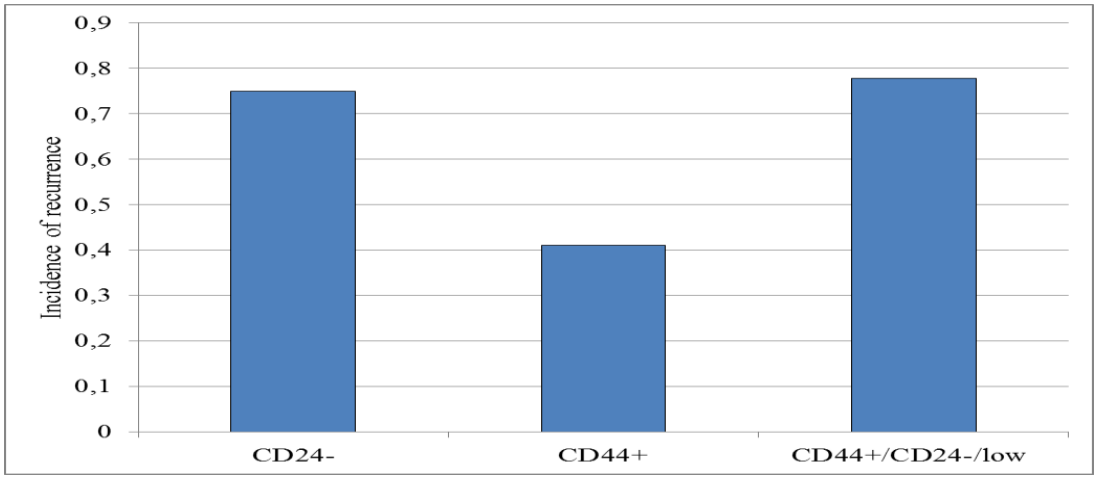

Fig. 5. The incidence of relapse in patients with prostate cancer dependent on expression of CSC markers in tumor tissue

The presented results show a multifunctionality of CSC and their relationship with tumor aggressiveness. However, the final question on the role of CSC in the development of prostate cancer, their relationship with the hormonal status of patients, microenvironment features and other important functions of these cells is not fully defined. At the same time, the information available in the scientific literature and our data allow discussing the possibility of using CSC markers for prognosis of the disease course, correction of the therapy or as targets for target therapy. For example, Liu *et al*. [16] showed that miR-34a reduces PC CSC counts and metastasis by direct inhibition of CD44 expression.

So, summing up the results we came to the conclusions that allow suggesting that CSC may be the key to solving a number of questions regarding tumor growth at various stages of tumorigenesis. It should also be noted that the identification of prostate cancer cells with the markers CD44+CD24-/low in the PC tissue may serve as an additional criterion for prognosis of the disease, particularly for predicting recurrence. The study of cancer stem cells can provide understanding of the mechanisms of tumor initiation, progression and metastasis.

CONCLUSION

So, CSCs may be the key to solving a number of questions regarding tumor growth at various stages of tumorigenesis. Identification of prostate cancer cells with the CD44+CD24-/low phenotype in the PC tissue may serve as an additional criterion for prognosis of the disease, particularly for predicting recurrence. Further studies are needed to understand the exact role of CSCs and studied CSC markers in mechanisms of tumor initiation, progression and metastasis.

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