Original paper

Prostate cancer with different ERG status may show different FOXP3-positive cell numbers

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Prostatic carcinoma is the most frequent cancer in males in the Western world. A significant proportion of these cancers have a recurrent translocation involving ETS family genes, which leads to the overexpression of ERG transcription factor. Prostate cancers, which bear this mutation, differ in a number of features, including tumor microenvironment. One of the components of the tumor microenvironment is FOXP3 positive lymphocytes, which may participate in breaking immunosurveillance and promoting tumor growth.

The aim of the study was to analyze the relationships between ERG expression, number of FOXP3 positive cells and other features of the tumor.

The study group consisted of 65 cases. Tissue microarrays composed of 2 mm tissue cores were used for immunohistological evaluation. Immunohistochemistry for ERG and FOXP3 was performed according to the routinely applied protocol. The FOXP3 positive cells were counted and the results were expressed as the number of cells per mm^2.

The average number of FOXP3 positive cells was 33.30/mm^2 for all cases, 21.43/mm^2 for the ERG negative and 42.28/mm^2 for the ERG positive group ($p < 0.02$). There were no significant relationships between FOXP3 positive cell count and any other parameters studied.

Our results suggest that the immune response may differ between ERG negative and ERG positive prostatic carcinomas.

Key words: prostate cancer, FOXP3, ERG.

Introduction

As Western society ages, the frequency of some cancers decreases, others become curable, but there are still some which occur even more often and remain a serious problem. One such tumor is prostate cancer. It has become the most prevalent cancer in some countries and an important cause of death [1]. In terms of prognosis, prostate cancer is a heterogeneous disease, but the number of established prognostic factors is limited, which often makes the therapy planning suboptimal. The most frequent single genetic event in prostate cancer is a translocation involving genes of the ETS family, most often ERG. Such translocation, which leads to ERG protein expression, is seen in about half of European and American prostate cancer cases. The prognostic significance of this phenomenon remains, however, con-

troversial. Prostatic carcinomas bearing the translocation involving ETS genes may differ in a number of features. For example, in one of the previous studies we demonstrated that they are characterized by higher microvessel density [2]. Regulatory T lymphocytes (Tregs), well identified by FOXP3 expression, are the negative regulators of the immune response. They are known to be involved in some physiological processes, e.g. the progression of labor [3], but were also shown to influence the prognosis in several cancers [4, 5, 6].

The aim of the present study was to analyze the relationships between the number of FOXP3 positive cells and ERG status as well as other basic parameters of prostate cancer.

Material and methods

The material of the study consisted of unselected prostatectomy specimens obtained from the files of the Pathology Department. The slides were reviewed by an urologic pathologist and reclassified according to the current Gleason system as well as the latest TNM criteria [7, 8, 9]. Nerve invasion, lymphovascular invasion, status of surgical margins, presence of multiple tumor foci and production of mucin were also reevaluated. The positive margins were classified as focal or extensive [10]. The approximate volume of the prostate was estimated using the ellipsoid volume formula v = a*b*c*0.523598, where a, b and c are the dimensions of the gland registered at gross examination. For each case, one representative section was chosen. On the slide, the region of interest containing carcinoma tissue was marked and its extent was subsequently copied to the surface of the paraffin block. For the tissue microarray (TMA) production a manual device (Histopathology Inc., Hungary) was used. On each paraffin block, from the area marked as cancer two 2 mm cores were obtained and transferred into a recipient block. The case numbers together with the respective location in the TMA were entered in an Excel (Microsoft Inc., USA) spreadsheet. The upper-left corner of the TMA was left empty to allow investigators proper orientation of the obtained slides. From the TMA paraffin blocks, 2 mm sections were prepared and stained with hematoxylin-eosin (HE) and immunohistochemically. HE slides were used to control quality of tissue selection and to determine the Gleason score of particular spots. Immunohistochemistry was processed according to the protocol used on a routine basis. For ERG staining (Fig. 1), a rabbit monoclonal antibody (clone EPR3864), produced by Abcam, was used in a 1 : 200 dilution. Antigen retrieval was performed by immersing the slides in citrate buffer and heating them for 30 minutes. For FOXP3 staining (Fig. 2), mouse monoclonal antibody (clone 236A/E7), produced by Abcam, was used in a 1 : 100 dilution. In this case, heating in EDTA for 30 minutes was performed for antigen retrieval. The LabVision detection system (Thermo Scientific, USA) was used for each staining. The results of the ERG staining were scored as positive when unequivocal nuclear staining was present; very faint nuclear as well as any cytoplasmic reaction was ignored, as previously reported [11]. The FOXP3 expressing cells were counted in each tissue microarray core and the results were expressed as the number of cells per mm^2. The person who counted the cells was blind to the ERG status as well as to other data under study. The results were collected in the Excel spreadsheet containing the case numbers. Statistics were calculated with Statistica 10 (StatSoft Inc., USA). Mann-Whitney U and Kruskal-Wallis ANOVA tests were used, as appropriate. The significance level was set to 0.05.

Results

The study group consisted of 65 cases. The average age of the patients was 61.26 years (ranging from 38 to 73 years, SD 6.62). The average PSA level was 12.22 ng/ml (ranging from 1.89 to 52.20, SD 9.71). The average volume of the prostate was 39.39 cm^3 (ranging from 12.57 to 91.89, SD 18.43). Only one case showed lymph node metastases. In 23 cases (35.38%) the disease was organ confined. Lymphovascular invasion was present in 47 cases (72.31%). The detailed characteristics are shown in Table I.

Twenty-eight cases (43.1%) were ERG negative and 37 (56.9%) were ERG positive (Fig. 1). The average number of FOXP3 positive cells (Fig. 2) was 33.30/mm^2 (SD 33.07). For ERG negative cases the average number of FOXP3 positive cells was 21.43/mm^2 (SD 16.90) while for ERG positive cases it was 42.28/mm^2 (SD 39.21). The difference was statistically significant ($p < 0.02$; Fig. 3). The number of FOXP3 positive cells did not show any correlations with other parameters included in the analysis, such as age, tumor stage, grade, prostate size or PSA level (data not shown).

Discussion

Prostatic cancer, being responsible for almost 30,000 deaths per year in the United States [1] and over 4000 a year in Poland [12], is among the most frequent and important cancers in men. Individual prognostication is difficult as in other malignancies, but in prostatic carcinoma particularly relevant, since not all the cases are aggressive. As a consequence, a significant proportion of patients receive therapy, which is of little, if any, benefit to them. On the other hand, patients who experience relapse and dissemi-

Table I. Pathologic data of the cases under study

Stage				No. (%)
	pT2a			2 (3.08%)
	pT2c			21 (32.31%)
	pT3a			36 (55.38%)
	pT3b			6 (9.23%)
Grade	Gleason's	No. (%)	grade group	No. (%)
	6 (3+3)	18 (27.69%)	1	18 (27. 69%)
	7 (3+4)	27 (41.54%)	2	27 (41.54%)
	7 (4+3)	12 (18.46%)	3	12 (18.46%)
	8 (3+5)	3 (4.62%)	4	4 (6.15%)
	8 (4+4)	1 (1.54%)		
	9 (4+5)	4 (6.15%)	5	4 (6.15%)
Margin status				No. (%)
	negative			30 (46.15%)
	positive – focal			17 (26.15%)
	positive – extensive			18 (27.69%)

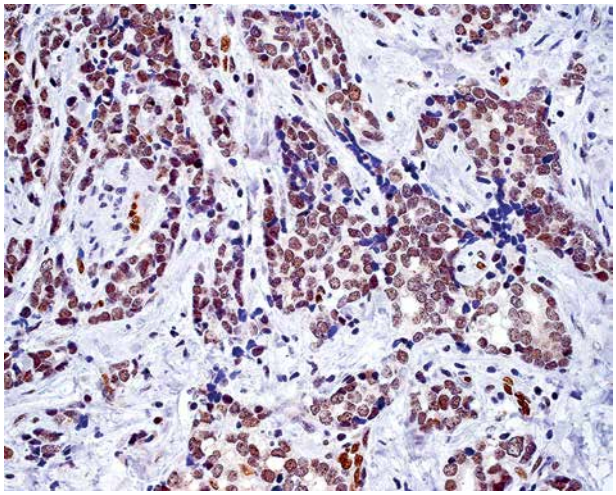

Fig. 1. Carcinoma of the prostate showing strong expression of ERG transcription factor. Immunohistochemistry, 400×

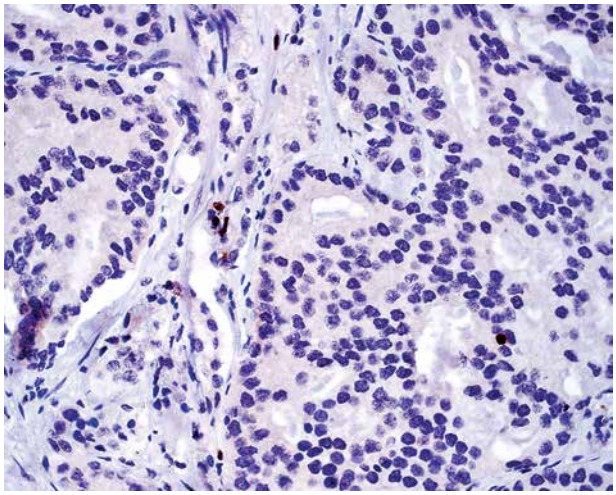

Fig. 2. Single FOXP3 positive cells in the stroma of a carcinoma of the prostate. Immunohistochemistry, 400×

nation of the disease may not be offered any definite treatment [13, 14]. It is thus of primary importance to recognize additional prognostic parameters, which could potentially be helpful in adjusting appropriate therapy. In this context, the immunological response seems to be worth investigating, and it has already been proposed to use tumor vaccines in prostate cancer [15]. Proper understanding of immunological processes is crucial, as many components of the tumor microenvironment, including macrophages, different classes of lymphocytes, NK cells, plasma cells, neutrophils, eosinophils and mast cells, participate in the immune response [16, 17, 18, 19].

A class of cells which has gained special interest and been extensively studied in cancers is regulatory lymphocytes [4, 5, 6, 20]. Merlo *et al.* [4] published an analysis of regulatory lymphocytes in breast carcinoma, in which they reported a significant survival benefit in FOXP3 negative cases. This effect was observed both in lymph node negative and positive cases, which was associated with a lower risk of metastatic disease, but not with the risk of local recurrence. Petersen *et al.* [5] reviewed cases of non-small-cell pulmonary carcinomas and found that patients with a higher FOXP3 positive/overall T lymphocyte ratio were at greater risk of relapse. Salama *et al.* [6] analyzed the popu-

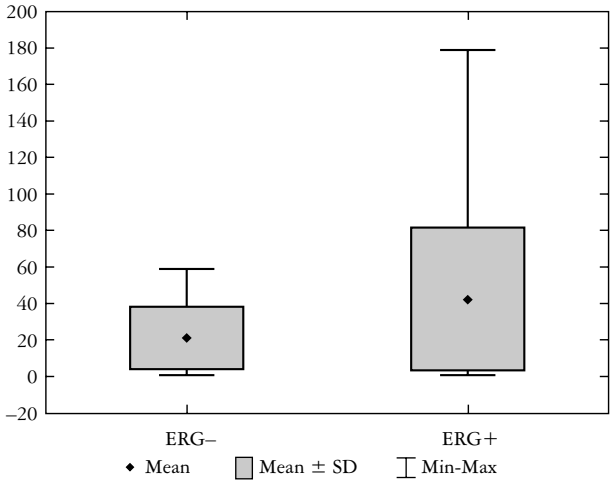

Fig. 3. Differences in the number of FOXP3 positive cells between ERG negative and ERG positive carcinomas of the prostate. Central point is arithmetic mean, box is mean ± standard deviation, whisker is range

lation of lymphocytes in colorectal cancer and found a relationship between FOXP3 positive cells and tumor stage, but there were no correlations with other clinicopathological parameters. FOXP3 positive cell number proved to be an independent prognostic factor, while other analyzed subsets of lymphoid cells did not. Interestingly, a higher number of FOXP3 positive cells correlated with better prognosis, and additionally these cells were not associated with the microsatellite instability status. Rubinkiewicz *et al.* [20], who analyzed colorectal adenoma and cancers, observed that the mean FOXP3/CD4 cell ratio increases during the progression to malignancy.

The number of studies on FOXP3 positive cells in prostatic carcinoma is more limited. Yokokawa *et al.* [21] analyzed peripheral blood Tregs in prostate cancer patients and found that the number of these cells was increased in metastatic disease. Tregs of these patients showed an increased ability to suppress antigen-dependent T-lymphocyte proliferation. Ebelt *et al.* [22] compared the lymphocyte populations in normal, hyperplastic and neoplastic prostate and reported that the foci of prostate cancer are surrounded by clusters of T lymphocytes. In these clusters, as well as in the sparse tumor infiltrating lymphocytes, the ratio of CD4 positive to CD8 positive cells was increased in comparison to benign tissue. Moreover, the cancer-associated lymphocytes were characterized by significantly reduced interferon gamma production and perforin expression, which might lead to a disturbed effector response. The same group of scientists [23] demonstrated that the peritumoral lymphocyte clusters contained regulatory lymphocytes, which actively silence the immune response against cancer. Miller *et al.* [24] found a higher number of regulatory T lymphocytes in peripheral blood of patients with prostatic carcinoma, as well as within tumor tissue. They also showed that these cells are indeed actively immunosuppressive in vitro, and that the prostate cancer supernatant has a chemotactic effect on Tregs. Additionally, Ebelt *et al.* [23] found no correlations between the number of regulatory lymphocytes in prostate cancer and tumor stage or grade, which is consistent with the results of our study. Sfanos *et al.* [25] found that tumor infiltrating T_H lymphocytes in prostate cancer include predominantly T_H1, T_H17 and Treg (FOXP3 positive) subtypes, while T_H2 are virtually nonexistent, although in some studies T_H2 cells were associated with the pathogenesis of this malignancy [26]. Interestingly, despite the fact that the level of interleukin 17 and, to some degree, the level of interferon gamma were dependent on Gleason score of the tumor, FOXP3 expression did not show any correlation with the tumor grade [25], which is in agreement with our findings. Furthermore, the same authors [27] found that in prostate cancer CD8 positive cells are oligoclonal and express high levels of inhibitory receptor PD-1, which is suggested to be a mechanism of tumor-induced tolerance. Kiniwa *et al.* [28] also analyzed the CD8 positive FOXP3 positive cells in prostate cancer. They cultured Treg cells derived from malignant tissue, purified them by fluorescence-activated cell sorting and analyzed them together with their location in the prostate cancer samples by using confocal microscopy. Their results indicate that CD8 positive FOXP3 positive Treg cells suppress immune responses and this phenomenon may be regulated by the toll-like receptor 8 ligands.

Di Carlo *et al.* [29] found that the prostate cancer cells lack interleukin 7 and BAFF/BLyS expression seen in non-neoplastic prostate and suggested that it may be the main mechanism of tumor escape from immunosurveillance. In addition, Sorrentino *et al.* [30] analyzed the changes in tumor infiltrating lymphocytes associated with hormonal neoadjuvant therapy. They found that the number of both effector and regulatory lymphocytes was increased, which let them deduce that the net effect of hormonal treatment on the immune response is insignificant. In contrast to the T cells, a proportion of which are involved in the anti-cancer response, the B cells are thought to have a tumor protecting and promoting effect. Woo *et al.* [31] found an increased number of B-lymphocytes in the immediate surroundings of prostatic cancer in comparison to the tissue areas distant from malignant cells.

To the best of our knowledge, this is the first study suggesting that different subtypes of prostatic carcinoma may differ in their ability to induce an immunologic response.

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The authors declare no conflict of interest.

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