Expression of *p65, DD3* and *c-erbB2* genes in prostate cancer*

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The expression of *p65*, *DD3* and *c-erbB2* genes was analyzed in 39 histologically verified human prostate cancers. The expression of *p65* and *DD3* genes was observed in significant percentage in well- and moderately-differentiated tumors. Both genes expression was lower in poorly differentiated tumors. On the contrary, *c-erbB2* gene expression increased with advanced histological grading and reached the highest percentage in poorly-differentiated cancers. In the all investigated groups straight dependence between *p65* and *DD3* genes expression occurred. Opposite dependence was noticed in expression of *p65*/*DD3* and *c-erbB2* genes.

Key words: p65, DD3, c-erbB2, PCR, RT-PCR, prostate cancer.

Prostate cancer is the second leading cause of cancer deaths in male population [16]. Therefore, there is need to develop sensitive and specific tests for its detection and also for accurate determination of the degree of malignancy. A number of prostate-specific markers are known, including prostate-specific membrane antigen [8], prostate stem cell antigen [17], and prostate-specific antigen [21]. The most useful method for screening and monitoring prostate cancer disease till now is prostatic acid phosphatase and prostate-specific antigen (PSA) estimation [3]. PSA serves as an indicator of metastatic involvement and as a good parameter for following the response to surgery and therapy. Both of these proteins are secreted to the serum and may serve as markers of the disease. However, on the basis of these markers is difficult to get straight answer about cancer development. An evaluation of additional markers may be helpful for routine diagnosis. To such markers belongs *c-erbB2* oncogene, which is known as a poor prognostic factor, *DD3* a newly described prostate-specific gene and also *p65* gene investigated by us.

c-erbB2 is homologous with the gene of epidermal growth factor receptor and codes of membrane receptor protein. Expression and amplification of *c-erbB2* are connected with more aggressive disease in many human cancers. The clinical importance of *c-erbB2* became apparent with the recognition of gene amplification and overexpression of the encoding protein in breast cancer patients, which are associated with poor prognosis. The same dependence was found in prostate cancers. Immunohistochemical study showed expression of protein *c-ErbB2* in benign prostate hyperplasia and in prostatic adenocarcinoma [24]. Oncogene *c-erbB2* is also amplified and/or overexpressed in both benign and malignant prostate tissue [5]. Overexpression/amplification of this oncogene in prostate cancer is associated with large tumor volume [20], high tumor grade and distant metastases [18].

DD3 gene is specifically and highly expressed in prostate cancer, none or very low expression was found in normal prostate tissue or in patients with benign prostate hyperplasia. Expression of this gene was not observed in any other normal human tissue and in some investigated tumors of breast, cervix, endometrium, ovary and testis. The role of this gene is unclear. *DD3* is expressed as a spliced and polyadenylated RNA molecule what suggests that it could be a pseudogene [2]. Verhaegh et al [23] identified the promoter elements that are responsible for the prostate cancer specific expression of *DD3*. It was shown that the high mo-

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bility group HMGI(Y) protein binds to one of the DNA ase I protected areas of *DD3* promoter region.

Another gene, which may be connected with prostate cancer development, is *p65* encoding 65 kDa oncofetal protein (*P65*) [11]. P65 was detected only in cancer tissue but not in benign tumors [13]. It was shown that P65 blood level in patients suffering from prostate cancer was significantly higher in comparison to healthy men [6]. Among 311 patients, non divided according to histological stage, about 85% had significantly increased serum *P65* level. In the same group of patients prostate specific antigen was detected in lower percentage.

In this study the expression of *p65* gene was studied in histologically differentiated human prostate cancers and related to the expression of well-known *c-erbB2* oncogene and for prostate cancer specific *DD3* gene.

Material and methods

Tissue. Paraffin embedded tissue specimens from 39 human prostate cancers were obtained from the Department of Pathomorphology, Central Teaching Hospital of the Military Medical University in Warsaw. The slides were divided according to WHO - Mostofy histological classification into 3 groups: well- (G1), moderately- (G2), and poorly-differentiated tumors (G3). According to our determination G1 was equivalent to 1–4, G2 to 5–7 and G3 to 8–10 Gleason classification.

RNA extraction. RNA was isolated by Total RNA Prep Plus Minicolumn Kit (A&A Biotechnology, Poland) based on RNA isolation method developed by Chomczynski and Sacchi [4]. The isolated RNA had an $A_{260/280}$ ratio of 1.6–1.8.

RT-PCR. cDNA was synthesized by RevertAid^{TM} cDNA Synthesis Kit (Fermentas, Lithuania). Reaction mixture (total volume 20 μl) containing 1 μl of total RNA (3 μg), 1 μl oligo(dT) 18 primer (0.5 μg) and 8 μl deionized nuclease free water was prepared. The mixture was spinned down and incubated at 70 ˚C for 5 min, then chilled on ice and the following components were added: 4 μl 5x reaction buffer, 1 μl ribonuclease inhibitor (20 U/μl) and 4 μl 10 mM dNTPs mix. The mixture was incubated at 37 ˚C for 5 min and 1 μl RevertAid M-MulV reverse transcriptase (200 U/μl) was added. The mixture was incubated at 42 ˚C for 60 min. The reaction was stopped by heating at 70 ˚C for 10 min. To verify the quality of synthesized cDNA, control reaction was performed using primers for *β-actin* gene (5'-TGTATGCCTCTGGTCGTACCAC-3', 5'ACAGAG-TACTTGCGCTCAGGAG-3') according to Tokunaga et al [22].

To study *p65* gene expression by RT-PCR the primers were designed on the basis of N-terminal domains of undigested P65 molecule (5'-GGTCCACGGCGGACCGGT-3') and its peptide, which after CNBr treatment migrated in electrophoresis as 51 kDa band (5'-GACCCCGA-GAACGTGGTGCGC-3') [12]. As a source of RNA for RT-PCR method optimalization human breast cancer cell line (MCF-7) was used [1]. Preliminary experiments allowed to find the best conditions (annealing temperature, Mg^{2+} concentration, amount of cycles and time of reaction) for the PCR. DNA for *p65* was amplified in 35 cycles using the following parameters: denaturation (94 ˚C; 30 s), annealing (57 ˚C; 30 s) and extension (72 ˚C; 30 s).

Sets of primers for *DD3* amplification (5'-AGATTT-GTGGTGCTGCAGCC-3', 5'-TCCTGCCCAATCTT-TAAGG-3') were planned according to Bussemakers et al [2]. The amplification of *DD3* was performed as follows: denaturation (94 ˚C, 30 s); annealing (60 ˚C, 30 s); extension (72 ˚C, 30 s) during 35 cycles.

Sets of primers for *c-erbB2* (5'-CCTCTGACGTCCAT-CATCTC-3', 5'-CGGATCTCCTGCTGCCGTCGT-3') were planned on the basis of article by Schwartz et al (1998) [19]. PCR was carried out as follows: denaturation (94 ˚C; 1 min.), annealing (55 ˚C; 1 min) and extension (72 ˚C; 2 min) for 30 cycles.

The PCR products were separated by electrophoresis on 2% agarose gel.

Statistics. Statistical analysis was done on the basis of non-parametric Chi-square independence test and Yula's factor (STATISTICA for Windows version 5.1, 97 Edition, StatSoft, Inc.).

Results

The investigated prostate cancers were divided into 3 groups according to histopathological degree of differentiation. In all cases *p65*, *DD3* and *c-erbB2* genes expression was checked by RT-PCR technique. In the first group, marked as G1 (well-differentiated tumors), the expression of *p65* gene in 6 and *DD3* in 7 out of 13 investigated cases was observed. The expression of *c-erbB2* oncogene occurred in 4 cases (Tab. 1). In this group straight dependence between *p65* and *DD3* genes expression (p=0.0047, Q>0) was noted. Opposite dependences in expression of *p65* and *c-erbB2* genes (p=0.04, Q<0) and also for *DD3* and *c-erbB2* genes (p=0.02, Q<0) were found.

In moderately-differentiated tumors (G2) *p65* gene was expressed in 8 out of 13 investigated cases and *DD3* in 6 cases. In this group *c-erbB2* oncogene was expressed in 6 cases (Tab. 2). In this group straight dependence between *p65* and *DD3* genes expression (p=0.01, Q>0) was found. Opposite dependence was found for *p65* and *c-erbB2* genes (p=0.004, Q<0) and also for *DD3* and *c-erbB2* genes (p=0.004, Q<0).

In poorly-differentiated tumors (G3) both *p65* and *DD3* genes were expressed in lower percentage (Tab. 3) and

Table 1. *p65*, *DD3* and *c-erbB2* positive (+) and negative (-) prostate cancer cases classified as grade G1 (well-differentiated tumors)

G1	*p65*	*DD3*	*c-erbB2*
1	–	–	+
2	–	–	+
3	+	+	–
4	–	+	–
5	–	–	+
6	+	+	–
7	+	+	–
8	–	–	+
9	+	+	–
10	–	–	–
11	+	+	–
12	–	–	–
13	+	+	–
Positive cases (%)	46	53	31

Table 2. *p65*, *DD3* and *c-erbB2* positive (+) and negative (–) prostate cancer cases classified as grade G2 (moderately-differentiated tumors)

G2	*p65*	*DD3*	*c-erbB2*
14	–	–	+
15	+	+	–
16	–	–	+
17	+	+	–
18	+	+	–
19	+	–	+
20	+	+	–
21	+	+	–
22	–	–	+
23	–	–	+
24	+	–	–
25	+	+	–
26	–	–	+
Positive cases (%)	61	46	46

Table 3. *p65*, *DD3* and *c-erbB2* positive (+) and negative (-) prostate cancer cases classified as grade G3 (poorly-differentiated tumors)

G3	*p65*	*DD3*	*c-erbB2*
27	–	–	+
28	+	+	–
29	–	–	+
30	–	–	+
31	–	–	+
32	–	–	+
33	+	–	–
34	+	+	–
35	–	–	+
36	–	–	–
37	–	–	+
38	–	–	–
39	+	–	–
Positive cases (%)	30	15	53

Table 4. Statistical dependences between *p65*, *DD3* and *c-erbB2* genes expression in histologically differentiated groups of prostate cancers (non parametric test, exact p Fisher two-way test)

Grade of malignancy	Genes	*p65*	*DD3*	*c-erbB2*
G1	*p65*	–	p=0.0047, Q>0	p=0.04, Q<0
	DD3	p=0.0047, Q>0	–	p=0.02, Q<0
	c-erbB2	p=0.04, Q<0	p=0.02, Q<0	–
G2	*p65*	–	p=0.01, Q>0	p=0.004, Q<0
	DD3	p=0.01, Q>0	–	p=0.004, Q<0
	c-erbB2	p=0.004, Q<0	p=0.004, Q<0	–
G3	*p65*	–	p=0.07, Q>0	p=0.02. Q<0
	DD3	p=0.07, Q>0	–	p=0.19 ND
	c-erbB2	p=0.02, Q<0	p=0.19 ND	–

G1 – well-, G2 – moderately- and G3 – poorly-differentiated prostate tumors; Q – Yula's factor (Q>0 – straight dependence, Q<0 – opposite dependence) ND – no dependence.

there was straight dependence between expressions of these genes (p=0.07, Q>0). The highest percentage of positive cases was observed for *c-erbB2* oncogene. The expression of *p65* gene was detected in 4 out of 13 investigated cases, *DD3* in 2 and *c-erbB2* in 7 cases. For *c-erbB2* and *DD3* no dependence (p=0.19) but for *c-erbB2* and *p65* opposite dependence (p=0.02, Q<0) were noted.

Statistical dependence between investigated genes in all histologically classified groups are summarized in Table 4.

The examples of *p65*, *DD3* and *c-erbB2* expression in well- (G1), moderately- (G2) and poorly-differentiated (G3) prostate cancers are illustrated in Figure 1.

It should be stated that in all analyzed cases no simultaneous expression of *c-erbB2* and *DD3* or *p65* genes was observed.

Discussion

p65 gene has been suggested as a novel member of the superfamily of genes that encode nuclear receptors for hydrophobic ligands like steroids, vitamin D, thyroid hormones and retinoic acid [7,12]. Some of genes from this superfamily appear to have relations with tumorgenesis. To this superfamily belong also genes for androgen, estrogen and progesterone receptors. These receptors are being investigated in prostate cancer because they might play a role in endocrine treatment and they could be used to identify prostate tumor patients who may respond to antiestrogen therapy [9,10].

In our previous study we evaluated the *p65* gene expression by RT-PCR in relation to grading and staging of human breast cancers. In the group of ductal and lobular cancers, where most were classified as G3 (poorly-differentiated tu-

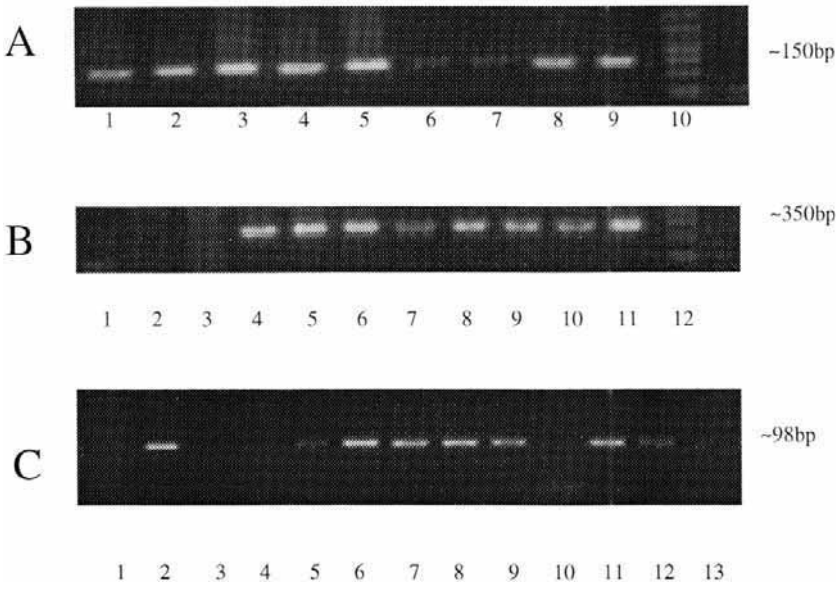

Figure 1. The examples of *p65* (A), *DD3* (B) and *c-erbB2* (C) genes expression in well- (G1), moderately- (G2) and poorly-differentiated prostate cancer (G3).
A (p65) – G1 – lane 1 to 3, G2 – lane 4 to 6, G3 – lane 7 to 9, lane 10 – DNA size markers.
B (DD3) – G1 – lane 6 to 8, G2 – lane 9 to 11, G3 – lane 1 to 5, lane 12 – DNA size markers.
C (c-erbB2) – G1 – lane 1 to 5, G2 – lane 10 to 13, G3 – lane 6 to 9.

mors), we observed *p65* gene expression in cases with small tumor size and in general, with absence of lymph nodes metastases. In this group we also found negative correlation between *p65* and *c-erbB2* genes expression. The later was expressed in cases with diameter of tumor above 2.5 cm and with node-positive status [1].

P65 and c-ErbB2 proteins were also checked in infiltrating ductal breast carcinoma by immunohistochemistry technique. It was established that in poorly-differentiated tumors high c-ErbB2 expression was connected with low P65 expression. The lack of c-ErbB2 expression was combined usually with high P65 oncoprotein level [15]. In the same type of breast carcinoma statistically significant correlation was stated between P65 protein assessed immunohistochemically and estrogen/progesterone receptors estimated immunoenzymatically. It was suggested that high levels of these receptors were accompanied by the presence of P65 protein in breast cancer [14].

The biological similarity between prostate and breast adenocarcinomas permits to suggest that the estimation of *p65* and *c-erbB2* genes expression may be also helpful in prostate cancer detection or in the development of new treatment modalities. It particularly concerns patients with advanced cancer or disqualified for surgical treatment.

In the present study *p65* gene expression was determined by RT-PCR technique in well-, moderately- and poorly-differentiated prostate cancers and compared to expression of other factors connected with prostate cancer. These were well-known oncogene *c-erbB2* (poor prognostic factor) and newly described prostate specific gene *DD3* highly overexpressed in prostate cancer tissue [2]. In all investigated stages of disease straight dependence between *p65* and *DD3* genes expression and opposite dependence between *p65* and *c-erbB2* expression were observed. Opposite dependence was also noted between *DD3* and *c-erbB2* genes expression in well- and moderately-differentiated cancers. It is interesting that in all analyzed cases no simultaneous expression of *c-erbB2* and *DD3* or *p65* genes was observed.

Bussemakers et al [2] noticed, that the regulation of *DD3* expression appeared to be an early event in prostate cancer development. They detected by RT-PCR *DD3* expression in well-, moderately- and poorly-differentiated tumors and observed a trend toward more expression in the latter. *DD3* gene expression was also present in the all analyzed samples of prostate metastatic lesions [2]. However, they carried out experiment on limited panel of prostate cancers. Our study was also based on limited panel of paraffin sections for which only histological classification was available. However, based on our results, we could make a preliminary conclusion that similar *p65* and *DD3* genes expression mainly connected with well-differentiated tumors may suggest better prognosis in older patients excluded from surgical treatment.

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