

HIF-1, GLUT1, endoglin, and BIRC5 expression in urine samples obtained from patients with bladder malignancies – after photodynamic diagnosis (PDD)

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KEY WORDS

bladder cancer ► photodynamic diagnosis ► genetic markers

ABSTRACT

Introduction. Bladder cancer (BC) is a serious medical problem. The high rate of recurrence and progression demands the development of new methods, such as genetic markers, which allow diagnosis and patient follow-up.

Objectives. The aim of this study was to compare expression of HIF-1, GLUT1, endoglin, and BRIC5 in patients without and those with BC. The second group was divided into sub-groups: those without a history of PDD (photodynamic diagnosis) in the diagnostic process and those after PDD.

Methods. Patients with BC were diagnosed using the PDD method using hexaminolevulinic acid (Hexvix®). The expressions of HIF-1, GLUT1, endoglin, and BRIC5 genes were established in urine specimens by real-time quantitative polymerase chain reaction (PCR).

Results. The expressions of all tested genes were higher in the group of patients with BC than in the group without BC. In the group after PDD, a statistically significant overexpression of HIF-1 was observed. In this group, changes were not observed in cases of the other three tested genes.

Conclusions. The differences between the group with PDD and the group without it can be connected with the direct influence of PDD on malignant tissue, which can cause overexpression of HIF-1 only. This is, however, only a hypothesis and needs further study.

cystoscopy with cytology. However, some lesions such as small, flat tumors (Cis) can easily be missed in white light cystoscopy. The most important disadvantage of urine cytology is low sensitivity especially, in low – grade tumors, at the beginning of the disease where sensitivity is a vital factor. The medium sensitivity of cytology is about 35% [2].

To improve standard diagnostic tools like white light cystoscopy and urine cytology, new options have been introduced.

Photodynamic diagnosis (PDD) is a precise method of diagnosis in patients with NIMBC (non muscle invasive BC). It allows diagnosing changes that cannot be seen in white light cystoscopy. In PDD of BC, a hexyl of 5-aminolevulinic acid (5-ALA, Hexvix) is instilled into the bladder. This photosensitizer joins all cells – tumor cells and healthy cells as well. In a short period of time, however, it is removed from normal urothelial cells because of hypoxia, worse blood circulation, and different composition of cell membrane. Hexvix is accumulated in malignant tissue. This leads to different concentration between normal tissue and cancer (mostly like 1:4). Using light with a beam length 405 nm, a red glow of tumor cells can be observed [3]. In 1992 the first PDD with 5-ALA instillation into the bladder was done in man. A few years of PDD has proved a high sensitivity of this method (97%) comparing to standard cystoscopy (72%) [4, 5]. The main advantage of PDD is its sensitivity. The disadvantage is low specificity, 35–66%. False positive results can be an effect of low experience of the operator, scars after TURBT and hyperplasia in urinary bladder that can emit a red glow in PDD. After intravesical therapy false positive results are also observed [6].

Studies to find urine biomarkers, which can allow for better and noninvasive diagnosis of BC, are conducted in many research centers. Comparing to cytology, the sensitivity of genetic tests is higher but specificity does not reach the approved level. Many types of cancer can occur as overexpression of the same mRNA. Sometimes, such as in the case of cyclooxygenase 2 (COX2), the level of expression can be similar in cancerous disease and in inflammation [7].

Hypoxia-inducible factor (HIF-1) is a factor that plays an important role in human oxygen homeostasis. HIF-1 is transcriptional activator. Because of its activity the transcription of many proteins begins, for example VEGF (vascular endothelial growth factor) and erythropoietin. These proteins promote erythropoiesis and angiogenesis, both of which increase oxygen availability. HIF-1 consists of two main units: HIF-1 β unit and one of three HIF- α units (HIF-1 α , HIF-2 α , or HIF-3 α). HIF-1 α is highly expressed and stable in hypoxic conditions and remains at a normal level in normoxic state. High activity on subunit HIF-1 α acts in the transcription of a series of genes that are connected with angiogenesis, cell proliferation, and glucose and iron transport and metabolism. Overexpression of HIF-1 α has been noticed in brain, breast, cervical, ovarian, and uterine cancers [8].

INTRODUCTION

Bladder cancer (BC) is a serious worldwide health problem. It is the most common malignancy of the urinary tract. Morbidity related to BC is 101 men in 1,000,000 and 25 women in 100,000. At the time of BC diagnosis, 75–85% cases are limited to the urothelium (Ta, Cis – *carcinoma in situ*) or to the submucosa (T1). During the first two years after initial treatment, the recurrence rate is 50 to 70% and the progression rate to invasive or metastatic BC is 10–25% of patients [1].

Early and sensitive diagnosis allows the introduction of appropriate treatment and aids in patient follow-up. So far, the gold standard for BC diagnosis in urology has been the use of urethro-

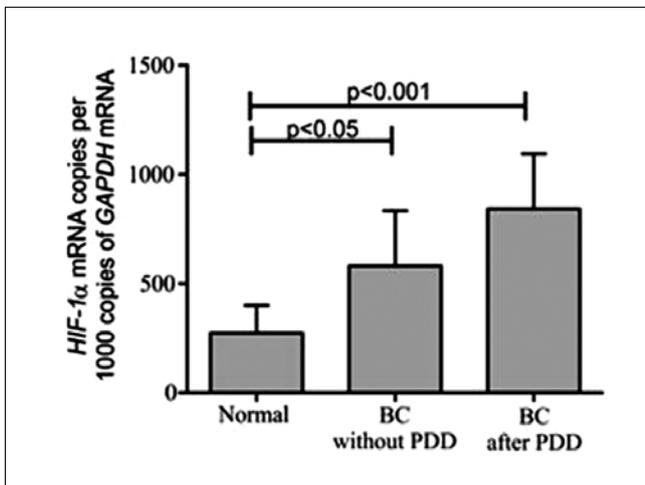


Fig. 1. HIF-1 α mean mRNA expression level in normal bladder tissue, bladder cancer tissue and bladder cancer tissue obtained from patients after PDD.

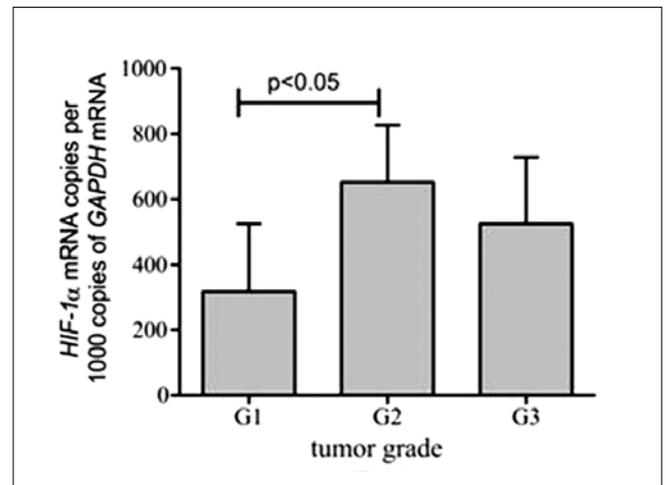


Fig. 2. HIF-1 α mean mRNA expression level in bladder carcinomas; a comparison between subgroups with different tumor grade

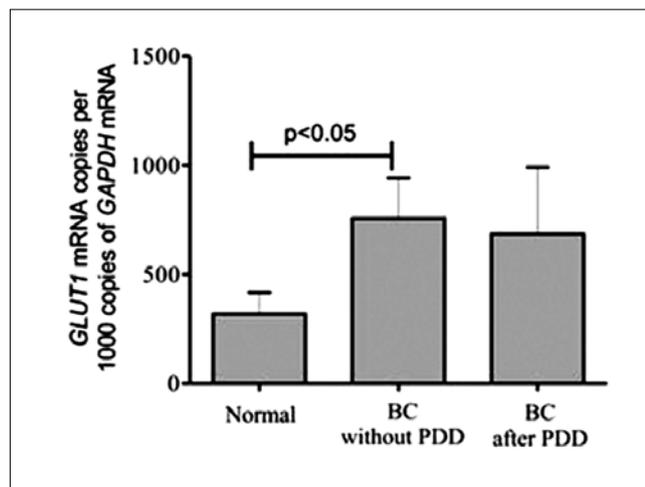


Fig. 3. GLUT1 mean mRNA expression level in normal bladder tissue, bladder cancer tissue, and bladder cancer tissue obtained from patients after PDD.

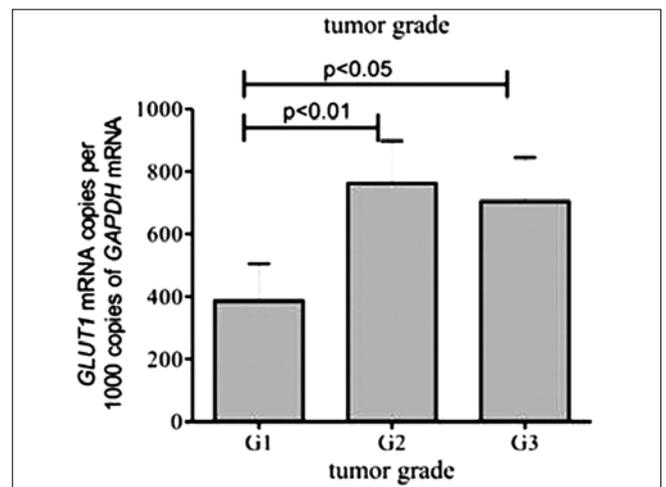


Fig. 4. GLUT1 mean mRNA expression level in bladder carcinomas; a comparison between subgroups with different tumor grade.

In hypoxic conditions, which is a common state in malignant tissue, glucose metabolism switches to the oxygen independent way. Glycogen and its degradation during glycolysis is the most important mechanism of energy production in hypoxia. During that process two molecules of ATP are produced. The HIF-1 target genes are responsible for glycolysis and high glucose uptake. Glucose transporters, GLUT1 and GLUT3, are overexpressed in glycolysis and that activity is regulated by HIF-1 [9, 10].

Endoglin (CD105) is one of the proteins of the transforming growth factor- β (TGF- β) receptor system. Endoglin is overexpressed on the endothelial cells in a few conditions, for example in healing wounds and inflammatory or malignant tissues. Thus, endoglin can be marked as a factor characteristic of activated endothelial cells. Hypoxia stimulates the endoglin expression in endothelium. So far, a high level of endoglin has been common in a few malignant tissues and it correlates with lower survival and the presence of metastases [11, 12].

Survivin, also called baculoviral inhibitor of apoptosis repeat-containing 5 or BIRC5, is a protein that, in humans, is encoded by the BIRC5 gene. BIRC5 is a protein whose high expression can be found in fetal and malignant tissues [13]. In case of BC, the level of BIRC5 is proportionate to the possibility of recurrence, progression, mortality, and staging of the disease [14]. The RT-PCR method (Real Time PCR) allows for BIRC5's mRNA detection. The specificity

of this method is almost 100% and sensitivity is about 64% to 94% (15).

The aim of this study was to estimate the value of urinary genetic markers in BC diagnosis especially after PDD. This study has also tried to answer the question if the genetic markers are really useful in clinical practice.

MATERIALS AND METHODS

A total of 187 urine sediments were collected from 72 healthy people 83 patients with recurrent BC (without PDD), and 32 patients with recurrent BC (with PDD). The median age of patients was 68.12 \pm 9.98 years. Among the patients, those without PDD totaled 57 males and 26 females. In the case of PDD, patients totaled 22 men and 10 women. All BC specimens underwent clinicohistopathological evaluations. The series included 38 cases classified as G1, 22 cases as G2, and 23 as G3 grade. In the group of patients after PDD, there were 16 G1 cases, five G2 cases, and three G3 cases. Eight patients were diagnosed with papillary urothelial neoplasm of low malignant potential (PUNLMP). In two cases, carcinoma in situ (Cis) was diagnosed (one case concomitant with T1G1 cancer, and one case as Cis alone).

For HIF-1, GLUT1, endoglin, and BIRC5 measurements, samples of 30-50 ml of spontaneously voided urine were centrifuged at

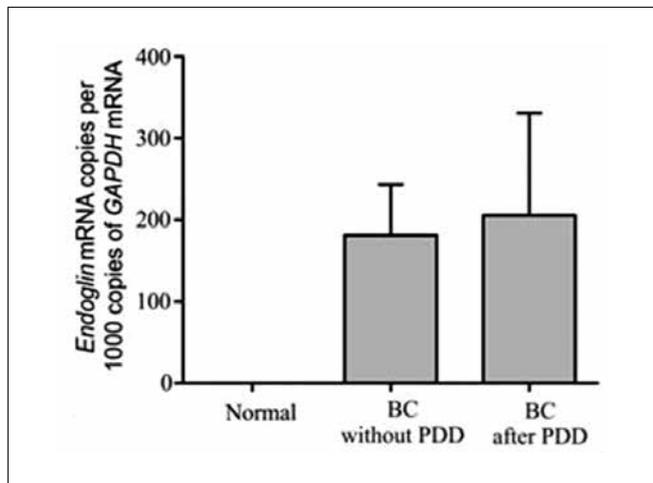


Fig. 5. Endoglin mRNA expression level in normal bladder tissue, bladder cancer tissue, and bladder cancer tissue obtained patients after PDD.

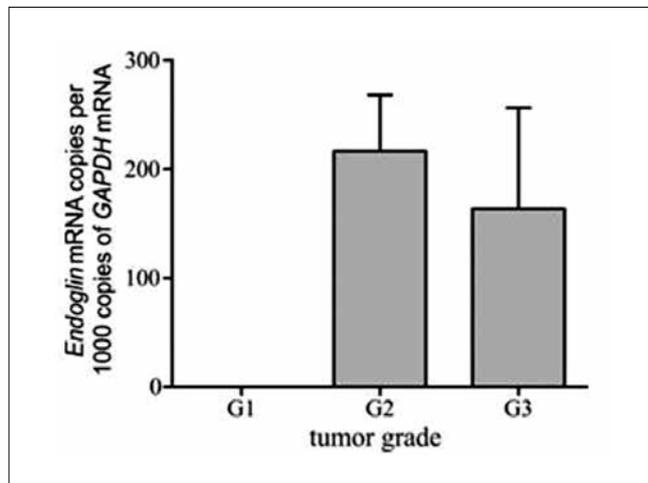


Fig. 6. Endoglin mean mRNA expression level in bladder carcinomas; a comparison between subgroups with different tumor grade.

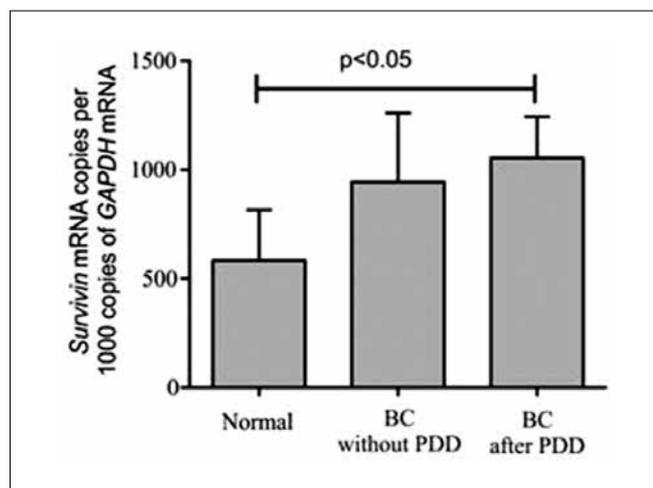


Fig. 7. Survivin mean mRNA expression level in normal bladder tissue, bladder cancer tissue, and bladder cancer tissue obtained from patients after PDD.

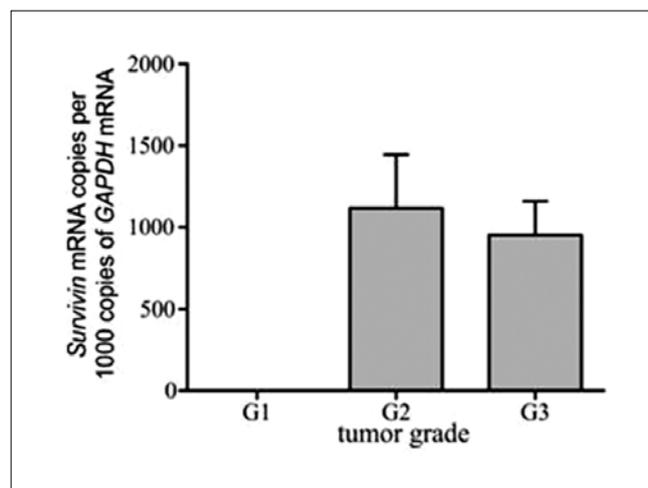


Fig. 8. Survivin mean mRNA expression level in bladder carcinomas; a comparison between subgroups with different tumor grade.

2.500 g for 10 minutes. The supernatant liquid was removed and the cell pellets were washed twice with cold PBS (1x). Finally, the pellets were stored with 1:5 RNA (Ambion) at -20°C until RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using TRI Reagent (Sigma Aldrich) according to the manufacturer's protocol. First-strand cDNAs were obtained by reverse transcription of 1 µg of total RNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas International) following the manufacturer's protocol.

Real-time quantitative PCR

Real-time amplification of the cDNA was performed using TaqMan® Gene Expression Assay (Applied Biosystems) according to the manufacturer's instruction. The fluorogenic, FAM-labeled probes and the sequence specific primers for HIF-1, GLUT1, endoglin, and BIRC5 as well as the internal control GAPDH were obtained as inventoried assays (Hs00383442_m1, Hs00892681_m1, Hs00923996_m1, Hs04194392_s1, and Hs99999905, respectively). The abundance of HIF-1, GLUT1, endoglin, and BIRC5 mRNA in samples was quantified using the ΔCt method. The ΔCt (Ctgene - CtGAPDH) values were recalculated into relative copy number values (number of HIF-1, GLUT1, endoglin, or BIRC5 mRNA copies per 1000 copies of GAPDH mRNA).

Statistical analysis

The statistical analyses were performed using STATISTICA version 9.0 (StatSoft, Poland). Since levels of expression in breast cancer specimens did not show normal distribution (Kolmogorov-Smirnov test), the non-parametrical statistical tests (Mann-Whitney U test, Kruskal-Wallis test, or the Spearman rank correlation test) were applied. A p-value <0.05 was considered statistically significant.

RESULTS

HIF-1 mRNA expression

In case of HIF-1 mRNA expression the results were as follows: in the group of 72 healthy people, the level of HIF-1 was 274.23 ±126.71 in 6 samples; and in the group with BC without PDD (83 patients), the level was 581.12 ±253.26 in 38 cases.

Dividing this group into subgroups according to the grading system: in grade G1, HIF-1 mRNA expression was 317.81 ±207.21 in five patients out of 38; in grade G2, HIF-1 mRNA expression was 652.45 ±174.34 in 16 patients out of 22; and in grade G3, HIF-1 mRNA expression was 524.67 ±203.45 in 17 patients out of 23.

In the set after PDD (16/32), the HIF-1 mRNA expression was on a level of 842.29 ±252.15 (Figs.1, 2).

A statistically significant difference in the level of HIF-1 expression was noticed between:

1. samples from healthy people and samples from patients with BC ($p < 0.05$),
2. samples from healthy people and samples from patients in grade G2 and G3 ($p < 0.05$ in both cases),
3. samples from patients after PDD and samples from healthy people ($p < 0.001$),
4. samples from patients after PDD and patients with BC without PDD in grade G1 ($p < 0.05$), and
5. samples from patients after PDD and patients with BC without PDD in grade G2 and G3 ($p < 0.05$ in both cases).

GLUT1 mRNA expression

GLUT1 mRNA expression in 58 out of the 72 healthy people was on the level 318.67 ± 98.34 and in 60 samples from the 83 patients with BC it was 756.47 ± 186.15 . In this group according to grading system the results were as follows: in grade G1, GLUT1 mRNA expression was 386.41 ± 119.25 in 24 patients out of 38; in grade G2, GLUT1 mRNA expression was 763.18 ± 134.22 in 19 patients out of 22; in grade G3, GLUT1 mRNA expression was 705.15 ± 139.12 in 17 patients out of 23.

In the group diagnosed with PDD, in 23 samples from 32 patients the level of expression was 685.43 ± 305.78 (Figs. 3, 4).

A statistically significant difference in the level of GLUT1 expression was noticed between:

1. samples from healthy people and samples from patients with BC ($p < 0.01$),
2. samples from healthy people and samples from patients with BC in grade G2 ($p < 0.05$ in both cases),
3. samples from patients after PDD and samples from healthy people ($p < 0.001$), and
4. samples from patients after PDD and samples from patients with BC in grade G1 ($p < 0.05$).

Endoglin mRNA expression

There was no endoglin mRNA expression in any samples taken from healthy people. In 14 cases from the 83 patients with BC, the level of endoglin was 181.06 ± 62.19 .

According to the grading system the results were as follows: in all patients with grade G1 expression of endoglin was not observed; in grade G2, endoglin mRNA expression was 216.36 ± 51.72 in three patients from 22; and in grade G3, endoglin mRNA expression was 163.67 ± 92.55 in 11 patients from 23.

In four samples taken from 32 patients after PDD, endoglin level was 205.48 ± 125.18 . (Figs. 5, 6).

There was no statistically significant difference in the level of endoglin expression between the tested groups.

BRIC5 mRNA expression

The results of BRIC5 mRNA expression were as follows: in 7/72 samples from healthy people 583.56 ± 231.98 ; and in 16

cases from 83 samples from patients with BC 943.87 ± 317.56 . According to the grading system: in all patients with grade G1, expression of BRIC5 was not observed; in grade G2, BRIC5 mRNA expression was 1117.98 ± 326.87 in nine patients out of 22; and in grade G3, BRIC5 mRNA expression was 952.59 ± 206.51 in seven patients out of 23.

In the group of patients after PDD, expression of BRIC5 was on the level of 1054.76 ± 189.65 in eight out of 32 cases (Figs. 7, 8).

A statistically significant difference in the level of BRIC5 expression was noticed between:

1. samples from healthy people and samples from patients with BC ($p < 0.01$),
2. samples from healthy people and samples from patients with BC in grade G2 and G3 ($p < 0.01$ in both cases), and
3. samples from patients after PDD and samples from healthy people ($p < 0.001$).

The obtained results from all groups (healthy people, patients with BC without PDD, and patients after PDD) are presented in table 1.

DISCUSSION

In this study we focused on HIF-1 and other genetic markers associated with it. We observed a statistically significant difference in HIF-1 mRNA expression. The lower the differentiation of cancer cells, the higher the level of HIF-1 expression that was found. In the group of patients after PDD, there was overexpression of HIF-1 even when compared to patients with BC without PDD. This, however, can be linked with the mechanism of PDD. The usage of a photosensitizer and light with beam length 405 nm results in additional hypoxic effects that can produce a high expression of HIF-1. Theodoropoulos et al. [16] performed a study searching for the dependence between expression of HIF-1 and prognosis for patients with BC. The authors presented it as a positive correlation between the level of expression and grade of BC cells ($p < 0.009$). Similar results were presented by Deniz et al. [17].

GLUT1 and GLUT3 are the genes that code for the proteins responsible for glucose transport. These genes are activated in hypoxic conditions. In our study the level of expression of GLUT1 increased with the grade of cancer cells. The positive correlation between the expressions of GLUT in urine samples from healthy people and those with BC shows that hypoxic conditions are present in malignant tissue. Hoskin et al. [18] in their study called "GLUT1 – the marker of hypoxia", the expression level correlated with the grade score ($p < 0.001$). Younes et al. [19] claim that the high level of GLUT1 is an independent prognostic factor in BC. The authors postulated that in the case of patients with overexpression of GLUT1, cystectomy should be considered.

One of the effects of a hypoxic condition is angiogenesis. The marker for this process is endoglin. In our study the expression of CD 105 antigen was observed only in the group of patients with BC. Endoglin is an independent prognostic factor and it correlates with metastatic disease and poor survival. These results were presented by Santos et al. [20] and Agrawal et al. [21]. Also, an elevated level of endoglin in blood serum, as well as in urine, can be typical of gastric (Salvensen et al. [22]) and endometrial cancers (Nikiteas et al. [23]).

In our study, overexpression of BRIC5 was discovered in urine samples from patients with BC and compared to BRIC5 level in healthy people and revealed a statistically significant difference. Wang et al. [24], in their study used confocal microscopy to prove that the fluorescent signal was higher in patients with BC than in healthy patients. The authors recommended the usage of this method for early detection of recurrence. Birkhahn et al.

Table 1. The results obtained for genes expression for HIF-1, GLUT1, endoglin, and BIRC5 from all groups (patients without BC, patients with BC with and without PDD)

	HIF-1	GLUT1	Endoglin	BIRC5
without BC n = 72	6	58	0	7
BC n = 83	38	60	14	16
G1 n = 38	5	24	0	0
G2 n = 22	16	19	3	9
G3 n = 23	17	17	11	7
PDD n = 32	16	23	4	8

[25] estimated the clinical effectiveness of 24 genetic biomarkers. BRIC5 proved to be an independent prognostic factor in disease progression.

In malignant tissue, the level of HIF-1, GLUT1, endoglin, and BRIC5 rises according to the grading score. In our material, in the group of patients with BC in whom PDD was not performed, the overexpression of all tested genes was observed. In the case of patients after PDD, only the level of HIF-1 was higher and it was a statistically significant dependence. The expression of GLUT1, endoglin, and BRIC5 in this group was not so high as it could result from the level of HIF-1. The results are different than the natural history of BC because molecular changes in malignant tissue lead to growth not only in case of HIF-1 but also GLUT1, endoglin, and BRIC5. We can try to formulate a hypothesis if the reason for this is PDD itself and its direct influence on cancer cells. Of course further studies need to be performed on a larger group of patients to confirm these preliminary results. Apart from this it is necessary to evaluate the level of expression of HIF-1 before PDD and after PDD.

In cases of BC, genetic markers seem to be useful diagnostic tools. However, considering the present state of knowledge on this subject, further studies are required to define those with the highest specificity and sensitivity for BC. Beside this, the high cost of laboratory equipment and tools and long time of analysis are serious limitations of the method. For now, PDD is in common use in many centers. It allows for early detection of recurrence, which leads to early and proper treatment. Genetic biomarkers can be a supplement for PDD in the future because even the best endoscopic method cannot reveal the molecular cancer changes in a small group of malignant cells.

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