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The *ESR1* and *GPX1* gene expression level in human malignant and non-malignant breast tissues

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Background: The aim of this study was to establish whether the gene expression of estrogen receptor alpha (encoded by *ESR1*) correlates with the expression of glutathione peroxidase 1 (encoded by *GPX1*) in the tumor and adjacent tumor-free breast tissue, and whether this correlation is affected by breast cancer. Such relationships may give further insights into breast cancer pathology with respect to the status of estrogen receptor. Methods: We used the quantitative real-time PCR technique to analyze differences in the expression levels of the *ESR1* and *GPX1* genes in paired malignant and non-malignant tissues from breast cancer patients. Results: *ESR1* and *GPX1* expression levels were found to be significantly down-regulated by 14.7% and 7.4% (respectively) in the tumorous breast tissue when compared to the non-malignant one. Down-regulation of these genes was independent of the tumor histopathology classification and clinicopathological factors, while the *ESR1* mRNA level was reduced with increasing tumor grade (G1: 103% *vs*. G2: 85.8% *vs*. G3: 84.5%; $p<0.05$). In the non-malignant and malignant breast tissues, the expression levels of *ESR1* and *GPX1* were significantly correlated with each other (Rs=0.450 and Rs=0.360; respectively). Conclusion: Our data suggest that down-regulation of *ESR1* and *GPX1* was independent of clinicopathological factors. Down-regulation of *ESR1* gene expression was enhanced by the development of the disease. Moreover, *GPX1* and *ESR1* gene expression was interdependent in the malignant breast tissue and further work is needed to determine the mechanism underlying this relationship.

Key words: estrogen receptor, antioxidant enzymes, gene expression, breast cancer tissue

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Abbreviations: ERs, estrogen receptors; ERα and ERβ, alpha and beta estrogen receptors; GPx's, glutathione peroxidases; ROS, reactive oxygen species; Trx, thioredoxin; TrxRs, thioredoxin reductases

INTRODUCTION

Breast cancer is the most common cancer among women worldwide. The number of diagnosed breast cancer cases among women has continued to rise since the 1980's, and now it constitutes 20% of all malignant tumors. Women aged 45–69 years are at the highest risk of developing breast cancer, and the incidence rate in that group is 50% of all the diagnosed breast cancer cases (Bojar *et al*., 2012).

Pathogenesis and development of breast cancer is often related to estrogen receptors (ERs) and their estrogen ligands. ERs belong to a large family of nuclear receptors that play a role of a transcription factors in cells. There are two types of ERs: alpha (ERα) and beta (ERβ) encoded by the *ESR1* and *ESR2* genes, respectively, and presenting opposite roles. Activation of ERα is associated with proliferation and growth of tumor cells (Au *et al*., 2007; Lin *et al*., 2007), while ERβ promotes apoptosis, suppresses malignant transformation and inhibits growth of tumor cells (Ström *et al*., 2004; Paruthiyil *et al*., 2004; Behrens *et al*., 2007). ERs regulate transcription by direct interaction and binding to DNA (Klinge, 2001) or indirectly through other transcription factors (e.g. AP-1 activator protein-1) (Kushner *et al*., 2000). ERs owe the ability to bind to DNA to specific zinc finger structures located in their DNA-binding domain. One zinc finger is responsible for binding to DNA, while the function of the other one is to stabilize the ER–ER homodimer (Schwabe *et al*., 1993). Zinc fingers are highly susceptible to oxidation, which for example may occur due to accumulation of reactive oxygen species (ROS) (Webster *et al*., 2001). Oxidation of cysteine thiol groups results in the release of zinc ions, causing change in the tertiary structure and loss of the protein ability to bind to DNA (Liang *et al*., 1998).

Cells are protected from oxidizing agents, such as ROS, by antioxidant enzymes: catalase, superoxide dismutases (soluble and extracellular Cu/ZnSOD and MnSOD) and selenoproteins, such as the family of glutathione peroxidases (GPx's) and thioredoxin reductases (TrxRs) which, with glutathione and thioredoxin (Trx), respectively, form an active ROS-reduction system and ensure redox homeostasis in a cell (Schafer & Buettner, 2001; Valko *et al*., 2007). Cu/ZnSOD is the first line of defense against ROS by catalyzing the dismutation reaction of the superoxide anion radical to hydrogen peroxide. TrxR, on the other hand, utilizes NADPH to reduce and activate Trx, as well as other proteins (Mustacich & Powis, 2000). Reduced Trx is an oxidative stress response protein that activates transcription factors in order to alter the expression of peroxiredoxin genes, so that cellular hydrogen peroxide can be diminished (Webster *et al*., 2001). H_2O_2 is also subsequently enzymatically reduced to water by peroxidases, including GPx-1 (encoded by *GPX1*) and catalase. GPx-1 is found in the cytosol, in mitochondria, and also in peroxisomes. It uses reducing equivalents of glutathione to detoxify organic and hydrogen peroxides, and its activity depends on the selenium availability (Lubos *et al*., 2011). It was previously reported by Shultz-Norton (2008) that TrxR and Cu/

ZnSOD are closely related to ERα by being a part of a large ERα-ERE (estrogen response element) protein complex in the nucleus, where they influence regulation of estrogen-responsive genes in the target cell (Rao *et al.*, 2009; Rao *et al.*, 2008). Apart from this, TrxR is involved in maintaining a reduced cellular environment and active transcription factors (Arnér & Holmgren, 2000). That observation provides evidence of its special function in protecting ERα against oxidative agents in the nucleus.

Due to strong antioxidant properties of GPx-1 and high sensitivity of zinc finger structures to ROS, and the presence of Cu/ZnSOD and TrxR in the nucleus protein complex, we decided to investigate the relationship between the *GPX1* mRNA level and the *ESR1* mRNA level in human breast tissue.

More specifically, the differences in constitutive expression levels of the above mentioned genes between the healthy non-malignant and paired tumorous breast tissue specimens, as well as their mutual associations in the healthy and/or tumorous breast tissues, were analyzed. Moreover, the effect of tumor grading and staging on the above mentioned differences and/or associations was determined. The investigated relationships between the expression levels of the targeted genes may give further insights into breast cancer pathology with respect to the estrogen receptor status.

MATERIALS AND METHODS

Patients and tissue specimens The study involved 37 breast cancer female patients aged 44–82 years (mean age 63.1 years; S.D. 9.9 years) undergoing a curative resection surgery without adjuvant chemotherapy or radiotherapy at the Department of Oncology Surgery, Regional Cancer Center in Lodz, Poland, between November 2011 and December 2013.

Of all the enrolled patients, 9 reported themselves as current-smokers, 9 as ex-smokers, 18 as non-smokers and 1 subject did not specify her smoking-status in detail. At the time of the study, none of the subjects received hormonal replacement therapy, but 9 of them declared hormonal treatment for more than 1 year in the past. Detailed characteristics of the investigated group of patients with respect to various clinicopathological factors (the histological grade (G), the primary tumor site (T) and the regional lymph node involvement (N), estrogen receptor (ER) and progesterone receptor (PR)

Table 1. Normalized expression of ESR1 and GPX1 genes in tumorous breast tissue when compared to the paired non–malignant breast tissue. Results of expression analysis stratified by various clinicopathological features of tumors and between–group comparisons.

	N	NRQ	
		ESR1	GPX1
All patients[a]	37	0.872 (0.691–1.154)*	0.931 (0.753–1.080)*
Histopathological classification[a]			
Ductal carcinoma	24	0.921 (0.711–1.172)*	0.947 (0.759–1.149)*
Non-ductal carcinoma	13	0.861 (0.699–1.091)*	0.860 (0.753–0.980)*
Estrogen receptor status[a]			
ER–	8	0.801 (0.732–0.913)	0.889 (0.809–0.933)*
ER+	29	0.934 (0.793–1.000)*	0.897 (0.833–0.982)*
Progesterone receptor status[a]			
PR–	14	**0.793 (0.739–0.925)***	0.901 (0.859–0.943)*
PR+	23	**0.944 (0.814–1.029)**	0.893 (0.832–0.984)*
Her/neu-2 status[a]			
HER2–	34	0.875 (0.739–0.989)*	0.901 (0.835–0.978)*
HER2+	3	0.938 (0.766–0.945)	0.866 (0.639–0.972)
Histological grade[b]			
G1	6	**1.038 (0.944–1.063)**	0.973 (0.898–1.008)
G2	19	**0.858 (0.747–0.966)**	0.893 (0.841–0.972)
G3	12	**0.845 (0.710–0.959)**	0.907 (0.819–0.942)
Tumor size[a]			
T1	18	0.901 (0.717–1.148)	0.777 (0.547–1.060)
T2	18	0.907 (0.693–1.140)*	0.895 (0.767–1.040)*
Lymph node involvement[a]			
N0	23	0.890 (0.721–1.105)*	0.875 (0.751–1.040)*
N1	12	0.915 (0.673–1.228)	0.756 (0.575–1.000)

Data presented as median normalized relative quantity (NRQ) of mRNA copies in paired tissue samples with respective interquartile range (in parentheses). In stratified analysis, NRQ values (i.e. ratio of normalized expression of a gene in tumorous breast tissue to paired non–malignant breast tissue) within all strata were tested for significance by means of the Mann–Whitney U test. Statistically significant NRQs are indicated by asterisks (*$p<0.05$); Between–group comparisons of *ESR1* and *GPX1* expression levels were tested for significances by [a]the Mann–Whitney U test or [b]Kruskal–Wallis test. Statistically significant differences between individual strata are presented **in bold** ($p<0.05$).

status, Her/neu-2 status) and their smoking status, are presented in Table 1. There were no statistically significant differences in the age and BMI between the above mentioned groups (data not shown).

Prior to analysis, a written and informed consent for participation in the study was obtained from each enrolled subject. The study was performed in accordance with the guidelines of the Helsinki Declaration for human research and was approved by the Local Bioethics Committee for Scientific Research (resolution no. 01/2011).

Thirty-seven primary breast tumor specimens (including 25 ductal carcinomas and 12 breast tumors of different types: 5 lobular carcinomas and 7 not specific type carcinomas) with paired non-malignant surrounding breast tissue samples, were removed intra-operationally and placed immediately at –20°C for 24 h, transported to the Nofer Institute of Occupational Medicine and stored at –80°C until further processing.

Gene expression analysis. Total RNA was isolated from the malignant and adjacent non-malignant breast tissue specimens using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Genomic DNA contamination was removed by the on-column digestion with the RNase-free DNase set (Qiagen, Hilden, Germany). Total RNA was further quantified and analyzed with regard to protein content using an Eppendorf BioPhotometer instrument (Eppendorf, Germany) and stored at –80°C. An aliquot of 200 ng of purified RNA was then reverse-transcribed in a 20 µl reaction mixture using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, on an MJ Research BioRad PTC-200 DNA Peltier thermal cycler (MJ Research, Watertown, MA, USA) and the cDNA samples were frozen at –20°C.

Expression levels of the *ESR1* and *GPX1* genes were evaluated by means of the quantitative real-time PCR (qPCR) technique with the BioRad's CFX96 Real Time PCR system (BioRad, Hercules, CA, USA) using an SsoAdvanced SYBR Green Supermix (BioRad, Hercules, CA, USA) and beta-actin (*ACTB*) as the reference gene. Real-time PCR reactions were performed in 10 µl reaction mixture containing 5 ng cDNA, 500 nM of each of the forward and reverse primers, and 1x SsoAdvanced SYBR Green Supermix. The primer sequences (Table 2) were designed by the Beacon Designer 7.0 (PREMIER Biosoft Int., Palo Alto, CA, USA) and cycling conditions comprised of 30 s of polymerase activation at 95°C, followed by 49 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. Products of the PCR reaction were analyzed by means of the Melt Curve technique using the Bio-Rad CFX Manager Software. qPCR efficiencies were calculated using dilutions of 5 randomly selected and pooled cDNA samples. All of the samples were measured in duplicate and the paired malignant and non-malignant breast tissue specimens were always analyzed in one analytical run in order to avoid between-run variations. As confirmed by the initial data analysis, expression of the reference gene (ACTB) was stable under experimental conditions.

Normalized relative expression level (NRQ) for a given gene of interest in the tumorous versus the paired adjacent non-malignant sample and the expression level of genes of interest normalized to the expression level of the housekeeping gene *ACTB* (NQ) was calculated utilizing a method described previously by Pfaffl (Pfaffl *et al.*, 2002), based on each sample's average CT value and each gene's average PCR efficiency.

Statistical analysis Normality of the data was evaluated by the Shapiro-Wilk's W-test. Experimental data showing departure from normality are presented as median and interquartile range (IQR; in parentheses). To test whether differences in the expression levels of genes of interest normalized to the expression levels of the reference gene between the non-malignant and tumorous breast tissues met the criterion of statistical significance, the Mann-Whitney U-test was utilized. The between-group differences in the measured parameters were tested by the Mann-Whitney U test or the Kruskal-Wallis test. Spearman's rank correlation coefficient (R_S) was used to assess simple associations between the variables. Analyses were performed using the STATISTICA 10 software package (StatSoft, Tulsa, OK, USA).

RESULTS

Expression level of ESR1and GPX1 genes in malignant and non-malignant breast tissues

We observed a statistically significant down-regulation of expression level of the *ESR1* and *GPX1* genes in tumorous breast tissue when compared to the adjacent non-malignant one. In the tumorous tissue samples, expression level of the *ESR1* gene was down-regulated when compared to the adjacent non-malignant one by 14.7% (NRQ(*ESR1*)=0.872, IQR: 0.691-1.154; $p<0.05$), whereas the expression level of *GPX1* was reduced by 7.4 % (NRQ(*GPX1*)=0.931, IQR: 0.753-1.080; $p<0.05$) (Table 1).

Expression level of the ESR1 and GPX1 genes in malignant and non-malignant breast tissue according to clinicopathological characteristics

We observed statistically significant differences in down-regulation of expression level of the *ESR1* gene between the group of patients with negative and positive progesterone receptor status (PR-:NRQ(*ESR1*)=0.793, IQR:0.739–0.925 *vs.* PR+:NRQ(*ESR1*)=0.944, IQR:0.814–1.029; $p<0.05$). The expression level of *ESR1* also depended on the tumor grade classification (G). We observed a statistically significant decline in *ESR1* mRNA level with an increasing tumor grade (G1:NRQ(*ESR1*)=1.038, IQR:0.944–1.063 *vs.* G2:NRQ(*ESR1*)=0.858, IQR:0.747–0.966 *vs.* G3:NRQ(*ESR1*)=0.845, IQR:0.710–0.959; $p<0.05$). We did not observe any statistically significant differences in down-regulation of expression level of *GPX1* or *ESR1* between

Table 2. List of the primer sequences used in the real-time PCR assays.

Gene	Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon length (bp)
ESR1	estrogen receptor alpha	aggctttgtggatttgac	ccaagagcaagttaggag	137
GPX1	glutathione peroxidase 1	caaccagtttgggcatcag	tctcgaagagcatgaagttgg	107
ACTB	beta-actin	ccaaccgcgagaagatgacc	ggagtccatcacgatgccag	125

the groups with various histopathological type of tumor, estrogen receptor status, Her/neu-2 status, tumor size or lymph node involvement (Table 1).

Stratified analysis Analysis of the experimental data revealed a statistically significant down-regulation of *ESR1* gene expression in the malignant breast tissue when compared to its non-malignant counterpart, regardless of the histopathological classification of breast cancer (NRQ(*ESR1*)=0.861, IQR:0.699–1.091; $p<0.05$ for non-ductal type and NRQ(*ESR1*)=0.921, IQR:0.711–1.172; $p<0.05$ for ductal carcinoma), as well as in the group of patients without lymph node metastases (N0) (NRQ(*ESR1*)=0.890, IQR:0.721–1.105; $p<0.05$), and larger tumor size (T2) (NRQ(*ESR1*)=0.907, IQR:0.693–1.140; $p<0.05$). Furthermore, decreased expression of *ESR1* in tumorous breast tissue when compared to the adjacent non-malignant breast tissue was observed in the group of patients with positive estrogen receptor status (NRQ(*ESR1*)=0.934, IQR:0.793–1.000; $p<0.05$), negative progesterone receptor status (NRQ(*ESR1*)=0.793, IQR:0.739–0.925; $p<0.05$) and negative Her/neu-2 status (NRQ(*ESR1*)=0.875, IQR:0.739–0.989; $p<0.05$).

Regarding the *GPX1* expression level in the malignant breast tissue when compared to its non-malignant counterpart, we observed a significant down-regulation of this gene's expression among patients with ductal carcinoma (NRQ(*GPX1*)=0.947, IQR:0.759–1.149; $p<0.05$) and non-ductal carcinoma (NRQ(*GPX1*)=0.860, IQR:0.753–0.980; $p<0.05$), as well as in the group of patients without lymph node metastases (N0) (NRQ(*GPX1*)=0.875, IQR:0.751–1.040; $p<0.05$) and larger tumor size (T2) (NRQ(*GPX1*)=0.895, IQR:0.767–1.040; $p<0.05$). Furthermore, a decreased expression of *GPX1* in tumorous breast tissue when compared to the adjacent non-malignant breast tissue was observed in the group of patients with positive and negative estrogen receptor status (ER+: NRQ(*GPX1*)=0.897, IQR:0.833–0.982 and ER–: NRQ(*GPX1*)=0.889, IQR:0.809–0.933; $p<0.05$), positive and negative progesterone receptor status (PR+: NRQ(*GPX1*)=0.893, IQR:0.832–0.984 and PR–: NRQ(*GPX1*)=0.901, IQR:0.859–0.943; $p<0.05$) and negative Her/neu-2 status (NRQ(*GPX1*)=0.901, IQR:0.835–0.978; $p<0.05$).

Down-regulation of *ESR1* and *GPX1* expression in the malignant breast tissue as compared to its non-malignant counterpart also concerns the non- and ex-smoker groups of patients (NRQ(*ESR1*)=0.902, IQR:0.700–1.174 and NRQ(*GPX1*)=0.919, IQR:0.741–1.112; $p<0.05$), and the current smoker group in the case of *GPX1* expression only (NRQ(*GPX1*)=0.924, IQR:0.821–1.045; $p<0.05$) (Table 1).

Correlation between the expression levels of the investigated genes in the malignant and non-malignant breast tissue samples

We found significant positive correlations between normalized relative expression levels (NRQ) of *ESR1* and *GPX1* ($R_S=0.454$, $p<0.05$) (Fig. 1), as well as the normalized expression level (NQ) of these genes in both, the non-malignant ($R_S=0.450$, $p<0.05$) (Fig. 2) and malignant ($R_S=0.360$, $p<0.05$) (Fig. 3), breast tissue samples analyzed separately. We also noted a positive correlation between mRNA level of the *ESR1* gene and estrogen receptor status ($R_S=0.438$, $p<0.05$).

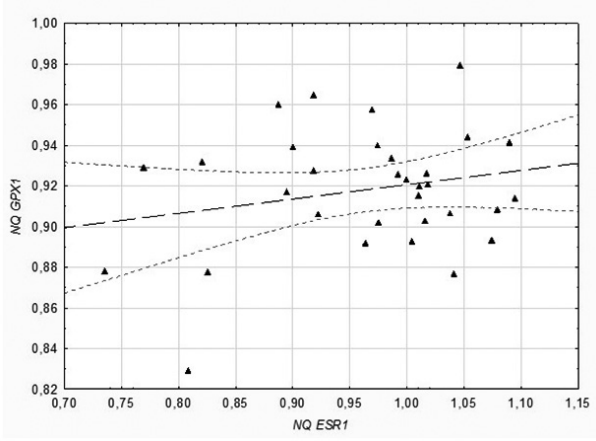

Figure 2. Correlation between the expression level of *ESR1* and *GPX1* normalized to expression level of the housekeeping gene (NQ) in non-malignant breast tissue samples.
Spearman's rank correlation analysis results: $R_s=0.450$; $p<0.05$. (dotted lines: 95% CI for regression line; n=35).

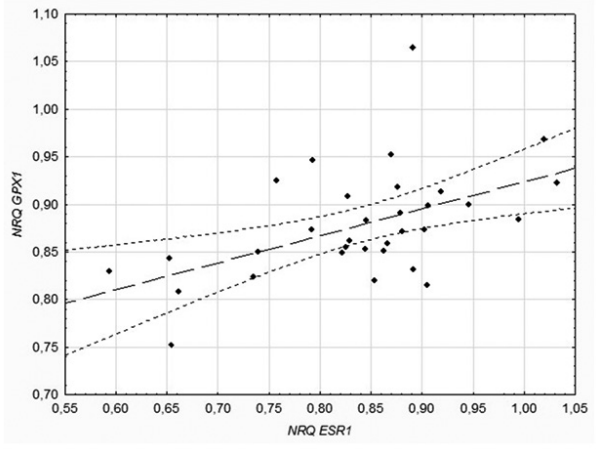

Figure 1. Correlation between the normalized relative expression level (NRQ) of *ESR1* and *GPX1* genes.
Spearman's rank correlation analysis results: $R_s=0.454$; $p<0.05$. (dotted lines: 95% CI for regression line; n=33).

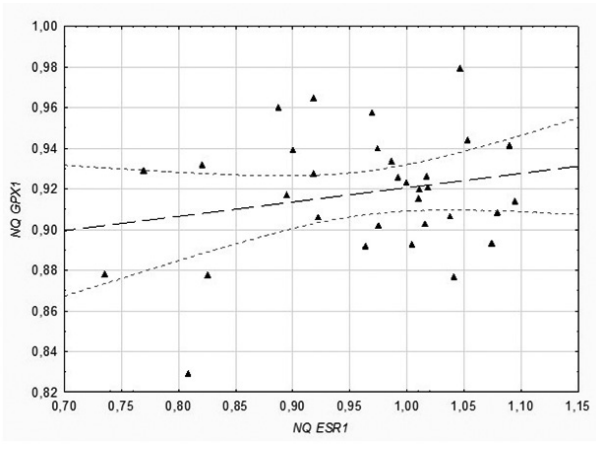

Figure 3. Correlation between the expression level of *ESR1* and *GPX1* normalized to expression level of the housekeeping gene (NQ) in tumorous breast tissue samples.
Spearman's rank correlation analysis results: $R_s=0.360$; $p<0.05$. (dotted lines: 95% CI for regression line; n=35).

DISCUSSION

This study analyzed association between mRNA expression level of the *GPX1* gene and mRNA level of the *ESR1* gene in both, non-malignant and tumorous breast tissues, and evaluated a possible role of such relationship in the development of breast cancer.

The antioxidant defense is very important for maintaining tertiary structure of ERα which has been previously described for human MCF-7 breast cancer cell line in the case of which antioxidant enzymes, like Cu/ZnSOD and TrxR, interact with ERα to form a large protein complex, which migrates to the nucleus following the receptor activation (Schultz-Norton *et al.*, 2008). This observation led us to investigate an association between mRNA level of the *ESR1* gene and *GPX1*, yet another crucial antioxidant enzyme, even though it is not involved in the abovementioned protein complex.

In the study presented here we demonstrated that expression level of the *ESR1* gene was significantly decreased in tumorous breast tissue when compared to the adjacent non-malignant one. This down-regulation of *ESR1* was found to be related to the histological grade of the tumor and decreased significantly with increasing grade of cancer. It is known that receptor status can change as the tumor progresses (Amir *et al.*, 2012). In very high-grade cancers this expression decreases or can even be lost (Huang *et al.*, 2014; Stierer *et al.*, 1993). Such trend was also observed in our study. In patients with the G1 breast cancer, the *ESR1* expression levels did not differ significantly between malignant and paired non-malignant breast tissue, nevertheless, the *ESR1* expression was significantly reduced in malignant breast tissue of the G2 and G3 breast cancer patients. The overall down-regulation of *ESR1* expression observed when all samples were analyzed together can be explained by the structure of the group of patients examined in this study, with G2 and G3 patients accounting for 84% of all patients, which may have significantly influenced the level of *ESR1* expression measured in the whole group of patients. The expression level of *ESR1* varies depending on the progesterone receptor status. We found that the down-regulation of *ESR1* expression in malignant breast tissue when compared to a paired non-malignant one was significantly much more pronounced among patients with a negative progesterone receptor status when compared to those with a positive progesterone receptor status. This observation may be related to the fact that activated estrogen receptor alpha induces transcription of the progesterone receptor (Kastner *et al.*, 1990). Reciprocally, the lack of progesterone receptor may thus be seen as a consequence of reduced activity of ERα, which in turn may results from reduced amount of ERα due to down-regulated expression of *ESR1*.

It is noteworthy that the down-regulation of *ESR1* expression relates particularly to the group of patients with positive estrogen receptor status, shortage of progesterone receptor activity, negative Her/neu-2 status and more advanced/bigger tumors (T2). We observed this down-regulation separately in all of the abovementioned subgroups, but considering the limited size of the study group we were unable to assess whether such *ESR1* down-regulation would be also observed in a group of patients presenting all of these clinicopathological features together. Such an observation would be very interesting and would allow one to answer the question if, from a genetics point of view, the bigger tumors with negative HER/neu-2 status and lacking the progesterone receptor, tend to transform into the triple-negative subtype (TN) of breast cancer (with negative estrogen/progesterone receptor and Her/neu-2 status) or into tumors with decreased expression of ERα (ER-) instead. Tumors transformed into the TN or ER- type are highly undesirable. These types of tumors are more aggressive than other subtypes of breast cancer and are characterized by poorer survival rates. This mainly follows from the fact that TN and ER- tumors are the most difficult ones to be treated because of the lack of benefits from the endocrine therapy and molecular targeted treatments for Her/nau-2 (Qiu *et al.*, 2016).

The level of enzymatic activity and protein concentration of GPx-1 in tumor tissue has been broadly investigated in relation to breast cancer (Tas *et al.*, 2005; Kumaraguruparan *et al.*, 2002; Punnonen *et al.*, 1994; Portakal *et al.*, 2000). Those studies have shown an increased activity in tumor tissue when compared to the normal one. Contrary to immunocytochemical research, we evidenced the down-regulation of *GPX1* mRNA expression in tumorous breast tissue as compared to the paired non-malignant tissue samples. This finding seems analogical to the results of other previous studies that have reported lowered expression of *GPX1* mRNA in colorectal (Nalkiran *et al.*, 2015) and gastric (Min *et al.*, 2012) cancer. In the case of gastric cancer, almost 25% of the cases even lacked the *GPX1* expression. These outcomes were associated with an advanced gastric cancer, lymphatic invasion, aggressiveness of this cancer and poor patient survival (Min *et al.*, 2012). In our study, the level of the *GPX1* transcript was found to be down-regulated independently of the clinicopathological factors.

Down-regulation of the *GPX1* gene in tumorous tissue may lead to decreased GPx-1 protein level and in consequence to reduction of its enzymatic activity. Shortage in the antioxidant defense may lead to increased oxidative stress in cells which may possibly have two mutually opposite effects: excessive levels of ROS may induce the carcinogenesis process and progression of cancer on one hand, but at the same time may be toxic to cancer cells on the other one (Barrera, 2012).

Induction of increased level of ROS in cancer cells is an often used chemotherapeutic approach. Chemotherapeutic agents, such as vinblastine, cisplatin, mitomycin C or doxorubicin, exert their anticancer activity by inducing the ROS-dependent apoptosis of cancer cells (Chiu *et al.*, 2012; Casares *et al.*, 2012; Kim *et al.*, 2012). Hence, declined antioxidant response in cancer cells, due to down-regulated *GPX1*, for example, may be of benefit for further treatment.

In addition to a separate analysis of *ESR1* and *GPX1* transcript levels, we also analyzed the relationship between these two genes. We found a significant positive correlation between the levels of *GPX1* and *ESR1* transcripts, regardless of the tissue type. These results allow us to hypothesize that expression levels of the *GPX1* and *ESR1* genes are mutually inter-related, even though GPx-1 has not been previously identified among proteins involved in the formation of the protein-ERα-ERE complex. Moreover, research studies performed up to date have not defined any interaction mechanism between ERα and GPx-1 at the protein level, as well as any molecular relationships between genes encoding these proteins (e.g. mediated by common transcription factors). Based on the data presented here, we hypothesize that the down-regulation of *GPX1* expression may lead to increased oxidative stress in tumorous breast tissue, which in turn may lead to a decreased expression of the *ESR1* gene. This may, however, be contradictory to a previous study, according to which the oxidative stress induced

by hydrogen peroxide, the main substrate of GPx-1, has only a minimal effect on the ERα level in MCF-7 cells (Tamir *et al*., 2002). On the other hand, interaction between the *GPX1* and *ESR1* gene expression can be opposite. In MCF-7 cells, it was observed that physiological concentration of 17-β-estradiol acts through the membrane-located estrogen receptors on activities of the MAP kinase (MAPK) and NFκB. It was shown that activation of MAPK and NFκB by estrogen, up-regulates expression of the Mn-SOD and GPx-1 antioxidant enzymes (Borras *et al*., 2005). Thus, this aspect definitely remains open and deserves further investigation.

The major weakness of the study concerns a relatively small sample size, which limited the possibility to perform a more advanced statistical analysis of the data. Also, the lack of information about the further course of treatment does not allow us to draw extensive conclusions about the influence of the *ESR1* and *GPX1* genes' expression level on the effectiveness of the therapy.

In summary, our study provides evidence in favor of the significant down-regulation of *ESR1* and *GPX1* expression in malignant breast tissue when compared to the adjacent non-malignant breast tissue. The correlation between these genes was significantly positive regardless of the type of tissue. The extent of down-regulation of *ESR1* in tumorous tissue as compared to the paired non-malignant breast tissue was dependent on clinicopathological factors and was mostly related to the histological grade and progesterone receptor status, while the *GPX1* expression was reduced in tumorous tissue when compared to the surrounding non-malignant one, independently of the clinicopathological breast cancer features.

Based on our data, it seems evident that further research is needed in order to fully elucidate the mechanism underlying association between expression level of the *ESR1* and *GPX1* genes in the malignant and adjacent non-malignant breast tissue.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (resolution no. 01/2011) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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