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Estrogen receptors and markers of oxidative stress in women with breast cancer.

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ABSTRACT

Breast cancer is the most common cancer among women in Poland and worldwide. In Poland, the incidence of this type of cancer has increased twice in the last three decades and, according to the data from 2015, breast cancer makes up 22% of all diagnosed cancers. The number of deaths caused by this disease has gradually been declining since the 1990s and currently accounts for ca. 13% of all deaths caused by cancer.

The main breast cancer risk factors are: age, obesity, exposure to estrogens (for example, in case of early menarche and late menopause or the application of hormone replacement therapy), childlessness, and alcohol consumption.

Due to the special role of estrogens in its etiology, breast cancer is classified as an estrogen-dependent cancer. Estrone and 17-β-estradiol activate estrogen receptors α and β (encoded by *ESR1* and *ESR2* genes, respectively). The studies of human breast cancer cell lines show that these receptors play the opposite roles in the initiation of the neoplastic process. The activation of the estrogen receptor α (ERα) is associated with the proliferation and growth of tumor cells, while the estrogen receptor β (ERβ) promotes apoptosis as well as inhibits the malignant transformation and the growth of tumor cells.

ERα and ERβ play the role of transcription factors in the cell due to the specific structure of the DNA-binding domain. The ligand-activated receptors are dimerized and then they bind to DNA at the ERE site by means of zinc fingers, thereby initiating the transcription of the selected genes. The structure of zinc fingers is very sensitive to reactive oxygen species (ROS), which oxidize zinc ions and thus, destroy the tertiary structure of the protein. In order to prevent this negative effect, antioxidant enzymes, such as zinc/copper superoxide dismutase (Zn/Cu-SOD, encoded by *SOD1*) and thioredoxine reductase (TrxR), are included in the large ERα-ERE protein complex in the nucleus.

Zn/Cu-SOD catalyzes the dismutation of the superoxide anion radical to hydrogen peroxide, while TrxR and Trx are responsible for maintaining the redox environment of the cell. Another significant antioxidant enzyme that is not included in the ERα-ERE complex is glutathione peroxidase

(GPx-1, encoded by *GPX1*), which reduces organic and hydrogen peroxides to alcohols and water using GSH (reduced glutathione) as co-substrate.

The imbalance between the amount of generated ROS and the activity of antioxidant enzymes leads to oxidative stress. This phenomenon is commonly used in anti-cancer therapy. The treatment with some chemotherapeutic drugs that increase the concentration of ROS in cancer cells causes considerable damage to cell structures, which induces the apoptotic process.

The presence of estrogen receptors and the activity of antioxidant enzymes play an important role in the induction and the development of breast cancer and also during the process of treatment. The profile of gene expression of these proteins in various types of biological material can be significant for determining the cause of the disease and for the effectiveness of the treatment.

The *ESR1* genetic variation also appears to be important. Numerous studies have shown that single-nucleotide polymorphisms that can be found in the gene of this receptor can increase or reduce the risk of breast cancer incidence.

The aim of this thesis is to look for the relationship between estrogen receptors α and β, and oxidative stress markers in women with breast cancer, taking into account various additional factors, such as the ERα gene polymorphism and the clinical characteristics of the disease. This was achieved by determining the expression profile of the *ESR1*, *ESR2*, *GPX1*, and *SOD1* genes in malignant and non-malignant breast tissues obtained intraoperatively from female breast cancer patients, and in peripheral blood leucocytes of women with breast cancer and the healthy ones, as well as by determining the relationships between the expression of *ESR1* and *ESR2* genes, and the expression of *GPX1* and *SOD1* genes in the examined biological material. In addition, the impact of the ERα genotype on the risk of breast cancer and its effect on oxidative stress markers in case of women with breast cancer and those from the control group were examined.

The gene expression profile was determined with the use of the Real-Time PCR technique in malignant and non-malignant tissues collected from 37 patients and in peripheral blood leucocytes obtained from 67 women with breast cancer and 71 healthy volunteers. The genetic polymorphisms of the *ESR1* gene were determined in the peripheral blood leucocytes of 223 women with breast cancer and 209 healthy ones, with the use of TaqMan probes. Furthermore, the plasma concentration of TBARS and the activity of GPx-1 and Zn/Cu-SOD in erythrocytes were measured.

A statistically significant decrease in the expression levels of *ESR1* and *GPX1* genes was found in tumorous tissues, as compared to the non-malignant ones. In the case of *ESR1* gene expression the difference was 12.8%, while for the *GPX1* gene 6.9% (NRQ(*ESR1*) = 0.872; NRQ(*GPX1*)=0.971; $p<0.05$). The decrease in the expression of *ESR1* gene was characteristic of all tumors of all histopathological types (ductal carcinoma: NRQ(*ESR1*)=0.921; other: NRQ(*ESR1*)=0.861; $p<0.05$), also of tumors larger than 2 cm in diameter (T2: NRQ(*ESR1*)=0.907; $p<0.05$), tumors with a positive estrogen receptor status (ER+: NRQ(*ESR1*)=0.934; $p<0.05$), tumors with a negative progesterone receptor status (PR-: NRQ(*ESR1*)=0.793; $p<0.05$), and tumors with a

standard HER2 receptor expression level (HER2-: NRQ(*ESR1*)=0.875; p<0.05). It was also observed in patients without lymph nodal metastases (N0: NRQ(*ESR1*)=0.890; p<0.05). As in the case of the *ESR1* gene, a decreased level of the expression of the *GPX1* gene was found in all tumors of all histopathological types (ductal carcinoma: NRQ(*GPX1*)=0.947; other: NRQ(*GPX1*)=0.860; p<0.05) and in tumors with a diameter larger than 2 cm (T2: NRQ(*GPX1*)=0.895; p<0.05). In addition, the expression level of the *GPX1* gene was significantly lower in malignant tissues, regardless of the status of the hormone receptors (ER+: NRQ(*GPX1*)=0.897; ER-: NRQ(*GPX1*)=0.889; PR+: NRQ(*GPX1*)=0.893; PR-: NRQ(*GPX1*)=0.901; p<0.05). This down-regulation was also observed in case of tumors with a standard expression level of HER2 receptor (HER2-: NRQ(*GPX1*)=0.901; p<0.05) and in case of patients with no metastases to lymph nodes (N0: NRQ(*GPX1*)=0.875; p<0.05).

A statistically significant correlation between the expression levels of *ESR1* and *GPX1* genes, and the expression levels of *ESR1* and *SOD1* genes was observed in both examined tissues (malignant tissues: *ESR1/GPX1*: R_S=0.360 and *ESR1/SOD1*: R_S=-0.389; non-malignant tissues: *ESR1/GPX1*: R_S=0.450 and *ESR1/SOD1*: R_S=-0.362; p<0.05). A positive correlation between the expressions of *GPX1* and *SOD1* genes was also found in tumor tissues (R_S=0.436; p<0.05).

Having examined the expression of *ESR1*, *ESR2*, *GPX1* and *SOD1* genes in peripheral blood leucocytes obtained from women with breast cancer and from the healthy ones, I observed that the former have a lower expression of the *ESR1* gene and a higher expression of *ESR2* and *SOD1* genes in leucocytes, as compared to healthy women. The expression of *ESR1* gene was lower in the group of breast cancer patients by 9.3% (NRQ (*ESR1*) = 0.907, p <0.05), while the *ESR2* gene expression was increased by 28.5% (NRQ (*ESR2*) = 1.285; p < 0.05), and the *SOD1* gene by 2.5% (NRQ (*SOD1*) = 1.025, p <0.05), compared to the expression level of these genes in leukocytes of healthy women.

In both examined groups, statistically significant correlations between the expression levels of all tested genes (*ESR1, ESR2, SOD1, GPX1*) were found. In the group of women with breast cancer, statistically significant correlations were: *ESR1/ESR2* R_S=0.3547, *ESR1/GPX1* R_S=0.8905, *ESR1/SOD1* R_S=0.9895, while in the control group: *ESR1/ESR2* R_S=0.5743, *ESR1/GPX1* R_S=0.6518, *ESR1/SOD1* R_S=0.6495 (p<0.05 for all correlations). Furthermore, in the control group there was a correlation between the TBARS concentration and the expression of *ESR2* (R_S=0.3436; p<0.05), *SOD1* (R_S=0.3653; p<0.05), and *GPX1* (R_S=0.3612; p<0.05) genes. The activity of GPx-1 was also significantly correlated to the expression levels of *ESR1* (R_S=0.2556; p<0,05), *ESR2* (R_S=0.3552; p<0.05), *SOD1* (R_S=0.3175; p<0.05) and *GPX1* (R_S=0.3472; p<0.05) genes in the control group. What is more, there was a correlation between the TBARS concentration and the expression of the *ESR2* gene (R_S=0.4126; p<0.05) and between the activity of GPx-1 and that of Zn/Cu-SOD (R_S=0.3975; p<0.05).

In addition, I found that GPx-1 has a significant impact on the TBARS concentration in the group of healthy women who participated in the research (β-coef= 0.278; p<0.05). The expression levels of *ESR1* and *ESR2* genes and the TBARS concentration also have an impact on expression level

of *GPX1* and *SOD1* genes (case: *ESR1/GPX1* β-coef= 0.989; *ESR2/GPX1* β-coef= 0.356; *ESR1* + TBARS/*GPX1* β-coef= 0.991; *ESR2* + TBARS/*GPX1* β-coef= 0.506; *ESR1/SOD1* β-coef= 0.908; *ESR2/SOD1* β-coef= 0.388; *ESR1* + TBARS/*SOD1* β-coef= 0.910; *ESR2* + TBARS/*SOD1* β-coef= 0.536; control: *ESR1/GPX1* β-coef= 0.642; *ESR2/GPX1* β-coef= 0.849; TBARS/*GPX1* β-coef= 0.388; *ESR1* + TBARS/*GPX1* β-coef= 0.599; *ESR2* + TBARS/*GPX1* β-coef= 0.815; *ESR1/SOD1* β-coef= 0.650; *ESR2/SOD1* β-coef= 0.901; TBARS/*SOD1* β-coef= 0.390; *ESR1* + TBARS/*SOD1* β-coef= 0.606; *ESR2* + TBARS/*SOD1* β-coef= 0.874; $p<0.01$).

Moreover, I discovered statistically significant differences in the distribution of *ESR1* rs3798577 genetic variants between the two groups of women in the recessive model of the experiment (case: *ESR1 TT*- 31.4% and *ESR1 CT+CC*- 68.6% vs control: *ESR1 TT*- 22.5% and *ESR1 CT+CC*- 77.5%; $p<0.05$). What is more, I found that the polymorphism of the rs3798577 gene has a statistically significant impact on the risk of breast cancer in the recessive genotype distribution model (OR=0.624; p=0.039).

A statistically significant difference between various genetic variants of the *ESR1* rs9340799 polymorphism was demonstrated for the TBARS concentration in the control group: women with the *AG* genotype have a lower TBARS concentration, as compared to women with the *AA* and the *GG* genotypes (*ESR1 AG*: 2.22 nmol/ml vs *ESR1 AA*: 2.32 nmol/ml and *ESR1 GG*: 2.32 nmol/ml; p=0.004). A lower concentration of TBARS was also observed among women with the *AG* genotype who had breast cancer, as compared to patients with the *GG* genetic variant (*ESR1 AG*: 2.25 (1.80-2.84) nmol/ml vs. *ESR1 GG*: 2.69 (2.02-3.50) nmol/ml; $p<0.05$). Moreover, the study showed that Zn/Cu-SOD was significantly more active in case of women with the *AA* genotype of *ESR1* rs9340799 and the *CT* genotype of *ESR1* rs3798577 polymorphisms, as compared to the control group. The Zn/Cu-SOD activity was significantly higher in the case group compared to the reference group (*ESR1* rs9340799 - *AA*: 6.613 (5.839-7.635) U/mg Hb vs. 6.169 (5.456-6.875) U/mg Hb; p=0.017 and *ESR1* rs3798577- *CT*: 6.578 (5.702-7.438) U/mg Hb vs. 6.299 (5.632-6.785) U/mg Hb; p=0.013).

On the basis of the results that I obtained, the following conclusions can be formulated:

1. There is a strong relationship between the expression level of the *ESR1* and *ESR2* genes and the expression level of the *GPX1* and *SOD1* genes, regardless of the woman's health condition.
2. There is a relationship between the expression level of the *ESR1* and *GPX1* genes and the *ESR1* and *SOD1* genes in both malignant and non-malignant breast tissues obtained from women with breast cancer, and the nature of this relationship is similar in both tissues.
3. Changes in the gene expression profiles of *ESR1*, *ESR2* and *SOD1* genes are evident in peripheral blood leucocytes of women with breast cancer, as compared to the healthy ones.
4. There is a connection between the *ESR1* rs3798577 polymorphism and breast cancer. The *TT* genotype was significantly more frequent in case of women with breast cancer than in the control group, which suggests that it has a role in the development of breast cancer.

5. The genetic variants of the *ESR1* rs3798577 and the rs9340799 polymorphisms had no effect on the activity of GPx-1 and Zn/Cu-SOD and the TBARS concentration in case of women with breast cancer and in the reference group.

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