Original Research Article

**Expression of Glutathione S-Transferase P1 in Women with Invasive Ductal Carcinoma**

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**ABSTRACT**

Breast cancer is the most frequent cancer in women worldwide. Prognostic markers are important for diagnosis, allowing therapeutic strategies to be defined more efficiently. We explored the relationship between the GSTP1 (Ile105Val) polymorphism, tumor GSTP1 protein expression, and clinicopathological parameters in breast carcinoma. Thirty paired of tissue biopsies and blood samples were collected from patients with invasive ductal carcinoma, which attended Oncology Teaching Hospital and Al-Yarmook Teaching Hospital in Baghdad–Iraq from June to September 2014. Tissue biopsies were kept in 10% buffered neutral formalin to prepare paraffin embedded blocks, which used in histopathological diagnosis and Immunohistochemistry. Deoxyribonucleic acid was extracted from blood samples for those patients that were enrolled in this study to detect polymorphism of GSTP1 by RFLP-PCR. GSTP1 polymorphism among breast cancer cases revealed, 40% homozygous (Ile/Ile), 50% heterozygous (Ile/Val) and 10% were homozygous mutant (Val/Val). There were (77%) of samples revealed GSTP1 positive breast tumors and (23%) revealed negative expression. The results showed that high GSTP1 expression was significantly associated with poorer prognostic characteristics including higher age (>50) years, grade III histology, stage III and large tumor size (>2cm), (p< 0.05). This study revealed that high expression of GSTP1 associated with the progression of the disease.

**Keywords**

Breast cancer, Glutathione S-transferase P1, Polymorphism, Immunohistochemistry

**Introduction**

Breast cancer is one of the most common cancers among women worldwide. Recent studies and modern genome-wide association study (GWAS) describe that the associations between breast cancer and polymorphisms in genes are involved in xenobiotic detoxification (Balmukhanov et al., 2013). Glutathione S-transferases (GSTs), a multigene family of phase-II metabolic enzymes, are active in the
detoxification of a wide variety of potentially toxic and carcinogenic electrophiles by conjugating them to glutathione. In mammals the eight classes of GSTs, i.e. alpha (GSTA), mu (GSTM), theta (GSTT), Pi (GSTP), zeta (GSTZ), sigma (GSTS), kappa (GSTK), and omega (GSTO) were classified, based on sequence homology and substrate specificity (Afrandet et al., 2015, Sharma et al., 2014). The GSTP1 gene is located on chromosome 11q13.2, spans 3 kb and contains seven exons, encodes a 210 amino acid protein. GSTP1 represents the major GST expressed in the oesophagus and lungs, with minimal expression in the liver (Hassen et al., 2014).

There are two polymorphic sites in the coding DNA sequence of the GSTP1 gene have been identified, the first polymorphic site is an A→G transition at nucleotide 313, translating an isoleucine → valine substitution at codon 105 (Ile105 → Val105) in exon 5 and second polymorphic site is C→T at nucleotide 341 resulting in replacement of alanine → valine at the amino acid position 114 (Ala114 → Val114) in exon 6. Hence, the human GSTP1 locus comprises of four different alleles: GSTP1*A (wild type Ile 105 → Ala114), GSTP1*B (Val105 → Ala114), GSTP1*C (Val 105 → Val 114) and GSTP1*D (Ile105 → Val114) (Lo et al., 2008). Glutathione S-transferase P1 is involved in the detoxification of base propenalsan metabolizes carcinogenic products such as benzo- (α)-pyrene dial epoxide and acrolein, which are derived from cigarette smoke (Rose-Zerilli et al., 2009). Biochemical studies indicated that GSTP1 Val 105 allele has a lower thermal stability than GSTP1 Ile105 allele, and Val homozygotes had a lower conjugating activity than Ile homozygotes, with heterozygotes displaying intermediate activity. Individuals with at least one Val allele at codon105 of GSTP1 enzyme don’t have ability to break down both chemotherapeutics and the carcinogen found in cigarettes. It was therefore claimed that variation in carcinogen breakdown among individuals forms a foundation for cancer development within humans (Sailaja et al., 2010, Karkucak et al., 2012), and might have an underlying predisposition to cancer when exposed to environmentally derived or endogenously formed GSTP1 substrates. Indeed, the GSTP1 codon 105Val allele was associated with a significantly increased risk of lung, bladder, testicular cancer and breast cancer (Rose-Zerilli et al., 2009). Several studies have reported an association of the GSTP1 (Val/Val) genotype with greater breast cancer risk (Helzlsouer et al., 1998, Gudmundsdottir et al., 2001, Egan et al., 2004), although subsequent studies reported mixed or conflicting results for the GSTP1 (Val/Val) polymorphism with respect to breast cancer (Zhao et al., 2001, Kimeta et al., 2004, Vogel et al., 2004). These inconsistencies in the relations between GSTP1 genetic polymorphism and breast cancer suggest that the effects of the GSTP1 genotype may depend on environmental factors such as cruciferous vegetable intake that are the primary source of isothiocyanates and other glucosinolate derivatives and that are known to induce phase II detoxifying enzymes, including GSTs. It has been found, that GSTP1 protein plays several critical roles in both normal and neoplastic cells. It also regulates cellular signaling through binding to a variety of important signaling proteins, including c-Jun NH2-terminal kinase, Apoptosis signal-regulating kinase 1 (ASK1), and TNF receptor-associated factor 2 (TRAF2), there by regulating their downstream signaling functions. Glutathione S-transferase P1 protein positive can be protecting the normal cells from the adverse effects of carcinogens. Reduction or
loss of GSTP protein expression has been reported to occur mainly by epigenetic mechanism in several forms of cancer, including breast, leading to suggestions that such loss may result in additional genetic damage in cancer cells and accelerated progression of disease. Conversely, patients with GSTP-positive breast cancer cells have shown to be resistant to treatment with chemotherapeutics, such as cyclophosphamide, doxorubicin, 5-fluorouracil, docetaxel or paclitaxel. The results of these studies suggest that GSTP-positive cancer cells neutralize the cytotoxic effects of chemotherapeutic agents by a mechanism similar to that of their normal counterparts against carcinogens (Su et al., 2003; Arai et al., 2008; Yu et al., 2009). For this reason, better predictive and prognosis markers are needed in clinical practice (Thomas and Berner, 2000). Studies of the clinical and molecular characteristics of tumors allow therapeutic strategies to be designed more efficiently and with less toxicity (Gonzalez-ngul0 et al., 2007; Hicks and Kulkarni, 2008; Duffy et al., 2011). Therefore, the expression of antioxidant proteins in tumor cells has been assessed as a predictive and prognosis factor of the response to cytotoxic treatments (Pastore et al., 2003). This is the first study to our knowledge, to give the expression of GSTP1 gene and its association toward clinical pathological parameters in Iraqi women with breast cancer.

Materials and Methods

Materials

Monoclonal mouse anti-Human GSTP1 antibodies were purchased from abcam, (United Kingdom). The antigen–antibody binding was visualized by means of the avidin–biotin complex (ABC-method) using DAB (Diaminobenzidinetetrahydrochloride) as Chromogen. DNA purification kit from Bioneer (Korea), BsmA1 (England bio lab).

Patients samples

This study consisted of 30 patients between 29-74 years. The mean of age was 50.2±12.88 years who were diagnosed with breast cancer (invasive ductal carcinoma) confirmed by histopathological examination and not to neoadjuvant–chemotherapy/radiotherapy, were included in this study. Samples were collected from patients treated in Oncology Teaching Hospital and Al-Yarmook Teaching Hospital in Baghdad– Iraq, between June to September 2014. All blood samples were collected in EDTA tubes taken from patients before surgical operation of breast cancer. Tissue specimens were fixed in 10% buffered formaldehyde and embedded in paraffin.

DNA extraction

The products of DNA extraction were verified by horizontal electrophoresis in 1% agarose. DNA concentrations were measured using Nano drop.

Analysis of the GSTP1 gene polymorphism

The GSTP1 (Ile105Val) gene polymorphism in exon 5 at 105 codon was determined by PCR- RFLP based on the method of Korytina et al., 2005.

The fragment of GSTP1 having the (Ile105Val) polymorphism was amplified using forward primer (F-5- ACCCCAGGGCTCTATGGGAA-3) and reverse primer (R-5 TGAGGGCACAAGAAGCCCCT-3) in a 25 µL PCR reaction containing 5µL (100-
150ng of template DNA, 12.5µL of Hotstar green Master Mix (Promega), 0.6µL of 10pmol of each primer, and 6.3µL of free DNAse distilled water. The PCR cycling conditions include: initial denaturation was carried out for 5 min. at 95 °C followed by 30 cycles at 94 °C for 30 s, Annealing at 60 °C for 30 s, then extension at 72 °C for 30 s, and a final extension was done at 72°C for 10 min. Ten microliters of amplified PCR product was incubated at 55°C for 2 hours with 5 U of BsmA1 (England bio lab) restriction enzyme, and electrophoresis on 2.5% agarose gels. Homozygous alleles wild type (Ile/Ile) yielded a band of 176bp, whereas the homozygous mutant alleles (Val/Val) yielded two bands at 91bp and 85 bp. In the heterozygous mutant alleles (Ile/Val) yielded three bands 176, 91 and 85 bp products (Figure 1).

**Immunohistochemical analysis**

Thirty sections with breast cancer (invasive ductal carcinoma exclusive lymph nodes) of 5μm were cut from paraffin blocks. All paraffin sections were dewaxed in xylene, rehydrated through a series of graded alcohol, placed in citrate buffer (pH 6.0) and submitted to heat retrieval for 35 min. After heating, the slides were allowed to cool at room temperature and washed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Protein block was used for 10 min in order to block nonspecific immunoreactions. Monoclonal mouse anti-Human GSTP1 was diluted 1: 500 in antibody diluents and was applied to all slides for 30 min at room temperature. Detection was performed using advance HRP detection system according to the manufacturer’s instructions, and then slides were visualized using DAB as chromagen. Hematoxylin was applied as a counter stain. The positive control for GSTP1 was a slide containing prostate tissue run simultaneously with the samples according to the manufacturer’s instructions (Figure 2G).

**Evaluation of immunohistochemical staining**

Evaluation of the immunostaining was done with the assistance of a histopathologist. Cells with positive immunostaining gave nuclear and/or cytoplasmic dark brown granules. For evaluation the expression for GSTP1 the number of positive cells was multiplied by the intensity of nuclear and cytoplasmic staining. Evaluation followed the German Immuno Reactive Score (Hass et al., 2006). The following score levels were used: for percentage of positive cells: 0% = 0, 1–10% = 1, 11–50% = 2, 51–80% = 3, >80% = 4. For intensity of staining: negative =0, weak = 1, moderate = 2, strong = 3. The maximum score level was 12 for GSTP1. Score levels 3–12 were defined as positive expression and score levels 6–12 were defined as strong expression in breast cancer with invasive ductal carcinoma.

**Statistical analysis**

Statistical analysis were carried out using SASS program (2012). Chi-square were used to analyze the association between GSTP1 gene expression and variables factors (age, grade, stage, and tumor size). P<0.05 was considered to indicate a statistically significant.

**Results and Discussion**

The presence of polymorphisms in several genes such as the GST family has been associated with an increased risk for developing bladder, head, larynx, breast, skin, colon, stomach, lung, and testicle cancer (Rodríguez et al., 2014). In this
study, the genetic polymorphisms of GSTP1 gene and the expression of this gene in breast cancer were investigated. The genotypic results of GSTP1 were presented in (Table 1 and Figure 1) among 30 patients with breast cancer, 12 (40%) were homozygous for the wild type allele (Ile/Ile), 15 (50%) were heterozygous (Ile/Val) and 3 (10%) homozygous for the mutant allele (Val/Val). GSTP1 expression is one of the known factors which associated with the development of resistance to chemotherapeutics in invasive breast cancer patients. It is widely reported to be down-regulated in cancer cells in high percent of cases of patients with invasive breast cancer (Chaiwun et al., 2011). In this study, IHC method was used to find the expression of GSTP1 gene for 30 tumor samples of patients with breast cancer. The results of immunohistochemistry show there were 23 cases (77%) of samples revealing GSTP1 positive. Ten cases with wild type allele (Ile/Ile) and 13 cases with heterozygous (Ile/Val) allele exhibited positive expression of GSTP1 (Table 1). Among (77%) positive expression of GSTP1 in tumors, there 47% of them exhibiting positive staining intensity score 3–12 (Figure 2D) and 30% of them exhibiting strong positive staining intensity score 6–12 (Figure 2F). Negative expression of GSTP1 was found in 7 cases (23%) of tumors (Figure 2B and Table 1). This finding is in line to that reported by Khabazet al., 2014. The absence of GSTP1 protein in the remaining homozygous wild type allele (Ile/Ile) in breast tumor due to epigenetic modification such as DNA methylation and chromatin remodeling (Vecanova et al., 2011). Haas et al., 2006 reported in their research that GSTP1 expression was consistently weaker in invasive carcinomas than in nonneoplastic mammary glands. Obviously, the ability to express GSTP1 was reduced or lost in the majority of breast cancers. In contrast, GST mu expression was frequently found to be stronger in tumors than in nonneoplastic breast tissue, indicating the capacity of the tumor to enhance GST mu expression. As for GST alpha was detected in only 3% of the tumors and nonneoplastic tissue and thus far less common than otherwise reported. This enzyme seems to be of minor importance for breast cancer progression. GSTP1 is considered to act as a tumor-suppressor gene, leading to tumor growth when it is inactivated. It has been reported that GSTP1 interferes with N-terminal c-Jun kinase signaling. If inactivated, GSTP1 may act as a caretaker gene leading to additional somatic genome alterations that promote tumor growth. GSTP1 binds non-covalently to steroids, allowing it to act as an intracellular buffer to minimize short-term changes in steroid levels. The breast is an important organ of the body which is continuously exposed to these steroids and it is therefore highly likely that estrogens act as endogenous tumor initiators in the breast tissue when GSTP1 is inactivated by promoter methylation. GSTP1 promoter region inactivation by hypermethylation is a common event in cancer and this epigenetic modification is often linked with a loss of GSTP1 expression (Saxena et al., 2012). Saxena et al., 2012 revealed an inverse correlation between GSTP1 methylation and GSTP1 expression. Methylated samples exhibited a loss of gene expression, suggesting that the silencing of the GSTP1 gene by CpG island DNA methylation plays an important role in the development of breast cancer. Moreover, they demonstrated that hypermethylation of GSTP1 occurs more frequently in advanced stage cancer cases than in early disease status cases. As well as they found there was no association between GSTP1 promoter hypermethylation and the GSTP1 genotype.
Table 1: Genotypes and expression of the GSTP1 gene in patients with breast cancer

<table>
<thead>
<tr>
<th>Genotypes of GSTP1</th>
<th>N(%)</th>
<th>Expression of GSTP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive (N)</td>
</tr>
<tr>
<td>Ile/Ile</td>
<td>12(40)</td>
<td>10</td>
</tr>
<tr>
<td>Ile/Val</td>
<td>15(50)</td>
<td>13</td>
</tr>
<tr>
<td>Val/Val</td>
<td>3(10)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>30(100%)</td>
<td>23(77%)</td>
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</table>

Table 2: Expression of GSTP1 and its relation with patients clinical and pathological characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>GSTP1 expression</th>
<th>Intensity</th>
<th>100% for positive cell</th>
</tr>
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<tbody>
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<td>+</td>
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<td>(40.00%)</td>
<td>(40.00%)</td>
<td>(20.00%)</td>
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<td>1</td>
<td>8</td>
<td>6</td>
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<td></td>
<td></td>
<td>(6.67%)</td>
<td>(53.33%)</td>
<td>(40.00%)</td>
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<tr>
<td>Chi-square P-value</td>
<td></td>
<td>8.934 **</td>
<td>5.254 *</td>
<td>9.271 **</td>
</tr>
<tr>
<td>Age, years</td>
<td>N=15</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>&lt;=50</td>
<td>6</td>
<td>6</td>
<td>3</td>
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<td></td>
<td></td>
<td>(40.00%)</td>
<td>(40.00%)</td>
<td>(20.00%)</td>
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<td></td>
<td>&gt;50</td>
<td>1</td>
<td>8</td>
<td>6</td>
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<td></td>
<td>(6.67%)</td>
<td>(53.33%)</td>
<td>(40.00%)</td>
</tr>
<tr>
<td>Chi-square P-value</td>
<td></td>
<td>9.215 **</td>
<td>11.946 **</td>
<td>1.274 NS</td>
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<td>Tumor size, cm</td>
<td>N=7</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>&lt;2</td>
<td>2</td>
<td>4</td>
<td>1</td>
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<td>(28.57%)</td>
<td>(57.14%)</td>
<td>(14.29%)</td>
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<tr>
<td></td>
<td>&gt;2</td>
<td>5</td>
<td>10</td>
<td>8</td>
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<td>(21.74%)</td>
<td>(43.48%)</td>
<td>(34.78%)</td>
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<tr>
<td>Chi-square P-value</td>
<td></td>
<td>9.215 **</td>
<td>11.946 **</td>
<td>1.274 NS</td>
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<tr>
<td>Clinical stages</td>
<td>N=4</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>I</td>
<td>2</td>
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<td>(50.00%)</td>
<td>(50.00%)</td>
<td>(0.00%)</td>
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<td>II</td>
<td>4</td>
<td>4</td>
<td>6</td>
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<td></td>
<td>(28.57%)</td>
<td>(28.57%)</td>
<td>(42.86%)</td>
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<tr>
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<td>III</td>
<td>1</td>
<td>8</td>
<td>3</td>
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<td></td>
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<td>(8.33%)</td>
<td>(66.67%)</td>
<td>(25.00%)</td>
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<tr>
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<td></td>
<td>11.425 **</td>
<td>13.084 **</td>
<td>2.547 NS</td>
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<td>Histological grade</td>
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<td>I</td>
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<td>0</td>
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<td>(50.00%)</td>
<td>(50.00%)</td>
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<td>II</td>
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<td>5</td>
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<td>(22.22%)</td>
<td>(50.00%)</td>
<td>(27.78%)</td>
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<td></td>
<td>III</td>
<td>2</td>
<td>4</td>
<td>4</td>
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<td>(20.00%)</td>
<td>(40.00%)</td>
<td>(40.00%)</td>
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<tr>
<td>Chi-square P-value</td>
<td></td>
<td>13.296 **</td>
<td>14.063 **</td>
<td>4.516 **</td>
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**Figure 1** PCR-RFLP analysis of GSTP1 gene polymorphism using BsmAI restriction enzyme: M: DNA molecular weight marker 25bp. Lane (1,2,3,8,9,12) represent Homozygous wild type (Ile/Ile), lane (5,6,10,11) represent Heterozygous mutant (Ile/Val), lane (4,7) represent Homozygous mutant (Val/Val)

**Figure 2** Images of immunohistochemical staining of GSTP1 in breast carcinoma. Immunostaining is evident in cytoplasm and at the nucleus of neoplastic cells. Figures (A,C,E) represent grade I, II, III respectively before immunohistochemical staining of GSTP1. (B,D,F) represent (negative, positive, strong positive Immunostaining of GSTP1) respectively. (G) represent the positive control (human prostate tissue)
The results of this study show that high GSTP1 expression and intensity were significantly correlated (p<0.05) with poorer prognostic characteristics, including higher age (>50), histological grade (III), stage (III), and tumor size (>2). While the number of positive cell were significantly correlated (p<0.05) with higher age and grade but not with tumor size and stage of disease (Table 2). These results agree with Jardim et al., 2012, which associated the highest GSTP1 expression with high histological levels of invasive ductal carcinomas. Nevertheless, other authors have demonstrated contrary results. Cairns et al., 1992 associated an absence of GSTP1 in tumor tissue with the highest histological grade, and Buser et al., 1997 showed that lower GST levels were associated with more advanced breast cancer. In addition, Haas et al., 2006 linked smaller tumor sizes with high GSTP1 expression. The reason for this difference between our results and those conflicting results may be due to the diversity of GSTP1 assessment methods and the difference in sample size.

Li et al., 2014 found that GSTP1 expression positively correlates with tumor grade. Furthermore, GSTP1 expression was elevated in estrogen receptor-negative breast cancer. This difference may be attributed to the various methods of assessment used and to the sample size and diversity of patients studied.

In vitro studies corroborate the participation of GSTP1 in this resistance. In a mammary carcinoma cell line, the development of resistance to the chemotherapeutic agent doxorubicin was followed by an increase in GSTP1 gene expression (Kalinina et al., 2007). It has also been observed that greater sensitivity to specific cytostatic drugs and ionizing radiation correlates with lower intracellular GSTP1 concentrations in tumor cells (Vibet et al., 2008). On the other hand, Miyake et al., 2012 found the pathological complete response rate for neoadjuvant chemotherapy was significantly higher in GSTP1 negative tumors (80.0%) than GSTP1-positive tumors (30.6%) (P=0.009) among estrogen receptor (ER)-negative tumors but not among estrogen receptor-positive tumors (P=0.267).

References


Li, W., Song, M. 2014. Expression of multidrug resistance proteins in invasive ductal carcinoma of the breast. Oncology Letters. 8: 2103-2109


