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Is it Useful to Determine Glutathione Peroxidase and Thioredoxin Reductase Activities for Comparisons of Malign and Benign Breast Diseases?

[İyi ve Kötü Huylu Meme Hastalıklarının Karşılaştırılmasında Glutatyon Peroksidaz ve Tiyoredoksin Redüktaz Enzimlerinin Aktivitesini Tayin Etmek Faydalı mıdır?]

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ABSTRACT

Objectives: The aim of this study is to investigate thioredoxin reductase and glutathione peroxidase activities in malignant and benign lesions of breast.
Methods: We have investigated thioredoxin reductase and glutathione peroxidase activities spectrophotometrically in 32 patients (breast cancer, fibroadenoma and fibrocystic disease) in malignant and benign lesions of breast. Tissue samples were taken from each patient from both tumor and healthy tissue surrounding the tumor. Enzyme activities in tumor tissues were compared with the healthy peritumoral tissue samples in both benign and malignant lesions.
Results: There was a statistically significant difference for both of the enzyme activities in tumoral tissue samples than healthy tissue samples in both benign and malignant lesions ($p < 0,001$).
Conclusion: We reported that thioredoxin reductase and glutathione peroxidase have been increased activities in patients with tumors of the breast when compared with healthy tissues. This study aimed these enzymes may be a marker in cancerous lesions with respect to breast tumors. Unfortunately higher enzyme levels in both benign and malignant lesions compared with normal tissues shows that these enzymes are not specific for only malign diseases in breast. High enzyme activity levels in breast tumors may be an adaptive response to oxidative stresses through enhanced antioxidant defense systems.
Key Words: Breast cancer, fibroadenoma, fibrocystic disease, thioredoxin reductase and glutathione peroxidase

ÖZET

Amaç: Bu çalışmanın amacı kötü ve iyi huylu meme lezyonlarındaki tiyoredoksin redüktaz ve glutatyon peroksidaz aktivitelerini araştırmaktır.
Yöntem: 32 hastadan (meme kanseri, fibroadenoma ve fibrokistik hastalık) elde edilen kötü ve iyi huylu meme lezyonlarındaki tiyoredoksin redüktaz ve glutatyon peroksidaz aktiviteleri spektrofotometrik olarak ölçüldü. Doku örnekleri her hastadan hem tümör hem de tümörü saran sağlıklı dokudan alındı. Tümör dokularındaki tiyoredoksin redüktaz ve glutatyon peroksidaz aktiviteleri kötü ve iyi huylu lezyonların sağlıklı kısımları ile karşılaştırıldı.
Bulgular: Tümör dokularının tiyoredoksin redüktaz ve glutatyon peroksidaz enzim aktivite düzeylerinde kötü ve iyi huylu lezyonların sağlıklı kısımlarına göre anlamlı bir faklılık bulundu ($p < 0,001$).
Sonuç: Bu çalışmada meme kanseri hastalarında sağlıklı dokularla karşılaştırıldığında tiyoredoksin redüktaz ve glutatyon peroksidaz aktivitelerinin arttığı sonucu ortaya çıkmıştır. Bu çalışmada tiyoredoksin redüktaz ve glutatyon peroksidaz enzimlerinin kanserli lezyonlarda bir belirteç olabileceğini hedefledik. Ancak, normal dokuya göre hem iyi hem de kötü huylu lezyonlarda enzim düzeylerinin yüksek oluşu bu enzimlerin memedeki malign hastalıklar için spesifik olamayacağını göstermektedir. Malign ve preneoplastik tümörlerde enzim aktivite düzeylerinin yüksek olmasının nedeninin oksidatif strese karşı antioksidan savunma sisteminin adaptasyonu olabilir.
Anahtar Kelimeler: Meme kanseri, fibroadenoma, fibrokistik hastalık, tiyoredoksin redüktaz, glutatyon peroksidaz

Introduction

Breast cancer is the third most common cancer and additionally to this high percentage every year approximately one million new cases is adding (1). Breast cancer etiology is multifactorial; hormonal, genetic and environmental factors appear to interplay in the pathogenesis of breast cancer. Breast cancer has been the subject of numerous scientific inquiries, but research has yielded inconsistent results (2). Several pathologic entities are associated with an enhanced risk of breast cancer. A recent report suggests that there is a slight increase in the risk of breast cancer among women more than 50 years of age with benign lesions that are in the lower category of risk: cyst, adenosis, mammary duct ectasia, fibrosis, metaplasia, fibroadenoma, mild-to-moderate or florid hyperplasia without atypia, and papilloma (3). Fibroadenoma is the most frequent lesion of breast which is generally seen in women before 50 years of age. Hormonal status is the primary factor in fibroadenoma (4). It has been reported that an imbalance in the redox status in patients with fibroadenoma (5). Some risk factors associated with breast cancer may exert their effects via generation of reactive oxygen species (ROS), which are interact with and modify cellular protein, lipid, and DNA, which results in altered target cell function and neoplastic transformation (6, 7). Oxidative stress occurs when the balance between the productions of ROS overrides the antioxidant capability of the target cell. The accumulation of oxidative damage has been implicated in both acute and chronic cell injury and may result in the formation of newly initiated preneoplastic cells (7). A highly co-ordinated glutathione pathway was strongly correlated in peritumoral tissues, suggesting that appeared disrupted in breast tumors. Researchers have been suggested that glutathione and glutathione dependent enzymes might be the key point in the occurrence of the resistance and progression in the tumor tissues (8).

Glutathione peroxidase (GPx) and thioredoxin reductase (TR) are crucial antioxidant enzymes which protects the cell from the harmful effects of ROS (9). It has been established that cytosolic GPx, involved in the development various types of cancer such as; breast, lung, head and neck (10). Glutathione peroxidases (EC 1.11.1.9 and EC 1.11.1.12) use reduced glutathione (GSH) to remove reactive oxygen intermediates, especially H_2O_2 and organic hydroperoxides (11). GPx plays a significant role in maintaining the redox status during acute oxidative stress in the cell. Other antioxidant enzymes and GPx are working in harmony to protect the cell from harmful effects various toxic chemicals (12). It has been established that breast cancer cell glutathione content, redox status, detoxification capacity are very significant in cell proliferation and cell resistance to oxidative stress (13). Oxidative stress plays a significant role in initiation, development and progression of many diseases including cancer. We have show the effect of oxidative stress on breast cancer in Figure 1.

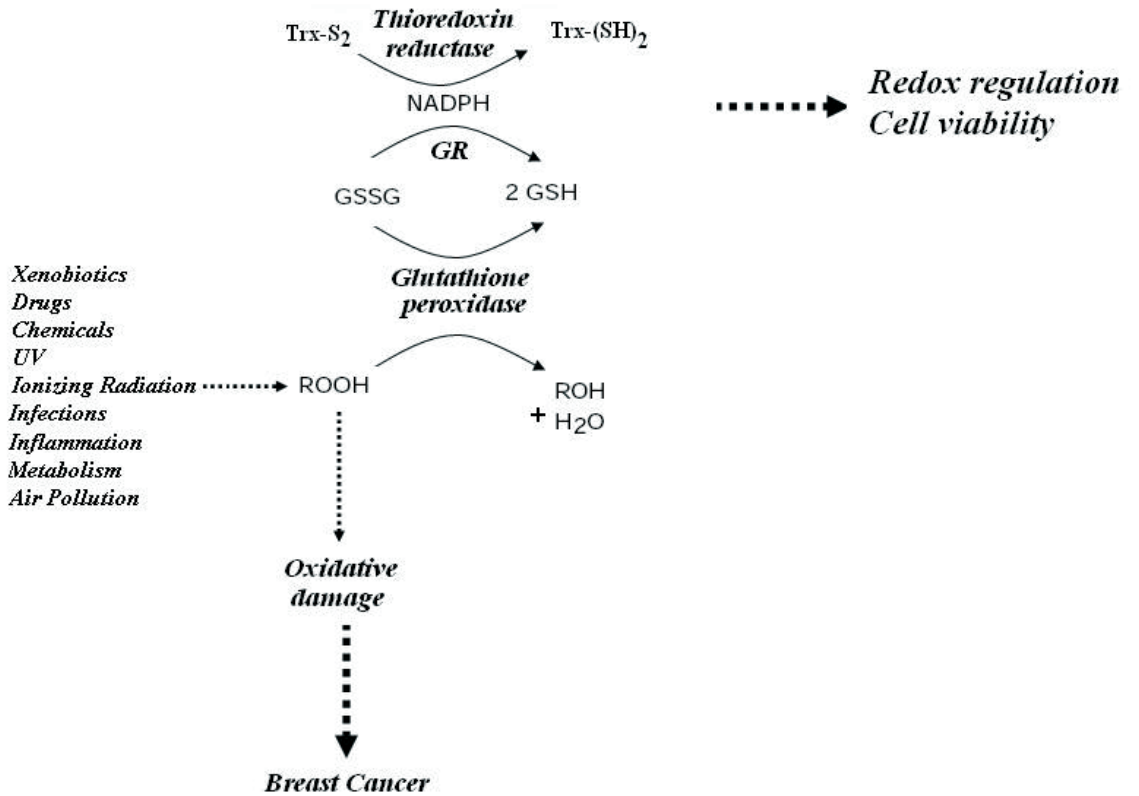

Figure 1. Antioxidative system and breast cancer

Thioredoxin reductase and thioredoxin (Trx) system is involved in many essential cellular processes including, cell proliferation, gene expression and signal transduction, regulation of the redox state, protection against oxidative stress, anti-apoptotic functions, growth factor and co-cytokine effects. In addition these numerous beneficial functions this system plays a critical role in oncogenesis and tumorigenesis (14). Expression and function of TR and the other oxidant and antioxidant enzymes are modulated by various pathological conditions, and therapeutic interventions. It has been showed that activity of TR decreased significantly in diabetic rat heart (15). It has been emphasized that aggressive tumors have a high proliferation capacity, a low apoptosis rate and high metastatic potential (14). According to the data Trx/TR system have central role in several human primary cancers (14, 16) It has been shown that (thioredoxin) Trx expression associated with aggressive tumor growth and decreased patient survival (16). Therefore, Trx/TR system is potential target for anticancer therapy for a wide range of human tumors (14)

Investigations of marker enzymes are very important in cancerous and precancerous tissues. In this study we aimed to examine TR and GPx activities in malignant and benign lesions of breast.

Material and Methods

Patients and methods

The study has been approved by the local ethical committee of the Medicine Faculty. The tissues were taken from Ankara University and homogenization, centrifugation and all the other biochemical anlyses were done in Hacettepe University Biochemistry Department.

Patients:

The studied population included 32 women diagnosed and treated for primary breast cancer and benign tumoral breast disease (8 fibroadenoma, 3 fibrocystic disease, 21 breast cancer) at the department of surgery in Ankara University Faculty of Medicine. All patients were recruited into the study after obtaining their informed consent.

The clinical diagnosis was confirmed by histopathological examinations at the Pathology Department of Ankara University Faculty of Medicine. They were selected for primary breast only, and they were obtained from previously untreated breast disease at the time of surgery.

Tissue collection and sample preparation

Fresh samples of tissues were taken at the time of surgery for biochemical analyses. The longer axis of sample were obtained approximately 1cm for analyzes. The samples didn't affect tumoral specimens for the histological diagnosis requirements. For each patient, tissue samples were taken from both tumor and healthy tissue surrounding the tumor. The tumoral tissues were sent to pathological examination after that other small part was sent to biochemistry department for biochemical analysis. The paired samples were immediately rinsed in + 4°C physiological saline and frozen in liquid nitrogen and stored at - 80°C until enzyme assays. The samples removal time never exceeded 15 min, so was taken care to prevent the samples exhibited histological denaturation.

Homogenization of tissues

The tissues were removed, and after washing with ice-cold sterile physiological saline solution, samples were weighed. Then each sample was homogenized by an ultra turax homogenizer with S18N-10G probe at 22,000 min for approximately 3 minutes with 3 volumes of 50 mM potassium phosphate, buffer pH 7.4. The homogenate was centrifuged at 105 000x g for 60 minutes at 4ºC by using Beckman L7-80 Ultracentrifuge, and supernatants were used for the measurement of enzyme activities. Enzyme activities and protein content were determined spectrophotometrically using an Ultraspec 2100 Pro UV/visible spectrophotometer, (Amersham, Biosciences). All assays were run in triplicate for each supernatant of the tissues. Enzyme activities are expressed as units per mg protein (U/mg protein).

Measurement of Glutathione Peroxidase Activity

Each 5 µl sample was incubated for 10 min at 37° C in a 495 µl incubation mixture containing 50 µl of 100 mM potassium phosphate buffer (pH 7.0), 5 µl of 100 mM GSH, 10 µl of 200 mM EDTA, 5 µl of 400 mM sodium azide, 50 µl of 2 mM NADPH, 320 µl distilled water and 50 µl GR (10 U/ml). After the 10 min incubation period, the reaction was initiated by the addition of 5 µl of 10 mmol/l of H_2O_2. The decrease in the absorbance of the system was measured for 30 s at 340 nm. A similar mixture excluding GSH was used as a blank (17). A unit of activity (U) was defined as the amount of enzyme that catalyzes the oxidation of 1 micromole of NADPH to $NADP^+$ in 1 min under these conditions.

Measurement of Thioredoxin Reductase Activity

Enzyme activity was determined spectrophotometrically by monitoring the NADPH-dependent production of 2-nitro-5-thiobenzoate (extinction coefficient of 13.600 M^{-1} cm^{-1}) at 412 nm and at 37ºC. The sample added to an assay mixture of 100 mM sodium phosphate pH 7.4, 2 mM EDTA, 3 mM DTNB. The reaction is initiated by addition of 0.2 mM NADPH (18). The activities were followed for 60 s. The reaction was linear entire the experimental period. A unit of thioredoxin reductase activity was expressed as 1 micromole of NADPH oxidized to $NADP^+$ in one min under assay conditions.

Measurement of Protein Concentrations

Protein concentrations were determined according to the methods of Bradford, using bovine serum albumin as a standard (19).

Statistics

All values are presented as median, minimum-maximum value. Statistical significance was judged by a p value of < 0.05 and $p<0.01$. Statistical procedures were performed using SPSS for Windows version 15.0 (SPSS

Inc., Chicago, IL, USA). We have used Mann Whitney, Kruskal-Wallis test and Wilcoxon Signed Rank tests were used in the statistical analysis.

Results

We have studied GPx and TR activities in 32 women diagnosed and treated for primary breast cancer and benign tumoral breast disease (8 fibroadenoma, 3 fibrocystic diseases, and 21 breast cancer). All the statistical results of the patients are given in Table 1. None of them showed any distal metastasis at the time of the diagnosis breast cancer, whereas axillary lymph nodes metastases were detected in 17 patients. Most of the malign tumoral samples were invasive ductal carcinoma histological type (2 cases of invasive lobular carcinoma and 2 cases of ductal carcinoma in-situ -DCIS). Some physiological and histological patient details are given in the Table 2. Tissue samples were taken from the peritumoral healthy tissues adjacent to the tumor from all patients. Thus we admired peritumoral tissues the nearest reference to normal tissue. The peritumoral tissues which were taken didn't show imflammatory changes. The tissue samples were taken from macroscopic healthy tissue.

Proliferative features, reported us to range the tumors in grade I; 3, grade II; 10, grade III; 6 patient and 2 samples were diagnosed higher- grade comedocarcinoma. The hormonal status of the samples was assessed by the presence of estrogen (ER) and progesterone (PR) receptors, which was determined by immunohistochemistry. The oncogene c-erbB-2 was appointed in 12 cases in Table 3.

Comparison of GPx activities in healthy peritumoral tissue samples

We divided all paired sample into three groups (fibroadenoma, fibrocystic disease and breast cancer). The results of GPx activities obtained in healthy peritumoral tissue samples were depicted in Table 1. As seen from table 1 there was no significant difference between healthy tissue samples of each groups' GPx levels with statistical analyses (p>0. 05).

Comparison of GPx activities in tumoral tissue samples

We compared GPx activities of tumoral tissue samples. According to the statistical analyses, although there was no difference in GPx activities between fibroadenoma and fibrocystic tissue samples (p>0.05) but there was a significant difference between benign disease and breast cancer (p<0.001) (Table 1).

Comparison of TR activities in healthy peritumoral tissue samples of each groups

We compared TR activities in healthy tissue samples of each groups and we saw that there was no crucial difference with statistical analyses (p>0.05) (Table 1).

Comparison of TR activities in tumoral tissue samples of each groups

On the other hand in the comparison of TR activities in tumoral tissue samples, we obtained unexpected results. While we were expecting some differences, we noticed

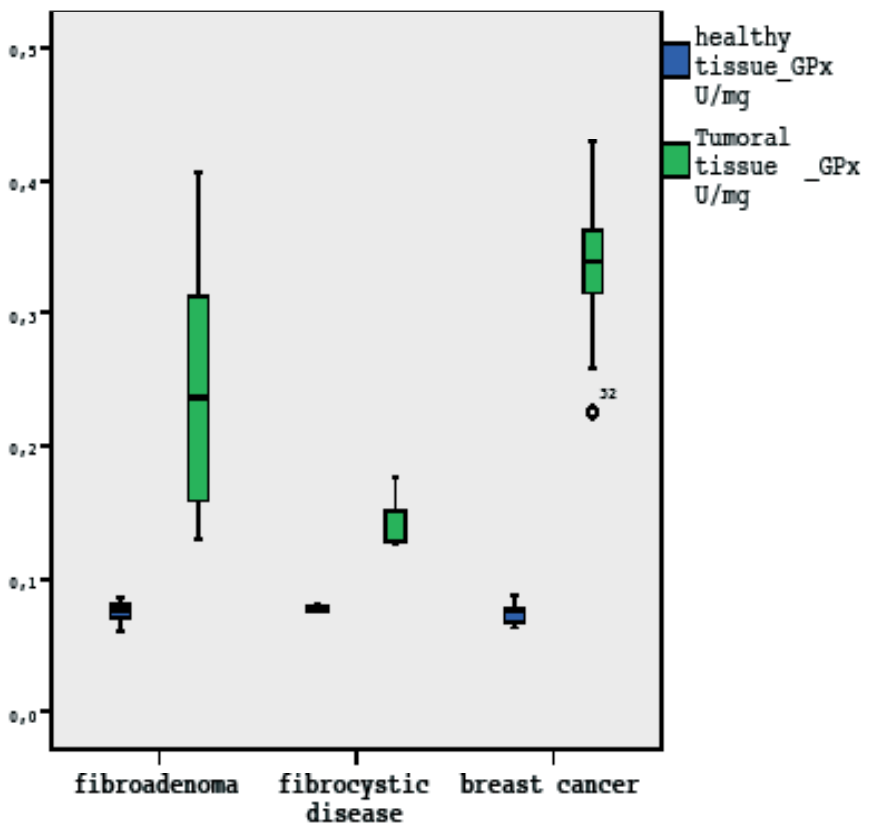

Figure 2 Comparison of GPx activities in tumoral and healthy tissue samples of each group

Table 1. Statistical results of healthy and tumoral tissue samples of fibroadenoma, fibrocystic and breast cancer patients

	Tissue	Groups	N	Median	Minimum	Maximum	Kruskal Wallis Sig.	Compared Groups	Wilcoxon Rank Sig.
GPx U/mg	Healhty tissue	Fibroadenoma	8	0.0770	0.0610	0.0860	p>0.05	Healthy &Tumoral	P<0.05*
		Fibrocystic Disease	3	0.0765	0.0764	0.0810			P>0.05
		Breast Cancer	21	0.0760	0.0764	0.0810			P<0.001**
	Tumoral tissue	Fibroadenoma	8	0.2361	0.1290	0.4060	p<0.001**		
		Fibrocystic Disease	3	0.1280	0.1260	0.1760			
		Breast Cancer	21	0.3390	0.2250	0.4300			
Tr U/ mg	Healhty tissue	Fibroadenoma	8	0.0066	0.0056	0.0078	p>0.05	Healthy &Tumoral	P<0.05*
		Fibrocystic Disease	3	0.0069	0.0067	0.0090			P>0.05
		Breast Cancer	21	0.0071	0.0057	0.0085			P<0.001**
	Tumoral tissue	Fibroadenoma	8	0.0172	0.0119	0.0260	p>0.05		
		Fibrocystic Disease	3	0.0161	0.0135	0.0172			
		Breast Cancer	21	0.0165	0.0129	0.0208			

Table 2. Some physiological and histological characteristics of patients

Variables	Number of patients (n)
Age	
≤40 41-51 51-60 61-70	2 8 8 3
Histopathological types	
Ductal AdenoCa Lobular AdenoCa Others	17 2 2
Histopathological Grade	
Grade I Grade II Grade III High Grade comedocarcinoma	3 10 6 2

Table 3. Some clinical characteristics of patients

Variables	Number of patients (n)
Estrogen receptors positive Estrogen receptors negative	17 4
Progesterone receptors positive Progesterone receptors negative	15 6
c-erbB-2 positive c-erbB-2 negative	12 9

that there was no significant difference between Benign and Malign tissue samples p>0.05 (Table 1).

Comparison of GPx activities in tumoral and healthy tissue samples

There was no difference in fibrocystic groups' healthy and tumoral tissue samples. There was a significant difference in healthy and tumoral tissue samples' GPx activities of breast cancer and fibroadenoma groups (p<0.001 and p<0.05 respectively, Figure 2 and Table 1).

Comparison of TR activities in tumoral and healthy tissue samples

Also in TR activities, we saw the same results like the GPx activities in both tumoral and healthy tissue samples' comparison (figure 3). There was significant difference between healthy and tumoral tissue of fibroadenoma and breast cancer tissue samples p<0.001 (Table 1).

We have investigated glutathione peroxidase in figure 2-3 and thioredoxin reductase in figure 4 and 5 activities in primary breast cancer and benign tumoral breast disease. We have found that glutathione peroxidase and thioredoxin reductase activity levels were elevated in cancer tissue.

Examination of enzymes activity levels with histopathology results in cancer tissue samples

Immunohistochemistry test results for estrogen receptor (ER) positive and negative [ER (+) ER (-)]; progesterone receptor (PR) positive and negative [PR (+) PR (-)]; c-erbB-2(+) and c-erbB-2(-) were compared with the

Table 4. Comparison of GPx and TR enzyme activity levels with estrogen receptor, progesterone receptor and c-erbB-2 positive and negative groups of breast cancer tissue samples

Histopathological property	Statistical analysis	GPx U/mg protein	Tr U/mg protein
ER (–)	Mean± Std. Deviation Number of patients (n) Median(minimum-maximum)	0.329 ± 0.054 4 0.335 (0.258 – 0.389)	0.017 ± 0 .003 4 0168 (0.014-0.208)
ER (+)	Mean± Std. Deviation Number of patients (n) Median(minimum-maximum)	0.335 ± 0.053 17 0.339 (0.225 – 0.430)	0.0167 ± 0.002 17 0,016 (0.013 – 0.207)
	p value	0.897	0.829
PR (-)	Mean± Std. Deviation Number of patients (n) Median(minimum-maximum	0.328 ± 0.048 6 0.338(0.258 – 0.389)	0.018 ± 0.002 6 0.0189(0.014 – 0.020)
PR (+)	Mean± Std. Deviation Number of patients (n) Median(minimum-maximum	0.336 ± 0.055 15 0.339(0.225 – 0.430)	0.016 ± 0.002 15 0.016 (0.013 – 0.019)
	p value	0.791	0.112
c-erbB-2 (-)	Mean± Std. Deviation Number of patients (n) Median(minimum-maximum	0.342 ± 0.050 9 0.357 (0.225 – 0.389)	0.016 ± 0.002 9 0.016(0.013 – 0.019)
c-erbB-2 (+)	Mean± Std. Deviation Number of patients (n) Median(minimum-maximum	0.327 ± 0.054 12 0.330 (0.258 – 0.430)	0.017 ± 0.002 12 0.017(0.014 – 0.021)
	p value	0.464	0.602

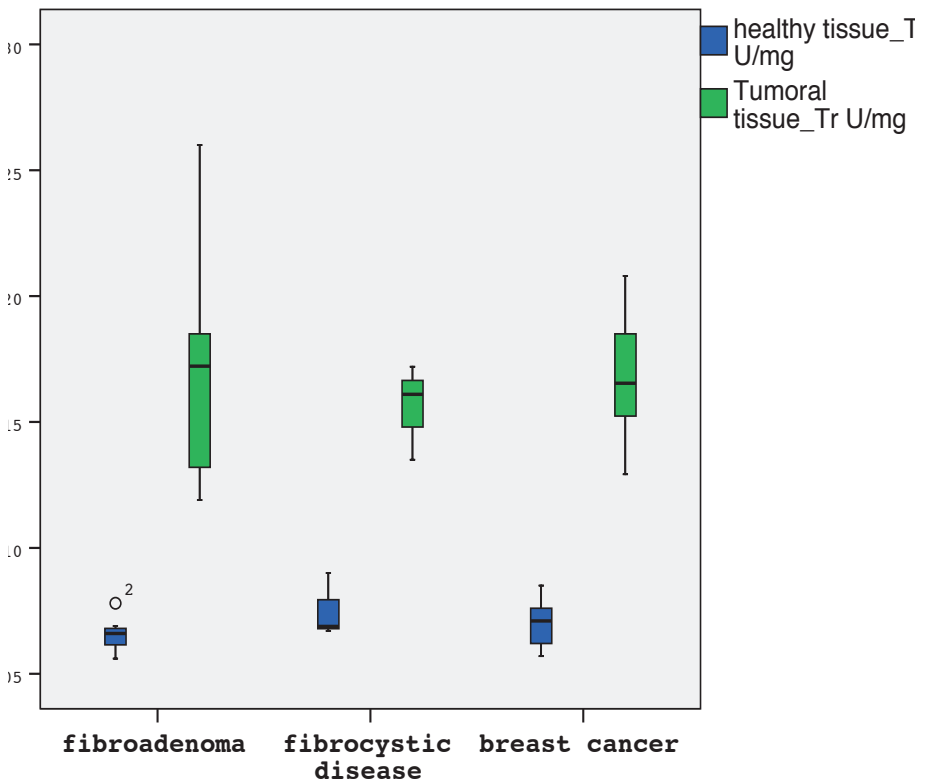

Figure 3 Comparison of TR activities in tumoral and healthy tissue samples of each group

enzyme activity results in Table 4. We want to find any correlation with the hormonal status of the patient's tissue enzyme levels. However we haven't found any correlation with histopathology results in breast cancer according to statistical analysis ($p>0.05$)

Discussion

Breast carcinoma is one of the most common neoplasms in women and is a leading cause of cancer-related deaths worldwide (20). It has been suggested that the common risk factor in the development of breast cancer is the increased lifetime exposure to endogenous or exogenous estrogens (6). A number of genes, including BRCA1 and BRCA2, HER-2/neu and p53, have been linked to breast cancer susceptibility and development (21). However, biological systems have evolved an array of enzymatic and non-enzymatic antioxidant defense mechanisms to combat the deleterious effects of free radicals. Oxidative stress is considered to be implicated in the pathophysiology of breast cancers. Some risk factors associated with breast cancer may exert their effects via generation of ROS, which are recognized to induce oxidative DNA damage and neoplastic transformation (22). To protect themselves from these damaging effects, cells have developed several enzymatic and non-enzymatic mechanisms include glutathione/GSH, GPx, and Trx/TR (23). Family history is accepted risk factor for breast cancer disease, however at the present time; researches are studying on to show the significance of oxidative stress on this disease (22, 24). It has been established that oxidants are important human carcinogens that are mutagenic and may participate in the activation of proto-oncogenes and the inactivation of tumor suppressor genes (25).

Oxidative stress has many affects on cell metabolism such as modification on intercellular communications, protein kinase activity, membrane structure and function, gene expression, and cell growth (7). Trx has been shown to be overexpressed and secreted from several types of human tumor cells compared to levels in the corresponding normal tissue and increased Trx could be a cause resistance to chemotherapy. These include carcinomas of the pancreas, mesothelioma, liver, stomach and uterine cervix (26-32). Oxidative stress activates phosphorylation of transcription factors which may contribute to tumor growth of breast carcinoma cells. In addition, Trx is capable of influencing the function of several transcription factors, such as p53, AP-1, c-fos, c-jun, TFIIIC and NF-kB by regulating their ability to bind to DNA (33- 36).

These findings implicated that TR and GPx are important role in the pathophysiology of breast cancers and prognosis. The reason why cancerous cells exhibit abnormal levels and activities of antioxidant enzymes is unknown. It is not known whether the changes in antioxidant defense observed in cancerous tissues play a role in carcinogenesis, or are formed as results of the disease (37).

In this study we have show that unlike normal tissue, in breast cancer tissue cells, enzyme activities are elevated many times. These results may be due to the cancer cell metabolism. These increased enzyme activities may affect the chemoresistance of these patients to drug therapy.

The present investigation was designed in order to relation of GPx and TR in breast tumors. In our study population of 32 patients GPx and TR enzymes activities appeared to be discretely enhanced in the breast tumors except fibrocystic disease. Our patients were not previously exposed to chemotherapy, so we couldn't investigate the correlations of these activities with a variable as complicated as chemotherapy.

The results could reflect important metabolic changes or detoxification processes against the pro-oxidant events that could have accompanied the promotion or progression of the breast tumors. Nonetheless this study demonstrated the breast tumor cells have apparently strengthened their detoxification capacities which are expected to provide resistance. Additional studies are now requested to bear out our analyses such characteristics increased GPx and TR activities with respect to the breast tumor aggressiveness.

Conclusion

After every research we need to do additional, supplementary and more complicated researches to understand mechanisms involved in this disease. The reason why cancerous cells exhibit abnormal levels and activities of antioxidant enzymes is unknown. The present study reported that there was a statistically significant difference in both TR and GPx levels in the healthy peritumoral tissue samples of benign and malignant lesions. GPx and TR activities are increased many times compared to the normal tissue in all of the patients. According to our hypothesis we were expecting a difference in GPx and TR activities between breast cancer and other tumoral tissues. However we could not find any difference between the malign and benign tissues. By novel supplementary methods this finding might be explained in the future studies. These tests may be helpful when the histopathological tests are inconclusive.

Acknowledgment

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