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Turk J Biol
33 (2009) 89-100
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doi:10.3906/biy-0808-12

Comparison of GST Isoenzyme Expression in Normal and Neoplastic Breast Tissue: Correlation with Clinical and Prognostic Factors

Serpil OĞUZTÜZÜN[1], Mesude İŞCAN[2], Müzeyyen ÖZHAVZALI[3], Serpil DİZBAY SAK[4]

[1]Department of Biology, Faculty of Arts and Sciences, Kırıkkale University, 71450 Yahşihan-Kırıkkale - TURKEY

[2]Department of Biological Sciences, Faculty of Arts and Sciences, Middle East Technical University 06531 Ankara - TURKEY

[3]Department of Mathematics, Faculty of Arts and Sciences, Kırıkkale University, 71450 Yahşihan-Kırıkkale - TURKEY

[4]Department of Pathology, Faculty of Medicine, Ankara University, Ankara - TURKEY

Received: 19.08.2008

Abstract: Glutathione S-transferases in breast tissue play an important role in the susceptibility to the mutagenic effects of chemical carcinogens and in the response of breast tumors to chemotherapy. In this study the immunohistochemical staining characteristics of glutathione S-transferase isoenzymes (alpha, mu, pi, and theta) were investigated in invasive duct carcinomas and in normal breast tissue of 43 patients. The relationships between the expression of the GST isoenzymes and some clinicopathological features were also examined. Diffuse cytoplasmic staining of varying intensity was observed for GST alpha, theta, and pi in normal and tumorous breast tissue in 100% of the samples. In normal epithelium there was a stronger intensity of staining for GST alpha, mu, and pi expression than in invasive tumor tissues ($P < 0.05$); however, it was statistically proven that in normal and tumor epithelial cells there was no significant difference in the GST theta isoenzyme staining scores ($P > 0.05$). In this study significant relationships were observed between microcalcification status and GST mu, between menopause status and GST alpha, and between tumor grade and GST mu expression ($P < 0.05$). The relationships between GST isoenzyme expression, and estrogen receptor status, tumor grade, smoking status, chemotherapy status, parity, patient's age, and hormone therapy status were not statistically significant ($P > 0.05$).

Key Words: Glutathione-S-transferase, breast cancer, immunohistochemistry

Normal ve Tümörleşmiş Meme Dokusunda GST İzozimlerinin Ekspresyonlarının Karşılaştırılması: Klinik ve Prognoz Faktörlerle İlişkisi

Özet: Meme dokusunda bulunan Glutatyon-S-transferazlar, meme tümörlerinin kemoterapiye verdiği cevapta ve karsinojenlerin mutajenik etkilerine duyarlılıkta önemlidir. Bu çalışmada, 43 invasif duktal kanser ve normal meme dokusunda glutatyon-S-transferaz enzimlerinin (alfa, mü, pi ve teta) immünohistokimyasal boyanma özellikleri araştırılmıştır. Ayrıca, GST izozimlerinin salınımları ve hastaların klinik özellikleri arasındaki ilişkiler de tespit edilmiştir. GST alfa, mü, pi ve teta izozimleri, vakaların % 100'ünde normal ve tümörlü meme dokularında farklı boyanma şiddetinde ve yaygın sitoplazmik olarak tespit edildi. GST alfa, mü ve pi enzimleri normal epitelde invasif tümör dokularına göre daha şiddetli boyanma göstermiştir ($P < 0,05$). Ancak, istatistiksel olarak normal ve tümör epitel hücrelerinde GST teta salınımlarında bir fark tespit edilmemiştir ($P > 0,05$). Bu çalışmada, mikrokalsifikasyon durumu ve GST mü; menapoz durumu ve GST alfa; tumor evresi ve GST mü salınımları arasında önemli bir ilişki gözlenmiştir ($P < 0,05$). GST izozimleri ile estrojen reseptör durumu, evre, sigara içimi, kemoterapi, çocuk sayısı, yaş ve hormon terapi durumu arasında istatistiksel olarak bir ilişki bulunamamıştır ($P > 0,05$).

Anahtar Sözcükler: Glutatyon-S-transferaz, meme kanseri, immünohistokimya

* This study was supported by grants from the Scientific and Technological Research Council of Turkey (TÜBİTAK), and the British Council.

Introduction

Breast cancer is the most common malignancy observed in women, with an incidence rate varying between 70 and 100 per 100,000 women (1). The known risk factors, such as higher than average life-time exposure to estrogens and family history of the disease, account for only 30% of the cases (2) and its etiology remains largely unknown. Dietary and/or environmental factors are suggested to play a role in the pathogenesis of breast cancer. The breast is an organ that is particularly susceptible to chemical carcinogenesis due to its anatomical features (2,3). Potent carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), aromatic and heterocyclic amines present in the diet, and occupational and environmental exposure are commonly lipophilic in nature, and so they can be stored and concentrated in the breast fat pad. These carcinogens are thought to induce tumor growth in the mammary gland, following metabolic activation to reactive derivatives that form DNA adducts (4).

PAHs activated by hydroxylation can be detoxified via glutathione conjugation with glutathione-S-transferases (GSTs), which are phase II metabolizing isoenzymes. They facilitate clearance of endogenous hydrophobic compounds, such as hormones, steroids, heme, bilirubin, and bile acids. Furthermore, they are essential for metabolism of environmental carcinogens, drugs, and pesticides, as they catalyze the conjugation of reactive chemical intermediates to soluble glutathione conjugates (5). In all, 7 classes of cytosolic GSTs are recognized in mammalian tissue: alpha, mu, pi, sigma, omega, theta, and zeta (5). In breast cancer polymorphic GST isoenzymes may play a role in tumorigenesis and resistance to chemotherapy (6). Using biochemical measurements, components of the glutathione pathway were observed to increase in breast tumor cells (7,8); however, immunohistochemical studies on the clinical relevance of GST expression in breast cancer have yielded inconsistent results. The absence of GST pi expression in tumors is associated with poor tumoral differentiation (9). Another study reported an inverse relationship between GST pi expression, and estrogen receptor (ER) and progesterone receptor (PR) status (10). No relationships have been observed between GST pi, alpha, or mu expression and response to mitoxantrone chemotherapy in advanced breast cancer cases (11).

In the present study we describe GST (alpha, mu, pi, and theta) isoenzyme expression in normal and neoplastic breast tissue in the same patient group, and discuss their role in conjunction with known breast cancer prognostic factors.

Materials and Methods

Patients

The study included 43 patients with invasive breast carcinomas with an accompanying intraductal component that were treated with lumpectomy, quadrantectomy, or mastectomy at the Liverpool Royal Hospital Trust. For each patient, age, tumor grade, and parity were known. Data concerning 42 patients' history of chemotherapy, 39 patients' smoking status, 43 patients' history of hormone therapy, and 19 patients' estrogen receptor status are shown in Table 2. Two tissue samples were obtained from each patient—1 from the tumor and 1 from macroscopically normal breast parenchyma—and were examined microscopically by a pathology technician.

Histopathology

The tissues were fixed in 10% buffered formalin and embedded in paraffin blocks. Then, 4-mm thick sections were cut and 1 section was stained with hemotoxylin-eosin (H&E) to observe the tissue morphology and tumor grade. A modified Bloom-Richardson grading system was used. For immunohistochemistry, endogenous peroxidase activity was blocked by incubating the sections in 1% hydrogen peroxide (v/v) in methanol for 20 min at room temperature (RT). The sections were subsequently washed in distilled water for 5 min and antigen retrieval was performed in a domestic pressure cooker for 3 min using 0.01 M citrate buffer (pH 6.0). The sections were transferred into 0.05 M Tris-HCl (pH 7.6), which contained 0.15 M sodium chloride (TBS). After washing in water the sections were incubated at RT for 30 min with either normal swine serum (for anti-GST alpha, mu, and pi) (1:50) or normal goat serum (for anti-GST theta) (1:50) diluted in TBS to block nonspecific binding. The sections were then covered with the primary antibodies diluted 1:400 for anti-GST alpha, mu, and theta, and 1:300 for anti-GST pi in TBS at 4 °C overnight. The monoclonal antibody against hGSTT1-1 was a gift from Dr. E. Juronen, Tartu, Estonia. Polyclonal antibodies against hGST alpha, mu, and pi raised in rabbits were purchased from Biotrin International, Limited, (Dublin,

Ireland). After washing in TBS (15 min) sections were incubated at RT for 1 h with secondary antibody (swine-anti-rabbit Ig-biotinylated for anti-GST mu, alpha, and pi, or goat-anti-rabbit Ig-biotinylated for anti-GST theta) diluted 1:200, which was followed by treatment with avidin-biotin peroxidase complex (Dakopatts, Denmark). Diaminobenzidine was used to visualize peroxidase activity in the tissues. Nuclei were lightly counterstained with hematoxylin and then the sections were dehydrated and mounted. Both positive and negative controls were included in each run. Positive controls consisted of sections of normal human liver for GST alpha, mu, and theta, and normal human small intestine for GST pi. TBS was used in place of the primary antibody for negative controls.

Immunohistochemically stained sections were examined under a light microscope while blinded to the clinical information of the patients, and the distribution, localization, and characteristics of immunostaining were recorded. A brown color in the cytoplasm and/or nucleus of the epithelial cells was evaluated as positive staining. Staining in the stromal cells was noted, if present, but was not evaluated further. Staining intensity was graded as 0 if no staining was observed, 1 if weak staining was present, 2 if moderate staining was observed, and 3 if strong staining was present.

For each isoenzyme, staining scores in invasive and normal epithelium were compared statistically. The relationships between GST isoenzyme expression and clinicopathological data were also examined. Wilcoxon signed rank and Spearman's rank tests were used to determine if the relationships were significant.

Results

In all, 43 samples of infiltrating breast carcinoma and normal parenchyma from the surrounding breast tissue of 43 patients were examined. Among the patients, 9 (21%) were under 50 years of age and 34 (79%) were over 50 years; 19% of the patients were in stage 2 and 3, and 81% were in stage 4; 28% of the patients never had a full-term pregnancy, 26% had had 1 child, and 46% had 2 or more children.

Histologic grade of the tumors was determined using slides stained with H&E; 5 cases were grade I, 36 cases were grade II, and 2 cases were grade III invasive ductal carcinoma.

In all the cases cytoplasmic staining for all 4 GST isoenzymes, both in normal and neoplastic compartments, was observed with varying intensity; however, staining for GST mu was usually weaker, patchier, and more heterogeneous in intensity. On the other hand, nuclear staining of the 4 isoenzymes was usually patchy, and different percentages of cells stained positively with varying intensity (Figures 1-4).

GST alpha expression was stronger in normal breast epithelium than in breast tumor epithelium (Table 1, $P < 0.05$); 48% of strong GST alpha expression was in normal epithelium and 82% of the tumors were considered to have moderate GST alpha expression. Similarly, GST pi and mu expression was higher in normal breast epithelium than in breast tumor epithelium (Table 1, $P < 0.05$); 37% of strong GST pi expression was in normal epithelium and 3% of the tumors were considered to have strong GST pi expression ($P < 0.05$). Thus, stronger GST alpha, mu, and

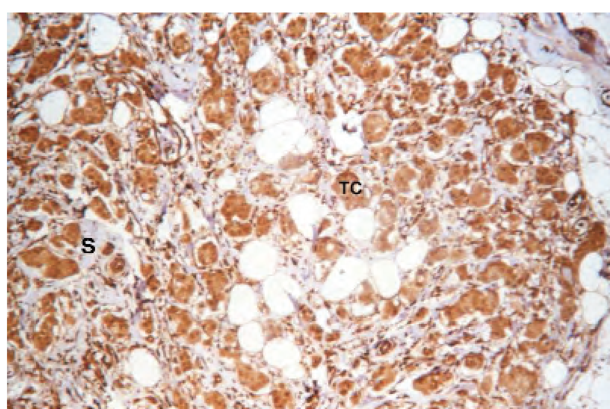

Figure 1. Grade II invasive duct carcinoma. Cytoplasmic staining score was 5 and nuclear staining score was 5 in this case (GST pi , 80). Stroma (S), tumor cells (TC).

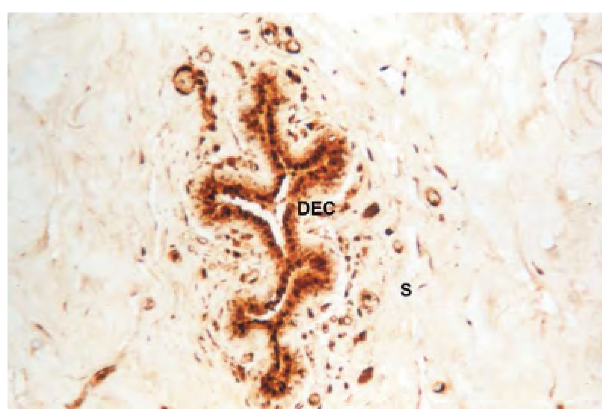

Figure 2. Normal breast tissue. Cytoplasmic score was 5 and nuclear score was 6 in this case (GST alpha, 80). Ductal epithelial cells (DEC), stroma (S).

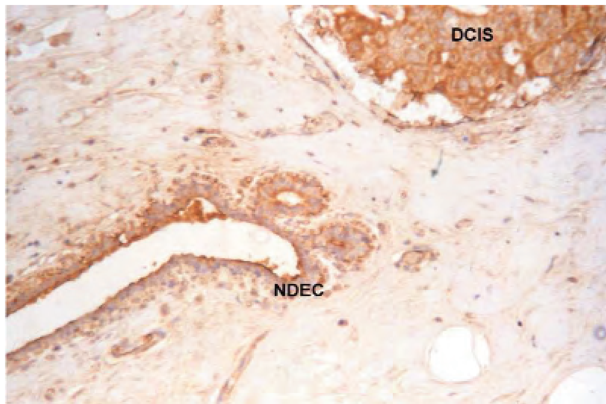

Figure 3. Normal ductal epithelial cells (NDEC) and ductal carcinoma in situ (DCIS), with weak cytoplasmic staining and negative nuclear staining of epithelial cells (GST mu, 80).

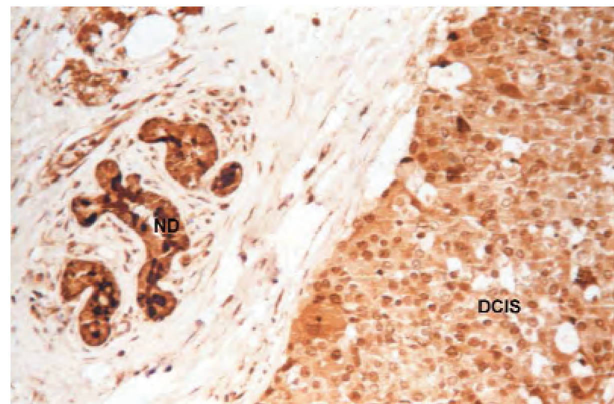

Figure 4. Normal duct (ND) and ductal carcinoma in situ (DCIS). Nuclear staining score of normal ductal epithelial cells was 5 and nuclear score of in situ carcinoma cells was 5 in this case (GST alpha, 80).

pi expression was observed in normal epithelium than in breast tumor epithelium; however, the difference in GST theta expression between normal and tumoral epithelium was not significant ($P > 0.05$).

In general, for a certain case, for a certain GST isoenzyme, there was little variation in the cytoplasmic GST staining intensity of individual tumor cells; however, in a few cases the cytoplasmic staining score of individual tumor cells varied (Figure 5). Generally, stronger cytoplasmic staining was observed in normal cells than in tumor tissue (Figure 5).

The clinical and pathological characteristics of the breast tumors, along with the GST alpha, mu, pi, and theta levels of expression are shown in Table 2. Spearman's rank test results show that there were weak positive associations between GST alpha, pi, and parity, between GST theta and age, between GST pi and theta, and menopausal status, between GST pi and mu, and tumor stage, between GST alpha, mu, and pi, and chemotherapeutic status, between GST pi and receptor status, and between GST pi and smoking status. There were weak negative associations between GST mu and theta, and parity, between GST alpha, mu, and pi, and age, between GST alpha and mu, and menopausal status, between GST alpha and theta, and tumor stage, between GST theta and chemotherapeutic status, between GST alpha, pi, and theta, and hormone therapy status, between GST alpha, mu, and theta, and receptor status, between GST alpha, mu, and theta, and smoking status, and between GST alpha, mu, pi, and theta, and microcalcification status. However, there was no relationship between GST mu and hormone therapy status.

The expression of GST alpha, mu, pi, and theta, and menopause status was compared in human breast tumors; 67% of strong GST alpha expression was pre-menopause status, and 83% of moderate GST alpha expression was post-menopause status. Thus, there was a significant relationship between GST alpha expression and menopausal status in human breast tumors ($P < 0.05$).

Similarly, there was a relationship between GST mu expression, and microcalcification status and tumor grade (Table 2, $P < 0.05$); 25 (61%) patients with moderate GST mu expression had a positive microcalcification status, whereas 2 (100%) patients with weak GST mu tumor expression had a negative microcalcification status.

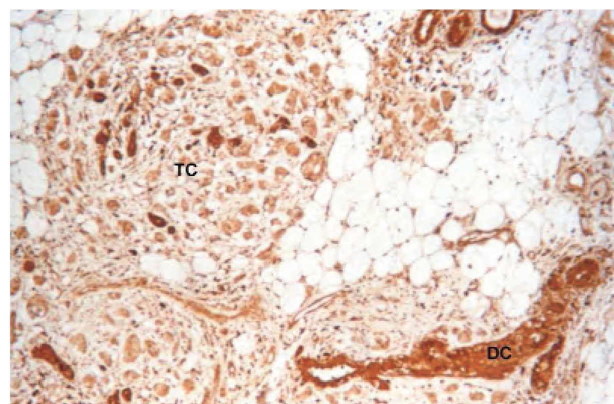

Figure 5. Infiltrating duct carcinoma. There is diffuse staining in the tumor cells and normal epithelial cells with heterogeneous intensity. Cytoplasmic score of normal ductal epithelial cells (DC) was 6 and cytoplasmic score of tumor cells (TC) was 4 (GST theta, 40) in this case.

Table 1. Relationships between the expression of GST alpha, mu, pi, and theta in normal epithelium and in breast tumor epithelium.

		GST alpha			Total	GST mu			Total	GST pi			Total	GST theta			Total
		Weak	Moderate	Strong		Weak	Moderate	Strong		Weak	Moderate	Strong		Weak	Moderate	Strong	
Normal epithelium	n	4	10	13	27	8	12	7	27	6	11	10	27	2	12	13	27
	%	15%	37%	48%	100%	30%	44%	26%	100%	22%	41%	37%	100%	7%	44%	48%	100%
Tumor epithelium	n	4	54	8	66	15	36	15	66	22	42	2	66	2	29	35	66
	%	6%	82%	12%	100%	23%	55%	23%	100%	33%	64%	3%	100%	3%	44%	53%	100%
Total	n	8	64	21	93	23	48	22	93	28	53	12	93	4	41	48	93
	%	9%	69%	23%	100%	25%	52%	24%	100%	30%	57%	13%	100%	4%	44%	52%	100%
		P = 0.005*				P = 0.000*				P = 0.000*				P = 0.164			

*Correlation is significant at the 0.05 level (2-tailed).

Table 2. Relationships between the expression of GST alpha, mu, pi, and theta in normal epithelium and in breast tumor epithelium.

Characteristic[a]			GST alpha			Total	GST mu			Total	GST pi			Total	GST theta			Total
			Weak	Moderate	Strong		Weak	Moderate	Strong		Weak	Moderate	Strong		Weak	Moderate	Strong	
Age (years)	≤ 50	n	1	5	3	9	1	6	2	9	2	7	0	9	0	5	4	9
		%n	11	56	33	100	11	67	22	100	22	78	0	100	0	56	44	100
	> 50	n	2	30	3	35	10	18	7	35	14	19	2	35	1	15	19	35
		%n	6	86	9	100	29	51	20	100	40	54	6	100	3	43	54	100
	Total	n	3	35	6	44	11	24	9	44	16	26	2	44	1	20	23	44
		%n	7	80	14	100	25	55	20	100	36	59	5	100	2	45	52	100
			r_s = −0.200, P = 0.198 > 0.000				r_s = −0.161, P = 0.303 > 0.000				r_s = 0.279, P = 0.070 > 0.000				r_s = −0.179, P = 0.251 > 0.000			
Tumor Grade	I	n	0	5	0	5	1	3	1	5	2	2	1	5	0	2	3	5
		%n	0	100	0	100	20	60	20	100	40	40	20	100	0	40	60	100
	1I	n	3	28	6	37	8	20	8	36	13	22	1	36	1	17	18	36
		%n	8	76	16	100	22	56	22	100	36	61	3	100	3	47	50	100
	III	n	0	2	0	2	1	1	0	2	1	1	0	2	0	0	2	2
		%n	0	100	0	100	50	50	0	100	50	50	0	100	0	0	100	100
	Total	n	3	35	6	44	10	24	9	43	16	25	2	43	1	19	23	43
		%n	7	80	14	100	23	56	21	100	37	58	5	100	2	44	53	100
			r_s = 0.032, P = 0.838 > 0.000				r_s = −0.163, P = 0.297 > 0.000				r_s = −0.104, P = 0.507 > 0.000				r_s = −0.066, P = 0.672 > 0.000			
Chemotherapy Status	U	n	0	6	1	7	1	4	2	7	2	5	0	7	0	4	3	7
		%n	0	86	14	100	14	57	29	100	29	71	0	100	0	57	43	100
	T	n	3	27	5	35	9	19	7	35	13	20	2	35	1	16	18	35
		%n	9	77	14	100	26	54	20	100	37	57	6	100	3	46	51	100
	Total	n	3	33	6	42	10	23	9	42	15	25	2	42	1	20	21	42
		%n	7	79	14	100	24	55	21	100	36	60	5	100	2	48	50	100
			r_s = 0.71, P = 0.0650 > 0.000				r_s = 0.175, P = 0.273 > 0.000				r_s = 0.019, P = 0.907 > 0.000				r_s = −0.144, P = 0.369 > 0.000			
Hormone Therapy Status	U	n	2	8	1	11	2	7	2	11	6	5	0	11	1	5	5	11
		%n	18	73	9	100	18	64	18	100	55	45	0	100	9	45	45	100
	T n	1	26	5	32	9	16	7	32	9	21	2	32	0	15	17	32	
		%n	3	81	16	100	28	50	22	100	28	66	6	100	0	47	53	100
	Total	n	3	34	6	43	11	23	9	43	15	26	2	43	1	20	22	43
		%n	7	79	14	100	26	53	21	100	35	60	5	100	2	47	51	100
			r_s = −0.206 P = 0.190 > 0.000				r_s = 0.000, P = 1.000 > 0.000				r_s = −0.227, P = 0.147 > 0.000				r_s = −0.066, P = 0.677 > 0.000			

Table 2. (Contunied).

Characteristic[a]			GST alpha			Total	GST mu			Total	GST pi			Total	GST theta			Total
			Weak	Moderate	Strong		Weak	Moderate	Strong		Weak	Moderate	Strong		Weak	Moderate	Strong	
ER Status	(−)	n	1	8	0	9	3	5	1	9	2	6	1	9	0	5	4	9
		%n	11	89	0	100	33	56	11	100	22	67	11	100	0	56	44	100
	(+)	n	0	8	2	10	1	6	3	10	3	7	0	10	1	3	6	10
		%n	0	80	20	100	10	60	30	100	30	70	0	100	10	30	60	100
	Total	n	1	16	2	19	4	11	4	19	5	13	1	19	1	8	10	19
		%n	5	84	11	100	21	58	21	100	26	68	5	100	5	42	53	100
			r_s = −0.394 P = 0.095 > 0.000				r_s = −0.162, P = 0.506 > 0.000				r_s = 0.169, P = 0.490 > 0.000				r_s = −0.258, P = 0.285 > 0.000			
Cigarette Smoking Status	non-smoker	n	1	12	2	15	4	8	3	15	3	11	1	15	0	8	7	15
		%n	7	80	13	100	27	53	20	100	20	73	7	100	0	53	47	100
	smoker	n	1	19	4	24	5	13	6	24	9	14	1	24	1	11	12	24
		%n	4	79	17	100	21	54	25	100	38	58	4	100	4	46	50	100
	Total	n	2	31	6	39	9	21	9	39	12	25	2	39	1	19	19	39
		%n	5	79	15	100	23	54	23	100	31	64	5	100	3	49	49	100
			r_s = −0.056, P = 0.738 > 0.000				r_s = −0.055, P = 0.744 > 0.000				r_s = 0.240, P = 0.146 > 0.000				r_s = −0.006, P = 0.973 > 0.000			
Tumor Grade[c]	II, III	n	0	7	1	8	3	5	0	8	4	4	0	8	0	2	6	8
		%n	0	88	13	100	38	63	0	100	50	50	0	100	0	25	75	100
	IV	n	3	28	5	36	8	19	9	36	12	22	2	36	1	18	17	36
		%n	8	78	14	100	22	53	25	100	33	61	6	100	3	50	47	100
	Total	n	3	35	6	44	11	24	9	44	16	26	2	44	1	20	23	44
		%n	7	80	14	100	25	55	20	100	36	59	5	100	2	45	52	100
			r_s = −0.073, P = 0.642 > 0.000				r_s = 0.301, P = 0.050 > 0.000*				r_s = 0.010, P = 0.950 > 0.000				r_s = −0.148, P = 0.344 > 0.000			
Number of Live Births	0	n	2	8	2	12	2	6	4	12	5	7	0	12	1	3	8	12
		%n	17	67	17	100	17	50	33	100	42	58	0	100	8	25	67	100
	1	n	0	10	1	11	1	9	1	11	6	5	0	11	0	5	6	11
		%n	0	91	9	100	9	82	9	100	55	45	0	100	0	45	55	100
	+2	n	1	17	3	21	8	9	4	21	5	14	2	21	0	12	9	21
		%n	5	81	14	100	38	43	19	100	24	67	10	100	0	57	43	100
	Total	n	3	35	6	44	11	24	9	44	16	26	2	44	1	20	23	44
		%n	7	80	14	100	25	55	20	100	36	59	5	100	2	45	52	100
			r_s = 0.071, P = 0.0650 > 0.000				r_s = −0.161, P = 0.303 > 0.000				r_s = 0.279, P = 0.070 > 0.000				r_s = −0.179, P = 0.251 > 0.000			

Table 2. (Contunied).

Characteristic[a]			GST alpha			Total	GST mu			Total	GST pi			Total	GST theta			Total
			Weak	Moderate	Strong		Weak	Moderate	Strong		Weak	Moderate	Strong		Weak	Moderate	Strong	
Microcalcification Status	(+)	n	4	32	5	41	8	25	8	41	14	25	2	41	1	20	20	41
		%n	10	78	12	100	20	61	20	100	34	61	5	100	2	49	49	100
	(−)	n	0	2	0	2	2	0	0	2	1	1	0	2	0	1	1	2
		%n	0	100	0	100	100	0	0	100	50	50	0	100	0	50	50	100
	Total	n	4	34	5	43	10	25	8	43	15	26	2	43	1	21	21	43
		%n	9	79	12	100	23	58	19	100	35	60	5	100	2	49	49	100
			r_s = −0.027, P = 0.865 > 0.000				r_s = −0.327, P = 0.34.000*				r_s = −0.016, P = 0.921 > 0.000				r_s = −0.200, P = 0.204 > 0.000			
Menopausal Status	Pre	n	0	1	2	3	0	2	1	3	2	1	0	3	0	2	1	3
		%n	0	33	67	100	0	67	33	100	67	33	0	100	0	67	33	100
	Post	n	3	34	4	41	11	22	8	41	15	24	2	41	1	18	22	41
		%n	7	83	10	100	27	54	20	100	37	59	5	100	2	44	54	100
	Total	n	3	35	6	44	11	24	9	44	17	25	2	44	1	20	23	44
		%n	7	80	14	100	25	55	20	100	39	57	5	100	2	45	52	100
			r_s = −0.368, P = 0.015 < 0.05*				r_s=−0.134, P = 0.392 > 0.000				r_s = 0.113, P = 0.469 > 0.000				r_s = 0.076, P = 0.630 > 0.000			

*Correlation is significant at the 0.05 level (2-tailed).

Abbreviations
U: previously untreated patients;
T: patients previously treated with chemotherapy or hormone therapy;
ER: estrogen receptor status, as determined by the hospital providing the tissue;
(-): negative;
(+): positive.

Discussion

It has been suggested that glutathione S-transferases are the most important class of enzymes involved in the protection of cells from the toxic effects of reactive electrophiles. It has been clearly established that these enzymes have an important role in mediating resistance to a wide variety of electrophiles, from the endogenous products of oxidative metabolism to environmental carcinogens and anticancer drugs. An ongoing goal is to identify which isoenzymes are important in the detoxification of specific electrophiles, and to determine if it would be possible to regulate those important isoenzymes using pharmacological agents.

Wide inter-organ and inter-individual variation in GST isoenzymes has been reported in different tissues based on biochemical methods (12,13). This variation may be responsible for inter-individual and inter-organ differences in susceptibility to tissue damage and carcinogenesis following exposure to certain xenobiotics. Both increased and reduced levels of expression of specific GST isoenzymes in tumors, particularly in those that have become resistant to anti-cancer drugs, suggest a role for these proteins in the development of resistance to chemotherapy. Determination of the GST isoenzyme profile of a cancer tissue could have prognostic value in the selection of treatment.

As breast cancer is a major cause of morbidity and mortality in women, and in consideration of the established role of GST isoenzymes in carcinogenesis and drug detoxification, it is of particular interest to identify the GSTs in normal breast tissue and breast tumors. The present study immunohistochemically examined the expression of different forms of GST in primary intraductal breast tumors. Using immunohistochemical techniques the specific cell types containing the different GST enzymes can be identified, and, in particular, tumor cells expressing different GST isoenzymes can be specifically recognized.

There have been several biochemical studies of GSTs in various tumors, including breast cancer, and those studies of breast cancer have identified frequent expression of GST pi (14-20). Nonetheless, biochemical studies of breast tumors have used tissue homogenates that contain a mixture of cell types, including neoplastic and non-neoplastic epithelium, and stromal cells, all of which may express GST. In addition, the cell lines derived do not necessarily reflect the xenobiotic enzyme composition of primary tumors. An immunohistochemical study (9) of GST in breast cancer reported specific localization of GST pi in tumor cells in 47% of cases. The expression of GSTs alpha and mu appears to be variable, and the results from different studies are contradictory. Shea et al. (15) identified GST mu in 48% of primary breast tumors, while GST alpha was not identified in any of the tumors. In contrast, Forrester et al. (14) failed to identify GST mu in any breast tumor and reported the presence of GST alpha in 89% of the breast tumors in their study group. Cairns et al. (9), using immunohistochemistry, detected GST alpha in 14% of tumors and GST mu in 42% of tumors.

In the present study GST expression in many of the cases was much stronger in the accompanying stromal cells and inflammatory infiltrate, while normal mammary epithelium also stained consistently and strongly. This observation emphasizes the usefulness of the immunohistochemical technique in demonstrating the distribution of enzymes and other proteins in tissues composed of a variety of cell types, such as tumors. To the best of our knowledge the present study offers the first comprehensive description of the 4 classes of GST in normal and tumoral human breast tissue. Other investigators have studied the distribution of GST alpha, mu, and, pi isoenzymes in the breast, but GST theta has never been studied in human tissues using immunohistochemical techniques, except in the liver and lungs (9,14,10,21). In the present study we observed staining of all the GST isoenzymes in 100% of our cases, both in neoplastic and non-neoplastic breast tissue. Stronger intensity of staining for GST alpha, mu, and pi was observed in normal epithelial cells than in tumor cells; however, the difference in GST theta expression between normal and tumoral epithelium was not significant. Cancer cells reveal multiple genetic alterations, resulting in morphological and functional differences from normal cells. Tumor cells may lose some of their functions (e.g. expression of some proteins) during the process of malignant transformation. It can be speculated that low-level GST expression in tumor cells might be a result of this transformation. Our results concerning GST alpha and pi isoenzymes in normal epithelium are in accordance with those of Forrester et al. (14). Staining of pi was observed in 91% of normal breast tissue samples (10); however, immunohistochemical staining for GST pi in

neoplastic epithelium was previously observed in 75%, 47%, 22%, and 15% of cases, respectively (9,14,22,23). GST alpha isoenzyme staining in neoplastic areas was observed in 19%, 11% and 1.8% of cases by Cairns et al. (9), Forrester et al. (14) and Haas et al. (23), respectively. Cairns et al. (9) observed only occasional staining of GST alpha in normal breast tissue, and observed GST mu expression in 48% of normal and 42% of neoplastic breast tissues. These different findings may be due to the use of different antibody sources and different detection systems.

Immunohistochemistry also provides information about intracellular localization. In the present study epithelial cells (malignant and non-malignant) stained with any of the 4 GST antibodies generally showed diffuse cytoplasmic staining and patchy nuclear staining. As GSTs are cytosolic proteins, the significance of nuclear staining is uncertain. They have shown nuclear staining in colon, breast, ovary, renal, pancreas, and uterine cervical carcinoma tissues, in addition to cytoplasmic staining (24-27); however, there were no comments concerning the significance of nuclear staining in the aforementioned studies. Rat GST YbYb, which is present in rat cell nuclei (28), is able to bind to steroid hormones (29). It has been suggested that there may be a similar role for GSTs in the human breast, given the importance of steroid hormones as regulators of the function of mammary epithelium (9). GST theta is known to have peroxidase activity and it is associated with the repair of oxidative DNA damage. This function of GST theta may offer an explanation for the observed nuclear localization. Nonetheless, the relationships between the nuclear localization of GST isoenzymes and their functions remain to be clarified further.

In the present study we observed that nuclear staining of GST isoenzymes was patchy and heterogeneous in intensity, even in an individual tumor section. The enzymatic techniques did not show this heterogeneity within tumors. This heterogeneity is an expected finding, which is most likely the result of tumor progression. Heterogeneity may influence the response of tumor cells to chemotherapeutic agents. It could be that tumor cells with high GST content may be more resistant to cytotoxic and chemotherapeutic agents.

We did not have the opportunity to study possible changes in GST levels before and after treatment, but we did observe a weak or non-significant positive correlation between GST alpha, mu, and pi expression in the treated and untreated patients. Nevertheless, our findings do not rule out the possibility of such a difference, which might be observed in a larger study group. Peters et al. (30) examined the relationships between pre-treatment GST alpha, mu, and pi levels in primary breast tumors, and the length of disease-free survival following adjuvant chemotherapy. They did not find any correlation and suggested that GSTs were not useful markers for predicting the response to adjuvant chemotherapy in human breast cancer. Silvestrini et al. (31) observed that in primary lymph node-negative breast cancer patients, after surgery alone the risk of local recurrence at 6 years was higher in the patients with breast tumors that had elevated levels of p53 and GST pi protein expression than in the patients with low levels. Conversely, in a series of patients that underwent conservative surgery followed by radiotherapy, there was no difference in local tumor recurrence rates between the patients with tumors that expressed or did not express p53, GST pi, and Bcl-2 proteins. Ambrosone et al. (32) reported that genetic polymorphisms in GSTs M1 and T1, known to be involved in the response to reactive oxygen species (ROS) and products of lipid peroxidation resulting from chemotherapy and radiation therapy, were associated with a significantly reduced risk of death and risk of recurrence following treatment for breast cancer. Women with null genotypes for both GST M1 and GST T1 had 33% lower risk of death than those with alleles for both genes. Huang et al. (33) reported a significantly lower disease-free survival rate in patients with GST pi-positive breast tumors that received adjuvant chemotherapy after surgery than in the patients that had GST pi-negative tumors.

Survival data for the patients in the present study are not currently available, but a correlation was observed between GST expression and known prognostic indicators. There was an association between GST mu and tumor grade; high grade cases (which are associated with a poorer prognosis) were more likely to express this isoenzyme. This significant correlation raises the possibility that determination of the expression of GST mu in a tumor might be used as an indicator of poor prognosis.

We observed that there was an association between GST mu expression and microcalcification status. Microcalcification status on a patient's mammogram

serves as an early diagnosis of in situ carcinoma of the breast. Thus, patients with a positive microcalcification status have low levels of GST mu expression and a good prognosis.

Perquin et al. (7) reported that glutathione peroxidase activity was greater in the tumors of premenopausal patients. Conversely, the results of the present study suggest that GST alpha expression was more strongly associated with breast tumors in postmenopausal women than in premenopausal women. This may be because premenopausal breast tumors generally tend to be more aggressive than breast tumors that develop in women after the onset of menopause. This is especially significant in countries like India, where the incidence of breast tumors is more common in premenopausal women (34).

The results presented herein indicate that the expression of GST pi is indeed positively related to estrogen receptor status in human breast cancer. The reason for this relationship between the expression of GST pi and estrogen receptor status is not clear. GST isoenzymes are capable of binding to hormones, including thyroid hormone and glucocorticoids (35,36). Although the physiologic significance of this effect is not known, the regulation of GST pi gene expression in hormone-sensitive tissues may be particularly important.

Wu et al. (37) suggest that environmental carcinogens, in addition to cigarette smoking and alcohol consumption, could induce breast cancer. Our findings also indicate a weak or non-significant negative relationship between GST alpha, mu, and pi expression and smoking status in breast cancer patients.

The number of breast cancer patients in the present study was small. Future studies with substantially larger numbers of breast cancer patients will be needed to prospectively examine the possible relationship between GST expression and prognostic factors.

Expression of xenobiotic metabolizing enzymes within tumors has been identified as a potentially important factor in determining anti-tumor drug resistance. Both the amount and proportion of different enzymes present in tumors play a role in determining anti-cancer drug resistance. Immunohistochemistry is a useful method for investigating the expression and cellular localization of GSTs within tumors, and may be useful in identifying those tumors that are potentially resistant to a specific anti-cancer drug. Such data are needed to improve our understanding of the role of these enzymes in neoplasias and in the resistance to cytotoxic drug therapy.

Corresponding Author:
Serpil OĞUZTÜZÜN
Department of Biology,
Faculty of Arts and Sciences
Kırıkkale University,
71450 Yahşihan, Kırıkkale - TURKEY
E-mail: soguztuzun@yahoo.com

References

1. Harris JR, Lippman ME, Veronesi U et al. Breast Cancer. N Engl J Med 327: 319-328, 1992.
2. Kelsey JL, Berkowitz GS. Breast Cancer Epidemiology. Cancer Res. 48: 5615-5623, 1988.
3. Henderson IC. Risk factors for breast cancer development. Cancer (suppl.) 71: 2127-2140, 1993.
4. Li D, Wang M, Dhingra K et al. Aromatic DNA adducts in adjacent tissues of breast cancer patients: Clues to breast cancer etiology. Cancer Res. 56:287-293, 1996.
5. Hayes JD, Flaagan JU, Jowsey IR. Glutathione-S-Transferases. Annu Rev Pharmacol Toxicol 45: 51-88, 2005.
6. Coles BF, Kadlubar FF. Detoxification of electrophilic compounds by glutathione S-transferase catalysis: determinants of individual response to chemical carcinogens and chemotherapeutic drugs? Biofactors 17: 115-130, 2003.
7. Perquin M, Oster T, Maul A et al. The glutathione-related detoxification system is increased in human breast cancer in correlation with clinical and histopathological features. J Cancer Res Clin Oncol 127: 368-374, 2001.
8. Rajneesh CP, Manimaran A, Sasikala KR et al. Lipid peroxidation and antioxidant status in patients with breast cancer. Singapore Med J 49: 640-3, 2008.
9. Cairns J, Wright C, Cattan AR et al. Immunohistochemical demonstration of glutathione S-transferases in primary human breast carcinomas. J Pathol 166: 19-25, 1992.
10. Gilbert L, Elwood LJ, Merino M et al. A pilot study of pi-class glutathione S-transferase expression in breast cancer-correlation with estrogen receptor expression and prognosis in node-negative breast cancer. J Clin Oncol 11: 49-58, 1993.

11. Wright C, Cairns J, Cantwell BJ et al. Response to mitoxantrone in advanced breast cancer: correlation with expression of c-erbB-2 protein and glutathione S-transferases. Br J Cancer 65:271-74, 1992.

12. Sherman M, Titmuss S, Kirsch RE. Glutathione S-transferase in human organs. Biochem Int 6: 109-118, 1983.

13. Corrigall AV, Kirsch RE. Glutathione S-transferase distribution and concentration in human organs. Biochem Inter 16: 3: 443-448, 1988.

14. Forrester LM, Hayes JD, Millis R et al. Expression of glutathione S-transferases and cytochrome P450 in normal and tumor breast tissue. Carcinogenesis 11-12: 2163-2170, 1990.

15. Shea TC, Kelley SI, Henner WD. Identification of an anionic form of GST present in many human tumors and tumor cell lines. Cancer Res. 48: 527-533, 1988.

16. Lewis AD, Forrester LM, Hayes JD et al. Glutathione enzymes in human tissues and tumor derived cell lines. Br J Cancer 60: 327-331, 1989.

17. Shea TC, Claflin G, Comstock KE et al. Glutathione transferase activity and isoenzyme composition in primary human breast cancer. Cancer Res 50: 6848-6853, 1990.

18. Moscow JA, Townsend AJ, Goldsmith ME et al. Isolation of the human anionic GST cDNA and the relation of its gene expression to estrogen receptor content in primary breast cancer. Proc Natl Acad Sci USA 85: 6518-6522, 1988.

19. Iscan M, Coban T, Cok I et al. The organochlorine pesticide residues and antioxidant enzyme activities in human breast tumors: is there any association? Breast Cancer Research and Treatment 72: 173-182, 2002.

20. Sreenath AS, Kumar KR, Reddy GV et al. Evidence for the association of synaptotagmin with glutathione S-transferases: Implications for a novel function in human breast cancer Clinical Biochemistry 38: 436-443, 2005.

21. Mainwaring GW, Williams SM, Foster JR et al. The distribution of theta-class glutathione S-transferases in the liver and lung of mouse, rat and human. Biochem J 318: 297-303, 1996.

22. Aliya S, Reddanna P, Thyagaraju K. Does glutathione S-transferase pi a marker protein for cancer? Molecular and Cellular Biochemistry 253: 319-327, 2003.

23. *Haas S*, *Pierl C*, *Harth V* et al. Expression of xenobiotic and steroid hormone metabolizing enzymes in human breast carcinomas. *Int J Cancer. 119: 1785-91, 2006.*

24. McKay JA, Murray GI, Weaver RJ et al. Xenobiotic metabolizing enzyme expression in colonic neoplasia. Gut 34: 1234-1239, 1993.

25. Hamada S-I, Kamada M, Furumoto H et al. Expression of glutathione S-transferase-pi in human ovarian cancer as an indicator of resistance to chemotherapy. Gynecol Oncol 52: 313-319, 1994.

26. Shiratori Y, Soma Y, Maruyama H et al. Immunohistochemical detection of the placental form of glutathione S-transferase in dysplastic and neoplastic human uterine cervix lesions. Cancer Research 47: 6806-6809, 1987.

27. Harrison DJ, Kharbanda R, Bishop D et al. Glutathione S-transferase isoenzymes in human renal carcinoma demonstrated by immunohistochemistry. Carcinogenesis 10: 1257-1260, 1989.

28. Bennet CF, Yeoman LC. Microinjected glutathione S-transferase Yb subunits translocate to the cell nucleus. Biochem J 247: 109-112, 1987.

29. Homma H, Listowsky I. Identification of Yb-glutathione S-transferase as a major rat liver protein labeled with dexamethasone 21-methanesulfonate. Proc Natl Acad Sci USA 82: 7165-7169, 1985.

30. Peters WHM, Roelofs HMJ, van Putten WLJ et al. Response to adjuvant chemotherapy in primary breast cancer: no correlation with expression of glutathione-S-transferases. Br J Cancer 68: 86-92, 1993.

31. Silvestrini R, Veneroni S, Benini E et al. Expression of p53, glutathione-S-transferase-pi, and bcl-2 proteins and benefit from adjuvant radiotherapy in breast cancer. Journal of the National Cancer Institute 89: 9, 639-645, 1997.

32. Ambrosone CB, Sweeney C, Coles BF et al. Polymorphisms in glutathione-S-transferases (GSTM1 and GSTT1) and survival after treatment for breast cancer. Cancer Research 61: 7130-7135, 2001.

33. Huang J, Tan P-H, Thiyagarajan J et al. Prognostic significance of glutathione S-transferase-pi in invasive breast cancer. Mod Pathol 16: 558-565, 2003.

34. Chunder N, Mandal S, Basu D et al. Deletion mapping of chromosome 1 in early onset and late onset breast tumors-a comparative study in eastern India. Pathol Res Pract 199: 313-21, 2003.

35. Listowsky I, Abramovitz M, Ishigaki S. Glutathione-S-transferases are major cytosolic thyroid hormone binding proteins. Arch Biochem Biophys 273: 265-272, 1989.

36. Kumaraguruparan R, Mohan KVPC, Nagini S. Xenobiotic-metabolising enzymes in patients with adenocarcinoma of the breast: Correlation with clinical stage and menopausal status. The Breast 15: 58-63, 2006.

37. Wu SH, Tsai SM, Hou MF et al. Interaction of genetic polymorphisms in cytochrome P450 2E1 and glutathione S-transferase M1 to breast cancer in Taiwanese woman without smoking and drinking habits. Breast Cancer Res Treat 100: 93-98, 2006.