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GST isoenzymes in matched normal and neoplastic breast tissue

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The potential to metabolize endogenous and exogenous substances may influence breast cancer development and tumor growth. Therefore we investigated GST activity and the protein expression of glutathione S-transferases (GSTs) isoenzymes known to be involved in the metabolism of endogenous and exogenous carcinogens in breast cancer tissue to obtain new information on their possible role in tumor progression.

The interindividual variation in the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) and of 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) with glutathione (GSH) by cytosolic glutathione S-transferases (GSTs) were investigated in human breast matched normal and tumor samples. The GSTA, GSTM, GSTP and GSTT isoenzymes from the crude extracts of matched breast normal and tumor tissues in terms of their immunological properties using western blotting were compared.

In most of the samples, the GST activities were higher in the tumor than in the normal cytosolic fractions against both CDNB and EPNP. In the western blotting analysis, it was proved statistically that in normal and tumor epithelial cells, there was difference between GST pi and theta isoenzymes expressions ($p<0.05$), but no difference between the staining scores of GST mu and alpha isoenzymes ($p>0.05$). In normal epithelium there was a stronger GST theta expression than in invasive tumor tissues ($p=0.013$). However, the stronger GST pi expression was observed in tumor epithelium than in normal epithelium in human breast cancers ($p=0.000$).

We found the GSTP protein level and GST activities were higher in the breast tumor than in the normal cytosolic fractions against both CDNB and EPNP, thus implicating a certain biological importance.

Key words: Breast cancer, glutathione S-transferases, theta, alpha, mu and pi classes of GSTs, western blotting

The influence of exogenous and endogenous factors on tumor growth partly depends on the individual potential to metabolize these substances. Glutathione S-transferases (GSTs) are a large family of multifunctional enzymes involved in phase II detoxification of exogenous and endogenous compounds [1]. Moreover, these enzymes are highly polymorphic, giving rise to variations in enzymatic activity [1]. This may influence predisposition to cancer as well as tumor development and progression [2]. Therefore, these enzymes are candidates for the investigation of a potential role in breast cancer.

Glutathione S-transferases are essential for metabolism of environmental carcinogens, drugs and pesticides by catalyzing the conjunction of reactive chemical intermediates to soluble glutathione conjugates [3]. Seven classes of cytosolic GSTs are recognized in mammalian tissues (alpha (GSTA), mu (GSTM), pi (GSTP), omega (GSTO), sigma (GSTS), theta (GSTT), and zeta (GSTZ) [4,5]. In breast cancer, polymorphic GST isoenzymes may play a role in tumorigenesis and resistance to chemotherapy [6]. By biochemical measurements, wide interindividual variations were found in enzyme activities in tumor and normal breast tissues. A number of studies have shown that the GST activity toward several substrates including 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EAA), 1,2-epoxy-3-(p-nitrophenoxy)-propane (ENPP), 1-menaphthyl sulfate (MS) in tumor tissue were significantly higher than those in normal breast tissue [7-9].

However, immunohistochemical studies investigating GST expression in breast cancers showed inconsistent results. A number of studies have shown that GSTP expression is higher in human tumor relative to non-tumor tissues [10-14]. Oğuztüzün and coworkers [15] showed that expression of GSTA, GSTM, and GSTP in normal epithelium was stronger than in invasive tumor tissues, but there was no significant difference in GSTT expression between normal and tumor tissue. Also, Haas

and coworkers [16] found that GSTP expression was weaker in invasive carcinomas than in nonneoplastic mammary glands.

This work was designed to compare the GSTA, GSTM, GSTP and GSTT isoenzymes from the crude extracts of matched breast normal and tumor tissues in terms of their immunological properties using western blotting with monoclonal and polyclonal antibodies. In addition, the comparison was made in terms of substrate specificity of the GSTs isoenzymes against the different substrates: 1-chloro-2, 4-dinitrobenzene, 1, 2-epoxy 3-(*p*-nitrophenoxy) propane.

Materials and methods

Samples, For GSTs activity assays and western blotting analysis, 21 samples of cancerous breast tissue from lump resection, specimens along with a portion of surrounding normal tissue, as well as matched far neighbor of tumor tissue were used. Samples were obtained from The Demeteveler Oncology Hospital – ANKARA, and stored at -80°C. A portion of each tissue was examined by a pathologist and confirmed as being cancerous or normal. Cancer samples selected for this study were invasive ductal carcinomas.

Preparation of cytosols from matched normal and cancer human breast tissues. Tissues were suspended (1:2 w/v) and homogenized in TED buffer (10 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, 1 mM DTT, and 100 μM PMSF). The homogenate was centrifuged at 12,000g for 25 minutes and the resulting supernatant fraction was centrifuged at 134,000g for 50 minutes and the supernatants obtained were passed through cheesecloth to remove floating lipid materials and stored at -80°C to be used later in protein determination, activity assays, SDS-PAGE, and Western blotting. Protein concentrations were measured by the method described by Lowry et al. [17] using bovine serum albumin as standard.

GST activity assays. GSTs activities in the cytosolic fractions prepared from 21 human matched control and tumor breast tissues were determined spectrophotometrically using CDNB and EPNP as substrates by monitoring the thioether formation at 340 nm and 360 nm, respectively, as described by Habig et al. [18]. The assay mixture in a volume of 1 mL consisted of 100mM phosphate buffer (pH 6.5), the sample, 1 mM GSH, and 1 mM 1-chloroform-2,4-dinitrobenzene or 1, 2-epoxy 3-(*p*-nitrophenoxy) propane (as a substrate).

Immunoquantification of GSTs. Samples of the cytosolic preparation each containing 40 μg protein were separated by SDS-PAGE in 12% (w/v) polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane. Transfer buffer was the Towbin transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol and transfer was for 50 min at 15-20V. Filters were then washed in 50 mM Tris-HCI, pH 7.9, containing 150 mM NaCl and 0.05% Tween-20 (TBST) (2x10 min) then blocked using low-fat milk 85% w/v in TBST). Polyclonal antisera against the three human GST alpha, mu and pi were diluted as 1:7500, the monoclonal anti GSTT1-1 was diluted 1:10000 and exposed to filters for 1 h. (Monoclonal antibody against hGSTT1-1 was a kind gift of Dr. E. Juronen, Tartu, Estonia. Polyclonal antibodies against hGST alpha, mu, pi raised in rabbit were purchased from Biotrin International Limited, Dublin, Ireland). The filter was then washed five times, each for 5 min with TBST and incubated with the secondary antibody (biotinylated goat anti-rabbit 1:7500 diluted in TBST) for 1 h. Following further washing then followed treatment with the streptavidin-biotinylated alkaline phosphatase complex. 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) was used to visualize alkaline phosphatase activity in the tissues. Human liver cytosols were used as internal control in the blots.

Staining of the mmunoblots was quantified by densitometric analysis, using SynGene GeneTools (File version: 4.00.00, Serial No. 16250 13621, zeydan mpcs SynGene Laboratories).

Statistics. Mann-Whitney test was used to assess statistical significance of differences in the GSTs activities, between control and tumor groups. Differences in the protein expression between control and tumor groups were analyzed using the paired t tests.

Results

GSTs activities, were determined under the optimized conditions against CDNB and EPNP in the 21 matched con-

Table 1. GSTs Activities against CDNB and EPNP in the matched control and tumor human breast cytosolic fractions.

Patient No.	CDNB GSTs (nmole/min/mg protein)		EPNP GSTs (nmole/min/mg protein)	
	Control	Tumor	Control	Tumor
1	38.0	96.2	38.9	47.8
2	6.4	38.2	21.1	22.7
3	24.2	70.5	0.0	0.0
4	22.7	140.7	16.4	44.5
5	48.2	54.8	21.5	25.8
6	67.7	74.7	17.1	18.8
7	18.8	2.1	11.0	32.0
8	30.2	191.1	0.0	0.0
9	47.5	41.6	59.8	69.2
10	197.6/	300.1	32.9	23.7
11	131.4	184.4	47.4	57.8
12	115.5	165.3	20.7	24.5
13	57.9	165.3	15.8	21.1
14	43.3	123.6	38.3	54.8
15	102.4	93.0	0.0	0.0
16	49.9	320.8	15.9	45.7
17	293.9	517.7	55.2	73.7
18	112.2	98.1	0.0	0.0
19	220.9	142.0	0.0	0.0
20	74.7	133.8	11.7	27.4
21	66.4	174.5	0.0	0.0
	84.2 ± 73.9	134.6 ± 111.9	20.1 ± 18.7	28.1 ± 23.7

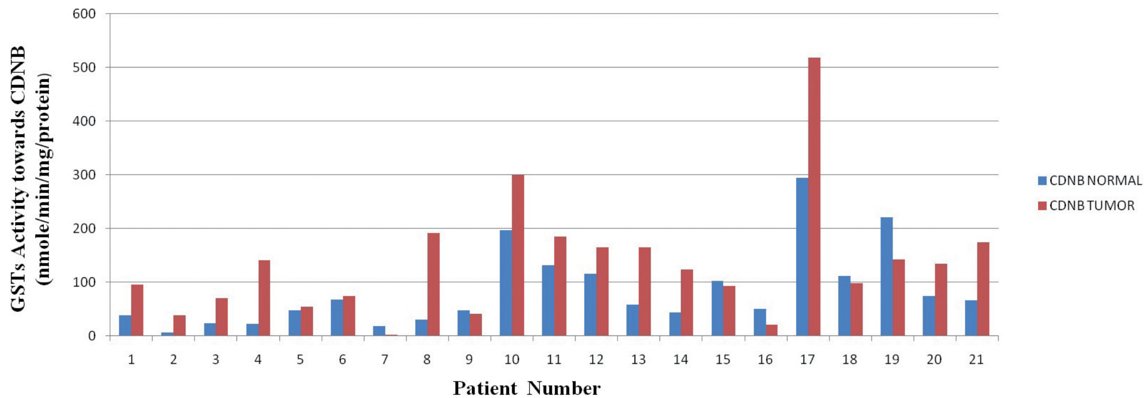

Figure 1. Comparison between the 21 matched breast control and tumor cytosolic fraction with respect to their GSTs activities against CDNB.

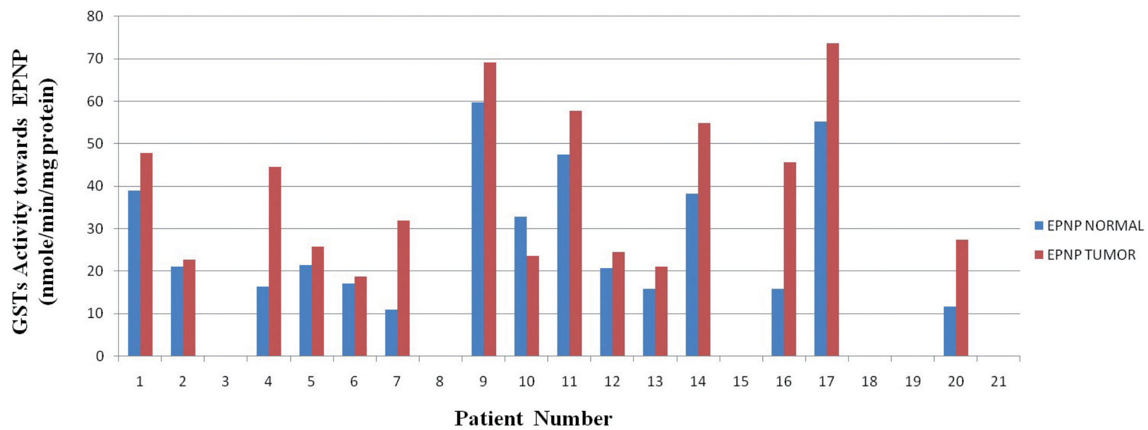

Figure 2. Comparison between the 21 matched breast control and tumor cytosolic fraction with respect to their GSTs activities against EPNP

trol and tumor human breast cytosolic fractions. The average specific activity against CDNB was calculated as 84.2 ± 73.9 nmole/min/mg protein for breast control GSTs, and 134.6 ± 111.9 nmole/min/mg protein (Mean ± S.E., n=21) for breast tumor GSTs (Figure 1). The average specific activity against EPNP was calculated as 20.1 ± 18.7 nmole/min/mg protein for breast control GSTs, and 28.1 ± 23.7 nmole/min/mg protein (Mean ± S.E., n=21) for breast tumor GSTs (Figure 2). The statistical analysis, using the Mann-Whitney test, of the data obtained from the two controls and tumor groups revealed that the difference between the two groups is statistically significant with respect to the CDNB GSTs activities as well as to the EPNP GSTs activities. As it is clear from Table 1, the GSTs activities were higher in the tumor cytosolic fractions, against both CDNB and EPNP, in the 16 and 14 of the 21 patients examined, respectively. However, while GSTs activity against CDNB as a substrate was detected in all of the matched 21 cytosolic fraction, 29 % of the cytosolic fractions (6 out of 21) had no activity of GSTs against EPNP as a substrate.

Western blots were carried out on the 21 matched breast controls and tumor samples to assess the relative GST isoenzyme content. Representative blots using polyclonal antibodies to GSTA, GSTM, GSTP, and monoclonal antibody to GSTT are shown in Figure 3. In tumor tissue significant upregulation of GSTP expression was detected (Table 2, Fig. 3) ($p<0.05$). Increased levels of GSTP were detected in tumor tissue in 13 of the 21 patients Moreover, the difference in GSTT expression between normal and breast tumor tissue was also significant ($p=0.013< 0.05$, Table 3). In normal breast tissue significant upregulation of GSTT expression was detected (Table 2, Fig. 3) ($p=0.000<0.05$). Increased levels of GSTT were detected in normal tissue in 14 of the 19 patients. The differences in GSTA and GSTM expressions between normal and breast tumor tissue were not statistically significant ($p> 0.05$, Table 3).

As it is clear from Figure 1 and 2, the GST activities were higher in the tumor cytosolic fractions, against both CDNB and EPNP, in the majority of the patients examined. The 13 samples have more GSTP protein in tumor than normal breast tissue and 14 samples have more GSTT protein in normal breast than tumor tissues in western blotting analysis (Table 2).

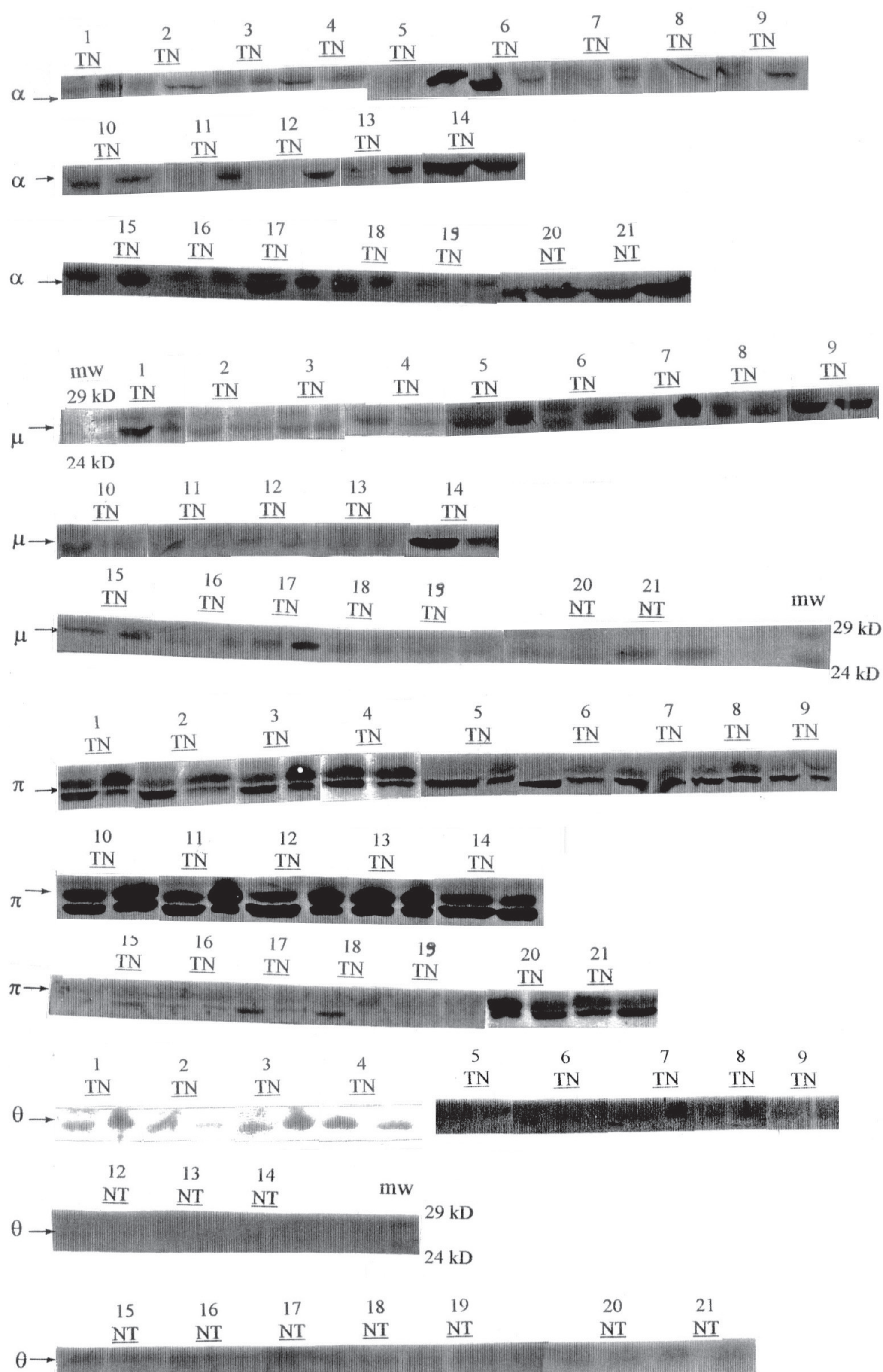

Figure 3. Immunodetection of glutathione S-transferases class alpha, mu, pi and theta in normal breast and corresponding tumor cytosolic fractions (~40μg protein) from breast normal (N) and tumor (T).

Discussion

In this study, the GST activities were measured against CDNB and EPNP in 21 matched normal and tumor breast cytosolic fractions. The statistical analysis, using the Mann-Whitney test, of the data obtained from the control and tumor groups revealed that the difference between the two groups was statistically significant with respect to the CDNB GSTs activities as well as to the EPNP GSTs activities. In most of the samples, the GST activities were higher in the tumor than those in the normal cytosolic fractions against both CDNB and EPNP. A number of studies showed that the GST activity toward several substrates including CDNB, DCNB, EAA, ENPP, MS in tumor tissue were significantly higher than those in normal breast tissue [7-9, 19]. Kelley et al. [20] reported that the average level of GSTs was substantially elevated in the cancer tissues than the levels in normal breast tissue from the same patient. An increasing body of evidence indicates that GSTs play a role in the intrinsic and acquired resistance of tumors to anticancer drugs [21]. Consequently, the increased activity of the GSTs in the breast tumor may be associated with the developed resistance of the tumors against the anticancer drugs.

The GST activities, in the normal and tumor breast cytosolic fractions, towards EPNP, ranged from 0 to 73.7 nmole/min/mg protein. Six of the samples (29 %) had no detectable GST activity against EPNP. This is most probably associated with the well-known polymorphic expression of the class theta GSTT1-1 where 30-40 % of the human population has been reported to be negative conjugators. The polymorphism in the genes that encode enzymes involved in the metabolism of carcinogens or environmental toxins may be related to an increased risk of cancer in some individuals [22]. As GSTs are involved in the metabolism of many carcinogens, environmental pollutants, anticancer drugs, and reactive oxygen species (ROS), it is reasonable to suppose that the absence of specific isoenzymes will have a profound effect on the tolerance of an organism to xenobiotics.

The overall GST activities were higher in the tumor cytosolic fractions, against both CDNB and EPNP, in 16 and 14 of the 21 patients, respectively. In the western blotting study of the samples, GST isoenzyme levels were higher in tumor tissue when compared with normal tissue in 57%, 50%, 86% and 26% of the samples for GSTA, GSTM, GSTP and GSTT respectively. This agrees with the positive correlation of GSTP content with CDNB activity (Table 1, 2). It is important to note however that GSTP was not the only GST subunit expressed in high levels and that the expression of other subunits made a significant contribution to overall GST content (Table 2).

Table 2. The classification of the matched breast normal and tumor tissues for each GST isoenzyme class on western blotting.

	GSTA		GSTM		GSTT		GSTP	
patient no	tumor	normal	tumor	normal	tumor	normal	tumor	normal
1	22633,76	27168,61	21449,31	14357,85	ND	ND	ND	ND
2	50570,43	10808,33	27681,99	56826,39	17928,51	30120,39	86576,37	32796,92
3	27599,24	13521,33	29090,41	26234,27	15059,92	33224,82	81581,07	51683,9
4	35608,27	17645,07	24513,07	24098,96	21229,18	28384,64	72512,12	57178,43
5	ND	ND	ND	ND	40605,11	36825,1	90943,84	57573,27
6	9405,88	95533,94	21898,82	19940,58	5318,08	8299,58	54142,15	18049,04
7	87069,17	24415,82	8111,68	26943,08	4477,82	6928,72	53979,64	18394,31
8	14947,49	24835,87	28605,66	26208,07	8427,39	8467,85	42491,85	39572,1
9	713,34	19254,78	6455,95	26586,01	3198,36	9708,67	29428,59	34995,96
10	18481,41	27674,29	58569,94	59122,02	7499,01	4589,7	23786,7	14209,95
11	148380,17	85475,92	28296,09	13994,53	ND	ND	130899,06	112212,87
12	1271,12	91490,86	15092,39	562,94	ND	ND	145432,98	64086,02
13	712,12	203806,45	14493,6	16106,15	4175,14	12483,91	193030,09	89757,35
14	28900,78	157565,53	ND	ND	9577,78	11660,15	140916,84	109748,7
15	474029,63	365397,63	120912,3	42855,3	1830,27	14277,91	188644,44	160384,23
16	37845,56	13236,29	18389,54	2724,64	14072,54	12011,63	562,45	3955,91
17	ND	ND	ND	ND	ND	ND	ND	ND
18	11824,44	13640,65	5173,2	11314,1	13312,91	8977,62	22042,72	448,4
19	30866,66	28578,14	12837,45	31768,42	8562,8	10845,91	23608,7	9209,79
20	409,12	5888,32	16293,14	10639,61	4165,39	12901,97	5749,51	6658,14
21	22020,1	38465,76	20471,23	22496,47	8437,7	5097,64	8900,88	11310,73
22	89323,1	20379,32	12278,88	19647,26	279,19	926,89	84556,98	66917,05
23	186104,7	71564,26	21876,49	28933,77	1112,54	10557,53	86459,37	53810,02

ND: Not determined

Table 3. Statistical analysis of the matched breast normal (C) and tumor (T) tissue for each GST isoenzyme class on western blotting.

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper			
Pair 1	GSTalphaT - GSTalphaC	-2744,32	75604,44060	16498,24	-37159,0	31670,41	-,166	20	,870
Pair 2	GSTmuT - GSTmuC	1482,415	21123,88790	4609,610	-8133,06	11097,89	,322	20	,751
Pair 3	GSTthetaT - GSTthetaC	-4053,74	6373,98998	1462,294	-7125,90	-981,571	-2,772	18	,013
Pair 4	GSTpiT - GSTpiC	26347,30	27136,74575	5921,723	13994,80	38699,80	4,449	20	,000

By western blotting analysis, in tumor tissue significant upregulation of GSTP expression was detected ($p=0.000<0.05$). Increased levels of GSTP were detected in tumor tissue in 18 of the 21 patients. In normal breast tissue, significant upregulation of GSTT expression was detected (Table 1, Fig. 3) ($p=0.013< 0.05$). Increased levels of GSTT were detected in normal tissue in 14 of the 19 patients. The differences in GSTA and GSTM expressions between normal and breast tumor tissue were not statistically significant ($p> 0.05$). Sreenath et al. [19] found also a significant elevation in GSTP levels in breast cancer tissues with no appreciable changes in GSTA and GSTM compared to normal breast tissue using western blot analysis.

The phenotypic absence of GSTM1 and GSTT1 activity is due to homozygosity for an inherited deletion of these genes, termed the null genotype [23, 24]. Six of the 21 samples had no detectable GSTs activity against EPNP. This is most probably associated with the polymorphic expression of the class theta GSTT1-1. However, according to the western blotting analysis of the same cases, GSTT protein was present in all of the samples. Our results may indicate that GSTM and GSTT protein in this study might be different gene products of GSTM and GSTT gene class family.

Overexpression of GSTs that participate in the detoxification of xenobiotics and antioxidant defenses are known to decrease the incidence of a variety of cancers, including lung [23] and breast [12, 15]. In the present study, the GSTP protein level and GST activities were higher in the breast tumor than those in the normal cytosolic fractions against both CDNB and EPNP, thus suggesting a certain biological importance. On the other hand, in normal breast tissue significant upregulation of GSTT expression was detected. Whether these GST isoenzyme expression patterns may influence a tumor's sensitivity or resistance to systemic treatment needs to be determined by the joint analysis with long-term clinical follow-up data.

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