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Research Article

Alterations of Glutathione and GSTM1 Mutation Induce Tumor Metastasis and Invasion Via EMT Pathway in Breast Cancer Patients

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Abstract

Objectives: Alteration in the Glutathione (GSH) and Glutathione S-Transferase (GST) family lead to various disorders including breast cancer. However, the role of GSH and GSTM1 in the onset of breast cancer is still not fully elucidated. In the present study we observed considerable deficiency in the levels of glutathione and genetic mutation in the GSTM1 enzyme that influence susceptibility to breast cancer metastasis and invasion via EMT pathway.

Methods: GSTM1 genotype was identified by multiplex polymerase chain reaction (PCR), real time (RT)-PCR and western blotting in breast tumor samples and adjacent normal control tissue (ANCT) samples. The endogenous glutathione levels were determined by high performance liquid chromatography (HPLC).The tumor metastasis, invasion and epithelial-mesenchymal transition (EMT) biomarkers were determined by RT-PCR.

Results: In present study genotyping analysis of GST investigated that genetic mutation in GSTM1 was detected in breast cancer tissue samples. mRNA and protein expression of GSTM1 was significantly downregulated in tumor samples as compared to adjacent normal control tissue (ANCT) samples in breast cancer patients. Significant reduction of total glutathione (GSHt $P<0.05$) was observed among correlation with patient ages, stages and histological grades of breast cancer patients. Additionally, downregulation of GSTM1 promotes EMT pathway that leads to enhanced the expression of tumor proliferation, invasion and metastasis in breast cancer patients ($p<0.05$).

Conclusion: The present findings suggest that GSTM1 genotype could be a potential biomarker for breast cancer pathogenesis.

Keywords: Breast cancer, Glutathione, Glutathione S-Transferase M1, Invasion, Metastasis, Proliferation;

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Breast cancer is most frequently diagnosed cancer among women and is the second leading cause of cancer deaths in females' worldwide.[1] It is initiated in the breast tissues possesses milk producing glands known as lobules and ducts connecting lobules to the nipple, whereas the other parts are composed of fatty, connective and lymphatic tissues. The onset of breast cancer occurs in both sexes but are comparatively rare in males.[1] Generally, breast cancer accounts for 23% of all cancer cases and 14% cancer death have been considered, and their incidence rate are usually higher in European countries.[2] Glutathione, a tripeptide composed of cysteine, glycine, and glutamic acid, provides protection against ROS either directly as an antioxidant agent or indirectly, by supporting other cellular antioxidants in a working state.[3] Glutathione (GSH) plays central role in a number of cellular processes such as differentiation, proliferation and apoptosis and aberration in GSH homeostasis are contributing in the progression and development of different human diseases including cancer. GSH forms complexes with a different variety of product of oxidative stress and carcinogens via reactions promoted by the enzyme glutathione S-transferase (GST). An equilibrium in GSH: GSSG ratio is important for survival of the cells and control regulation of the system is

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therefore very necessary. Alteration in GSH or GSH activity has also been studied in the trabecular mesh work and aqueous humor of patients with glaucoma.[4] Glutathione (GSH) and glutathione-S-transferases (GSTs) are important first lines of defense system against both acute and chronic toxicities of electrophiles and reactive oxygen/nitrogen species. Both GSH concentrations and GST enzyme functions are under equilibrium homeostatic control. Glutathione S-Transferase (GST) conjugates with glutathione and confer anti-oxidant protection through neutralization of toxic carbonyl, epoxide, and peroxide containing metabolites produced within the cell by oxidative stress.[5] GSTs are responsible for substantial proportion of total glutathione peroxidase activity in humans.[6]

GST family genes play a critical role in various biochemical and molecular aspects of different cellular resistance process. Metabolic detoxification is one of the basic mechanism of cellular resistance govern by GST family.[7] The GST family include GSTT1, GSTM1 and GSTP1 genes that encode most essential phase II detoxifying enzymes involved in the conjugation of substrates that are toxic to cancer cells, including anthracyclines as chemotherapeutic agents used in breast cancer treatment. Both GSTT and GSTM1 classes posses polymorphic null forms (GSTT1 null and GSTM1 null), which lack their two alleles and are therefore unable to encode the detoxifying enzymes.[8] Thereof, homozygous deletion of glutathione S-transferase M1 (GSTM1), results in accumulation of oxidative stress byproducts which determine its protective role against antioxidant as well.[9] Reduction in GSTT1 and GSTM1 activities closely associated with increased risk of developing different cancers. [10] Numerous studies reveal that null genotype closely associated with increased risk of developing breast cancer.[11] According to meta analysis, GSTM1 null genotype significantly increased the risk of breast cancer (1:10) ratios varies in different ethnicity, such as Asian (1:21) and Caucasian (1:05) especially in postmenopausal woman (1:11).[12] Furthermore, D. N. Chirilã et al investigate that null genotype of GSTM1 is a potential risk factor to develop synchronous breast cancers and for breast cancer linked with one extra mammary cancer. The presence of GSTM1 and GSTT1 null genotype lead to increase risk for multiple breast cancer (bilateral or synchronous).[11] A functional role of GSTM1 is determine in vascular smooth muscle cells (VSMC), showing that decrease expression of GSTM1 results in increase oxidative stress, cell migration and proliferation.[13] In breast cancer, genetic polymorphisms of GSTT1, GSTM1, and GSTP1 may be involved in modifying the response to neoadjuvant chemotherapy, given that null alleles or the alteration in the genes expression resulting to enzyme deficiency, which is associated with the inability of the chemotherapy drug to export cells.[14] However, the association of GSTM1 within Pakistani breast cancer populations have not been investigated so far. The present study attempts to elucidate the role GSTM1 in breast cancer tissues and to highlight its possible role in regulation of EMT pathway that associated with breast cancer pathogenesis, which may be used as early prognosis biomarker of breast cancer in clinical settings.

Methods

Subject Enrollment and Sample Collection

Tumor tissue samples were collected from one hundred and ninety eight breast cancer patients and adjacent normal control tissue (ANCT) samples in RNAlater® stabilization solution (Thermo Fisher, Waltham, USA) at the time of surgery from different hospital at Khyber Pakhtunkhwa, Pakistan. ANCT was selected from the area about 2 cm away from the affected site by an oncologist on the basis of histopathological observations. Patients with history of other infectious and familial diseases were excluded from this study. This study was conducted after prior approval from the Ethical Review Committee of the collaborating hospital. Prior to samples collection, written informed consent was obtained from patients participated in the study and the collected samples were stored at -80°C for further analysis.

DNA Extraction, Gel Electrophoresis, and Quantification

DNA was extracted from the breast tumor tissue samples and ANCT samples using the standard phenol-chloroform method.[15] PCR was performed to determine the genotype of GSTM1. The PCR products were electrophoresis using 2% agarose gel. Thereafter, stained gel with ethidium bromide and visualized under the UV illuminator BioDoc Analyze TM (Biometra, Göttingen, Germany).

Total RNA Extraction, cDNA Synthesis and Expression Analysis

Total RNAs were extracted from breast cancer tissue samples and ANCT samples using total RNA kit 1 (OMEGA) and reversely transcribed into complementary DNA (cDNA) using commercially available cDNA reverse transcriptase kit (TAKARA, ohtsu,Japan). Then, qPCR detection of gene expression was performed with specific primers and SYBR Green master mix (Bio-Rad) using GAPDH as an internal control. The thermal cycling conditions included initial denaturation step at 95 ºC for 30 s, 40 cycles at 95 ºC for 10 s, 60 ºC for 20 s, 72 ºC for 5s. Gene specific primers for respective genes (sense primer) and (antisense primer)

were design (Table 1). Then CFX96TM quantitative RT- PCR were performed to analyze the expression of target genes in breast cancer tissue samples and ANCT samples according to manufacture protocol. Data was analyzed by 2-ΔΔCt method.

Protein Extraction and Western Blot Analysis

Western blot was performed in tissue lysates using GAPDH as an internal control. Briefly, total proteins were extracted from breast cancer tissues samples and ANCT samples in lysis buffer. Proteins concentrations were determined using BCA method and then proteins were resolved on 8-10% SDS-PAGE and consequently transferred to a PVDF membrane. The membrane was incubated in 5% skim milk for 1 h. Then membrane was incubated using primary antibodies for overnight at 4°C. GSTM1 Rabbit polyclonal antibody (Cat No; 12412-1-AP, dilution; 1: 1000) and GAPDH Rabbit polyclonal antibody (Cat No; 10494-1-AP; dilution 1:8000) were used as primary antibodies after diluted in 1X TBST. The membranes were washed three times followed by incubation with HRP Goat anti Rabbit IgG antibody (Cat No; SA00001-2, dilution 1:3000)) for 2 h at room temperature. Finally, measure the blots using the enhance chemiluminescence (ECL) kit according to the manufacturer's protocols.

Table 1. List of primers used in this study

Gene	Direction	5'_3' sequence
GSTTI	Sense	TGCCGCGCTGTTTACATCTT
	Antisense	GTGCTGACCTTTAATCAGATCCA
GSTM1	Sense	TCTGCCCTACTTGATTGATGGG
	Antisense	TCCACACGAATCTTCTCCTCT
E-cadherin	Sense	ATTTTTCCCTCGACACCCGAT
	Antisense	TCCCAGGCGTAGACCAAGA
HER2	Sense	TGCAGGGAAACCTGGAACTC
	Antisense	ACAGGGGTGGTATTGTTCAGC
CEA	Sense	CTGTCCAATGACAACAGGACC
	Antisense	ACGGTAATAGGTGTATGAGGGG
MMP-2	Sense	TACAGGATCATTGGCTACACACC
	Antisense	GGTCACATCGCTCCAGACT
MMP-9	Sense	TGTACCGCTATGGTTACACTCG
	Antisense	GGCAGGGACAGTTGCTTCT
Ki-67	Sense	ACGCCTGGTTACTATCAAAAGG
	Antisense	CAGACCCATTTACTTGTGTTGGA
VEGF	Sense	AGGGCAGAATCATCACGAAGT
	Antisense	AGGGTCTCGATTGGATGGCA
BCL2	Sense	GGTGGGGTCATGTGTGTGG
	Antisense	CGGTTCAGGTACTCAGTCATCC
MUC-1	Sense	TGCCGCCGAAAGAACTACG
	Antisense	TGGGGTACTCGCTCATAGGAT
Lamine	Sense	TGACTTTCAAGACATTCCGTCC
	Antisense	AGGCGAAGTATCTATACACACCC
Vimentin	Sense	TGCCGTTGAAGCTGCTAACTA
	Antisense	CCAGAGGGAGTGAATCCAGATTA
Fibronectin	Sense	AGGAAGCCGAGGTTTTAACTG
	Antisense	AGGACGCTCATAAGTGTCACC
Snail	Sense	ACTGCAACAAGGAATACCTCAG
	Antisense	GCACTGGTACTTCTTGACATCTG
GAPDH	Sense	GGAGCGAGATCCCTCCAAAAT
	Antisense	GGCTGTTGTCATACTTCTCATGG

HPLC Separation, Quantification and Determination of GST Subunits and Activity

All the procedures were performed at 4 °C. The tumors tissue samples and ANCT samples (weights ranging from 35-65 mg) were homogenized in 3.0 ml of Tris/HCl (25 mM, pH 7.4), using an ultra-turrax. To reduced contamination of the connective tissues, epithelium layer from the cystadenomas were removed from the cyst wall and used for additional analysis. For cytosolic preparation, homogenized samples were centrifuged at highest speed for 85 minutes. Cytosolic glutathione was purified following the Bogaards et al., 1989 protocol.[16] The equilibration and condition of column were performed in 5 minutes obtaining the initial conditions. 200 ul of the sample extract and the standards were injected into the chromatographic column maintained at 35 °C. In brief, selected amount of cytosol was used to a 2 ml S-hexylgluthathione-agarose affinity column, wash with 16 ml buffer containing 0.4 M NaCl solution, and eluted in the same buffer solution containing 5 mM of S-hexylglutathione. Elutes precursory of the S-hexylglutathione alpha were analyzed for total glutathione activity, and mostly less than 5% of the total applied enzymatic activity were present. The concentration of elutes were changed to approximately 0.2 ml with the help of centricon PM 10 ultra filtration tube. The glutathione subunits were eluted with incline of acetonitrile in water, both including 0.1% trifluoroacetic acid (from 40 to 50% acetonitrile in 18 min, proceeded by an additional increase to 53% in 5 min and isocrating separation for another 7 min). The detection of elution profile was observed by measuring the absorbance at 214 nm.

Data Analysis

Data was analyzed using OriginPro 2015 statistics software (OriginLab, Northampton, USA). The correlation among different factors were assessed at 95% confidence intervals (CI) using the Mann-Whitney, Kruskal Wallis, and ANOVA test. Specific comparisons were undertaken by standard descriptive analysis, and p-value of <0.05 was considered significant.

Results

Genotyping Determination of GSTM1 in Breast Cancer Patients

The distribution of GSTM1 genotype in the breast cancer tissues and ANCT tissues were determined by standard PCR as shown in figure 1a and table 1. The frequency of null GSTM1 genotype was significantly higher (0.733) in breast cancer tissues samples compared with the ANCT samples (0.134) (***p=0.004), Further, we categorized the breast cancers patients based on their age, it was revealed that the null-GSTM1 genotype frequency was significantly higher in patients with age less than 50 (<50) years as compared with patients of greater than 50 (>50) years age of patients (0.705 vs 0.582,**p=0.007). These data indicate that mutation in GSTM1 genotype has significant association with early development of breast cancer in Pakistani population. In addition, HPLC result determined that the level of endogenous glutathione (GSHt, GSH, GSSG) were decreased in patients with age less than 50 (<50) years compared with patients having age greater than 50 (>50) years as shown in figure 2.

Determination of GSTM1 Expression in Breast Cancer Patients

Western blotting and RT-PCR were performed to evaluate the protein and mRNA expression levels of GSTM1 in both tumor and control individual tissues. The relative expression of GSTM1 mRNA was significantly decreased (***p=0.001) in the tumor tissues samples compared to the ANCT samples, irrespective of the histological and disease status of the patient (Fig. 1b). Furthermore, the concentration of GSTM1 proteins were determined by western blot. The result concluded that the protein concentration of GSTM1 was significantly decreased in breast cancer tissue samples as compared with ANCT samples as shown in figure 1c. The results were highly significant.

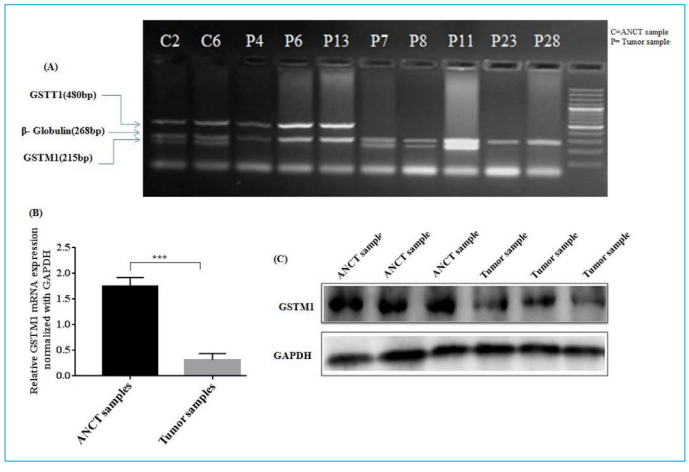

Figure 1. Genotyping determination of GST in breast tumor tissue samples compared with ANCT samples **(a)** PCR identification of GSTM1 genotype **(b)** mRNA expression of GSTM1(***p=0.001) **(c)** Protein expression of GSTM1 compared with GAPDH as a internal control in breast cancer patients.

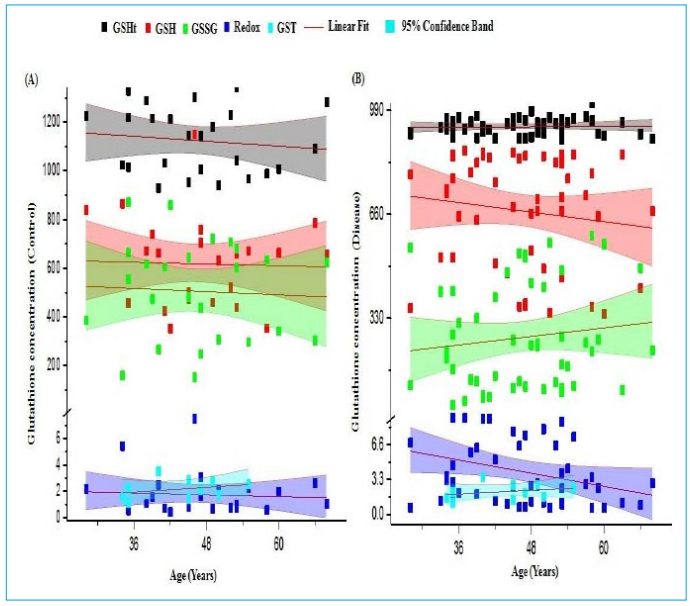

Figure 2. Correlation of endogenous level of GSH with age of breast cancer patients.

Determination of GSH Concentration Among Patients with Different Age Groups

Glutathione (GSH) plays crucial role in a multitude of cellular processes, including cell proliferation, differentiation, and apoptosis and alteration in GSH homeostasis are participating in the development and progression of different diseases, including cancer. The deficiency of GSH or decrease in the ratio of GSH/glutathione disulphide (GSSG) leads to enhanced susceptibility to oxidative stress implied in the development of cancer. Therefore, the endogenous GSH (GSHt, GSH, GSSG) concentrations were determined by HPLC and compared between two study cohorts of breast cancer, <45 and >45 years. Interestingly, in both study cohorts, the GSHt concentration was higher in the control tissues (135.206±39.031 and 138.59±38.438 respectively) compared to that of tumor tissues (22.478±4.792 and 30.468±5.758 respectively). Statistical analysis showed that GSHt concentrations were significantly associated with both tissue types (***p=0.003, **p=0.005), while GSH and GSSG concentrations were also decreased in tumor tissues of both study cohorts, but no significant difference was observed (p>0.05) between tumor tissue samples and ANCT samples (Fig. 3, Table 2). These data indicated that endogenous GSH, especially GSHt was significantly downregulated in tumor tissues in both study cohorts.

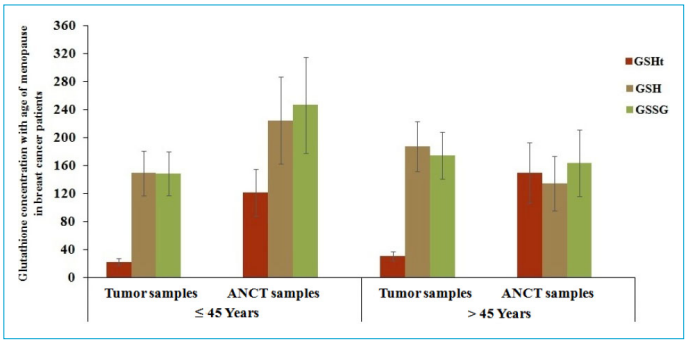

Figure 3. Determination of endogenous level of GSH associated with above and below 45 years of patients age.

GSH Concentration with Premenopausal and Postmenopausal Status of Breast Cancer Patients

Further, the endogenous concentration of GSH were investigated among premenopausal and postmenopausal breast cancer patients. Of the total study cohorts (n=198), 115 belonged to the premenopausal group and 83 belonged to the postmenopausal group. The GSHt concentration was significantly lower in the tumor tissues samples of premenopausal group (25.52±4.361) compared to ANCT samples (133.479±32.372, **p=0.006). Similarly, significant reduction in GSHt was observed in the tumor tissues samples of postmenopausal group (30.602±7.422) compared with the ANCT samples (145.183±51.330, *p=0.009). In addition, the concentration of GSH and GSSG were also significantly decreased in premenopausal tumor tissues samples ($p<0.05$), while no difference has been observed in postmenopausal tumor tissues samples as compared with the ANCT samples. The data obtain suggested that GSHt was significantly downregulated in the tumor tissues samples of both premenopausal and postmenopausal patients (Fig. 4, Table 3).

Determination of GSH Among Different Stages and Grades in Breast Cancer Patients

Moreover, the endogenous GSH levels were determined in different stages and grades of breast cancer. Accord-

Table 2. Analysis of endogenous GSH levels in breast cancer patients and its correlation with above and below 45 years age of patients

Variables	Analysis	Samples	N	Mean	SEM	95% CI of Mean		Skewness	p
						Lower	Upper		
Overall									
	GSHt	Control	198	1124.31	26.86	1068.87	1179.76	0.0997	0.002
		Diseased	198	937.25	3.821	929.578	944.937	0.2849	
	GSSG	Control	198	618.511	36.75	542.658	694.363	0.8631	0.001
		Diseased	198	670.941	24.73	621.234	720.647	-0.666	
	GSH	Control	198	505.808	41.31	420.54	591.071	-0.090	0.021
		Diseased	198	268.512	23.52	221.241	315.783	0.578	
	Redox	Control	198	1.740	0.327	1.065	2.416	2.407	0.004
		Diseased	198	4.076	0.503	3.0656	5.088	1.2238	
≥45									
	GSHt	Control	78	1117.07	38.43	1033.32	1200.82	0.361	0.005
		Diseased	78	938.516	5.758	926.702	950.331	0.3502	
	GSSG	Control	78	651.044	54.85	531.522	770.565	1.0655	0.201
		Diseased	78	637.066	35.57	564.068	710.064	-0.410	
	GSH	Control	78	466.025	55.64	344.79	587.26	-0.054	0.060
		Diseased	78	299.61	33.14	231.602	368.618	0.439	
	Redox	Control	78	1.979	0.514	0.858	3.101	2.485	0.051
		Diseased	78	3.066	0.489	2.062	4.071	0.972	
<45									
	GSHt	Control	120	1132.17	39.03	1046.27	122.079	-0.183	0.003
		Diseased	120	935.656	4.792	925.689	945.623	-0.12	
	GSSG	Control	120	583.267	48.64	476.21	690.327	0.497	0.201
		Diseased	120	714.055	31.86	647.794	780.317	-0.96	
	GSH	Control	120	548.906	61.43	413.699	690.327	-0.227	0.054
		Diseased	120	228.934	31.67	163.057	294.809	0.70	
	Redox	Control	120	1.481	0.402	0.595	2.366	2.359	0.103
		Diseased	120	5.362	0.900	3.491	7.234	0.87	

Table 3. Association of GSH concentration with menopausal status of breast cancer patients

Variables	Analysis	Samples	N	Mean	SEM	95% CI of Mean		Skewness	p
						Lower	Upper		
Pre- menopause									
	GSHt	Control	85	1127.1	32.37	1058.4	1195.6	-0.1093	0.006
		Diseased	85	934.55	4.361	925.67	943.43	0.11986	
	GSSG	Control	85	629.77	49.28	525.30	734.24	0.9783	0.008
		Diseased	85	679.38	29.46	619.36	739.39	-0.6634	
	GSH	Control	85	497.25	54.24	382.26	612.25	0.04134	0.002
		Diseased	85	260.05	28.03	202.97	317.15	0.48514	
	Redox	Control	85	1.9214	0.462	0.9409	2.9018	2.09283	0.006
		Diseased	85	4.63	0.681	3.2424	6.0176	1.0409	
Post- menopause									
	GSHt	Control	113	5.645	51.33	997.17	1239.9	0.5751	0.004
		Diseased	113	2.115	7.422	926.77	958.24	1.0136	
	GSSG	Control	113	1118.5	50.56	475.02	714.12	-0.6228	0.608
		Diseased	113	942.50	45.99	557.06	752.05	-0.6857	
	GSH	Control	113	594.57	62.64	375.85	672.10	-0.5364	0.202
		Diseased	113	654.55	43.69	192.31	377.54	0.7243	
	Redox	Control	113	523.97	0.278	0.697	2.0141	0.691	0.06
		Diseased	113	284.92	0.608	1.713	4.292	6.287	

GSHt= glutathione total; GSSG= glutathione oxidation form; GSH=Glutathione; Redox= Glutathione reduction form.

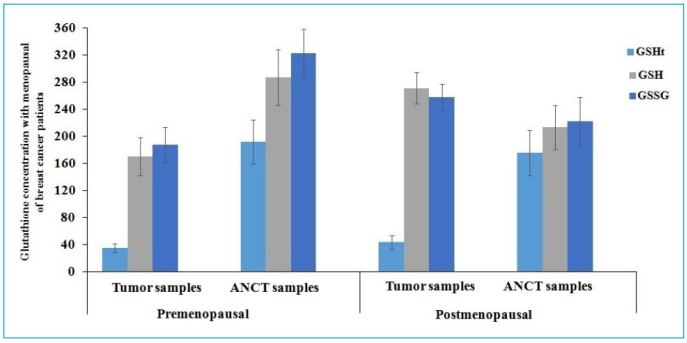

Figure 4. Association of GSH concentration with menopausal status of breast cancer patients.

ing to stages wise stratified, 86 samples belong to stage I, 64 belong to stage II and 48 belong to stage III of the study subjects as shown in table. The data reveal that the GSHt levels were decreased in breast cancer tissue samples in tumor stages (I, II and III) (24.499±24.527, 29.838±7.459, 28.556±14.428) compared to ANCT samples (134.955±34.488, 110.811±41.883, 136.776±96.714). The results obtained were statistically more significant in stages I and II of breast cancer (*p=0.014, ***p=0.003) as that of stage III in breast cancer patients (*p=0.031). The concentration of GSH, and GSSG was decreased as advancement in stages of breast cancer, however, the results were statistically not significant (p>0.05) (Fig. 5A, Table 4). Further, their levels were also correlated with different grades of breast cancers. Of total study cohorts (n=198), 78 grade I, 63 grade II, and 57 individuals belong to grade III. Similarly, the levels of GSHt were higher in breast cancer tissue samples in tumor grades (I, II and III) (211.675±122.27, 128.452±29.468, 142.11±82.047) as compared to ANCT samples (19.013±7.762, 26.19±4.194, 26.798±11.984). These result was highly significant in grades I and II (*p=0.03, *p=0.036) as compared to grade III individuals (*p=0.05) of breast cancer patients. Hence, the concentration of GSH and GSSG has no significant correlation with tumor grades (Fig. 5B, S Table 5).

Downregulation of GSTM1 Promotes Tumor Proliferation, Metastasis and Invasion in Breast Cancer Patients

Present study further evaluate the expression of various biomarkers, including tumor, proliferation, and invasion and metastasis in cancer tissue and ANCT samples of breast cancer patients. The result showed that the mRNA expression of tumor biomarkers (HER2 and CEA) were significantly upregulated in tumor tissue samples (***p=0.001, *p=0.03) as compared with ANCT samples (Fig. 6a). Similarly, tumor proliferation biomarkers were also analyzed in breast cancer tissues using RT- PCR. The result showed that mRNA expression of tumor proliferation markers (Ki-67, P-AKT, and Bcl2) were significantly increased in tumor tissues samples as compared with ANCT samples (**p=0.006, *p=0.009,

Table 4. Association of GSH levels with different stages of breast cancer patients

Variables	Analysis	Samples	N	Mean	SEM	95% CI of Mean		Skewness	p
						Lower	Upper		
Stage 1									
	GSHt	Control	86	1148.68	34.48	1075.17	1222.2	-0.18418	0.014
		Diseased	86	934.53	4.527	925.270	943.790	-0.1187	
	GSSG	Control	86	642.069	51.23	532.86	751.278	0.8634	0.065
		Diseased	86	677.978	33.10	610.27	745.688	-0.76445	
	GSH	Control	86	506.619	55.76	387.75	625.483	-0.08641	0.21
		Diseased	86	259.948	31.27	195.99	323.904	-1.11701	
	Redox	Control	86	1.8661	0.408	0.9957	2.73648	2.53881	0.003
		Diseased	86	4.7636	0.727	3.27604	6.25116	0.9853	
Stage II									
	GSHt	Control	67	1050.78	41.88	948.30	1153.26	0.96546	0.003
		Diseased	67	945.013	7.459	929.14	960.914	0.65063	
	GSSG	Control	67	534.841	46.92	420.03	649.651	-0.19222	0.06
		Diseased	67	663.138	43.38	570.657	755.619	-0.653	
	GSH	Control	67	515.942	72.63	338.21	693.677	-0.22875	0.31
		Diseased	67	281.875	42.20	191.91	371.841	0.64354	
	Redox	Control	67	1.2747	0.289	0.5652	1.98427	0.40614	0.22
		Diseased	67	3.3743	0.691	1.9005	4.84799	1.57434	
Stage III									
	GSHt	Control	45	1186.73	96.71	-42.123	2415.60	2.3683	0.031
		Diseased	45	930.737	14.42	884.82	976.654	-0.1378	
	GSSG	Control	45	722.887	64.14	-92.213	1537.98	1.5421	0.045
		Diseased	45	663.535	88.47	381.97	945.097	-0.84194	
	GSH	Control	45	463.852	160.8	-1580.1	2507.81	0.5491	0.61
		Diseased	45	267.201	79.74	13.428	520.974	1.13752	
	Redox	Control	45	1.82602	0.771	-7.9775	11.6295	1.8262	0.002
		Diseased	45	1.9652	0.560	0.1813	3.74927	-0.0463	

*p=0.014) (Fig. 6b). The result was highly significant. For further validation, tumor metastasis and invasion were determined in the tumor tissue samples and ANCT samples. The results revealed that the mRNA expression of tumor metastasis and invasion biomarkers (MMP-2 and MMP-9) were significantly increased in tumor tissue samples as compared with ANCT samples (*p=0.007, *p=0.009) (Fig. 6c).

Downregulation of GSTM1 Induces Epithelial to Mesenchymal Cell Transition Pathway

Epithelial to mesenchymal cell transition (EMT) plays a crucial role in appropriate morphogenesis during development. Deregulation of this process has been concerned as a key events in fibrosis and the progression of carcinomas to a metastatic state. However we analyzed EMT process in tumor tissue samples and ANCT samples. Both RNA and proteins were isolated to determined EMT pathway through RT-pcr and western blot. We finalized that downregulation of GSTM1 induced the mRNA expression of mesenchymal marker i.e vimentin (*p=0.019) Snail (**p=0.0074) and Fibronectin (**p=0.0089) via decreased the expression of epithelial marker i.e E-cadherin (**p=0.009), MUC-1 (**p=0.0082) and lamine (**p=0.0097) in breast cancer tissue samples as compared to their ANCT samples as shown in figure 7. Each results were repeated as triplicate.

Discussion

Several studies indicated that oxidative stress has been involved in the development and progression of cancer, clarifying that antioxidant treatment may contribute in protection from cancers.[17] Oxidative stress mostly implicated by a malignant cell is greater than their respective benign counterpart. Glutathione (GSH) plays central role in a number of cellular processes, such as differentiation, proliferation, and apoptosis. Aberration in GSH homeostasis are contributing in the progression and development of numerous human diseases, including cancer. The deficiency of GSH or reduction in the ratio of GSH/glutathione disulphide (GSSG) leads to promote susceptibility to oxidative stress

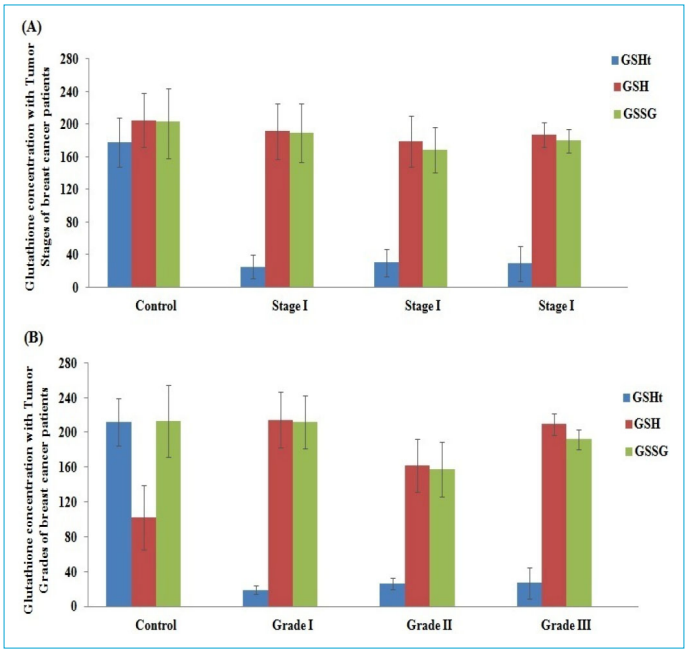

Figure 5. Association of GSH concentration with tumor stage and grade of breast cancer patients **(a)** Correlation of GSH level with different stages of breast cancer **(b)** correlation of GSH level with different grades of breast tumor tissue samples compared with ANCT samples.

intimated in the development of cancer. The depletion in GSH or blocking of GST results to enhanced cytotoxicity via oxidative stress in melanoma cells.[18, 19]

In the current study, the levels GSHt, GSH and GSSG were decreased according to the patients age, histological status and tumor stages and grades. Thereof, the concentration GSH total were significantly correlated with premenopausal status of patients. High levels of oxidative stress and decreased antioxidant status is strongly associated with age of individual in most of population.[20] The following studied also support our hypothesis that the GSH concentration is decreased in patient with age less than 50 (<50) as shown in figure 2. Previous study concluded that the amount of GSSG produced as a results of reaction among GSH and ROS is quick revert back to normal GSH, thus stabilizing the normal redox cycle.

In normal situations GSSG plays key role, to stable GSH in its reduced form.[21] Our results also supports the hypothesis that GSH levels were significantly decreased in breast cancer patients compared with control subjects, determining the mechanism that protection against ROS controlled by GSH could be lowered in breast cancer patients, pos-

Table 5. Association of GSH levels with different grades of breast cancer patients

Variables	Analysis	Samples	N	Mean	SEM	95% CI of Mean		Skewness	p
						Lower	Upper		
Grade I	GSHt	Control	89	1165.2	122.27	639.193	1691.31	-1.17745	0.002
		Diseased	89	915.25	7.762	895.303	935.207	0.61864	
	GSSG	Control	89	720.55	59.113	466.21	975.901	1.73049	0.045
		Diseased	89	601.01	87.437	376.242	825.766	-0.8931	
	GSH	Control	89	444.69	122.79	-83.616	973.001	1.13459	0.061
		Diseased	89	314.25	86.425	92.089	536.412	0.10275	
	Redox	Control	89	1.874	0.461	-0.109	3.857	-1.43487	0.071
		Diseased	89	4.849	1.452	1.115	8.582	0.15529	
Grade II	GSHt	Control	70	1117.5	29.468	1055.58	1179.41	0.15566	0.006
		Diseased	70	940.61	4.194	932.125	949.104	0.35828	
	GSSG	Control	70	589.01	45.549	493.309	684.701	1.31505	0.013
		Diseased	70	686.61	25.974	634.025	739.192	-0.79957	
	GSH	Control	70	528.49	49.278	424.958	632.018	-0.33417	0.06
		Diseased	70	254.01	25.27	202.844	305.167	0.70031	
	Redox	Control	70	1.699	0.424	0.807	2.592	2.35101	0.021
		Diseased	70	4.584	0.589	3.39	5.777	1.05052	
Grade III	GSHt	Control	39	1126.6	82.047	773.59	1479.64	1.08201	0.04
		Diseased	39	926.41	11.984	893.135	959.68	0.48935	
	GSSG	Control	39	703.34	41.879	523.147	883.531	1.72083	0.06
		Diseased	39	599.63	93.701	339.484	859.781	-0.21048	
	GSH	Control	39	423.29	101.35	-12.782	859.351	1.63559	0.052
		Diseased	39	326.78	85.815	88.522	565.031	0.35207	
	Redox	Control	39	1.864	0.447	-0.059	3.788	-0.44259	0.24
		Diseased	39	3.0236	1.2862	-0.5474	6.5947	1.45917	

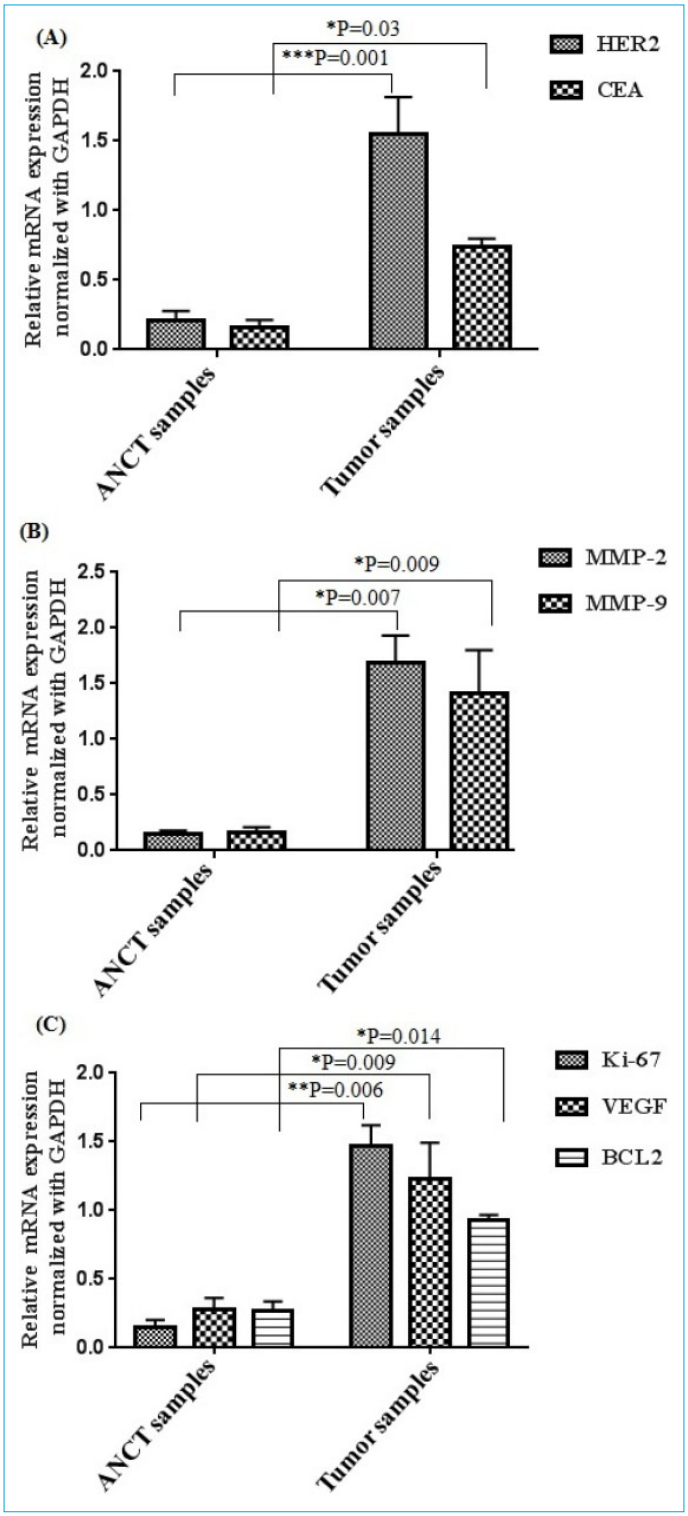

Figure 6. Determination of tumor proliferation, metastasis and invasion biomarker in breast cancer tissues **(a)** Relative expression of Her2 (***P=0.001) and CEA (*P=0.03) tumor markers, **(b)** Relative expression of MMP-2 (*P=0.007) and MMP-9 (*P=0.009) markers, **(c)** Relative expression of Ki-67(**P=0.006), VEGF (*P=0.009) and BCL2 (*P=0.014) markers in tumor tissue samples compared with ANCT samples.

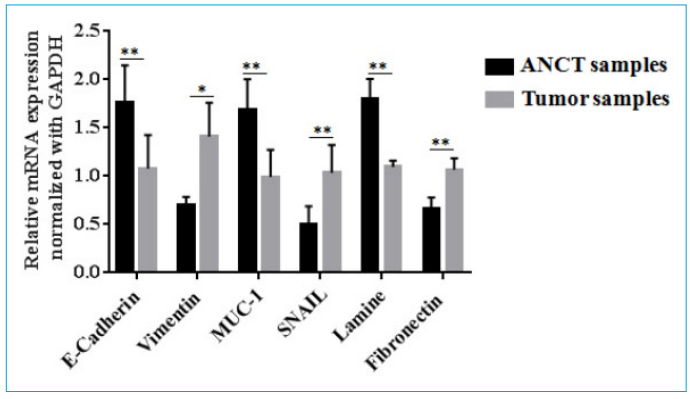

Figure 7. Aberrant expression of GSTM1 promote EMT pathway in breast cancer (A) Relative mRNA expression of EMT biomarkers, E-cadherin (**P=0.009), Vimentin (*P=0.019), MUC-1 (**P=0.0082), Snail (**P=0.0074), Lamine (**P=0.0097), Fibronectin (**P=0.0089) in tumor tissue samples as compared to ANCT samples in breast cancer patients.

sibly due to a abnormal redox cycle. The intracellular oxidation and redox level is well be balanced in the cells. The alteration in the redox level would cross this balance and occupied the potential to promote the oxidation level.[22] Didziapetriene J, et al. investigated that the antioxidant pathways and especially GSH levels are downregulated in breast cancer tissues.[23] Reduction in the ratios of GSH to GSSG were investigated in both serum and tissue samples of breast cancer patients as compared to serum and ANCT samples of the same patients. Our results are in agreement with already reported studies that the GSH and GSSG levels were decreased in breast cancer patients.[23]

In current study, we also determined the possible association of GSTM1 genetic polymorphism with development of breast cancer. The GSTs enzymes play important role in the detoxification of different products implied by cancer therapy, excited us to determined the prognostic efficiency of GSTs genotype deletion in breast cancer. Several studies have investigating the role of deletion in GST genotyping in correspondence with the development of breast cancers but still the results are conflicting.[24] Our finding support the previous result that the GSTM1 genotype was deleted in the tumor tissue samples of breast patients as shown in figure 1a.

The GSTM1 null genotype has been closely associated with the development of brain, stomach and lung cancer.[25, 26] With regard to breast carcinoma, the GSTM1 null genotype has not been closely linked with the elevated risk but revealed as a high risk factor for the eldest postmenopausal women also supported the present hypothesis. Vaury et al investigated that the GSTM1 genotype is involved to provoke CYPJA1 gene transcription.[27] The deletion in GSTM1 and GSTT1 alone or in combination with CYPIA1 polymorphisms leads to develop breast cancer. The deletion of both alleles of the GSTM1 gene is exist in 30-60% of mostly pop-

ulation, leading in complete deficit of the concerned enzyme.[28] In addition another study determined that GSTM1 null genotype or CYPJA1 polymorphism might be linked to increased the risk of breast cancer in postmenopausal Caucasian women.[29]

Revival of the EMT process is commonly regarded to be a core component of tumor progression and development. EMT is an embryonic network that relaxed cell-cell adhesion complexes and increased cells invasiveness and migration. EMT signaling pathway has also been considered as therapeutic targets in different disease, considering in a breast cancer in vitro models murine pancreatic cancer model, and even in clinical settings.[30] EMT is the course in which breast cancer cells lose epithelial features and gain mesenchymal phenotypes. Therefore, the present study determined the expression of EMT associated proteins in breast cancer tissue samples. Consistent with the reported results, we also found that the expression of epithelial markers was decreased, while the expression of mesenchymal markers were increased in breast cancer tissue samples, suggesting that GSTM1 might regulate the growth and aggressiveness of breast cancer through EMT process. A significant role of GSTM1 was determined in vascular smooth muscle cells (VSMC), identified that the downregulation of GSTM1 expression in these cells leads to enhanced oxidative stress, cell migration, metastasis and proliferation.[13] These studies support our results that the expression of tumor proliferation, metastasis and invasion biomarker is upregulated via EMT pathway in breast cancers tissues.

Conclusion

In conclusion, our outcomes identified that GSTM1 was downregulated in breast cancer tissues, and contributed to invasion, metastasis and migration abilities of breast cancer. Moreover, low expression of GSTM1 led to reduced the level of total GSH and mainly associated with poor prognosis in breast cancer patients. Simultaneously, the implementation of GSTM1 role was also correlated with the expression of proliferation, metastasis and invasion as well as EMT-related biomarkers in breast cancer tissues. These findings suggested new insights into the regulatory mechanism of GSTM1 in breast cancer. A limitation of our study was the lack of in vitro experiments to confirm the findings on breast cancer cells, and the lack of specific signaling pathway involved in GSTM1 regulation of breast cancer proliferation and apoptosis. Additional studies need to further elucidated the role GSTM1 in lymph node metastasis. In the next plan, we will focus on solving these problems.

Disclosures

Ethics Committee Approval: Kohat University of Science and Technology. Hospital name and date: Khyber Teaching Hospital (KTH-12-07-017) (25-05-018).

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – Ar.A.; Design – Ar.A., A.A.; Supervision – Ar.A.; Materials – Ar.A., A.A., SA..; Data collections processing – A.A., S.A.; Analysis and interpretation – Ar.A., A.A., S.A.; Literature search – A.A., S.A.; Writing – Ar.A., A.A., S.A.; Critical review – Ar.A., A.A., S.A.

References

1. Malik FA, Sanders AJ, Jones AD, Mansel RE, Jiang WG. Transcriptional and translational modulation of KAI1 expression in ductal carcinoma of the breast and the prognostic significance. Int J Mol Med 2009;23:273–8.
2. Ward E, Jemal A, Thun M. Regarding "Increase in breast cancer incidence in middle-aged women during the 1990s". Ann Epidemiol 2005;15:424–5; author reply 6–7. [CrossRef]
3. Gherghel D, Griffiths HR, Hilton EJ, Cunliffe IA, Hosking SL. Systemic reduction in glutathione levels occurs in patients with primary open-angle glaucoma. Invest Ophthalmol Vis Sci 2005;46:877–83. [CrossRef]
4. Ferreira SM, Lerner SF, Brunzini R, Evelson PA, Llesuy SF. Oxidative stress markers in aqueous humor of glaucoma patients. Am J Ophthalmol 2004;137:62–9. [CrossRef]
5. Strange RC, Spiteri MA, Ramachandran S, Fryer AA. Glutathione-S-transferase family of enzymes. Mutat Res 2001;482:21–6. [CrossRef]
6. Huang J, Tan PH, Tan BK, Bay BH. GST-pi expression correlates with oxidative stress and apoptosis in breast cancer. Oncol Rep 2004;12:921–5. [CrossRef]
7. The diabetes prevention program: evaluation and management of diabetes. Response to Adler and Turner and Singer et al The DPP Research Group. Diabetes Care 1999;22:1757–8.
8. McIlwain CC, Townsend DM, Tew KD. Glutathione S-transferase polymorphisms: cancer incidence and therapy. Oncogene 2006;25:1639–48. [CrossRef]
9. Suvakov S, Damjanovic T, Stefanovic A, Pekmezovic T, Savic-Radojevic A, Pljesa-Ercegovac M, et al. Glutathione S-transferase A1, M1, P1 and T1 null or low-activity genotypes are associated with enhanced oxidative damage among haemodialysis patients. Nephrol Dial Transplant 2013;28:202–12.
10. Rebbeck TR. Molecular epidemiology of the human glutathione S-transferase genotypes GSTM1 and GSTT1 in cancer susceptibility. Cancer Epidemiol Biomarkers Prev 1997;6:733–43.
11. Chirila DN, Balacescu O, Popp R, Oprea A, Constantea NA, Vesa S, et al. GSTM1, GSTT1 and GSTP1 in patients with multiple breast cancers and breast cancer in association with another type of cancer. Chirurgia (Bucur) 2014;109:626–33.

12. Qiu LX, Yuan H, Yu KD, Mao C, Chen B, Zhan P, et al. Glutathione S-transferase M1 polymorphism and breast cancer susceptibility: a meta-analysis involving 46,281 subjects. Breast Cancer Res Treat 2010;121:703–8. [CrossRef]
13. Yang Y, Parsons KK, Chi L, Malakauskas SM, Le TH. Glutathione S-transferase-micro1 regulates vascular smooth muscle cell proliferation, migration, and oxidative stress. Hypertension 2009;54:1360–8. [CrossRef]
14. Mishra A, Chandra R, Mehrotra PK, Bajpai P, Agrawal D. Glutathione S-transferase M1 and T1 polymorphism and response to neoadjuvant chemotherapy (CAF) in breast cancer patients. Surg Today 2011;41:471–6. [CrossRef]
15. Strauss WM. Preparation of genomic DNA from mammalian tissue. Curr Protoc Neurosci. 2001;Appendix 1:Appendix 1H.
16. Bogaards JJ, van Ommen B, van Bladeren PJ. An improved method for the separation and quantification of glutathione S-transferase subunits in rat tissue using high-performance liquid chromatography. J Chromatogr 1989;474:435–40.
17. Cabello CM, Bair WB, 3rd, Wondrak GT. Experimental therapeutics: targeting the redox Achilles heel of cancer. Curr Opin Investig Drugs 2007;8:1022–37.
18. Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronzato MA, et al. Role of glutathione in cancer progression and chemoresistance. Oxid Med Cell Longev 2013;2013:972913.
19. Benlloch M, Ortega A, Ferrer P, Segarra R, Obrador E, Asensi M, et al. Acceleration of glutathione efflux and inhibition of gamma-glutamyltranspeptidase sensitize metastatic B16 melanoma cells to endothelium-induced cytotoxicity. J Biol Chem 2005;280:6950–9. [CrossRef]
20. Knight JA. The biochemistry of aging. Adv Clin Chem 2000;35:1–62. [CrossRef]
21. DeLeve LD, Kaplowitz N. Glutathione metabolism and its role in hepatotoxicity. Pharmacol Ther 1991;52:287–305. [CrossRef]
22. Liang H, Zhou Z, Luo R, Sang M, Liu B, Sun M, et al. Tumor-specific activated photodynamic therapy with an oxidation-regulated strategy for enhancing anti-tumor efficacy. Theranostics 2018;8:5059–71.
23. Didziapetriene J, Kazbariene B, Tikuisis R, Dulskas A, Dabkeviciene D, Lukoseviciene V, et al. Oxidant/antioxidant status of breast cancer patients in pre- and post-operative periods. Medicina (Kaunas) 2020;56:70. [CrossRef]
24. Gudmundsdottir K, Tryggvadottir L, Eyfjord JE. GSTM1, GSTT1, and GSTP1 genotypes in relation to breast cancer risk and frequency of mutations in the p53 gene. Cancer Epidemiol Biomarkers Prev 2001;10:1169–73.
25. Kano SI, Choi EY, Dohi E, Agarwal S, Chang DJ, Wilson AM, et al. Glutathione S-transferases promote proinflammatory astrocyte-microglia communication during brain inflammation. Sci Signal 2019;12:eaar2124. [CrossRef]
26. Chen ZH, Xian JF, Luo LP. Association between GSTM1, GSTT1, and GSTP1 polymorphisms and gastric cancer risk, and their interactions with environmental factors. Genet Mol Res 2017;16. [CrossRef]
27. Vaury C, Laine R, Noguiez P, de Coppet P, Jaulin C, Praz F, et al. Human glutathione S-transferase M1 null genotype is associated with a high inducibility of cytochrome P450 1A1 gene transcription. Cancer Res 1995;55:5520–3.
28. Pacholak LM, Amarante MK, Guembarovski RL, Watanabe MAE, Panis C. Polymorphisms in GSTT1 and GSTM1 genes as possible risk factors for susceptibility to breast cancer development and their influence in chemotherapy response: a systematic review. Mol Biol Rep 2020;4:5495–501. [CrossRef]
29. Bailey LR, Roodi N, Verrier CS, Yee CJ, Dupont WD, Parl FF. Breast cancer and CYPIA1, GSTM1, and GSTT1 polymorphisms: evidence of a lack of association in Caucasians and African Americans. Cancer Res 1998;58:65–70.
30. Marcucci F, Stassi G, De Maria R. Epithelial-mesenchymal transition: a new target in anticancer drug discovery. Nat Rev Drug Discov 2016;15:311–25.