Comparison of NAT1, NAT2 & GSTT2-2 activities in normal and neoplastic human breast tissues

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Received April 29, 2005

In this study, arylamine N-acetyltransferases, NATs (E.C.2.3.1.5) and glutathione-S-transferase-T2-2, GSTT2-2 (E.C.2.5.1.18) enzyme activities in the breast tumor and surrounding tumor-free tissues of 22 female breast cancer patients with infiltrating ductal carcinoma were measured. The possible impacts of grade of malignancy, chemotherapy treatment, estrogen receptor status and menopausal status on all enzyme activities were evaluated. The results showed that, both NAT2 and GSTT2-2 display significant differences between tumor and tumor-free breast tissues, while no difference was observed in NAT1. Grade of malignancy seems to be positively associated with NAT1 and negatively associated with GSTT2-2. Though, both NAT2 and GSTT2-2 have increased mean tumor activities, the grade of malignancy, chemotherapy status, menopausal status or estrogen receptor status are not correlated statistically.

Key words: breast cancer, arylamine N-acetyltransferases, NAT1, NAT2, GSTT2-2

Breast cancer is the most common malignancy among 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 etiology still remains largely unknown. Dietary and/or environmental factors were suggested to play a role in initiation of breast cancer and breast is an organ that is more susceptible to chemical carcinogenesis due to its anatomical features [2, 3]. Potent carcinogens like polycyclic aromatic hydrocarbons (PAHs), aromatic and heterocyclic amines present in the diet, occupational and environmental exposures are commonly lipophilic in nature, so they can be stored and concentrated in the breast fat pad. Those carcinogens are thought to induce tumors in the mammary gland, after metabolic activation to reactive derivatives that form DNA adducts [4].

The PAHs activated by hydroxylation can be detoxified via glutathione conjugation by glutathione-S-transferases (GSTs). GSTs are a superfamily of enzymes composed of six major gene families (-A, -M, -P, -S, -T, and -Z) with overlapping substrate specificities [5]. Aromatic and heterocyclic amines are either directly detoxified by N-acetylation or transformed to more potent carcinogens by O-acetylation activity of N-acetyltransferases (NAT1 and NAT2) [6, 7]. Both NAT1 and NAT2 have genetic variants which have been correlated with biochemical phenotypes ranging from slow to fast acetylators [8]. Similarly, polymorphism in GST genes have resulted in low or absent enzyme activity [9]. Several studies related to GSTs and NATs were carried out in order to find evidence for gene-environment action in the breast cancer etiology. The GST activities towards 1-chloro, 2-4 dinitrobenzene (CDNB), the general substrate for the demonstration of multiple forms of GST excluding Theta class GSTs, has a tendency for elevation in breast tumors [10, 11]. However, contradictory data exists in regard to behaviour of different GST isozymes in breast tumors compared to their controls. In this context, besides GSTM1 which can detoxify carcinogenic PAHs like benzo[a]pyrene and mycotoxin aflatoxin, the Theta class GSTT1-1 isozyme is of particular interest and both isozyme activities are absent from approximately half of all Asians due to homozygous deletions of the respective genes [12]. Human Theta class GSTs (GSTT1-1 and GSTT2-2) display activity against a broad range of com-

pounds including carcinogens like methyl halides, sulfate esters or small reactive hydrocarbons such as ethylene oxide and diepoxybutane [8]. In rats, induction of Theta class isozymes by a variety of chemopreventive agents has been demonstrated [13, 14]. In addition, they have distinct lipid peroxidase activity [15]. The GSTM1 and GSTT1 null-genotype was significantly associated with increase of breast cancer risk for alcohol-consuming premenopausal women [16], while other studies suggested an association between GSTM1 null genotype and breast cancer risk in postmenopausal women [17, 18]. Besides GSTs, the role of NATs in heterocyclic amine activation within human breast and development of breast cancer were investigated in several studies. We had detected a higher mean activity of NAT2 in human breast cancer tissues compared to their controls [19. Similarly, AMBROSONE et al, had suggested that extensive acetylation may be related to lobular breast cancer [20]. LEE et al have demonstrated that tamoxifen (antiestrogen drug) decreases NATs activities in human breast cancer tissue [21]. WILLIAMS et al suggested an association of NAT1 but not NAT2 with DNA adduct formation [22]. Recent studies have focused on the potential association of GST isozymes and NATs in breast cancer initiation, but the outcomes have been inconsistent. In this regard, FIROZI et al suggested that besides GSTM1, polymorphism of NAT2 significantly affected either the frequency or the level of DNA adducts in normal breast tissues of women having breast cancer, especially in smokers [23]. However, in a genotype study conducted on French-Canadian population, the results argue agaist a major role for GSTM1, GSTT1 and GSTP1, but suggest an increased risk among women who consume well-done meat with NAT1*10 allele and increased frequency of NAT2 rapid acetylators among smokers [24]. The associations of the GSTs and NATs in relation to breast cancer are still not clear. There are few studies on phenotypic expression of NATs in human breast cancer tissues. While, characteristics of Theta class make it reasonable to assume that the isozyme GSTT2-2 might also have a role in the carcinogenesis and in the sensitivity of tumors against anticancer drugs, there is no data on the activity level of GSTT2-2 in the human breast tissue, and it is not known whether there is an association between NATs and GSTT2-2 activities. We, therefore, wanted to measure the enzyme activities of NAT1, NAT2 and GSTT2-2 in the breast tumor and surrounding tumor-free tissues of 22 female breast cancer patients with infiltrating ductal carcinoma. We also evaluated the possible impacts of grade of malignancy, chemotherapy treatment, estrogen receptor status, menopausal status on enzyme activities.

Material and methods

Patients and tumor samples. Breast tumors and surrounding tumor-free (normal, taken as control; up to 3 cm from the tumor) tissues were obtained from 22 female breast cancer patients with infiltrating ductal carcinoma aged between 37–76 (52±13, mean ±SD) who had undergone mastectomy in Oncology Hospital, Demetevler, Ankara, Turkey. Tissue samples were fixed in 10% formalin for histological examination and remaining tissue was used for kinetic analysis. Histopathological examination was undertaken to differentiate normal and malignant tissues. To avoid contamination of surrounding normal tissue, the central parts of the tumors were utilized. After fixation, tumors and normal tissues were dehydrated, embedded, sectioned (5 μm) and stained with hematoxylin and eosin. All patients completed a questionnaire on factors that might have influenced the expression of NATs and GSTT2-2, such as medication, smoking and alcohol consumption. Six of the patients received 2–3 cycles of adjuvant standard combination chemotherapy (cyclophosphamide, methotrexalate, classical CMF, cyclophosphamide, epirubicin, 5-fluorouracil, CEF, Navelbine and Adriablastin) before mastectomy. Three of the patients were current smokers (10–20 cigarettes/day). None of the patients were alcohol drinkers. The menopausal status, estrogen receptor status and grade groupings of the patients were recorded. The study was approved by the regional Ethics Committee.

Enzyme assay procedures. Human breast tumor and tumor-free tissues were minced and suspended in three volumes times its mass of 20 mM Tris-HCl buffer of pH 7.5 containing 0.25 mM PMSF, 1mM EDTA and 1 mM DTT. The homogenates were centrifuged at 10,000 g to remove cell debris, nuclei and mitochondria. Cytosols were prepared by subsequent centrifugation at 133,537 g at 4 °C for one hour. Supernatants (3 ml aliquots) were stored at –80 °C for enzyme assays. The activity of the NATs was determined spectrophotometrically as explained previously [25]. The substrate 1-menaphthyl sulfate (MS) for GSTT2-2 was prepared according to the method of CLAPP and YOUNG [26]. The conjugation product formation was monitored spectrophotometrically to determine the activity of the GSTT2-2 by using modified method of HABIG et al [27]. The estrogen receptors in tumors were determined using immunocytochemical assay described by PERTSHUCK et al [28]. Protein was determined by the method of LOWRY et al [29].

Statistical analysis. The results were expressed as the mean ± standard error (SE). Differences between the means were compared with the Wilcoxon rank-sum test. P-value of 0.05 was taken to denote significance. Correlations were assessed by the Spearman rank tests.

Results

The distribution of enzyme activities from human breast cancerous and matched control samples and the percent change in tumor activities compared to controls for NAT1, NAT2 and GSTT2-2 are given in Table 1. Large interindividual variations were found in both tumor and tumor-free tissues. Mean enzyme activities in breast tumor tissues were significantly higher than their matched controls excluding NAT1 isozyme. NAT1 activity of tumors and tumor-free

Table 1. Arylamine NATs and GSTT2-2 activities in tumors and surrounding tumor-free (control) breast tissues of 22 breast cancer patients with infiltrating ductal carcinoma[a]

		NAT1				NAT2				GSTT2-2			
	n	Control	%	Tumor	%	Control	%	Tumor	%	Control	%	Tumor	%
Total	22	14±3[b]	**100**	19±3	**135**	10±2	**100**	33±7[c]	**340**	272±61	**100**	424±79[c]	**155**
		(0–53)[d]		(0–52)		(0–35)		(0–132)		(0–1200)		(0–1250)	
Chemotherapy[e]													
+	6	11±3	**100**	18±6	**163**	9±4	**100**	30±15[c]	**330**	261±190	**100**	295±194	**113**
		(0–19)		(0–42)		(0–25)		(0–102)		(0–1200)		(0–1250)	
–	16	16±4	**100**	20±4	**125**	10±3	**100**	36±7[c]	**370**	276±53	**100**	472±81[c]	**171**
		(0–53)		(0–53)		(0–35)		(0–132)		(0–710)		(0–1153)	
ER Status[f]													
+	5	13±3	**100**	20±6	**154**	10±4	**100**	51±21[c]	**216**	236±89	**100**	422±140	**197**
		(0–20)		(0–42)		(0–25)		(0–132)		(0–541)		(0–793)	
–	17	15±4	**100**	20±4[c]	**133**	9±3	**100**	28±4[c]	**361**	285±77	**100**	420±97[c]	**103**
		(0–53)		(0–53)		(0–63)		(0–13)		(0–1200)		(0–1250)	
Grade													
1	2	7±3	**100**	23.2±9	**331**	13±3	**100**	93±25	**715**	161±61	**100**	359±137	**225**
		(0–13)		(0–42)		(8–21)		(21–132)		(0 – 0.292)		(0 – 65)	
2	13	11±3	**100**	15±4	**136**	9±3	**100**	27±5[c]	**300**	319±89	**100**	483±103[c]	**151**
		(0–40)		(0–38)		(0–25)		(0–63)		(0–1200)		(0–1250)	
3	7	17±3	**100**	21±3	**123**	8±5	**100**	24±6[c]	**300**	251±107	**100**	394±159[c]	**158**
		(6–24)		(6–27)		(0–35)		(6–46)		(0–710)		(145 –1153)	
Premenopause	11	12±4	**100**	19±4[c]	**158**	10±3	**100**	29±5[c]	**298**	336±109	**100**	501±130[c]	**149**
		(0–26)		(0–38)		(0–35)		(0–63)		(0–1200)		(0–1250)	
Postmenopause	11	17±5	**100**	19±5	**112**	10±3	**100**	40±12	**420**	207±55	**100**	347±89[c]	**168**
		(0–53)		(0–53)		(0–25)		(0–132)		(0–541)		(0–793)	

[a]Activities are given as pmol/min/mg protein; [b]mean±SE; [c]significantly different from respective control with $p<0.05$; [d]minimum and maximum range; [e]+: chemotherapy received, –: chemotherapy not received; [f]estrogen receptor status, +: positive, –: negative.

breast tissues ranged from 0–53 and 0–52 pmol/min/mg protein. Although, more than half of the tumor samples (12/22, 55%) had higher NAT1 activity than their corresponding normal tissues, no significant difference was noted between the mean NAT1 activity of tumor and matched control samples. In three patients (3/22, 13.6%) tumor and tumor-free breast tissue NAT1 activity was non-detectable. Smoking did not seem to stimulate the NAT1 activity as for the three patients activities detected in tumor tissue were either indifferent or not significantly changed when compared to matched control samples. There was no significant difference between tumor and tumor-free tissues' mean NAT1 activities in different grades or within chemotherapy treated and untreated group. However, menopausal state of the patient seems to have an effect on the mean tumor NAT1 activity. Mean NAT1 activity of tumor tissues of premenopausal patients (11/22, 50%) was significantly higher than in control group. Similarly, among estrogen receptor negative patients NAT1 mean tumor tissue activity was significantly higher compared to tumor-free tissues. When grade 2 tumor NAT1 activity was compared to tumor activity in grade 3, a significant increase was observed. The increase in mean NAT1 activity in grade 3 compared to mean NAT1 activity in grade 2 was also observed among tumor-free tissues. This indicated a possible positive relationship between grade of the malignancy and NAT1 activity, as tumor and tumor-free tissue NAT1 mean activities were significantly increasing with increase in grade.

NAT2 activities of tumor and tumor-free tissues ranged from 0–35 and 0–132 pmol/mg protein/min, respectively. NAT2 displayed higher activity in 91% (20/22) of tumor tissues compared to their respective controls, mean breast tumor NAT2 activity was significantly higher than corresponding tumor-free breast tissues with $p<0.05$. Among control tissues, the percentage of measurable NAT2 activity was 68% (15/22), while in tumor tissues it increased 91% (20/22). Smoking did not seem to stimulate the NAT2 activity as for the three patients' activities detected in tumor tissue were either indifferent or not changing significantly compared to

matched control samples. When other factors like chemotherapy status, estrogen receptor status, grade of malignancy or menopausal state of the patients are considered, it is observed that the mean NAT2 normal (tumor-free) breast tissue activities are not different from each other (8±5–13±3, mean ±SE). Mean tumor NAT2 activity among estrogen receptor positive patients was determined to be 1.8-fold higher than of estrogen receptor negative group. Also, postmenopausal patients displayed about 1.4-fold higher mean NAT2 activity in the tumor tissues compared to premenopausal group. However, those increases in mean NAT2 activities among tumor tissues didnot reach statistical significance.

Theta class isozyme GSTT2-2 activities detected in tumor and tumor-free tissues ranged from 0–1200 and 0–1250 pmol/mg protein/min, respectively. GSTT2-2 activities were higher than their respective control tissues in 82% of breast tumor tissues (Tab. 2). Similar to arylamine NAT2, mean tumor GSTT2-2 activity was significantly higher when compared to tumor-free controls with $p<0.05$. Among control tissues, the percentage of measurable GSTT2-2 activity was 82% (18/22), while in tumor tissues it increased to 86% (19/22). When data is grouped according to chemotherapy treatment status of the patients, it was observed that the control GSTT2-2 activities were not significantly changed in either group but, mean GSTT2-2 tumor activities in chemotherapy group were 1.6-fold higher than tumor activities of those who actually received chemotherapy. This tendency was also observed with GSTT2-2 tumor activity in between histological grades. Mean tumor activities of this isozymes was shown to decrease significantly, as grade increases from II to III with $p<0.05$. Tumor GSTT2-2 activities seemed to be affected also by the menopausal status of the patients. Premenopausal patients were showing significantly higher GSTT2-2 activity compared to mean GSTT2-2 activity in postmenopausal group. No such association could be detected when estrogen receptor status of the patients was considered. Similarly, smoking did not seem to stimulate the GSTT2-2 activity as for three patients' activities detected in tumor tissue were either indifferent or not changing significantly when compared to matched control samples.

Table 2. The number and percentage of tumor samples that have higher values than their corresponding tumor-free (control) breast tissues in 22 paired samples

	n	Percentage
NAT1	12	55
NAT2	20	91
GSTT2-2	18	82

Discussion

In this study, low activities and wide interindividual variations were found in NAT1, NAT2 and GSTT2-2 activities in tumor and tumor-free breast tissues of 22 female breast cancer patients with infiltrating ductal carcinoma. The reason for wide distribution of all enzyme activities investigated could be due to the genetic variability of the isozymes among the patients, or to variable exposure to inducers and thus different level of induction or to a combination of these factors. In the current study, no significant difference in the NAT1 activity was noted between tumors and tumor-free breast tissues. This confirms our previous study conducted in a smaller group of breast cancer patients (n=12) with infiltrating ductal carcinoma where NAT1 mean tumor activities were not different from respective controls. KRAJINOVIC et al [24] reported a 4-fold increased risk of breast cancer with NAT1*10 allele among women who consumed well-done meat. NAT1*10 is an allele with a mutation at 3' polyadenylation site, resulting in rapid acetylator phenotype, but its prevalence in our population is not known. However, when data is grouped according to grade of malignancy and menopausal state of the patients, significant differences are noted in NAT1 activities. Mean NAT1 tumor and tumor-free breast tissues were not significantly different from each other in either grade. When grade 2 tumor NAT1 activity was compared to tumor activity in grade 3, a significant increase was observed. The increase in mean NAT1 activity in grade 3 compared to mean NAT1 activity in grade 2 was also observed among tumor-free tissues. Among premenopausal patients mean NAT1 activity exhibited a significant increase in tumor tissues, but among postmenopausal patients values were not statistically significant. This is the first study investigating the activities of NAT1 in tumor and tumor-free tissues in patients of breast cancer according to menopausal status. Previous studies had found associations of slow NAT2 acetylation among postmenopausal breast cancer patients who are active smokers. However, there existed no significant differences between the NAT1 activity of tumor and tumor-free breast tissue, regardless of chemotherapy treatment or estrogen receptor status.

As, 91% of tumor tissues (20/22) among patients possessed higher NAT2 activities than controls, mean NAT2 activity tumor tissues were significantly higher than the mean tumor-free NAT2 activity. This tendency was not changing when chemotherapy status, estrogen receptor status, grade of malignancy or menopausal status is considered. Although, WILLIAMS et al [22] have detected NAT2 mRNA to be two to three-fold lower than NAT1 mRNA in healthy breast tissue obtained from reduction mammoplasty operations, we detected significant increases in NAT2 but not in NAT1 activities in tumor tissues compared to tumor-free tissues [19]. When chemotherapy given patients' tumor NAT2 activities were compared to tumor activities in the group without treatment, it was observed that there existed no significant

change. This was also the case for tumor-free NAT2 activities in both groups. In human lung cell-line, both NAT1 and NAT2 were shown to be inhibited by the chemopreventive agent, paclitaxel [30], as no such data exists in literature for NATs in human breast cancer tissues, we are unable to compare our results. Mean tumor NAT2 activity of estrogen receptor positive patients (n=5) were found to be 1.8 fold higher than mean tumor activity of estrogen negative patients, the difference was not significant. LEE et al [21] in his work has shown that estrogen receptor positive patients NAT1 and NAT2 activities are more inhibited via the antiestrogenic drug, tamoxifen, compared to estrogen receptor negative patients. There is no information whether those patients who are estrogen receptor positive had usage of antiestrogenic drugs in their treatment and also, the estrogen receptor positive group is quite small in sample size. Due to these reasons the increase in NAT2 mean tumor activity might not be reaching significance. Similarly, a 1.4 fold increase in mean tumor NAT2 activity in postmenopausal patients was observed compared to NAT2 tumor activity in premenopausal patients without statistical significance. Therefore, no clear association between menopausal status and NAT2 activities could be established.

Theta class isozyme, GSTT2-2, had significantly higher tumor activity than matched controls, which was also reflected as increased percentage (82%) of tumor GSTT2-2 activities that are higher than their respective controls. This is the first report about GSTT2-2 isozyme in human breast cancer where tumor and tumor-free tissue activities were investigated. Previously, with GSTT1-1 we couldn't detect any difference between tumor and tumor-free breast tissues [10]. In a study conducted on human colorectal and gastric cancer tissues, both GSTT1-1 and GSTT2-2 were found to be present at high levels in tumor-free tissues, whereas after malignant transformation, expression is not influenced or even down-regulated [31]. Regulation of the isozymes in target organs might be different depending on the tissue specific factors and relative exposure to xenobiotics and degree induction might differ in different cancer sites. As far as chemotherapy status is considered, a significant difference between tumor and tumor-free tissues was noted only among patients who didn not receive any chemotherapy treatment. Moreover, it was observed that mean tumor GSTT2-2 activity among patients who had chemotherapy treatment was 1.6 fold lower than tumor activity among patients who were not treated with chemotherapy. On contrary, in rats, a variety of chemopreventive agents were shown to be inducers of Theta class isozymes. GSTT2-2 activity does not seem to be affected by the estrogen receptor status, as the mean tumor activity in estrogen receptor positive group is not different from mean tumor activity in estrogen receptor negative group. In terms of comparison of the tumor and tumor-free GSTT2-2 activities within two groups, a significant difference is noted only in the estrogen receptor negative group. In relation to grade of malignancy, a possible association, which is opposite to the one observed with NAT1 is detected. When grade 2 mean tumor or tumor-free GSTT2-2 activity is compared to grade 3 tumor and tumor-free GSTT2-2, respectively, there is a trend for significant decrease in both mean tumor and tumor-free GSTT2-2 activity in grade 3.

In conclusion, NAT2 and GSTT2-2 display significant differences between tumor and tumor-free breast tissues, while NAT1 exhibits no differences in a total of 22 female breast cancer patients with infiltrating breast carcinoma. Grade of malignancy seems to be positively associated with NAT1 and negatively associated with GSTT2-2. Both NAT2-2 and GSTT2-2 have a trend for increased mean tumor activities, but the role of these isozymes in chemical carcinogenesis could not be clearly established in breast cancer irrespective of grade of malignancy, chemotherapy status, menopausal status or estrogen receptor status.

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