Genetic polymorphisms and transcriptional pattern analysis of CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 genes in breast cancer

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Abstract. In order to detect the contribution of cytochrome P450 1A1 (CYP1A1), aryl hydrocarbon receptor (AhR), glutathione S-transferases M1 (GSTM1), P1 (GSTP1), and T1 (GSTT1) genes in breast cancer, genetic analysis was performed, as well as transcriptional analysis in sporadic primary tumours and corresponding adjacent normal tissues from the same patient. CYP1A1 3'-untranslated region (3'-UTR) termed as m1 (*Msp*I) polymorphism and the null(-) deletions of both GSTM1 and GSTT1 genes were examined in genomic DNA from blood samples of 207 female breast cancer patients and 171 age and sex matched controls. The frequencies of the m1 genotype of the CYP1A1 gene in cases and controls were 0.13 and 0.15, while the frequencies of homozygotes with GSTM1(-) were 0.52, in each, and for homozygotes with GSTT1(-) were 0.14 and 0.10, respectively. Statistical analysis of these genotypes in combinations did not reveal any significant difference between the breast cancer population and the control group. Expression of mRNA levels of CYP1A1, GSTM1, GSTP1, GSTT1 and AhR genes in 31 breast cancer patients, revealed inter-individual variation in an independent manner to patient age, genotype, or tumour stage. Eighty-seven percent of the tumour specimens tested were deregulated, compared to their normal counterparts, in at least one locus. Up-regulation of CYP1A1 was observed only when one of the GSTM1 or GSTP1 was down-regulated while the other remained constant. Genotyping analysis did not show any correlation to breast cancer risk. However, RT-PCR analysis provided evidence that CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 genes are frequently deregulated in breast cancer and could be used as molecular biomarkers for better clinical management of such patients, with respect to chemotherapy.

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Introduction

Environmental chemicals, such as polycyclic aromatic hydrocarbons (PAH) and dioxin, as well as high levels of endogenous compounds, such as estrogens have been associated with increased breast cancer risk. The main mechanism of their metabolism includes activation of procarcinogens by cytochrome P450s (CYPs, phase I enzymes) and enhancement of the detoxification of these activated carcinogens by glutathione S-transferases (GSTs, phase II enzymes). Most of the human metabolizing enzymes are genetically polymorphic, and these polymorphisms may affect the enzyme activity, transcriptional regulation or mRNA stability (1).

Cytochrome P450 1A1 (CYP1A1) polymorphism m1 in the 3'-untranslated region (3'-UTR) is an *Msp*I restriction fragment length polymorphism (RFLP), stemming from a T→C transition, 250 bp downstream of the polyadenylation site, which implies differences both in its regulation and transcript half-life in cytosol (2,3). This mutation determines three genotypes, namely wt/wt, which is the wild-type lacking the *Msp*I cleavage site, wt/m1 and m1/m1, which are heterozygous and homozygous respectively for the polymorphic allele with the *Msp*I site. The m1 allele has been associated with higher induction of CYP1A1 (4,5). The higher enzyme activity would result in increased levels of carcinogenic intermediates, leading to greater risk for cancer development. However, CYP1A1 catalyses the hydroxylation of 17ß-estradiol (E_2) and its induction could serve as protection against breast or endometrial tumourigenesis (6-8). Several studies associated this genotype with a higher risk for lung (2,9), oral (10), and colorectal cancer (11), while the results in breast cancer were contradictory (12,13).

Perhaps the largest body of mechanistic information centres on the induction of CYPs 1A by the aromatic hydrocarbon receptor (AhR) (14). CYP1A1 transcription depends on both AhR and AhR nuclear transporter (ARNT) gene products (15). Since AhR is bound to its chemical ligands and is transported in the nucleus by ARNT and binds on to the CYP1A1 promoter, where it induces gene transcription. Besides CYP1A1, AhR and ARNT regulate CYP1A2 and four other non-P450 genes, which also have an AhR response element (AHRE) DNA motif at their promoters (16). AhR affects both cell cycle arrest and apoptosis, via direct interaction on the retinoblastoma (pRb) master regulator and tumour

suppressor gene (17) and by an indirect effect of oxidative stress on p53 protein (16). AhR interacts with estrogen response elements suggesting cross talk between AhR and estrogen receptor (ER), an issue of importance in breast cancer biology (18,19).

The cytosolic glutathione S-transferase (GST) enzyme family activity is due to the presence of multiple isoenzymes. These multifunctional dimeric proteins catalyse many reactions between glutathione (GSH) and lipophilic compounds with electrophilic centres (20). However, mutagenicity of several substances (haloalkanes and haloalkenes) is increased by the action of GSTs (21,22). GSTM1, GSTP1 gene products can detoxify PAHs while GSTT1 detoxifies smaller hydrocarbons and all of them may also play a role in the metabolism of lipids, chemotherapeutic agents and reactive oxygen species (23). GSTM1 and GSTT1 are both polymorphic due to large deletions of their structural gene (24,25). Although considerable ethnic differences have been observed, the homozygous null genotype of GSTM1 is in general more frequent than GSTT1 (24).

We investigated in a case-control study whether the genotyping profiles for m1 polymorphism of CYP1A1, as well as GSTM1 and GSTT1 null deletions are involved in susceptibility to sporadic breast cancer. Moreover, we compared the mRNA expression patterns of CYP1A1, AhR, GSTM1, GSTP1 and GSTT1, between sporadic breast cancer primary tumours and adjacent normal tissues from the same patient, in order to eliminate inter-individual variations previously reported (26). We examined statistically the correlations between the gene transcription patterns, patient or tumour clinical parameters and CYP1A1, GSTM1 and GSTT1 genotypes. Genotyping analysis did not show any correlation to breast cancer risk. However, RT-PCR analysis provided evidence that in 87% of the breast tumour specimens tested, CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 genes were deregulated compared to the adjacent normal correspondent tissues. This finding is of clinical importance, with respect to chemotherapeutic management of breast cancer patients.

Materials and methods

Specimens. Blood samples were collected from 207 Greek female patients, aged 31-79 (56.3±12.5) years, with breast cancer and 171 Greek healthy female controls aged 29-82 (56.7±14.1) years at the 'Prolepsis' Centre of Breast Cancer Diagnosis and Research, Athens, Greece, and stored at 4°C. Thirty-one tumour specimens, from breast cancer patients, aged 36-76 (56.0±10.3) years, paired with their corresponding adjacent normal tissue were surgically obtained and stored at -70°C (27), all corresponded to primary tumours. Clinical data (stage, grade, histological subtype, age) were available for all the specimens tested. The tumours were all characterised as sporadic, while familial histories of the healthy individuals were cancer-free. The University of Crete ethics committee approved this study and all the patients gave written informed consent.

DNA and RNA extraction. Genomic DNA was extracted from blood and tissue using proteinase K, followed by phenol extraction and ethanol precipitation according to standard procedures (28). DNA was resuspended in 50 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Working stocks were prepared by 10-fold dilution in double distilled H_2O. Total RNA extractions were performed from tissues using the TRIzol reagent (Life Technologies Ltd., UK) according to standard procedures (29) followed by digestion with RNase-free DNase I. DNA and RNA concentrations and purity were determined on a Hitachi Model U-2001 UV/Vis spectrophotometer (Hitachi Instruments Inc., USA) by the 260 nm absorbance and 260-280 nm absorbance ratio respectively. One % agarose gel electrophoresis and ethidium bromide staining were used to examine DNA and RNA integrity.

Restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) methods. PCR assays were performed by introducing 100 ng of genomic DNA in a PCR reaction mixture containing 1X PCR buffer, 200 μM dNTPs, 2.0 mM $MgCl_2$ and 0.35 U Taq DNA polymerase (Life Technologies Ltd., UK) to a 15 μl total reaction volume. Amplification parameters were: 3 min for initial denaturation at 94°C; 30 sec at 94°C, 30 sec at annealing temperature, 72°C for 30 sec, these steps repeated for the indicated cycles; final extension step at 72°C for 10 min. The oligonucleotide primer sequences for the amplification of CYP1A1 3'-UTR and for the co-amplification of either GSTM1 or GSTT1 with ß-globin as an internal positive control were used to a final concentration of 0.3 μM as shown in Table I. PCR assays were carried out in a PTC-100 programmable thermal controller (MJ Research Inc., USA). For the CYP1A1 m1 polymorphism the 340 bp PCR product underwent complete digestion with *Msp*I (New England Biolabs Inc., USA) (30). If the product remained uncut then the sample were characterized as wt/wt and as wt/m1 if the digestion produced a pattern constituted from two DNA restriction fragments sized 134 bp and 206 bp, followed by the 340 bp PCR product, if only the former fragments were observed as m1/m1.

Reverse transcriptase-polymerase chain reaction (RT-PCR) method. Reverse transcription reactions for the preparation of first strand cDNA from 3 μg of total RNA, were performed for 1 h at 52°C, using 15 U Thermoscript reverse transcriptase, 40 U RNaseOut, 50 ng of random hexamers and 1.0 mM of each dNTP in a total volume of 20 μl of 1X first strand cDNA synthesis buffer containing 5 mM dithiothreitol (DTT), ensued by incubation for 20 min at 37°C with 2 U of *E. coli* RNase H to avoid RNA contamination of cDNA, according to manufacturer's protocol (Life Technologies Ltd., UK). Multiplex PCR assays, in three panels (Table II), were carried out in a PTC-100 programmable thermal controller; 2 μl of cDNA was amplified in a total volume of 10 μl containing, 1X PCR reaction buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, and 0.6 U Platinum Taq DNA polymerase, with 30 pmol of each primers set. Cycling parameters were as follows: 3 min for initial denaturation at 94°C; 30 sec at 94°C, 30 sec at 55°C for primers annealing, 40 sec at 72°C for primers extension, these steps repeated for 30 cycles; final extension step at 72°C for 10 min. The novel primer set for GSTM1 mRNA amplification (Table II) was designed in order to avoid co-amplification of other GSTMs mRNA using the J03817 as

Table I. Oligonucleotide primers used for genetic polymorphism analysis.

Gene	Primer sequences	bp	Ta (˚C)[a]	Cycle	Refs.
CYP1A1	FP: CAGTGAAGAGGTGTAGCCGCT RP: TAGGAGATCTTGTCTCATGCCT	340	65	30	(30)
GSTM1	FP: GAACTCCCTGAAAAGCTAAAGC RP: GTTGGGGTCAAATATACGGTGG	218	55	36	(31)
GSTT1	FP: ATGACCCCCACACCCACAGT RP: CCACATTCCCAGCCTCACCT	187	55	36	(31)
ß-globin	FP: CAACTTCATCCACGTTCACC RP: GAAGAGCCAAGGACAGGTAC	267	55	36	(31)

[a]Annealing temperature. FP, forward primer; RP, reverse primer.

Table II. Oligonucleotide primers used at transcriptional level and in genomic alteration analysis.

Gene	Primer sequences	bp	Panel[a]	Refs.
CYP1A1	FP: CTCTTAGGTGCTTGAGAGCCC RP: CATCAGCATCTATGTGGCCC	244	A	UniSTS[b] name: sts-K03191
AhR	FP: GTCTATTTATCTCTATCCTG RP: TTTTACTATCTTGAAAGAGCCC	212	B	UniSTS name: sWSS2972
GSTM1	FP: TGAGAAACTGAAGCCAAAGT RP: AGCAGTTGGGCTCAAATATA	164	B	Novel set designed for this study
GSTP1	FP: AGAAAGGAAGGCAAACTCTGC RP: TGATCCATGAGGTCCTAGCC	194	A	UniSTS name: SHGC-11734
GSTT1	FP: ATGACCCCCACACCCACAGT RP: CCACATTCCCAGCCTCACCT	187	C	(31)
ß2-microglobulin	FP: TCCAACATCAACATCTTGGT RP: TCCCCCAAATTCTAAGCAGA	122	A, B, C	UniSTS name: mp0554

[a]Panel of 3-plex and 2-plex reaction. [b]Sequence tagged sites, source http://www.ncbi.nlm.nih.gov/genome/sts. FP, forward primer; RP, reverse primer.

reference sequence and http://www.ncbi.nlm.nih.gov/BLAST. ß2-microglobulin was used as an internal control in all the above reactions.

To evaluate the above conditions for specific and optimum amplification of each primer set of a given panel, and to achieve integrated density measurements at the exponential amplification phase, we performed kinetic analysis increasing the number of PCR cycles from 24 to 38, for different annealing temperatures 53˚C, 55˚C and 57˚C (data not shown). For this purpose a mixture of all cDNA preparations were used as reference sample. These pilot optimization experiments helped to eliminate the incidence of competition between the different primer sets in multiplex PCR assays.

Genomic PCR analysis for the detection of copy number alterations. To determine whether alterations of gene expression of CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 were due to gene amplification or caused by other events, genomic DNA was isolated from the same tissues and proceeded to PCR (32). The above multiplex PCR assays (Table II) were performed under the same conditions with 100 ng of genomic DNA. To eliminate the possibility of mRNA contamination, the DNA preparations from tissues were treated with *E. coli* RNase H (Life Technologies Ltd., UK) for 20 min at 37˚C. The internal control for this procedure was ß2-microglobulin.

Table III. Distribution of individual genotypes.

	CYP1A1			GSTM1		GSTT1		Total
	wt	wt/m1	m1	+	-	+	-	
Normal cases	123	45	3	82	89	154	17	171
Frequency	0.72	0.26	0.02	0.48	0.52	0.90	0.10	1.00
%	74.11	23.21	2.68	47.95	52.05	90.06	9.94	100.00
Breast cancer cases	156	48	3	99	108	177	30	207
Frequency	0.75	0.23	0.01	0.48	0.52	0.86	0.14	1.00
%	69.42	29.75	0.83	47.83	52.17	85.51	14.49	100.00

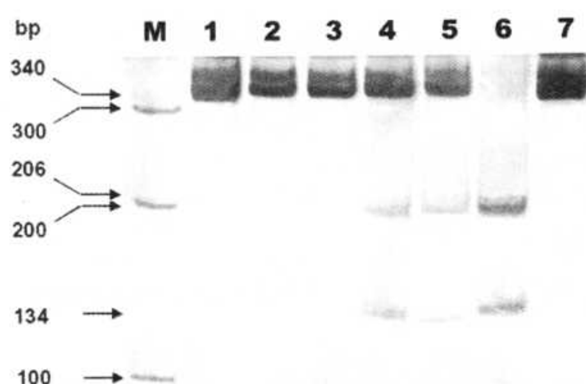

Figure 1. Representative examples of CYP1A1 *Msp*I 3'-UTR polymorphism analysis. M, DNA molecular size standard. Lanes 1 and 7, Uncut CYP1A1 PCR product; lanes 2 and 3, wt/wt; lanes 4 and 5, wt/m1; lane 6, m1/m1.

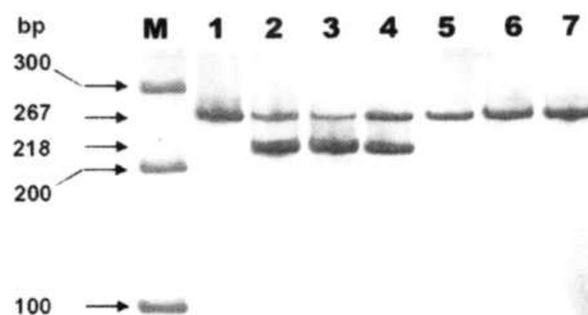

Figure 2. Representative examples of multiplex PCR assays for GSTM1 polymorphism and ß-globin. M, DNA molecular size standard. Lane 1, ß-globin (singleplex); lanes 2-4, GSTM1(+); lanes 5-7, GSTM1(-).

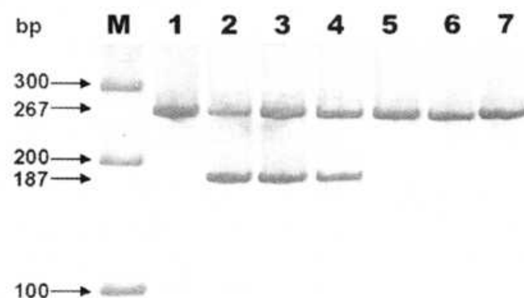

Figure 3. Representative examples of multiplex PCR assays for GSTT1 polymorphism and ß-globin. M, DNA molecular size standard. Lane 1, ß-globin (singleplex); lanes 2-4, GSTT1(+); lanes 5-7, GSTT1(-).

Electrophoresis. PCR products were analysed by 8% polyacrylamide gel electrophoresis (29:1 ratio acrylamide/bis-acrylamide) and silver stained. Gels were scanned on an Agfa SnapScan 1212u (Agfa-Gevaert N.V., Belgium). Integrated density of the bands was used as quantitative parameter and was calculated by digital imaging using the Adobe Photoshop 6.0 software (Adobe Systems Inc., USA). The ratio of the integrated density of each gene tested to that of ß2-microglobulin were used to semi-quantify the results.

Statistical analysis. Non-parametric procedures, χ^2 test and Mann-Whitney test for the evaluation of statistical significant differences in population genetic polymorphism analysis were applied. Statistical analysis for mRNA expression patterns was performed by using Student's t-test or analysis of variance, and χ^2 test (33). The statistical calculations were carried out by the SPSS 8.0 (SPSS Inc., USA) program.

Results

Genetic analysis. We assayed 207 genomic DNA samples, extracted from blood of sporadic breast cancer patients and 171 aged and sex matched controls by PCR and RFLP-PCR techniques in order to obtain the frequencies of m1 CYP1A1 3'-UTR *Msp*I polymorphism and the null deletions of GSTM1 and GSTT1 genes, in each population. The frequency of m1 was 0.13 in breast cancer cases and 0.15 in controls. The frequency of GSTM1(-) genotype was 0.52 in both groups, while that of GSTT1(-) was 0.14 and 0.10 in cases and controls, respectively. The distribution of each genotype is provided in Table III. Characteristic examples of these experiments are shown in Figs. 1-3. Statistical analysis of these genotypes, separate or in combination, did not reveal any significant difference between the breast cancer population

Table IV. Distribution of combined genotypes.

Genotype			Normal population			Breast cancer		
CYP1A1	GSTM1	GSTT1	Cases	Frequency	%	Cases	Frequency	%
wt	+	+	53	0.31	30.99	63	0.30	30.43
wt/m1	+	+	21	0.12	12.28	20	0.10	9.66
m1	+	+	2	0.01	1.17	2	0.01	0.97
wt	-	+	57	0.33	33.33	71	0.34	34.30
wt/m1	-	+	20	0.12	11.70	20	0.10	9.66
m1	-	+	1	0.58	0.89	1	0.00	0.48
wt	+	-	4	0.02	2.34	11	0.05	5.31
wt/m1	+	-	2	0.01	1.17	3	0.01	1.45
m1	+	-	0	0.00	0.00	0	0.00	0.00
wt	-	-	9	0.05	5.26	11	0.05	5.31
wt/m1	-	-	2	0.01	1.17	5	0.02	2.42
m1	-	-	0	0.00	0.00	0	0.00	0.00
Total			171	1.00	100.00	207	1.00	100.00

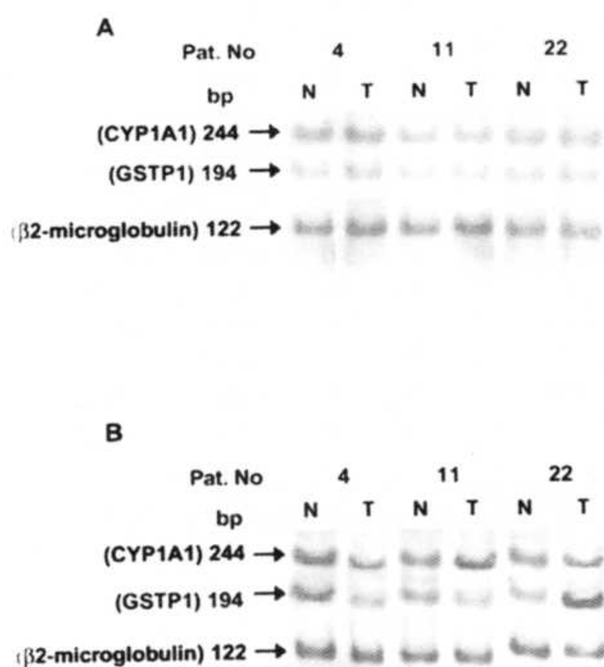

Figure 4. Representative examples of multiplex PCR assays (A) for genomic alteration analysis and (B) multiplex RT-PCR assays for transcriptional levels. N, adjacent normal tissue; T, breast tumour. Numbers indicate sample label as shown in Table V.

and the control group. The distribution of combined genotypes of these genes is shown in Table IV. The most frequent combinations were CYP1A1 wt/wt, GSTM1(-), GSTT1(+) with 34.3% in cases and 33.3% in controls, followed by CYP1A1 wt/wt, GSTM1(+), GSTT1(+) with 30.4% in breast cancer patients and 31.0% in healthy individuals.

We also assayed 62 samples of genomic DNA extracted from 31 breast tumour specimens and their adjacent normal tissues. The genotypes obtained are provided in Table V.

Transcriptional level analysis. To investigate the functional role of CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 genes in breast cancer, we examined 31 tumour specimens with their corresponding adjacent normal tissues, for their mRNA transcriptional patterns. The genes were separated into 3 panels of multiplex RT-PCR reactions (Table II) where ß2-microglobulin was used as reference control (Figs. 4-6). The expression of any given gene was represented by the integrated density (ID), calculated by digital imaging as ID = [mean density-background]*pixels.

The ß2-microglobulin expression was determined in this way for 3 independent experiments from different cDNA preparations for each panel and the ratio of its RT-PCR product to that of another experiment was calculated. Student's t-test 2-tailed was performed and a 99% confidence interval was determined between 0.82 and 1.20, while the mean difference was 1.01 ± 0.07 ($t=13.708$, $df=182$, $p<0.0001$). Thus, we accepted as down-regulation any value <0.67 and as up-regulation any value >1.72.

The ratios of RT-PCR product of each gene tested to the reference control were calculated. The transcriptional levels of CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 genes, varied between normal tissues from patients by approximately 11-fold, 24-fold, 10-fold [with GSTM1(-) carriers excepted], 9-fold and 27-fold [with GSTT1(-) carriers excepted] respectively, in an age, stage or grade independent manner. Among the primary tumour tissues variation of CYP1A1 mRNA levels was 13-fold, AhR 18-fold, GSTM1 5-fold [with GSTT1(-) carriers excepted], GSTP1 5-fold and GSTT1 48-fold [with GSTT1(-) carriers excepted], in an age, stage or grade independent manner.

We estimated the ratio of each gene transcription between tumour and correspondent adjacent normal tissue (Table V).

Table V. Transcriptional analysis in breast cancer compared to normal adjacent tissue.

No.	CYP1A1	AhR	GSTM1	GSTP1	GSTT1	Age	Stage	Grade	Genotype		
									CYP1A1	GSTM1	GSTT1
1	1.60 -	0.16 ↓	3.84 ↑	0.27 ↓	1.18 -	50	II	III	wt/wt	+	+
2	2.22 ↑	1.08 -	1.03 -	0.39 ↓	4.50 ↑	49	I	II	wt/wt	+	+
3	0.98 -	0.41 ↓	1.96 ↑	0.44 ↓	0.97 -	56	II	II	wt/wt	+	+
4	0.18 ↓	0.21 ↓	2.64 ↑	0.42 ↓	2.18 ↑	62	I	I	m1/m1	+	+
5	0.63 ↓	8.32 ↑	0.75 -	0.85 -	0.80 -	39	II	III	wt/wt	+	+
6	1.62 -	0.31 ↓	0.88 -	0.91 -	5.31 ↑	67	II	II	wt/wt	+	+
7	0.96 -	0.38 ↓	1.10 -	1.08 -	0.75 -	48	II	II	wt/wt	+	+
8	0.63 ↓	0.31 ↓	Null	2.28 ↑	0.79 -	72	II	II	wt/wt	-	+
9	0.84 -	10.64 ↑	0.36 ↓	2.42 ↑	5.34 ↑	65	I	II	wt/wt	+	+
10	1.36 -	0.32 ↓	Null	3.6 ↑	1.36 -	44	I	II	wt/wt	-	+
11	4.22 ↑	7.51 ↑	0.26 ↓	0.94 -	0.27 ↓	47	II	II	wt/wt	+	+
12	1.34 -	0.86 -	0.53 ↓	2.18 ↑	0.97 -	57	II	III	wt/wt	+	+
13	0.51 ↓	2.28 ↑	Null	2.05 ↑	0.96 -	53	I	II	wt/wt	-	+
14	0.30 ↓	0.96 -	0.79 -	1.16 -	1.29 -	65	II	III	wt/wt	+	+
15	2.85 ↑	0.94 -	1.34 -	0.21 ↓	0.73 -	56	I	II	wt/wt	+	+
16	0.60 ↓	0.95 -	0.78 -	1.27 -	0.33 ↓	76	I	II	wt/wt	+	+
17	3.20 ↑	0.29 ↓	Null	0.88 -	1.16 -	36	III	II	wt/wt	-	+
18	0.74 -	0.94 -	1.08 -	1.03 -	0.97 -	48	I	II	wt/wt	+	+
19	1.32 -	0.95 -	0.78 -	1.12 -	1.30 -	61	I	II	wt/m1	+	+
20	0.99 -	3.11 ↑	0.40 ↓	2.35 ↑	Null	53	II	II	wt/wt	+	-
21	1.44 -	0.67 -	0.80 -	0.95 -	0.76 -	75	III	II	wt/wt	+	+
22	0.56 ↓	0.96 -	Null	3.4 ↑	0.64 ↓	66	II	II	wt/wt	-	+
23	1.02 -	10.55 ↑	1.38 -	0.87 -	4.49 ↑	50	II	II	wt/wt	+	+
24	2.85 ↑	1.99 ↑	0.73 -	0.28 ↓	3.94 ↑	65	I	II	wt/m1	+	+
25	1.33 -	0.40 ↓	0.91 -	1.23 -	7.90 ↑	48	II	III	wt/wt	+	+
26	3.39 ↑	0.18 ↓	1.05 -	0.33 ↓	5.27 ↑	57	III	III	wt/wt	+	+
27	0.31 ↓	1.13 -	Null	3.24 ↑	Null	52	I	II	wt/wt	-	-
28	3.64 ↑	1.05 -	Null	1.15 -	9.96 ↑	62	III	II	wt/wt	-	+
29	1.87 -	1.08 -	1.30 -	1.35 -	Null	40	II	II	wt/m1	+	-
30	0.39 ↓	1.05 -	2.22 ↑	0.35 ↓	0.68 -	59	II	II	wt/wt	+	+
31	0.89 -	0.79 -	Null	2.11 ↑	Null	47	I	II	wt/wt	-	+

↑, up-regulation; ↓, down-regulation; -, constant expression in tumours compared to corresponding normal tissues.

CYP1A1 was deregulated in 51.6%, AhR also in 51.6% but not in accordance to CYP1A1, except 4 cases (samples 5, 11, 13, 24), GSTP1 in 54.8% of the breast cancer specimens compared to their corresponding normal tissues, while GSTM1 and GSTT1 in 34.8% and 44.4% of the tumours express them, respectively. The deregulation patterns ranged between 6-fold up and 3-fold down. Correlations between the above transcriptional patterns failed to show any accordance with the exception of CYP1A1, GSTM1 and GSTP1. Our data show that up-regulation of CYP1A1 is possible only when one of the GSTM1 or GSTP1 is down-regulated or absent and the other is constant.

Genomic alteration analysis. We examined whether the deregulations of CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 were the consequences of gene amplification or loss (Figs. 4-6). Since the above primer sets (Table II), target unique sequences of the human genome, they were used also in this analysis. No alterations in gene copy number were observed which suggest that the deregulations of mRNA levels observed are due to transcriptional apparatus modifications.

Discussion

PCR-based assays were applied for the determination of CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 genes in sporadic breast cancer. We focused on these genes because cytochrome P450-glutathione S-transferase biochemical pathway of cell detoxification is predominant in this process (24,34), and there is general agreement that environmental factors and somatic events are the main contributors to sporadic cancer (35). With regard to breast cancer susceptibility, the frequency

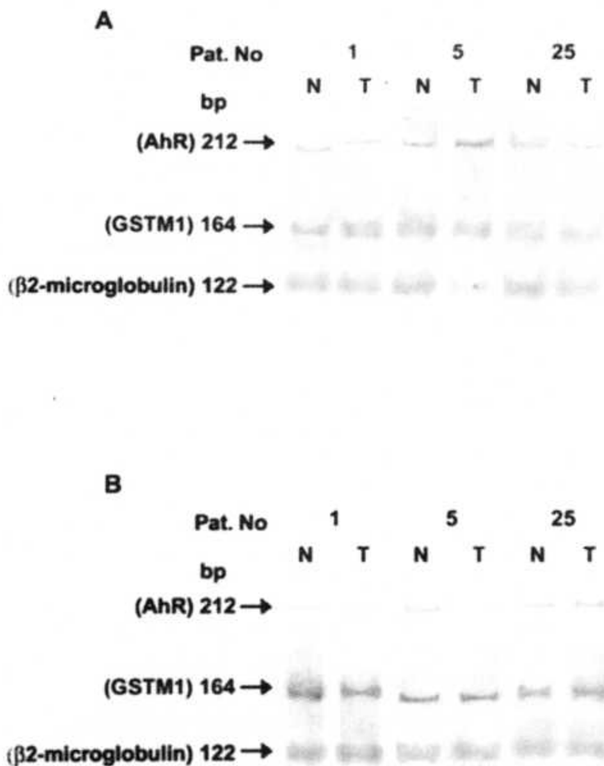

Figure 5. Representative examples of multiplex PCR assays (A) for genomic alteration analysis and (B) multiplex RT-PCR assays for transcriptional levels. N, adjacent normal tissue; T, breast tumour. Numbers indicate sample label as shown in Table V.

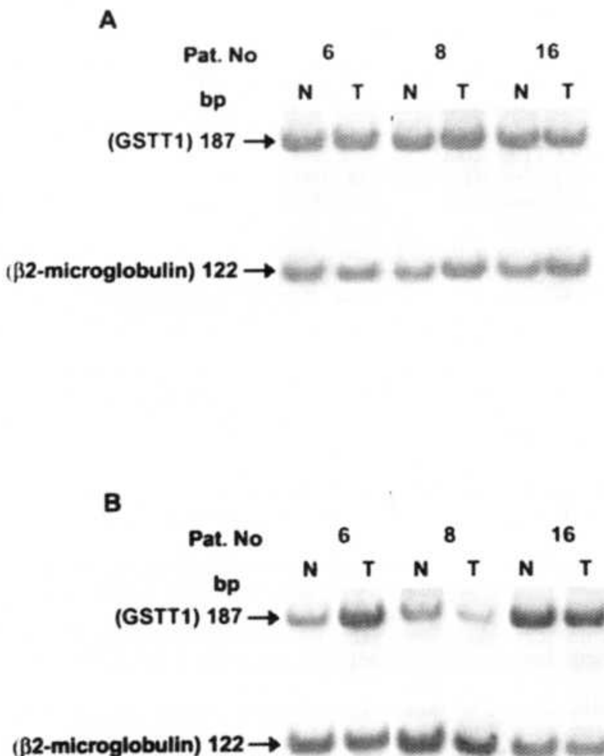

Figure 6. Representative examples of multiplex PCR assays (A) for genomic alteration analysis and (B) multiplex RT-PCR assays for transcriptional levels. N, adjacent normal tissue; T, breast tumour. Numbers indicate sample label as shown in Table V.

of mutations in the known high-risk genes, BRCA1 and BRCA2, is too low to explain more than a fraction of the genetic effects in the development of this type of tumours (36).

We determined the allelic frequencies of the CYP1A1 3'-UTR *Msp*I genetic polymorphism, termed as m1 and those of the null deletions of GSTM1 and GSTT1, as well as of their genotypes in combination. Previous studies have estimated the above frequencies for Caucasian populations at 12%, 50%, and 11-16% respectively (24,37,38). The differences in the allelic frequencies observed in this study and the previous reports, are attributed to ethnic variety. There was no statistically significant difference between the control group and breast cancer patients, when the polymorphism was analysed independently or in combination, within each cancer group according to the onset age of the disease or menstrual status. These results are in accordance with previous reports on breast cancer risk and the above polymorphisms (13,23,39).

To assess the functional role of CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 genes in breast cancer biology we examined their transcriptional levels in 31 sporadic tumour specimens compared to their adjacent normal tissues. Normal tissues from breast cancer patients exhibited 11-fold variation in CYP1A1 mRNA expression, while the reciprocal breast tumours 13-fold, both regardless of age, stage or grade. A previous study reported that in a group of normal breast specimens from both cancer-free and affected persons, variation of CYP1A1 transcription is approximately 400-fold independent of age (26). The most variable transcriptional levels between adjacent normal tissues and breast cancer were those of GSTT1, approximately 28-fold and 48-fold, respectively. GSTM1 and GSTP1 showed the least variant mRNA levels in the normal tissue, 10-fold and 9-fold, respectively. These data suggest less tolerance of breast tumours to extreme transcriptional deregulation of GSTM1 and GSTP1 compared to their normal counterparts.

The deregulation schemes in the expression of CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 between normal breast and tumours are complex. Eighty-seven per cent of tumours were transcriptionally regulated. These patterns seem to be the result of transcriptional regulation rather than gene amplification or loss, since no alterations were detected on DNA copy number.

CYP1A1 was not correlated to AhR expression, except in four cases. This may be the consequence of AhR dependence on ligand charge, in order to activate CYP1A1 transcription (14), rather than binding to ARNT, since the levels of the later does not alter dramatically between different human breast epithelial cell lines (40). Moreover, AhR function could be suppressed by H-*ras*, via signal transduction pathways and lead to down-regulation of CYP1A1 (41). Moreover, a previous study has shown that H-*ras* was up-regulated in 37% of breast tumours (42). In tumour cases where AhR is up-regulated and estrogen-dependent, a diindolylmethane treatment could lead to their regression (43). Transcriptional deregulation of CYP1A1 was correlated to GSTM1 and GSTP1 expression.

Whereas GSTM1 or GSTP1 mRNA levels were elevated in breast tumours compared to their normal counterparts, those of CYP1A1 were constant or lower. Although GSTM1

and GSTP1 could provide protection against neoplasia, due to their function as phase II detoxification enzymes, it should be considered that in the case of breast cancer especially when the tumours are estrogen-dependent, their increased activity could lead to the reverse effect, due to the observed CYP1A1 down-regulation and to decreased hydroxylation of 17ß-estradiol (E_2).

mRNA levels were 5-fold higher in 33.3% of the tumours tested, with GSTT1 expression, compared to the normal breast tissues from the same individual and 2-fold lower in 11.1%. This fact together with the observation of its variability among cancer patients in both normal and tumour tissues implies different degree of sensitivity to haloalkanes and haloalkenes such as brominated-trihalomethanes (THMs), which are present frequently in chlorinated drinking water (44). Moreover, tumour cells exhibiting elevated GSTT1 expression may be prone to sister chromatid exchanges, due to its metabolic activity (45).

Considering their functional significance in the metabolism of a series of chemotherapeutic compounds and the variety of transcriptional patterns exhibited by different breast tumours in an independent manner to age, stage or grade, CYP1A1, AhR, GSTM1, GSTP1 and GSTP1 mRNA levels could be useful molecular biomarkers. The complexity of their transcriptional patterns, possibly because of the diversity of their natural histories, attributes to different breast tumour properties, in a well-defined biochemical pathway. Knowledge of these patterns in each case could lead to better disease management, such as adequate use of chemotherapeutic compounds.

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