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EPIDEMIOLOGY

TGFβ1 (Leu10Pro), p53 (Arg72Pro) can predict for increased risk for breast cancer in south Indian women and TGFβ1 Pro (Leu10Pro) allele predicts response to neo-adjuvant chemo-radiotherapy

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Abstract The breast cancer incidence has been increasing in the south Indian women. A case ($n = 250$)–control ($n = 500$) study was undertaken to investigate the role of Single Nucleotide Polymorphisms (SNP's) in GSTM1 (Present/Null); GSTP1 (Ile105Val), p53 (Arg72Pro), TGFβ1 (Leu10Pro), c-erbB2 (Ile655Val), and GSTT1 (Null/Present) in breast cancer. In addition, the value of the SNP's in predicting primary tumor's pathologic response following neo-adjuvant chemo-radiotherapy was assessed. Genotyping was done using PCR (GSTM1, GSTT1), Taqman Allelic discrimination assay (GSTP1, c-erbB2) and PCR-CTPP (p53 and TGFβ1). None of the gene SNP's studied were associated with a statistically significant increased risk for the breast cancer. However, combined analysis of the SNP's showed that p53 (Arg/Arg and Arg/Pro) with TGFβ1 (Pro/Pro and Leu/Pro) were associated with greater than 2 fold increased risk for breast cancer in Univariate ($P = 0.01$) and Multivariate ($P = 0.003$) analysis. There was no statistically significant association for the GST family members with the breast cancer risk. TGFβ1 (Pro/Pro) allele was found to predict complete pathologic response in the primary tumour following neo-adjuvant chemo-radiotherapy (OR = 6.53 and 10.53 in Univariate and Multivariate analysis respectively) ($P = 0.004$) and was independent of stage. This study suggests that SNP's can help predict breast cancer risk in south Indian women and that TGFβ1 (Pro/Pro) allele is associated with a better pCR in the primary tumour.

Keywords Breast cancer · SNP · GSTM1 (Present/Null) · GSTP1 (Ile105Val) · p53 (Arg72Pro) · TGFβ1 (Leu10Pro) · c-erbB2 (Ile655Val) · GSTT1 (Null/Present) · Response to neo-adjuvant therapy

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Introduction

The incidence of breast cancer has risen in the Madras Metropolitan Tumour Registry (MMTR) by nearly 200% since 1982. The age standardized rate (ASR) is 29/100,000 in 2002 in Chennai (formerly Madras) [1]. Risk factors associated with breast cancer include family history, early onset of menarche and late onset of menopause, nulliparous or first childbirth after 30 years of age, post-menopausal status and consanguineous marriage [2]. While 5–10% of the breast cancers could have a hereditary background, the vast majority are sporadic. Our own data on hereditary breast cancers has shown that deleterious mutations in high risk genes, BRCA1 and BRCA2, are seen in 15% of the cases studied, which compares with other published data [3, 4]. The risk for a carrier of a deleterious mutation for breast cancer ranges from 50 to 80% by 70 years of age, indicating that other factors/genes may modulate this risk.

In sporadic cancers, such gene-environment and gene-gene interactions also play a role. Dunning et al. [5] had

examined the effect of common alleles of 18 genes on breast cancer risk in a meta-analysis. Their study found CYP19 (TTTA)n polymorphism, GSTP1 (Ile105Val) polymorphism, p53 (Arg72Pro) polymorphism and GSTM1 deletion to be statistically significantly associated with increased risk of breast cancer. Rad51 (135g to c) has been associated with increased risk of breast cancer and a lower risk of ovarian cancer in BRCA2 mutation carriers [6]. The Ile655Val polymorphism in c-erbB2 gene was associated with increased risk of breast cancer, particularly among young women [7]. The Pro allele of TGFβ1 (Leu10Pro) has been associated with increased secretion of TGFβ1 and an increased risk of breast cancer [8].

Attempts are being made to identify the low risk genes, which although associated with a small individual risk, cumulatively could increase the risk substantially. Genome wide association studies have started to provide information on the role of these "low-risk genes" in several chronic diseases including cancer [9–11]. We had conducted a case–control study to identify the role of Single Nucleotide Polymorphism's (SNP's) in breast cancer risk. The data with GSTM1, GSTP1 and p53 was published earlier [2]. This paper presents the data from three additional SNP's in TGFβ1 (Leu10Pro), c-erbB2 (Ile655Val), and GSTT1 (Null/Present) and looks at their association with regard to breast cancer risk. In addition, the study also provides results on the association of the SNP's with treatment results, particularly with the pathologic response to neo-adjuvant concurrent chemo-radiotherapy.

Materials and methods

Patients

The detail of the case control study has been published [2]. The study was approved by the Institutional Ethical Committee. Briefly, 250 breast cancer cases and 500 healthy controls were matched on 5-year age category in the ratio of 1:2. The Inclusion criteria for the healthy controls were, no prior diagnosis of benign breast diseases; no history of hysterectomy or mastectomy or oophorectomy; no relatives with breast or ovarian or endometrial or prostate cancer; no physical or mental disability which would preclude their participation in the study. Inclusion criteria for the cases were histological confirmation of breast cancer; no previous cancer treatment. Informed consent was mandatory for both groups.

A detailed questionnaire on their personal history and food habits and 15 ml of heparinized blood were collected from the cases and the controls.

Treatment protocol

Patients with stage I, IIA and IIB with tumours $\leq$3 cm and who were clinically node negative were taken up for breast conserving surgery (BCS) or modified radical mastectomy (MRM) followed by adjuvant therapy based on the histopathological examination and estrogen receptor (ER) and progesterone receptor (PR) status. In the pre-menopausal women, whose tumour was ER/PR positive, bilateral salphingo-oopherectomy (BSO) was done.

In patients with stage IIA or IIB, with tumours >3 cm and in those with N1 disease, or in stage IIIA and IIIB initial neo-adjuvant chemo-radiotherapy followed by BCS/MRM was done. This was followed by further post-operative chemotherapy $\pm$ hormonal therapy based on the ER/PR status. In the pre-menopausal women, with ER/PR positivity, BSO was done.

Chemotherapy consisted of either Flurouracil-Adriamycin-Cyclophosphamide (FAC) or Cyclophosphamide, Methotrexate and 5-Flurouracil (CMF) regimen. Of the 250 cases, 101 underwent neo-adjuvant chemo-radiotherapy followed by surgery. Chemotherapy regimen consisted of either CMF or FAC given concurrently on days1, 22 and 43 with radiotherapy. Radiotherapy to the breast was delivered using the Theratron Cobalt beam unit to a total dose of 40 Gy. Surgery (Patey's mastectomy) was done 3–4 weeks after the third cycle of chemotherapy. BSO was done if the patient was pre-menopausal and her tumour was ER and PR positive. Patients were continued on further chemotherapy (three more cycles) and Tamoxifen was added on completion of chemotherapy in ER+ tumour patients.

Genotyping

DNA extraction and processing was done as described earlier [2]. Genotyping was done for TGFβ1 (Leu10Pro) by Polymerase Chain Reaction with Confronting Two-Pair Primers (PCR-CTPP) [12]. c-erbB2 (Ile655Val) genotyping analysis was performed by the Taqman Allelic Discrimination method (Applied Biosystems, Foster City, CA). Primers and probes mix were obtained directly from Applied Biosystems Assays-on-Demand^{TM}. Genotyping for GSTT1 was performed by PCR using exon specific primers (Forward 5′-GCCCTGGCTAGTTGCTGAAG-3′ and Reverse 5′-GCATCTGATTTGGGGACCACA-3′) [13] with modifications. PCR reaction were carried out in 25 µl aliquots containing 50 ng of genomic DNA, 10 pico-moles of each primer, 100 mM of d-NTPs, 10× reaction buffer (GE Healthcare, Hong Kong) and 0.5 unit of Taq polymerase (GE Healthcare, Hong Kong). Amplification was done for 30 cycles with initial denaturation at 94°C for 10 min, and denaturation, annealing, extension at 94°C for

15 s, 59°C for 30 s, 72°C for 45 s and final extension at 72°C for 7 min. The presence of the gene was determined by the presence of 110 bp band, while the null genotype lacks the band when the PCR products were run on the 2% agarose gel electrophoresis stained with ethidium bromide.

Statistical analysis

Descriptive statistics was used to present the distribution of case and control subjects with respect to the factors studied. Chi-squared test was used to test for statistical significance in the difference in the proportion of subjects between cases and control groups, for the factors measured on a nominal scale. Analysis was conducted as a matched case control study. Univariate conditional logistic regression was performed to calculate the odd ratios (OR) always accompanied by 95% Confidence Interval and *P* values. Pathological tumor remission was the intermediate outcome studied among the cases and the OR was estimated using logistic regression analysis [14]. Disease free survival was estimated using Kaplan Meier method [15].

Results

The distribution of the demographic characteristics, known major risk factors for breast cancer and the distribution of GSTM1, GSTP1 and p53 have been published [2]. The allelic frequencies for the genes and the risk for breast cancer by cases and controls are given in Tables 1 and 2. Gene groups were combined on their basic characteristics and combined analysis was carried out separately for all the permutations of the allele in the group.

Group 1: p53, TGFβ1 and c-erbB2
Group 2: GST family—GSTM1, GSTT1 and GSTP1.

The GSTM1 (Present), GSTP1 (Ile/Ile), GSTT1 (Present), p53 (Pro/Pro), TGFβ1 (Leu/Leu) and c-erbB2 (Ile/Ile) were taken as the reference category. With regard to the Group 1, Univariate analysis showed p53 (Arg/Arg and Arg/Pro) + TGFβ1 (Pro/Pro and Pro/Leu) + c-erbB2 (any form) to be statistically significantly associated with increased breast cancer risk (Odds ratio 2.22 (c-erbB2 reference category) and 2.32 (c-erbB2 Val/Val and Val/Ile), with a $P = 0.03$ and 0.04, respectively) (Table 3A). In addition, TGFβ1 (Pro/Pro and Pro/Leu) with p53 and c-erbB2 reference category was also statistically significant with an Odds ratio of 2.44 ($P = 0.03$). In multivariate analysis, a similar picture was seen with increased Odds Ratio. In addition, variant p53 alone seemed to increase the risk, in multivariate analysis, after adjusting for religion, age at menarche, age at first child birth, menopausal status and consanguineous marriage. We then analyzed the data for two genes at a time—p53 and c-erbB2, p53 and TGFβ1 and c-erbB2 and TGFβ1. The combination of TGFβ1

Table 1 Distribution of allelic frequencies of GSTM1, GSTP1, GSTT1, P53, TGF β1 and c-erbB2 polymorphism in breast cancer cases and controls

Polymorphism	Group	Number of subjects	Genotypes		
GSTM1 (Null/Present)			Null		Present
	Cases	250	65 (26.0%)		185 (74.0%)
	Controls	500	110 (22.0%)		390 (78.0%)
GSTP1 (Ile105Val)			Ile/Ile	Ile/Val	Val/Val
	Cases	250	118 (47.2%)	103 (41.2%)	29 (11.6%)
	Controls	500	230 (46%)	219 (43.8%)	51 (10.2%)
GSTT1 (Null/Present)			Null		Present
	Cases	250	44 (17.6%)		206 (82.4%)
	Controls	500	84 (16.8%)		416 (83.2%)
p53 (Arg72Pro)			Arg/Arg	Arg/Pro	Pro/Pro
	Cases	250	66 (26.4%)	125 (50.0%)	59 (23.6%)
	Controls	500	135 (27.0%)	224 (44.8%)	141 (28.2%)
TGF β1 (Leu10Pro)			Leu/Leu	Leu/Pro	Pro/Pro
	Cases	250	80 (32.0%)	44 (17.6%)	126 (50.4%)
	Controls	500	190 (38.0%)	76 (15.2%)	234 (46.8%)
c-erbB2 (Ile655Val)			Ile/Ile	Ile/Val	Val/Val
	Cases	250	181 (72.4%)	62 (24.8%)	7 (2.8%)
	Controls	500	363 (72.6%)	119 (23.8%)	18 (3.6%)

Table 2 Effect of gene factors on breast cancer risk by Univariate and Multivariate conditional logistic regression analysis

Variables[b]	Cases	Controls	Odds ratio (95% CI)	
			Univariate	Multivariate
p53				
Pro/Pro	59	141	1.00[a]	1.00[a]
Arg/Arg	66	135	1.18 (0.77–1.81)	1.23 (0.78–1.95)
Pro/Arg	125	224	1.34 (0.92–1.95)	1.46 (0.97–2.20)
TGF_β				
Leu/Leu	80	190	1.00[a]	1.00[a]
Pro/Pro	44	76	1.38 (0.87–2.17)	1.47 (0.88–2.46)
Leu/Pro	126	234	1.28 (0.91–1.80)	1.22 (0.84–1.77)
c-erbB2				
Ile/Ile	181	363	1.00[a]	1.00[a]
Val/Val	7	18	0.77 (0.32–1.89)	0.89 (0.35–2.30)
Ile/Val	62	119	1.01 (0.74–1.50)	1.16 (0.79–1.70)
GSTM1				
Present	185	390	1.00[a]	1.00[a]
Null	65	110	1.26 (0.88–1.80)	1.22 (0.83–1.82)
GSTT1				
Present	207	415	1.00[a]	1.00[a]
Null	43	85	1.01 (0.68–1.52)	1.08 (0.70–1.68)
GSTP1				
Ile/Ile	118	230	1.00[a]	1.00[a]
Val/Val	29	51	1.11 (0.67–1.83)	1.16 (0.68–1.98)
Ile/Val	106	219	0.92 (0.67–1.26)	0.92 (0.65–1.30)

[a] Reference category

[b] Adjusted for religion, age at menarche, age at first child birth, menopausal status and consanguineous marriage

(Pro/Pro and Leu/Pro) and p53 (Arg/Arg and Arg/Pro) was associated with more than 2 fold increased risk for breast cancer in Univariate ($P = 0.01$) and Multivariate ($P = 0.003$) analysis (Table 3A). The other gene interactions (p53 and c-erbB2 and TGFβ1 and c-erbB2) were not associated with any significant risk of breast cancer. There was no statistically significant association for the GST family members with the breast cancer risk (Table 3B).

One hundred and one patients underwent neo-adjuvant chemo-radiotherapy followed by surgery. Post surgical histo-pathological evaluation of the primary tumour revealed that 25/101 (25%) primary tumours did not have any residual disease after the neo-adjuvant therapy. The pathologic complete response (pCR) in the primary tumour was 47% (9/19) for the TGFβ1 Pro/Pro allele, 25% (12/49) for the Pro/Leu and 12% (4/33) for the Leu/Leu, indicating that the individuals with the Pro/Pro allele were 10 fold likely to achieve a pathologic CR in the primary tumour ($P = 0.004$), (Table 4), emerging as an independent predictive factor after adjustment of stage of the disease and other factors. We further analyzed to see if the genotype influenced the response to the chemo-therapeutic regimens used. While the TGFβ1 Pro/Pro allele was associated with a better pCR rate in the primary with the FAC regimen ($P = 0.04$), the numbers were too few in the CMF group (data not shown). There was no significant association between the other gene alleles and the primary tumour pCR rates.

The duration of follow up is only 30 months and hence it would be premature to comment on the impact of the gene factors on DFS and OS.

Discussion

This manuscript presents information on three additional genes (TGFβ1, c-erbB2 and GSTT1) in the case control study done [2]. Individually, none of the genes were found to increase the risk for breast cancer.

Cox et al. [16] have demonstrated a weak association for TGFβ1 Leu10Pro (OR1.07 and 1.16 for heterozygotes and Pro/Pro homozygotes, respectively) in their study comprising 11,391–18,290 cases and 14,753–22,670 controls. Studies have also shown that the Pro/Pro genotype is associated with a 2.8 fold increased TGFβ1 secretion compared to the Leu/Leu genotype and therefore an increased risk for breast cancer [8]. Dunning et al. [5] had shown a positive association for Cyp19 (TTTA)n polymorphism [(TTTA)10 carrier OR 2.33)], GSTP1 (Ile105Val—Val carrier OR 1.33), p53 (Arg72Pro—Pro carrier OR 1.27) and GSTM1 (Null—OR 1.33) in their metanalysis of 46 studies on 18 different genes.

The Her2 (Ile655Val) SNP has been studied in the past and the variant Val allele was found to be associated with an increased risk for familial breast cancer [17–19]. A case control study involving 2,192 cases and 2,257 controls in white British population did not find any significant association for c-erbB2 (Ile655Val) polymorphism and breast cancer risk [20]. A Korean study also did not find any significant association between c-erbB2 (Ile655Val) and risk for breast cancer [21]. Two studies have also reported an inverse statistically significant association between Val/Val genotype and breast cancer risk, with OR of 0.63 and 0.68 [22, 23].

Variable results have been obtained with regard to the GST genes (GSTM1, GSTP1and GSTT1). Some studies [24, 25] did not find any significant association for the polymorphisms in these genes with breast cancer risk. However, other studies [26, 27] have found significant association. Studies looking for gene interactions among the GST family and other genes, have also shown a greater risk for women carrying deletions of both GSTM1 and GSTT1 genes [26, 28]. Our study, did not find any significant associations for the SNP's studied in the GST family with breast cancer risk.

Table 3 Effect of gene combinations on breast cancer risk by univariate and multivariate conditional logistic regression analysis

Variables[b]			Cases	Controls	Odds ratio (95% CI) Univariate	*P* value	Odds ratio (95% CI) Multivariate	*P* value
A								
p53	TGFβ1							
Pro/Pro	Leu/Leu		16	60	1.00[a]		1.00[a]	
Pro/Pro	VAR		43	81	2.02 (1.03–3.94)	0.04	2.82 (1.33–5.98)	0.007
VAR	Leu/Leu		64	130	1.89 (1.00–3.59)	0.05	2.83 (1.38–5.80)	0.005
VAR	VAR		127	229	2.11 (1.16–3.84)	0.01	2.76 (1.41–5.41)	0.003
p53	TGFβ1	c- erbB2						
Pro/Pro	Leu/Leu	Ile/Ile	11	43	1.00[a]		1.00[a]	
VAR	Leu/Leu	Ile/Ile	46	100	1.89 (0.87–4.11)	0.11	3.10 (1.28–7.27)	0.01
VAR	VAR	Ile/Ile	89	162	2.22 (1.07–4.57)	0.03	3.14 (1.38–7.13)	0.006
Pro/Pro	VAR	Ile/Ile	35	58	2.44 (1.09–5.44)	0.03	3.99 (1.61–9.89)	0.003
Pro/Pro	Leu/Leu	VAR	5	17	1.21 (0.35–4.04)	0.75	1.75 (0.48–6.45)	0.4
Pro/Pro	VAR	VAR	8	23	1.39 (0.49–3.95)	0.53	1.99 (0.65–6.14)	0.22
VAR	Leu/Leu	VAR	18	30	2.42 (0.98–5.98)	0.06	4.53 (1.66–12.34)	0.003
VAR	VAR	VAR	38	67	2.32 (1.06–5.09)	0.04	3.58 (1.50–8.56)	0.004

Variables[b]			Cases	Controls	Odds ratio (95% CI)	
					Univariate	Multivariate[b]
B						
GSTM1	GSTT1	GSTP1				
Pres	Pres	Ile/Ile	78	144	1.00[a]	1.00[a]
Null	Pres	Ile/Ile	23	41	1.08 (0.59–1.96)	1.07 (0.56–2.05)
Null	Null	Ile/Ile	0	9	0	0
Pres	Null	Ile/Ile	17	36	0.84 (0.44–1.62)	0.88 (0.43–1.78)
Pres	Pres	VAR	74	180	0.77 (0.53–1.13)	0.75 (0.49–1.13)
Pres	Null	VAR	16	30	1.01 (0.52–1.96)	1.24 (0.6–2.54)
Null	Pres	VAR	32	50	1.18 (0.7–1.98)	1.23 (0.7–2.17)
Null	Null	VAR	10	10	1.91 (0.77–4.74)	1.86 (0.67–5.13)

[a] Reference category; VAR, Variant and Heterozygous

[b] Adjusted for religion, age at menarche, age at first child birth, menopausal status and consanguineous marriage

In contrast, gene interactions between p53, TGFβ1 and c-erbB2, showed a statistically significant association with breast cancer risk. This was also seen with p53 (Arg/Arg and Arg/Pro) and TGFβ1 (Pro/Pro and Leu/Pro) gene interaction. We are unable to find studies which have done combined genotype analysis for TGFβ1, p53 and c-erbB2, for us to compare our results with.

We then analyzed the role of the SNP's studied in the response to neo-adjuvant chemo-radiotherapy, wherein concurrent FAC or CMF chemotherapy was given with radiotherapy to breast and axilla. These patients underwent surgery after this and the specimen was histo-pathologically assessed for residue in the primary and the axillary nodes. TGFβ1 (Pro/Pro) followed by the (Leu/Pro) alleles were more likely to achieve a pathological complete response in the primary. TGFβ1 (Pro/Pro) has been shown earlier to be associated with greater radiation toxicity response in the breast [29]. Ours is the first study to demonstrate the association of the TGFβ1 (Lue10Pro) with pathological primary tumour response in breast cancer, following neo-adjuvant chemo-radiotherapy. None of the other genes studied were found to have any significant association with pathological response in the primary tumour. This effect was independent of stage, reproductive factors and socio-economic factors.

Other studies have shown that patients with null genotype for GSTM1 and GSTT1 have a better response to chemotherapy [30]. Edvardsen et al. [25] had shown that the GSTP1 (Ile105Val) was associated with greater radiation toxicity following radiotherapy to the breast. The GSTP1 105Val genotype was found to confer a better survival following treatment with chemotherapy compared

Table 4 Univariate and Multivariate logistic regression analysis to study the effect of TGFβ1 and stage of disease on primary tumour response following neo-adjuvant chemo-radiotherapy

Variables[b]	pCR in primary tumour		Odds ratio (95% CI)	
	Not achieved	Achieved	Univariate	Multivariate
TGFβ1				
Leu/Leu	29	4	1.00[a]	1.00[a]
Pro/Pro	10	9	6.53 (1.64–25.93)*	10.53 (2.11–51.48)*
Leu/Pro	37	12	2.35 (0.68–8.06)	2.82 (0.74–10.7)
STAGE				
II A and II B	23	6	1.00[a]	1.00[a]
III A and IIIB	53	19	1.37 (0.49–3.89)	2.03 (0.57–7.28)

[a] Reference category; *$P = 0.004$

[b] Adjusted for religion, age at menarche, age at first child birth, menopausal status and consanguineous marriage

with the 105Ile genotype [31]. These results suggest that while toxicity may be greater with these alleles, they could also enhance the response/survival rate.

The duration of follow up is 30 months in our series of patients and hence may be premature to comment on the DFS and OS rates.

In conclusion, our study has shown that TGFβ1, p53 polymorphisms are associated with increased breast cancer risk and that TGFβ1 10Pro allele can predict response to neo-adjuvant chemo-radiotherapy.

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