

Association of single nucleotide polymorphisms in CYP1B1 and COMT genes with breast cancer susceptibility in Indian women

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Abstract. Cytochrome P450 1B1 (CYP1B1) and catechol-*O*-methyltransferase (COMT) enzymes play critical roles in estrogen metabolism. Alterations in the catalytic activity of CYP1B1 and COMT enzymes have been found associated with altered breast cancer risk in postmenopausal women in many populations. The substitution of leucine (Leu) to valine (Val) at codon 432 increases the catalytic activity of CYP1B1, however, substitution of Val to methionine (Met) at codon 158 decreases the catalytic activity of COMT. The present study was performed to evaluate the associations of CYP1B1 Leu⁴³²Val and/or COMT Val¹⁵⁸Met polymorphisms with total, premenopausal and postmenopausal breast cancer risks in Indian women. COMT and CYP1B1 polymorphisms in controls and breast cancer patients were analyzed employing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) followed by gel electrophoresis. Although CYP1B1 and COMT genotypes did not exhibit statistically significant association with breast cancer risks when analyzed individually, COMT wild type (Val¹⁵⁸Val) in combination with CYP1B1 heterozygous variant (Leu⁴³²Val) [OR: 0.21; 95% CI (0.05–0.82), *p* value; 0.021] and COMT heterozygous variant (Val¹⁵⁸Met) in combination with CYP1B1 wild type (Leu⁴³²Leu) [OR: 0.29; 95% CI (0.08–0.96), *p* value; 0.042] showed significant protective association with premenopausal breast cancer risk. The results demonstrate that CYP1B1 wild type in combination with COMT heterozygous or their inverse combination offer protection against breast cancer in premenopausal Indian women.

Keywords: Breast cancer, genetic polymorphism, CYP1B1, COMT

1. Introduction

Circulating level of estrogens regulates the incidences of breast cancer in postmenopausal women [1, 2]. Xenobiotic metabolizing genes involved in estro-

gen metabolism act as important contributors in the regulation of circulating level of estrogens [3]. Single nucleotide polymorphism in cytochrome P450 1B1 (CYP1B1) and catechol-*O*-methyltransferase (COMT) have been associated with increased incidences of breast cancer risk in many populations, however, lack of such associations have been also reported [4–8]. CYP1B1 and COMT play an important role in estrogen metabolism. CYP1B1 gene, located on short arm of chromosome 2, consists of three exons and four introns, spans 8546 base pairs and encodes a protein of

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543 amino acids [9,10]. COMT gene is localized on long arm of chromosome 22 and contains two separate promoters on the same chromosome for its two isoforms [11,12]. COMT gene encodes either a soluble protein containing 221 amino acid residues or a membrane bound protein containing 271 amino acids along with 50 additional residues at its N-terminal end [13, 14].

CYP1B1 enzyme catalyzes 4-hydroxylation of estrogens and COMT converts catechol derivatives of estrogens mostly into inactive metabolites [15,16]. Hydroxylated intermediates are generally metabolized into water-soluble methyl derivatives and easily excreted from human body [17]. Hydroxylated products produce estrogenic and carcinogenic effects, if not metabolized, however, 4-hydroxyestradiol exhibits more pronounced effects than 2-hydroxyestradiol [18,19]. Leucine (Leu) to valine (Val) substitution at codon 432 (C/G) in exon 3 of CYP1B1 gene exhibits three times higher activity than wild type *in vitro*, however, Leu substitution is associated with altered protein activity in presence of other CYP1B1 polymorphisms [20,21]. Substitution of Val to methionine (Met) (Val108/158Met) at position 158 (G/A) in exon 4 of membrane bound COMT causes the protein to become 2–3-fold less active *in vitro*, as compared with its wild type protein [22].

Postmenopausal breast cancer is associated with obesity and estrogen levels that are directly or indirectly influenced by life style, environmental exposure and food habits [3]. Despite variability in life style, multiple ethnicity, environmental exposure and food habits [23–25], the association studies on estrogen metabolism with breast cancer risk in north Indian women have been rarely studied. Associations of polymorphisms in some of the estrogen metabolizing genes with breast cancer risk were evaluated recently in Indian population [25]. Despite established association of polymorphisms of estrogen metabolizing genes, such as CYP1A1 with postmenopausal breast cancer risk in several populations, lack of significant associations with postmenopausal breast cancer risk were observed in Indian women, although heterozygosity of CYP1A1 M2 polymorphism was found protective against postmenopausal breast cancer [25]. Variability in association studies of one of the estrogen metabolizing genes with breast cancer risk in Indian women has prompted us to look into the contribution of genetic polymorphisms of other estrogen metabolizing genes, such as CYP1B1 and COMT in breast cancer risks. The exploratory character of the study was to assess whether

premenopausal or postmenopausal breast cancer risks in Indian women are associated with the CYP1B1 and COMT polymorphisms or not.

2. Material and methods

2.1. Selection of subjects, blood collection and genotyping

The blood was collected from female controls and breast cancer patients by clinicians at Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow, on the basis of inclusion and exclusion criteria, as described previously [24,25]. In brief, 166 controls and 154 breast cancer patients between the ages of 25–65 years were recruited for this study. Cases and controls were from the same ethnic group and were residents of northern parts of India. Patients were classified in premenopausal and postmenopausal groups on the basis of clinical history and information given by them. The medical ethics committees of the concerned institutions approved the study. A written pre-informed consent was obtained prior to blood collection from the individuals involved in this study.

Blood was drawn through venous puncture from controls and breast cancer patients and collected in vials containing tri sodium citrate (3.8%; 9:1 v/v; pH 6.5) and genomic DNA was extracted [25, 26]. Genotyping of CYP1B1 Leu432Val and COMT Val108/158Met polymorphisms were performed employing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) followed by gel electrophoresis. PCR primers were designed and amplification was performed, as reported elsewhere [7]. The desired segment of CYP1B1 and COMT genes was amplified using optimal conditions of amplification (Mastercycler gradient thermocycler, Eppendorf, Germany). The reported forward (5'CACTGCAACACCTCTGTCT3') and reverse (5'GCAGGCTCATTGGGTTG3') primers were used to amplify the desired segment of CYP1B1 gene. Similarly, the reported forward (5'ACTGTGGCTACTCAGCTGTG3') and reverse (5'CCTTTTCCAGGTCTGCAAC3') primers were also used to amplify the desired segment of COMT gene. In brief, the PCR amplification reaction was performed in 25 μ l of assay mixture, containing genomic DNA (100 ng), 10x buffer (2.5 μ l), MgCl₂ (15 mM), dNTPs (0.16 mM each), Hot Start Taq DNA polymerase (1.5U) (Fermentas, Canada) and

forward and reverse primers (20 pM each) (Hysel, India). The reaction mixture was initially denatured at 94°C for 15 min, followed by 35 cycles of denaturation at 94°C for 45 sec. The annealing temperature was 60°C for CYP1B1 for 45 sec and 65°C for COMT for 45 sec. Final extension temperature for both genes was 72°C for 45 sec.

PCR amplicon of CYP1B1 was digested with Eco57I restriction endonuclease (Fermentas, Canada) at 37°C for 16 h. The digested sizes of the fragments were 187bp and 107bp in wild type, 294bp in homozygous variant and 187bp, 107bp and 294bp in heterozygous variant. The PCR amplicon of COMT was digested with NlaIII at 37°C for 16 h. The digested sizes of the fragments were 114bp, 29bp and 26bp in wild type, 96bp, 29bp, 26bp, 18bp in homozygous variant and 114bp, 96bp, 29bp, 26bp and 18bp in heterozygous variant.

The PCR amplicon of CYP1B1 and its digested products were resolved in 1.5% agarose gel. COMT and its digested products were resolved in 12% polyacrylamide gel. Ethidium bromide using UV transilluminator visualized the bands.

2.2. Clinical and pathological information

The mean ages of premenopausal patients and controls were 40.41 ± 0.82 and 32.60 ± 0.60 years and postmenopausal patients and controls were 53.72 ± 0.93 and 52.50 ± 0.88 years respectively. The average ages of menarche were 13.75 ± 0.09 years in patients and 13.70 ± 0.05 years in controls. The average body mass indexes of patients and controls in terms of Kg/m^2 were 25.31 ± 0.42 and 20.20 ± 0.20 respectively. Among selected subjects, 59 patients (38.31%) and 99 controls (59.64%) were premenopausal and 95 patients (61.69%) and 67 controls (40.36%) were postmenopausal women. Mostly non-smokers samples could be collected, as females in Indian population rarely possess smoking habit. The numbers of non-smokers were 149 in cases (96.76%) and 160 in controls (96.39%). The tumor was diagnosed at first stage in 6 patients, second stage in 78 patients and third stage in 70 patients. Among diagnosed patients, radiotherapy was given to total 117 (75.97%), neo-adjuvant chemotherapy to 34 (22.08%), tamoxifen therapy to 29 (18.84%) and chemotherapy to 112 (72.72%) individuals. Surgery was performed in 136 subjects (88.31%) at some or other stages. The lobular tumor was identified in 4 patients, ductal in 146 patients and mixed/unclassified in 4 patients. The nodal

Table 1
Clinical information of cases and controls

<i>Age (Years)</i>		
Premenopausal	40.41 ± 0.82	32.60 ± 0.60
Postmenopausal	53.72 ± 0.93	52.50 ± 0.88
<i>Menarche age</i>	13.75 ± 0.087	13.70 ± 0.053
<i>Menopausal status (Number)</i>		
Premenopausal	59	99
Postmenopausal	95	67
<i>Stage (Number)</i>		
≤ Stage I	06	
Stage II	78	
≥ Stage III	70	
<i>Type</i>		
Tumour of duct	146	
Tumour of lobule	04	
Unclassified	04	
<i>Nodal status</i>		
+	102	
-	52	
<i>Surgery</i>		
+	136	
-	18	
<i>Chemotherapy</i>		
+	112	
-	42	
<i>Tamoxifen therapy</i>		
+	29	
-	125	
<i>Neo-adjuvant chemotherapy</i>		
+	34	
-	120	
<i>Radiotherapy</i>		
+	117	
-	37	

status of tumors was detected positive in 102 patients (66.23%) and negative in 52 patients (33.77%) [Table 1]. The lymph node status was identified either by surgical resection or by fine needle aspiration cytology (FNAC) [27].

2.3. Statistical analysis

Allelic and genotypic frequencies were calculated; unadjusted odds ratio (OR) was calculated by 2×2 contingency table with 95% confidence intervals and statistical significance for OR was calculated using the chi-square test using Epi Info-5 software. Fisher exact test was used, when expected cell frequency was less than five. Minimum detectable OR with 80% power significance level (0.05) and 100 samples each in cases and controls with prevailing percent of homozygous wild type in controls were considered 0.5 in case of protective association and 2.0 in case of increased risk [28]. The data presented for the study were not corrected for multiple testing as correction bares the risk to ignore important findings and the ex-

Table 2
Allelic and genotypic frequencies of CYP1B1 polymorphism in Indian female breast cancer patients and controls considered for analysis

	Patients <i>n</i> = 154	Case controls <i>n</i> = 166	Odds Ratio (95%CI)	p-value
<i>Total women</i>				
Allele frequency (number of alleles)				
C	0.68(208)	0.69(229)	1.0 (reference)	
G	0.32(100)	0.31(103)	1.07(0.76–1.51)	0.758
Genotypic frequency (number of genotypes)				
C/C	0.45(69)	0.42(69)	1.0 (reference)	
C/G	0.45(70)	0.55(91)	0.77 (0.47–1.25)	0.312
G/G	0.10(15)	0.03(6)	2.50 (0.85–8.30)	0.110
C/G + G/G	0.55(85)	0.58(97)	0.88 (0.55–1.40)	0.637
<i>Premenopausal women</i> <i>n</i> = 59 <i>n</i> = 99				
Allele frequency (number of alleles)				
C	0.71(84)	0.70(140)	1.0 (reference)	
G	0.29(34)	0.30(58)	0.98(0.57–1.66)	0.970
Genotypic frequency (number of genotypes)				
C/C	0.53(31)	0.44(44)	1.0 (reference)	
C/G	0.37(22)	0.53(52)	0.60 (0.29–1.25)	0.190
G/G	0.10(6)	0.03(3)	2.84 (0.55–18.65)	0.173
C/G + G/G	0.47(28)	0.56(55)	0.72 (0.36–1.45)	0.411
<i>Postmenopausal women</i> <i>n</i> = 95 <i>n</i> = 67				
Allele frequency (number of alleles)				
C	0.65(124)	0.66(89)	1.0 (reference)	
G	0.35(66)	0.34(45)	1.05(0.64–1.72)	0.922
Genotypic frequency (number of genotypes)				
C/C	0.40(38)	0.37(25)	1.0 (reference)	
C/G	0.51(48)	0.58(39)	0.81 (0.40–1.65)	0.644
G/G	0.09(9)	0.04(3)	1.97 (0.43–12.33)	0.517
C/G + G/G	0.60(57)	0.63(42)	0.89 (0.45–1.78)	0.855

ploratory character of the study. Hardy-Weinberg equilibrium genotypic frequencies were calculated using Hardy-Weinberg calculator (<http://www.genes.org.uk/software/hardy-weinberg.shtml>).

3. Results

3.1. Allelic and genotypic frequency of C/G in CYP1B1 gene (Leu⁴³² Val)

Although lack of statistically significant protection or risk was seen in total, premenopausal or postmenopausal women as compared with respective controls on the basis of probability score, the frequencies of C allele [68.5%; 71.5% and 64.5%] were approximately twice as compared with G allele [31.5%; 29.5% and 36.5%]. Similarly, genotypic frequency of G/G homozygous was 2–3 folds higher in patients [10%; 10% and 9%] of all the groups as compared with respective controls [3%; 3% and 4%] (Table 2). Polymorphisms of CYP1B1 in estrogen receptor positive and estrogen receptor negative breast cancer patients were also analyzed separately (data not shown), but no statistically

significant difference was observed. The patterns of association were similar as observed with total breast cancer patients.

3.2. Allelic and genotypic frequency of G/A in COMT gene (Val¹⁵⁸ Met)

The frequency of G allele (62%) was higher as compared with A in premenopausal women; however, no statistically significant change was observed in total and postmenopausal women (Table 3).

3.3. Hardy-weinberg equilibrium

The genotypic frequencies for each SNP in cases and controls did not show statistically significant deviation from expected frequencies except for CYP1B1. The observed genotypic frequencies of COMT G/G, G/A and A/A in cases and controls did not show statistically significant deviation from expected frequencies. The observed genotypic frequencies of CYP1B1 C/C, C/G and G/G in total, premenopausal and postmenopausal patients also did not show significant deviation from expected frequencies, however, significant deviation was

Table 3
Allelic and genotypic frequencies of COMT polymorphism in Indian female breast cancer patients and controls considered for analysis

	Patients <i>n</i> = 154	Case controls <i>n</i> = 166	Odds Ratio (95%CI)	p-value
<i>Total women</i>				
Allele frequency (number of alleles)				
G	0.55(170)	0.57(189)	1.0 (reference)	
A	0.45(138)	0.43(143)	1.07 (0.78–1.48)	0.717
Genotypic frequency (number of genotypes)				
G/G	0.29(44)	0.32(52)	1.0 (reference)	
G/A	0.53(82)	0.51(85)	1.14 (0.67–1.95)	0.701
A/A	0.18(28)	0.17(29)	1.14 (0.56–2.32)	0.820
G/A+A/A	0.71(110)	0.68(114)	1.14 (0.69–1.89)	0.678
<i>Premenopausal women</i> <i>n</i> = 59 <i>n</i> = 99				
Allele frequency (number of alleles)				
G	0.64(76)	0.60(117)	1.0 (reference)	
A	0.36(42)	0.40(81)	0.80 (0.48–1.31)	0.413
Genotypic frequency (number of genotypes)				
G/G	0.39(23)	0.33(32)	1.0 (reference)	
G/A	0.51(30)	0.53(53)	0.79 (0.37–1.68)	0.622
A/A	0.10(6)	0.14(14)	0.60 (0.16–1.98)	0.508
G/A + A/A	0.61(36)	0.67(67)	0.75 (0.36–1.55)	0.498
<i>Postmenopausal women</i> <i>n</i> = 95 <i>n</i> = 67				
Allele frequency (number of alleles)				
G	0.49(94)	0.54(72)	1.0 (reference)	
A	0.51(96)	0.46(62)	1.19 (0.74–1.89)	0.520
Genotypic frequency (number of genotypes)				
G/G	0.22(21)	0.30(20)	1.0 (reference)	
G/A	0.55(52)	0.48(32)	1.55 (0.68–3.53)	0.345
A/A	0.23(22)	0.22(15)	1.40 (0.52–3.79)	0.615
G/A + A/A	0.78(74)	0.70(47)	1.50 (0.69–3.25)	0.350

observed for CYP1B1 C/C, C/G and G/G genotypes in total (p value = 0.0003), premenopausal (p value = 0.0077 and postmenopausal (p value = 0.0126) controls. The significant deviation of control group from equilibrium could be due to variability of environmental conditions, life style and food habits of Indian women and could play a role in offering protection against breast cancer in Indian women.

3.4. Association of COMT (Val¹⁵⁸ Met) and CYP1B1 (Leu⁴³² Val) polymorphisms in combination

It was observed that homozygous wild type (G/G) of COMT and CYP1B1 heterozygous variant (C/G) in combination exhibited a significant protection against breast cancer risk in total and premenopausal women [OR = 0.29; 95% CI, 0.11–0.76, p value = 0.009 and OR = 0.21; 95% CI, 0.05–0.82; *p* = 0.021]. COMT G/A heterozygous variant in combination with CYP1B1 wild C/C showed significant protective association in premenopausal women [OR = 0.29; 95% CI, 0.08–0.96, p value = 0.042]. Homozygous variant (A/A) of COMT and CYP1B1 heterozygous variant (C/G) in combination exhibited a trend of protection al-

though not statistically significant against breast cancer risk in premenopausal women [OR = 0.15; 95% CI, 0.01–1.11, p value = 0.054] (Table 4). The association patterns of COMT polymorphism between estrogen receptor positive and estrogen receptor negative breast cancer patients did not exhibit statistically significant change, when analyzed separately (data not shown). The patterns of association were statistically similar as observed with total breast cancer patients.

4. Discussion

Substitution of Leu to Val in CYP1B1 increases its catalytic activity by 2.4–3.4 folds, while Val to Met substitution in COMT decreases its catalytic activity of COMT by 2 to 3 fold. In this study, the polymorphisms of COMT (Val¹⁵⁸Met) and CYP1B1 (Leu⁴³²Val) genes were performed to assess their contribution in the breast cancer risk in Indian women. Although such studies have been performed in some populations globally [7, 29–34], keeping into consideration of the variability in food habit, life style, environmental exposure and ethnicity [23,24], it was worthwhile to perform such study in Indian women.

Table 4
 Combinational genotypic frequencies of COMT and CYP1B1 polymorphism in Indian female breast cancer patients and controls considered for analysis. Former genotype given in table indicates COMT and later CYP1B1

	Patients n = 154	Case controls n = 166	Odds Ratio (95%CI)	p-value
<i>Total women</i>				
Genotypic frequency (number of genotypes)				
G/G, C/C	0.15(23)	0.09(15)	1.0 (reference)	—
G/G, C/G	0.10(16)	0.22(36)	0.29(0.11–0.76)	0.009
G/G, G/G	0.04(5)	0.01(1)	3.26(0.31–164.34)	0.392
G/A, C/C	0.21(32)	0.26(43)	0.49(0.20–1.16)	0.110
G/A, C/G	0.26(40)	0.23(39)	0.67(0.28–1.57)	0.419
G/A, G/G	0.06(10)	0.02(3)	2.17(0.45–14.1)	0.336
A/A, C/C	0.09(14)	0.07(11)	0.83(0.26–2.61)	0.924
A/A, C/G	0.09(14)	0.10(16)	0.57(0.19–1.67)	0.371
A/A, G/G	00(0)	0.01(2)	—	—
<i>Premenopausal women</i>				
n = 59 n = 99				
Genotypic frequency (number of genotypes)				
G/G, C/C	0.22(13)	0.08(8)	1.0 (reference)	—
G/G, C/G	0.14(8)	0.23(23)	0.21(0.05–0.82)	0.021
G/G, G/G	0.03(2)	0.01(1)	1.23(0.05–81.57)	1.000
G/A, C/C	0.24(14)	0.30(30)	0.29(0.08–0.96)	0.042
G/A, C/G	0.20(12)	0.21(21)	0.35(0.10–1.25)	0.120
G/A, G/G	0.07(4)	0.02(2)	1.23(0.14–16.47)	1.000
A/A, C/C	0.07(4)	0.06(6)	0.41(0.07–2.44)	0.441
A/A, C/G	0.03(2)	0.08(8)	0.15(0.01–1.11)	0.054
A/A, G/G	00(0)	00(0)	—	—
<i>Postmenopausal women</i>				
n = 95 n = 67				
Genotypic frequency (number of genotypes)				
G/G, C/C	0.11(10)	0.10(7)	1.0 (reference)	—
G/G, C/G	0.08(8)	0.20(13)	0.43(0.09–1.92)	0.344
G/G, G/G	0.03(3)	00(0)	—	—
G/A, C/C	0.19(18)	0.20(13)	0.97(0.25–3.79)	0.799
G/A, C/G	0.29(28)	0.27(18)	1.09(0.30–3.88)	0.886
G/A, G/G	0.06(6)	0.01(1)	4.20(0.35–220.30)	0.352
A/A, C/C	0.11(10)	0.07(5)	1.40(0.26–7.59)	0.927
A/A, C/G	0.13(12)	0.12(8)	1.05(0.23–4.79)	0.792
A/A, G/G	00(0)	0.03(2)	—	—

Although lack of significant association with individual genotype was observed in total, premenopausal or postmenopausal women, the genotypic frequency of CYP1B1 G/G homozygous was 2–3 folds higher in patients as compared with respective controls. The absence of significant association with breast cancer in Indian women is contrary to Caucasian, English, Swedish and Turkish populations, where either positive or negative associations with breast cancer risk are reported [29,30,32]. The frequency of C allele was found higher in Indian women as compared with G allele. This is in accordance with American Caucasians but higher than that of Chinese [35,36]. The higher frequency of C allele was also observed in premenopausal Indian women, as observed in Turkish and Chinese [7, 32]. The frequency of C/G genotype in Indian patients exhibited almost similar pattern as observed in African American and Caucasian American but lower than Chi-

nese and higher than Turkish and Taiwanese [7,31,32, 35].

The frequency of G allele (62%) in COMT was slightly higher in premenopausal women, as observed in other Asians [37] but contrary to Caucasians [38]. However, lack of statistically significant difference was noted in the frequency of alleles-G and A both in total and postmenopausal women as observed in Finnish women [39].

COMT wild type and CYP1B1 heterozygous variant in combination exhibited significant protection against breast cancer risk in total and premenopausal women. COMT homozygous variant and CYP1B1 heterozygous variant in combination also exhibited a significant protection against breast cancer risk in premenopausal women. The protective association in premenopausal women could be a compensatory phenomenon that regulates the accumulation of toxic metabolites under the conditions of increased 4-hydroxylation and decreased

methyl transfer reactions, as Leu to Val substitution in CYP1B1 increases its catalytic activity and substitution of Val to Met at codon 158 decreases the catalytic activity of COMT [20,33,34]. Combination of increase in 4-hydroxylation activity and decrease in COMT activity could be important to protect premenopausal women with breast cancer susceptibility. In postmenopausal Indian women, polymorphisms in estrogen-metabolizing genes possibly do not play important role, as reported in many other populations [6, 7,29–32] due to variable life style, environmental exposure and ethnicity of Indians.

In conclusion, the results demonstrate that individually COMT or CYP1B1 polymorphism may not be very important contributors; in combination they may act as important determinants of breast cancer incidences among premenopausal Indian women.

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