RESEARCH COMMUNICATION

CYP1A1 Gene Polymorphisms: Lack of Association with Breast Cancer Susceptibility in the Southern Region (Madurai) of India

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Abstract

The cytochrome P 450 1A1 gene encoding a phase I metabolic enzyme appears to be a candidate for breast cancer risk. It is involved in the phase I detoxification of polycyclic aromatic hydrocarbons (PAHs) and 2-hydroxylation of estrogens and mammary carcinogens into 2-hydroxy catechol metabolites. Several studies have investigated polymorphisms in *CYP1A1* and breast cancer risk with inconsistent results. We here carried out a population based case-control study of the CYP MspI (CYP1A1*1/M1) and Ile462Val (CYP1A1*2/M2) polymorphisms in *CYP1A1* to clarify their importance in determining breast cancer susceptibility in a South Indian population. A total of 50 cases and 50 controls were genotyped for both polymorphisms. We also investigated putative interactions with exposure to pollution, radiation and intake of tobacco and *CYP1A1* genotype and breast cancer risk using a case only study design. The genotype distribution of *CYP1A1*1* in cancer patients was 6% for homozygous (*CYP1A1* M1 [C/C], 34% for heterozygous CYP1A1 M1 [T/C] and 60% for wild type (*CYP1A1* M1 [T/T] (OR: 0.583, CI-95% (0.252-1.348). The genotype distribution of M2 genotypes in patients was 24% of homozygous (*CYP1A1* M2 [Val/Val], 4% for heterozygous (*CYP1A1* M2 [Ile/Val] and 72% for wild type allele (*CYP1A1* M2 [Ile/Ile] [OR: 0.720, CI-95% (0.606-0.856)]. Our results suggest that there is no significant correlation between *CYP1A1* M1/ *CYP1A1* M2 polymorphism and occurrence of breast cancer in South Indian women.

Keywords: CYP 1A1 - M1 and M2 polymorphisms - breast cancer - South Indian population

Asian Pacific J Cancer Prev, **12**, 2133-2138

Introduction

Breast cancer is the major type of cancer which affects women in India. The etiology of breast cancer is complex and still not clear, whereas a small proportion of breast cancer cases is attributed purely to genetic reasons (Colditz, 1993; Kelsey & Bernstein, 1996), but for a vast number of cases, there is compelling evidence for the role of other factors such as family, and reproductive history, diet, alcohol consumption and exposure to environmental carcinogens. Among the environmental factors, exposure to polycyclic aromatic hydrocarbons (PAHs) has been suggested to contribute to breast cancer formation (Perera et al. 1995). PAHs are procarcinogens which are metabolically activated by CYP1A1, generating reactive metabolites which reacts with DNA possibly inducing tumorogenesis (da Fonte de Amorim et al., 2002).

CYP1A1, encoded by *CYP1A1* gene is one of the most important phase I enzymes (with aryl hydrocarbon hydroxylase activity) expressed in breast tissue (Li et al., 2004). In humans, *CYP1A1* is under the regulatory control of the aryl hydrocarbon receptor, a transcription factor that regulates gene expression (Masson et al., 2005). Formation of aryl epoxides by aryl hydrocarbon hydroxylase is the first step in the metabolism of PAHs. The activity of aryl hydrocarbon hydroxylase encoded by the *CYP1A1* gene has been observed in both normal and neoplastic human breast epithelium. Some studies suggest that heterocyclic amines are activated by CYP1A1 via N-hydroxylation in breast tissue. CYP isoforms metabolize estrogens to catecholoestrogens (CEs), semiquinones and quinines by oxidation reactions. These carcinogens metabolites are capable of forming either stable or depurinating DNA adducts, thus having the potential to result in permanent nucleotide mutation (Syamala et al. 2010). *CYP1A1* gene polymorhisms have been extensively studied, especially in relation to cancer susceptibility (Mahmoud et al., 2010) and CYP1A1 is an interesting candidate factor that might influence susceptibility to breast cancer risk (Chen et al., 2007).

The *CYP1A1* gene, located at 15q22-q24, comprises seven exons and six introns and spans 5,810 base pairs (Masson et al., 2005). Four common polymorphisms of the *CYP1A1* gene have been identified: M1, a T-C substitution

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at nucleotide 3801, giving rise to a *MspI* restriction site in the 3'-noncoding region (Kawajiri et al., 1990); M2, nucleotide 2455 A/G, resulting in an amino acid change at codon 462 of isoleucine to valine within the heme-binding domain of exon 7 (Hayashi et al., 1991); M3, nucleotide 3205 T-C, creating a *MspI* restriction fragment length polymorphism (RFLP) in the 3'-noncoding region (Crofts et al. 1993); and M4, nucleotide 2453 C/A, resulting in an amino acid substitution at codon 461 of threonine to asparagine (Cascorbi et al., 1996).

Though the functional significance of variant CYP1A1 genotypes is unclear (Chen et al., 2007), studies of CYP1A1 in cultured human lymphocytes showed significantly elevated levels of inducible enzyme activity among M2 genotypes compared with the wild-type genotype. Crofts et al. (1994) reported that M2 alleles appeared to be associated with CYP1A1 inducibility at the level of transcription followed by threefold elevation in aryl hydrocarbon hydroxylase enzyme activity. The M1 allele was also reported to be more readily inducible than the CYP1A1 wild-type allele (Kiyohara et al., 1996; Li et al., 2004). Since studies concerning the polymorphism of CYP1A1 in South Indian breast cancer patients are very limited, in the present study we have evaluated the correlation between CYP1A1*1 and CYP1A1*2 polymorphism and breast cancer risk from South Indian population

Materials and Methods

Chemicals

The restriction enzyme *NcoI* was obtained from New England Biolabs, UK. *MspI*, Primers, *Taq* polymerase, dNTPs, and the chemicals used for PCR amplification was obtained from Genie (Bangalore, India). Other chemicals used were of analytical grade.

Collection of blood samples

Blood samples were collected from cancer patients of Guru Cancer Hospital, Devaki Multispeciality Hospital, Dr. G. K. Mohana Prasad Laboratories, Madurai, Tamil Nadu, India, using EDTA coated vacutainers. 50 samples each were collected from breast cancer patients and non-cancer individuals as controls.

DNA extraction from blood

Blood samples anticoagulated with EDTA were stored at 4 ˚C until use. DNA was extracted from 200μl of whole blood by the method of (Jeffreys & Morton 1987).

Exon 4 (codon 72) and exon 7 (codon 249) amplification

The CYP1A1 3' non coding region was amplified using 20 pmol each of the primers (5' TAGGAGTCTTGTCTCATGCCT 3' and 5' CAGTGAAGAGGTGTAGCCGCT 3') in a 25µl reaction volume containing 200µM of dNTPs, 1X concentration of standard PCR buffer, 1U *Taq* polymerase, 2.5mM $MgCl_2$, and 50 ng DNA template. The thermocyling conditions were 95°C for 5 min, amplification was carried out by 35 cycles of 95°C for 30 sec, 66°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 10 min in a Autorisierter Thermocycler (Eppendorf India Limited, Chennai). The amplification product (343 bp) was visualized by staining with ethidium bromide, after electrophoresis on 1% agarose gels (Figure 1a).

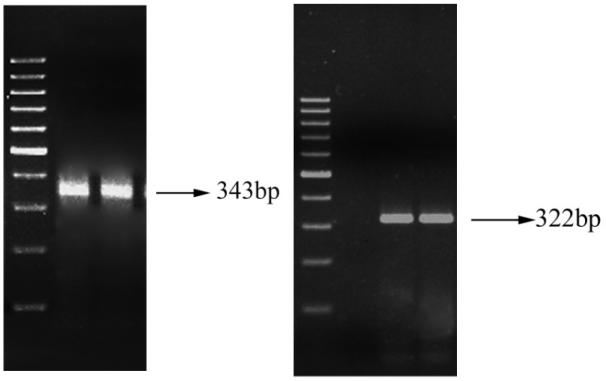

Figure 1. Agarose Gel Electrophoresis. Left) CYP1A1*1 genotype amplicons; Right) CYP1A1*2 genotype amplicons

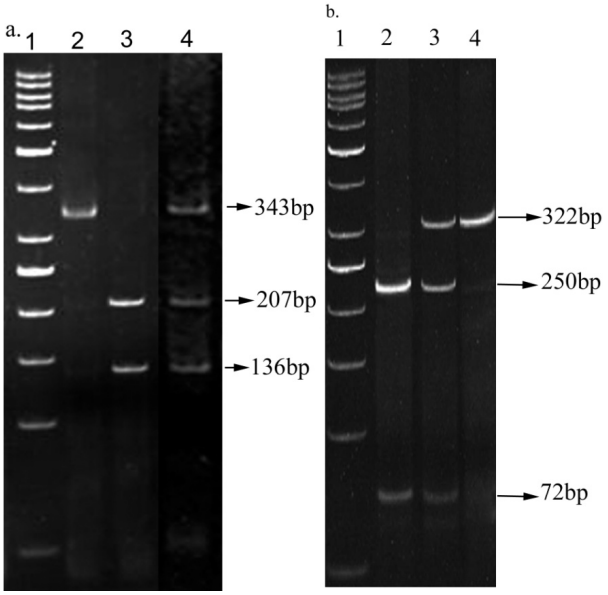

Figure 2. RFLP Analysis. a) *CYP1A1*1*. Lanes 1, 50bp DNA molecular weight markers; 2, wild type (TT); 3, homozygous variant (CC); 4, heterozygous variant (T/C)amplicons; b) *CYP1A1*2*. Lanes 1, 50bp DNA molecular weight markers; 2, homozygous variant (Val/Val); 3, heterozygous variant (Ile/Val) wild type (TT); 4, wild type (Ile/Ile)

The CYP1A1 exon 7 region was amplified and the expected size of the PCR product was 322 bp. The exon 7 of the CYP1A1 gene was amplified using 25 pmol of each primer (5' AAAGGCTGGGTCCACCCTCT 3' and 5' AAAGACCTCCCAGCGGGCCA3') in a 25µl reaction volume containing 200μM of dNTPs, 1X concentration of standard PCR buffer, 1U polymerase, 1.5 mM $MgCl_2$ and 50 ng DNA template. The thermocyling conditions were 94°C for 5 min, amplification was carried out by 35 cycles of 94°C for 1 min, 63°C for 30 sec, and 72°C for 2 mins, with a final extension at 72°C for 7 min in a Autorisierter Thermocycler (Eppendorf India Limited). The amplification product (322 bp) was visualized by staining with ethidiumbromide, after electrophoresis on 1% agarose gels (Figure 1b).

Detection of polymorphism by restriction analysis

The 3' non coding region of 343bp DNA fragment,

Table 1. Clinical Characteristics and Risk Factors of Breast Cancer Patients by CYP1A1*1 Status

	N (%)	T/T(%)	T/C (%)	C/C (%)	P
Age (n=50)					
≤ 49	34 (68)	17 (100)	17 (56.7)	0 (0)	0.984
50-59	8 (16)	0 (0)	8 (26.7)	0 (0)	
≥ 60	8 (16)	0 (0)	5 (16.7)	3 (100)	
Body mass index (n=50)					
< 18.5	6 (12)	6 (35.3)	0 (0)	0 (0)	0.604
18.5-24.9	23 (77)	11 (64.7)	12 (40)	0 (0)	
25-29.9	18 (36)	0 (0)	18 (60)	0 (0)	
≥ 30	3 (6)	0 (0)	0 (0)	3 (100)	
Tobacco (n=50)					
Yes	21 (42)	14 (82.3)	4 (13.3)	3 (100)	0.001
No	29 (58)	3 (17.6)	26 (86.7)	0 (0)	
Residence (n=50)					
Urban	20 (40)	17 (100)	3 (10)	0 (0)	0.534
Rural	30 (60)	0 (0)	27 (90)	3 (100)	
Diet (n=50)					
Veg	5 (10)	5 (29.4)	0 (0)	0 (0)	0.037
Non Veg	45 (90)	12 (70.6)	30 (100)	3 (100)	
Indoor pollution (n=50)					
Yes	36 (72)	3 (17.6)	30 (100)	3 (100)	0.001
No	14 (28)	14 (82.4)	0 (0)	0 (0)	
Radiation (n=50)					
Yes	35 (70)	2 (11.8)	30 (100)	3 (100)	0.001
No	15 (30)	15 (88.2)	0 (0)	0 (0)	
Exposure to asbestos (n=50)					
Yes	26 (52)	16 (94.1)	7 (23.3)	3 (100)	0.159
No	24 (48)	1 (5.9)	23 (76.7)	0 (0)	

Table 2. Clinical Characteristics and Risk Factors of Breast Cancer Patients by CYP1A1*2 Status

	N (%)	Ile/Ile (%)	Ile/Val (%)	Val/Val (%)	P
Age (n=50)					
≤ 49	34 (68)	34 (94.4)	0 (0)	0 (0)	0.640
50-59	8 (16)	2 (5.6)	1 (50)	5 (41.6)	
≥ 60	8 (16)	0	1 (50)	7 (58.3)	
Body mass index (n=50)					
<18.5	6 (12)	6 (16.7)	0 (0)	0 (0)	0.042
18.5-24.9	23 (46)	15 (41.6)	2 (100)	6 (50)	
25-29.9	18 (36)	12 (33.3)	0 (0)	6 (50)	
≥ 30	3 (6)	3 (8.33)	0 (0)	0 90)	
Tobacco (n=50)					
Yes	29 (58)	20 (55.6)	1 (50)	8 (66.7)	0.001
No	21 (42)	16 (44.4))	1 (50)	4 (33.3)	
Residence (n=50)					
Urban	20 (40)	18 (50)	0 (0)	2 (16.7)	0.009
Rural	30 (60)	18 (50)	2 (100)	10 (83.3)	
Diet (n=50)					
Veg	45 (90)	31 (86.1)	2 (100)	12 (100)	0.037
Non Veg	5 (10)	5 (13.9)	0 (0)	0 (0)	
Indoor pollution (n=50)					
Yes	36 (72)	22 (61.1)	2 (100)	12 (100)	1
No	14 (28)	14 (38.9)	0 (0)	0 (0)	
Radiation (n=50)					
Yes	35 (70)	21 (58.3)	2 (100)	12 (100)	0.001
No	15 (30)	15 (41.7)	0 (0)	0 (0)	
Exposure to asbestos (n=50)					
Yes	26 (52)	24 (66.7)	2 (100)	0 (0)	0.313
No	24 (48)	12 (33.3)	0 (0)	12 (100)	

which is derived from CYP1A1 gene, was subjected to restriction enzyme *MspI* digestion as follows:0.5 µl (10 units) of MspI enzyme, 2 µl of 1x buffer, 15 µl of DNA fragment, 2.5 µl sterile MilliQ water (20 µL total reaction volume). These reactions were kept for 37°C overnight in a water bath. Enzyme *MspI* cleaves a C/CGC sequence at CYP1A1 3' non coding region, generating 207 bp and 136 bp from the 343 bp DNA product of the PCR reaction. Electrophoresis was carried out on non-denaturing 15% polyacrylamide gels in 1xTBE buffer at 100 V for 8 hours. The genotype was determined under UV-illumination using a gel documentation system after staining with ethidium bromide (Figure 2a).

The 322 bp DNA fragment, which is derived from exon 7 of *CYP1A1* gene, was subjected to restriction enzyme digestion as follows: 2 units of enzyme *NcoI*, 2 µl of buffer, 15 µl of DNA fragment, 2.5 µl sterile milliQ water (20 µL total reaction volume). These reactions were kept for 2h at 37°C in a water bath. Enzyme *NcoI* cleaves a TC/CA sequence at exon7, generating 250 bp and 72 bp from the 322 bp DNA product of the PCR reaction. If there is a polymorphism at exon7, it results in a cleaved 322 bp fragment to 250 bp and 72 bp and this feature will be distinguished from that of normal samples (uncleaved) on 15% non-denaturing polyacrylamide gels stained with ethidium bromide (Figure 2b).

Statistical analysis

The statistical analysis was performed using the SPSS software version 17. χ^2 was used to analyze categorical variables and the Student t-test was used to compare the continuous variable age. The association between the CYP1A1 polymorphism and breast cancer was determined using the logistic regression method to assess odds ratio (ORs) and 95% confidence intervals (95% CI). The association between the CYP1A1 polymorphism and risk factors (both epidemiological and environmental) of the breast carcinomas was assessed using the $\chi 2$ test (categorical variables). P-values less than 0.05 were considered statistically significant.

Results

In total, 50 breast cancer patients and 50 healthy controls were genotyped for detecting *CYP1A1 MspI* G to C transversion (CYP1A1*1) and exon 7 codon 462 (CYP1A1*2) polymorphism. The frequency of M1 allele was 6% in patients (n=50) and 4% in controls (n = 50). The frequency of wild type allele was 60% in patients (n =50) and 72% in controls (n = 50). Statistically no significant association was found between the presence of M1 genotype and decreased risk of breast cancer [P< 0.205, OR: 0.583, CI-95% (0.252-1.348)]. But, OR proved that 50% of population are susceptible to breast cancer having M1 genotypes. In addition, people with consumption of tobacco, intake of fatty diet, living in urban areas, exposure to indoor pollution and radiation have increased breast cancer risk with M1 allele (Table 1). The present study shows that, there is no association in the presence of M1 polymorphism in breast cancer but there is association between environmental pollution and the presence of M1 allele in breast cancer.

For M2 polymorphism, all controls had the wild-type allele (Ileu). In 12 cases of patients having homozygous

(Val), only 4 of south Indian breast cancer patient was heterozygous (Ile/Val). The frequency of the Val allele was 24% for cancer cases and 0% in controls. The frequency of the Ileu was 72% in breast cancer cases and 100% in controls. Statistical analysis was performed and the results proved that there is a significant increase in the presence of Val genotype in breast cancer patients compare to control (P<0.001). OR was estimated for the presence of CYP1A1*2 genotype and breast cancer which proved that OR for M1 genotypes with cancer is not significantly increased (OR: 0.720, CI-95% (0.606-0.856)).

We also performed χ^2 test for categorical variables, which includes age, BMI, tobacco intake, diet, residential areas i.e., exposure to indoor pollution, radiation and asbestos. There is a highly significant increase in M2 genotype in the population with intake of tobacco (P<0.001). Similarly, there is a significant increase in the presence of M2 genotype in the population exposed to radiation (P<0.001). In addition, people living in the urban areas have a significant association between M2 genotype and cancer. Increased distribution of M2 genotypes was also observed in women with more intakes of fatty foods (Table 2). Overall, statistical analysis proves that M2 polymorphism is not linked with breast cancer susceptibility because of the observed less frequency of M2 polymorphism in our population.

Discussion

CYP1A1 is an important Phase I enzyme that is induced by, and acts upon many of the potent mammary carcinogens. The genotype and phenotype pattern of CYP1A1 may modulate the qualitative and quantitative pattern of products (Chacko et al., 2005). Crofts et al. (1994) demonstrated that the exon 7 polymorphism has a role in gene function by increasing both enzyme activity and mRNA levels in Asians. (Camitta et al. 1997)found that the polymorphisms in the CYP1A1 gene enhance the activity and inducibility of the enzyme. Although, CYP1A1 detoxifies or causes toxicity depends on many factors, such as sub-cellular content and location, amount of Phase II metabolism, degree of coupling to Phase II enzymes, and cell type- and tissue-specific context, as well as pharmacokinetics (Nebert et al., 2004; Chen et al., 2007).

CYP1A1*1 polymorphism is located in the noncoding region, (Masson et al. 2005) proposed that polymorphisms in noncoding sequences may influence gene function by altering the level, location, or timing of gene expression or messenger RNA stability. *CYP1A1*1* polymorphism affect the basal and/or induced CYP1A1 messenger RNA expression in lymphocytes and placenta. Apart from that, *CYP1A1*1* (C) variant has been associated with higher levels of DNA adducts in breast tissue in some studies, but not others. For *CYP1A1*2* polymorphism, the enhanced enzyme activity of the *CYP1A1* Val variants leads to increased production of the reactive intermediates from drugs as well as the carcinogens. *CYP1A1 M2* polymorphism alter the enzyme kinetic properties to produce both the diol metabolites form B(a)P and diol epoxide 2 from B(a)P -7,8-dihydrodiol. *CYP1A1 M2* polymorphism might affect the function of estrogenic metabolism, which decreases the risk of breast cancer by influencing the level of estrogen or competing with the 16a-hydroxylation pathway (Chen et al., 2007).

In the present study we found no evidence for an association between *CYP1A1* genotype and breast cancer risk for both the *CYP1A1 M1* and *CYP1A1 M2* polymorphisms. In contrast, several studies conducted on the association between polymorphisms of the *CYP1A1*1* and breast cancer risk revealed that in New York (Ambrosone et al., 1995), African-American, white women (Li et al., 2004), China (Shen et al., 2006), Korea (Shin et al., 2007) and India (Syamala et al., 2010), *CYP1A1*1* had a risk factor for breast cancer. *CYP1A1*1* variant genotype with an increased enzyme activity emerged as an important independent risk factor for sporadic as well as familial breast cancer in the South Indian population (Kiyohara et al., 1996).

However, in the present study there no risk was found to be associated for the presence of CYP1A1*1 polymorphism and breast cancer. We also found that there is a link between usage of tobacco, exposure to pollution and presence of *CYP1A1*1* polymorphism with breast cancer. Toxicological studies support that environmental toxicants like polychlorinated biphenyls induce CYP1A1 to metabolize environmental carcinogens into highly reactive intermediates, potentially resulting in DNA damage and ultimately carcinogenesis (Zhang et al. 2004). Thus, the effect of gene environment interaction between *CYP1A1* genotype and individual exposed to environmental contaminants on breast cancer risk merits further investigation. Our results are in concordance with studies from Caucasian and African American (Bailey et al. 1998), Canada (Krajinovic et al., 2001), Japan (Miyoshi et al., 2002), Germany, Austria (Hefler et al., 2004), USA (Le Marchand et al., 2005), China (Boyapati et al., 2005) and India (Singh et al., 2007a; 2007b), where no risk was found between presence of *CYP1A1*1* polymorphism and breast cancer.

Overall the lack of significant association between the studied *CYP1A1*2* polymorphisms and breast cancer risk is not in agreement with most of the previous studies among Indian population. Reports show that there is a significant association between *CYP 1A1*2* polymorphism and breast cancer risk (Chacko et al., 2005; Singh et al., 2007b; Surekha et al., 2009). Similarly other studies from Connecticut (Zhang et al., 2004), China (Huang et al., 1999), USA (Taioli et al., 1999) also found that presence of *CYP1A1*2* polymorphism had increased risk for breast cancer. The frequency of presence of Val allele was found to be slightly increased in breast cancer patients with respect to stage of the disease (Surekha et al., 2009). Our results are concordant with the previous study from Korea (Shin et al., 2007), African-American white women (Li et al., 2004), UK (Basham et al., 2001), Brazil (da Fonte de Amorim et al., 2002), Caucasian and African Americans (Bailey et al., 1998) and North Indians (Singh et al., 2007b), wherein *CYP1A1*2* polymorphism was not observed to be a risk factor to breast cancer. Overall, the *CYP1A1* 2* polymorphism was found to show a null or weak association with breast cancer risk (Rozati

et al., 2008). Subgroup analysis in this study between tobacco use and presence of *CYP1A1*2* polymorphism confirmed that higher incidence of presence of *CYP1A1*2* polymorphism in populations with tobacco intake.

To summarise, the discrepancy between the earlier studies (where the *CYP1A1 M1* and *CYP1A1 M2* polymorphism is associated with breast cancer) and our results may be due to several factors including difference in ethnicity, diet, study subject number and perhaps, most importantly ascribed to geographic variation and difference in the study population and other environmental exposures.

Acknowledgement

The authors gratefully acknowledge the computational and bioinformatics facility provided by the Alagappa University Bioinformatics Infrastructure Facility (funded by Department of Biotechnology, Government of India; Grant No. BT/BI/25/001/2006).

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