PREVALENCE OF CYP2D6*4 GENOTYPE AND ITS ASSOCIATION WITH TAMOXIFEN INDUCED HOT FLASHES IN PAKISTANI FEMALE BREAST CANCER PATIENTS

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ABSTRACT

Objective: To investigate the frequency of CYP2D6*4 in Pakistani breast cancer patients for the first time and also investigate its association with tamoxifen induced hot flashes.

Methodology: A retrospective study carried out in Nuclear Medicine, Oncology and Radiotherapy Institute (NORI) Islamabad and Combined Military Hospital Rawalpindi (CMH). Pre and postmenopausal breast cancer women who were advised 20mg/day of tamoxifen as adjuvant therapy were recruited for the study. The data from January 2000 to September 2013 was collected from the medical records of the outpatient breast cancer clinics. 232 women who fulfilled the eligibility criteria were initially recruited and their peripheral whole blood samples were taken. CYP2D6*4 was determined by using PCR-RFLP, allele*4 was not identified in 9 women and study was conducted on 223 women. None of the women died during the study period.

Results: Data of 223 women was analysed and the allele frequency of CYP2D6*1 was 86% and that of CYP2D6*4 was14 %. Women with CYP2D6*4/*4 did not experience mild to moderate or severe hot flashes as compared to women heterozygous or homozygous for wild type allele *1.

Conclusion: The frequency of CYP2D6*4 allele in Pakistani breast cancer women is 14% which is comparable to the Caucasians moreover CYP2D6*4/*4 genotypes have lower incidence of hot flashes, but the results are not statistically significant.

Key words: CYP2D6, Breast neoplasm, Hot flashes, Pakistan.

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INTRODUCTION

The CYPD6 gene is CYP450, family 2, subfamily D, polypeptide 6 and locates on the long arm of chromosome 22[1]. CYP2D6 is one of the key metabolizing enzymes involved in the metabolism of many endogenous and exogenous substances and about 20% to 25% of the drugs[2]. The gene shows polymorphism and there is marked ethnic disparity in the frequency of alleles. CYP2D6*4 a poor metabolizer (PM) allele is more common amongst Caucasians with a frequency of 12-23%, CYP2D6*17 is commonly seen in Africans while CYP2D6*10 is frequently observed amongst Asians[3]. Depending upon the activity of the enzyme and the allele permutations the individuals are categorized as poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM) and ultra-rapid metabolizers (UM) of CYP2D6 substrates. PM are individuals who are homozygous for one deficient allele or heterozygous for two variant deficient alleles, IM are heterozygous for one deficient allele or are carriers of two alleles with reduced enzyme activity; extensive metabolizers (EMs), who have two wild type alleles and UM are those who have multiple functional copies of the gene[4]. All these phenotypes have significant effects on the clinical efficacy of a drug and also on its untoward effects. A number of clinical studies have been carried out on the breast cancer patients who were advised adjuvant tamoxifen. Most of these studies show that patients taking tamoxifen and carry genetic variants with decreased (CYP2D6*10) or absent (CYP2D6*4) activity have reduced levels of endoxifen[5]. Research studies mainly on Caucasian breast cancer patients[6, 7, 8] and a few on Asian women [9,10] treated with adjuvant tamoxi-

fen show that those with CYP2D6 poor or intermediate metabolizer status are associated with decreased disease free survival or have increased risk of recurrence. A retrospective study has concluded that moderate or severe hot flashes are considerably less common in women homozygous for the *4 null allele of CYP2D6[11]. As patients with decreased CYP2D6 activity have poorer breast cancer outcomes and are less expected to complain hot flashes, it has been proposed that existence of hot flashes during tamoxifen therapy may forecast for better breast cancer results as this means that the woman is a carrier of EM or wild type gene and absence of hot flashes may indicate that the women is having a decreased or absent activity allele. In Pakistan there is no such pharmacogenomics information regarding frequency of CYP2D6 genotypes and their association with hot flashes, to our knowledge has never been reported. The rationale of our study is to find out the frequency of CYP2D6*4 and its association with hot flashes, as absence of hot flashes may predict the poorer outcome of tamoxifen therapy. We therefore have conducted this research for personalized healthcare in oncology, based on Pakistani women profiles and this will serve as important baseline information for further studies in cancer patients.

METHODOLOGY

Thirteen years and eight months retrospective data from January 2000 to August 2013 was reviewed using medical records of the patients. A questionnaire proforma was given to the patients along with a detailed educational letter, proclaiming the goal of this study. Patients were enquired about their experiences of any hot flashes before initiating tamoxifen therapy, and also if they experienced hot flashes during tamoxifen treatment. In both cases the patients were asked to document the frequency of the flashes per week and the average severity of the experienced hot flashes (severity grades: mild, <5 minute duration; moderate, 5 to 15 minute duration; severe, 15 to 20 minute duration; very severe, >20 minute duration, Appendix 1). These patients were coming for their follow ups and we interviewed all the enrolled patients in our study. All those women who reported hot flashes were asked to give the details about the frequency and intensity of hot flashes and then those were graded accordingly. The study was carried out in accordance with the current Good Clinical Practices (GCP). The protocol of the study was approved by Ethical Committee of Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College, Rawalpindi, Pakistan.

All the patients incorporated in the study provided a written informed consent. We recruited 232 Pakistani females with diagnosed breast cancer who received 20mg/day of adjuvant Tamoxifen from breast cancer clinics at NORI and CMH Rawalpindi belonging to different regions of Pakistan to provide representation from all areas. The exclusion criteria included Endocrine treatment other than tamoxifen; Chronic use of corticosteroid therapy; Use of medicines known to inhibit or induce CYP2D6; Hepatic or renal disease; and Pregnant or lactating women

The analytical procedures were carried out at Institute of Biomedical and Genetic Engineering (IBGE), Islamabad. A 5 ml of blood sample was taken from all the patients included in the study.

DNA extraction and CYP2D6*4 genotyping: Genomic DNA was isolated from the peripheral blood by the standard organic phenol–chloroform method[12]. Genotypes were determined using PCR or PCR-RFLP method as described [13]. The PCR for 1846G>A SNP genotyping was carried out containing 2.5uL of 10X PCR buffer (Fermentas UAB, Vilnius, Lithuania), 1uL of 25mM MgCl2 (Fermentas UAB, Vilnius, Lithuania) 1uL of 2mM dNTPs (Fermentas UAB, Vilnius, Lithuania), 0.2uL of 5U/uL Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania), 1uL of 100mM forward 5'- TGC CGC CTT CGC CAA CCA CT-3' and reverse primers 5'-TCG CCC TGC AGA GAC TCC TC-3' and 20ng genomic DNA. The final volume was adjusted to 25uL with PCR water. The PCR reaction was carried out under following thermal and cycling profile: (i) initial denaturation at 95°C for 1min

Appendix 1: Hot Flashes Definitions for the Patients

	Duration	Physical Symptoms	Emotional Symptoms
Mild	Less than 5 minutes	Warmth, Feeling uncomfortable, Red face	Not expected
***Moderate**	Lasts upto 15 minutes	Head, neck, ears or whole body feels warm, Tense, tight muscles, Clammy skin, Change in heart rate and rhythm, Sweating & Dry mouth	Feelings of irritation and restlessness, Feeling tired, Drained out, Annoyed and embarrassed.
****Severe**	Lasts upto 20 minutes	Warmth, Change in heart rate and rhythm, Severe sweating, Chest heaviness, Headache, Pricking or stinging sensation on skin	Anxiety, Feelings of having panic attack.

* Actions needed: Needs to use fan, open window, and remove the covers and wear light clothes
** Actions needed: Needs to use fan, open window, and remove the covers and sudden awakenings during night.

(ii) 35 cycles each of denaturation at 95°C for 45sec, primer annealing at 62°C for 45 sec and extension at 72°C for 45 sec and (iii) final extension at 72°C for 08 min. After the PCR of target DNA region containing the *4, RFLP was performed and the amplified DNA fragment was digested with the Bst N1 restriction enzyme (Fermentas UAB,Vilnius,Lithuania) at 37°C for 16 hrs. The digested products were analyzed on 2.5% w/v agarose gel through electrophoresis. After digestion the patients homozygous for wild type had two fragments of 201bp, 108bp. The individuals who were heterozygous contained both the wild and mutant allele yielded fragments of 309bp, 201bp, and 108bp. The mutant homozygous individuals produced a single fragment of 309 bp.

Hot flashes: A retrospective analysis of the data has been done for hot flashes and the women were given the questionnaire for grading of the hot flashes according to the National Cancer Institute Common Toxicity Criteria (version 1) as follows: 0, none or no change; 1, mild; 2, moderate; or 3, severe. Patients undergoing treatment with tamoxifen and taking 20 mg tamoxifen orally per day for breast cancer were registered. Participants were asked about the hot flashes before initiation and then after 1 month of tamoxifen therapy.

Genotypes were tested for Hardy-Weinberg equilibrium and the frequency of genotypes was calculated by direct counting. A p value of less than 0.05 was considered significant. Demographic data were determined and presented as mean, median, percentage or frequency where suitable for qualitative or quantitative variables. Hot flashes were graded as follows: 0, none or no change; 1, mild; 2, moderate; or 3, severe. To ascertain whether the severity of hot flashes (0 to 3) differed with respect to genotype, the Wilcoxon rank sum test was used and to evaluate whether the proportion of women with moderate or severe hot flashes was smaller for those with the CYP2D6*4/*4 genotype than those without the CYP2D6*4/*4 genotype one-sided Fisher's exact test was used.

RESULTS

Out of 232 CYP2D6*4 was unidentified in 9 women so 223 women who were diagnosed cases of breast cancer and were advised 20mg/day of tamoxifen with mean age 47.09 ± 9.9 years were inducted in the study. Among the 223 patients, a total of 56.9% (n = 127) were premenopausal and 43.04% (n = 96) were postmenopausal women. There was no perimenopausal woman in our study. Other demographic data of the patients is presented in Table 1.

Amongst the three genotypes group, 3.1% of the patients was CYP2D6 homozygous variant genotype (CYP2D6*4/*4), 22% was CYP2D6 heterozygous genotype (CYP2D6*1/*4), and 74.9% was CYP2D6 homozygous wild type genotype (CYP2D6*1/*1). The allele frequency of CYP2D6*1 was 86% and CYP2D6*4 was 14%, as shown in Table 2.

Hot flashes: Out of 223 patients, a total of 25% (n =54) reported having hot flashes, with 27.7% (n =15) reporting mild (grade 1), 70.3% (n=38) reporting moderate (grade 2), and 1.85% (n =1) reporting severe (grade 3) hot flashes. Association between CYP2D6*4 genotypes and hot flashes is shown in table 3 and Figure 1. However, for CYP2D6*4, none (0 of 7) of the women with the CYP2D6 *4/*4 genotype had mild to moderate or severe hot flashes compared with 25% (54 of 216) for patients with either the *1/*4 or *1/*1 genotypes. The

Figure 1: Association between CYP2D6*4 genotypes and hot flashes

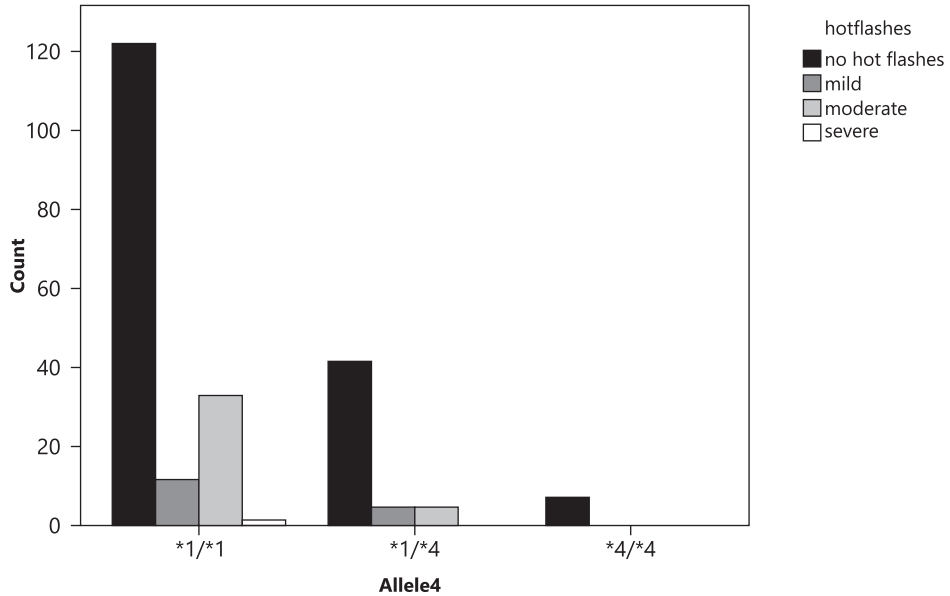

Table 1: Demographic data of the patients

Descriptive data	N	%
Number of patients	223	100
Age (years) Mean ± SD	47.09 ± 9.9	
Height (cm) Mean ± SD	156.05 ± 5.49	
Weight (Kg) Mean ± SD	66.3 ± 11.6	
Body mass index		
≥ 28	50	22.5
< 28	173	77.5
Menopausal status		
Premenopausal	127	56.9
Post menopausal	96	43.04
Stage		
1	25	11.2
II	120	53.8
III	78	35
Marital status		
Married	215	96.4
Unmarried	8	3.6

Table 2: Frequency of CYP2D6 genotypes

223 patients X 2= 446 Alleles (N)			Observed		Predicted	
Alleles	**N= 446**	**%**	**Genotypes**	**N= 223**	**%**	**(HWE)**
*1	383	86	*1 / *1	167	74.9	164
*4	63	14	*1 / *4	49	22.0	54
			*4 / *4	7	3.1	5
Chi-square =1.98; p = 0.1591 (HWE) Hardy-Weinberg Equilibrium						

Table 3: CYP2D6*4 genotypes and differential frequency of hot flashes

Genotypes		hot flashes				Total
		none	**mild**	**moderate**	**severe**	
*1/*1	Count	121	11	34	1	167
	%	72.5%	6.6%	20.4%	.6%	100.0%
*1/*4	Count	41	4	4	0	49
	%	83.7%	8.2%	8.2%	.0%	100.0%
*4/*4	Count	7	0	0	0	7
	%	100.0%	.0%	.0%	.0%	100.0%
Total	Count	169	15	38	1	223
	% within Allele4	75.8%	6.7%	17.0%	.4%	100.0%

Fisher exact test statistic value is 0.199724. The result is not significant at $p < 0.05$. When women with CYP2D6 carrying wild type allele were compared with women carrying heterozygous variant *1/*4 and homozygous variant* 4 / *4 (*1/*4 + *4/*4) the results show a significant association P= 0.048.

Frequency of CYP2D6*4 calculation: Allelic frequencies of CYP2D6 genotypes were in Hardy-Weinberg Equilibrium (HWE), p = 0.159. The calculation if allelic frequencies were in HWE:

The number of the *1 allele = (167 x 2) + (49 x 1) = 383 alleles

The number of the *4 allele = (7 x 2) + (49 x 1) = 63 alleles

The frequency of the *1 allele = p = 383/ (383 +63) = 0.8587

The frequency of the *4 allele = q = 63 / (383+ 63) = 0.14

The proportion of expected *1/*1, *1/*4 and *4/*4 genotypes could be predicted from HWE: p+q = 1 and (p + q)2 = 1 or p2 + 2pq + q2 = 1

p2 = 0.8587 x 0.8587 = 0.737

2pq = 2 x 0.8587 x 0.14 = 0.240

q2 = 0.14 x 0.14 = 0.0196

The total number of patients included to this study was 223

Expected number of *1/*1 = 0.737 x223 = 164.56≈ 165

Expected number of *1/*4 = 0.240 x 223 = 54

Expected number of *4/*4 = 0.0196 x 223 = 4.3 ≈ 4

The observed number of *1/*1 = 167

The observed number of *1/*4 = 49

The observed number of *4/*4 = 7 Chi-square =1.98; p = 0.1591. Therefore, we accepted the null hypothesis that the observed and expected values are not significantly different, and that our population is indeed in Hardy Weinberg equilibrium.

DISCUSSION

Quite a number of studies have established a correlation between CYP2D6 polymorphisms and the clinical efficacy of adjuvant tamoxifen treatment in ER-positive breast cancer[5]. Studies carried out in Caucasian women demonstrated that the CYP2D6*4 causes a PM phenotype and poorer clinical outcome of tamoxifen therapy in these women[6, 14]. Comparatively few studies were conducted in the Asians reporting that CYP2D6*4/*4 is a rare genotype in Asians with a frequency of 1% to 3%[10, 15]. However, frequency of CYP2D6*4 genotypes were not explored in Pakistani breast cancer women before this study. We conducted this study to investigate the prevalence CYP2D6*4 genotypes in Pakistani female breast cancer patients. Our Study found that the frequency of CYP2D6*4/*4 genotype is 3.1% which is synonymous to other Asians. We also explored the association of CYP2D6*4 genotypes with tamoxifen induced hot flashes. Women with homozygous CYP2D6*4 genotype have a PM status and therefore do not metabolize tamoxifen to its active metabolite endoxifen and hence no antiestrogenic effect and no hot flashes. None of the women (0/7) in our study with the *4/*4 genotype experienced mild to moderate or severe hot flashes, compared with 25% (54/ 216) of the women with either the *4/*1 or*1 /*1 genotypes. In a study by Goetz[11] 20% (36/177) women with normal enzyme activity reported hot flashes compared to 0% (0/13) with PM genotype *4/*4. This discovery increases the likelihood that adverse effects are an indirect measure of CYP2D6 activity. Women with CYP2D6*1 wild type genotype were compared with women carrying heterozygous variant *1/*4 and homozygous variant* 4 / *4 (*1/*4 + *4/*4) the results showed a significant association of PM genotype with hot flashes P= 0.048. Our data maintain the possibility of a noteworthy connection between hot flashes and CYP2D6 genotype and propose that CYP2D6 activity is liable but not the sole determinant of tamoxifen associated hot flashes. This study presents the first data in Pakistan that self-reported hot flashes may be prognostic of the effectiveness of tamoxifen and long term survival in women with early stage breast cancer. Our study had some limitations mainly, owing to the retrospective nature of the study design and lack of data association between CYP2D6 polymorphism and the plasma concentration of tamoxifen metabolites.

CONCLUSION

The prevalence of CYP2D6*4/*4 found in Pakistani breast cancer women according to our study was 3.1% which is similar to general Asian populations and the allele*4 frequency is 14%. CYP2D6 activity may be a weak prognostic factor for tamoxifen-induced hot flashes. As Tamoxifen efficacy should not be determined by the absence or presence of hot flashes. For a better understanding of the relationship between hot flashes and breast cancer outcome and to establish factors involved in tamoxifen induced hot flashes, more research studies and clinical trials are required.

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CONTRIBUTORS

NN conceived the idea, planned the study and wrote the manuscript. AW, KF, MI, QM and NQ helped in acquisition of data, statistical analysis and interpretation of data. All authors contributed significantly to the submitted manuscript.