RESEARCH ARTICLE

Tamoxifen Resistance and CYP2D6 Copy Numbers in Breast Cancer Patients

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Abstract

Background: Breast cancer accounts about one million from total annual ten million new diagnosed cases of neoplasia worldwide and is the main cause of death due to cancer in women. Tamoxifen is the most popular selective estrogen receptor modulator used in anti estrogen treatments. Tamoxifen must be converted into its metabolite endoxifen for biologic effects; this conversion process is catalysed by highly polymorphic cytochrome P450 2D6 (CYP2D6). This study surveyed copy number variation of the CYP2D6 gene and its possible correlation with Tamoxifen resistance in breast cancer patients. **Methods:** This case control study was performed on samples taken from 79 patients with breast cancer who used tamoxifen in Yazd and Tehran Cities, Iran. Real time reactions were conducted for 10 healthy samples using the comparative C_t (Cycles threshold) method, each pair of genes being compared and samples with ratios around 1 were taken as control samples. Proliferation reactions were done by Real-Time PCR ABI Prism 7500. All registered data were transformed into SPSS 15 program and analyzed. **Results:** Efficiency of PCR for both CYP2D6 and ALB genes was 100%. From all 23 drug resistant patients 21.7% had one copy, 47.8% two copies and 30.4% had three copies. Also from all 56 drug sensitive patients, 26.8% had one copy, 51.8% two copies and 21.4% had three copies. The percentage of patients with one and two copies was similar between two groups but patients with three copies were more likely to belong to the drug resistant group more. Odd ratios for one and two copies were 0.759 and 0.853 respectively, indicating possible protective effects while that for three copies was 1.604. **Conclusions:** Based on our study there is no significant link between *CYP2D6* gene copy numbers and tamoxifen resistance in women with breast cancer. But more studies considering other influencing factors appear warranted.

Keywords: Tamoxifen - CYP2D6 gene - copy numbers - drug resistance

Introduction

From total annual ten million new diagnosed cases of neoplasia worldwide one million are breast cancer who is the main cause of death due to cancer in women (Mousavi et al.,2009). Based on reports in year 2000 about 375000 deaths due to breast cancer was reported and in 2010 more than 1.5 million new cases of breast cancer is diagnosed (National cancer institute, 2009). Breast cancer is more prevalent in urban areas residents and also in Caucasian women. Prevalence of breast cancer according to Age Standardized Rate (ASR) in developed areas like North America, Australia and Japan is 67.8 in 100000 of population wile in areas with lower development like Africa, Asia (Except Japan) and Central America is about 23.8 in 100000. But during three past decades the mortality due to breast cancer had increasing pattern. Based on reports from countries of Middle East, breast cancer is the most common cancer among women of this regions and includes about 12-13% of all cancer reports (Mousavi et al., 2009). Its prevalence had been increasing in Iran too. In Iran breast cancer is more common among 50-54 years women (about 23.16 in 100000 of population) (Mousavi et al., 2009).

In patients with estrogen receptors (ER+) tumoral cells growth is depended to estrogen. Selective modulators of estrogen receptors inhibit adhesion of estrogen to its receptor and decrease or block the proliferation induced by estrogen in ER+ tumors (Torresan., 2008; Janelle et al., 2009). Tamoxifen is the most popular selective estrogen receptor modulator using in anti estrogen treatments (Wu et al., 2009). This product is using extensively in the world even for women in premenopausal or postmenopausal period with metastatic breast cancer. Also it benefited for adjuvant therapy of primary breast cancer and as part of chemotherapy for high risk women (Osborne et al., 1998; Bradford et al., 2002; Wegman et al., 2005). Recent reports shows that tamoxifen converts to its metabolite endoxifen for its biological effects; the conversion process catalyses by a highly polymorphic enzyme, cytochrome

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P450 2D6 (CYP2D6). Resistance to tamoxifen has been reported in some patients. The resistance is a complicated multifactorial process that genetic processes are one of effective factors (Bonanni et al., 2006). Using genotypic information of CYP2D6 can be effective to understand the causes of treatment resistance and possibly is helpful as a pharmocogenetic instrument to optimize treatment of breast cancer. In European general population, 6-10% of people had deficiency in metabolism of CYP2D6 (Ozawa et al., 2004) that causes weak ability of converting tamoxifen to endoxifen and then lack of pharmacological effect and resistance. Genomic diversity of CYP2D6 includes single nucleotide polymorphism and copy numbers variation (CNV) (National cancer institute, 2009; Yu et al., 2009).

CNV means one Kb or higher pieces of DNA with various numbers of copies in contrasting with a reference genome that is a part of genomic variation with single nucleotide polymorphism can be a factor in phenotype variation related to drug response (Schaeffeler et al., 2003; Yu et al., 2009). Copies of CYP2D6 can vary from 0-13 repeats of gene that possibly cause phenotypic variation in metabolism of drugs substrate of this enzyme. All CNVs will not act in similar fashion. In CYP2D6, CNV can cause decreased enzyme activation (gene deletion) or increased enzyme metabolism (Ingelman-Sundberg et al., 1991; Johansson et al., 1993; Schaeffeler et al., 2003; Ingelman-Sundberg et al., 2007; Hosono et al., 2009). The aim of this study was to detect copy numbers variation of the CYP2D6 gene and its possible correlation with Tamoxifen resistance among breast cancer patients.

Materials and Methods

After approval of institutional ethics committee this case control study was done during April 2010-February 2011. Peripheral blood samples of 79 patients with breast cancer who used tamoxifen were taken in Yazd and Tehran Cities, Iran. Written consent was taken from all participants.

All participants after six months of treatment with tamoxifen were divided into two groups of sensitive and resistant to tamoxifen. Patients were allocated based on age and metastasis. Genomic DNA of blood samples were extracted using promega kits (catalogue no. LA1120(USA)) based on factory instructed protocol.

Primers for CYP2D6 gene from exon nine and ALB (albumin) gene (as internal reference gene) from exon twelve were designed using Gene runner and Primer express 3.0 software. List of primers are indicated in Table 1.

For drawing standard curve for PCR performance, both two extracted pieces of genes (CYP2D6 and ALB) from PCR were transferred into PTZ57R plasmid of TA cloning kit (InsTA cloning kit, fermentas) and proliferated by E.coli bacteria (TOP10F'). Proliferated plasmids were extracted from bacteria by miniprep Bioneer kits and then were undergone Real-Time PCR with similar 1/4 attenuation and standard curves were drawn for both two genes.

Selecting control samples

Samples of ten normal subjects without any clinical manifestations were taken. Real time reaction was done for them. By using comparative C_t (Cycles threshold) method, each couple of genes was compared and samples with ratio≈1 were found and took as control samples.

Proliferation reactions implemented Real-Time PCR ABI Prism 7500. Each reaction was consisted of 40 nanograms of genomic DNA, 1X master mix buffer (PrimerDesign Ltd, UK) and 500 Nm from each primer (Cinna Gen, Iran) in a 25 μl volume. PCR was done under ten minutes 95°C of primary denaturation, 40 cycles in 15 seconds and 95°C and 30 seconds in 60°C for primers connection and 72°C for proliferation by polymerase enzyme during 20 seconds. Then on Cycles thresholds (Ct) and using $2^{-\Delta\Delta Ct}$ formula, ratio of target and reference genes were calculated and compared under Excel software. All registered data were transformed into SPSS-15 program and analyzed.

Results

Mean age of participants in resistant group was 46.2±9.5 years while in sensitive group was 53.3±13.9. Resistant patients had lower ages significantly (P=0.026). Curve was drawn by ABI 7500 showed highly effective proliferation of ALB and CYP2D6 genes (Efficiency of CYP2D6 102% and r^2=1, Efficiency for ALB 107% and r^2=0.999) (Figures 1, 2). Mean age in drug resistant group was lower than drug sensitive group.

Mean *CYP2D6* gene numbers of copies in both groups has been indicated in Table 2. From all 23 drug resistant patients 21.7% had one copy, 47.8% two copies and 30.4% three copies. Also from all 56 drug sensitive patients, 26.8% had one copy, 51.8% two copies and 21.4% three copies. The percentage of patients with one and two copies was similar between two groups but patients with three

Table 1. CYP and ALB and Local Primers of Exon Nine that CYP Primers are Designed from it

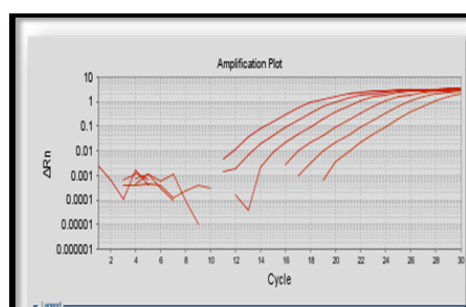| Name	Sequence	Mer	Amplicon size
ALB -F	5´-TGCATGAGAAAACGCCAGTAAGT-3´	20	102bp
ALB -R	5´-ATGTTTCATCGACTTCCAGAGCT-3´	20	
CYP -F	5´-CTTCCTCTTCTTCACCTCCCTG-3´	19	99bp
CYP -R	5´-CACCAGGAAAGCAAAGACACC-3´	21	

Figure 1. Amplification Curve for CYP2D6 Gene in Different Attenuations. X axis shows numbers of elapsed cycles during PCR and axis Y is log of florescence. The serial ordered pattern amplification shows high amplification efficacy
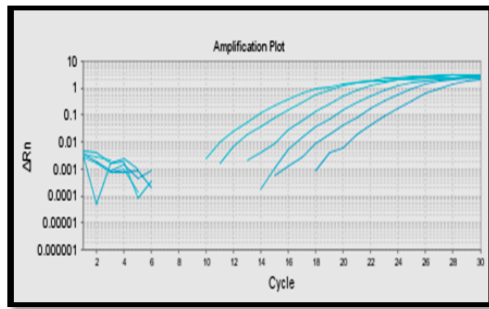
Figure 2. Amplification Curve for ALB Gene in Different Attenuations (with 1.4 coefficient). X axis shows numbers of elapsed cycles during PCR and axis Y is log of florescence

Table 2. Copy Numbers in Sensitive and Resistant Patients to Tamoxifen

Copy numbers	Sensitivity to tamoxifen		Total
	Resistant	sensitive	
1	5	15	20
	21.7%	26.8%	25.3%
2	11	29	40
	47.8%	51.8%	50.6%
3	7	12	19
	30.4%	21.4%	24.1%
Total	23	56	79
	100%	100%	100%

Table 3. Odd Ratio of Resistance to Tamoxifen for Each Copy Number with Maximum and Minimum Values of 95% Confidence Interval (Odd ratio more than one shows higher possibility of drug resistance)

Copy numbers	Value	95% of confidence interval (CI)	
		Min	Max
3	1.604	0.537	4.789
2	0.853	0.323	2.255
1	0.759	0.239	2.407

copies were more in drug resistant group. Odd ratio for one and two copies were 0.759 and 0.853 respectively that shows protective effect of them in drug resistance while odd ratio for three copies was 1.604 that shows the higher risk of patients with three copy numbers for tamoxifen resistance (Table 3).

Discussion

There are several studies on effect of CYP2D6 polymorphism on clinical outcomes of different drugs but the results are contradictory. A group of studies concluded that this enzyme polymorphism causes metabolic and pharmacokinetic diversity in ER+ breast cancer patients who get tamoxifen. Gotez et al. in 2005 surveyed on menopaused ER+ breast cancer women and found that poor metabolizers give lower benefits from tamoxifen treatment and are at higher risk of recurrence contrasting with extensive metabolizers (Goetz et al., 2005).

During a study in Korea on women (menopaused and non menopaused) with metastatic breast cancer patients with intermediate metabolizer (IM) genotype (IM/IM) had faster progression contrasting with others. Two other studies in Japan and china confirmed these results

(Hoskins et al., 2009; Hosono et al., 2009; Lim et al., 2007). But some other studies reject effect of CYP2D6 on metabolism of tamoxifen. These studies did not find any difference between poor, intermediate and extensive metabolizer genotypes with recurrence and metastasis (Wegman et al., 2005; Okishiro et al., 2005; 2009; Wegman et al., 2007; Pułczyńska et al., 2011).

New achievements on discovering effects of gene copy numbers on phenotypic diversity show the importance of studies to find relation between the CYP2D6 copy numbers and drugs pharmacokinetics (Sebat et al., 2004; Yu et al., 2009). Schaeffele et al in Germany used Real-time PCR for finding CYP2D6 copy numbers. Results were acceptable and similar to results of long-distance PCR and southern blot methods without any overlap on non metabolizer, poor metabolizer, intermediate metabolizer and extensive metabolizer alleles (Schaeffeler et al.,2003). In 2005 in France, Bodin et al used CYP2D6 Real-time PCR for 43 samples that the long term PCR and Southern blot methods were done for them previously. They found range of 1.02-1.28 for samples with one copy, 1.85-2.21 for two copies and 2.55-3.3 for three copies that shows sensitivity of real-time PCR to detect deletions and duplications (Bodin et al., 2005). Other studies in Germany (Duc et al., 2009) and Japan (Hosono et al., 2009) confirmed the efficacy of Real-time PCR for detecting copy numbers of CYP2D6 gene.

Based on our study, there are no studies on effect of numbers of CYP2D6 gene copies on enzyme pharmacokinetics and drugs metabolism. We in our study selected an area of exon 9 of CYP2D6 for PCR. Cause this position of exon 9 distinguishes between this exon and pseudo genes that is genes CYP2D7 and CYP2D8 preventing making pseudo copies (Schaeffeler et al., 2003). We choose a region (6q as internal reference gene. Although some mutation in ALB gene on chromosome 4 and 13 have been detected; but are not considered concordance with general polymorphism of CYP2D6. Although this study did not find relationship between CYP2D6 copy and tamoxifen resistance but some important key points have been achieved. Out of all, odd ratio=1.6 for three copy numbers that implicate higher risk of tamoxifen resistance in this group. This can be due to faster metabolization of drug by enzyme in this group (Kirchheiner et al., 2005; et al 2011). Another possible reason on enzyme forms that can decrease enzyme activity such as variable in three copies. We did not assume patients genotype and this matter needs another study; conservations on numbers of copy are only preventative for planning accurate and possible other confounding factors in prediction must be taken into consideration. The key influencing factors are expression and function level of estrogen receptors (ER) and dominant type of ER (ERα or ERβ), isoforms of ER and junction between growth factors signals and ER and also genetic diversity of metabolizing genes of tamoxifen that CYP2D6 is the most important (but not the only) of them.

Another important finding of this study was positive relation between increased age and numbers of *CYP2D6* copies (P<0.001). These results were concomitant with

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Gjerde et al in Norway that found relation between SULT1A1 gene copy numbers and age (Gjerde et al., 2008). This phenomenon could be due to increased duplications and unequal crossovers between sister chromatids with age (Lundqvist et al., 1999).

In conclusion, based on our study there is no significant relation between *CYP2D6* gene copy numbers and tamoxifen resistance in women with breast cancer. But more studies with considering other influencing factors must be done to find out the actual effect of this phenomenon on tamoxifen resistance.

Acknowledgements

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