RESEARCH ARTICLE

Screening for the 3’ UTR Polymorphism of the PXR Gene in South Indian Breast Cancer Patients and its Potential role in Pharmacogenomics

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Abstract

Background: Breast cancer, the commonest cancer among women in the world, ranks top in India with an incidence rate of 1,45,000 new cases and mortality rate of 70,000 women every year. Chemotherapy outcome for breast cancer is hampered due to poor response and irreversible dose-dependent cardiotoxicity which is determined by genetic variations in drug metabolizing enzymes and transporters. Pregnane X receptor (PXR), a member of the nuclear receptor superfamily, induces expression of drug metabolizing enzymes (DMEs) and transporters leading to regulation of xenobiotic metabolism. Materials and Methods: A genomic region spanning PXR 3’ UTR was amplified and sequenced using genomic DNA isolated from 96 South Indian breast cancer patients. Genetic variants observed in our study subjects were queried in miRSNP to establish SNPs that alter miRNA binding sites in PXR 3’ UTR. In addition, enrichment analysis was carried out to understand the network of miRNAs and PXR in drug metabolism using DIANA miRpath and miRwalk pathway prediction tools. Results: In this study, we identified SNPs rs3732359, rs3732360, rs1054190, rs1054191 and rs6438550 in the PXR 3’ UTR region. The SNPs rs3732360, rs1054190 and rs1054191 were located in the binding site of miR-500a-3p, miR-532-3p and miR-374a-3p resulting in the altered PXR level due to the deregulation of post-transcriptional control and this leads to poor treatment response and toxicity. Conclusions: Genetic variants identified in PXR 3’ UTR and their effects on PXR levels through post-transcriptional regulation provide a genetic basis for inter-individual variability in treatment response and toxicity associated with chemotherapy.

Keywords: Pregnane X receptor - 3’ UTR variation - MiRSNPs - drug metabolism - doxorubicin - cardiotoxicity

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Introduction

Human Pregnane X Receptor (also called NR1I2), a member of orphan nuclear receptor superfamily, maps to chromosome 3q11-13 with ten exons coding for 434 amino acids (Zhang et al., 2001). PXR is a xenobiotic sensor and acts as a transcription factor of drug metabolizing enzymes (DMEs) and drug transporters. It can potentially affect the efficacy of chemotherapeutic drugs given during cancer treatment by transcriptionally regulating genes like P-glycoprotein, CYP3A, CYP2B6, UGT1A1, ABCB1 multidrug resistance proteins and glutathione S-transferases which accelerate the metabolism and elimination of chemotherapeutic agents like cyclophosphamide, tamoxifen and taxol (Honkakoski et al., 2000). PXR predominantly expressed in liver and intestine and to a lesser extent in other organs like kidney and lung, transcriptionally regulates the most abundant cytochrome enzyme CYP3A4 that catalyses the metabolism of more than 50% of drugs. Inter-individual genetic variations in PXR gene significantly contribute to the variability in the induction of CYP3A4 influencing both baseline and inducible metabolism of drugs and altered clinical response (Conde et al., 2008; Takagi et al., 2008; Kotta et al., 2013).

In breast cancer treatment, drug resistance and toxicity worsen the treatment outcome. Forced expression of PXR in breast cancer cell lines MCF-7 and MDA-MB-231 resulted in increased expression of drug resistance proteins like MDR1 (Multi Drug Resistance Protein) and BCRP (Breast Cancer Resistance Protein) and reduced response to tamoxifen, cisplatin and paclitaxel treatment whereas its downregulation restored cell cycle regulation and apoptosis. In chemoresistant cells, PXR localized more...
in the nucleus than in its usual cytoplasmic localization (Masuyama et al., 2007; Chen et al., 2009; Qiao et al., 2014). PXR overexpression and altered subcellular location due to genetic variations is commonly observed in various cancers like breast, endometrial, prostate and colorectal cancers, reducing not only the clinical efficacy of antineoplastic drugs but also has implications in proliferation, apoptosis and metastasis of cancer cells (Pondugula et al., 2013).

Single Nucleotide Polymorphisms in the 3’ UTR of PXR may influence its protein level by altering microRNA mediated post-transcriptional regulation. The 3’ UTR SNPs, located in the miRNA target sequence (MiRSNPs), interfere with miRNA-mRNA binding efficiency and alter the miRNA function. Analysing MiRSNPs in major drug metabolizing genes becomes pharmacologically relevant as they contribute to interindividual variability in drug response and toxicity (Swart et al., 2013). Therefore, we screened the 3’ UTR of PXR in 96 breast cancer patients by PCR amplification and direct sequencing to identify MiRSNPs and performed in silico analysis to understand their effect on miRNA mediated post-transcriptional regulation of PXR and treatment resistance in breast cancer.

Materials and Methods

Study subjects & DNA isolation

Ninety six pathologically confirmed breast cancer patients visiting Arignar Anna Government Hospital, Kancheepuram were selected for the present study. Blood samples were collected from all the patients with proper informed consent and Institutional Ethical approval. Genomic DNA was isolated from the collected blood samples following Proteinase K digestion and Phenol:Chloroform isolation method.

Screening of genetic variants at PXR 3’UTR by sequencing

Genomic region spanning the PXR 3’UTR was amplified using the primers PXR Fwd: 5’-GTAGGTCAAGGACATCAGAGG-3’ and PXR Revs: 5’-CAGCGTAGCCTTGTCACAGAGC-3’. PCR amplification was carried out in 100 μL total volume using 100 μM dNTPs, 80 nM each of forward and reverse primers and 0.5 Units of Taq polymerase with proper informed consent and Institutional Ethical approval. Genomic DNA was isolated from the collected blood samples following Proteinase K digestion and Phenol:Chloroform isolation method.

Identification of germ line PXR 3’UTR genetic variants predicted

PXR 3’ UTR SNPs Identified in our Study Population with their Allelic Frequencies

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Sequence</th>
<th>Allele</th>
<th>Allele Frequency</th>
<th>Genotype</th>
<th>Genotype Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3732359</td>
<td>aggat(G/A)gggeca</td>
<td>G/A</td>
<td>0.3882/0.6117</td>
<td>GG/GA/AA</td>
<td>0.14/0.50/0.36</td>
</tr>
<tr>
<td>rs3732360</td>
<td>ggcgggt(T/T)gcagtc</td>
<td>C/T</td>
<td>0.3882/0.6117</td>
<td>CC/CT/TT</td>
<td>0.14/0.50/0.36</td>
</tr>
<tr>
<td>rs1054190</td>
<td>agcac(C/T)gtataa</td>
<td>C/T</td>
<td>0.9946/0.0053</td>
<td>CC/CT/TT</td>
<td>0.98/0.01/0.00</td>
</tr>
<tr>
<td>rs6438550</td>
<td>caaac(A/G)aatttg</td>
<td>A/G</td>
<td>0.9787/0.0212</td>
<td>AA/AG/GG</td>
<td>0.97/0.00/0.02</td>
</tr>
<tr>
<td>rs1054191</td>
<td>atggc(G/A)gggac</td>
<td>G/A</td>
<td>0.9734/0.0266</td>
<td>GG/GA/AA</td>
<td>0.94/0.05/0.00</td>
</tr>
</tbody>
</table>
Deregulation of miRNAs binding to PXR 3' UTR

The genetic variations observed in the 3' UTR of PXR alter the miRNA binding site affecting the microRNA mediated PXR regulation. The base changes either abolish/

Table 2. PXR 3' UTR Genetic Variants Altering miRNA Binding and Regulation of PXR

<table>
<thead>
<tr>
<th>SNP ID</th>
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<th>Enhance</th>
<th>Decrease</th>
<th>Break</th>
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</thead>
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<tr>
<td>rs3732360</td>
<td>hsa-miR-500a-3p</td>
<td>-</td>
<td>hsa-miR-532-3p</td>
<td>hsa-miR-4763-5p</td>
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<tr>
<td></td>
<td>hsa-miR-501-3p</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>rs1054190</td>
<td>hsa-miR-374a-3p</td>
<td>hsa-miR-214-3p</td>
<td>-</td>
<td>hsa-miR-1250-5p</td>
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<tr>
<td></td>
<td>hsa-miR-5094</td>
<td>hsa-miR-3619-5p</td>
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<td>hsa-miR-1250-3p</td>
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<td>hsa-miR-520f</td>
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<td>rs1054191</td>
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</tr>
<tr>
<td></td>
<td>hsa-miR-519e-3p</td>
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</table>

Figure 1. Experimental Workflow Employed to Identify miRNAs Whose Binding Sites are Altered by 3' UTR SNP of PXR gene. Among the 12 SNPs located in PXR 3' UTR, five SNPs showed variations in the present study population. Further analysis using miRBase database indicated three SNPs altering miRNA binding in PXR 3' UTR. Enrichment analysis of altered miRNAs identified miR 500a-3p, miR 532-3p and miR 374a-3p involve in doxorubicin induced cardiotoxicity in heterozygous conditions with a frequency of 0.005 and 0.02 respectively. In the case of rs6438550, the proportion of minor allele in homozygous condition is 0.01 and no heterozygosity was observed.

Deregulation of miRNAs binding to PXR 3' UTR

The genetic variations observed in the 3' UTR of PXR alter the miRNA binding site affecting the microRNA mediated PXR regulation. The base changes either abolish/
by the minor allele of PXR miRSNPs were predicted to be associated with treatment response and cardiotoxicity, a major complication of breast cancer patients undergoing doxorubicin treatment.

The pathways modulated by the selected miRNAs were further interpreted using miRwalk pathway prediction tool (Dweep et al., 2015) and found that miRNAs hsa-miR-500a-3p, hsa-miR-374a-3p and hsa-miR-374a-3p were associated with KEGG pathways related to cardiotoxicity and cancer suggesting that the expression of these miRNAs in breast cancer tissue in the presence of SNPs rs3732360, rs1054190 and rs1054191 may change the dynamics of doxorubicin metabolism.

Additional in depth analysis of research studies (Table 3) provided evidence that the miRNAs hsa-miR-500a-3p, hsa-miR-374a-3p and hsa-miR-374a-3p were involved in all the three crucial events - breast cancer pathogenesis, doxorubicin treatment response and cardiac pathology (Figure 3).
Discussion

Breast cancer, the most frequent cancer among women with an estimated 1.67 million new cases diagnosed worldwide. In India, nearly 1.45 million new cases are being diagnosed every year with a mortality rate of 70,000 women making it the top most common cancer (Ferlay et al., 2013). Chemotherapy is the most suggested treatment options for majority of the breast cancer patients in which anthracycline based drugs like doxorubicin are commonly used but the main limitations are irreversible dose dependent cardiotoxicity (Singal et al., 1998), myelosuppression, veno-occlusive liver disease and minor complications like nausea, vomiting. About 4 - 36% of the patients receiving chemotherapy are suffering from cardiotoxicity (Schlitt et al., 2014). Genetic factors are the main determinants of chemotherapy response and accounts for 20-95% of the observed inherited variability in therapeutic efficacy and toxicity of drugs in individual patients (Evans et al., 2003).

Genetic variations in DMEs and alterations in the expression level of receptors of the signal transduction pathways of drug metabolism and elimination mainly involve in poor response to chemotherapy (Lal et al., 2010). PXR, a dominant nuclear receptor, in response to the drugs, transcriptionally activates downstream drug metabolizing enzymes and transporters and its expression level influences the drug metabolism and elimination. Genetic variations in the untranslated region of PXR mRNA are known to alter the expression level of PXR (Hughes et al., 2006). These polymorphisms at the 3' UTR of PXR alter the expression level of PXR and results in inter-individual variability in CYP3A activity, the major drug metabolizing enzyme and downstream effector gene of PXR (Oleson et al., 2010).

In this study, we analysed PXR 3' UTR variants in South Indian Breast cancer patients and identified SNPs rs3732359, rs3732360, rs1054190, rs1054191 and rs6438550 of which rs3732360, rs1054190 and rs1054191 were predicted to alter the miRNA mediated post-transcriptional regulation of PXR. SNPs rs1054190 and rs1054191 may not result in any drastic change of miRNA binding as wild type allele was observed in majority of the patients and no homozygous mutant was seen in any individual of our study population. But the effect of microRNA deregulation in heterozygous condition of these SNPs has to be considered. Interestingly, in the case of rs3732360 and rs3732359, the linked polymorphisms, the proportion of mutant allele was higher than that of the wild type and it creates a new binding site for miRNA 500a-3p and decreases binding of miRNA 532-3p which are shown to play a role in doxorubicin induced cardiac cell apoptosis (Wang et al., 2015).

When we investigated for the involvement of the abrogated miRNAs through DIANA miRpath analysis followed by miRwalk pathway prediction, we found that these miRNAs, in addition to the regulation of PXR, also participate in drug metabolism induced by CYP3A activity, cancer associated pathways and cardiotoxicity. The predicted function of these miRNAs were further investigated individually in various studies for their role in breast cancer, doxorubicin metabolism and cardiac pathology. This analysis revealed that the miRNAs, miR-500a-3p, miR-501-3p, miR-502-3p, miR-374a-3p, miR-520f and miR-515-3p were upregulated and miR-532-3p and miR-1271-3p were downregulated in breast cancer. miR-500a-3p is downregulated in doxorubicin treated breast cells with cardiomyopathy and miR-532-3p is increased in doxorubicin treated cardiomyocytes and induces cardiac cell apoptosis. MiR-1250 was reported to be downregulated in cells treated with doxorubicin and is not reported to be involved in breast cancer and cardiotoxicity (Janssen et al., 2010; Nilsson et al., 2011; Lee et al., 2011; Zhang et al., 2013; Cai et al., 2013; Feliciano et al., 2013; Chavali et al., 2014; Chang et al., 2015; Van Schooneveld et al., 2015; Wang et al., 2015). MiR-4763, downregulated in cells treated with 5 Fluorouracil, was found to be involved in atherosclerosis by inducing vascular smooth muscle cell contraction and in cardiac hypertrophy (Wang et al., 2013; Karagiannis et al., 2013).

While these miRNAs were involved in either breast cancer treatment or cardiotoxicity, three miRNAs miR-500a-3p, miR-532-3p and miR-374a-3p were found to play key role in breast cancer proliferation, cardiac pathology as well as in doxorubicin treatment (Figure 3). The above miRNAs were also shown to be induced by doxorubicin treatment and determines the PXR expression level affecting the metabolism of doxorubicin through enhanced or reduced CYP3A activity. These data suggest that the miRNAs predicted to deregulate PXR expression, possess direct role in doxorubicin induced cardiac dysfunction as well as breast cancer proliferation (Figure 4).

In summary, germline genetic variations in the xenobiotic sensor PXR and drug metabolizing enzymes influence the drug clearance not only in the respective organ but also extend its impact on drug induced irreversible toxicity in other organs. Therefore analysing genetic variations at non-coding regions of genes like

Figure 4. Schematic Representation of Network of PXR 3' UTR SNPs, miRNA and Cardiotoxicity. Doxorubicin induces the expression of miR 500a-3p, miR 532-3p and miR 374a-3p whose binding on PXR 3' UTR is altered by SNPs rs3732360, rs1054190 and rs1054191. The expression level of these miRNAs in synergistic with the SNPs increases Doxorubicin induced cardiotoxicity. In addition, these miRNAs independently have the potential to induce cardiac dysfunction.
PXR and the deregulation of miRNA control may provide insights of complex drug metabolism network and further opportunities for elucidating mechanism of doxorubicin induced cardiotoxicity in cancer patients.

Acknowledgements

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