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

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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'-GTAGGTCAGGACCATCAGAGAGG-3' and *PXR* Rvs: 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 the following thermal cycling parameters: 94°C for 10 min once; 40 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec followed by 72°C for 7 min and 4°C hold. The amplicon was then purified and sequenced using BigDye Terminator Reaction Chemistry v3.1 on Applied Biosystems 3730 DNA analyzers (Applied Biosystems, USA) using M/s Macrogen Inc, Korea sequencing service.

In silico analysis of miRNA binding site alteration due to PXR MiRSNPs

The identified *PXR* 3' UTR genetic variants predicted to alter miRNA binding were identified using miRSNP database (http://compbio.uthsc.edu/miRSNP/) and classified into one of the following four categories based on their effect on miRNA binding: (i) create - the derived allele creates a new miRNA binding site in the variant mRNA, (ii) enhance - the derived allele enhances the binding of the originally targeting miRNA to the variant mRNA (iii) break - the derived allele completely disrupts the miRNA binding site and (iv) decrease - the derived allele decreases the binding efficacy of the originally targeting miRNA to the variant mRNA (Liu et al., 2012).

Enrichment analysis of miRNA regulating PXR

To understand the network between miRNAs and *PXR* in drug metabolism and cancer, an enrichment analysis of above predicted miRNAs was performed in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway v58.1 in the web-based tool "DNA Intelligent Analysis (DIANA) - miRPath v2.0". The biological pathways enriched were identified using the algorithm microT-CDS, with a p-value threshold of 0.05 and the graphical output provides an overview of the pathways modulated by the miRNAs and facilitates the selection of crucial pathways for further interpretation (Vlachos et al., 2012). The complete workflow of identifying the genetic variations in 3' UTR of *PXR* and miRNA regulation is given in Figure 1.

Results

Identification of germ line PXR 3'UTR genetic variants in South Indian Breast Cancer Patients

The sequencing chromatograms of *PXR* 3'UTR of 96 breast cancer samples were analysed and the sequences were aligned with reference sequence to identify the nucleotide variations across the samples (Figure 2). SNPs located in the 3' UTR of *PXR* were retrieved from miRdSNP (http://mirdsnp.ccr.buffalo.edu/) (Bruno et al., 2012) and NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). Among the twelve SNPs reported in the 3' UTR of *PXR* gene, 5 SNPs were observed in our study population (Table 1).

The SNPs rs3732359 (G>A) and rs3732360 (C>T) were found to be linked SNPs with a genotype frequencies 0.14, 0.50 and 0.36 for GG/GA/AA and CC/CT/TT respectively. The major allele of the other three SNPs rs1054190 (C/T), rs1054191 (G/A) and rs6438550 (A/G) were found to be predominant with the frequency of 0.99, 0.97 and 0.98 respectively whereas the minor alleles of rs1054190 and rs1054191 were observed only

Table 1. List of *PXR* 3' UTR SNPs Identified in our Study Population with their Allelic Frequencies

SNP ID	Sequence	Allele	Allele Frequency	Genotype	Genotype Frequency
rs3732359	aggat(G/A)ggcca	G/A	0.3882/0.6117	GG/GA/AA	0.14/0.50/0.36
rs3732360	ggcagg(C/T)gcatg	C/T	0.3882/0.6117	CC/CT/TT	0.14/0.50/0.36
rs1054190	agcac(C/T)gataa	C/T	0.9946/0.0053	CC/CT/TT	0.98/0.01/0.00
rs6438550	caaac(A/G)atttg	A/G	0.9787/0.0212	AA/AG/GG	0.97/0.00/0.02
rs1054191	atggc(G/A)ggcac	G/A	0.9734/0.0266	GG/GA/AA	0.94/0.05/0.00

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

SNP ID	Create	Enhance	Decrease	Break
rs3732360	hsa-miR-500a-3p hsa-miR-501-3p hsa-miR-502-3p	-	hsa-miR-532-3p	hsa-miR-4763-5p hsa-miR-4787-3p
rs1054190	hsa-miR-374a-3p hsa-miR-5094 hsa-miR-520f	hsa-miR-214-3p hsa-miR-3619-5p	-	hsa-miR-1250-5p hsa-miR-1250-3p
rs1054191	hsa-miR-371b-3p hsa-miR-4258 hsa-miR-4707-3p	hsa-miR-1825	hsa-miR-3614-5p	hsa-miR-1271-3p hsa-miR-33b-3p hsa-miR-4722-3p hsa-miR-4763-5p hsa-miR-4787-3p hsa-miR-515-3p hsa-miR-519e-3p

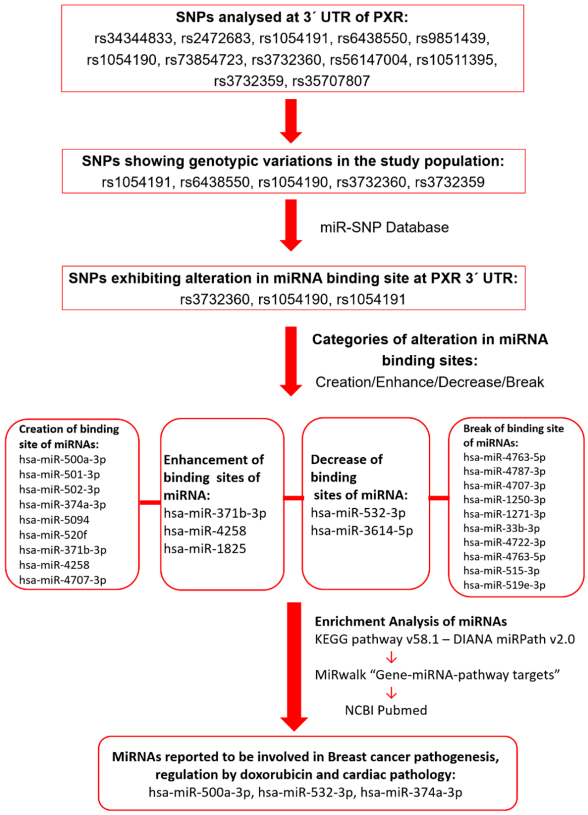

Figure 1. Experimental Workflow Employed to Identify miRNAs Whose Binding Sites are Altered by 3' UTR SNP of *PXR* gene. Among the 12 SNPs located in *PXR* 3' UTR, five SNPs showed variations in the present study population. Further analysis using miRbase database indicated three SNPs altering miRNA 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 cardiotoxocity

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/

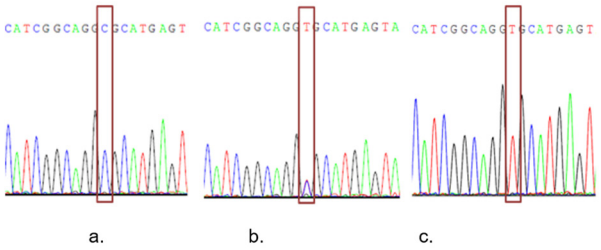

Figure 2. Representative Sequencing Chromatograms Showing the Genotypes of *PXR* SNP rs3732360. PXR 3' UTR SNPs were identified by PCR amplification and direct sequencing of *PXR* 3' UTR region. The chromatogram of SNP rs3732360 (C>T) which showed variation in our study subjects is represented here with the genotypes. Panel a) CC Homozygotes; b) CT Heterozygotes and c) TT Homozygotes

create a new binding site or enhance/reduce the binding efficiency of existing binding sites for microRNAs thereby disrupting the normal post transcriptional regulation.

When we searched miR-SNPs database to know the effect of genetic variations identified in this study, three SNPs rs3732360, rs1054190 and rs1054191 were found to alter miRNA mediated regulation of *PXR* by creating new binding sites for 9 miRNAs; abolishing the existing binding sites of 11 miRNAs; enhancing the binding efficiency of existing binding sites for 3 miRNAs; and reducing the binding efficiency of 2 microRNAs. The complete details of creation/loss of binding sites of miRNAs as well as enhanced/reduced binding efficiency of existing miRNA binding sites resulting due to the minor allele of SNPs are listed in Table 2.

Enrichment analysis of miRNAs targeting PXR:

Mapping of the miRNA binding sites altered by the five SNPs observed in this study revealed that three SNPs created a new binding site for nine new miRNAs and 3 enhanced binding of existing sites thereby bringing *PXR* under the control of additional miRNAs and strong binding of existing miRNAs, resulting in further down-regulation of *PXR* level. MicroRNAs altering *PXR* expression can delay drug clearance due to reduced transactivation of down-stream drug metabolizing enzymes and may also result in drug toxicity. DIANA miRPath pathway enrichment analysis revealed the molecular networks and canonical pathways related to these miRNAs. Interestingly the miRNAs whose binding sites are altered

Table 3. Involvement of miRNAs Altered by *PXR* 3' UTR Genetic Variants in Breast Cancer, Doxorubicin Metabolism and Cardiotoxicity

MiRNAs	Breast cancer	Reference	Doxorubicin treatment	Reference	Cardiac pathology	Reference	Effect of 3' UTR SNP on its binding site
miR500a-3p	↑	Janssen et al., 2010	↓	Zhang et al., 2013	↓ Diastolic dysfunction diabetic cardiomyopathy	Chavali et al., 2014	Create (rs3732360)
miR501-3p	↑	Chang et al., 2015	-				Create (rs3732360)
miR502-3p	↑	Janssen et al., 2010 Chang et al., 2015	-		↑ Coronary Artery Disease	Wang et al., 2014	Create (rs3732360)
miR374a-3p	↑	Cai et al., 2013	↑ (resistance to cisplatin)	Li et al., 2015	↑ Cardiac dysfunction	Xing et al., 2015	Create (rs1054190)
miR520f	↑	Van Schooneveld et al., 2015	-				Create (rs1054190)
miR532-3p	↓	Janssen et al., 2010; Nilsson et al., 2011	↑	Wang et al., 2015	↑ Doxorubicin induced apoptosis in cardiomyocytes	Wang et al., 2015	Decrease (rs3732360)
miR519e	-	Janssen et al., 2010	-		↑ Non-ischemic heart failure	Van et al., 2015	Break (rs1054191)
miRNA 33b-3p	↓	Lin et al., 2015	-		Congenital Heart Defect	Omran et al., 2013	Break (rs1054191)
miR515-3p	↑	Lee et al., 2011	-				Break (rs1054191)
miR1271-3p	↓	Feliciano et al., 2013	-				Break (rs1054191)
miRNA-1250	-		↓	Zhang et al., 2013			Break (rs10541910)
miR4763	-		↓ after 5 Flurouracil	Wang et al., 2013	Atherosclerosis	Karagiannis et al., 2013	Break (rs1054191)
					Cardiac hypertrophy		
miR371b-3p	-		-		Congestive heart failure	Cakmak et al., 2015	Enhance (rs1054190) Create (rs1054191)
miRNA 1825	-		-		Congestive heart failure	Cakmak et al., 2015	Enhance (rs1054191)

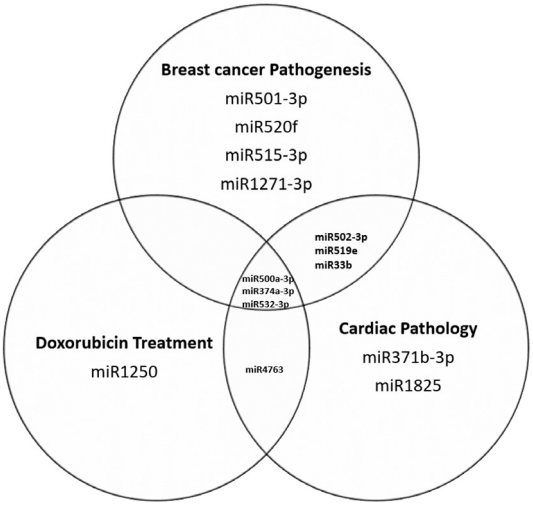

Figure 3. Venn Diagram for the Distribution of miRNAs altered by *PXR* 3' UTR SNPs in Breast cancer pathogenesis, doxorubicin treatment and cardiac pathology. Three miRNAs - miR 500a-3p, miR 532-3p and miR 374a-3p were found to play role in breast cancer proliferation as well as doxorubicin induced cardiotoxicity

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-532-3p, hsa -miR-374a-3p, hsa-miR-520f, hsa-miR-214-3p, hsa-miR-3619-5p, hsa-miR-1250-3p, hsa-miR-1271-3p, hsa-miR-33b-3p, hsa-miR-519e-3p and hsa-miR-3614-5p 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-532-3p 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 1,45,000 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* influence transport, localization and the stability of *PXR* mRNA (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*.

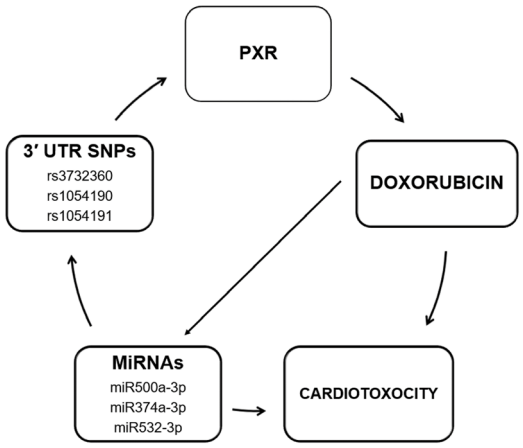

Figure 4. Schematic Representation of Network of *PXR* 3' UTR SNPs, miRNA and Cardiotoxicity. Doxorubicin induces the expression of miR 500a-3p, miR 532-3p and miR 374a-3p whose binding on *PXR* 3' UTR is altered by SNPs rs3732360, rs1054190 and rs1054191. The expression level of these miRNAs in synergistic with the SNPs increases Doxorubicin induced cardiotoxicity. In addition, these miRNAs independently have the potential to induce cardiac dysfunction.

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 microRNAs, 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 cells and 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

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.

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