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RNA Expression of Cytochrome P450 and its Clinical Impact in Breast Cancer Patients

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Abstract

Objectives: The expression pattern of cytochrome P450 genes (CYPs) affected by tumorigenesis may have an important role in the progression of cancer and in the metabolism of anticancer drugs. The aim of the present study was to determine the mRNA expression pattern of two cytochrome P450 genes (*CYP3A4*, and *CYP3A5*) in breast cancer patients.

Methods: Tumor samples were collected from 38 patients recently diagnosed with breast cancer along with corresponding non-malignant tissues. Quantitative polymerase chain reaction (qPCR) was used to quantify the levels of gene expression in all the samples. The association between CYPs expression and clinico-pathological parameters was also analyzed.

Results: In the present study, our results showed that *CYP3A5* gene expression was not significantly associated with breast cancer ($p=0.14$). Significant differences in *CYP3A4* mRNA expression were observed between tumor tissue and the corresponding normal tissue ($p<0.003$). There was no statistical difference between *CYP3A4* and *CYP3A5* expression and clinical and pathological features.

Conclusion: *CYP3A4* expression has a potential role as a breast cancer prognosis marker. We conclude that increased expression levels of the examined *CYP3A4* may promote breast cancer progression and also may associate with resistance to drug treatment by inactivation of anticancer drugs. Moreover, one potential therapeutic approach may be to block *CYP3A4* function.

Keywords: Breast cancer; Cytochrome P450; *CYP2D6*; *CYP3A4*; *CYP3A5*; Gene expression; Drug gene interactions

Introduction

Breast cancer is the most common cancer in the female population responsible for 15% of cancer-related deaths [1]. Cytochrome P450 (*CYP450*) is a super family of hemoprotein that can be found in all species and are essential for survival. *CYP450* contains monooxygenases and are involved in the metabolism of a wide range of exogenous and endogenous compounds including drugs, toxins and carcinogens [2]. The gene expression of *CYP450*s in different organs alter pharmacological outcomes of medication [3]. Interindividual variability in the expression of cytochrome p450 may contribute to interindividual differences in drug efficacy and adverse reactions. More than 2 million adverse drug reaction cases were reported annually in the United States, because only 25-60% patients are responding to specific medication. Drugs are usually approved based on safety and efficacy data in a limited number of patients that are thought to represent the entire population. However, individuals in a population show differences in drug sensitivity, efficacy, toxicity, and dosing [4]. Anticancer agents such as cyclophosphamides, anthracyclines, taxanes, tamoxifen and irinotecan are metabolized by *CYP450*s. The efficacy and toxicity of these agents are greatly influenced by *CYP450* genetic polymorphisms and differential enzymatic activities. The expression of tumor specific proteins in mammary glands may play a critical role in the development of breast cancer as well as in the success of chemotherapy treatment. To date, very few critical markers have been validated for the prediction of drug efficiency in breast cancer. Thus, the aim of the present study was to determine the mRNA expression pattern of three representative CYPs (*CYP3A4*, and *CYP3A5*) in paired tumor and normal tissue of breast cancer patients [5].

Materials and Methods

Tissue samples, ethics statement and consent form

Fifty tumor and corresponding non-tumor tissues from adjacent regions were obtained from patients diagnosed with breast cancer from MNJ Institute of Oncology and Regional Cancer Centre, Hyderabad, between 2016 and 2017. Tumor and non-tumor tissue samples had been verified as tumor or non-tumor by histopathological evaluation. The study protocol was approved by the Institutional Ethics Committee and written informed consent was obtained from all patients. A group

of candidate genes were chosen, and the expression was analyzed by qRT-PCR.

RNA extraction

Total RNA was isolated using Isolation Kit (Qiagen) its integrity was verified by 1.5% agarose gel plus ethidium bromide and the ratio of optical density (OD) at 260 nm and 280 nm was also measured. RNA was quantified using a NanoDropND-1000 Spectrophotometer (Thermo Scientific) and single-stranded cDNA was synthesized from 300 ng of RNA in each sample.

Reverse transcription

Reverse transcription was performed in a personal Master Cycler (Bio-Rad CFX 96), using 1 µg of total RNA in the presence of Random Hexamer (50 ng/µl) and reverse transcriptase (50 U/µl) in a total volume of 20 µl, including: 10 × TaqMan RT buffer, $MgCl_2$ solution (25 mM), dNTPs mixture (10 mM), an RNAse inhibitor (20 U/µl) and nuclease-free water. The reaction mixture was incubated for 10 minutes at 25°C, 60 minutes at 42°C, heated for 5 minutes to 95°C and then at 4°C for a minimum of 2 minutes. The resulting cDNA was stored at –20°C until further use.

SYBR–Green quantitative reverse transcription-PCR (qRT-PCR)

The *CYP2D6*, *CYP3A4*, and *CYP3A5* levels were quantified by quantitative reverse transcription-PCR (qRT-PCR) using SYBR-Green. Quantification of the expression of miR- *CYP2D6*, *CYP3A4*, and *CYP3A5* genes was performed using Bio-Rad CFX96. The relative quantification method was used, where we compared the gene expression in each patient with the gene expression in the control group. In assessing the relative gene expression, quantitative real time - polymerase chain reaction (qRT-PCR) was performed with 1 µl of cDNA, 12.5 µl SYBR Green, and with specific primers (Table 1) were synthesized at Bioserve Biotechnologies Ltd (Hyderabad, India).

Gene	Oligonucleotide	Sequence	PCR product size
CYP3A4	5-Primer	AACGGCACCTCCATGTG	186 bp
	3-Primer	GCGTTGTGATCTCCTTCTGA	
CYP3A5	5-Primer	GCCAAGCCCATAACTCAG	152 bp
	3-Primer	AATCTCAGGTTCACAGGTTAAGG	
GAPDH	5-Primer	AATCCCATCACCATCTTCCAG	121 bp
	3-Primer	AAATGAGCCCCAGCCTTC	

Table 1: Oligonucleotide primer sequence used for RT-PCR.

The program used was: 5 min of initial denaturation and enzyme activation at 95C, followed by 35 cycles composed of 30 s at 95C, 30 s at 60C for annealing and 40 s at 72C for elongation. Amplification and melting curve analysis were carried out to verify the specific product according to its specific melting temperature (Tm). Each reaction was performed in triplicate. Gene expression was defined based on the threshold cycle (Ct), and relative expression levels were calculated as 2-[(Ct of RNA)–(Ct of reference gene)] after normalization with reference to expression of U6. The results were analyzed by the melting curve analysis software. Gene expression values were expressed as Ct, Ct being the point at which the fluorescence rises significantly above baseline or background fluorescence and comparing the Ct of the genes in tumors with the Ct of the genes in controls.

Statistical analysis

Statistical analyses for RT-PCR results were performed on ΔCT data. *p*-Values<0.05 were considered statistically significant.

Results

Clinical and pathological characteristics

We obtained data on 38 female patients with breast cancer. Thirty-eight breast biopsy samples were compared with normal pooled breast tissue. The clinicopathological data for the patients recruited in this study are provided in Table 2.

Clinical Characteristics	N=38
Menopausal Status	
Premenopausal	24 (63%)
Postmenopausal	14 (37%)
Tumor Size	
>2	24 (63%)
<2	14 (37%)
Tumor Grade	
Grade II	28 (74%)
Grade III	10 (26%)
Lymph Nodes	
No Nodes	20 (53%)
1-5	14 (37%)
Above 5	4 (10%)
Hormonal Status	
ER/PR Positive	14 (37%)
ER/PR Negative	24 (63%)
Her-2 Positive	14 (37%)
Her-2 Negative	24 (63%)
Ki-67 Positive	28 (74%)
Ki-67 Negative	10 (26%)

Table 2: Clinical and pathological characteristics of breast cancer patients.

The mean age of the breast cancer breast cancer patients was 47 years (Ranging from 29-73). Most of the breast cancer patients of invasive ductal carcinomas were grade II (74%) and grade III (26%). The percentage of ER/PR negative tumors (63%) was high when

compared to ER/PR positive tumors (37%). Her-2 negative tumors were found high frequency (63%) than Her-2 positive tumors (37%). 74% (28 of 38) cases were Ki-67 positive and 26% (10 of 38) were Ki-67 negative.

Frequency of expression of CYP3A4

In the group of 38 pairs of breast cancer tumor tissue and the corresponding normal tissue obtained from the same patient, in most cases expression of the CYP3A4 gene was higher in tumor than in the control tissue. Significant differences in CYP3A4 mRNA expression were observed between tumor tissue and the corresponding normal tissue ($p<0.003$). The mean relative expression for *CYP3A4* was significantly, two-fold higher in breast tumor tissues (Table 3).

Analyses		Tumor	Normal	p-Value
CYP3A4	Altered Expression	16 (42%)	10 (26%)	0.14
	Normal Expression	22 (58%)	28 (74%)	
CYP3A5	Altered Expression	20 (52%)	0 (0%)	0.003
	Normal Expression	18 (48%)	38 (100%)	

Table 3: RT-PCR analyses of CYP3A4 and CYP3A5 mRNA expression in breast cancer patients.

Frequency of expression of CYP3A5

In 16 of the 38 (42%), *CYP23A5* was over expressed in the breast cancer tissue when compared with its expression in corresponding normal tissue. Normal expression was demonstrated in 58% of tumor tissues. In corresponding normal tissue expression was detected in 10 of 38 (26%). Therefore, *CYP3A5* gene expression was not significantly associated with breast cancer (p=0.14).

CYP3A4 and CYP3A5 over expression and their correlation with clinic pathologic factors

In this study, several key clinicopathological factors were collected to access the correlation with the expression level of *CYP3A4* or *CYP3A5*. Detailed clinicopathological factors (including age, gender, and lymph node metastasis) are summarized in Table 4. The level of *CYP3A4* and *CYP3A5* mRNA expression was found to be high in premenopausal women. The *CYP3A4* and *CYP3A5* mRNA expression level was higher in tumor size, tumor <2 cm showed a higher expression of *CYP3A4* and *CYP3A5* than tumors of >2 cm. The level of *CYP3A4* mRNA expression was found to be higher in high grade group (grade III) compared to intermediate grade (grade II), Whereas *CYP3A5* showed high expression in intermediate grade group (Grade II). The *CYP3A4* (87%, 14 of 16) and *CYP3A5* (88%, 18 of 20) expression was higher in Ki-67 expressed tumors.

Clinical characteristics	CYP3A4 altered expression (n=16)	CYP3A5 altered expression (n=20)
Menopausal Status		
Premenopausal	10 (62%)	12 (60%)
Postmenopausal	6 (38%)	8 (40%)
Tumor Size		
Above-2	10 (62%)	16 (80%)
Below-2	6 (38%)	4 (20%)
Tumor Grade		
Grade III	6 (38%)	16 (80%)
Grade II	10 (62%)	4 (20%)
Lymph Nodes		
No Nodes	10 (62%)	12 (60%)
1-5	4 (26%)	6 (30%)
Above 5	2 (12%)	2 (10%)
Hormonal Status		
ER/PR Positive	2 (13%)	10 (50%)
ER/PR Negative	14 (87%)	10 (50%)
Her-2 Positive	4 (25%)	8 (40%)
Her-2 Negative	12 (75%)	12 (60%)
Ki-67 Positive	14 (87%)	14 (88%)
Ki-67 Negative	2 (13%)	6 (12%)

Table 4: Correlation of the CYP3A4 and CYP3A5 mRNA levels with breast cancer clinical data.

Discussion

Enzymes of the cytochrome P450 (CYP) subfamily 3A comprise the largest portion of the liver and small intestinal CYP protein and they are involved in the metabolism of 45% to 60% of all currently used drugs. In addition to drugs, *CYP3A* isozymes metabolize a variety of other compounds including steroid hormones, toxins and carcinogens *CYP3A4* and *CYP3A5* plays an important role in cell regulation *via* its involvement in the metabolism of a wide variety of endogenous metabolites active in cellular signaling [6]. In the present study we defined the gene expression of *CYP3A4* and *CYP3A5* in south Indian women with breast cancer. There was no statistical difference between *CYP3A4* and *CYP3A5* expression and clinical and pathological features.

The results obtained in the present study, showed that *CYP3A5* gene expression was not significantly associated with breast cancer (p=0.14). Iscan [5], reported that *CYP3A5* mRNAs were expressed neither in tumors nor in control tissue. *CYP3A5* is highly expressed in normal prostate cells but absent in prostate cancer. Significant differences in *CYP3A4* mRNA expression were observed between tumor tissue and the corresponding normal tissue ($p<0.003$). Hence, we determined that *CYP3A4* expression has a potential role as a breast cancer prognosis marker. In other work, *CYP3A4* gene expression was observed, although in low levels, in breast cancer. The authors suggest that *CYP3A4* expression is predictive value for any estimation of response to anticancer drugs metabolically inactivated by this cytochrome [7]. Yasuo Miyoshi [8-10], found that high *CYP3A4* mRNA expression is significantly associated with a low response rate to DOC treatment in breast cancer patients [11-13].

Conclusion

CYP3A4 expression has a potential role as a breast cancer prognosis marker. We conclude that increased expression levels of the examined *CYP3A4* may promote breast cancer progression and also may associate with resistance to drug treatment by inactivation of anticancer drugs. Moreover, one potential therapeutic approach may be to block *CYP3A4* function. However, there were still some limitations that should be mentioned. The number of samples was limited [14,15]. There was lack of multi-center samples to validate our findings in particular. Second, the precise role of two genes in carcinogenesis remained unclear. Further validation with a larger number of samples was warranted to strengthen our results.

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