RESEARCH COMMUNICATION

Down-Regulation of CYP1A1 Expression in Breast Cancer

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Abstract

    Breast cancer is a major cause of death in women worldwide. Mammary tissue expressing xenobiotic metabolizing enzymes metabolically activate or detoxify potential genotoxic breast carcinogens. Deregulation of these xenobiotic metabolizing enzymes is considered to be a major contributory factor to breast cancer. The present study is focused on the expression of the xenobiotic metabolizing gene, CYP1A1, in breast cancer and its possible relationships with different risk factors. Twenty five tumors and twenty five control breast tissue samples were collected from patients undergoing planned surgery or biopsy from different hospitals. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and western-blotting were used to investigate the expression of CYP1A1 in breast cancer control and disease samples. mRNA expression of CYP1A1 was down-regulated in 40% of breast tumor samples. Down-regulation was also observed at the protein level. Significnat relations were noted with marital status and tumour grade but not histopathological type. In conclusion, CYP1A1 protein expression was markedly reduced in tumor breast tissues samples as compared to paired control tissue samples.

Keywords: CYP1A1 - genotoxic - expression - mRNA

Introduction

    Breast cancer is the most common cancer among Pakistani women, accounting for 34.6% of all female cancers (Bhurgri, 2004). Approximately one in every nine Pakistani women is likely to suffer from breast cancer. Only 10 percent of women are diagnosed and out of them about 75 percent women do not get treatment and die within five years. This is one of the highest incidence rates in Asia (Baloch and Iqbal, 2006). Factors for determining an individual's susceptibility to breast cancer are still largely unknown. One possible reason may be an imbalance in the detoxification enzymes such as phase I enzymes like Cytochrome P450 and phase II enzymes like glutathione S transferases.

    CYP1A1 is a heme containing mono oxygenase that is involved in the metabolism of endogenous and exogenous compounds (Gonzalez and Gelbion, 1994). The transcriptional activation of the CYP1A1 gene is mediated by the binding of environmental pollutants (benzo[a] pyrene) to the cytosolic receptor ,aryl hydrocarbon receptor (AhR) and then translocates to the nucleus where it heterodimerizes with another protein aryl hydrocarbon nuclear tranlocator (ARNT). This heterodimer binds to consensus regulatory sequences termed as aryl hydrocarbon response elements (AhREs) located in the promoter region of AhR target genes such as CYP1A1 and CYP1A2 and initiate their transcription by recruiting RNA polymerase II (Puga et al., 2009). In addition to the well known positive regulation of CYP1A1, the expression of this gene is repressed by several agents and conditions.

Inflammatory cytokines as well as oxidative stress have been shown to down-regulate CYP1A1 gene expression (Abdel et al., 1994; Morel et al., 1998; Paton et al., 1998). Several studies have reported down-regulation of CYP1A1 in various cancer such as esophageal cancer (Murray et al., 1994) and head and neck cancer (Nosheen et al., 2011). Present study was under taken to analyze the expression of CYP1A1 in breast cancer.

Materials and Methods

    RNA Later (Ambion, USA), Trizol (Invitrogen,California,USA), SuperScript III First Strand cDNA synthesis kit (Invitrogen,USA), PCR Mastermix (Fermentas,USA), Agrose (Invirogen, USA), Ripa buffer (Sigma,USA), Bradford reagent (Sigma,USA), Primer (Fermentas,Canada),Anti-CYP1A1 (Novus Biologicals,USA),Secondary antibody (Novous Biologicals,USA), Nitrotetrazolium Blue Chloride Solution (Sigma,USA), Bromo-4-Chloro-3-indolyl phosphate disodium salt (Sigma,USA) were used in this study.

Tissue collection and Storage of Samples

    25 tumors and 25 control breast tissue samples were collected from patients undergoing planned surgery or biopsy from two different hospitals; Military Hospital Rawalpindi (MH) and Pakistan Institute of Medical Sciences (PIMS). Normal tissues in the course of the operation dwere also collected as control samples. This study was approved by the ethical committee of hospitals

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and department. Informed consent was obtained from patients prior to surgery and interview. Specimens were stored in RNA Later and rapidly frozen at -86ºC to preserve the mRNA, proteins, lipids and DNA in the samples for future analysis.

Information regarding medical history was collected from patient medical records including specific information about laboratory test results, tumor measurements and clinic o pathological risk factors for statistical analysis.

RNA isolation

Total RNA was extracted from breast specimens using Trizol reagent (Invitrogen). Precautionary measures were taken to avoid contaminations. RNA was run on 1% agarose gel to check its quantity by visualizing the intensity of the band under ultra violet light.

Semi-quantative reverse transcription polymerase chain reaction (RT-PCR)

In order to evaluate expression analysis of CYP1A1, semi quantative RT-PCR was used. RNA was reverse transcribed using Super First Strand Synthesis System cDNA kit (Cat no.11904-018, Invitrogen, USA). cDNA was further amplified using CYP1A1 gene specific primers (Table 1). The expression of CYP1A1 was analyzed in 25 tumor breast tissue samples along with their control samples and Beta actin as standard reference.

Amplification of CYP1A1

$2\mu l$ of RT products was used for subsequent PCR reaction. The optimized thermal cycling conditions comprised an initial denaturation step at 95C for 2 minutes, second step denaturation at 94ºC for 15 seconds, annealing at 55ºC for 30 minutes and extension at 72ºC for 1 minute. PCR amplicons of CYP1A1 were analyzed on 2% agarose gel. Electrophoresis was performed at 120 volts/cm (80 m A) for 30 minutes in 1XTBE (Tris Borate-EDTA) buffer. Amplified products were visualized under ultraviolet light.

Protein extraction and estimation

Radio-immune precipitation assay (Ripa) buffer was used to extract proteins from breast tissue samples. Phenyl methane sulfonyl fluoride (PMSF) was added as protease inhibitor. Bradford assay was used to estimate total proteins concentration in each tissue sample (cancerous and non cancerous tissues).

SDS-PAGE and Immuno Blot detection

Proteins ($50\mu g$) were separated using SDS PAGE (10 %) by the method of Laemilli (Laemilli, 1970). The proteins were transferred on to nitrocellulose membrane (0.25µm). The Western blots were used to immuno-detect CYP1A1. Using anti-CYP1A1 (Novus Biologicals, USA). Immunoreactive proteins were visualized using NBT/ BCIP substrate solution.

Statistical analysis

SPSS software (version 16.0) was used to determine any association of socio demographic and prognostic risk factors with the expression of CYP1A1.

Table 1. Primers for CYP1A1 and Beta Actin

Gene	Function	Sequence
CYP1A1	Forward	CTCTTAGGTGCTTGAGAGCCC
	Reverse	CATCAGCATCTATGTGGCCC
Beta Actin	Forward	AGCGAGCATCCCCCAAAGTT
	Reverse	GGGCACGAAGGCTCATCATT

Results

Table 2 explains correlations between the expression pattern of CY1A1 gene with socio demographic and prognostic features of the breast cancer. The mean age of breast cancer patients was 47.2(±10.7). About 92% females were married and 8% patients were unmarried. Analysis of tumor grade illustrated that the most dominant tumor grade in the study population was grade III (56%). Grade II (24%) and stage I (20%) were comparatively less common. The most frequent histopathological type was invasive ductal carcinoma (52%). Invasive lobular carcinoma (8%), mixed invasive lobular ductal carcinoma (8%), infiltrating carcinoma (12%), medullary carcinoma (12%), fibroadenoma (8%) were found in low frequency. Lymph node involvement was seen in 92% cases.

Expression of CYP1A1 was markedly reduced in tumor samples as compared to control tissue samples (Figure 1). Immunoblot analysis also showed down-regulation of CYP1A1 protein in tumor tissue samples as compared to non cancerous tissue samples (Figure 2). Association studies of socio-demographic and prognostic characteristics of breast cancer patients with CYP1A1 expression are shown in Table 3. CYP1A1 mRNA expression also revealed a stage specific pattern where down-regulation was at a higher rate in later stages of breast cancer as compared to early stages. CYP1A1 expression was down-regulated in 10% in tumor tissue stage of stage I, 40% in stage II and 50% in stage III. Married women (80%) showed more down-regulation as compared to unmarried women (p<0.01). No significant association of age, age at menarche, age at first full term

Table 2. Comparison of Socio Demographic Features of Breast Cancer Patients Showing Down Regulation of CYP1A1 Gene and Those Patients Having Normal Expression of CYP1A1 Gene

Variables	Patients with CYP1A1 gene Down regulation Mean (±S.tD)	Patients with normal CYP1A1 gene expression Mean (±S.tD)	P value
Age	48.2 (±8.3)	46.4 (±1.23)	0.4123
Age at menarche	14.1 (±1.9)	14.6 (±1.6)	0.7106
Age at first full term pregnancy	21.9 (±3.9)	22.1 (±3.7)	0.1269
Age at menopause	46.5 (±4.3)	43.4 (±3.7)	1.9245
Marital status			p<0.001
Married	100%	80%	Chi Square
Unmarried	0%	20%	test χ²=22.2
Side of breast			
Right breast	50%	54%	p=0.57
Left Breast	50%	46%	χ²=0.32

Table 3. Comparison of Prognostic Features of Breast Cancer Patients Showing Down Regulation of CYP1A1 Gene and That Patients Having Normal Expression of CYP1A1 Gene

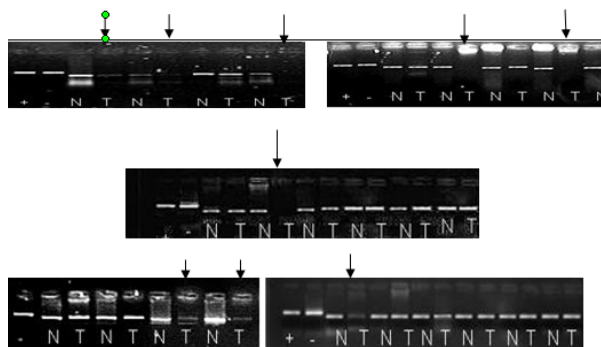| Tumor Grade	% of patients with CYP1A1 down regulation	% of Patients with CYP1A1 normal expression	P value
Grade I	10%	30%	Chi Square χ^2
GradeII	40%	16%	P<0.001
GradeIII	50%	53%	χ^2=20.37
Histopathological Type:			P=0.63
Invasive ductal carcinoma			
	50%	53%	χ^2=2.58
Mixed invasive ductal and lobular carcinoma.			
	20%	13%	
Invasive lobular carcinoma			
	10%	13%	
Medullary type	10%	13%	
Miscellaneous	10%	8%	
Lymph node involvement			
Yes	90%	93%	P=0.45
No	10%	7%	χ^2=0.58

Figure 1. Electropherogram of Ethidium Bromide stained 2% Agarose Gel showing Amplified PCR Product of CYP1A1 (product size, 246bp). Lane 1, (+) refers to the expression of housekeeping gene (Beta actin) in control breast tissue sample. Lane 2, (-) refers to the expression of Beta actin in tumor sample of breast Lane 3, (N) refers to the expression of CYP1A1 gene in controls samples of breast where as Lane 4, (T) refers to the expression of CYP1A1 gene in tumor samples of breast. Arrows indicate the down regulation of CYP1A1 gene
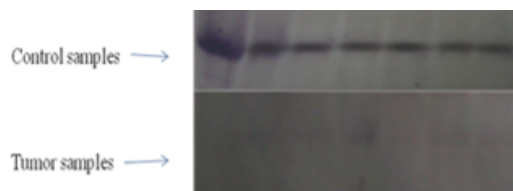

Figure 2. Western blots of Normal and Tumor (Breast Cancer) Samples with anti-human CYP1A1 using NBT /BCIP Substrate

pragnacy, age at menopause with down regulation of CYP1A1 expression was found (p>0.01)

Discussion

Carcinoma of breast is the commonest malignancy of females and leading cause of death among females worldwide with more than 1 million cases occurring worldwide per annum (Kelsey and Horn, 2007). Thirty percent of all cancers in women occur in the breast. There is a striking variation in the incidence rate of breast cancer in different countries (Forbes et al., 1997; Jørgensen et al., 2004). While data for developing countries are limited cancer registries suggest that age-standardized incidence rates are rising even more rapidly in low-incidence regions such as Africa and Asia (Sasco, 2001).

Breast cancer is a multi factorial disease. Increased risk may be associated with exposure to genotoxic agents during the breast development because the undifferentiated ductal elements of breast are more susceptible to the action of genotoxin early in life (Martin et al., 1996). Members of a number of classes of environmental chemicals have been found to be mammary carcinogens. Most of the potential human mammary carcinogens require multiple enzyme catalyzed steps to affect biotransformation to DNA reactive metabolites (Malfatti et al., 1999). Most common mammary xenobiotic metabolizing enzymes are CYP enzymes, epoxide hydrolase, glutathione S transferase. Peroxidase and lipooxgnase involved in the metabolism of carcinogens (Li et al,. 1996). Impairment in these enzymes may lead to altered DNA structure. Different studies reported breast cancer in relation to different xenobiotic metabolizing enzymes expression. Limited number of studies has been published in context to Pakistani population on the xenobiotic metabolizing genes. The objective of the present study was to carry out expression analysis of xenobiotic metabolizing enzyme CYP1A1 in breast cancerous tissues using semi-quantative RT-PCR and western blotting. This study was carried out on 25 fresh tumor samples along with their adjacent control tissues. From the statistical analysis it is apparent that frequency of breast cancer was higher in middle age. Earlier studies have also observed the similar pattern in Pakistani population (Siddiqui et al., 2000; Baloch et al., 2006). Most common histopathological carcinoma type found in the present study was invasive ductal carcinoma. The same type has also been reported in some of other studies in Pakistani population (Batool et al., 2005, Aslam et al., 2006, Quershi et al., 2007). The most dominant tumor grade in the study population was grade III (56%). Grade II (24%) and Grade I (20%) were less common as compared to grade III. Late presentation of breast cancer in our society has also been highlighted in a study by Shaukat Khanum Memorial Cancer Hospital who reported stage III as most dominant stage in cancer patients (Stage III 43%) (Gilani et al., 2003). This suggests that the disease is still being presented at a late stage in Pakistan and thus making curative treatment difficult whereas according to Western statistics where only 10% of patients are presented in stage III & stage IV (Klonoff et al., 1998).

At expressional level, CYP1A1 was significantly down-regulated in breast tumor tissues as compared to control. These results were in accordance with previously published studies in different carcinoma such as head and neck cancer, lung cancer and esophageal cancer (Murray et al., 1994; Wei et al., 2001; Masood et al., 2011). CYP1A1 enzyme also showed down regulation in urinary bladder tumor and their expression correlated with bladder tumor grade (Murray et al., 1995). Immunoblotting analyses also demonstrated the down regulation of CYP1A1 in breast

tissue samples .Numerous studies have investigated the expression pattern of CYP1A1 in extra hepatic tissues which are largely exposed to environmental carcinogens. CYP1A1 has been detected in lungs microsomes from human subjects by western immune blotting, although the expression was weak (Michele et al., 1998; Bernauer et al., 2006). This provides a critical confirmation of the down regulation of CYP1A1 in breast cancer patients.

In conclusion, expressional variation of CYP1A1 was reflected, in this preliminary study, showing down regulation of CYP1A1 in breast cancer. A correlation with stages of cancer was also found with down regulation at advance stages of breast cancer. However, to confirm CYP1A as a prognostic marker for staging of breast cancer further studies with larger sample size are being planned.

Acknowledgements

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