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Differences in Gene Expression Profiles between Human Breast Tissue and Peripheral Blood Samples for Breast Cancer Detection

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Abstract

The purpose of this study is to check the similarities of differential gene expression of 11 genes in breast cancer tissue and blood samples from the same individual for early detection of breast cancer. We had investigated differential gene expression by qRT-PCR in 20 breast cancer patients' tumoral tissues and corresponding blood sample. In our analysis BRCA2, HER-2, ER, PR, MET and BRAF mRNA levels were significantly over expressed in tumoral tissues. ER and PR mRNA levels were not detected in any of the peripheral blood samples, whereas KRAS and PTEN mRNA levels were not detected in any of the tumoral tissues. HER-2 (45%), EGFR (40%) and PI3KCA (30%). KRAS and PTEN mRNA levels were significantly over expressed in peripheral blood. In the correlation analysis expression of most of the genes were significantly altered in grade II and III in the tissues, where as in premenopausal women mRNA expression was significantly high in Grade II and III and ER/PR negative tumors. Our results suggests that BRCA2, ER, PR, PI3KCA, MET and BRAF differential gene expression at mRNA levels showed no diagnostic value as a marker of circulating tumour cells in breast cancer. qRT-PCR may be suitable alternative method for the determination of HER-2, EGFR, PI3KCA KRAS and PTEN mRNA status in the blood of breast cancer patients. Premenopausal women with high grade (Grade II and III) and ER/PR negative cases may be associated with proliferation/metastasis, high recurrence rate, and poor prognosis.

Keywords: Real-time PCR; Breast cancer; Differential gene expression

Introduction

Breast cancer is the most common cancer in women in developed countries [1]. As breast cancer is a heterogeneous disease [2], determination of prognosis by algorithms are mainly based on the factors like tumour size, histological grade and the metastatic disease level. Breast cancer survival depends mainly on early detection, treatment and stage. The survival rate of breast cancer patients was reported to be 100% for early stages (zero and one), and for stage four is nearly 20% for a period of five years. This emphasizes the importance of early detection so that treatment can be initiated as early as possible during tumour development. Early detection of breast cancer can reduce the morbidity and mortality of breast cancer patients [3]. In addition to the mammography, ultrasonography and magnetic resonance Imaging (MRI), sensitive molecular techniques may be used to detect early stage breast cancer. Presently molecular techniques are promising tools which may address the major problems with early detection of breast cancer. In general molecular markers will be helpful in defining risks and identifying the early stages of cancer. The search for molecular markers that can be used for the early diagnosis of breast cancer has become one of the most important objectives in clinical investigation, particularly because the current methods are invasive, show low specificity and require additional procedures for therapeutic decisions [4]. Gene expression is a common event in the progression of human cancer detection and quantification of potential tumour marker. Gene expression profiling has the potential to change the prognostication and treatment options for breast cancer patients.

qRT-PCR is a high-throughput technology that utilises a sensitive online fluorescence detecting system for quantification of gene expression in the cancer samples. qRT-PCR also allows for the early detection of cancer when the tumor burden is small and the disease is potentially more curable [5]. Extensive clinical literatures is available on gene expression analysis in breast cancer diagnosis.However, most published work has involved either in Blood or in tissue samples, but never both from the same individual. In the present study, our aim was to look for similarities of differential gene expression patterns in breast cancer tissue and corresponding blood sample from the same individual for early detection of breast cancer. For the said purpose, we had selected eleven breast cancer related genes BRCA1, BRCA2, PR, ESR, EGFR, Her2neu, MET, K-RAS, PI3KCA, PTEN and B-RAF and one reference gene GAPDH from the genome database and published literature.

Materials and Methods

Clinical sample collection

Based on the above criteria, twenty Blood samples and freshly frozen human breast tumour samples were obtained from Bibi Cancer Hospital between November 2010-February 2012. Tissue samples were preserved after surgery in RNA later and stored at 37°C overnight. Informed consent was obtained from all the individuals prior to collection of samples. The study was approved by the ethics committee, from where the samples were obtained. The patient cohort consisted of 10 invasive ductal carcinomas and 10 Infiltrative ductal Carcinomas.

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Normal tissue away from the tumour was included as controls for tissue samples and blood samples from age and sex matched individuals were collected for blood expression analysis. Control tissue away from the tumor, is the best matched control for relative gene expression purposes and for blood, since the same is not possible, age and sex matched controls had to be included.

Total RNA isolation

From each woman, 2.5 ml blood was drawn by trained phlebotomist directly in PAXgene tubes (PreAnalytiX). PAX tubes were left overnight at room temperature and then RNA was isolated from the sample the next day according to manufacturer's instructions. Total RNA was isolated from 30 mg tumour tissue and the corresponding control tissue, using the RNeasy Mini Kit method (QIAGEN). The concentration and purity of extracted RNA were determined by measuring the absorbance at 260 nm and 280 nm. All samples whose concentration was a minimum of 100 ng/µl and 260/280 between 1.8 to 2 were included in the study. Alternately, the samples were run on agarose to check for the integrity. All the 20 samples qualify the established criteria.

Reverse transcription assay

Reverse transcription was performed in a personal Master cycler (Bio-Rad), using 1 µg of total RNA in the presence of Random Hexamer (50 ng/µl) and Reverse Transcriptase (200 U/µl) in a total volume of 20 µl, including also: 10×TaqMan RT Buffer, $MgCl_2$ solution (25 mM), dNTPs mixture (10 mM), 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.

Real time PCR

For the gene expression profiles of BRCA1, BRCA2, PR, ESR, EGFR, Her2neu, MET, K-ras, PI3KCA, PTEN and B-RAF, real time-polymerase chain reaction (qRT-PCR) was performed, with 1 µl of cDNA, 12.5 µl Eva green, and with specific primers (Table 1) which were synthesized at Bioserve Biotechnologies Ltd. (Hyderabad, India). A three-step PCR assay was standardized using an Bio-Rad thermo cycler and carried out with initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s. A final extension at 72°C for 5 min was carried out. Amplification products were visualised by ethidium bromide staining following separation by electrophoresis through agarose gels. A melt curve was also added to the PCR program for confirming a single intact PCR product without any non specific amplification. Primers were designed in different exons for all the genes to avoid amplification from contaminating DNA.

Statistics

The data analysis was performed by using MedCalc software for Windows (version 7.4.1.0; Mariakerke, Belgium) P-value of <0.05 was considered statistically significant.

Results

Clinical and pathological features of 20 invasive breast cancer samples in qRT-PCR analysis

The present study was carried out in 20 sporadic breast cancer patients, fresh tumour tissues and corresponding normal tissue and blood samples from the same individual. The distribution of patient's biological characteristics and risk factors are shown in table 2. Age range for breast cancer patients was 30-73 years. The mean age at which breast cancer identified was 43 years in south Indian population. Depending on the menopausal status, breast cancer patients were categorized into Premenopausal (35%).and postmenopausal groups (65%). It was observed that high frequency of sporadic breast cancer cases were in postmenopausal group. In the current study, Infiltrating duct cell carcinomas (IFDC) showed the highest frequency 85% (n=17) as compared to Invasive Duct cell carcinomas (IDC) 10% (n=2), other types of breast disease like Medullary carcinoma 5% (n=1) showed very low frequency as compared to IDC. Estrogen and progesterone receptor status in breast cancer patients serves as a good prognostic and predictive marker for response to therapy. ER, PR by immunohistochemistry (IHC) is routinely done in all the centers from where samples were collected; The categorization according to hormone receptor status which was determined by IHC was as follows ER-/PR-, ER+/ PR+, ER+/PR- and ER-/PR+. The percentage of ER+PR+ (40%) and ER-PR- (55%) breast tumours were high when compared to other tumour subtypes like ER+/PR-(5%). The percentages of Her-2neu positive and negative were equal in number. The high number of nodes 16/22 was present in triple negative postmenopausal women. In the present study, more number of nodes was present in grade III tumors. Breast Cancer patients from the current study received combinations of chemotherapy. FAC (5-Fluorouracil Adriamycin

S.NO	Genes	F-Primer 5'→3'	R-Primer 5'→3'	Annealing Temp (°C)	size
1	BRCA1	TGCTTGAAGTCTCCCTTG	CTTCCATTGAAGGGTCTG	58	267bp
2	BRCA2	ACCCTTTCAGGTCTAAATGG	TGCCTGCTTTACTGCAAG	58	268bp
3	PR	CGCGCTCTACCCTGCACTC	TGAATCCGGCCTCAGGTAGTT	60	121bp
4	ESR	CTTGTGCAGGATTGTTGTG	GCCAATTGTAGGAACACAG	61	269bp
5	EGFR	TTAGCAGGAAAGGCACTG	CAGCTTCATCCTACACAAG	58	269bp
6	Her-2*neu*	AGTACCTGGGTCTGGACGTG	CTGGGAACTCAAGCAGGAAG	51	194bp
7	MET	GAAGACCTTCAGAAGGTTG	TGGGGAGAATATGCAGTG	54	267bp
8	PI3KCA	GGACAATCGCCAATTCAG	TGGTGGTGCTTTGATCTG	57	268bp
9	PTEN	GACAGACTGATGTGTATACG	GTGTAAATAGCTGGAGATGG	58	269bp
10	BRAF	ATGGTGATGTGGCAGTGAAA	TAGCCAGTTGTGGCTTTGTG	58	150bp
11	K-RAS	AGGGCAGTTTGGATAGCTCA	CACCACCCCAAAATCTCAAC	51	153bp
12	GAPDH	AATCCCATCACCATCTTCCAG	AAATGAGCCCCAGCCTTC	54-60	121bp

Table 1: Details of qRT-PCR primers, along with the target band size and annealing temperatures.

Age	
Age Range	30-73
Mean	43
Nuclear Grade	
Grade-I	10(50%)
Grade-II	7(35%)
Grade-III	2(10%)
No Data	1(5%)
Lymph Node Positive	
0	7(36.84%)
1-3	6 (31.5%)
4-9	4(21.05%)
Above 10	2 (10.5%)
Hormonal Status	
ER+/PR+	8(40%)
ER-/PR-	11(55%)
ER+/PR-	1(5%)
Her-2 Status	
Her-2 Positive	10(50%)
Her-2 Negative	10(50%)
Menopausal Status	
Postmenopausal	13(65%)
Premenopausal	7(35%)
Chemotherapy	
Adriyamycine, 5FU, Endoxane	20(100%)

No cases of ER−/PR+ were identified in this group

Table 2: Clinical and pathological features of 20 invasive breast patients in qRT-PCR analysis.

and Cyclophosphamide,) was given to 100% (n=20) of breast cancer patients, In the present study FAC Combination chemotherapy showed high frequency than other combinations.

Differential gene expression profiles of the selected 11 gene panel in breast cancer patients

In this study, BRCA1 mRNA levels showed down regulation in 10% (2 samples) of tissues. (-1.12 and -1.62). BRCA2 mRNA levels showed up regulation in 25% (5 samples) of tissues (range 2.01 to 4.56 folds). Her-2 mRNA expressions by qRT-PCR was significantly high (P=0.05) in tissues (75% (15 cases) when compared with blood sample analysis. 45% (9 samples) samples were positively amplified by qRT-PCR in both blood and tissue samples, in those nine samples two samples were negative by IHC. Six samples were showed under expression in blood in those six samples; three samples were over expressed by qRT-PCR, which were negative by IHC. PCR index for Her-2neu amplification indicating in blood ranged from 3.33–9.96 folds, where as in tissue ranged from 2.1-5.55 folds. In Her-2neu gene one sample showed 5.3 folds over expression in blood than tissue (9.96 and 4.57 folds). In both ER and PR genes, 45% (9 samples) of breast cancer tumoral samples detected ER and PR altered mRNA expression by qRT-PCR. ER gene is over expressed in 9 samples in tissues by qRT-PCR and IHC, out of 9 samples one case, showed 14 folds strong over expression seen in tissue than blood (range 0.94 and15.96 folds). In ER gene one sample with ER negative by IHC is over expressed by qRT-PCR in both blood and tissue (3.11 and 2.78), where as in PR gene, one sample with PR negative and two samples with PR positive by IHC were showed detectable mRNA expression in both blood and tissue (2.76 and 3.15, 4.15 and 3.98, 6.78 and 5.58 folds). PI3KCA gene 30% (6 samples) of blood and 45% (9 samples) of tumoral tissues had over expression. PI3KCA gene was uniformly over expressed in three samples in both blood and tissue, out of these 3 samples one sample showed one fold over expression was observed in tissue than blood (range 3.2 and 2.3 folds). PI3KCA is highly expressed in tissues when compared to blood, but statistically not significant (P=0.32). The MET mRNA expression was detectable by qRT-PCR in 50% (10 samples) breast cancer tissue samples; whereas only 20% (4 samples) were detected by qRT-PCR in both blood and tissue. The MET mRNA copies had amplification range 2.10-5.52 folds. The Increased expression of MET was significantly (P=0.05) greater in tumoral tissues than blood. In this study we also found that 25% (5 samples) samples were MET under expressed in blood. In our study, we found that 55% (11 samples) tissue and 40% (8 samples) blood samples were over expressed EFGR mRNA levels. One breast tissue showed 15 folds strong altered mRNA expression than blood (range 0.94 and 15.96 folds). 40% (8 samples) metastatic breast cancer Patients expressed EGFR mRNA in both blood and tissue samples. We had also found correlation between EGFR positivity in blood and tumoural tissue. In BRAF 20% (4 samples) samples were significantly (P=0.001) over expressed in tissue. In K-RAS 75% (15 samples) samples were significantly (P=0.001) under expressed in blood, where as in tissue samples, all 20 samples were displayed normal mRNA levels. PTEN expression was reduced in 15 (75%) of 20 BC blood samples (range-1.05 to -3.33) and 6 (30% of 20 in tumoral BC samples (range-1.10-to-7.14). The results of qRT-PCR showed that 46% (7 samples) samples were detected lower mRNA expression in both blood and tumorals of BC samples, PTEN showed significantly reduced (P=0.006) mRNA expression in blood (45%) when compared with tissue samples (Table 3).

Correlation of qRT-PCR markers by menopausal status

In the present study BRCA1 (12.5%), BRCA2 (37.5%), ER and PR (50%), HER-2 and PTEN (87.5%), EGFR and MET (62.5%) genes were expressed high frequency in premenopausal women when compared with postmenopausal women (Table 4). KRAS showed equal expression in both pre and postmenopausal women (75%). PI3KCA is the single gene in our panel expressed highly (58.3%) in postmenopausal women. The above results were not statistically significant.

Correlation of qRT-PCR markers by lymph node status

In total of 12/20 (60%) patients showed metastasis to one or more lymph nodes. lymph node positive patients were positive to qRT-PCR markers. In the present study ER, PR, Her-2neu, EGFR markers are commonly positive in node positive tumours, whereas at least one of the qRT-PCR marker were positive in node negative tumours, in our study, out of seven node negative tumours five showed altered *Her-2neu* mRNA expression.

Correlation of qRT-PCR markers by tumour grade

Tumour grade is a method used to classify cancer cells in terms of how abnormal they look. In the present study out of 20 cases, 2 (10%) cases were classified as having Grade I (well differentiated), 10 were classified as Grade II (moderately differentiated) and 7 (35%) were classified as Grade III poorly differentiated) tumours (Table 5). In the present study, 50% grade I cases showed reduced BRCA1 expression and 100% (2 Patients) grade I patients showed high expression in *Her-2 neu* and MET in tumoural tissues but not in blood. In BRCA2, 30% (3 cases) grade II cases showed reduced expression in tumoral tissue, 70% (7 patients) grade II cases were displayed strongly elevated mRNA levels of ER, PR, *Her-neu*, and EGFR markers in tissue and In PTEN and KRAS 80% grade II patients detected in blood. In Grade III tumours, 71.4% cases expressed in *Her-neu* in tissue and 57.1% Grade III tumours expressed in blood, In PTEN and KRAS low level of mRNA expression were displayed in 71% (5 cases) cases. 40% (4 cases) grade II patients expressed EGFR in blood, 50% (5 cases) grade II cases

expressed MET, where as in grade III 42.8% patients expressed both EGFR and MET.

Correlation of qRT-PCR markers by hormone receptor status (IHC)

ER/PR status was available for 20 patients. In the present study BRCA1 is expressed in 12.5% (1 patient) of ER/PR positive cases in tissue and 10% (1patients) ER/PR negative cases. BRCA2 is expressed in 37.5% (3 patients) of ER/PR positive cases in tissues. 18.1% (2 patients) expressed in ER/PR negative cases in tissues. 87.5% (7 patients) ER/PR Positive patients expressed *Her-2neu* in tissues, where as 63.6% (7 patients) ER/PR negative and 45% (2 patients) ER/PR negative patients expressed *Her-2neu* in blood. 100% (8 patients) ER/PR Positive patients expressed ER and PR genes, 54.5% (6 patients) ER/PR negative patients were highly expressed PI3KCA in tissues, whereas, 37.7% (3 patients) ER/PR positive were expressed in both blood and tissue. 62.5% (5 patients) ER/PR positive patients were expressed EGFR in tissues and 45.45% (5 patients) ER/PR negative patients expressed EGFR in both blood and tissue. 75% (patients) ER/PR positive patients expressed MET gene in tissues. 87.5% (7 patients)

Genes	Tissue (%)	Blood (%)	P Value
BRCA1 mRNA Altered Expression Normal Expression	 2(10%) 18(90%)	 0(15%) 20(100%)	 P=0.002
BRCA2 mRNA Altered Expression Normal Expression	 5(25%) 15(75%)	 0(0.0%) 20(100%)	 P=0.001
ER mRNA Altered Expression Normal Expression	 9(45%) 11(55%)	 1(5%) 19(95%)	 P=0.014
PR mRNA Altered Expression Normal Expression	 9(45%) 11(55%)	 3(15%) 17(85%)	 P=0.046
HER-2 mRNA Altered Expression Normal Expression	 15(75%) 5(25%)	 9(45%) 11(55%)	 P=0.057
EGFR mRNA Altered Expression Normal Expression	 11(55%) 12(60%)	 8(40%) 9(45%)	 P=0.961
MET mRNA Altered Expression Normal Expression	 10(50%) 10(50%)	 4(20%) 16(80%)	 P=0.052
PI3KCA mRNA Altered Expression Normal Expression	 9(45%) 11(55%)	 6(30%) 14(70%)	 P=0.329
B-RAF mRNA altered Expression Normal Expression	 5(25%) 15(75%)	 0(0%) 20(100%)	 P=0.001
PTEN mRNA Altered Expression Normal Expression	 6(30%) 14(70%)	 15(75%) 5(25%)	 P=0.006
K-RAS mRNA Altered Expression Normal Expression	 0(0%) 20(100%)	 15(75%) 5(25%)	 P=0.001

Table 3: qRT-PCR analysis of BRCA1, BRCA2, HER-2, ER, PR, PI3KCA, EGFR, MET, K-RAS and BRAF mRNA expression.

Genes	Post -12	Pre -8
BRCA1 mRNA Altered Expression Normal Expression	 1(8.3%) 11(91.6%)	 1(12.5%) 7(87.7%)
BRCA2 mRNA Altered Expression Normal Expression	 2(16.6%) 10(83.3%)	 3(37.5%) 5(62.5%)
ER mRNA Altered Expression Normal Expression	 5(41.6%) 7(63.6%)	 4(50%) 4(50%)
PR mRNA Altered Expression Normal Expression	 5(41.6%) 7(63.6%)	 4(50%) 4(50%)
HER-2 mRNA Altered Expression Normal Expression	 8(66.6%) 4(33.3%)	 7(87.5%) 1(12.5%)
EGFR mRNA Altered Expression Normal Expression	 6(50%) 6(50%)	 5(62.5%) 3(37.5%)
MET mRNA Altered Expression Normal Expression	 5(41.6%) 7(58.3%)	 5(62.5%) 3(37.5%)
PI3KCA mRNA Altered Expression Normal Expression	 7(58.3%) 5(41.6%)	 2(25%) 6(75%)
B-RAF mRNA Altered Expression Normal Expression	 2(16.6%) 10(83.3%)	 3(37.5%) 5(62.5%)
PTEN mRNA Altered Expression Normal Expression	 8(66.6%) 4(33.3%)	 7(87.5%) 1(12.5%)
K-RAS mRNA Altered Expression Normal Expression	 9(75%) 3(25%)	 6(75%) 2(25%)

Table 4: Correlation between multi gene expressions with menopausal status.

ER/PR positive patients expressed PTEN in blood, and 45.4% ER/PR negative patients expressed in tissues. 37.5% ER/PR positive patients over expressed BRAF in tumoral tissues (Table 6).

Discussion

Breast cancer is a major cause of cancer deaths in women and its incidences is increasing. One of the possible reasons for this scenario is that, we still lack a complete picture of the breast cancer heterogeneity with respect to molecular alterations. This difficulty not reflected by clinical parameters and pathological markers which are commonly used in the clinic to select treatment and to stratify patient's prognostic prediction. Biomarkers with IHC like hormone receptor status (ER/PR status), *Her-2 neu* expression and other parameters like lymph nodes, grades, direct the therapeutic decision. The presence or absence of such markers can be useful for the choice of patients for a certain treatment, but does not predict the response to this treatment. Immunohistochemistry is less sensitive than qRT-PCR for Hormone Receptor Status. Breast cancer develops as a result of altered expression of multiple genes and abnormal cellular pathways. In both blood and tissue gene expression patterns are helping to define the complex

biological processes associated with profiles of genetic disease. It has long been the source tissue used in various tests for the identification of disease and the monitoring of disease progression. The gene expression responses of circulating tumour cells can potentially provide an early warning of the threats they discover. Thus, peripheral blood has the potential to be used diagnostically as surrogates for direct sampling of sites of other disease processes. Blood is a critical issue for the understanding of disease and the development of disease treatments. It is a ubiquitous tissue that interacts throughout the body and literally acts as a sensor of physiological conditions [6,7]. This study is the first to report an evaluation of 11 qRT-PCR breast cancer markers (BRCA1, BRCA2, PR, ESR, Her2neu, EGFR, MET, PI3KCA, PTEN, K-RAS, and B-RAF). Comparison of mRNA levels in total RNA obtained from whole blood and corresponding tissues has been done in the present study, to look for similarities in the expression patterns in tissue and blood, so that blood can be used as source of testing for early detection as it is minimally invasive as compared to the tissue.

Genes	Altered expression blood	Genes	Altered expression tissue
BRCA1(N=0/20) Grade-I Grade-II Grade-III	 0(0%) 0(0%) 0(0%)	**BRCA1(N=2/20)** Grade-I Grade-II Grade-III	 1(50%) 0(0%) 0(0%)
BRCA2(N=0/20) Grade-I Grade-II Grade-III	 0(0%) 0(0%) 0(0%)	**BRCA2(N=5/20)** Grade-I Grade-II Grade-III	 1(20%) 3(60%) 1(20%)
HER-2(N=9/20) Grade-I Grade-II Grade-III	 0(0%) 4(50%) 4(50%)	**HER-2(N=15/20)** Grade-I Grade-II Grade-III	 2(14.3%) 7(50%) 5(35.7%)
ER(N=1/20) Grade-I Grade-II Grade-III	 0(0%) 0(0%) 1(100%)	**ER(N=9/20)** Grade-I Grade-II Grade-III	 1(11.1%) 6(66.7%) 2(22.2%)
PR(N=3/20) Grade-I Grade-II Grade-III	 0(0%) 2(66.7%) 1(33.3%)	**PR(N=9/20)** Grade-I Grade-II Grade-III	 1(11.1%) 7(77.7%) 1(11.1%)
PI3KCA(N=6/20) Grade-I Grade-II Grade-III	 1(16.6%) 3(50%) 2(33.4%)	**PI3KCA(N=9/20)** Grade-I Grade-II Grade-III	 1(12.5%) 3(37.5%) 4(50.0%)
EGFR(N=8/20) Grade-I Grade-II Grade-III	 1(12.5%) 4(50%) 3(37.5%)	**EGFR(N=11)** Grade-I Grade-II Grade-III	 1(9.1%) 7(63.6%) 3(27.3%)
MET(N=4/20) Grade-I Grade-II Grade-III	 0(0%) 2(50%) 2(50%)	**MET(N=10/20)** Grade-I Grade-II Grade-III	 2(20%) 5(50%) 3(30%)
B-RAF (0/20) Grade-I Grade-II Grade-III	 0(0%) 0(0%) 0(0%)	**B-RAF(5/20)** Grade-I Grade-II Grade-III	 1(20%) 4(80%) 0(0%)
K-RAS(15/20) Grade-I Grade-II Grade-III	 2(13.3%) 8(53.3%) 5(33.4%)	**K-RAS(0/20)** Grade-I Grade-II Grade-III	 0(0%) 0(0%) 0(0%)
PTEN (15/20) Grade-I Grade-II Grade-III	 1(7.1%) 8(57.2%) 5(35.7%)	**PTEN(6/20)** Grade-I Grade-II Grade-III	 0(0%) 3(50%) 3(50%)

Table 5: Correlation between multi gene expressions with histological grades.

BRAC1 and BRCA2 genes are co-ordinately regulated during proliferation. The present study, observed 10% down regulation in BRCA1 and 25% up regulation in BRCA2 of tumoral tissues, whereas BRCA1 and BRCA2 mRNA was not detected in any of the peripheral blood samples obtained. Down regulation of BRCA1 is reported in many studies, suggesting a possible role in BRCA1 pathogenesis of breast cancer. Reduced expression of BRCA1 mRNA could thus, be the cause of the genetic instability. Similar to our study, Bieche et al. [8]. reported that, BRCA2 mRNA expression is up regulated in breast cancer. BRCA2 mRNA expression may be induced by proliferation and, high proliferation might result in the up-regulation of BRCA2 mRNA levels.

ER, PR and HER-2 genes are important biological markers in breast cancer. According to this study, ER, PR and HER-2 mRNA levels were showed significantly over expressed in tumoral tissues. High ER mRNA levels were associated with absence of necrosis and vascular invasion together with absence or minimal level of tumour lymphocytic infiltration. ER and PR mRNA levels were able to predict overall survival. Over expression of Her2 receptor is associated with poor prognosis in patients with breast cancer, as well as with aggressive tumour growth and metastases [9]. ER and PR mRNA was not detected in any of the peripheral blood samples. In HER-2 only 5% over expression concordance was observed between blood and tissue. HER2 is usually not over expressed in all CTC [10]. CTCs is clearly associated with worse prognosis, it should be of great importance to develop therapeutic approaches to target these cells before the development of overt metastases. ER, PR and Her-2, expression has great value, which can be used to judge the prognosis of the tumour and predict the effectiveness of therapy for breast cancer.

Over expression of EGFR has been reported in 55% of tumoral tissues. EGFR over expression might take part in tumour development and it may predict poor prognosis in breast cancer. qRT-PCR positivity in breast cancer for EGFR was associated to a greater degree with visceral sites of metastasis [11]. Whereas, 40% of cases detected EGFR mRNA in blood. Detection of EGFR mRNA in peripheral blood might be useful to select patients that are likely to respond to EGFR targeting drugs. Our results on EGFR mRNA expression in blood were consistent with previous studies. De Luca et al. [12], found that 59% of the patients with metastatic breast carcinoma expressed EGFR RNA in blood samples and Mitsuhashi et al. [13], detected EGFR mRNA in 26.7% (12 of 45) of cervical patient's peripheral blood. In our study 40% of tumour tissues were mRNA expressed EGFR and Her-2. MET gene expression was found to be significantly elevated in 40% of tumour biopsies expression of MET was significantly (P=0.05) greater in tissue samples than blood. Over-expression of MET in cells promotes growth, survival, migration and invasion, all hallmarks of malignancy. High levels of MET expression were associated with a less favourable clinical course. An Earlier study Beviglia et al. [14], has indicated that over-expression of c-Met in moderately differentiated carcinoma cells may be one of the several attributes that contribute to an invasive phenotype during the progression of breast cancer. The variable up-regulation of EGFR and MET expression suggests that metastases may be more sensitive to therapies directed against these tyrosine kinases than a primary tumour. The PI3K pathway is mutated more commonly in

GENES	Normal Expression	Altered Expression	P Value	GENES	Normal Expression	Altered Expression	P Value
BRCA1 ER/PR+ ER/PR-	 8(100%) 11(100%)	 0(0%) 0(10%)	0.882	**BRCA1** ER/PR+ ER/PR-	 7(87.5%) 10(90%)	 1(12.5%) 1(10%)	0.811
BRCA2 ER/PR+ ER/PR-	 8(100%) 11(100%)	 0(0%) 8(0%)	0.096	**BRCA2** ER/PR+ ER/PR-	 5(62.5%) 9(81.8%)	 3(37.5%) 2(18.1%)	0.353
HER2 ER/PR+ ER/PR-	 5(62.5%) 6(54.54%)	 3(37.5%) 5(45.45%)	0.729	**HER2** ER/PR+ ER/PR-	 1(12.5%) 4(36.3%)	 7(87.5%) 7(63.63%)	0.263
ER ER/PR+ ER/PR-	 8(100%) 10(90%)	 0(0%) 1(10%)	0.601	**ER** ER/PR+ ER/PR-	 0(0%) 10(90%)	 8(100%) 1(10%)	0.004
PR ER/PR+ ER/PR-	 6(75%) 10(90%)	 2(25%) 1(10%)	0.365	**PR** ER/PR+ ER/PR-	 0(0%) 10(90%)	 8(100%) 1(10%)	0.004
PI3KCA ER/PR+ ER/PR-	 5(62.5%) 9(81.8%)	 3(37.5%) 2(18.1%)	0.353	**PI3KCA** ER/PR+ ER/PR-	 5(62.5%) 5(45.4%)	 3(37.5%) 6(54.5%)	0.465
EGFR ER/PR+ ER/PR-	 5(62.5%) 6(54.5%)	 3(37.5%) 5(45.4%)	0.729	**EGFR** ER/PR+ ER/PR-	 3(37.5%) 5(45.4%)	 5(62.5%) 6(54.5%)	0.465
MET ER/PR+ ER/PR-	 7(87.5%) 8(72.7%)	 1(12.5%) 3(27.2%)	0.445	**MET** ER/PR+ ER/PR-	 2(25%) 7(63.6%)	 6(75%) 4(36.3%)	0.107
PTEN ER/PR+ ER/PR-	 1(12.5%) 4(36.36%)	 7(87.5%) 7(63.3%)	0.263	**PTEN** ER/PR+ ER/PR-	 7(87.5%) 6(54.5%)	 1(12.5%) 5(45.4%)	0.151
BRAF ER/PR+ ER/PR-	 8(100%) 11(100%)	 0(0%) 0(0%)	0.882	**BRAF** ER/PR+ ER/PR-	 5(62.5%) 9(81.8%)	 3(37.5%) 2(16.6%)	0.353

Table 6: Correlation between multi gene expressions with ER and PR status.

women cancer. Genetic alterations of **PIK3CA** gene results in increase expression of **PIK3CA** gene. In the present study, 45% patients showed increased expression of PI3KCA in tissues. High PI3KCA mRNA expression appears to be adverse prognostic significance in patients with high risk of breast cancer [15].

BRAF gene is over expressed in 25% tumoral tissue. BRAF mRNA was not detected in any of the peripheral blood samples. This study is the first investigation to examine expression of BRAF in breast cancer. The significant over expression of BRAF in breast cancer activates RAS-BRAF kinase pathway which subsequently increases the possible invasive of breast cancer. Similar to our study, previous studies also detected BRAF mRNA expression in different types of cancers [16,17]. In our study KRAS and PTEN mRNA was significantly (P=0.001 and P=0.006) detected reduced expression in blood when compared with tumour tissue. A decreased expression of KRAS and PTEN in blood suggests that the gene might be involved in the progression of the breast cancer. 30% cases showed reduced expression in both blood and tumoral tissues. Reduced expression of PTEN may leads to genetic and epigenetic changes. It is unclear about the exact molecular mechanism of PTEN effect on peripheral blood. Our significant results suggest that regulation of KRAS and PTEN expression of mRNA binding proteins is important to breast cancer progression.

Relation between multi gene expressions with clinic-pathological parameters

Gene expression levels were correlated with menopausal status of breast cancer patients to see the effect of expression profile in modulating the risk of breast cancer. In our analysis mRNA expression is high in premenopausal women with high grade (Grade II and III) and ER/PR negative. Premenopausal women with high grade and ER/PR negative cases may be associated with aggressive tumour, high recurrence rate, and poor prognosis. Identifying the distinguishing characteristics may help in developing more effective treatment to the premenopausal women. The hormone receptor status observed here is consistent with that in other studies. The ER and PR mRNA levels had statistically significant (P<0.004 and P<0.004) correlation with ER/PR positive in tissue. The frequency of HER-2 mRNA levels was showed equal distribution between ER/PR positive and negative subtype tumours. Estrogen and progesterone receptors are most important

prognostic and predictive markers for breast cancer. Approximately 75% of ER/PR-positive tumors respond positively to endocrine therapy [18].

Conclusion

The results suggests that BRCA2, Her-2 neu, ER, PR, PI3KCA, EGFR, MET and BRAF mRNA levels showed no diagnostic value as a marker of circulating tumour cells in breast cancer, because the expression levels of these makers were significantly elevated in tumoral tissues than blood. qRT-PCR may be suitable alternative method for the determination of KRAS and PTEN mRNA status in the blood of breast cancer patients. The level of expression of most genes is significantly altered in grade III and grade II patients of tissues. In our panel, mRNA expression was found high in young women with high grade. Premenopausal women with high grade (Grade II and III) and ER/PR negative cases may be associated with aggressive tumour, high recurrence rate, and poor prognosis. Monitoring of this 11 gene panel may significantly improve the sensitivity and specificity for detection of breast cancer in both tumoral tissue and blood. However, more studies with large number of patients are required to confirm the clinical application of these molecular biomarkers.

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