Real-time PCR analysis of the mRNA expression of PIK3CA gene in breast cancer tissues showed a significant increase in expression compared to the control tissues (p=0.001).

Conclusions: PIK3CA mutations are an emerging tumor marker that, in the future, might be used in the process of choosing a treatment. The detection of PIK3CA mutation might have important clinical implications for diagnosis, progression and therapy.

Keywords: Phosphatidylinositol 3-kinase - breast cancer - gene expression - mutations
of PI3KCA in both primary breast tumoral tissue and corresponding normal tissue.

Materials and Methods

Study population
Breast carcinoma patients were assessed on the basis of clinical and pathological examinations. This Study is a Hospital-based case-control study conducted in South Indian population. All incidents of breast cancer cases were newly diagnosed during the study period Ethical committee approved the study for the benefit of humans in general. The procedures followed were in accordance with the ethical standards of responsible committee of the Institutes/Hospitals, to participate in a face-to-face interview using a structured questionnaire.

Selection criteria
Senior pathologists confirmed all diagnoses. We interviewed and collected the data about the patient’s demographic factors; we collected the information on age, smoking, chewing, usual alcohol intake, and previous cancer diagnoses. Participants were also asked about their family history of cancer, and the clinical information for these cases was obtained from medical records like tumor size, stage, and whether they were receiving chemotherapy, and radiotherapy. Patients were recruited following certain inclusion and exclusion criteria, which were determined before the beginning of the study.

Inclusion and exclusion criteria
All new cases of clinically confirmed breast cancer would be taken for study. Patients of confirmed breast cancer who give their consent were included. All patients who refuse to give consent were excluded.

Sample collection
For mutation analysis, based on the above criteria, a total of 38 breast cancer patients were enrolled in the study. About 3ml Blood samples were collected in from pathology lab after diagnosis. All the samples diagnosed mainly as invasive Ductal Carcinoma (IDLS). For gene expression study, human breast tumor tissues and corresponding normal tissues were collected after surgery in RNAlater (Ambion). All collected tissue and blood samples were preserved and stored at 37°C overnight. All samples were obtained from the Indo American cancer Hospital & NIMS Hospital, Hyderabad, between August 2011 - June 2012. The patient cohort consisted of 38 Invasive ductal carcinoma. Informed consent was obtained from all the individuals prior to collection of samples. The ethics committee approved the study from where the samples were collected.

DNA isolation
DNA was isolated from the tissue samples from breast cancer patients and blood samples from healthy volunteers by a rapid non-enzymatic method by salting out cellular proteins with saturated solution and precipitation by dehydration (Alluri et al., 2008). The red blood cells were lysed completely using RBC lyses solution. The lysate were then treated with cell lysis solution in order to lyse the cell components. The protein content is removed by protein precipitation solution. The precipitated DNA was suspended in 70% ethanol in order to remove the salts. The DNA was then dissolved in TE buffer and stored at 4°C. Cell lysis, protein precipitation, DNA precipitation and DNA hydration were carried out in the experiment.

PCR and direct sequencing
One sample of the genomic DNA was used per 25m l of PCR mixture. PCR buffer with 15 mM MgCl₂, 0.15 mM dNTPs, 1U/μL Taq polymerase and 12.5 pmol of each primer (Table 1). After mixing all the contents, PCR tubes were kept in thermal cycler and a 3 step PCR was performed with an initial denaturation at 94°C for 5 minutes followed by cycling at 94°C for 30 seconds, annealing for 30 seconds at appropriate temperature, 72°C for 45 seconds and a final extension at 72°C for 5 minutes was carried out for about 35 cycles. PCR products were analyzed on a 2% agarose gel and photographed on a UV light transilluminator. PCR products were purified by QIAquick PCR purification kit (Qiagen) and sense and antisense sequences were obtained by using forward and reverse internal primers, respectively. Each exon was sequenced using the BigDye Terminator Cycle sequence following the PE Applied Biosystem strategy and Applied Biosystems ABI PRISM3100 DNA Sequencer (Applied Biosystem, Forster City, CA). All mutations were confirmed by performing two independent PCR amplifications.

RNA extraction and cDNA synthesis
The samples were thawed and homogenized using omini THq electric homogeniser (Inkarp) Total RNA was extracted from biopsy of breast tumors and corresponding non-tumor tissues using the RNEasy Mini Kit (Qiagen, Germany). According to the manufacturer’s instructions. RNA was digested with DNase I (Invitrogen). The concentration and purity of extracting RNA were determined by measuring the absorbance at 260 nm and 280 nm. All samples whose concentration was a minimum of 100ng/μl and 260/280 between 1.8-2 were included in the study. Alternately the samples were run on agarose to check for the integrity. Reverse transcription was performed in a personal Master cycler (Bio-Rad CFX 96), using 1μg of total RNA in the presence of Random Hexamer (50ng/μl) and Reverse Transcriptase (50U/μl) in a total volume of 20μl, including also: 10×TaqMan RT Buffer, MgCl₂ solution (25mM), dNTPs mixture (10mM),

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3KCA mutation analysis</td>
<td>PI3KCA Exon 9</td>
<td>F: GGGAAAAAATATGACAAAGAAAAAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTGAGTCAGAAATTCAGG</td>
</tr>
<tr>
<td>PI3KCA gene expression</td>
<td>PI3KCA cDNA</td>
<td>F: GGACAAATCGCAATTCAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGGTCATGTGCTTGTGATC</td>
</tr>
</tbody>
</table>
an RNase Inhibitor (20U/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.

Gene expression analysis

In assessing the relative gene expression on the SYBR chemistry of, PI3KCA gene, real time - polymerase chain reaction (qRT-PCR) was performed, with 1 μl of cDNA, 12.5μl SYBR Green, and with specific primers (Table 1) were synthesized at Bioserve Biotechnologies Ltd. (Hyderabad, India). Primers were designed in different exons for all the genes to avoid amplification from contaminating DNA. A three-step PCR assay was standardized using a Bio-Rad thermo cycler and carried out with an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s, and extension at 72°C for 45s. A final extension at 72°C for 5 min was carried out. Each sample was run in triplicates and each PCR experiment included one non-template control wells. A melt curve was also added to the PCR program for confirming a single intact PCR product without any non-specific amplification. An NTC was also added with all the experimental setups. Gene expression levels were normalized to the expression of the housekeeping gene GAPDH. In addition to SYBR green fluorescent detection, the amplified products were also resolved on 1.8% agarose gels and visualized by staining with ethidium bromide. In the present study, increased mRNA expression was defined as ≥2.0 folds, “normal” expression was ranging from 0.5001-1.9999 folds and decreased mRNA expression was ≤0.5 folds The data collection step was performed at a temperature that was 3°C lower than the melting temperature of each primer pair. This cycle was followed by a melting curve analysis, ranging from 55°C to 95°C, with temperature increasing steps of 0.5°C every 10 s. Baseline and threshold values were automatically determined for all plates using the Bio-Rad iQ5 Software 2.0.

Statistical analysis

The fisher’s exact test (2x2 only) was performed by

Table 2. Clinical and Pathological Characteristics of Breast Cancer Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Patients</td>
<td>38 (100%)</td>
</tr>
<tr>
<td>Age Range</td>
<td>29-73</td>
</tr>
<tr>
<td>Median</td>
<td>47</td>
</tr>
<tr>
<td>Nuclear Grade</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>28 (74%)</td>
</tr>
<tr>
<td>III</td>
<td>10 (26%)</td>
</tr>
<tr>
<td>Tumor Size</td>
<td></td>
</tr>
<tr>
<td>4 above</td>
<td>32 (80%)</td>
</tr>
<tr>
<td>Lymph Node</td>
<td>6 (15%)</td>
</tr>
<tr>
<td>Hormonal Status</td>
<td></td>
</tr>
<tr>
<td>ER+/PR+</td>
<td>20 (53%)</td>
</tr>
<tr>
<td>ER-/PR-</td>
<td>18 (47%)</td>
</tr>
<tr>
<td>Her-2 Status</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>14 (37.5%)</td>
</tr>
<tr>
<td>Negative</td>
<td>24 (63%)</td>
</tr>
<tr>
<td>Menopausal Status</td>
<td></td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>12 (37%)</td>
</tr>
<tr>
<td>Premenopausal</td>
<td>25 (63%)</td>
</tr>
</tbody>
</table>

Results

Clinical and pathological characteristics of breast cancer patients

The clinical and pathological data of breast cancer patients were showed in Table 1. The median of patients age was 47 (ranged from 29-73). All 38 cases were invasive ductal carcinomas. The majority samples were of histological grade II with 15 cases, and ten cases which were grade III. The percentages of ER/PR positive tumors (63%) were higher when compared with ER/PR positive tumors (37.5%) (Table 2).

Table 3. List of PI3KCA exon 9 and exon 20 Mutations Identified in Breast Cancer

<table>
<thead>
<tr>
<th>Exon</th>
<th>PI3KCA Mutation</th>
<th>Cases (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon-9</td>
<td>1633G&gt;A (E545K)</td>
<td>9 (18.5%)</td>
</tr>
<tr>
<td>Exon-20</td>
<td>3140A&gt;G</td>
<td>4 (10.5%)</td>
</tr>
</tbody>
</table>

Expression of PI3KCA gene in breast cancer tumoral tissue and corresponding normal tissue

The Relative gene expression patterns of PI3KCA gene were quantified in 38 breast cancer tumor samples and corresponding blood samples of the same individual by real time PCR. Gene expression levels were shown ratio between PI3KCA gene and the reference gene GAPDH to correct for the variation in the amount of RNA. The mRNA expression of the investigated genes of breast cancer tissue and corresponding normal tissue was stored at -20°C until further use.

Table 4. RT-PCR Analyses of mRNA Expression in Breast Cancer Patients

<table>
<thead>
<tr>
<th>PI3KCA Expression</th>
<th>Tumoral Tissue (%)</th>
<th>Normal Tissue (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Altered Expression</td>
<td>17 (64%)</td>
<td>6 (16%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Normal Expression</td>
<td>14 (36%)</td>
<td>32 (84%)</td>
<td></td>
</tr>
</tbody>
</table>
mutations in exon 9 and exon 20 were showed increased expression of \textit{PI3KCA} in tumoral tissues when compared with corresponding normal tissues.

\textbf{Correlation of the \textit{PI3KCA} mRNA levels with clinical data}

In the present study, Postmenopausal women with ER positive tumors showed high frequency of mutations in exon 9 (66%, 6 of 9) and exon 20 (75%, 3 of 4). \textit{PI3KCA} gene expression is high in grade II tumors, these tumors also showed high frequency of exon 9 and exon 20 mutations in grade II cases. Tumor size between 1-4 and lymph node positive showed high frequency of gene expression and lymph node negative tumor showed high frequency of exon 20 mutations (Table 5).

\textbf{Discussion}

The \textit{PIK3CA} pathway is frequently activated in different human cancers \textit{PIK3CA} gene is mutated in an average of 15\% of human cancers it is evident that cancer of the breast harbor the most \textit{PIK3CA} mutation with average mutation frequency of 25\%. In this study we have reported the presence of \textit{PIK3CA} gene mutation in 28\% of breast cancer tumors. The incidence of \textit{PIK3CA} mutations found in our study 28\% is high compared with previous reports. These somatic mutations may increase the kinase activity of \textit{PIK3CA} contribution to cellular transformation. These mutations were predominantly found in kinase and helical domain of \textit{PIK3CA}. Frequently occurring mutation were found in exon-9 and exon-20 Bachman et al. (2004). Reported that average 25\% of breast cancers harbors mutation in either kinase or helical domain. Campbell et al. (2004) reported 40\% of mutation frequency in all 20 coding exons (new as well as previously reported mutations). Bachman et al. (2004) and Campbell et al. (2004) studies noted no association of the presence of \textit{PIK3CA} mutations with other clinical factors. Saal et al. (2005) reported 25\% of \textit{PIK3CA} mutation rate, in this study author found significant correlation between \textit{PIK3CA} mutation and ER/PR positive and lymph node metastasis and Her2. Levine et al. (2005) reported 18\% of mutation in exon-9 and exon-20 for breast cancer with no correlation between \textit{PIK3CA} mutation and clinical data. Kang et al. (2005) found over expressed cDNA containing the common E545K mutations in chick embryo fibroblast. Sammul (2005) reported that common mutation of \textit{PIK3CA} may be in increased activity of the \textit{PIK3CA} enzyme as maintained by increased cell signaling, cell growth and invasion. \textit{PIK3CA} mutation is an important pathway in cancer cell growth (Areumnuri, 2013).

The frequency of PI3K pathway mutations are different among the different breast cancer subtypes. A recent study has reconfirmed that the \textit{PIK3CA} mutations occur almost exclusively in invasive tumors whereas upstream mutations of the PI3K–AKT pathway (PTEN and K-RAS mutations) occur with equal frequency in early- and advanced-stage tumors. This finding suggests that the \textit{PIK3CA} mutations cooperate with these alterations in the malignant transformation (Oda et al., 2008). In our study Estrogen receptor positive tumors show high frequency of exon 9 and exon 20 mutations. Tumors with PI3K mutations were more likely to be ER/PR positive (Cooper et al., 2013). \textit{PIK3CA} mutation status as an independent prognostic value in patients with ERBB2+ breast cancer. \textit{PIK3CA} mutation status could serve as a new independent

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
All Patients & Exon 9 & Exon 20 & Altered Expression \\
\hline
Nuclear Grade & II & 6 (66\%) & 2 (50\%) & 11 (64\%) \\
& III & 3 (34\%) & 2 (50\%) & 6 (36\%) \\
Tumor Size & 1-4 & 9 (100\%) & 4 (100\%) & 13 (70\%) \\
& ≥4 & 0 (0\%) & 0 (0\%) & 5 (30\%) \\
Lymph Node & Negative & 5 (55\%) & 3 (75\%) & 8 (41\%) \\
& Positive & 4 (45\%) & 1 (25\%) & 10 (59\%) \\
Hormonal Status & ER+/PR+ & 7 (77\%) & 3 (75\%) & 10 (59\%) \\
& ER-/PR- & 2 (23\%) & 1 (25\%) & 8 (48\%) \\
Her-2 Status & Positive & 7 (77\%) & 4 (100\%) & 11 (64\%) \\
& Negative & 2 (23\%) & 0 (0\%) & 9 (52\%) \\
Menopausal Status & Postmenopausal & 6 (66\%) & 3 (75\%) & 9 (52\%) \\
& Premenopausal & 3 (34\%) & 1 (25\%) & 4 (23\%) \\
\hline
\end{tabular}
\caption{Correlation of the \textit{PI3KCA} exon 9 and exon 20 Mutations and mRNA Levels with Clinical Data}
\end{table}
prognostic tool when selecting targeted therapies for patients with ERBB2+ breast cancer (Magdalena et al., 2012). A HER2-positive patient treated with trastuzumab is significantly worse in patients with PIK3CA-mutated compared with wild-type tumours (Cizkova et al., 2013). This breast cancer subtype specificity suggests that PIK3CA mutations and other PI3K pathway aberrations may play a distinct role in the pathogenesis of breast diseases. PIK3CA mutations were also associated with sensitivity to tamoxifen. PIK3CA mutations will be important as these patients may respond well to hormonal therapy (Sherene et al., 2010). The presence of helical domain mutations may predict poor outcome whereas the presence of kinase domain mutations predicted an improved outcome. Magdalena et al. (2012) gene expression profiling study suggests that over-expression of genes belonging to the Wnt signaling pathway may also play a pivotal role in PIK3CA-mutated breast tumors.

Gene expression profiling is an important tool to evaluate genetic heterogeneity in carcinoma and is useful to develop expression based classification for different type of cancer. PIK3CA gene expression is associated with biological effects of aberrant cells proliferation and apoptosis, both of which are directly linked to tumor formation. In this study, we have analyzed the gene expression of PIK3CA gene in 38 breast carcinoma tumor and corresponding control tissue gene expression analyses performed at mRNA level by RT-PCR. In our study, we have clearly found significantly higher expression of PIK3CA in breast cancer tissues in comparison with control tissues. Moreover genetic alteration of PIK3CA gene been proved to increase expression of PIK3CA gene. Gene expression and gene mutation in PIK3CA gene have been reported in many human cancer types including breast cancer. We also demonstrated a significant correlation between the presence of PIK3CA mutation and the presence of PIK3CA expression. Sherene, 2013, data reported that the high levels of the PIK3CA-Gene Signatures are associated with thePIK3CA mutant genotype. It may facilitate better selection of responsive patient populations for mTOR inhibition in combination with letrozole. PIK3CA gene expression is associated with tumor aggressiveness in breast cancer pattern interfering that PIK3CA deregulation may have important biologic and cellular consequences in oncogenic development pathway from transforming through progression. Poor prognostic impact of PIK3CA mutation in breast cancer PIK3CA over expression may show short sequence and disease free survival. Brain and bone metastatic recurrence tend to over express PIK3CA than those who developed metastatic recurrence. PIK3CA expression was increased and was associated with high functional (PI3 kinase) activity.

In conclusion, genomic aberrations can predict responsiveness to targeted therapies, and because multiple PI3K pathway members are frequently aberrant in human breast tumors through mutation and other anomalies, this creates an expectation that targeting this pathway may provide an effective therapeutic approach in breast cancer. PIK3CA mutation is also an emerging tumor marker that, in the future, might be used in the process of choosing a treatment. However the detection of PIK3CA mutation has important clinical implications for diagnosis, progression and therapy.

References


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