Materials and Methods
Subjects

In the present study, 165 women with breast cancer were enrolled who were diagnosed and treated at Gujarat Cancer & Research Institute, Ahmedabad. The clinical information regarding age, menopausal status, tumor size, lymph node status, stage, histological type and grade and treatment offered were gathered from the case files maintained at Medical Record Department. Disease staging was done according to TNM Staging system. Majority of the patients (93%) were treated with surgery followed by chemotherapy (CMF or FAC or AC) and/or radiotherapy and/or hormonal therapy (Tamoxifen). The biomarkers related to apoptosis (Bcl2, BAG-1 and p53) and proliferation (Topo II alpha and ki67) were studied by immunohistochemistry from paraffin blocks. Of 165 patients, follow-up data of 92 patients were available and were followed for five year or until their death within that period.

The patient and tumor characteristics are shown in Table -5. Of 165 patients, 50% (82/165) patients were below 48 years, 47% (57/122) patients had T2 tumor size, 46% (73/165) patients had lymph node negative status, 38% (59/165) patients had early stage disease, 90% (149/165) patients had invasive ductal carcinoma and 34% (54/165) had histological grade III tumors. Among them, 55% (91/165) patients had ER negative tumors and 61% (101/165) patients had PR negative tumors.
**Immunohistochemistry - Principle**

Immunohistochemistry is the process of localizing proteins in cells of a tissue sections exploiting the principle of antibodies binding specifically to antigens in biological tissues. It is widely used to understand the distribution and localisation of biomarkers and differentially expressed proteins in different parts of a biological tissue. The unlabelled primary antibody reacts with tissue antigen and a labelled secondary antibody which reacts with primary antibody (The secondary antibody must be raised against the immunoglobulin of the animal species in which primary antibody has been raised). This biotinylated secondary antibody is coupled with Streptavidin horseradish peroxidase. This is reacted with DAB (3,3’ Diaminobenzedine) to produce a brown staining. (Figure-9)

**Figure-9: Antigen- Antibody Reaction by Immunohistochemistry - Indirect method applied in Immunohistochemistry**

![Antigen-Antibody Reaction](image)
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Reagents Used

- 0.2M Citrate solution
  2.1 gm Citric acid (Merk)
  1 litre DW
  pH-6 with 1N NaOH

- 0.001M EDTA
  0.37gm EDTA in 1000ml DW
  pH 9 with 1N NaOH

- 0.2gm Trypsin powder
  0.2gm Cacl2
  200ml DW
  pH-7.8 by N/10 NaOH

- 0.6% H2O2
  1ml 30% H2O2 +49ml Methanol

- 0.5M Tris Buffer Saline
  6gm Tris(hydroxymethylaminomethane)
  8gm Nacl
  1000 ml DW
  pH - 7.6 with 1N Hcl -4.4ml(17ml conc.Hcl in 200ml DW)

- Acid Alcohol
  700ml Alcohol absolute
  300ml DW
  7ml conc. Hcl

- Tris Hcl
  48ml - 0.2M Tris (2 42gm in 100ml DW)
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76ml - 0.1N HCl (1.7ml conc. HCl in 200ml DW)
76ml - DW
pH-7.6 with 1 N NaOH

- Haematoxylin
  4gm - Haematoxylin powder
  40ml - Absolute Alcohol
  80gm - Aluminium Alum
  800ml - DW
  2gm - Mercury Oxide (HgO2)

- 5%Eosin
  5gm Eosin powder in 100DW

- 3,3' Diaminobenzidine (DAB)
  0.1 gm DAB powder in 10ml DW on ice
  Dispense 500μl in each vial

Table-2: List of Antibodies procured from different companies and their dilution used were as under:

<table>
<thead>
<tr>
<th>N</th>
<th>Antibodies</th>
<th>Company</th>
<th>Clone</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bcl2</td>
<td>Novocastra, UK</td>
<td>NCL-bcl-2</td>
<td>1:40</td>
</tr>
<tr>
<td>2</td>
<td>P53</td>
<td>Dakocytomation CA USA</td>
<td>DO-7</td>
<td>1:25</td>
</tr>
<tr>
<td>3</td>
<td>BAG-1</td>
<td>Dakocytomation, CA, USA</td>
<td>KS-6C8</td>
<td>1:100</td>
</tr>
<tr>
<td>4</td>
<td>Topoisomerase II-</td>
<td>Dakocytomation, CA, USA</td>
<td>SWT3D1</td>
<td>1:50</td>
</tr>
<tr>
<td>5</td>
<td>Ki67 Antigen</td>
<td>DAKO, Glostrup, Denmark</td>
<td>MIB-1</td>
<td>1:25</td>
</tr>
<tr>
<td>6</td>
<td>CerbB2</td>
<td>Novocastra</td>
<td>CB11</td>
<td>1:30</td>
</tr>
</tbody>
</table>
Instruments Used

- Microtome
- Moist chamber
- Microscope

Procedure for Immunohistochemical localisation of Biomarkers

From paraffin embedded tissues blocks 3μ Sections were cut by microtomy

Then the slides were incubated overnight in Hot-air oven at 60°C (to fix the tissue on slide)

Then the slides were Deparaffinised and Rehydrated (Three Xylene washes and two isopropyl alcohol washes each for 5 min) (To clean the tissue from wax and unwanted material)

Antigen Retrieval by Citrate Solution was carried out (For unmasking Antigen)
Then the slides were placed in Tris Buffer for 5 minutes

Then the slides were dipped in H2O2 Blocking for 10-15 minutes to block endogenous peroxidase already present in the tissues)

TBS wash was given for 5 minutes

After that the tissues were covered with normal rabbit serum for 15 minutes (To avoid non-specific staining)

Rinsed with TBS for 5 minutes

After rinsing 100μl of respective Primary Antibody (as detailed in table) were added and the slides were incubated for overnight at 4°C.

The slides were given TBS wash again for 5 minutes and
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Then Secondary Antibody (Biotinylated - has high affinity Biotin sites) were added and kept for 1 hour (to link primary antibody and tertiary antibody to form a Ag-Ab complex) ↓

Again TBS wash was given for 5 minutes and Tertiary Antibody (Streptavidin) was added for 1 hour (which contain Horseradish peroxidase enzyme which binds with biotin) ↓

Again TBS wash was given for 5 minutes and then 3'3-Diaminobenzidine (DAB) was added for 2-3 minutes (Which acts as a Chromogen and give color reaction) ↓

Then the slides were rinsed in running Tap Water and stained with Harry's Hematoxyline for 2-3 minutes (For staining) ↓

After that slides were rinsed in Tap Water properly, and dipped in Acid-alcohol (For Decolourisation) ↓

After decolourisation the slides were dipped in Absolute-alcohol (For Dehydration) ↓

And then the slides were mounted in DPX ↓

Finally, the slides were viewed under Microscope

The Positive control used was tissue sample of breast carcinoma and Negative control used was without primary antibody

Scoring Interpretation

Cytoplasmic staining was observed for Bcl2 and BAG-1, and nuclear staining was observed for P53, Topo II alpha and Ki67 and membranous staining for Her2/Neu. The semi quantitative scoring was done as follows: tumours with marker staining in <10% cells was considered negative, staining in 10-20 % cells was considered 1+ and staining in 20-50% cells was considered 2+ and staining in >50% cells was considered 3+. All 165 patients were also scored into molecular IHC subtypes by using ER, PR
and Her2 Neu in to (i) Basal type (ER-/PR-/Her2-) , (ii) ER-/PR-/Her2+, (iii) Luminal A (ER+/PR+Her2-) and (iv) Luminal B (ER+/PR+/Her2+).

**Figure 10- Expression of Her2neu in Breast Carcinoma**

Among positive group, 08% (13/165) patients expressed 1+ staining, 30% (50/165) patients expressed 2+ staining and 36% (60/165) patients were having Her2Neu 3+ score positive membranous staining.

Among receptors 45% (74/165) and 39% (64/165) ER and PR positive nuclear staining seen respectively.
Figure 11- Expression of ER in Breast Carcinoma

Figure 12- Expression of PR in Breast Carcinoma
Gene Expression analysis by Polymerase chain reaction (PCR)

Subjects

To study gene expression by PCR 44 women with breast cancer were enrolled who were diagnosed and treated at GCRI, Ahmedabad. Frozen tissues were collected at the time of surgery. In addition to routine pathology examination, samples used for DNA extraction were examined histologically and were snap frozen in liquid nitrogen and preserved at -70°C till analysis. The DNA was extracted, quantitated and subjected to PCR.

DNA Extraction Procedure

Figure -13 DNA extraction process

Source-The Agencourt AMPure system

Principle

The cells are lysed during a short incubation with proteinase K in the presence of chaotropic salt (guanidine HCl), which immediately in actives all nucleases and enhances the binding of DNA onto a glass surface.
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Reagents Used

- QIAamp DNA Mini Kit – QIAGEN

The kit contained following reagents,

(i) Protease or Proteinase K, (ii) Purification on QIAamp spin columns and (iii) Phosphate-Buffered Saline

Procedure

Isolation of DNA from breast cancer tissues

25 mg of the tissue sample was cut into small pieces in Petridish and placed in a clean 1.5 ml microcentrifuge tube and 180 µl of Tissue Lysis buffer was added.

Then 20 µl of Proteinase K was added, vortexed by vortex mixer, and incubated at 56°C for about 1 hr, and tissue was lysed.

Then the tubes were centrifuged, 200 µl of Binding buffer was added, it was mixed by pulse-vortexing for 15 sec., and incubated at 70°C for 10 min. Then the tubes were briefly centrifuged.

200 µl of ethanol was added to the sample and it was briefly centrifuged.

The lysate was carefully transferred to the QIAamp Spin Column in 2ml tube. The cap was closed and centrifuge at 8000rpm for 1 min. Place the QIAamp Spin Column in a clean 2ml collection tube and the tube containing the filtrate was discarded.

Then carefully open the QIAamp Spin Column and 500µl Buffer AW1 was added. The cap was closed and centrifuge at 8,000 rpm for 1 min. The QIAamp Spin Column was kept in a clean 2ml collection tube and the tube containing the filtrate was discarded.

Then carefully open the QIAamp Spin Column and 500µl Buffer AW2 was added. The cap was closed and centrifuge at full speed 14,000 rpm for 3 minutes.
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Place the QIAamp Spin Column in a clean 1.5ml microcentrifuge tube and discard the collection tube containing filtrate. Carefully open the QIAamp Spin Column and add 200μl Buffer AE (10mM Tris-Cl; 0.5mM EDTA; pH 9.0) or Distill water. Incubate at RT for 1 min. and then centrifuge at 8000 rpm for 1 minute and stored at -20°C.

Principle of PCR

PCR is a method used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size. The amplification of a double stranded DNA-fragment occurs in repeating cycles using defined temperatures to perform the reaction.

The 3 steps of a cycle involve:

a) Denaturation: Separation of double-stranded DNA at 95°C.

b) Hybridisation: Annealing of primers to target DNA at 58°C. The temperature is dependant on the GC nucleotide content of the primer pair.
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c) Polymerisation (Extension): Synthesis of double-stranded DNA by Taq polymerase at 72°C.

During these cycles, the DNA strands repeatedly undergo denaturation, annealing of primers and formation of double-stranded DNA by Taq polymerase. The amplified DNA fragments can be separated by gel electrophoresis techniques and can be visualized by various staining procedures.

**Protocol** QIAGEN PCR Kit

For each tube reaction mixture prepared were as follows:-

**Table – 3: Preparation of Reaction Mixture**

<table>
<thead>
<tr>
<th>For each tube</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>5µl</td>
<td>10X PCR buffer</td>
</tr>
<tr>
<td>10µl</td>
<td>5x Q solution</td>
</tr>
<tr>
<td>2µl</td>
<td>25mM Mgcl2</td>
</tr>
<tr>
<td>1.5µl</td>
<td>dNTPs Mix</td>
</tr>
<tr>
<td>0.5µl</td>
<td>Taq polymerase</td>
</tr>
<tr>
<td>5µl</td>
<td>Primer A Forward</td>
</tr>
<tr>
<td>5µl</td>
<td>Primer B Reverse</td>
</tr>
<tr>
<td>29µl</td>
<td>Total volume mixture</td>
</tr>
<tr>
<td>100ng</td>
<td>DNA sample</td>
</tr>
<tr>
<td>Make final volume 50µl</td>
<td>by adding remaining Autoclave Distill Water</td>
</tr>
</tbody>
</table>
First master mix was prepared
Then aliquots were made (3.5 µl Taq polymerase) on ice
And 35 µl 10 x solutions was added from PCR kit in 1.5ml vial
Then 70 µl Q solution was added
Then 14µl MgCl₂ solution was added
And after that 10.5µl dNTPs was added in the same 1.5ml vial
Then vials were vortex gently and distributed in a thin-walled seven
PCR tubes 0.2ml, on ice
(29µl + DNA sample calculated 100ng/µl + Autoclave Distill Water
(ADW))
Then samples were placed in a thermal cycler and 40 cycles of PCR
were run

Reagents Used

- Primer Forward and Reverse
- PCR Quiagen kit contains Taq-Polymerase, 10x solution, Q solution,
  MgCl₂, dNTPs

Table- 4: Primer Sequence of Markers Used

<table>
<thead>
<tr>
<th>Markers</th>
<th>sequence</th>
<th>Base pair</th>
</tr>
</thead>
</table>
| P53 Exon5 | **Exon 5**
  
F -TGACTTTCAACTCTGTCTCCT
  
R-TCAGTGAAGGATGAGAGGCC
  
1) Denaturing at 95°C - 2mins.
  
(2) Annealing at 55°C - 1min.
  
(3) Elongation at 72°C.-1min.
  
No. of Cycles 40 | 290bp |
| P53 Exon7 | **Exon 7**
  
F-TCGCGCACTGGCCTCATCTT
  
R-TCAGCGGCAAGCAGGCTG
  
1) Denaturing at 95°C - 2mins.
  
(2) Annealing at 57°C - 1min.
  
(3) Elongation at 72°C.-1min.
  
No. of Cycles 40 | 210bp |
| Bcl2 F-CAAG AGAT GGCC ACGGCTGCT
  
R-TCTTCTGCATCTGTCGGCA
  
1) Denaturing at 95°C - 2mins.
  
(2) Annealing at 57°C - 1min.
  
(3) Elongation at 72°C.-1min.
  
No. of Cycles 40 | 390bp |
**Instrument used**

Thermal cycler for PCR: Eppendrof gradient PCR system.

**Figure-14 : Gel Electrophoresis for PCR Products**

**Agarose Gel Electrophoresis**

**Principle**

Gel electrophoresis is a technique used for the separation of deoxyribonucleic acid, ribonucleic acid, or protein molecules through an electric charge. It is usually performed for analytical purposes, but may be
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used as a preparative technique to partially purify molecules prior to use of other methods such as mass spectrophotometry, RFLP, PCR, Cloning, DNA sequencing, or southern blotting for further characterization. In contrast to proteins, which can have either a net positive or net negative charge, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode. Proteins and nucleic acids are electrophoresed within a matrix or "gel". Most commonly, the gel is cast in the shape of a thin slab, with wells for loading the sample. The gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value.

**Equipments Used**

- Electrophoresis equipment (power supply and gel chamber)
- Eppendorf-Micropipettes 0.5-20 μl
- 0.5ml vials (Tarson mfg. Co.)

**Reagents Used**

- TBE buffer-(Tris-10.8gm,Boric acid-5.5gm, EDTA(0.5M-4ml,pH-8))
- 0.5M EDTA-(18.61gm in 100ml Dissolve by adjusting pH - 8 with NaOH)
- Agarose Gel-2% gel (1gm Agarose powder in 50ml TBE (Tris Borate EDTA) Buffer)
- 5mg Ethydiun Bromide in 1ml ADW
- 0.4gm Sucrose solution in 10ml ADW a pinch of Bromphenol blue
- 2 μl Lambda-DNA and10μl Bromophenolblue dye in 20μl ADW
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Procedure

2% Agarose gel was prepared in TBE buffer with 8 wells comb. 20μl PCR product obtained was mixed with 10μl bromophenol blue dye and loaded in each well. 30μl of ethidium bromide dye was added to the TBE buffer in the tank. Then we run Electrophoresis was carried out at 60 Volts for 2hrs.

Gel Visualization

PCR products were electrophoresed on agarose gel and viewed under gel documentation system (BioRad, USA) and image was stored.

Statistical analysis

The data were statistically analyzed using SPSS statistical software version 13. The two-tailed chi-square test was used to assess the association between two parameters. Correlation between two parameters was calculated using Spearman's correlation coefficient (r) method. Overall Survival (OS) were evaluated using the Kaplan-Meier method. The log rank test was used to assess the prognostic significance of overall survival and relapse free survival. p values less than 0.05 were considered statistically significant.
Everything takes longer than you think
Murphy’s law