Discussion
The incidence of breast cancer is rising in every country of the world especially in developing countries such as India. Breast cancer, like other forms of cancer, is considered to result from multiple environmental and hereditary factors. Lesions to DNA such as genetic mutations, exposure to estrogen has been experimentally linked to the mutations that cause breast cancer. Beyond the contribution of estrogen, research has implicated viral oncogenesis and the contribution of ionizing radiation, failure of immune surveillance, which causes malignancies. Abnormal growth factor signaling in the interaction between stromal cells and epithelial cells, for example in the angiogenesis necessary to promote new blood vessel growth near new cancers. Inherited defects in DNA repair genes, such as BRCA1, BRCA2 and p53 and more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis. The term apoptosis was coined by Kerr et al in 1972, which from a Greek word describing the natural cyclical dropping of leaves from trees or petals from flowers. Although the occurrence of this type of cell death in breast cancer was first reported in 1892 (Clada et al 1995).

The relationship between apoptosis and breast cancer has been realized only recently. It has been suggested that anti hormone- induced mammary tumor cell death was by apoptosis (Krommer et al 1997) Apoptosis is a normal physiological cell death process by which multicellular organisms maintain cellular hemostasis. Typically apoptosis is characterized by the condensation of chromatin around the nuclear periphery and the cleavage
Significance of molecular markers in carcinoma of breast

of DNA into oligonucleosome size fragments by an endogenous endonuclease. Cell death in tumors is commonly attributed to the induction of apoptosis (Wyllie AH et al 1992). The role of apoptosis in tumorigenesis is currently being studied extensively. Increasing evidence shows that apoptosis may be involved in the development and progression of the cancer (Marx J et al 1993). A preliminary study showed that chemotherapy for breast cancer resulted in a reduction in proliferation and apoptosis, with upregulation of the Bcl2 gene (Ellis PA et al 1996).

The clinical data support the hypothesis that Bcl2 may be involved in chemo resistance through the inhibition of drug-induced apoptosis. In a study of transforming growth factor beta-1 (TGF-beta1), which induces angiogenesis and capillary morphogenesis, it has been found that capillary morphogenesis; in vitro is associated with apoptosis (Choi ME et al 1995). The inhibition of angiogenesis limits tumor growth by elevating the incidence of apoptosis in micrometastasis is significantly reduced after induction of angiogenesis, although the high proliferation rate remains unchanged. If angiogenesis is suppressed, the metastasis remains dormant and exhibit a high incidence of apoptosis is concomitantly reduced and the metastasis grows in size (Murray C et al 1995). We have already studied the prognostic significance of tumor angiogenesis in advanced breast carcinoma. Angiogenesis in advanced breast carcinoma correlates with poorer survival and high micro vessel count was independent predictive factor for metastasis and poor survival (Karelia NH,
Desai NS et al, Neoplasma vol.44, 97-104, 1997). In continuation of this study we also found that microvessel density, DNA aneuploidy and Rb oncoprotein were independent prognosticators for node negative breast cancer and combination of these indicators was found to be useful in selection of a subgroup of patients with node negative breast cancer who are at high risk for occult metastasis and would benefit from more aggressive therapy (Desai NS et al 2001). The utility of Bcl2 as a marker of favorable outcome in breast cancer has been established in both node negative and node positive disease (Silvestrini et al, 1994; Silvestrini et al, 1996). Given that Bcl-2 is a potent blocker of apoptosis, higher levels of this antiapoptotic protein is correlated with better clinical outcome. Patients with BAG-1 protein were found to be more likely to have long-term survival as compared to BAG-1 protein negative tumors (Turner et al, 2001). Various large Immunohistochemical Studies have documented clinical significance of BAG-1 in breast cancer have been reported (Tang et al 1999, Turner et al 2001, Sjostrom et al 2002, Townsend et al 2002). A consistent finding is that relatively high levels of cytoplasmic BAG-1 expression are detected in 2/3rds. or more cases of breast cancer. A strong relationship between nuclear BAG-1 and tumor grade /differentiation has been identified in 2 studies, with relatively high levels of nuclear BAG-1 expression in low grade tumors. (Tang et al 1999, Townsend 2002). Turner et al (2001) reported no correlation between tumor grade and BAG-1 expression. They reported an overall 10 years survival for women with early stage breast cancer of 82% with high cytoplasmic
activity versus 42% survival with low BAG-1 levels. 2/3rd of tumors in non-small cell lung cancer expressed high level of BAG-1. Cytoplasmic expression of BAG-1 independently correlated with improved overall survival. Several groups have studied expression of BAG-1 in human squamous cell carcinoma (Yamauchi et al 2001) found that in contrast to breast and lung cancer, nuclear expression of BAG-1 in laryngeal tumors conferred a worse disease free survival after radiotherapy. In oral squamous cell carcinoma (Shindoh et al 2000) demonstrated increased BAG-1 expression in tumor tissue relative to adjacent normal epithelium in 60-80% of samples-revealed reduced nuclear BAG-1 expression in oral sq. cell ca. compared to normal oral epithelium. BAG-1 expressions has been reported to enhance metastasis in experimental models (Takaoka et al 1997, Yawata et al 1998). The authors suggested that there is increasing evidence that BAG-1 expression is frequently altered in human cancer. Prospective trials are required to confirm the exciting possibility that BAG-1 expression which might be used as a prognostic marker in early breast cancer (Turner et al 2001). Larger prospective studies should be more representative of the spectrum of breast cancer as a whole and have increased power to detect independent prognostic predictors in multivariate analysis in presence of tumor grade and ER alpha status. Studies have shown that altered expression of p53 occurs in 0-40% of patients with DCIS and approx. 50% of patients with IDC (Davidoff AM et al 1991, Allred et al 1998). P53 expression has also been extensively evaluated for predictive significance. Recent studies examined alterations
of p53 expression and successful treatment with tamoxifen and standard systemic chemotherapy. In the tamoxifen studies, patients with ER positive/lymph node negative tumors and higher expression of p53 responded better to treatment than those with lower p53 expression, but the difference was not statistically significant (Elledge RM et al 1997).

In the present study, clinical significance of Bcl2, BAG-1 and P53 was assessed in early and advanced breast cancer. Their expression was correlated with conventional prognostic factors such as age, tumor size, lymph node status, stage, histology, tumor grade, and survival. Our study showed that over expression of Bcl2, BAG-1 and p53 was noted in 57%, 68%, and 61% of breast carcinomas respectively. In these patients, comparison of marker expression was also done by grouping the patients according to age (i.e. <48 years and >48 years i.e younger and older age group) and lymph node status (negative vs. positive). A significant decrease in Bcl2 expression was observed with increase in tumor size, disease stage and tumor grade. Several investigators have noted a significant decrease in Bcl2 expression during the evolution from breast epithelium cells to intraductal carcinoma and from intraductal carcinomas to invasive carcinomas. They stated that during development and progression of breast cancer down regulation of Bcl2 is necessary which is important to induce apoptosis of cancer cells for the maintenance of increased cell turnover and balance between tumor growth and apoptosis. Further, its expression is influenced by estradiol in breast cancer. Also,
withdrawal of sex hormones stimulates apoptosis in breast cancer cells. Majority of the studies have shown a positive correlation between Bcl2 expression and ER status, with ER positivity in >60% of tumors. In our study no such correlation was found between Bcl2 and ER status, the reason could be low incidence of ER positivity (45%) with higher incidence of grade II or III tumors (95%). Further, Bcl2 overexpression proved to be favorable prognostic indicator in lymph node negative patients. These finding are consistent with various published reports.

Our study demonstrated cytoplasmic positivity of BAG-1 in breast tumors and none of them showed nuclear staining. Like Bcl2, lowest BAG-1 expression was found in advanced stage patients and histological grade III tumors as compared to their respective counterparts. Earlier reports have also noted high percentage of cytoplasmic staining (85%) and a very low percentage of nuclear staining (0.5%) with nuclear positivity in 18% patients. Several reports have shown nuclear and/or cytoplasmic staining pattern of BAG-1 in breast tumors. Further loss of nuclear BAG-1 staining in poorly differentiated tumors has also been reported. Regarding the prognostic value of BAG-1, various studies have reported divergent results. Our study demonstrated no association of cytoplasmic over expression with disease outcome. Regarding p53, over expression was seen in 61% of breast carcinoma. In older age group higher p53 positivity was noted in lymph node positive patients and advanced stage patients. Further p53 expression did not discriminate between patients with better or
worse overall survival. Thus, Bcl2 over expression was not associated with apoptosis status in primary breast tumors as it was found as a better prognosticator associated slow growing phenotype. Further, in trivariate survival analysis, patients with three markers positivity had reduced overall survival as compared to patients with one or two marker positivity followed by negative subgroup. Hence, breast tumors with Bcl2 negativity, BAG-1 positivity and p53 positivity identify patients with aggressive phenotype.

Several studies have documented strong correlations between ER positivity and Bcl-2 immunostaining in primary adenocarcinomas of the breast. Furthermore, consistent with the generally more aggressive nature of tumors which have progressed to an ER-negative, hormone-independent state, patients with ER-positive, Bcl2 positive tumors typically have longer disease-free and overall survivals.

In the present study, breast cancer patients were also classified according to recently introduced molecular classification into Basal type (tumors with ER-PR- and Her2- ) type, (ER-PR-Her2+), Luminal A (ER+ PR+ Her2-) and Luminal B (ER+ PR+ Her2+). Of them 18%(29/165) tumors expressed basal type (ER-PR-Her2-), 38%(63/165) Her2 +ve and ER/PR -ve and 16%(26/165) tumors showed Luminal A( ER+PR+ Her2-) and 28%(47/165) tumors showed Luminal B type( Her2+ER+/PR+). Between Luminal A and B, Luminal B group showed higher incidence of Bcl2 and BAG-1 expression as compared to Luminal A type. No such difference
Significance of molecular markers in carcinoma of breast was noted between Basal type (ER-PR and Her2-) and ER-PR- and Her2+ breast tumors in relation to Bcl2 and BAG-1. Also there was no such observation noted with p53.

Topoisomerases constitute a family of highly conserved essential enzymes, which exist in all investigated living prokaryotic and eukaryotic cells (Kellner et al 2001). Topoisomerases were first studied in the 1970s and found to be involved in the relaxation of super coiled DNA (Champoux et al 1978). They are indispensable for the control of DNA topology and play a key role in DNA replication (Maxwell, Osher et al 1986, 1989). Because of the important role of topoisomerases in the maintenance and replication of DNA during proliferation, cells become highly vulnerable when these functions are lost. Humans possess four types of topoisomerases, Topo II, which is one of the most studied and the focus of this class of biomarkers, belongs to the type-II enzymes and is represented in humans by two highly homologous isoforms, namely alpha (170kDa) and beta (180kDa). Topo II alpha protein, which is encoded by a gene located on chromosome 17 (17q21.2) and positioned telomeric to the ERBB2 oncogene, was first isolated and purified in 1981 (Miller et al). It has long been suggested that top2 alpha may have prognostic significance in breast cancer because its expression correlates with proliferative state of the cell cycle (Tsai et al 1988). The top2alpha gene is transcriptionally active in late S-phase and plays major role in DNA replication, cell cycle progression, and chromosome segregation (Miller, Chan, Roca et al
A major mode of regulation of topo II alpha activity appears to be phosphorylation. Although the mechanisms of phosphorylation are not fully elucidated, phosphorylation appears to stimulate topo II alpha in S-G2 cell cycle phases and inhibit topo II alpha during mitosis. Once stimulated, topoisomerase will form a non-covalent bond with double stranded DNA, thereby initiating the conversion of storage form DNA into a more relaxed form that is adequate for transcription. The report that the topo II alpha gene is co-amplified in breast tumors has led to the widespread examination of topo II alpha as a potential molecular marker in breast cancer patients. It has been reported that over expression of topo II alpha is correlated with poorly differentiated DCIS (Boege et al 1991, Spitz et al 2000, Sampson et al 1992). Breast tumors with Topo2 alpha amplification frequently have ERBB2 amplification, but not vice versa (DiLeo et al 2002), and the coamplification of ERBB2 and Top2 alpha is significantly associated with favourable local response to anthracycline-based therapy (Coon et al 2002).

Proliferation is an important cancer-associated phenomenon that has been widely investigated in relation to breast cancer progression. The pathways associated with proliferation have been well studied and there are many reports that show that proliferative potential of breast tumors has both prognostic and predictive significance. Many of the biomarkers already discussed play a role in the proliferative potential of breast tumors.
However Ki67 and bcl2 are probably the most widely studied and evaluated as cellular and proliferative biomarkers for breast cancer.

\textbf{MIB-1 Ki67} has become very important because tumor proliferation is known to be inversely associated with survival in patients with breast carcinoma (Weidner et al 1994). A study of 27 benign tissues and 70 breast carcinomas showed that the MIB-1 Ki67 scores (positive cells / total tumor cells) ranges from 0-4% in benign and 3-98% in malignant tissue (Devan et al 1989). Although a high level MIB-1 Ki67 immunostaining is often associated with early recurrence of breast cancer after mastectomy, mki67 levels appear to be independent of tumor size, lymphnode status and ER1 expression (Bouzar et al 1989). Recent studies have demonstrated that a correlation exists between MIB-1ki67 expression and malignancy (p<0.001), but no association was observed with increase or decrease of other cellular and proliferation markers such as bcl2 (Midulla et al 2002). Trihia et al 2003 (MIB-1) was associated with a poor DFS and OS in node +ve patients where as statistical significance was not reached in node -ve patients. While Sheshadri et al 1996 investigated MIB-1 positivity were associated with a significantly increased risk of relapse and death from cancer both in univariate and multivariate analysis. The median MIB-1 value in the present study is 16% which is considerably higher than that in the study by Sheshadri et al, tertile at 10%. The predictive value of MIB-1 estimates was evaluated recently by Faneyte et al, high Ki67 scores were significantly correlated with tumor response and were the only
parameter that significantly decreased after therapy. This may imply that in the future MIB-1 estimates can be used as predictive markers to guide the choice of chemotherapy. Petit et al 2004 showed patients with -ve HR status, high grade breast tumors with a high tumor cell proliferation (Ki67> 20%) were much more likely to respond to neoadjuvant anthracycline based chemotherapy. The proliferative activity of a tumor may be assessed in a number of ways. Mitotic counts are already an intrinsic part of the histological grade, where as proliferation rate measured as S-phase fraction and MIB-1 estimates represent other methods which have only reached limited clinical applicability. Present study showed that out of 165 breast cancer patients, 63% (104/165) of the patients showed nuclear expression of topo II alpha while 37% (61/165) of the patients did not expressed topo II alpha. Among the positive group, 35% (58/165) patients expressed 1+ staining, 15% (24/165) patients expressed 2+ staining and 13% (22/165) patients expressed 3+ staining. In the present study clinical significance of topo II alpha and Ki67 expression was evaluated in 165 patients with breast carcinoma. Topo II alpha expression was noted in 63% of the tumors among which 28% tumors exhibited high levels ie. 2+ or 3+ staining intensity. The biological role of topo II alpha is unknown. This enzyme is known to be a marker of cell proliferation in normal tissues. High topo II alpha expression may be related to high Proliferative rate. This view is supported by our finding that topo II alpha expression was found more common with advancement of disease and in high grade tumors. Therefore its expression relates to a more aggressive phenotype.
The cells lacking topo II alpha are not capable of finishing a normal cell cycle and should therefore not be viable. Also, the cells stained with low intensity are not cells with a complete lack of topo II alpha function. Cells with a low concentration of topo II alpha protein form fewer topo II alpha mediated DNA strand brakes are less sensitive to topo II alpha inhibiting drugs than cells containing a high concentration of topo II alpha. In addition it has been shown that experimental overexpression of topo II alpha in different human cell lines causes apoptosis. However in the present study topoll alpha expression was associated with BAG-1, Bcl2 and p53 over expression. This supports that the enzyme causes defects in apoptotic pathway in breast carcinoma. With hormone receptor status, no significant correlation was found. Further in this study topo II alpha expression showed no prognostic value. Contrary to our findings several studies have shown higher topo II alpha expression showed greater mortality and higher recurrence rate. In general topo II alpha expression is associated with high cellular proliferation and poor histological differentiation of the tumor. The data suggests that topo II alpha targeted drugs might be useful in the therapy of breast carcinoma. Ki-67 MIB-1 clone, a measurement of cell proliferative activity was analysed in patients with breast carcinoma. MIB-1 is expressed throughout the cell cycle in proliferating cell, but not in cells in either the G0 or early G1 phase. Nuclear expression of Ki67 was noted in breast carcinoma. The cut off of 20% was used to classify ki67 negative and ki67 positive breast tumors. In these patients overexpression of ki67 was noted in 44% (>20%) of the tumors while 66% tumors showed ki67 negative
Significance of molecular markers in carcinoma of breast

(<20%). Many authors have reported an association with histologic grade, lymph node status, patient age, tumor size, ER and PR status, ploidy, p53 status and epidermal growth factor receptor expression. An association between ki67 staining and both disease free interval and survival has been reported. In the present investigation, Ki67 expression when correlated with apoptotic markers BAG-1, Bcl2, p53 and topo II alpha a significant positive correlation was noted with Bcl2. 41% Bcl2 positive tumors showed by ki67 overexpression while only 24% Bcl2 negative tumors exhibited ki67 positive had apoptotic resistance as showed bcl2 overexpression. Therefore ki67 expression alone is of limited value but in association with Bcl2 identifies an aggressive phenotype.

In the present study, breast cancer patients were also classified according to recently introduced molecular classification (Nielsen et al IHC sub types) into Basal type (tumors with ER-PR- and Her2-) type, (ER-PR-Her2+), Luminal A (ER+ PR+ Her2-) and Luminal B (ER+ PR+ Her2+). Of them 18%(29/165) patients tumor express basal type (ER-PR-Her2-), 38%(63/165) Her2 +ve and ER/PR -ve and 16%(26/165) patients tumor showed Luminal A( ER+PR+ Her2-) and 28%(47/165) patients tumor showed Luminal B type( Her2+ER+/PR+). Between Luminal A and B, Luminal B group showed higher incidence of topo II alpha and ki67 expression as compared to Luminal A type. No such difference was noted between Basal type (ER-PR-and Her2-) and ER-PR- and Her2+ breast tumors in relation to topo II alpha and ki67 expression.
P53 is the most commonly mutated gene in human cancers. Approximately 90% of the p53 gene mutations are localized between domains encoding exons 5 to 8. The gene is located on chromosome 17 (17p13.1) and encodes a nuclear phosphoprotein that plays a role as a marker in breast cancer. Lukas J. et al (2000) studied p53 mutations and expression in breast carcinoma in situ. They demonstrated genetic alterations of p53 as well as overexpression of the p53 protein. Most p53 mutations in breast occur before invasion of the breast stroma. In combination with the lack of p53 mutations in benign epithelium in cases with p53 mutation in carcinoma components, it appears that p53 may be altered during formation of the CIS lesion in the pathogenesis of breast cancer. Second, identical mutations in both CIS and invasion components of the same tumor support a precursor product relationship between CIS and invasive disease consistent with a clonal relationship between CIS and invasive disease. Finally, p53 mutations occur in most histological types of intraductal breast cancer. These results suggest that p53 alterations are important in the pathogenesis of early breast neoplasia.

The present study was designed to determine the frequency of p53 gene mutations in breast cancer, to correlate the presence of p53 expression with established clinicopathological parameters, including the ER and PR status, and to assess the prognostic significance of p53 gene expressions. We found 37% (11/30) p53 exon 5 expressions, 11% (4/38) p53 exon 7 expressions and 33% (10/30) Bcl2 expressions was noted. When correlated with established clinicopathological parameters higher
incidence was noted between older age group and p53 exon 5 and Bcl2 expression. Also higher incidence was noted with high grade tumors and p53exon5 and p53 exon7. However a trend of inverse correlation (P =
0.09) was found between tumor size and p53exon7 and no correlation was noted with ER, PR and p53 exon 5. Higher incidence was also noted between older age group and Bcl2 expression while no such correlation was found between ER, PR and Bcl2. When Inter correlation was done A trend of positive correlation was observed between p53 exon 5 &7 (P=0.069) while no such correlation was observed between Bcl2 gene and Exon 5&7. when sub grouped according to Age and LN status a trend of positive correlation was noted between p53 exon 5 and 7 in younger age group (<48 years; P=0.089). With in LN status subgroup patients a significant positive correlation was noted between LN positive patients and p53 exon 5 and 7 positivity (P=0.027). A trend of positive correlation was also observed with Bcl2 gene and protein within Older age group patients (>48 years; P=0.068).No such correlation was observed with in LN status sub group patients. No such observation was seen between bcl2 gene and hormonal receptors.