Prologue
Breast cancer is a complex disease that still imposes a significant healthcare burden on women worldwide. The advanced molecular biology techniques have opened up new opportunities for discovery of novel biomarkers for early detection and prognostication of breast cancer. Unfortunately, none of the present biomarkers in use have sufficient diagnostic, prognostic and/or predictive power across all categories and stages of breast cancer (Arciero et al 2003). However, the relation between prognostic factors and response to adjuvant chemotherapy is less clear. Even though having node negative status and early stage many patients have relapsed with in short time. Several studies have shown that apoptosis and angiogenesis are novel prognostic markers in breast cancer, and they may have predictive value of the response to anticancer treatments. A recent data suggest that the progressive inhibition of apoptosis and induction of angiogenesis may contribute to tumor initiation, growth and metastasis in the pathogenesis of breast cancer. Apoptosis is a normal physiological cell death process by which multicellular organisms maintain cellular hemostasis. The role of apoptosis in tumorigenesis is currently being studied extensively. Increasing evidence suggests that apoptosis may be involved in the development and progression of the cancer. Several molecular markers have been associated with a poor prognosis with breast cancer and it is widely assumed that the presence of these markers is an indication for adjuvant therapy. Breast cancer patients with node negative lymph nodes are generally considered to have a relatively good prognosis. This is particularly true when other good
Significance of molecular markers in carcinoma of breast

prognostic factors are present such as small tumor size, estrogen (ER) and progesterone receptor (PR) positivity, and favourable nuclear grade. Approximately 30% to 40% of patients with apparently localized breast cancer probably have micrometastatic disease, which is clinically undetectable at the time of diagnosis and which accounts for most instances of distant relapses and disease-related deaths. Predictive biomarkers are greatly needed that can help guide clinicians and patients in treatment related decisions about the necessity (or lack thereof) for adjuvant chemotherapy, hormonal therapy, and new treatments as they become available. Therefore in the present study we evaluated clinical significance of the biomarkers including Estrogen Receptor (ER), Progesterone Receptor (PR), BAG-1, Bcl2, p53, Topoisomerase II alpha, ki67 and Her2-neu in early and advanced breast cancer. However the relation between prognostic factors and response to adjuvant chemotherapy is less clear We studied prognostic significance of these biomarkers by two different mechanisms i.e. apoptosis and proliferation.

1. **Apoptosis**- Apoptotic markers Bcl2, BAG-1 and p53

2. **Proliferation**- Proliferative markers Topoisomerase II alpha and Ki67

Homeostasis in normal tissue is regulated by a balance between proliferative activity and cell loss by apoptosis. Apoptosis is a physiological mechanism of cell loss that depends on both pre-existing proteins and de novo protein synthesis (Steller H, Thompson 1995). The process of apoptosis is integral to normal mammary gland development (Medina D
1996), metamorphosis, and organ involution in many diseases, including cancer (Steller H, Thompson 1995). Apoptosis is a highly regulated process, one important regulator of apoptosis is Bcl2.

Bcl2 is a proto oncogene that is located on chromosome 18 (18q21.33) and encodes a mitochondrial protein involved in multiple cellular functions, including the inhibition of programmed cell death (Hensel M 2002, Silustini R 1996). The Bcl2 oncoprotein is a 26 KDa integral membrane protein localized to the membranes of the endoplasmic reticulum, mitochondria, and nuclear envelope (Kroemer G 1997). It can function to suppress or delay the induction of apoptosis in a number of systems, including prostate (Beham A 1997), skin (Rodiguez 1998), lymphoid tissues (Strausser A 1994), and mammary gland (Jager R 1917, Lu PJ 1995). Differential regulation of these genes, especially Bcl2, may contribute to the biological nature of clinically more aggressive and highly proliferative breast cancers. Bcl2 is expressed in nearly 80% of breast cancers derived from women with primary tumors (Zhang GJ 1998). Since Bcl2 inhibits apoptosis, its low expression should correlate with highly aggressive tumor biology and resistance to hormonal/cytotoxic therapy. Interestingly high Bcl2 expression has been shown to be associated with a number of favourable prognostic factors including ER positivity, PR positivity, low histological grade, well differentiated tumors and absence of Her-2 neu and p53. Moreover, numerous studies have shown that patients with high Bcl2
expression in their tumors are more responsive to hormonal therapy and have more favourable disease-free and overall survival (Krajewski 1999).

BAG-1 is a recently identified Bcl2-binding anti apoptotic protein that may play an important role in the pathogenesis of breast cancer. BAG-1 interacts with Bcl2 and enhance the antiapoptotic activity of Bcl2 \textit{in vitro} experiments (Takyama S 1995). It also binds to hepatocyte growth factor receptor and enhances the protection from apoptosis by hepatocyte growth factor receptor (Bardelli A et al 1996). It is reported that over expression of BAG-1 resulted in sustained cell viability and proliferation, with minimal apoptosis and growth factor–independent state (Clevenger CV, Seikya 1997). In addition, a synergistic antiapoptotic effect was noted when Bcl2 and BAG-1 were cotransfected. Increased BAG-1 expression has been noted in breast cancer cell lines and breast cancer tumors, and it expression was correlated with that of Bcl2 proteins (Terada S, Takaoka A 1997). The exact mechanism by which BAG-1 cooperates with Bcl2 to suppress apoptosis is currently unknown.

p53, a proapoptotic tumor suppressor gene, located on chromosome 17 (17p13.1) encodes a nuclear phosphoprotein that plays a role as a marker in breast cancer. The function of the p53 protein is widespread and involves various aspects of the cell cycle; the protein also plays a role in the inhibition of abnormal DNA replication and facilitation of apoptosis, blocking angiogenesis through binding to various parts of the genome, stimulating or inhibiting gene expression (Alledge RM 1998, Yang Q 2001).
The role of p53 in cell cycle regulation has been extensively characterized and it is believed that this gene regulates the transition from G1 to S-phase and G2 to mitosis (Hermeking H 1997). The p53 tumor suppressor gene is the most frequently mutated and extensively studied gene associated within human cancers. Studies have shown that altered expression of p53 occurs in 0-40% of patients with ductal carcinoma in situ and approximately 50% of patients with infiltrating ductal carcinoma (Barbareschi M 1996, Tsu Tsui 2006). Further, mutant P53 is associated with resistance to chemotherapy or radiation therapy and shorter disease-free and overall survival. P53 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 1997). Therefore, the present study analyzed expression of Bcl2, BAG-1 and P53 by immunohistochemistry and evaluated their relationship to established prognosticators and their prognostic significance.

Topo II alpha is a very efficient marker for cell proliferation in breast carcinoma (Depowski PL 2000). The alpha isoform of topoisomerase is a key enzyme in DNA replication and also a target for various chemotherapeutic agents such as anthracyclines or epipodophyllotoxins.
The protein expression status of topo II alpha has been implicated in the prediction of clinical outcome and response to chemotherapy in breast cancer (Park K, Vilman K 2002, Carduso F 2004, Coon JS 2002). There are two Topo isomers in mammalian cells, alpha and beta. The Topo II alpha isomer weighs 170 kDa and the Topo II beta isomer weighs 180 kDa (Lynch BJ 1997, Austin 1993). These isomers are made from two different genes and have different tasks (Dileo 2002). The gene that is responsible for coding isomer alpha was mapped to chromosome 7q21-22 and isomer beta was located on chromosome 3q24 (Tsai 1988, Jarvinen JA 1996). Topo II alpha is a cell cycle-related enzyme that functions in the segregation of the newly replicated chromosome pair in chromosome condensations and altering DNA superhelicity. The enzyme performs these functions by cleaving and opening 1 DNA duplex, passing the second duplex through the opening and then releasing the break (Durbeq V 2004). Topo II alpha exists only in the S, G2, and M phase of the cell duplication. Immediately at the end of the mitosis, there is a decrease in Topo II alpha levels. Thus the percentage of cells that were positive for immunohistochemical staining represents the proliferating part of the lesion (Sutriou C 2003). Various studies have found that the use of Topo II alpha is a better prognostic indicator. It participates in the DNA replication process and could be a useful marker for malignant cell proliferation in many types of malignancies (Stathopoulos GP, Lee A 2000, Di Leo 2003, Holden JA 1995).
Ki67 is a nuclear protein that is tightly linked to the cell cycle. It is a marker of cell proliferation and has been used to stratify good and poor prognostic categories in invasive breast cancer. Its correlation with gene expression patterns has not been fully elucidated. Ki67 is a labile, nonhistone nuclear protein that is tightly linked to the cell cycle. It is expressed in proliferating cells during mid G1 phase, increasing in level through S and G2, and peaking in the M phase of the cell cycle. It is rapidly catabolized at the end of the M phase, and is undetectable in resting (G0 and early G1) cells (Rodriguez J W 2002). It is reported that Ki67 expression has a good relationship with growth fraction in several model systems (Petit T 2004, Helen Trihia 2002) and does not appear to be expressed during DNA repair processes. Hence, it is regarded as a marker of cell proliferation, and in invasive breast cancer, has been used to stratify patients into good and poor prognostic categories (Rodriguez J W 2002). It has also been reported to correlate with clinical response to chemotherapy (Trihia 2002, Stump J 1992). The appropriate cutoff values could distinguish between high and low proliferative activity in a clinically relevant manner using Ki67 immunohistochemistry in breast cancer, however, has not been universally established (Bouzubar N, Wrba F 1989). The relationship of Ki67 protein expression with gene expression profiles is also not fully investigated. In this study, we evaluated topo II alpha and Ki67 immunohistochemical protein detection in series of invasive breast cancers using formalin-fixed, paraffin-embedded tissue. The second aim of our study was to evaluated the expression of Topo II alpha and Ki67 antigen in breast cancer to find
Significance of molecular markers in carcinoma of breast

its prognostic significance with other prognostic factors. p53 mutations and the resulting accumulation of the protein have been extensively studied in human breast cancer with many reports suggesting an association with well known indicators of high malignant potential, such as high proliferative activity, high histological grade, and absence of estrogen and progesterone receptors (Cattoretti G et al 1988). There have been two reports that p53 protein over expression may represent an independent marker of poor prognosis in breast cancer patients (Allred et al 1998). Most of these studies, however, have concentrated on invasive ductal breast carcinomas which constitute 65-80 % of malignant breast neoplasms. Only a few cases of the less common histological types of breast cancer, some of which are known to have a relatively good prognosis, have been investigated for p53 abnormalities (Davidoff, Varley et al 1991). Based on this consideration we decided to undertake a systematic analysis of p53 mutation in breast carcinomas. PCR p53 and an immunohistochemical staining were used to assess p53 mutation and p53 protein accumulation.

Aims and Objectives

The major aims and objectives of the present study were:

1. To investigate expression of apoptosis related proteins such as Bcl2, BAG-1 and p53 in breast tumors by Immunohistochemistry and their correlation with established clinicopathologic prognostic factors and disease outcome.
2. To evaluate clinical significance of proliferation related proteins Topoisomerase II alpha and Ki67 in breast cancer patients and its correlation with clinicopathological parameters, and apoptotic markers such as Bcl2, p53, BAG-1 by Immunohistochemistry.

3. To assess correlation between the apoptotic markers (Bcl2, BAG-1, p53) and proliferative markers (TopoII alpha and Ki67) in breast cancer

4. To investigate clinical significance of mutations in
   - P53 exons 5
   - p53 exons 7
   - Bcl2

5. To evaluate correlation between the results obtained by PCR and IHC analysis
Nature always sides with the hidden flaw

Murphy’s law