DISCUSSION-SECTION 1A

Over several decades a wide variety of morphology-based and molecular based prognostic markers have been identified and validated to improve risk stratification of breast cancer. Following the original study by Slamon et al in 1987, many investigators have considered the prognostic potential of the Her-2/neu gene and protein in breast cancer. Additionally Her-2/neu (p185) had been shown to be predicative for response to certain chemotherapeutic agents (i.e doxorubicin, adriamycin) and Her-2 targeted therapies trastuzumab and lapatinib. Both morphology-based and molecular-based techniques have been used to measure Her-2/neu status in breast cancer clinical samples. A study by Ross et al (2009) have summarized results of 107 studies with 39,730 patients and observed the differences in the study conclusions may be attributed to a multitude of factors including differences in the number of patients, patient population including those receiving systemic adjuvant therapy, length of follow up, and most importantly Her-2/neu full length status determination and interpretation techniques. Of the various methods for determining Her-2/neu protein expression immunohistochemistry has been the predominant method utilized. Some portion of the prognostic significance of Her-2/neu may be due to associated expression of p95 Her-2/neu (Saez et al, 2006). However, p95 Her-2/neu a truncated form of Her-2/neu lacking the extracellular domain presumed to be the remnant of metalloprotease mediated proteolytic cleavage of the Her-2/neu ECD (Christianson et al, 1998) have shown to predict trastuzumab resistance and requires alternative or additional anti Her-2/neu therapies.
The present study evaluated two forms of Her-2/neu internal domain membranous and cytoplasmic by CB11 antibody and compared with membranous Her-2/neu external domain detected by SP3 antibody, in a series of 154 breast cancer patients. Further, the cytoplasmic form of Her-2/neu internal domain as truncated form was confirmed by double staining immunohistochemistry method using three different antibodies, in a combination of mouse monoclonal Her-2/neu internal domain (CB11) antibody with rabbit polyclonal cytokeratin antibody, mouse monoclonal Her-2/neu internal domain (CB11) antibody with rabbit monoclonal Her-2/neu external domain antibody (SP3) antibody and rabbit monoclonal Her-2/neu external domain (SP3) antibody with mouse monoclonal cytokeratin (AE1/AE3) antibody. Cytoplasmic expression of Her-2/neu internal domain was noted in 18% of the patients, membranous Her-2/neu internal domain expression in 41% of patients whereas Her-2/neu external domain expression was seen only in 19% of patients. In diagnostic histopathology, the membranous staining of Her-2/neu is considered to term tumor as Her-2/neu positive and not the cytoplasmic expression of Her-2/neu. Ricardo et al (2007) have with CB11 clone against Her-2/neu internal considered cytoplasmic staining as non specific staining and stated that SP3 clone against Her-2/neu external domain gives specific membranous staining and could be better predictor of patient’s response to trastuzumab therapy than CB11 clone. Contradictory to the numerous recent reports have shown cytoplasmic staining as truncated form of Her-2/neu and confirmed as specific staining by different methodologies (Christianson et al, 1998; Molina et al, 2002; Saez et al, 2006; Scaltriti et al, 2007). The initial study of Jose Baselga group used immunoblot method for detection of p95 Her-2/neu, a truncated form of Her-
2/neu and later on developed an immunofluorescence assay using cocktail of CB11 antibody and anti cytokeratin antibody for confirmation of cytoplasmic Her-2/neu expression as p95 Her-2/neu of and compared with findings of immunoblot method. Of the 25 tumors with p95 Her-2/neu expression detected by immunoblot, 21(84%) were positive for p95 Her-2/neu expression by immunofluorescence assay. Also, tumors that expressed only full-length p185 Her-2/neu by immunoblot did not show cytoplasmic staining of Her-2/neu by immunofluorescence (Scaltriti et al, 2007, Figure 39). Immunoblot method for p95 Her-2/neu detection may be considered as the gold standard, however it requires large amount of fresh-frozen tumor tissue and therefore immunofluorescence based assay was developed for FFPE tissue.

**Figure 39:** Detection of p95 Her-2/neu by Immunofluorescence (Adapted from Scaltriti et al, 2007)

Stained with internal internal domain antibody CB 11 and external domain antibody CBE1.

Tumor A: Shows membranous staining with both antibodies

Tumor B: Shows membrane staining with CBE1 but combined membrane and cytoplasmic staining with anti-cytokeratin antibody (yellow)
Based on this study for evaluation of truncated Her-2/neu by immunofluorescence our retrospective study adopted double staining immunohistochemistry to detect truncated Her-2/neu which is cheaper than immunofluorescence method and can be done in day to day practice along with diagnostic immunohistochemistry. A different assay, a Vera Tag assay for quantification of p95 Her-2/neu expression in FFPE tumor specimens was developed by the group of Sperinde et al (2010) that can specifically detect p95 Her-2/neu. Another study of Heriguchi et al (2010) demonstrated 34 of 1053 (4%) cases had cytoplasmic staining but lacked membranous staining with Hercep test and CB11 antibody correlated with neuroendocrine differentiation of breast carcinoma. However, TAB 250 and SV2-61V antibodies against Her-2/neu external domain showed no cytoplasmic reactivity in the same study. All these findings supports cytoplasmic staining of Her-2/neu by CB11 antibody as specific cytoplasmic staining of truncated Her-2/neu. The generation of truncated form p95 Her-2/neu could be either by alternative splicing, missense mutations, or proteolytic shedding. Proteolytic shedding of Her-2/neu from cultured tumor cells is membrane associated, has in vitro kinase activity, and is tyrosine phosphorylated and constitutively active. It has been indicated that p95 Her-2/neu has distinct biological properties in primary and metastatic breast cancer. (Christainsion et al, 1998; Molina et al 2001, Zwick, et al 2002; Bernasroune et al, 2004).

In present study, cytoplasmic positivity was observed in only those patients who showed membranous Her2/neu internal domain positivity, and a significant positive correlation observed between cytoplasmic Her-2/neu internal domain with
membranous Her-2/neu internal domain, and also with Her-2/neu external domain. A similar significant positive correlation between Her-2/neu internal domain and external domain was observed by Ricardo et al (2007) with the use of CB11 and SP3 antibodies. Cytoplasmic Her-2/neu internal domain expression was noted in 18% of patients of the present study which was comparable with the studies of Saez et al (2006), Sclariti et al (2007) and Molina et al (2002), the different studies conducted by the Jose Baselga group where p95 expression was noted in 9%, 20% and 27% of the patients respectively. It has been shown that external domain shed from tumor cells into peripheral blood that can be detected by ELISA kits. However, in this retrospective study peripheral blood was not available and therefore in cytoplasmic Her-2/neu internal domain positive group, mean and median of percentage of positive tumor cells for membranous Her-2/neu internal domain and Her-2/neu external domain was evaluated. The lower mean and median of percentage of Her-2/neu external domain (mean = 53.46, median = 225) was observed than percentage of membranous Her-2/neu internal domain (mean = 78.85, median = 240) in cytoplasmic Her-2/neu internal domain positive group indicated the shedding of Her-2/neu external domain. In some of the patients, number of cells showing membranous positivity of Her-2/neu internal domain was higher than cells showing Her-2/neu external domain positivity (Figure 40 A&B). In a study by Pallaud et al (2005) lower concentrations of Her-2/neu external domain were constantly observed with in tumors showing cytoplasmic staining. Further cytoplasmic Her-2/neu internal domain, membranous Her-2/neu internal domain, and Her-2/neu external domain expression was correlated with clinicopathological parameters, disease status and treatment offered.
incidence of cytoplasmic Her-2/neu internal domain was noted in T4 tumors, NG III tumors and medullary carcinoma in total patients and similar findings were observed along with a significant inverse correlation with ER and PR in patients with luminal A, luminal B and Her-2 positive subtypes. The study by Saez et al (2006) also observed an inverse correlation of truncated Her-2/neu internal domain with ER, PR and extent of nodal involvement. Unlike our study, Molina et al (2002) observed p95 Her-2/neu was not differentially expressed in tumors <2cms versus large tumors, but noted an increasing incidence of p95Her-2/neu with an extent of node involvement.

![Figure 40A: Higher positivity of membranous Her-2/neu internal domain](image1)

![Figure 40B: Positivity of membranous Her-2/neu external domain](image2)

Figure 40A: Higher positivity of membranous Her-2/neu internal domain

Figure 40B: Positivity of membranous Her-2/neu external domain

Regarding Her-2/neu external domain expression in total patients, its expression was found to be significantly higher in early stage patients as compared to advance stage patients. An important observation noted that only infiltrating ductal carcinoma overexpressed membranous Her-2/neu external domain whereas none of the lobular or medullary carcinoma showed its presence. Also, a trend of higher expression of membranous Her-2/neu external domain was observed in NG III tumors and HG III.
tumors. In luminal A, luminal B and Her-2 positive subtypes, a similar trend was seen with disease stage, histologic type, HG and NG. Additionally, a higher incidence of membranous Her-2/neu external domain was noted in LN positive patients and an inverse trend with ER and PR status. Like us, the group of Pallaud et al (2005) observed higher incidence of Her-2/neu external domain positivity in NG III tumors and in intraductal component, and also observed a significant correlation between Her-2/neu detected by IHC with serum Her-2/neu levels by ELISA.

Regarding membranous Her-2/neu internal domain expression in total patients, an inverse correlation was seen with LN status and a trend of higher incidence with advancement of NG and BR score of the tumor. Further, a higher incidence was in medullary carcinoma (83%) was seen than infiltrating ductal carcinoma (38%) and lobular carcinoma (25%). In patients with luminal A, luminal B and Her-2 positive subtypes, similar expression of membranous Her-2/neu internal domain was noted in LN negative and LN positive patients and a significant inverse correlation was observed with ER and PR status. Similar to this finding Ristimaki et al (2002) also observed lower rate of Her-2/neu gene amplification in invasive lobular carcinoma compared with invasive ductal carcinoma. Her-2/neu amplification also strongly correlated with tumor grade in both invasive ductal carcinoma and invasive lobular carcinoma, In the study by Rosenthal et al (2002), only one of 73 grade I invasive ductal carcinoma cases and one of 67 low-grade classic invasive lobular carcinoma cases showed Her-2/neu amplification detected by FISH. Further in accordance with our findings Castori et al (2001), Tsutsui et al (2002), Taucher et al (2003) reported that Her-2/neu overexpressing breast cancer had lower levels of ER and PR protein.
expression which could be due to activation of the PI3K-AKT-mTOR pathway. Similarly, in our study a significant inverse correlation of ER and PR status with Her-2/neu expression could be due activation of the PI3K-AKT-mTOR pathway, and a significant positive correlation has been observed between Her-2/neu protein expression and mTOR expressions which has been described in detail in the next section.

The survival analysis was done in patients with luminal A, luminal B and Her-2 positive patients by excluding triple negative patients. The higher incidence of disease relapse was observed with triple negative subtype (49%) followed by Her-2/neu positive (21%), luminal A (15%) and luminal B (14%) subtypes. This observation of association of triple negative subtype with disease aggressiveness was consistent with findings of findings of Smid et al (2008), Van de et al (2002) and Foulkes et al (2010).

In univariate survival analysis in patients with luminal A, luminal B and Her-2 positive subtypes membranous Her-2/neu internal domain and external domain expression did not discriminate patients with better or worse DFS or OS. The results were in accordance with that of some the studies where in Her-2/neu expression was not of univariate significance (Ko et al, 2007; Badve et al, 2007; Von Minckwiz et al, 2008; Bektas et al, 2008). However, the results were in discordance with many of the study groups where Her-2/neu positive patients were associated with reduced survival evaluated by immunohistochemistry on paraffin embedded tissue sections (Wright et al, 1989; Paik et al 1990; Battifora et al, 1991; McCann et al, 1991; Winstanley et al, 1991; Molina et al 1992; Press et al, 1993; Muss et al, 1994; Quenel et al, 1995; Sunblad et al, 1996; Querzoli et al, 1998; Sjogren et al, 1998; Kakar et al, 2000;

Interestingly, patients with cytoplasmic Her-2/neu internal domain positivity showed significantly reduced DFS and OS in univariate analysis and reduced DFS in multivariate analysis as compared to patients without cytoplasmic Her-2/neu internal domain expression. Further in Her 2 positive group, cytoplasmic Her-2/neu internal domain overexpression was associated with reduced DFS and OS. Similar to our findings Saez et al (2006) also have reported p95 Her-2 predicts worse outcome in Her-2/neu positive breast cancer. The association of overexpression of p95 Her-2/neu with reduced disease-free survival could also be related to its biological properties which are distinct from p185Her-2/neu, such as increased signaling activity and enhanced oncogenic potential (Molina et al, 2002). Singhai et al (2011) also found decreased survival in patients with elevated serum Her-2/neu external domain, a truncated form. The other two studies evaluated p95 Her-2/neu has correlated mainly with clinical outcome to identify response to anti-Her-2/neu therapies (Sclariti et al 2007; Sperinde et al, 2010).

Her-2/neu is currently considered not only as a marker of poor prognosis in node-positive patients, but also as a useful determinant of susceptibility to chemotherapy. With the availability of trastuzumab (Herceptin), a humanized monoclonal antibody against the extracellular portion of the Her-2/neu receptor protein, there has been a growing clinical demand for accurate determination of Her-2/neu status, as the presence of gene amplification or protein overexpression is by far the major criterion
for trastuzumab eligibility. Patients with Her-2/neu amplification or over expression are eligible for treatment with trastuzumab, a monoclonal antibody directed against Her-2/neu is being used in metastatic breast cancer and is also indicated in adjuvant therapy in primary breast cancer. Trastuzumab targets Her-2/neu receptor, binds to external domain and cause degradation of it, thereby inhibits signal transduction pathway. p95 has often been cited as a likely determinant of trastuzumab resistance (Ross et al, 2009) because it lacks the Her-2/neu external domain, which is the binding domain for trastuzumab. In the present study only two patients received trastuzumab due to affordability of treatment cost and one of two expressed cytoplasmic Her-2/neu developed liver metastasis within 18 months while one patient with cytoplasmic Her-2/neu internal domain negativity is disease free till date for 60 months. In the study of Sclatriti et al (2007) a series of patients with Her-2/neu positive advance breast cancer who were treated with trastuzumab, the presence of p95 Her-2/neu was associated with clinical resistance to trastuzumab, whereas tumors expressing only full-length receptor exhibited a high response rate to trastuzumab. Within a cohort of trastuzumab treated metastatic breast cancer high levels of p95 Her-2/neu determined by Vera Tag Her-2/neu assay were found to correlate with shorter progression free survival and overall survival (Sperinde et al, 2010). There are several other mechanisms responsible for trastuzumab resistance such as PTEN inactivation or loss and activation of IGF-IR. The proposed mechanism for trastuzumab action include inhibition of Her-2/neu shedding, inhibition of PI3K-AKT pathway, antibody dependent cellular toxicity and inhibition of tumor angiogenesis. Also an alternative strategy to reverse trastuzumab resistance would be a combined therapy with a metalloprotease
inhibitor and trastuzumab. The monoclonal antibody used to detect truncated form of Her-2/neu by immunohistochemistry specifically recognizes the 611 CTF only the truncated form and not the total Her-2/neu. Their findings were contradictory to these findings Loibl et al (2011) observed better response to neoadjuvant trastuzumab containing treatment in primary Her-2/neu positive breast cancer.

In relation to treatment offered the number of patients treated with surgery followed by CMF alone and CMF with adjuvant therapy was too small to draw any statistical conclusion and therefore the treatment efficacy was evaluated only in patients treated with surgery followed by FAC and adjuvant treatment.

Her-2/neu internal domain and external domain positive patients showed a better DFS when treated with surgery followed by FAC and a better OS when treated with surgery followed FAC +RT and FAC alone than with addition of TMX in these groups indicating that Her-2/neu positivity was associated with TMX resistance. In cytoplasmic Her-2/neu internal domain positive patients treated with FAC+RT showed better DFS and OS as compared to other treatment groups. Some studies have shown benefits of anthracycline based chemotherapy in Her-2/neu positive tumors (Muss et al, 1994; Paik et al, 1998; Thor et al, 1998). In a large trial including 1572 patients with node positive early breast cancer who receiving high, moderate and low doses of cyclophosphamide, doxorubicin and fluoracil and 442 random samples were assessed of Her-2/neu expression where patients with high Her-2/neu (>50%) expression who received high-dose chemotherapy had a significantly longer disease-free survival and overall survival as compared to patients without Her-2/neu expression (<50%) (Muss et al, 994). Some studies have correlated membranous Her-2/neu internal domain
expression with hormonal treatment. Experimental and clinical evidence have suggested an association between Her-2/neu overexpression and resistance to endocrine therapies. In a study of 516 patients clinical outcome after 5-10 years following tamoxifen based adjuvant therapy was compared between hormone receptor positive/Her-2/neu negative group and hormone receptor positive/Her-2/neu positive group and concluded that DFS and OS in patients receiving TMX alone or in combination with chemotherapy was significantly lower in and hormone receptor positive/Her-2/neu positive group compared to hormone receptor positive/Her-2/neu negative group (Gago et al, 2006).

Some studies have correlated Her-2/neu external domain expression with treatment. Leitzel et al (1995) Colomer et al (2004) demonstrated elevated levels of Her-2/neu external domain adversely affected the efficacy of chemotherapy associated with a lower response rate, decreased time to progression and shortened survival. James et al (2008) and Singhai et al (2011) have observed hormonal resistance in patients with elevated Her-2/neu like us. Yamuchi et al (1997) obtained a similar result in response to an antiestrogen droloxifene as first-line endocrine therapy; however, specimens from only 94 (25%) of 369 patients who were enrolled on to the therapeutic study were available for the Her-2/neu analysis. A different result was obtained by Willsher et al (1996) using a serum enzyme-linked immunosorbent assay (ELISA) in 77 patients with stage III or IV breast cancer. No significant correlation was observed between pretreatment serum Her-2/neu levels and response to first-line tamoxifen therapy. Her-2/neu overexpression is also associated with enhanced phosphorylation of both serine and tyrosine residues in the ER (Pietras et al, 1995; Kato et al 1995). Both alterations
may be significant for ligand independent ER activation with loss of the inhibitory effect of tamoxifen on ER-mediated transcription, providing available mechanism to explain the association of Her-2/neu with tamoxifen resistance (Lipton et al, 2002). Also another mechanism for tamoxifen resistance could be that activation of growth factor receptor receptors such as Her-2/neu can result in direct phosphorylation and activation of ER in an estrogen independent manner. This being a retrospective study we could only evaluate the effect of the treatment offered on patients.

However during the last few years the new drug lapatinib, dual inhibitor of Her-2/neu and EGFR has been approved by FDA in 2007 has come in for use in previously treated advanced metastatic breast cancer. Lapatinib, has exhibited clinical efficacy when combined with capecitabine in a randomized phase III trial of patients with HER-2 over-expressing breast cancer. Lapatinib has also shown clinical activity as a monotherapy in women with heavily pretreated HER-2 overexpressing invasive breast cancer which overexpressed Her-2/neu in combination with Capecitabine (Spector et al, 2007). Combination proliferation assays were performed in two HER-2 positive breast cancer cell lines, SKBR-3 and BT-474. Trastuzumab combined with lapatinib was also tested in BT-474 xenografts. Trastuzumab combined with lapatinib further enhanced apoptosis induction suggesting that inhibition of both EGFR and HER-2 may be required to induce apoptosis (ODonovan et al, 2011). Other Her-2/neu targeting agents are still being developed in preclinical testing stages include Pertuzumab (Albanell et al, 2003) which binds Her-2 and sterically hinders the recruitment of Her-2/neu into heterodimers and Ertumaxomab, a specific antibody targeting Her-2/neu and CD 3 (Kiewe et al, 2006).
Additionally, Her-2/neu expression when studied in the benign breast diseases, none of them showed cytoplasmic Her-2/neu internal domain expression by CB11 antibody and Her-2/neu external domain expression by SP3 antibody. However, 13% of patients with fibroadenoma and 27% of patients with fibrocystic disease exhibited membranous Her-2/neu staining which was significantly lower than expressed in breast cancer patients. Low-level Her-2/neu overexpression has been identified in benign breast disease biopsies and is associated with a greater risk for subsequent invasive breast cancer (Stark et al, 2000). The study by Allred et al (1992) showed that benign proliferative lesions and atypical hyperplasia do not generally show amplification or overexpression of Her-2/neu. In a study by Latta et al (2002) Her-2/neu was evaluated in pure atypical ductal hyperplasia wherein no Her-2/neu positivity was observed, however they concluded that Her-2/neu alterations are undoubtedly important in ductal carcinoma in situ and are usually maintained in an adjacent invasive ductal carcinoma. However, in our study, the follow up after surgery was not available for patients with benign breast disease and therefore it is not known if any of the Her-2/neu positive patient developed malignancy.

In summary, cytoplasmic Her-2/neu internal domain expression, a truncated form identifies an aggressive phenotype of breast cancer and its determination is of utmost importance along with membranous Her-2/neu internal domain and external domain evaluation. Double staining immunohistochemistry technique may provide a unique tool for evaluation of truncated Her-2/neu in breast tumors till the antibody to detect p95 Her-2/neu becomes commercially available as it is cheaper than the method
available for determination of truncated Her-2/neu and can be routinely performed in the laboratory along with routine Her-2/neu analysis.
The single nucleotide polymorphism (SNP) in the human her-2/neu gene was identified in the transmembrane coding region of the gene at codon 655, encoding either isoleucine (Ile) or Valine (Val, Papewalis et al, 1991). Changing the existing isoleucine (Ile ATC) to Valine (Val GTC) at codon 655 suggest an increased dimerisation, autophosphorylation of Her-2 and tyrosine kinase activity which may cause the transformation of cells (Nakajima, 1999). Some of the reports have revealed presence of development of Ile655Val polymorphism is associated with increased risk of breast cancer risk (Cox et al, 2006; Frank et al 2005; Lee et al, 2008; Tommasi et al; 2007, Xie et al, 2000). On the other hand others have shown that this correlation is controversial (Akisik et al, 2004; An et al 2005; Benusigilo et al, 2005; Kalemi et al, 2005; Naidu et al, 2008; Kallel et al, 2010; Kara et al, 2010). One reason for these contradictory results might be the sustained differences in genetic polymorphism in Her-2/neu codon 655 between ethical groups. In a study by Papadopoulou E et al (2007) Val/Val or Val/Ile genotype was associated increased breast cancer risk than Ile/Ile in Christian population and no such significant association was observed in Muslim population of Thrace. This inconsistent association between SNP and breast cancer risk across these two different ethnic groups and supported that polymorphism varies according to racial decent.

In the present study, SNP of Her-2/neu gene had been studied at codon 655 in FFPE extracted DNA of 127 out of 154 breast cancer patients. In the remaining 27 patients
SNP of Her-2/neu gene was not analyzed as housing keeping gene B-actin was not expressed.

Of these 127 patients, 20% (25/127) patients showed expression of Val/Val and Ile/Ile genotype each and 60% (75/127) of the patients showed Val/Ile heterozygous genotype. The frequency of the incidence of Val/Ile heterozygous genotype was higher in breast cancer patients in the present study. However, majority of the studies have observed higher frequency of Ile/Ile genotype than Val/Ile and Val/Val genotypes. Cox. et al (2005) in a series of 1271 breast cancer patients observed Ile/Ile genotype in 60%, followed Val/Ile heterozygous genotype in 35.5%, and Val/Val genotype in 4.5%. Similarly Nelson et al (2005) demonstrated higher incidence of Ile/Ile genotype (58.2%) followed Val/Ile heterozygous genotype (36%) and Val/Val genotype (5%). Zubor et al (2006) in 47 Caucasian population observed higher frequency of Ile/Ile genotype (47%) and Val/Ile heterozygous genotype in (47%) than Val/Val genotype in (6%). Kallel et al (2010) observed Ile/Ile genotype in 87%, Val/Ile heterozygous genotype in 9% and Val/Val genotype expression in 3% in patients of Tunisia. In these studies Ile/Ile genotype was observed in range of 47% to 80%, Val/Ile genotype was 9% to 47% and Val/Val genotype was 3% to 6%. Compared to these studies a higher incidence of Val/Val and Val/Ile genotypes and lower incidence of Ile/Ile genotype was observed in the present study. The observed difference could be of geographic and ethnic variations (Parkin et al, 1997). Further, there was no data on Her-2/neu SNP available from India. Majority of the studies have determined Her-2/neu protein by IHC and gene by FISH. The lower frequency
of the Val/Val genotype in continental Africans corresponds with lower incidence and lower risk of breast cancer in African women compared with Caucasian women. However, meta analysis of 22 studies by Yanlei et al (2011) indicated a modest association between the Her-2/neu Ile polymorphism and Asian population, suggested that difference in genetic background, environment do not influence the Her-2/neu Ile655Val polymorphism and breast cancer susceptibility. Contrary to that, Tao et al (2009) indicated that SNP at Her-2 codon 655 could be considered as a susceptibility biomarker for Asian women age 45 years or younger.

In the present study, the uniform distribution was observed in genotype frequencies when correlated with clinical parameters such as age and menopausal status. Whereas studies of Xie et al (2000) and Montgomery et al (2003) and Tao et al (2009) demonstrated that women with age less than 40 years with homozygosity for valine allele (Val/Val genotype) had an increased risk of early onset of breast cancer. Contrary to that, Tao et al (2009) indicated that SNP at HER2 codon 655 could be considered as a susceptibility biomarker for breast cancer for Asian women of age 45 years or younger. In postmenopausal women with age > 45 years Cowdin et al (2001) and Xie et al (2000) demonstrated that Ile655Val variant confers a modest increase in breast cancer risk among women for all stages of disease. Moreover, women with germline Val genotypes are more likely to develop localized disease and less likely or slower to progress to high stage breast cancer than women with Ile genotype. Unlike our study observed increasing incidence of Val/Val genotype with disease advancement. Higher frequency of heterozygotes for Val allele was
observed among premenopausal breast cancer patients and patients with, positive for HER2/neu status and advanced stage in a study by Surekha et al (2009). Satiroglu et al (2006) observed similar association of Ile/Val genotype with stage IV gastric carcinoma. A higher frequency of Val allele was demonstrated by Wu Ch et al (2009) in cases having nodal metastasis and tumor recurrence. In a study of Papadopoulou E et al (2007) on breast cancer, invasive carcinomas, low differentiation tumors, advanced stage, positive lymph nodes, high number of lymph nodes and HER-2/neu overexpression were more frequent in patients with allele Val than those with allele Ile. The association of Val/Val Her-2/neu genotype with clinicopathological characteristics and Her-2/neu expression indicated its possible implication on more aggressive phenotype.

Interestingly all patients with triple negative subtype, exhibited Her-2/neu gene expression with an incidence of 64% of Val/Ile and 18% each of Val/Val and Ile/Ile genotypes. Among molecular subtypes luminal A had higher frequency of Val/Val genotype (40%) and lower frequency of Val/Ile genotype than luminal B, Her-2 positive and triple negative subtypes.

The incidence of Val/Val genotype was found to be low in patients with expression of membranous Her-2/neu internal domain, cytoplasmic Her-2/neu internal domain and membranous Her-2/neu external domain positive group. When the correlation was evaluated in luminal A, luminal B and Her-2 positive subtype the incidence of Val/Val genotype was significantly low in patients with expression of membranous Her-2/neu
internal than without expression of membranous Her-2/neu internal domain. The observation of lower incidence of Val/Val genotype in Her-2/neu protein negative tumors remained unexplained. It has been hypothesized by Cowdin et al. (2001) that germ-line variant initially increases cellular proliferation while subsequently decreasing the likelihood that Her-2 gene will undergo amplification or protein overexpression. However, over expression of Her-2/neu in large number of cases indicates that activation of this gene is an important step in breast carcinogenesis.

With disease status, breast cancer patients with Ile/Ile, Val/Ile and Val/Val genotypes showed similar incidence of relapse and death. However, Val allele expression in oral squamous cell carcinoma was associated with increased risk of tumor recurrence and with poorer recurrence free survival of patients (Wu CH et al., 2009). Also, in a study by Papadopoulou E et al. (2010) suggested that TNF-alpha remained as an independent prognostic factor of worse overall survival however in combination with Val/Val genotype predicted a worse prognosis than high TNF-alpha alone.

In relation to treatment the number of patients expressing Val/Val genotype and Ile/Ile genotype the number was too small in each treatment group to draw any statistical conclusion. In patients with Val/Ile heterozygous genotype patients treated with FAC showed reduced DFS than addition of TMX in the treatment arm. Like the Her-2/neu protein expression heterozygous Val/Ile genotype may be associated with TMX resistance.
The study also compared Her-2/neu SNP genotypes with benign breast diseases. The incidence of Ile/Ile genotype was higher (54%) than Val/Ile genotype (17%) and Val/Val genotype (29%). Of them, fibroadenoma had similar distribution of these genotypes like breast cancer, but fibrocystic disease had two times higher incidence of Ile/Ile genotype than fibroadenoma and three times than breast carcinoma. However, incidence of Val/Val genotype of fibrocystic disease is similar to fibroadenoma and breast carcinoma. However, incidence of Val/Val genotype of fibrocystic disease is similar to fibroadenoma and breast carcinoma focused on predisposition on Her-2/neu SNP in studies available for Val/Val genotype. Zubor et al (2008) demonstrated similar incidence of Val/Ile genotype (35%) and Val/Val genotype (10%) in fibroadenoma and indicated that Her-2/neu polymorphism may play some role in the etiology of fibroadenoma formation. The frequency of Her-2/neu SNP genotypes in fibroadenoma and breast carcinoma as well as Val/Val genotype expression in fibrocystic disease supports, not only its involvement in breast cancer but it also suggests a role in benign breast disease.

The results suggest that Her Ile655Val polymorphism especially in homozygous form might play a role in etiology of breast carcinoma and benign breast disease.
DISCUSSION-SECTION II

The human tumors are characterized by their great heterogeneity and histopathologic variable. In addition to the variability of the histopathologic subtypes, molecular study of the tumors is even more complex. Tumorigenesis occurs due to synergistic interactions from a complex of signal transduction processes, including multiple oncoproteins and tumor suppressors such as Ras, Myc, PI3K/AKT/mTOR, Her-2/neu, p53 and PTEN. The mutation in the oncogene, that translocates a abnormal signaling protein tend to promote formation of its active state, while suppressing its ability to be inactivated. Conversely, tumor suppressor mutations can inactivate protein that normally attenuate signaling proteins. Both the type of mutations can lead to the activation of pathways that promote cell growth, survival, proliferation and invasion. The Her-2/neu signaling pathway which has a very important role in breast cancer with alteration is involved in several cellular process associated with malignant behavior including cell proliferation, differentiation, apoptosis, migration and migration via signal transduction pathway and the major signal transduction cascade include the PTEN/PI3K/AKT/mTOR pathway and the RAS/MEK/ERK pathways. Many components of this pathway have been described as genetically altered in breast cancer which include mutations causing loss of PTEN function or oncogenic activation of PI3K/AKT. Novel therapeutics targeting different components of this pathway are demonstrating efficacy in an array of human cancer types in preclinical studies, and these drugs are being carried forward into clinical trials. With the detailed evaluation of Her-2/neu expression in the previous section we have further tried to explore the PTEN/PI3K/AKT/mTOR pathway by evaluating its molecules.
PTEN methylation was evaluated in 91 breast cancer patients. Of these none of the patients showed PTEN methylation. The old FFPE blocks used in the study was one of the possible reasons why PTEN methylation was not observed in any of the patients. Several studies have evaluated PTEN methylation using methylation specific PCR which have been reported at a variable rate in the range of 6% to 70% and have also described PTEN methylation as an important change in breast cancer (Garcia et al, 2004; Khan et al, 2004; Sadeq et al, 2011; Gallardo A, 2012). However, Tserga et al (2012) demonstrated PTEN methylation in only 6% of patients with breast cancer which was close to our findings. In an Indian study by Shetty et al (2011) promoter methylation was evaluated by Methylation Specific Restriction Assay from formalin fixed tissue indicated that only 20% of the tumors showed PTEN methylation.

Further, clinical significance of protein expression of PTEN, AKT and mTOR was evaluated. PTEN protein was immunolocalised in 60% of the patients, whereas its loss was observed in 40% of patients. Our finding was similar to many studies which used immunohistochemistry method where PTEN loss was present in about one third of breast cancer patients which ranged from 15% to 48% (Feilotter et al, 1999; Bose et al, 1999; Perren et al, 1999; Depowoski et al 2000, Bose et al, 2002; Lee et al, 2004; Tsutsui et al, 2005; Wang et al, 2011; Gallardo et al, 2012). In relation to pathological parameters of our study, a significant higher PTEN loss was observed in patients with high grade tumors, larger tumor size and with lymphatic permeation than their counterparts. Similarly, Janssen et al (2007) observed PTEN loss in high grade tumors whereas Tenorio et al (2007) reported loss of PTEN with small tumor size which
remained unexplained. Our observation of PTEN expression with ER and PR status was in accordance with Engin et al (2006) where no significant association was observed with ER and PR status and in discordance with Parren et al (1999) and Janssen et al (2007) where PTEN loss was seen in ER negative breast tumors. A study by Deepowoski et al (2001) showed loss of PTEN expression predicted lymph node metastasis and correlated with receptor negativity but did not correlate with stage, tumor grade, disease recurrence, or loss of progesterone receptor. However, Noh et al (2008) observed no association of PTEN loss with any of the clinicopathological parameter. The loss of PTEN expression is considered to reflect the loss of PTEN function induced by a variety of mechanisms such as homozygous deletion, nonsense mutation with LOH, and promoter methylation (Mcmenamin et al, 1999).

With metastatic site in the present study, PTEN loss was observed in 60% patients with brain metastasis which was higher than other metastatic site. In other metastatic sites except ovarian metastasis higher PTEN loss was noted in more than 30% of the patients with liver, bone or lung metastasis and local recurrence. Wikman et al (2012) observed higher PTEN loss in primary tumors with brain relapse (59%) compared to primary tumors from patients without relapse (18%) or relapse other than brain tumors (12%), suggesting that brain metastasis often show very complex genomic aberration patterns and further suggesting a potential role loss of PTEN in development of brain metastasis.
With molecular subtypes, PTEN loss was higher in luminal A subtype (58%) followed by Her-2 positive (48%) and triple negative subtype (34%). The lowest PTEN loss was observed in luminal B subtype (24%). Lopez et al (2010) reported higher PTEN loss in basal like phenotype than luminal phenotype.

Further, univariate survival analysis indicated no difference in DFS or OS between two PTEN subgroups of PTEN loss and PTEN expression. However, in multivariate analysis with all conventional parameters, Her-2/neu protein and SNP and PTEN, AKT and mTOR, PTEN loss entered at step one and was found to be an independent prognosticator for disease free survival. The results of univariate analysis were in accordance with several studies of (Panigrahi et al, 2004, Slipveic et al, 2005; Tenorio et al 2007; Gori et al 2009; Yenemori et al 2009) where PTEN loss was not associated with poor prognosis. The results were in discordance with many studies (Fabi et al, 2002; Terakawa et al, 2003; Chung et al, 2004; Yang J et al, 2010; Milovanovic et al, 2011) where PTEN loss was associated with either tumor progression or poor prognosis.

Regarding AKT expression, two types of staining patterns were observed where nuclear AKT expression was observed in 40% and cytoplasmic AKT in 30% of the patients. Different types of AKT expression evaluated by immunohistochemistry which included phosphorylated AKT and AKT isoforms. Phosphorylated AKT is active form of AKT and regulates cellular function through the phosphorylation of a series of downstream players. In this study commercially available pan-AKT antibody was used which detects all the isoforms of AKT (AKT1, AKT2, AKT3). Santi et al (2010)
evaluated different isoforms and observed that AKT1 was mainly localized in cytoplasm, AKT2 mainly localized at mitochondria and AKT3 in the nucleus.

A variable range of AKT (Tenorio et al, 2002; Bose et al, 2006; Ackakant et al, 2008; Lee et al, 2012) and phosphorylated AKT (Tenorio et al, 2002; Zhou et al, 2004; Tokunaga et al, 2006; Andre et al, 2007; Gori et al, 2009; An J et al, 2010; Aleskandraranany et al, 2011; Adamo et al, 2011, Gallordo et al, 2012) expression have been demonstrated by various studies in the range of 15% to 76%. In our study higher nuclear AKT expression was observed in low grade tumors, in patients without lymphatic permeation and ER and PR positive tumors. This nuclear AKT showed an inverse trend in comparison with PTEN loss. The expression of cytoplasmic AKT was found to be higher in advance stage tumors, patients without lymphatic permeation, moderate nuclear grade tumors and intermediate BR score tumors. Zhou et al (2004) and Aleskandraranany et al (2011) reported a positive correlation between AKT and ER status. In a study by An J et al (2010) the expression of pAKT was associated with expression of estrogen receptors, progesterone receptors, lymph node metastasis and disease stage. However, Tokunaga et al (2006) reported an inverse correlation between pAKT and PR. A positive correlation between ER and AKT constituted a powerful evidence of ER mediated activation of AKT and Bose et al (2006) suggested that Her-2/neu may play a role in mediating the effects of ER and AKT rather than acting as an independent upstream regulator of the latter. Schmitz et al (2004) reported pAkt, in 57% breast cancers and showed mainly weak cytoplasmic immunostaining and 7% cases exhibited a strong cytoplasmic as well as nuclear
immunostaining. Further, no correlation was found between pAKT and tumor size, histologic grade and receptor status. Tenorio et al (2002) did not observe any significant correlation with any of the prognostic factors as tumor size or disease stage.

The results of the present study indicated association of nuclear AKT with favorable prognosticators and cytoplasmic AKT expression with unfavorable prognosticators. It has been observed that AKT in the cytoplasm translocates to the inner leaflet of plasma membrane, where it is activated by phosphorylation. Santi et al (2010) have shown that AKT1 localized mainly in the cytoplasm where a substantial portion was detected in plasma membrane. AKT2 localized mainly at mitochondria which was an active form. AKT3 is localized in the nucleus and stated that AKT3 is the only isoform present in the nucleus. The cytoplamic AKT in our study might be AKT1 and nuclear AKT might be AKT3 and this was further confirmed by the experiment of Santi et al (2010) where both AKT1 and AKT2 did not translocate into the nucleus on administration of ionizing radiation of EGF stimulation. They also observed that ablation of one or two AKT isoforms by siRNA did not significantly alter the subcellular localization of remaining AKT isoform suggesting subcellular localization of the AKT isoforms is unaffected by the presence or absence of other AKT isoform and the major function of an AKT isoform does not generally compensate for that of other AKT isoforms. Our study strengthens these findings where we observed different correlation, nuclear AKT was associated with favorable and cytoplasmic AKT with unfavorable prognostic factors. Also a positive correlation was observed between
PTEN and nuclear AKT only and not with cytoplasmic AKT suggesting a unique role of each of the isoform. This was further confirmed as a positive correlation was observed between cytoplasmic AKT and mTOR and cytoplasmic AKT and Her-2/neu suggesting the role of cytoplasmic AKT in mTOR activation.

With molecular subtypes, a trend of high nuclear and cytoplasmic AKT expression was observed in luminal tumors as compared to triple negative tumors. The results were similar to that of Aleskandrarany et al (2011) who showed higher proportion of luminal tumors positive for pAKT as compared to triple negative tumors. However, Moulder (2010) in a review stated triple negative tumors demonstrate higher levels of AKT activation compared with non triple negative breast cancers. This suggested a possible role for targeting the PI3K pathway for the treatment of this subset of aggressive cancers.

In univariate survival analysis, no significant difference in DFS or OS was observed in patients with and without AKT expression. Many of the studies have described a decreased survival in AKT positive patients (Tenori et al, 2002; Schmitz et al, 2004; Zhou et al, 2004; Cicenas et al, 2005; Gori et al, 2009). However, Aleskandrarany et al (2011) reported that AKT overexpression was not associated with metastasis-free survival (MFS) and indicated that although AKT is an oncogene correlated with poor prognostic variables, it was not a prognostic marker. Andre et al (2007) found no association of pAKT with survival. An J et al (2010) and Badve et al (2010) reported in univariate analysis expression that of pAkt was associated with longer disease-free survival. Though expression of AKT in the study was not associated with favorable
prognosis it was associated with favourable prognostic factors whereas cytoplasmic AKT was associated with unfavorable prognostic factors.

Further, mTOR expression was observed in 28% of the patients. Our results were similar to that of Bose et al (2006) wherein its expression was observed in 32% of patients with DCIS and 24% of patients with IDC. Further, mTOR expression has been observed in wide range from 23% to 98% by various study groups (Chan et al, 2009; Mutee et al, 2010; An J et al, 2010; Bakarokos et al, 2010; Gallardo et al, 2012; Lee et al 2012). Noh et al (2008) reported activation S6K1 a downstream regulator of mTOR in 36% of the breast cancers, whereas Rojo et al (2007) reported 4e-binding protein another downstream regulator of mTOR in 81% of the cases. In the present study the expression of mTOR was found to be high in T4 tumors, stage IV disease, high grade tumors, medullary carcinoma with no relation with nodal status and histological grade. It was also found to be high in patients who developed brain metastasis. Lee et al (2012) have reported higher mTOR expression with well to moderately differentiated tumors. Bakarokos et al (2010) showed a positive association of mTOR with lymph node status. An J et al (2010) observed pmTOR expression associated with expression of ER, PR, tumor size and stage. Mutee et al (2010) demonstrated no significant association of mTOR with age, disease stage, ER, PR and Her-2/neu status. Rojo et al (2007) found p4EBP1 was mainly expressed in poorly differentiated tumors, metastatic lymph node and locoregional recurrences.

With molecular subtype, high mTOR expression was noted in patients with Her-2 positive subtype and was low in triple negative subtype. This strengthens the findings
that Her-2/neu mediates PI3K/AKT/mTOR pathway. Contrary to our findings with Walsh et al (2012) studied pmTOR was found more frequent in triple negative than non triple negative breast cancers and suggested that mTOR may play a more important role in the progression of triple negative breast cancers compared to non triple negative breast cancers and may be a target for treatment of triple negative breast cancers.

In univariate survival analysis, no significant difference in DFS and OS was observed in patients with or without mTOR expression. This result was dissimilar with several studies where the mTOR positive tumors showed shorter disease free survival or overall survival (deGraffenried et al, 2004; Zhou et al, 2004; Bose et al, 2006; Bakarokos et al 2010). Also in another study by Rojo et al (2007) 4e-binding protein an another downstream regulator of mTOR was associated with locoregional recurrence. However, Lee et al (2012) have shown five years overall survival rate was significantly higher for the patients whose tumors overexpressed PTEN, pAKT1 and pmTOR and suggested AKT to be associated with favorable prognosis along with its downstream regulator which also correlated with tumors of low grade and without metastasis.

Additionally the expression of PTEN, AKT and mTOR was correlated with treatment offered. In patients with loss of PTEN, nuclear or cytoplasmic AKT expression and mTOR expression had a better response when treated with surgery followed by either FAC or FAC+RT as compared to those who were treated with addition of TMX in these treatment arms. Response to tamoxifen has been shown to be directly related to ER levels, low ER levels in these tumors cause a lesser effect of tamoxifen. In our study
68% patients treated with surgery followed by FAC+TMX or FAC+TMX+RT had low ER levels. Studies have shown correlation between tamoxifen resistance and tumors with high EGFR and HER-2 levels, and the crosstalk between membranous ER and growth factors seems responsible for the agonist activity of tamoxifen (Cui et al, 2005). Also high AKT levels are seem to be associated with TMX resistance (Kirkegaard et al, 2005; Tokunaga et al 2006). Stal et al (2003) reported that patients with a negative status of AKT (no overexpression of Akt1, Akt2 or pAkt) showed significant benefit from tamoxifen. However, in our study this trend was not seen.

A positive correlation of PTEN with nuclear AKT and total AKT was observed. No such correlation was observed with cytoplasmic AKT. However, loss of PTEN with AKT was observed by Mutee et al (2012). Similar to our findings Panigrahi et al (2004) failed to detect the inverse relationship between PTEN and AKT and similar to our findings have even reported a positive correlation. Also a positive correlation was observed in the expression scores of PTEN and pAKT by Mundhenk et al 2011 in bladder cancer. This can be accounted for by the fact that several other proteins in addition to PTEN are involved in AKT activation. AKT activation occurs independently of PTEN loss or retention, resulting in PTEN-independent constitutive activation of the PI3K/AKT pathway (Zhou et al, 2004; Panigrahi et al 2004). The reason is that each of the AKT isoform may be activated in a unique way suggesting that PTEN might be playing a role in the acitvation of one isoform and not the other. Since, the phosphorylation of all three AKT isoforms differ only by one amino acid residue the examination of the isoform specific function may be difficult. Thus our study supports
the idea of a complex relationship between PTEN, AKT and their regulators and effectors.

A positive correlation between cytoplasmic AKT and Her-2/neu protein expression further strengthens that the activation of AKT signaling pathway may occur via alternative pathways that are independent of PTEN, mediated via Her-2/neu. The findings were similar to that of Zhou et al (2004) who postulated that AKT activation occurs through ER and Her-2/neu and it was further confirmed in our study where the activation of AKT was independent of PTEN. Yet, the conventional model of the AKT/mTOR/S6KB2/4EBP1 pathway, except for the upstream effect of PTEN, still seems to be valid if studied with each of the AKT isoform.

Furthermore, no correlation of PTEN protein expression was observed with Her-2/neu protein expression, unlike Tenorio et al (2007) where PTEN correlated with low Her-2/neu status. However, when correlated with Her-2/neu polymorphism the incidence of PTEN loss was found to be significantly higher in patients with Val/Val genotype as compared to other genotypes further suggesting their role in aggressiveness, of the disease.

Cytoplasmic AKT also showed a positive correlation with Her-2/neu protein expression and not with nuclear AKT which was similar to the findings of Kirkegard et al (2005). A correlation of AKT with Her-2/neu overexpression has been observed by Stal et al (2003), Schmitz et al (2004) and Tokunaga et al (2006). Cicenas et al (2005) has shown that high levels of pAKT correlated with poor prognosis, and the significance of
this correlation increased in the subset of patients with ErbB-2 overexpressing tumors. Rojo et al (2007) reported that tumors with HER2 overexpression showed higher pAKT as compared with negative tumors. This further, suggests that cytoplasmic AKT detected in our study may be its active form. In a study by Macaskill et al (2011) in 31 post-menopausal women with early breast cancer were given 5 mg RAD001 once daily for 14 days prior to surgery and found a reduction in pAKT (cytoplasmic) and reduction in pmTOR following treatment and confirmed that tumors with high Ki67, high pAKT, and HER-2 positivity may be more responsive to mTOR inhibition with RAD001.

mTOR expression when correlated with Her-2/neu higher incidence was seen in Her-2/neu positive tumors. Zhou et al (2004) reported that Phosphorylated AKT, mTOR, and 4E-BP1 were positively associated with ErbB2 overexpression. Survival analysis showed that phosphorylation of each of these three markers was associated with poor disease free survival independently. In vitro, they further confirmed the causal relationship between ErbB2 overexpression and mTOR activation, which was associated with enhanced invasive ability and sensitivity to a mTOR inhibitors and concluded a link between ErbB2 and the AKT/mTOR/70S6K/4E-BP1 pathway in breast cancers in vitro and in vivo, and further confirmed a possible role of AKT/mTOR activation in ErbB2-mediated breast cancer progression.

In our study a significant correlation of mTOR was observed cytoplasmic AKT and not with nuclear AKT. In a study by Rojo et al (2007) levels of pAKT correlated with the, p4EBP1 and p70S6K both of which are the downstream molecules of mTOR. Also in a
study by Janssen et al (2007) pAKT correlated with mTOR. However, Yoshizawa et al (2010) reported a lack of association between pAKT1 and pmTOR expression in non small cell lung cancer. Creighton et al (2007) focused on breast cancer through gene signature and reported the AKT gene signature could possibly be used as a better indicator of pathway dysfunction in tumors. Genes activated by mTOR branch of AKT pathway were associated with increasing tumor grade and size, loss of ER expression and poor clinical outcome, whereas these associations were weaker or not present for the mTOR independent genes in the AKT pathway. These findings suggested a correlation and further indicated mTOR as an essential therapeutic target of the AKT pathway.

In benign breast disease only PTEN and AKT expression was evaluated. High PTEN loss was observed in fibroadenoma and fibrocystic disease than breast carcinoma, however the reason for higher loss in fibroadenoma and fibrocystic disease remains unknown. Further, AKT expression was significantly higher in fibrocystic disease than fibroadenoma and breast carcinoma. In comparison with our study, another study observed higher PTEN expression (80%) with low incidence of PTEN loss (20%) in patients with fibroadenoma (www.tumorres.com/tumor-stem-cell/36646.htm,2012). Interestingly none of the patient with benign breast disease expressed cytoplasmic AKT, further confirming a unique role of each of the AKT isoform and suggesting AKT3 to be associated with favourable prognosis. Zhou et al (2004) also evaluated AKT expression in normal breast epithelium, fibroadenoma, intraductal hyperplasia, and ductal carcinoma in situ and discovered phosphorylation of AKT, increased
progressively from normal breast epithelium to hyperplasia and abnormal hyperplasia to tumor invasion. None of the eight fibroadenoma patients in their study expressed pAKT. Balsara et al (2004) detected AKT activation in preneoplastic bronchial lesions with high risk of developing lung cancer and suggested that AKT activation might have a role in the conversion from a benign to a malignant tumor.

The loss of PTEN identifies a subgroup of breast carcinoma patients with aggressive phenotype. The difference in correlation of nuclear and cytoplasmic AKT with clinical parameters suggests that each of the AKT isoform has its unique function that is not shared by other isoform and hence a positive correlation of nuclear AKT with PTEN and, cytoplasmic AKT with and Her-2/neu was observed in the present study. This suggests that AKT activation of some isoform nuclear (AKT3) occurs in a PTEN independent manner and ER may play a role in nuclear AKT activation. Furthermore, mTOR expression correlated with advance tumors size and Her-2/neu expression suggests addition of mTOR inhibitors in Her-2/neu positive breast cancer. However, a larger study with each of the unique AKT isoform is required to confirm our preliminary findings.