SUMMARY AND CONCLUSION:
The past decade has witnessed a major leap in the understanding of the molecular mechanism involved in breast cancer pathogenesis and progression. Several signaling pathways that play a critical role in these processes have been identified and are now recognized as potential therapeutic targets. In parallel, a wide array of new agents of different classes and with diverse mechanism of action has been synthesized and is now under clinical evaluation. The most frequently mutated and aberrantly amplified oncogenic pathway in breast cancer is Her-2/neu - PI3K pathway. Her-2/neu is the most potent stimulator of the PI3K/AKT anti-apoptosis pathway, and an aberrant form of Her-2/neu missing extracellular domain so called p95 Her-2/neu can cause resistance to trastuzumab.

To understand the role of Her-2/neu – PI3K/AKT pathway in breast cancer, we analysed protein expression of Her-2/neu intracellular and extracellular domains, Her-2/neu Val655Ile SNP, PTEN methylation and protein expression of PTEN, AKT and mTOR and their clinical relevance. Further, cytoplasmic form of Her-2/neu protein as truncated form of Her-2/neu (p95) was confirmed by double staining immunohistochemistry. Further, these molecules were studied in patients with benign breast diseases and compared with breast carcinoma patients.

Summarizing the results:-

Section I-A

- In total patients, the incidence of membranous Her-2/neu internal domain was 41%, cytoplasmic Her-2/neu internal domain was 18%, and membranous Her-
2/neu external domain was 19%. Further, a trend of high incidence of membranous Her-2/neu internal domain expression was observed in LN negative and early stage patients along with a significant reduced DFS in patients with no expression of membranous Her-2/neu internal domain. This trend could be due to inclusion of patients with triple negative subtype. Therefore, patients with luminal A, luminal B and Her-2/neu positive subtypes were selected for Her-2/neu protein study. In them, the incidence of membranous Her2/neu internal domain was 70%, cytoplasmic Her-2/neu internal domain was 30%, and membranous Her-2/neu external domain was 33%.

Membranous Her-2/neu internal domain expression:

- A trend of higher incidence of membranous Her-2-neu internal domain expression was seen in stage II disease, HG grade III tumors, high BR score tumors and in lobular carcinoma.
- A significant higher incidence of membranous Her-2-neu internal domain expression was seen in ER negative and PR negative tumors.
- Its incidence was higher in patients who developed lung metastasis, brain metastasis and local recurrence. However, no significant correlation was observed with DFS and OS.

Cytoplasmic Her-2/neu internal domain expression:

- A trend of higher incidence of cytoplasmic Her-2/neu internal domain expression was seen in T4 tumors, NG III tumors, high BR score tumors and in medullary carcinoma.
SUMMARY AND CONCLUSION

- A significant high incidence of cytoplasmic Her-2/neu internal domain expression was seen in ER negative and PR negative tumors.
- Its incidence was higher in patients who developed brain metastasis and multiple metastasis.
- In univariate survival analysis, cytoplasmic Her-2/neu internal domain expression found to be a significant prognosticator for DFS and OS.
- In multivariate survival analysis cytoplasmic Her-2/neu internal domain expression found to be an independent predictor of DFS.

_Her-2/neu external domain expression:_

- A trend of higher Her-2/neu extracellular domain expression was found in patients with early stage disease, low size tumors, LN positivity, NG III tumors, high BR score tumors and in tumors without vascular permeation.
- With histological type, an important observation noted that expression of Her-2/neu extracellular domain was observed only in invasive ductal carcinoma.
- A significant high incidence of membranous Her-2/neu external domain expression was seen in ER negative and PR negative tumors.
- Its incidence was found higher in patients who developed lung metastasis.
- In univariate survival analysis, only a trend of reduced overall survival was seen in patients with membranous Her-2/neu external domain expression.
**Correlation between membranous Her-2/neu internal domain, cytoplasmic Her-2/neu internal domain and membranous Her-2/neu external domain:**

- A significant positive correlation was observed between membranous Her-2/neu internal domain, cytoplasmic Her-2/neu internal domain and membranous Her-2/neu external domain.

**Benign Breast Disease**

The incidence of membranous Her-2/neu internal was significantly lower in fibroadenoma (14%) and fibrocystic disease (27%) as compared to breast carcinoma (41%).

**Section I-B**

- The incidence of Ile/Ile and Val/Val genotype was found in 20% patients each, whereas the prevalence of Val/Ile heterozygous genotype was higher and observed in 60% of patients.
- The interesting observation noted that patients with triple negative subtype also exhibited Her-2/neu genotypes.

**Ile/Ile genotype**

- A trend of higher incidence of Ile/Ile genotype was observed in patients with early stage disease, HG III tumors, high BR score tumors, high NG (II+III) tumors and lobular carcinoma.

**Val/Ile heterozygous genotype**

- A trend of higher incidence of Val/Ile genotype was observed in patients with HG I tumors, NG I tumors, T1 tumors and stage I disease.
Summary and Conclusion

Val/Val genotype

- A trend of higher incidence of Val/Val genotype was demonstrated in patients with LN positivity, stage IV disease, low BR score tumors and in ER positive and PR positive tumors.
- In luminal A subtype the incidence of Ile/Ile and Val/Ile genotypes was low and Val/Val genotype was high in comparison with other three subtypes where the incidence of these genotypes was found similar.
- In patients with luminal A, luminal B and Her-2 positive subtypes the incidence of Val/Val genotype was significantly low in patients with membranous Her-2/neu internal domain expression. Similar trend was seen in patients with cytoplasmic Her-2/neu internal domain expression and membranous Her-2/neu external domain expression. Further, the incidence of Val/Ile genotype was significantly high in patients with membranous Her-2/neu internal domain expression.
- None of the genotype showed correlation with DFS and OS.
- In benign breast disease, the incidence of Ile/Ile genotype was higher in fibrocystic disease and Val/Ile heterozygous genotype in fibroadenoma, with a similar incidence of Val/Val genotype in both the groups.
- Patients with breast carcinoma had a low incidence of Ile/Ile genotype and high incidence of Val/Ile and Val/Val genotype than benign breast disease.

Section II

PTEN

- PTEN protein loss was observed in 40% of the patients.
- None of the patient expressed PTEN methylation.
• A significant higher incidence of PTEN loss was observed in HG II and HG III tumors than HG I tumors.

• A trend of higher PTEN loss was seen in patients with high BR score tumors, vascular permeation in tumors and lobular carcinoma.

• In luminal A tumors patients who developed brain metastasis had higher loss of PTEN.

• In univariate survival analysis, loss of PTEN expression was not correlated with DFS and OS.

**AKT**

• Two staining pattern-nuclear and cytoplasmic was observed for AKT.

• Nuclear expression of AKT was observed in 40% of the patients with a significant higher expression in patients with HG I tumors, patients with absence of lymphatic permeation in the tumors and PR positive tumors and a trend in patients with ER positive tumors and lobular carcinoma.

• Cytoplasmic AKT expression was observed in 30% of the patients with a trend of higher cytoplasmic AKT expression in patients with T4 tumors, intermediate BR score tumors, absence of lymphatic permeation and medullary carcinoma.

• Total AKT (nuclear+cytoplasmic) expression was observed in 42% of the patients with a trend of higher incidence in patients with HG I tumors, NG I tumors, low and intermediate BR score tumors, absence of lymphatic permeation and presence of vascular permeation.
• Nuclear, cytoplasmic and total AKT expression was found higher in Luminal B tumors.

• In univariate survival analysis, a trend of higher incidence of disease relapse was noted only in patients with total AKT expression.

**mTOR**

• Cytoplasmic expression of mTOR was observed in 28% of the patients with a trend of higher mTOR expression in patients with T4 tumors, intermediate BR score tumors, absence of lymphatic permeation, presence of vascular permeation and medullary carcinoma.

• mTOR expression was higher in Her-2 positive subtype and in patients who developed brain metastasis.

• In univariate survival analysis, no significant difference was observed in DFS or OS with mTOR expression.

• A significant positive correlation was observed between PTEN and nuclear AKT, PTEN and total AKT, and cytoplasmic AKT and mTOR.

• With Her-2/neu internal (membranous + cytoplasmic) and external domain expression a significant positive correlation of cytoplasmic AKT and mTOR was observed.

• A higher PTEN loss was observed with Val/Val genotype.

*Benign Breast Disease*

• In benign breast disease PTEN loss was observed in 58% of the patients and nuclear AKT expression was observed in 60% of the patients. None of the patients expressed cytoplasmic AKT expression.
• Loss of PTEN was significantly higher in breast cancer as compared to fibroadenoma and fibrocystic disease.

• The incidence of nuclear AKT was significantly higher in fibrocystic disease as compared to fibroadenoma and breast cancer.

**Markers in relation to treatment**

• Patients with Her-2/neu protein expression, Her-2/neu SNP, PTEN loss, nuclear AKT expression, cytoplasmic AKT expression and mTOR showed a trend of better response when treated with surgery followed by FAC or FAC+RT than treated with addition of TMX in these subgroups.

**Multivariate survival analysis in breast cancer patients**

• In multivariate analysis of all parameters including Her-2/neu internal domain, cytoplasmic Her-2/neu internal domain, membranous Her-2/neu external domain, genotype frequency, PTEN, AKT and mTOR expression, along with conventional parameters was carried out PTEN loss entered at step 1 for disease free survival in patients with luminal A, luminal B and Her-2 positive subtypes.

**CONCLUSION:**

The present study identified cytoplasmic Her-2/neu internal domain expression as truncated form of Her-2/neu (p95 Her-2/neu) by double staining immunohistochemistry, as an independent prognosticator in breast cancer and thereby defines a group of patients with Her-2/neu positive breast cancer with significantly worse outcome. The measurement of p95Her-2/neu in breast tumor section may be useful in guiding treatment for patients with Her-2/neu positive breast cancer. Double staining immunohistochemistry may provide a unique tool for the evaluation of truncated Her-
2/neu on FFPE tissues till the antibody to detect p95 Her-2/neu becomes commercially available. The double staining is cheaper and feasible than the methods available currently for the detection of truncated Her-2/neu and can be routinely performed on FFPE tissues along with ER, PR and Her-2/neu evaluation.

The similar frequency of Her-2/neu gene Ile655Val single nucleotide polymorphism observed in fibroadenoma and breast cancer as well as presence of Val/Val genotype in fibrocystic disease supports its involvement not only in breast cancer but it also suggests a role in benign breast disease.

PTEN protein might be of prognostic significance as loss of PTEN emerged as significant prognostic factor for disease free survival in multivariate survival analysis. A positive correlation between PTEN and AKT suggests the activation of AKT in PTEN independent manner which may be through Her-2/neu and ER. Each of the AKT isoform has a unique function which is not shared by other isoform and therefore nuclear aKT expression correlated with favourable prognosticators and cytoplasmic AKT expression with unfavourable prognosticators. A positive correlation of cytoplasmic AKT with mTOR was demonstrated. Further, with Her-2/neu a significant positive correlation of cytoplasmic AKT and mTOR protein expression suggests involvement of Her-2/neu mediated PI3K/AKT/mTOR pathway in the development of breast cancer. Targeting Her-2/neu/PI3K/AKT/mTOR pathway with mTOR antagonists alongwith conventional therapy may increase the therapeutic efficacy of breast cancer.