"HER 2 NEU AND BMI 1 GENE EXPRESSION IN INVASIVE DUCTAL CARCINOMA BREAST AND ITS CORRELATION WITH HORMONE RECEPTORS AND OTHER KNOWN PROGNOSTIC FACTORS"

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CERTIFICATE

This is to certify that this Dissertation entitled **"HER 2 NEU AND BMI 1 GENE EXPRESSION IN INVASIVE DUCTAL CARCINOMA BREAST AND ITS CORRELATION WITH HORMONE RECEPTORS AND OTHER KNOWN PROGNOSTIC FACTORS"** is the bonafide original work of Dr.M.VENNILA, in partial fulfillment of the requirement for M.D., (Branch III) in Pathology examination of the Tamilnadu Dr.M.G.R Medical University to be held in April 2011.

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DECLARATION

I Dr. M. Vennila, solemnly declare that the dissertation titled **"HER2NEU AND BMI-1 GENE EXPRESSION IN INVASIVE DUCTAL CARCINOMA BREAST AND ITS CORRELATION WITH HORMONE RECEPTORS AND OTHER KNOWN PROGNOSTIC FACTORS"** is the bonafide work done by me at Institute of Pathology, Madras Medical College under the expert guidance and supervision of Prof.Dr. A. Sundaram, M.D., Professor and Director of Institute of Pathology and Electron Microscopy, Madras Medical College. The dissertation is submitted to the Tamilnadu Dr.M.G.R Medical University towards partial fulfillment of requirement for the award of M.D., Degree (Branch III) in Pathology.

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ABBREVIATIONS

ER	:	Estrogen Receptor
PR	:	Progesterone Receptor
HER 2 NEU	:	Human epidermal growth factor receptor 2
CK 5/6	:	Cytokeratin 5/6
EGFR	:	Epidermal growth factor receptor 1
DNA	:	De oxy ribonucleic acid
BMI1	:	B cell specific Moloney murine leukemia virus insertion site gene
IHC	:	Immunohistochemistry
PCR	:	Polymerase chain reaction
RT PCR	:	Reverse Transcriptase Polymerase Chain Reaction
IDC NOS	:	Invasive ductal carcinoma not otherwise specified
ICMR	:	Indian Council of Medical Research
WHO	:	World Health Organisation
FISH	:	Fluorescent in situ hybridization
cDNA	:	complementary deoxy ribonucleic acid
mRNA	:	Messenger ribonucleic acid
GADPH	:	Glyceraldehyde 3 phosphate dehydrogenase
ACTB1	:	Actin beta 1
ASCO CAP	:	American Society of clinical oncologists College of American Pathologists
CT	:	Threshold cycle
ΔΔCT	:	Delta delta threshold cycle
N	:	Number of cases
SD	:	Standard deviation
SS	:	Statistically significant
NSA	:	No significant association

CONTENTS

ANNEXURE – I

PROFORMA

Case number : Name :

HPE number : Age :

IP number : Sex :

Clinical diagnosis : Menstrual status :

Risk factors if any :

Side of breast : Right/Left

Specimen : Simple Mastectomy / Modified radical mastectomy / Radical Mastectomy / Toilet mastectomy / Others

GROSS

Specimen size :

Nipple areola : Skin :

Tumor size : Tumor margin:

Appearance :

Resected margins : Superior : Inferior :

Medial : Lateral :

Posterior :

Associated findings :

Total number of nodes dissected :

Largest node size :

MICROSCOPY

Histological subtype:

Histological score: Nuclear score: Mitotic score:

Modified Scarf Bloom Richardson Grade: I / II / III

Skin	:	Free	/	Involved		
Nipple & Areola	:	Free	/	Involved		
Margins	:	Superior	:	Free	/	Involved
		Inferior	:	Free	/	Involved
		Medial	:	Free	/	Involved
		Lateral	:	Free	/	Involved
		Posterior	:	Free	/	Involved
Lymphatic invasion:		Present	/	Absent		
Vascular invasion	:	Present	/	Absent		
Lymphocytic infiltration:		Present	/	Absent		
Necrosis	:	Present	/	Absent		

Associated breast lesions :

Total number of nodes dissected :

Number of nodes involved:

ESTROGEN RECEPTOR STATUS

Proportion score :

Intensity score :

Total score :

PROGESTERONE RECEPTOR STATUS

Proportion score :

Intensity score :

Total score :

HER 2 NEU SCORE :

Relative expression of Bmi 1 in relation to ACTB1 gene :

FOLLOW UP

Chemotherapy :

Radiotherapy :

Hormonal therapy :

Follow up period :

Present status:

ANNEXURE II

WHO HISTOLOGICAL CLASSIFICATION OF EPITHELIAL BREAST TUMORS

INVASIVE BREAST CANCERS

Invasive ductal carcinoma not otherwise specified
- Mixed type carcinoma
- Pleomorphic carcinoma
- Carcinoma with osteoclastic type of giant cells
- Carcinoma with choriocarcinomatous features

Carcinoma with melanotic features
Invasive lobular carcinoma
Tubular carcinoma
Invasive cribriform carcinoma
Medullary carcinoma
Mucinous carcinoma
Cystadenocarcinoma
Signet ring carcinoma
Neuroendocrine tumors
- Solid neuroendocrine carcinoma
- Atypical carcinoid tumor
- Small cell/oat cell carcinoma
- Large cell neuroendocrine carcinoma

Invasive papillary carcinoma
Invasive micropapillary carcinoma
Metaplastic carcinoma
- Pure epithelial metaplastic carcinoma
  - Squamous cell carcinoma
  - Adenocarcinoma with spindle cell metaplasia
  - Adenosquamous carcinoma
  - Mucoepidermoid carcinoma
- Mixed metaplastic carcinoma

Lipid rich carcinoma
Secretory carcinoma
Oncocytic carcinoma
Adenoid cystic carcinoma
Acinic cell carcinoma
Glycogen rich carcinoma
Sebaceous carcinoma
Inflammatory carcinoma
Intraductal papillary carcinoma
Intracystic papillary carcinoma
Microinvasive carcinoma

NON INVASIVE BREAST CANCERS

Ductal carcinoma in situ
Lobular carcinoma in situ
Atypical papilloma

BENIGN EPITHELIAL TUMORS

Tubular adenoma
Lactating adenoma
Apocrine adenoma
Pleomorphic adenoma
Ductal adenoma
Papilloma

FIBROEPITHELIAL TUMORS

Fibroadenoma
Phyllodes tumor
- Benign
- Borderline
- Malignant

Periductal stromal sarcoma
Apocrine carcinoma
Mammary hamartoma

INTRADUCTAL PROLIFERATIVE LESIONS

Atypical ductal hyperplasia
Flat epithelial atypia
Usual epithelial hyperplasia

METASTATIC TUMORS

ANNEXURE III

NOTTINGHAM MODIFICATION OF SCARF BLOOM

RICHARDSON GRADING SYSTEM

TUBULE FORMATION	**SCORE**
Tubule formation in >75% of the tumor	1
Tubule formation in 10 to 75% of the tumor	2
Tubule formation in <10 % of the tumor	3
NUCLEAR PLEOMORPHISM	**SCORE**
Minimal variation in size and shape of nuclei	1
Moderate variation in size and shape of nuclei	2
Marked variation in size and shape of the nuclei	3
MITOTIC RATE	**SCORE**
<10 Mitosis per 10 high power field	1
10 to 20 mitosis per 10 high power field	2
>20 mitosis per 10 high power field	3

GRADE	***SCORE***
Grade 1	3,4,5
Grade 2	6,7
Grade 3	8,9

ANNEXURE IV

IMMUNOHISTOCHEMISTRY PROCEDURE

1. 4µ thick sections were cut from formalin fixed paraffin embedded tissue samples and transferred to gelatin-chrome alum coated slides.
2. The slides were incubated at 58ºC for overnight.
3. The sections were deparaffinized in xylene for 15 minutes x 2 changes.
4. The sections were dehydrated with absolute alcohol for 5 minutes x 2 changes.
5. The sections were washed in tap water for 10 minutes.
6. The slides were then immersed in distilled water for 5 minutes.
7. Heat induced antigen retrieval was done with microwave oven in appropriate temperature with appropriate buffer for 20 to 25 minutes.
8. The slides were then cooled to room temperature and washed in running tap water for 5 minutes.
9. The slides were then rinsed in distilled water for 5 minutes.
10. Wash with appropriate wash buffer (phosphate buffer) for 5 minutes x 2 changes.
11. Apply peroxidase block over the sections for 10 minutes.
12. Wash the slides in phosphate buffer for 5 minutes x 2 changes.
13. Cover the sections with power block for 15 minutes.
14. The sections were drained (without washing) and appropriate primary antibody was applied over the sections and incubated for 45 minutes.
15. The slides were washed in phosphate buffer for 5 minutes x 2 changes.
16. The slides were covered with SuperEnhancer for 30 minutes.
17. The slides were washed in phosphate buffer for 5 minutes x 2 changes.
18. The slides were covered with SS Label for 30 minutes.
19. Wash in phosphate buffer for 5 minutes x 2 changes.
20. DAB substrate was prepared by diluting 1 drop of DAB chromogen to 1 ml of DAB buffer.
21. DAB substrate solution was applied on the sections for 8 minutes.
22. Wash with phosphate buffer solution for 5 minutes x 2 changes.
23. The slides are washed well in running tap water for 5 minutes.
24. The sections were counterstained with Hematoxylin stain for 2 seconds (1 dip).
25. The slides are washed in running tap water for 3 minutes.
26. The slides are air dried, cleared with xylene and mounted with DPX.

ANNEXURE V

QUICK SCORING SYSTEM FOR ESTROGEN AND PROGESTERONE RECEPTOR EXPRESSION

SCORE FOR PROPORTION

Proportion of nuclei stained	Score
No nuclear staining	0
<1% nuclear staining	1
1 – 10% nuclear staining	2
11 – 33% nuclear staining	3
34 – 66% nuclear staining	4
67 – 100% nuclear staining	5

SCORE FOR INTENSITY

Intensity of nuclear staining	Score
No staining	0
Weak staining	1
Moderate staining	2
Strong staining	3

The score for proportion and the score for intensity are summated to a maximum total score of 8. Score of more than 2 is considered as positive.

ANNEXURE VI

HER 2 NEU SCORING SYSTEM – ASCO CAP GUIDELINES

Staining pattern	***Score***	***Her2neu expression***
No staining in tumor cells	0	Negative
Weak, incomplete membrane staining in any proportion of tumor cells	1+	Negative
Weak, complete membrane staining in less than 10% of tumor cells	1+	Negative
Weak, complete membrane staining in more than 10% of the tumor cells	2+	Borderline
Complete intense membrane staining in less or equal to 30% of tumor cells	2+	Borderline
Uniform intense, complete membranous staining in more than 30% of tumor cells	3+	Positive

ANNEXURE VII

RNA EXTRACTION

1. 10 Sections of formalin fixed paraffin embedded tissue samples with 10 µ thickness were collected in micro centrifuge tube.
2. Sections were cleared with 1 ml xylene, vortex, centrifuged for 2 minutes and supernatant was removed.
3. Sections were dehydrated with absolute ethyl alcohol, vortex, centrifuged for 2 minutes and supernatant was removed.
4. The air dried tissue pellet was resuspended in 240 µl Buffer PKD & 10µl proteinase K mixture to reverse the formaldehyde modification of nucleic acid.
5. Incubate the centrifuge tube at 55ºC for 15 minutes and 80ºC for 15 minutes.
6. 500µ of Buffer RBC was added to the mixture and vortex to adjust the binding condition.
7. All the centrifuge tube contents were transferred to gDNA eliminator spin column placed in 2 ml collection tube.
8. The tube is centrifuged at 13,000 rpm for 30 sec to filter the genomic DNA in the tissue sample.
9. The column with genomic DNA was discarded and the flow through was saved.
10. 1200ml of 100% ethanol was added to the flow through and mixed well to enable precipitation of RNA in sample.
11. RNA precipitates were then filtered in RNeasy Minelute spin column placed in 2 ml collection tube by centrifuging at 13,000 rpm for 15 seconds.
12. The column with RNA precipitate was transferred to another collection tube and 500 µl of Buffer RPE added and centrifuged at 13,000 rpm for 15 seconds and then the flow through was discarded.
13. Step 12 is repeated for 2 minutes.
14. Transfer RN easy Minelute spin column to a new 2 ml collection tube and centrifuged at 13,000 rpm for 5 minutes with their lids open to remove the residual ethanol form RNA and the flow through was discarded.
15. Transfer RNeasy Minelute spin column to a new 1.5 ml collection tube and 30 µl of RNAse free water was added and centrifuged for 1 minute at 13,000 rpm to elute total RNA.
16. The total RNA was stored at -20ºC to -70ºC.

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KEY TO MASTERCHART

SI	-	Skin infiltration
LI	-	Lymphatic invasion
VI	-	Vascular invasion
LCI	-	Lymphocytic infiltration
NEC	-	necrosis
LNS	-	Lymph node status
ER	-	Estrogen receptor total Quick score
PR	-	Progesterone receptor total Quick score
HER2	-	Human epidermal growth factor receptor score
CK5/6	-	Cytokeratin 5/6
BMI 1	-	Relative concentration of Bmi 1 mRNA in relation to actin B-1 gene
MRM	-	Modified radical mastectomy
SM	-	Simple mastectomy
RM	-	Radical mastectomy
TM	-	Toilet mastectomy
PSM	-	Palliative simple mastectomy
A	-	Absent
P	-	Present / positive
M	-	Male
F	-	Female
R	-	Right
L	-	Left
NAC	-	Neoadjuvant chemotherapy
IDC NOS	-	Invasive ductal carcinoma not otherwise specified
Sup	-	superior
Inf	-	inferior
Med	-	medial
Lat	-	lateral
Post	-	posterior
G	-	Grade
ACC	-	Adenoid cystic carcinoma
MPC	-	Micropapillary carcinoma
N	-	Negative

INTRODUCTION

Carcinoma of breast is one of the most common human neoplasms both in developed and developing countries accounting for 23% of all the cancers in females.[1] There is a steady rise in the incidence of breast cancer worldwide. It is the leading cancer among Asian women. Early detection and advances in treatment have begun to reduce mortality rates.

Breast carcinoma exhibits a wide range of morphological phenotypes. The histological appearances of tumor cannot fully reveal the prognosis. Exploration of the molecular pathways of carcinogenesis provides explanation for the different morphologic phenotypes and behavior. A large number of genetic alterations have been identified in breast carcinoma, many of which have potential prognostic and predictive values. Thus estrogen and progesterone receptor expression predict response to tamoxifen therapy and Her2neu over expression predict response to trastuzumab.

Recent research in breast biology has provided support for the cancer stem cell hypothesis. Tumor originates from the tissue stem cells through dysregulation of the normally tightly regulated process of self renewal.[2] Cancer stem cells have the potential to self renew and differentiate to generate phenotypically derived cancer cells.[3]The polycomb gene Bmi1 is the critical regulator of self renewal of stem cells and is over expressed in breast cancer.[4]

Genomic studies provide a new method of classification of breast cancers based on gene expression patterns[5,6,7,8,9]. Recently immunohistochemical markers such as ER, PR, Her 2 neu, CK5/6 and EGFR have been used as surrogates for DNA microarray in subtyping breast cancer[10]. These subtypes predict outcome, patient response to chemotherapy or targeted therapy.[7, 8, 11]

Thorough use of molecular techniques like immunohistochemistry and polymerase chain reaction to study the protein and DNA expression profiles may help to predict clinical outcome in individual patients and thus guides to plan personalized therapy.

In this study of 60 cases, an attempt is made to assess molecular subtypes of breast cancers and to compare the Bmi 1 gene and Her 2 neu oncoprotein expression with other known prognostic factors.

AIMS AND OBJECTIVES

1. To identify the relative frequency and distribution of breast carcinoma in population.

2. To study the histomorphological features of breast carcinoma including grade, lymph node status, lymphovascular invasion, lymphocytic response, and necrosis.

3. To study the immunohistochemical expression of estrogen and progesterone receptors in invasive ductal carcinoma breast.

4. To study the immunohistochemical expression of Her2neu protein in invasive ductal carcinoma breast.

5. To study the Bmi1 gene expression in invasive ductal carcinoma breast with respect to grading.

6. To determine the correlation of Her2neu and Bmi1 gene expression with hormone receptor status and other known prognostic factors such as tumor size, histological grade, axillary node status, presence of tumor necrosis, lymphocytic response, lymphatic and vascular invasion by tumor.

REVIEW OF LITERATURE

Invasive breast carcinomas are heterogeneous groups of malignant epithelial tumors characterized by invasion of adjacent tissues and have a marked tendency to metastasize to distant sites.[12]

The Edwin Smith Surgical Papyrus (3000-2500 BC) was the first document that referred to carcinoma of the breast and concluded that there was no treatment for cancer of the breast. In the second century AD, Galen gave the classical clinical observation as "the breast tumors exactly resemble the animal crab". Various authors all over the world followed different classification of breast cancers.

In 1925, Greenhough was the first to evaluate grading system for breast cancer. In 1928, Scarff EW et al proposed tubule formation, nuclear pleomorphism and hyperchromasia as criteria to grade breast cancers. In 1957, Bloom and Richardson introduced numerical scoring system based on tubule formation, nuclear pleomorphism and mitotic rate and that was adopted as preferred grading system by WHO.[13] In 1983, Bloodgood et al recognized ductal carcinoma in situ where neoplastic cells are limited within the terminal duct lobular unit. [14]

EPIDEMIOLOGY:

In 1997, Indian Council of Medical Research (ICMR) documented the rise in incidence of breast cancer in India mainly in urban population. It

reported that one in 22 women in India has lifetime risk of developing breast cancer compared to 1in 8 women in America. Population based survival studies revealed five years relative survival for female breast carcinoma patients in Chennai was only 45.6%.[15]

A study conducted by WHO in 1999 stated that Chennai has the highest incidence (26/1,00,000) of breast cancer among all leading cities in India.[16] Globocan 2008 stated that 1.38 million new cases were diagnosed in that year worldwide out of which 8.33% (1,15,000) of the cases were reported from India. The reported age standardized incidence rate of breast cancer in India is 22.9 per lakh population per year. The mortality rate is relatively high accounting for 11.1 per lakh population per year.[1]

The presenting symptoms include breast lump, nipple discharge, retraction or eczema. Breast abnormalities are evaluated by triple assessment including clinical examination, imaging and tissue sampling either by fine needle aspiration cytology or core needle biopsy.

RISK FACTORS:

The risk factors strongly associated with breast cancers include early menarche, late menopause, nulliparity, older age at first child birth, sedentary life style with high caloric diet, obesity, use of exogenous estrogens in the form of oral contraceptive pills or hormone replacement therapy and positive family history of breast cancer in any first degree relative. [12]

AETIOLOGY:

The main etiological factors of breast carcinoma include hormone excess and genetics.

The **estrogen excess hypothesis** states that the increased breast tissue proliferation and inhibition of apoptosis occur in the background of hyperestrogenism.[12] Bernstein et al postulated the higher risk of breast carcinoma in women with elevated tissue estrogen and progesterone. **Classical genetic models of carcinogenesis** states that any cell in the breast, can be transformed to be malignant by the right combination of mutations.[17] *BRCA1*gene plays an important role in DNA repair, activation of cell-cycle checkpoints, maintenance of chromosome stability and in differentiation of stem cells.[18] Women with BRCA 1 gene mutation carry 80% lifetime risk of developing breast cancer.[19]

Recent research in breast biology has provided support for the cancer stem-cell hypothesis which was first proposed more than 150 years ago.[20] Kordon et al. first demonstrated the existence of mammary stem cells in mice.[21] **The cancer stem cell hypothesis** states that tumors originate in tissue stem and/or progenitor cells through the dysregulation of the normally tightly regulated process of self-renewal.[2] Cancer stem cells have the potential to self renew and some of them undergo differentiation into ductal epithelial cells, lobular cells or myoepithelial cells contributing to tumor cellular heterogeneity.[22]

Invasive ductal carcinoma is a group of breast carcinoma in which the stromal invasion of malignant cells is evident beyond the epithelial component. Current histomorphological sub typing of breast carcinoma is based on world health organisation classification. (Annexure II)

INVASIVE DUCTAL CARCINOMA NOT OTHERWISE SPECIFIED (NOS):

Elston and Ellis et al stated that this is the most common type of invasive carcinoma of breast accounting for 75%. WHO defines this as a heterogeneous group of tumors that fail to exhibit sufficient characteristics to achieve classification as a specific histological type in more than 50% of the tumor mass. Grossly, they present as firm to hard grey white mass with irregular borders. Microscopy show the tumor cells arranged in cords, clusters, trabeculae, syncytial sheets or as tubules with central lumen. These tumors are graded using Nottingham modification of Scarf Bloom Richardson system. (Annexure III). 80% of these cases are associated with foci of Ductal carcinoma in situ. 70 to 80% of these tumors express estrogen and progesterone receptors and 15 to 30% of these tumors express Her2neu protein.[12]

Mixed carcinoma show ductal not otherwise specified pattern in 10 to 49% of the tumor mass and the rest of tumor show recognized special type. Other rare variants of ductal carcinoma NOS include pleomorphic carcinoma and those exhibiting osteoclast type of giant cells, choriocarcinomatous features and melanotic features.

INVASIVE LOBULAR CARCINOMA:

In 1941, Foote and Stewart et al described the histological features of lobular carcinoma in situ and also introduced the term infiltrating lobular carcinoma.[23] WHO defines this tumor as an invasive carcinoma usually associated with lobular carcinoma in situ with more than 90% of tumor mass is composed of non cohesive cells individually dispersed or arranged in single file linear pattern in a fibrous stroma. These tumors show loss of E cadherin expression due to deletion of chromosome 16q. These tumors represent 5 to 15 % of breast carcinomas. 70 to 80% of these tumors express estrogen receptors and 60 to 70% express progesterone receptors.[12]

TUBULAR CARCINOMA:

Fisher et al (1977) was the first to describe the tubular carcinoma of breast where the tumor cells form microtubules and also arranged in cords. It accounts for 2% of invasive ductal carcinomas. Grossly, these tumors measure 0.2 to 2 cm in diameter. Histologically, 90% of tumour mass show presence of open tubules lined by a single layer of small and regular epithelial cells set in a desmoplastic stroma. These tumors are nearly always show positivity for ER, PR and negativity for Her2neu, EGFR.[12]

INVASIVE CRIBRFORM CARCINOMA:

These tumors accounts for 0.8 to 3.5% of breast carcinomas. Histologically, more than 90% of tumor mass show tumor cells arranged in

islands in which well defined spaces are formed by arches of cells (sieve like or cribriform pattern). 100% of these tumors show estrogen positivity and 69% of these tumors show progesterone positivity.

MEDULLARY CARCINOMA:

WHO defines this tumor as "well circumscribed carcinoma composed of poorly differentiated cells arranged in large sheets, scant stroma and a prominent lymphoplasmacytic infiltrate". It accounts for 1 to 7% of breast cancers. Grossly, it has well defined margins, soft consistency. Histological criteria for diagnosis include syncytial growth pattern more than 75%, absence of glandular structures, diffuse lymphoplasmacytic infiltrate, marked nuclear pleomorphism and complete histological circumscription. Omar Hameed et al stated that these tumors usually lack the expression of estrogen and progesterone receptors.[24] It is frequently associated with BRCA1 and TP53 gene mutations.

MUCINOUS CARCINOMA:

This tumor accounts for 2% of all breast carcinomas. Grossly, they have glistening gelatinous appearance, pushing margins and soft consistency. Histologically, it is characterized by proliferation of clusters of uniform round cells floating in lakes of extracellular mucin which is mucicarmine positive. These tumor cells are typically estrogen positive and 70% show progesterone positivity.

INVASIVE PAPILLARY CARCINOMA:

These tumors accounts for less than 1 to 2 % of breast carcinoma. Fisher et al first reported that invasive papillary carcinoma is grossly circumscribed.[25] Microscopically, these tumors are circumscribed with cells arranged as delicate or blunt papillae lined by cells with amphophilic cytoplasm.100% of these tumors show estrogen positivity and 80% show progesterone positivity.

INVASIVE MICROPAPILLARY CARCINOMA:

WHO defines this tumor as "carcinoma composed of small clusters of tumor cells lying within clear stromal spaces resembling dilated vascular channels". They account for less than 2% of invasive breast cancers.

APOCRINE CARCINOMA:

This tumor shows cytological and immunohistochemical features of apocrine cells in >90% of the tumor cells. It contains two types of cells – Type A cells with abundant granular eosinophilic cytoplasm and Type B cells with clear/foamy cytoplasm. Immunohistochemically, they show positivity for GCDFP-15 and negative for bcl2 protein, ER and PR.[12, 24]

METAPLASTIC CARCINOMA:

It is a heterogeneous group of neoplasm characterized by intimate admixture of adenocarcinoma with dominant areas of spindle, squamous and/or mesenchymal differentiation ranging from chondroid and osseous

differentiation to frank sarcoma. It account for less than 1% of the breast cancers. Grossly, they present as well delineated firm pearly white glistening mass. Most of them were estrogen and progesterone receptors negative.

NEUROENDOCRINE CARCINOMA:

Primary neuroendocrine carcinomas express features of neuroendocrine differentiation such as cell arrangement in solid sheets and nests with peripheral pallisading with rare rosette like structures and immunocytochemical expression of neuroendocrine markers such as chromogranin and synaptophysin in more than 50% of the cell population. Most of them were ER and PR positive.

Other rare variants of breast carcinoma include lipid rich carcinoma, Secretory carcinoma, oncocytic carcinoma, adenoid cystic carcinoma and acinic cell carcinoma.

PROGNOSTIC FACTORS:

Prognostic factor is defined as any variable that provides information useful in assessing the outcome at the time of diagnosis of the disease. Predictive factor is defined as any variable that predicts the response to a given therapy. The prognostic factors are classified as clinical factors, morphological factors and genetic/molecular factors. The clinical factors with poor prognosis include premenopausal age group, pregnancy, larger tumor size, involvement of medial quadrant of breast. The pathological factors play more useful role in assessing prognosis.[26] It includes the following

1. Lymph node status: Metastasis in lymph node is the most important prognostic factor. The overall survival rate declines as the number of positive nodes increases. Sentinel node is the first lymph node in the direct drainage pathway. Sentinel node biopsy plays essential role in modern surgery as this node negative patients can be spared the morbidity of complete axillary dissection.

2. Tumor size: Pathological tumor size denotes the measurement of invasive component of the tumor. Survival of the patients decrease and risk of axillary node metastasis increase with the increase in tumor size.

3. Histological type: Women with tubular, cribriform and mucinous types show excellent prognosis with 30 year survival of 60% compared to 20 % survival of ductal carcinoma NOS patients.

4. Histological grade: Nottingham modification of Scarf Bloom Richardson grading system (Annexure III) classifies breast cancers into three groups. Ellis et al reported this grading system to have excellent correlation with patients' survival and rate of metastasis.[27]

5. Lymphatic invasion is a poor prognostic factor strongly associated with the presence of lymph node metastasis and poor patient survival.

6. Vascular invasion denotes the infiltration of tumor cells into vascular spaces and it predicts the risk of recurrence and visceral metastasis.

Other factors reported to have poor prognosis include tumor necrosis, lymphocytic infiltration and skin infiltration. The presence of stromal elastosis was reported to have favorable prognostic role. Clark et al reported the use of Nottingham prognostic index to classify patients of good, intermediate and poor prognostic group with annual mortality rate of 3, 7 and 30%.[28] After the advent of Immunohistochemistry and polymerase chain reaction, more molecular biomarkers play significant predictive as well as prognostic role in breast cancer management.

Estrogen and progesterone receptors predict response to hormonal therapy. 80% of women with ER/PR positive tumors respond to hormone therapy compared to 10% response in ER/PR negative subgroup.[28] Her 2 neu is a transmembrane epidermal growth factor receptor protein also known as c erb2. Its overexpression was reported to have poorer outcome and it predicts the response to trastuzumab, lapatinib and anthracycline based chemotherapy. Recently gene expression profiling has been shown to predict survival, recurrence free interval and to identify appropriate therapy for individual patients to which they respond better.

Patients with TP53 mutation were reported to have short survival and poor response to treatment. Higher proliferation indices and aneuploid DNA status are also assessed to play poor prognostic role in breast cancer patients.

IMMUNOHISTOCHEMISTRY:

Albert Coons et al in 1941 first labeled antibodies directly with fluorescent isocyanate. Nakane and Pierce et al in 1966, introduced indirect labeling technique in which unlabelled antibody is followed by second antibody or substrate. Various stages of development of Immunohistochemistry include peroxidase – antiperoxidase method (1970), alkaline phosphatase labeling (1971), avidin biotin method (1977) and two layer dextrin polymer technique (1993).[29]

USES OF IMMUNOHISTOCHEMISTRY IN BREAST PATHOLOGY[30]

1. Assessment of Estrogen and Progesterone receptor status using specific antibodies to receptor proteins.
2. Assessment of HER-2 neu protein overexpression using specific antibodies to the HER-2 neu protein.
3. Distinguishing insitu and invasive carcinoma using antibodies to myoepithelial markers and basement membrane proteins.
4. Assessment of metastatic lesions of possible breast origin by using antibodies to ER, GCDFP, CK7/CK20 and other markers.
5. Distinguishing ductal from lobular carcinoma in situ using antibodies to E-cadherin.
6. Evaluation of spindle cell lesions to distinguish metaplastic carcinoma from mesenchymal lesions.

ANTIGEN RETRIEVAL:

Antigen retrieval can be done by the following different techniques to unmask the antigenic determinants of fixed tissue sections.

1. Proteolytic enzyme digestion

2. Microwave antigen retrieval

3. Pressure cooker antigen retrieval

4. Microwave and trypsin antigen retrieval

PROTEOLYTIC ENZYME DIGESTION:

Huank et al in 1976 introduced this technique to breakdown formalin cross linkages and to unmask the antigen determinants. The most commonly used enzymes include trypsin and proteinase.[31] The disadvantages include over digestion, under digestion and antigen destruction.

MICROWAVE ANTIGEN RETRIEVAL:

This is a new technique most commonly used in current practice. Microwave oven heating involves boiling formalin fixed paraffin sections in various buffers for rapid and uniform heating. Antibodies against Ki67 and MIB-1 work well after heat pretreatment in this method.[29]

PRESSURE COOKER ANTIGEN RETRIEVAL:

Miller et al in 1995 compared and proved that pressure cooking method has fewer inconsistencies, less time consuming and can be used to retrieve large number of slides than in microwave method.[32]

PITFALLS OF HEAT PRETREATMENT:

Drying of sections at any stage after heat pretreatment destroys antigenicity. Nuclear details damage in poorly fixed tissues. Fibers and fatty tissues tend to detach from slides while heating. Not all antigens are retrieved by heat pretreatment and also some antigens like PGF9.5 show altered staining pattern.

DETECTION SYSTEMS:

After addition of specific antibodies to the antigens, next step is to visualize the antigen antibody reaction complex. The methods employed are direct and indirect methods.

In the direct method, primary antibody is directly conjugated with the label. Most commonly used labels are fluorochrome, horse radish peroxidase and alkaline phosphatase. Indirect method is a two step method in which labeled secondary antibody reacts with primary antibody bound to specific antigen. The use of peroxidase enzyme complex or avidin biotin complex further increases the sensitivity of immunohistochemical stains.[29]

In 1993, Pluzek et al introduced enhanced polymer one step staining, in which large numbers of primary antibody and peroxidase enzymes are attached to dextran polymer back bone. This is the rapid and sensitive method.[33]

Dextran polymer conjugate two step visualization system is based on dextran technology in Epos system. This method has greater sensitivity and is less time consuming.

ESTROGEN RECEPTORS:

In 1950, Elwood V Jensen et al identified Estrogen receptor[34] and in 1996, Kuiper et al identified Estrogen receptor β gene.[35] Estrogen and Progesterone receptors are localized in nuclei of approximately 7% of epithelial cells of normal breast tissue and it is expressed more in lobular than in ductal cells. It shows variation in expression during menstrual period.[36]

Estrogen and progesterone receptors belong to super family proteins. These nuclear transcription factors are involved in breast development, growth and tumorigenesis.[35] There are two forms of Estrogen receptors – Estrogen receptor α and Estrogen receptor β encoded by 6p25.1 and 14q genes respectively.[37] Estrogen receptor α is found in endometrium, breast, ovarian stroma and hypothalamus. Estrogen receptor β is seen in kidney, brain, bone, heart and lungs. Estrogen receptors regulate the expression of progesterone and bcl2.[35] Walker D et al in 1999 proposed that estrogen receptors are cytoplasmic in unliganded state. During activation, estrogen receptor diffuses into the cytoplasm and migrates to nucleus. After dimerisation of the receptor, it binds to Hormone Responsive Elements in DNA and activate MAPK/P13K pathway to induce cell proliferation.

Katrina Bauer et al in 2010 studied the ER, PR and HER 2 expression in the California Cancer Registry based breast cancer population and demonstrated the better five year survival in ER positive group when compared to ER negative group.[38]

PROGESTERONE RECEPTORS:

Progesterone receptor is an intracellular steroid receptor that specifically binds to progesterone. Progesterone is encoded by PGR gene in 11q22 gene which is regulated by estrogen receptor. Progesterone binding to its receptor results in structural changes to induce cell proliferation. In 1988, Clark et al demonstrated progesterone receptor by Immunohistochemistry in formalin fixed paraffin embedded sections.[39]

SCORING SYSTEM:

Estrogen and progesterone receptors show nuclear positivity. They are scored by the proportion of tumor cells showing positivity and intensity of the reaction. Both are summated to give a total score. Different scoring systems used to score estrogen and progesterone receptors include H scoring system[40], Quick scoring system[41] and Allred scoring system[42]. Quick scoring system proposed by Barnes et al in 1998 is widely used worldwide as the score correlates well with the chance of patient response to endocrine therapy.[41] (Annexure V)

Madhuri Kakarala et al (2010) analysed 3,60,933 breast cancer cases in American population through SEER program and reported the higher frequency of breast cancers with ER/PR negativity in Asian Indian Pakistani women when compared to Caucasian women.[43]

HER 2 NEU RECEPTORS:

HER2 neu (Human Epidermal growth factor Receptor 2) otherwise known as c-erbB2 is an oncogene of EGFR family that encodes a transmembrane glycoprotein with tyrosine kinase activity. The proto-oncogene is encoded in 17q11.2 – 12 gene.[44]Overexpression of this gene has been reported in 18 to 30% of breast cancers and is associated with high recurrence and worst prognosis.

Slamon et al (1987) first reported the potential of Her 2 neu to predict the time to relapse and overall survival[45] using Southern blot technique. Berger et al (1988) reported the correlation of Her 2 neu protein overexpression with lymph node metastasis and high tumor grade by using Immunohistochemistry. Muss et al (1994) reported significant predictive value for Her 2 neu overexpression on the response to cytotoxic chemotherapy and overall disease outcome. Valone et al[46] (1995) reported 10% response to monoclonal antibody against Her 2 protein in the clinical trial study. Cobleigh (1998) demonstrated 15 % response rate for Herceptin when used alone in 222 metastatic breast cancer patients.[47]

Her 2 neu can be assayed by Immunohistochemistry for protein over expression and by fluorescent in situ hybridization for gene amplification. Recent guidelines[48] for reporting Her 2 neu protein expression is given in Annexure VI.

FISH is used as secondary test in equivocal 2+ IHC categories to clarify HER2 status of these cases. HER 2 FISH testing results are expressed as the ratio of HER2 signals to chromosome 17 signals. The ratio of more than 2.2 is considered as positive for amplification. Other methods of HER 2 testing techniques include chromogenic in situ hybridization, polymerase chain reaction, enzyme linked immunosorbent assay and Southern blotting.

Over expression of Her 2 neu receptors predicts resistance to treatment with alkylating agents and endocrine therapy and predicts response to Herceptin therapy and anthracycline based treatments.[49]

POLYMERASE CHAIN REACTION:

PCR is a recently developed procedure for in vitro amplification of specific nucleic acid sequence from a complex DNA template in a simple enzymatic reaction. In 1971, Gobind Khorona et al described the basic principle of replicating a piece of DNA using two primers. In 1983, Kary Mullis et al proposed in vitro amplification of piece of DNA using Klenow fragment of thermo labile E coli DNA polymerase enzyme from single stranded DNA prepared by heat denaturation. But the product was incompletely pure with only 1% being target sequence and also there was need for addition of enzyme after denaturation step of each cycle.[50]

In 1988, Saiki et al proposed to use thermo stable DNA polymerase from thermos aquaticus to withstand repeated heating during consequent cycles of amplification. This technique showed increased specificity with 40 % of amplified DNA fragments showing altered base due to absence of proof reading activity. In 1996, Cline J et al developed Pyrococcus furiosus (Pfu) DNA polymerase and Thermococcus Litoralis (VENT) as alternative heat stable DNA polymerases with associated 3` to 5` exonuclease activity. This technique showed only 3.5% of DNA with altered base. In 1983, Higuchi et al first documented real time PCR that enabled the quantification of gene expression and DNA copy measurements.[51]

The process of PCR requires target DNA, molar excess of 2 oligonucleotide primers complementary to opposite strands of DNA, heat stable DNA polymerases, equimolar mixture of deoxy nucleotide triphosphates, Magnesium chloride, potassium chloride, 10mM Tris Hcl buffer. PCR cycle consists of 3 steps – denaturation, annealing and extension.

Denaturation refers to separation of 2 strands of target DNA at temperature of 94ºC to 96ºC. The reaction mixture is then cooled to 72ºC to permit annealing of oligonucleotide primer to target DNA. The DNA polymerase then initiates extension of each primer at its 3` ends. The primer extension product is dissociated from target DNA by heating. Each extension product as well as original target act as templates for subsequent cycle. At the end of each cycle, PCR products are doubled. Thus after n PCR cycles, 2^n target sequence can be amplified. The whole procedure is carried out in thermal cycler that precisely controls the temperature.[52]

Reverse transcriptase PCR is a process developed to amplify RNA targets. The complementary DNA is first produced from RNA target by reverse transcription and then the cDNA is amplified by PCR. Initially, both heat labile avian myeloblastosis virus reverse transcriptase and a thermo stable DNA polymerase were used for RT PCR. In 1991, Myers et al. proposed the DNA polymerase from Thermus thermophilus can function efficiently as both reverse transcriptase and a DNA polymerase.[53]

In 1988, Haqqi et al. developed Nested PCR with increased sensitivity and specificity. In this method, first primer is used for first round PCR of 15 – 30 cycles. The products of amplification are then subjected to second round amplification with second set of primer which anneal to sequence internal to the sequence amplified by the first primer set. In 1988, Chamberlain et al. described multiplex PCR where different target sequence can be amplified simultaneously in a single reaction using different primers. Competitive PCR is a process of co amplification in same reaction tube of 2 different templates of equal length and with same primer binding sequences.

Real time PCR is a technique in which target amplification and detection steps occur simultaneously in a same tube. It requires special thermal cyclers with precision optics that can monitor the fluorescence emission from sample wells through computer software at every cycle. The amplification show little change in fluorescence with initial cycles referred as baseline plot. Any increase above the baseline indicates detection of

accumulated PCR product. The PCR product is detected by using fluorescent dyes such as SYBR green I that emit enhanced fluorescence when bound to double stranded DNA. The PCR product can also be detected using hybridization probes such as Taqman probe, FRET probe, molecular beacon or dark quencher probe.[53]

In 1991, Wittwer et al. introduced rapid cycle PCR where thin walled tubes or capillaries are used to enable rapid thermal transfer rate. It incorporates 35 to 100 nucleotides / second. In case of mRNA quantification, gene expression has to be related to some house keeping genes that are expressed throughout the cells such as GADPH, β actin or PGP9.5.[54]

Advantages of PCR include high speed, ease of use, high sensitivity and ability to amplify DNA from formalin fixed paraffin embedded tissue samples. A limitation of PCR is that only 0.1 – 5 kb size DNA sequences can be amplified. The amplification product levels off due to finite enzyme.

HIERARCHICAL MODEL OF BREAST EPITHELIUM:

Molecular studies support the hierarchical arrangement of human breast epithelial cells. The stem cell gives rise to committed progenitor cells for either the myoepithelial or luminal epithelial lineages. The luminal progenitor subpopulation can commit to either a ductal or alveolar cell fate, depending on the developmental stage (puberty or pregnancy). Stem like cells in the human breast have an asymmetric distribution, primarily restricted to the ducts rather than lobules.[55]

MOLECULAR SUBTYPES OF BREAST CANCER

The histological appearances of tumors cannot fully reveal the underlying complex genetic alterations and biologic events involved in their development and progression. This promoted the development of a new classification based on key molecular events involved in process of carcinogenesis, thereby providing a molecular explanation for the different morphologic phenotypes and behavior.

On the basis of recent DNA microarray studies, Sorley et al identified distinct molecular subtypes of breast carcinoma on the basis of gene expression profiling. These include the luminal A, luminal B, basal-like, Her2 overexpressing and claudin-low normal-breast-like subtypes. The differences in tumor subtypes are hypothesized to reflect different mutation profiles, as well as differences in the cell of origin.[5] (Figure 1)

Large scale gene expression profiling from formalin fixed, paraffin embedded samples is not feasible. Therefore, immunohistochemical markers have been used as surrogates for DNA microarray in subtyping breast cancers. Nielsen et al was the first to define IHC subtypes using four antibody panel including ER, HER1, HER2 and CK5/6.[56] Carey et al. updated IHC subtype definition as luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), HER2 (ER-, PR-, HER2+), basal like (ER-, PR-, HER2-, CK5/6+) and unclassified (ER-, PR-, HER2-, CK5/6-, HER1-).[10] These molecular differences correlate with clinical features such as survival, prognosis and treatment sensitivity.

Common types of invasive ductal and invasive lobular carcinomas, display evidence of luminal cell differentiation. Luminal A and B subtypes are generally associated with a good prognosis.

Tumors that over express HER2 (or exhibit amplification) usually display luminal features, but are associated with poor overall survival.[57] These tumors have a distinct molecular profile and aggressive clinical course associated with the propensity to develop metastasis in brain.[58]

The basal-like subtype is very heterogeneous and comprises 15%–20% of breast cancers.[59] This group of tumors is the most clinically aggressive and tends to exhibit a triple-negative phenotype (i.e., lack expression of ER, PR, and HER2). In combination with EGFR and cytokeratin 5/6, these markers provide high specificity in identifying basal-like tumors.[60] Carey et al in 2006 studied 496 invasive breast cancer cases from California breast cancer study and demonstrated poor ten year survival of basal like and HER2 molecular type patients when compared to luminal type patients.[10]

ROLE OF GENES IN MAMMARY GLAND DEVELOPMENT & CARCINOGENESIS:

Pietersen and co-workers described the expression of Bmi1 in all mammary epithelial cells and its role in regulating stem cell proliferation but opposing its differentiation.[61] NOTCH signaling pathway regulates the mammary cell fate decisions. NOTCH4 gene directs progenitor cells to myoepithelial cell commitment. NOTCH3 direct progenitor cells to luminal cell commitment.[62] Gata3 controls differentiation along the ductal and alveolar luminal lineages.

In hereditary breast cancers, deletion of a allele of BRCA1 and in sporadic cancers, activation of other pathways such as Notch, Hedgehog, Wnt or amplification of Her 2 neu or deletion of PTEN lead to dysregulation of stem cell self renewal. These aberrant stem cells are targets for further carcinogenic mutations.[21](Figure 2)

Liu et al demonstrated that hedgehog signaling acting through BMI1 regulates the self-renewal of both normal and malignant human mammary stem cells. This process is blocked by specific inhibitors such as cyclopamine.[63]

ROLE OF BMI 1 GENE IN BREAST CANCERS:

B-cell-specific Moloney murine leukemia virus insertion site 1 gene (Bmi-1) is a transcriptional repressor that belongs to the polycomb-group family of proteins involved in hematopoiesis and regulation of proliferation. Bmi-1 is proved to be expressed in various human malignant tumors such as brain tumors, breast cancers, leukemia and also gastric cancers.[64]

Hyun Jo Youn et al (2005) suggested cell cycle deregulation by Bmi 1 gene alteration to play a role in lymph node metastasis of breast cancer patients.[65] Silva et al (2007) demonstrated poor overall and disease free survival in breast cancer patients with circulating Bmi-1 mRNA.[66] Young Jin Choi et al (2007) found Bmi-1 protein to be associated with favorable survival in ER positive breast cancer patients.[67]

In breast Bmi-1 acts as the critical regulator of stem cell self renewal and also maintains the proliferation of committed mammary progenitor cells.[63] Bmi-1 gene repress INK4a/ARF locus, which encodes two tumor suppressors (p16 & p19) and activates human telomerase reverse transcriptase, which extend the replicative life span and immortalize the cells.[68] The longevity of many adult stem cells and restricted progenitors makes them likely candidates for accumulating genetic mutations. These aberrant stem cells are highly resistant to all the current adjuvant therapies such as chemotherapy, radiation therapy and hormonal therapy. Newer treatment approaches to target this stem cell population is the emerging need of this era.

MATERIALS AND METHODS

This study is a retrospective descriptive study of invasive breast cancers conducted in the Institute of Pathology, Madras Medical College and Government hospital, Chennai during the period between July 2008 and May 2010.

Source of data:

The invasive ductal carcinoma cases reported in mastectomy specimen received in the Institute of pathology, Madras Medical College between July 2008 to May 2010 from the Department of Surgery, Oncology, Plastic surgery and Geriatrics, Government General Hospital. A total of 238 mastectomy specimens (simple, modified radical or radical mastectomy) were received during this period.

Inclusion criteria

All the invasive ductal carcinoma cases reported in mastectomy specimens irrespective of the age and sex were included for the study.

Exclusion criteria

- Non neoplastic lesions and benign tumors of breast.
- Ductal carcinoma breast reported in incision/excision biopsy and completion mastectomy specimens.
- Cases with inadequate material from the tumor for doing both immunohistochemistry and polymerase chain reaction were not included in the study.

METHOD OF DATA COLLECTION:

Detailed history of the cases regarding age, sex, menstrual history, side of the breast, type of procedure, history of neo adjuvant therapy, details of gross characteristics such as tumor size, nodal status details were obtained for all the 238 mastectomy cases reported during the period from Surgical Pathology records. Freshly cut and Hematoxylin Eosin stained 4 µ thick sections of the paraffin tissue blocks of mastectomy specimens were reviewed and graded using the Nottingham modification of the Scarf Bloom Richardson Grading system (Annexure III) and they are further evaluated for the presence of necrosis, lymphocytic response and lymphovascular invasion by tumor. 20 cases of each grade from Invasive ductal carcinoma NOS subtype were randomly selected from the total cases and their representative formalin fixed paraffin embedded tissue samples were subjected to Immunohistochemistry for a panel of 4 markers and to real time reverse transcriptase polymerase chain reaction for gene analysis. The results were recorded with photographs. Follow up data of breast cancer patients regarding the adjuvant therapy, dose, duration, recurrence, disease free survival were obtained from Medical Records Section of Department of Oncology.

IMMUNOHISTOCHEMICAL EVALUATION:

Immuohistochemical analysis of a panel of markers including estrogen receptor, progesterone receptor, Human epidermal growth factor receptor 2 (Her 2 neu) and Cytokeratin 5/6 were done in paraffin embedded tissue

samples using Supersentive polymer HRP system based on non biotin polymeric technology. Due to economic constraints, immunohistochemistry for Cytokeratin 5/6 was done only in triple negative cases to differentiate between basal and unclassified subtypes of breast cancer.

4 µ thick sections from selected formalin fixed paraffin embedded tissue samples were transferred onto gelatin coated slides. Heat induced antigen retrieval was done. The antigen is bound with mouse monoclonal antibody (Biogenex) against Estrogen and Progesterone receptors, Cytokeratin 5/6 and rabbit monoclonal antibody against Her2 neu protein and then detected by the addition of secondary antibody conjugated with horse radish peroxidase-polymer and Diaminobenzidine substrate. The step by step procedure of Immunohistochemistry is given in Annexure IV.

Antigen	Vendor	Species	Dilution	Positive control
ER	BIOGENEX	Mouse	Ready to use	Breast
PR	BIOGENEX	Mouse	Ready to use	Breast
Her 2 neu	BIOGENEX	Rabbit	Ready to use	Breast cancer
Cytokeratin 5/6	LABVISION	Mouse	1:50	skin

INTERPRETATION & SCORING SYSTEM:

The immunohistochemically stained slides were analyzed for the presence of reaction, cellular localization (nuclear/cytoplasmic /membranous / combinations), percentage of cells stained and intensity of reaction. Nuclear

staining was assessed for ER and PR, membranous staining for Her 2 neu and cytoplasmic staining was assessed for CK5/6. Immunohistochemical scoring for estrogen and progesterone receptors were done with Quick score (Annexure V). Her2neu scoring was done as per recent ASCO CAP guidelines (Annexure VI). In this study, Her 2 neu score of 0 and 1+ were considered as negative and score 2+ and 3+ were considered as positive. An estimation of more than 10 % of tumor cells with cytoplasmic staining is considered as positive reaction for CK5/6.

IHC SUBTYPING CRITERIA

The IHC-based definition of breast cancer subtypes used in this study was as follows: Luminal A (ER+ and/or PR+, HER2 -), Luminal B (ER+ and/or PR+, HER2+), HER2 (ER-, PR-, HER2+), basal-like (ER-, PR-, HER2-, CK5/6+) and unclassified (negative for all markers).

REAL TIME POLYMERASE CHAIN REACTION:

ISOLATION OF TOTAL RNA:

10 sections of 10μ thickness were collected from all the formalin fixed paraffin embedded tissue samples. The total RNA from the samples were purified with RNeasy kit (Qiagen) and stored in collection tubes at -20ºC to -70ºC. (Step by step procedure given in appendix VII). The concentration of total RNA isolated from each sample was estimated using Eppendorf Biophotometer. The volume of each sample containing 5ng of total RNA was calculated.

REAL TIME PCR AMPLIFICATION:

The real time one step polymerase chain reaction is carried out with Rotor Gene Q system using Rotor gene SYBR green RT PCR kit. Normal breast tissues obtained from autopsy material were used as controls to study the relative gene expression in breast cancer. β actin 1 (ACTB1) is used as house keeping gene in this study. Samples were run in duplicates for both BMI1 and ACTB1 gene.

To check the efficiency of the RT PCR a standard curve with log concentration obtained by dilution of control sample was generated for both BMI1 and ACTB1 gene simultaneously. The concentration dilutions were 100ng, 10ng and 1 ng respectively.

BMI 1 gene and β actin gene were amplified using the extracted RNA as templates and the following forward and reverse primers.

BMI1 forward (5' GCTTCAAGATGGCCGCTTG 3') primers,

BMI1 Reverse (5' TTCTCGTTGTTCGATGCATTTC 3') primers,

β actin forward (5'CCCCTGGCCAAGGTCATCCATGACAACTTT-3') primers&

β actin reverse (5'GGCCATGAGGTCCACCACCCTGTTGCTGTA-3') primers.

The Polymerase chain reaction was carried out as follows,

- Thaw 2x Rotor gene SYBR green RT PCR master mix, template RNA, primer and RNAse free water to prevent premature complementary DNA synthesis.

- The reaction mix was prepared in PCR tubes as follows
    - 2xRotor gene SYBR green RT PCR master mix - 12.5 ml
    - Rotor gene RT mix - 0.25 ml
    - Primer for BMI 1/β actin - 2.5 ml
    - Template RNA of each sample with concentration of 5 ng
    - The total reaction volume was made into 25 µl with RNAse free water.
- The PCR tubes were placed in Rotor Gene cycler and the cycling conditions were programmed as follows

STEP	TEMPERATURE	TIME
Reverse transcription	55ºC	10 minutes
PCR Initial activation step	95ºC	5 minutes
One step PCR cycling x 40 cycles		
Denaturation	95ºC	5 seconds
Combined annealing/extension	60ºC	10 seconds

- Melting curve analysis was performed at the end of reactions with temperature range of 55ºC to 95ºC to check the specificity of the reaction.

RELATIVE GENE EXPRESSION ANALYSIS:

The BMI1 RNA levels were calculated in the breast cancer samples and normal breast tissue in a relative quantification approach by using a reference gene ACTB1. Relative expression was calculated by deriving the delta delta CT values for BMI1 with reference to ACTB1 gene expression.

These values were generated automatically by Rotor Gene Q 2 Plex series software version 1.74. Relative concentration of BMI1 RNA in tumor samples to that of control samples was calculated as a linear value from the equation $2^{-\Delta\Delta CT}$. These values were subjected to statistical analysis.

STATISTICAL ANALYSIS:

The statistical analysis is performed using statistical package for social science software version 11.5. Correlation between Her2 neu expression and clinicopathological prognostic factors such as tumor size, nodal status, necrosis and lymphovascular invasion was analyzed using Pearson chi square test.

The efficiency of the real time PCR experiment derived from standard curve was within a range of 0.82 and 0.86 for BMI1 and ACTB1 reference genes respectively elucidating the validity of experiment. Standard curve from first set was used for the other set runs subsequently. Melt curve analysis showed minimized range of primer dimmers with peaks at 75ºC.

The expression of BMI 1 gene was non parametric, so the correlation with other prognostic factors was done using Mann Whitney U test, student t test and Pearson correlation co efficient analysis.

OBSERVATION AND RESULTS

In the study period of 23 months from July 2008 to May 2010, a total of 16,028 specimens were received in the Institute of Pathology, Madras Medical College for histological examination. Total numbers of breast specimens received were 969 cases, of these breast tumors accounted for 676 cases with a percentage of 4.22 %. The relative frequency of breast cancers among the specimen received was 2.1%. The total number of non neoplastic, benign and malignant cases was 293, 325 and 351 respectively. Thus the distribution of non neoplastic breast lesions were 30.24 %, of benign tumors were 33.54% and of malignant tumors were 36.22%. Breast cancers had a peak incidence in the age group of 41-50 years. (Table 1 and Chart 1)

TABLE 1 AGE WISE DISTRIBUTION OF BREAST CANCERS

AGE GROUP	NUMBER OF CANCERS	PERCENTAGE
21 – 30 years	12	5%
31 – 40 years	52	22%
41 – 50 years	89	38%
51 – 60 years	48	20%
61 – 70 years	29	12%
More than 70 years	8	3%
Total cases	238	100%

The youngest age of presentation of breast cancer is at 25 years in this study. The distribution of histological subtypes of breast carcinoma is shown in Table 2 & Chart 2.

TABLE 2 DISTRIBUTIONS OF HISTOLOGICAL SUBTYPES OF BREAST CANCERS

S.No	Histological subtypes	Number of cases	Percentage
1	Invasive ductal carcinoma NOS	205	86.13%
2	Invasive lobular carcinoma	3	1.26%
3	Mucinous carcinoma	10	4.20%
4	Papillary carcinoma	6	2.52%
5	Micropapillary carcinoma	2	0.84%
6	Medullary carcinoma	1	0.42%
7	Cribriform carcinoma	2	0.84%
8	Metaplastic carcinoma	3	1.26%
9	Mixed carcinoma	4	1.68%
10	Adenoid cystic carcinoma	1	0.42%
11	Apocrine carcinoma	1	0.42%
12	Total number of cases	238	100%

Among the 238 cases, 235 (99%) cases were reported in females and 3 (1%) cases were reported in male breast. (Table 3 & Chart 3)

TABLE 3 SEX DISTRIBUTION IN INVASIVE DUCTAL CARCINOMA

SEX	TOTAL NUMBER OF CASES	PERCENTAGE
Male	3	1%
Female	235	99%
Total	238	100%

116 cases of Invasive ductal carcinoma were reported in left breast, 122 cases were reported in right breast and 1 case had cancer in both the breasts. (Table 4 and Chart 4)

TABLE 4 DISTRIBUTION OF SIDE OF INVOLVEMENT IN BREAST CARCINOMA

SIDE OF BREAST CANCER	NUMBER OF CASES	PERCENTAGE
Left	117	49.1%
Right	120	50.5%
Both breasts	1	0.4%
Total cases	238	100%

56 cases (23%) had tumor less than 2 cm in size, 123 cases (52%) were of 2 to 5 cm in size and 59 cases (25%) were more than 5 cm in size. (Table 5 & Chart 5)

TABLE 5 - DISTRIBUTION OF SIZE IN INVASIVE DUCTAL CARCINOMA

SIZE OF TUMOUR	NUMBER OF CASES	PERCENTAGE
<2 cm (T1)	56	23%
2-5 cm (T2)	123	52%
>5 cm (T3)	59	25%

There were 206 Invasive ductal carcinoma NOS type breast cancers in the study sample which were graded according to Modified Scarf Bloom Richardson grading system (Annexure II) out of which 60 cases(29.12%) were in grade I, 109 cases (52.92%) were in grade II and 37cases (17.96%) were in grade III. (Table 6 & Chart 6)

TABLE 6 DISTRIBUTION OF HISTOLOGICAL GRADE IN INVASIVE DUCTAL CARCINOMA NOS TYPE

GRADE	NUMBER OF CASES	PERCENTAGE
GRADE I	60	29.12%
GRADE II	109	52.92%
GRADE III	37	17.96%
TOTAL	206	100%

68 cases (29%) had up to 3 nodes with metastatic ductal carcinomatous deposit, 50 cases (21%) had 4 to 10 involved nodes, 5 cases (2%) had more than 10 involved nodes while 115 cases (48%) had no lymph node involvement. (Table 7 & Chart 7)

TABLE 7 DISTRIBUTION OF LYMPH NODE METASTASIS IN BREAST CANCERS

LYMPH NODE STATUS	NUMBER OF CASES	PERCENTAGE
Negative	115	48%
1 - 3 positive nodes	68	29%
4 – 10 positive nodes	50	21%
>10 positive nodes	5	2%
Total	238	100%

137 cases (58%) had lymphatic invasion as against 101cases (42%) without lymphatic invasion. (Table 8 & Chart 8)

TABLE 8 DISTRIBUTION OF LYMPHATIC INVASION IN INVASIVE DUCTAL CARCINOMA BREAST

LYMPHATIC INVASION	NUMBER OF CASES	PERCENTAGE
Present	137	58%
Absent	101	42%
Total	238	100%

52 cases (22%) showed vascular invasion while 186 cases (78%) cases had no vascular invasion. (Table 9 & Chart 8)

TABLE 9 DISTRIBUTION OF VASCULAR INVASION IN INVASIVE DUCTAL CARCINOMA BREAST

VASCULAR INVASION	NUMBER OF CASES	PERCENTAGE
Present	52	22%
Absent	186	78%
Total	238	100%

17 % of the cases had skin infiltration (Table 10), 65% of the cases had lymphocytic infiltration (Table 11), 48% of the cases had necrosis (Table 12) as shown in Chart 9.

TABLE 10 DISTRIBUTION OF SKIN INFILTRATION IN INVASIVE DUCTAL CARCINOMA BREAST

SKIN INFILTRATION	NUMBER OF CASES	PERCENTAGE
Present	41	17%
Absent	197	83%
Total	238	100%

TABLE 11 DISTRIBUTION OF LYMPHOCYTIC INFILTRATION IN INVASIVE DUCTAL CARCINOMA BREAST

LYMPHOCYTIC INFILTRATION	NUMBER OF CASES	PERCENTAGE
Present	151	65%
Absent	83	35%
Total	238	100%

TABLE 12 - DISTRIBUTION OF NECROSIS IN BREAST CANCER

NECROSIS	NUMBER OF CASES	PERCENTAGE
Absent	124	52%
Present	114	48%
Total	238	100%

RESULTS OF IMMUNOHISTOCHEMICAL AND MOLECULAR STUDIES

In this study, 41% expressed positive reaction for estrogen receptors, 51% expressed positive reaction for progesterone receptors and 30% showed Her 2 neu over expression. (Table 13 & Chart 10)

TABLE 13 - DISTRIBUTION OF HORMONE RECEPTORS AND HER 2 NEU EXPRESSION IN INVASIVE DUCTAL CARCINOMA NOS

IHC PARAMETER	POSITIVE (%)	NEGATIVE (%)
Estrogen receptor	25 (41%)	35 (59%)
Progesterone receptor	31 (51%)	29 (49%)
Her 2 neu	18 (30%)	42 (70%)

Estrogen receptors were expressed in 80% of grade 1, 35% of grade 2 and 10% of grade 3 tumors while progesterone receptors were expressed in 80% of grade 1, 65% of grade 2 and 10% of grade 1tumors. Thus the receptor status was found to be comparatively reduced with increasing grade of the breast cancer. (Table 14 & Chart 11)

TABLE 14 - DISTRIBUTION OF HORMONE RECEPTORS IN DIFFERENT GRADES OF INVASIVE DUCTAL CARCINOMA NOS

	ER POSITIVE	PR POSITIVE
Grade I	16	16
Grade II	7	13
Grade III	2	2

The mean quick score for estrogen receptors was found to be 5 for grade 1, 1.55 for grade 2 and 0.45 for grade 3 while for progesterone receptors, it was found to be 4.9 for grade 1, 2.9 for grade 2 and 0.45 for grade 3 cases respectively. (Chart 12)

TABLE 15 DISTRIBUTION OF QUICK SCORE Vs GRADE OF BREAST CANCER

	MEAN ER QUICK SCORE	MEAN PR QUICK SCORE
GRADE I	5	4.9
GRADE II	1.55	2.9
GRADE III	0.45	0.45

TABLE 16 DISTRIBUTION OF MOLECULAR SUBTYPES IN INVASIVE DUCTAL CARCINOMA NOS TYPE

MOLECULAR SUBTYPE	NUMBER OF CASES	PERCENTAGE
LUMINAL A	27	45%
LUMINAL B	7	12%
HER 2 TYPE	11	18%
BASAL	5	8%
UNCLASSIFIED	10	17%

Molecular subtyping of breast cancers was done in accordance with Carey et al[10]. 27 cases (45%) were luminal A type, 7 cases (12%) were luminal B type, 11 cases(18%) were Her 2 type, 5 cases (8%) were basal type and 10 cases (17%) were of unclassified type. (Table 16 & Chart 13)

CORRELATION OF HER 2 NEU WITH OTHER PROGNOSTIC FACTORS:

Her 2 neu over expression was noted in 30.4% of premenopausal women and 30.5% of postmenopausal women. There was no significant correlation between the menstrual status and Her 2 neu over expression. (Table 17 & Chart 14)

TABLE 17 CORRELATION OF MENSTRUAL STATUS WITH HER 2 NEU EXPRESSION

Menstrual status	Her 2 neu positive (%)	Her 2 neu negative (%)	Total	Pearson chi square test
Premenopausal	7(30.4%)	16(69.6%)	23	$\chi 2$=0.01 P=0.99
Postmenopausal	11(30.5%)	25(69.5%)	36	

Her 2 neu oncoprotein expression was noted in 33.33% of T1 size tumors, 25% of T2 size tumors and 36.84% of T3 size tumors. There was no significant correlation between the tumor size and Her 2 neu over expression. (Table 18 & Chart 15)

TABLE 18 CORRELATION OF TUMOR SIZE AND HER 2 NEU EXPRESSION

Average size	Her 2 neu positive (%)	Her 2 neu negative (%)	Total	Pearson chi square test
<2 cm (T1)	3(33.33%)	6(66.67%)	9	$\chi 2$=0.85 P=0.65
2 -5 cm (T2)	8(25%)	24(75%)	32	
>5 cm (T3)	7(36.84%)	12(63.16%)	19	

Her 2 neu overexpression was noted in 31.57% of nodal metastasis positive group as against 27.27% of nodal metastasis negative group. Receptor positivity increases with nodal metastasis but no significant correlation was found in statistical analysis. (Table 19 & Chart 16)

TABLE 19 CORRELATION OF NODAL METASTASIS AND HER 2 NEU EXPRESSION

Nodal metastasis	Her 2 neu positive (%)	Her 2 neu negative (%)	Total	Pearson chi square test
Present	6(31.57%)	16(68.43%)	22	$\chi 2=0.01$
Absent	12(27.27%)	26(72.73%)	38	P=0.99

5% of grade 1, 55% of grade 2 and 30% of grade 3 tumors were found to be positive for Her 2 neu oncoprotein expression (Table 20 & Chart 17). This explained the higher receptor expression in higher grade tumors and the association was found to be statistically significant.(p=0.003).

TABLE 20 CORRELATION OF GRADE AND HER 2 NEU EXPRESSION

Grade	Her 2 neu positive (%)	Her 2 neu negative (%)	Pearson chi square test
Grade 1	1(5%)	19(95%)	$\chi 2=0.85$ P=0.65
Grade 2	11(55%)	9(45%)	
Grade 3	6(30%)	14(70%)	

There was no statistically significant association noted between Her 2 neu oncoprotein overexpression and other prognostic factors such as skin infiltration, lymphatic invasion, vascular invasion, lymphocytic infiltration and necrosis as shown in Table 21.

TABLE 21 CORRELATION OF HER 2 NEU WITH OTHER HISTOLOGICAL PROGNOSTIC FACTORS

Patient characteristics		HER2NEU		Pearson chi square test
		Negative	Positive	
Skin infiltration	Present	9	6	$\chi 2=0.95$ P=0.33
	Absent	33	12	
Lymphatic invasion	Present	25	12	$\chi 2=0.27$ P=0.60
	Absent	17	6	
Vascular invasion	Present	14	3	$\chi 2=1.72$ P=0.19
	Absent	28	15	
Lymphocytic infiltration	Absent	10	4	$\chi 2=0.02$ P=0.89
	Present	32	14	
Necrosis	Present	29	14	$\chi 2=0.49$ P=0.69
	Absent	13	4	

Her 2 neu overexpression was noted in 12% of ER positive group as opposed to 42.85% of ER negative group (Table 22 & Chart 18). There was inverse relationship between estrogen receptor expression and Her 2 neu protein expression and the correlation was found to be statistically significant. (p=0.01)

TABLE 22 CORRELATION OF ESTROGEN RECEPTOR STATUS AND HER 2 NEU EXPRESSION

ER status	Her 2 neu positive (%)	Her 2 neu negative (%)	Total	Pearson chi square test
Positive	3(12%)	22(88%)	25	$\chi 2=6.61$ P=0.01**
Negative	15(42.52%)	20(57.48%)	35	

Her 2 neu receptor positivity was observed in 19.35% of PR positive group as against 41.37% of PR negative group (Table 23 & Chart 19). Thus there was inverse relationship between progesterone receptor and Her 2 neu overexpression and the negative correlation was found to be statistically significant. (p=0.05)

TABLE 23 CORRELATION BETWEEN PROGESTERONE RECEPTOR AND HER 2 NEU EXPRESSION

PR status	Her 2 neu positive (%)	Her 2 neu negative (%)	Total	Pearson chi square test
Positive	6(19.35%)	25(80.65%)	31	$\chi 2=3.81$
Negative	12(41.37%)	17(58.63%)	29	P=0.05*

CORRELATION OF BMI 1 GENE EXPRESSION WITH OTHER KNOWN PROGNOSTIC FACTORS

Bmi-1 gene was upregulated in 12 cases while downregulated in 47 cases. Table 24 and Chart 20 shows increased mean relative concentration of Bmi 1 gene in premenopausal women as opposed to lesser relative concentration in postmenopausal women. The correlation was found to be statistically significant.

TABLE 24 CORRELATION OF MENSTRUAL STATUS AND BMI 1 GENE EXPRESSION

Menstrual status	N	Mean relative concentration of Bmi 1 mRNA	Standard Deviation	Mann whitney U test
Premenopausal	23	4.68	13.38	Z=1.96
Postmenopausal	36	1.97	6.45	P=0.05*

The mean relative concentration of Bmi 1 mRNA was higher in small size tumors when compared to large size tumors. The correlation was found to be statistically significant (p=0.007). (Table 25 & Chart 21)

TABLE 25 CORRELATION OF TUMOR SIZE AND BMI 1 GENE EXPRESSION

Size	N	Mean relative concentration of Bmi 1 mRNA	Standard Deviation	Kruskal wallis H test
<2 cm	9	12.0084700	21.74571466	
2 to 5 cm	32	0.5752878	1.54953139	Z=9.79
>5 cm	19	2.7442032	6.37071976	P=0.007**
Total	60	3.19	9.972	

The relative expression of Bmi 1 mRNA was higher in patients with axillary node metastasis but the correlation was not statistically significant (Table 26 & Chart 22).

TABLE 26 CORRELATION OF NODAL METASTASIS AND BMI 1 GENE EXPRESSION

Lymph node metastasis	N	Mean relative concentration of Bmi 1 mRNA	Standard Deviation	Mann whitney U test
Absent	22	0.5188127	1.49036185	Z=0.92
Present	38	4.4003005	11.91313924	P=0.35

Higher concentration of Bmi 1 mRNA was found in grade 3 tumors when compared to grade 1 tumors but the correlation was not statistically significant. (Table 27 & Chart 23)

TABLE 27 CORRELATION OF GRADE AND BMI 1 GENE EXPRESSION

Grade	N	Mean relative concentration of Bmi 1 mRNA	SD	Kruskal wallis H test
1	17	2.09	5.852	Z=1.77 P=0.41
2	19	2.40	7.907	
3	20	4.87	13.970	
Total	56	3.19	9.972	

There was no statistically significant correlation between the Bmi 1 mRNA concentration and other prognostic factors such as skin infiltration, lymphatic invasion, vascular invasion, lymphocytic infiltration and necrosis.(Table 28)

TABLE 28 CORRELATION OF BMI 1 GENE AND OTHER HISTOLOGICAL PROGNOSTIC FACTORS

Tumor characteristics		N	Mean	SD	Mann whitney U test
Skin infiltration	Absent	45	2.74	9.75	Z=0.33 P=0.79
	Present	15	3.69	9.69	
Lymphatic invasion	Absent	23	2.65	8.43	Z=0.97 P=0.33
	Present	37	3.18	10.46	
Vascular invasion	Absent	43	2.32	6.80	Z=0.14 P=0.89
	Present	17	4.64	14.79	
Lymphocytic infiltration	Absent	14	4.92	10.69	Z=0.54 P=0.88
	Present	46	2.39	9.37	
Necrosis	Absent	17	2.58	6.74	Z=1.32 P=0.18
	Present	43	3.14	10.67	

TABLE 29 CORRELATION OF ESTROGEN RECEPTOR AND BMI 1 GENE EXPRESSION

ER	N	Mean relative concentration of Bmi 1 mRNA	Standard Deviation	Mann whitney U test
Negative	35	4.20	11.969	Z=1.98 P=0.05*
Positive	25	1.51	5.020	

Table 29 shows higher concentration of Bmi 1 mRNA in estrogen receptor negative group when compared to estrogen receptor positive group. The inverse correlation between the estrogen receptor status and Bmi 1 gene expression was found to be statistically significant (p=0.05) (Chart 24)

Higher mean relative concentration of Bmi 1 mRNA was found in progesterone receptor positive group when compared to progesterone receptor negative group but the correlation was not statistically significant. (Table 30 & Chart 25)

TABLE 30 CORRELATION OF PROGESTERONE RECEPTOR AND BMI 1 GENE EXPRESSION

PR	N	Mean relative concentration of Bmi 1 mRNA	Standard Deviation	Mann whitney U test
Negative	29	2.26	6.666	t=0.71 P=0.47
Positive	31	4.19	12.669	

Higher mean Bmi 1 mRNA concentration was associated with Her 2 neu negative group when compared to that of Her 2 neu positive group. The inverse correlation was found to be statistically significant (p=0.05). (Table 31 & Chart 26)

TABLE 31 CORRELATION OF HER 2 NEU AND BMI 1 GENE EXPRESSION

HER2NEU	N	Mean relative concentration of Bmi 1 mRNA	SD	Mann whitney U test
Negative	38	3.52	10.826	Z=1.97P=0.05
Positive	18	2.50	8.118	

TABLE 32 CORRELATION ANALYSIS OF BMI 1 GENE WITH KNOWN PROGNOSTIC FACTORS

BMI 1 gene	Age	Size	Grade	Node status	ER	PR	HER2NEU
Pearson Correlation	-.108	-.230	0.132	0.054	-.113	-.014	-0.060
Sig. (2-tailed)	.413	.05*	0.316	0.681	.388	.915	0.651

Interpretation for r-value

Pearson correlation coefficient is denoted by "r". "r" always lies between -1 to +1

0.0 – 0.2 poor correlation

0.2 - 0.4 fair correlation

0.4 - 0.6 moderate correlation

0.6 – 0.8 substantial correlation

0.8 - 1.0 strong correlation

TABLE 33 CORRELATION OF MOLECULAR SUBTYPES OF INVASIVE DUCTAL CARCINOMA NOS AND BMI 1 GENE EXPRESSION

Subtype	N	Mean	Std. Deviation	Kruskal wallis H test
Luminal A	27	3.9531744	12.70713437	Z=9.99 P=0.05*
Luminal B	7	0.9897729	1.86134640	
Basal	5	2.7824000	4.15567357	
HER	11	3.4577845	10.36134463	
Unclassified	10	1.3013550	2.62116660	
Total	60	2.9770883	9.66183337	

The higher mean relative concentration of Bmi 1 mRNA was associated with luminal A subtype and Her 2 neu subtype. The correlation was found to be statistically significant (p=0.05). (Table 33 & Chart 27)

The study population was followed up till October 2010 for a maximum period of 17 months. Follow up data was available only for 34 out of 60 cases. 25 patients received chemotherapy while 6 of them defaulted before completion of the course. 7 patients received hormonal therapy and 10 patients received radiotherapy. 3 of the Her 2 type tumors presented with metastatic deposits in lung, chest wall and ribs while 2 of the luminal A subtype patients presented with lytic lesion in the skull bone and with recurrent cancer in opposite breast.

DISCUSSION

Breast cancer is the most frequent malignancy in both developed and developing countries at the present era. It accounts for 23% of all malignancies of women. India bears 8.3% of world burden of newly diagnosed breast cancer population with the incidence rate of 22 per lakh population[1].

In the present study, immunohistochemical evaluation and gene expression analysis were done in 60 invasive ductal carcinoma cases and an attempt has been made to correlate the Her 2 neu and Bmi 1 gene expression with the known prognostic factors of breast cancers.

Madras Medical College being a tertiary referral centre, the relative frequency of breast cancers among the other surgical cases was 2.1%. Among the entire breast specimen received for histopathological examination, 36.22% of the cases were reported to be malignant.

The age of breast cancer patients ranged from 25 years to 75 years with the mean age of 48.75 years. The highest incidence of breast cancer occurred in 40 to 50 year age group. This is in concurrence with the study done by Tanuja et al.[69]

The most common histological subtype of breast cancer in this study is Invasive ductal carcinoma NOS type. This is almost similar to the study of Dixon et al[70] and Elston CW Ellis et al[71]. The incidence of invasive ductal carcinoma NOS type is higher in Indian population than that of western population (86.13%) accounting for the worse prognosis (Table 34).

TABLE 34 - COMPARISON OF DISTRIBUTION OF HISTOLOGICAL SUBTYPES OF BREAST CANCERS

Histological subtypes	Dixon JM et al[70]	Omar Hameed et al[24]	Elston CW Ellis et al[71]	Current study
Invasive ductal carcinoma NOS	79%	>70%	40 to 75%	86.13%
Lobular carcinoma	10%	5 to 15%	5 to 15 %	1.26%
Mixed carcinoma	-		10 to 49%	1.68%
Mucinous carcinoma	2%	1 to 5%	2%	4.2%
Micropapillary carcinoma	-	2%	<2%	0.84%
Papillary carcinoma	1%	2%	1 to 2%	2.52%
Metaplastic carcinoma	<1%	rare	<1%	1.26%
Tubular carcinoma	6%	1 to 8%	2%	-
Cribriform carcinoma		1 to 3%	0.8 to 3.5%	0.84%
Medullary carcinoma	2%	1 to 7 %	1 to 7%	0.42%
Apocrine carcinoma	-	0.5 to 4%	0.4%	0.42%
Adenoid cystic carcinoma	-	rare	<0.1%	0.42%

In the study done by Dana Carmen et al[72], the incidence of breast cancer in premenopausal and postmenopausal women was 38.15% and 61.85% respectively.[72] In concurrence with the above mentioned study, the incidence of breast cancer in postmenopausal group was higher (61.67%) than that of premenopausal group (38.33%) in the present study. 1% of the total breast cancers were reported in males. 49 % of the breast cancers were found in left breast, 51% in right breast and 1 case was found to have bilateral breast cancer with apocrine carcinoma in right breast and invasive ductal carcinoma NOS in the left breast.

TABLE 35- COMPARISON OF DISTRIBUTION OF AJCC STAGING IN BREAST CANCERS

Stage	Madhuri et al	Katrina et al	Carey et al	Al moundhri et al	Current study
Stage I	36.3	46.9	39	6.94	6.66
Stage II	47.5	42.8	51	43.05	35
Stage III	11.3	10.4	8	47.22	55
Stage IV	4.9	NA	3	NA	3.33

Most of the cases presented in stage III followed by stage II which was similar to that of Al moundhri et al[73] who studied 72 breast cancers in Omani population and reported the increased incidence of higher stage tumors in their population when compared to the western studies done by Madhuri et al[43], Katrina et al[38] and Carey et al[10] in US women.(Table 35)

A higher proportion of T2 sized tumors (52 %) followed by T3 and T1 size tumors was seen (Table 36) similar to the study of Lobna Ayadhi et al[74] (Pakistan), Kakil Rassul et al[75] (Quatar) and Lakmini et al[76] (India).

TABLE 36 COMPARISON OF SIZE OF TUMORS

Size	Lobna Ayadhi et al	Kakil rassul et al	Lakmini et al	Current study
T1	12.9	20	14.5	23
T2	63.2	47.14	74	52
T3	23.9	32.86	11.5	25

The Grade II tumors were more frequent than other grades of breast cancers. This observation was similar to the study carried out by Katrina et al[38], Carey et al[10] and Madhuri et al[43] (Table 37).

TABLE 37 COMPARISON OF GRADE OF TUMOR

Grade	Katrina et al	Carey et al	Madhuri et al	Current study
Grade I	21.9%	25%	13.5%	29.12%
Grade II	42%	26%	40.8%	52.92%
Grade III	36.2%	49%	45.7%	17.96%

Nodal metastasis was noted in 48 % of the cases while it was not seen in 52%. This result correlates with the study done by Katrina et al[38] and Madhuri et al[43] who reported nodal metastasis in 36.7% and 40.5% of their cases.

58% of cases had lymphatic invasion which was similar to the observation made by Fiona blows et al[11], who reported 59.1% lymphatic invasion in his study.

Gloria Perio et al[77] conducted a study in 238 Spanish breast cancer patients and reported vascular invasion in 31.4 % and necrosis in 38.1% of his study population. In comparison with the above mentioned study, this study also showed the vascular invasion in 22% and necrosis in 52% of the cases.

There were lymphocytic infiltration in 65% and skin infiltration in 17% of invasive ductal carcinoma cases, in concurrence to the 33% skin infiltration reported in the study conducted by Chanda Bewtra et al[78].

TABLE 38 COMPARISON OF HORMONE RECEPTORS AND HER 2 EXPRESSION IN WORLD STATISTICS

	ER positive (%)	PR positive (%)	Her2neu positive (%)
Madhuri et al[43]	71.9	62.2	NA
Katrina et al[38]	77.9	66.8	21.7
D yassin et al[81]	53.1	44.9	79.6
Carey et al[10]	60	56	22
Kakil rassul et al[75]	51.42	44.28	26
Ratnatunga et al[80]	53.2	50	20.32
Lobna Ayadhi et al[74]	59.4	52.3	18.1
Taucher et al[84]	77.2	52.7	17.2
Rodriguez et al[85]	95.4	78.5	9.2
Glorio Perio et al[77]	73.6	67.1	27.8
Sughayer et al[86]	50.8	57.5	17.5
Lakmini et al[76]	45.7	48.3	19.1
Tanuja shet et al[69]	50.5	42	NA
Vikash kumar et al[79]	NA	NA	46.3
Current study	41	51	30

The expression of estrogen receptor, progesterone receptor and Her 2 neu was observed in 41%, 51% and 30% respectively. This proportion was similar to the studies conducted by Lakmini et al[76], Tanuja et al[69] and Vikash kumar et al[79] in Indian population, by Ratnatunga et al[80] in Srilankan population and by Kakil rassul et al[75] in Quatari population. (Table 38)

Her 2 neu over expression and hormone receptor negativity is more frequent in Indian population than the western population study data.

Luminal A is the most frequent subtype observed in this study in comparison to the other studies mentioned in the table 39. However, Her 2 subtype is more frequent and Basal type is less frequent in the Indian population when compared to the western population based studies.

TABLE 39 - COMPARISON OF MOLECULAR SUTYPES

	Luminal A %	**Luminal B %**	**Her 2 %**	**Basal %**	**Unclassified %**
D Carmen et al[72]	53.1	18.5	2.9	21	3.5
Dalal tamini et al[87]	25.1	3.4	18.6	10	42.8
Aitken et al[88]	56.2	5.23	5.7	18.6	14.3
Fiona Blows et al[11]	71	6	6	9	7
Chin hung lin et al[89]	61.77	8.75	11.77	12.84	4.86
Current study	45	12	18	8	17

CORRELATION OF HER 2 NEU EXPRESSION WITH OTHER KNOWN CLINICOPATHOLOGICAL PROGNOSTIC FACTORS

Ake borg et al (1990) studied 280 breast cancer patients in Sweden and found direct statistically significant relationship between Her 2 neu expression and number of lymph nodes involved, clinical stage, size, absence of hormone receptor expression while no association was seen with menopausal status.[90] He reported Her 2 neu as a significant predictor of early relapse and death in node positive patients.

D Yassin et al (2003) studied the Her family expression in 49 Egyptian breast cancer patients and reported statistically significant direct association between Her2 expression and positive lymph node metastasis, an inverse association with hormone receptor status and no association with size, grade and menopausal status.[81]

Susanne Taucher et al (2003) studied Her 2 expression in 923 Austrian breast cancer patients and demonstrated significant association of Her 2 expression in hormone receptor negative status, higher histological grade, larger tumor size and younger patients less than 50 years. There was no association between Her 2 expression and lymph node status.[84]

Mahmoud S Al Ahwal (2006) et al studied 145 Saudi Arabian breast cancer patients for Her 2 positivity using Immunohistochemistry and correlated with other histopathological features. He reported no correlation of Her 2 with age, race, subtype, size, grade, nodal status, lymphovascular invasion and progesterone receptor status. He found positive correlation of Her 2 neu only with estrogen receptor status.[83]

Ratnatunga et al (2007) studied hormone receptor and Her 2 neu expression in 124 Srilankan breast cancer patients and reported negative correlation of hormone receptors with Her 2 expression. There was no significant association found between Her 2 expression and tumor grade or age at diagnosis in his study.[80]

Lobna Ayadhi et al (2008) studied the correlation of Her 2 overexpression with clinicopathological parameters of 155 Tunisian breast cancer cases and reported no correlation of Her 2 with age, tumor size, histological subtype but it correlated well with tumor grade, lymph node metastasis and inversely correlated with hormone receptor expression.[74]

Mohammad Naeem et al (2008) studied Her 2 expression in 24 Pakistani breast cancer cases and demonstrated Her 2 neu positivity to be significantly associated with tumor size, negative ER & PR status while no association was found with subtype, number of lymph nodes involved and grade.[82]

In comparison with the above mentioned studies, this present study also showed no correlation of Her 2 neu expression with menopausal status, tumor size, stage, lymphovascular invasion, lymphocytic infiltration, skin infiltration or nodal status.

There was a direct significant association between Her 2 neu overexpression with histological grade of breast cancer which is in parallel to the study done by Susanne Taucher et al.[84]

There was a significant inverse correlation between Her 2 neu overexpression and Hormone receptor status as shown in Table 40.

TABLE 40 COMPARISON OF HER 2 NEU CORRELATION WITH HORMONE RECEPTOR STATUS

	Her 2 neu positivity % in hormone receptor status					
	ER +	**ER-**	**Association**	**PR+**	**PR-**	**Association**
D Yassin et al	73.07	91.3	SS	72.72	88.88	SS
Mahmud ahwal et al	19.5	80.5	SS	36.6	63.4	NSA
Mohamad naeem et al	9.09	76.92	SS	16.62	75	SS
Susanne Taucher et al	9.8	37.1	SS	8	25.4	SS
Lobna aayadhi et al	7.6	33.3	SS	12.34	24.65	SS
Ake borg et al	11	32	SS	11	37	SS
Ratnatunga et al	10.76	31.03	SS	9.8	30.65	SS
Current study	12	42.85	SS	19.35	41.37	SS

*SS-statistically significant association; NSA-no significant association

CORRELATION OF BMI 1 GENE EXPRESSION WITH OTHER CLINICOPATHOLOGICAL PROGNOSTIC FACTORS

Hyun Jo Youn et al (2005) studied the expression of Bmi 1 gene in 71 Korean patients using both IHC and PCR techniques and demonstrated 62.6% expression of the gene in breast cancer. He reported significant increase in the expression of Bmi1 mRNA in human breast cancers with strong correlation with lymph node metastasis and positive ER status.[65]

Javier Silva et al (2006) studied 134 Spanish breast cancer patients and reported Bmi 1 expression to be positively correlated with hormone receptor status and p53 negativity while no correlation was found with lymph node status, size or Ki67.

Feng yang et al (2007) studied the expression and significance of Bmi 1 in Chinese patients by using IHC and reported 82.8% expression in his study population. The expression of Bmi 1 positively correlated with higher clinical stage, lymph node metastasis while no correlation found with tumor size, ER, PR, Her 2 neu expression.[91]

Javier Silva et al (2007) studied 111 Spanish breast cancer patients for the expression of Bmi 1 gene in plasma by using RT PCR. He reported higher expression of Bmi 1 mRNA in cancer patients than healthy controls. The higher expression of Bmi 1 correlated with poor prognostic factors such as PR negativity and p53 positivity. He suggested to use Bmi 1 gene as a non invasive diagnostic marker of poor prognosis.[66]

Alexandra M Pieterson et al (2008) studied the expression of EZH2 and Bmi 1 gene expression in 295 stage 1 and stage 2 breast cancer samples in Netherlands. He reported higher expression of Bmi 1 gene with lesser grade, ER positivity and luminal A subtype while no association found with lymph node status or age at diagnosis.[92]

Young Jin Choi et al (2009) studied the expression of Bmi 1 protein in 960 breast cancer specimen from Korean patients and first reported the favourable prognostic association of this gene in breast cancer patients. He demonstrated the expression rate of Bmi 1 was significantly higher in patients with good prognostic factors such as small tumor size, negative lymph node, earlier stage, low nuclear grade and with positive hormone receptor status while no significant association was found between Bmi 1 expression and age or Her 2 status.[67]

Significant correlation of higher relative expression rate of Bmi 1 mRNA was observed in the present study with small size tumors and luminal A molecular subtype similar to the study done by Young Jin Choi et al[67] and Alexandra M Pieterson et al[92]. Correlation was also found with premenopausal age group, ER and Her 2 negativity. (Table 41) No correlation of Bmi 1 mRNA was found with lymph node status, grade, lymphovascular invasion, skin infiltration, lymphocytic infiltration, necrosis or PR status which is similar to the observation made by Alexandra M Pieterson et al[92].

TABLE 41 COMPARISON OF RELATIVE BMI 1 GENE EXPRESSION

Tumor characteristics		**AM Pietersen et al[92]**	**J Silva et al[66] (2006)**	**Current study**
Grade	Grade1	0.0067		2
	Grade 2	-0.15		2.4
	Grade 3	-0.41		4.8
ER	Positive	-0.08	1.349	1.5
	Negative	-0.56	0.715	4.2
PR	Positive	NA	1.447	4.1
	Negative	NA	0.68	2.2
Molecular subtype	Luminal A	0.11		3.9
	Luminal B	-0.246		0.9
	Her 2	-0.512		2.7
	Basal	-0.614		3.4
	Unclassified	-0.614		1.3

SUMMARY

60 breast cancer samples were subjected to immunohistochemistry and polymerase chain reaction to assess the Her2neu and Bmi 1 gene expression and the results were correlated with hormone receptor status and other known clinicopathological prognostic factors.

- The relative frequency of breast carcinoma among other surgical cases of Madras Medical College is 2.1%.
- The non neoplastic breast lesions form 30.24 %, benign tumors 33.54% and malignant tumors 36.22%.
- Breast carcinoma had a peak incidence in the age group of 41 to 50 years.
- The most common histological subtype was Invasive ductal carcinoma NOS type which constituted 86.13%.
- 52% of the breast cancer presented with T2 size (2 to 5 cm) tumors.
- Grade II was the most common grade observed accounting for 52.92%.
- Nodal metastasis observed in 52 %.
- Lymphatic invasion and vascular invasion were seen in 58% and 22% respectively.

- Skin infiltration was seen in 17 % of the tumors.
- Lymphocytic infiltration was observed in 65% of the tumors.
- Necrosis was found in 52% of the tumors.
- Estrogen and progesterone receptor expression was seen in 41% and 51% of the tumors.
- Her 2 neu over expression was found in 30 % of the study population.
- Immunohistochemical molecular subtyping revealed 45% luminal A type, 12% luminal B subtype, 18% Her 2 type, 7% basal type and 17% unclassified type of tumors.
- Her 2 neu over expression showed statistically significant association with high grade tumors and hormone receptor negativity.
- No association was found between Her 2 neu expression and menopausal status, size, nodal status, lymphovascular invasion, skin infiltration, lymphocytic infiltration or necrosis.
- Higher relative Bmi 1 gene expression was significantly correlated with premenopausal age group, small size tumors, ER negativity, Her 2 neu negativity and luminal A molecular subtype.

- No correlation was found between Bmi 1 gene expression and grade, lymph node status, PR status, lymphovascular invasion, skin infiltration, lymphocytic infiltration and necrosis.

- Bmi 1 gene expression of this study was not in concordance with the results of the studies conducted in Spain [66] and Netherlands [92] while higher expression was observed in small size tumors similar to the study conducted in Korean population.[67] The variation in expression could be hypothesized as due to many reasons including racial differences between European and Asian population which needs extensive and larger study sample .

- Further studies have to be conducted in future to define the clinicopathological importance of Bmi 1 gene expression and the possibility of using this gene as an novel prognostic marker in breast cancer patients.

CONCLUSION

The incidence of Invasive ductal carcinoma NOS was higher in this study. Many of our patients presented in younger age with large sized tumors accounting for aggressive nature of breast cancer in our population. Her 2 molecular types were more frequent and Basal type tumors were less frequent in the present study when compared to the western literature. Her 2 overexpression was common with high grade, hormone receptor negative tumors. Higher Bmi 1 gene expression was associated with premenopausal, small size, ER negative, luminal A subtype tumors in concordance to the study conducted in Korean population.[67]

Hormone receptor negativity and Her 2 neu overexpression is more common in breast cancers of Indian women than the western population. Due to high prognostic significance, Her 2 neu should be checked in all breast cancer patients so that positive cases can benefit by herceptin therapy and anthracycline based chemotherapy which can improve the survival in these patients.

The ethnical difference between European and Asian races may explain the variation in the expression of Bmi1 gene expression. However, further studies have to be conducted in future on a larger number of samples to assess the actual role of Bmi 1 gene expression in Indian population and to explore the possibility of using this gene as a novel prognostic marker in breast cancers.

CHART 1- AGE DISTRIBUTION IN BREAST CANCER

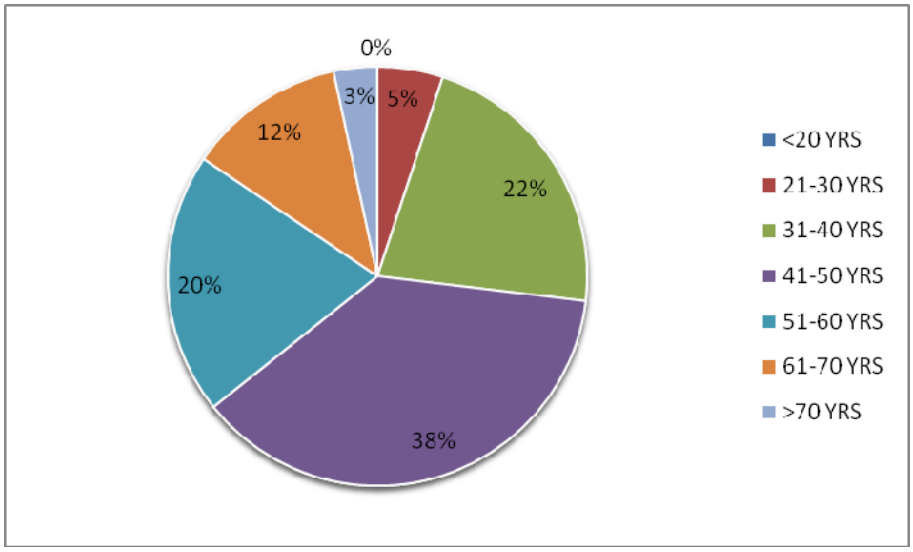


CHART 2 DISTRIBUTION OF HISTOLOGICAL TYPES IN BREAST CANCER

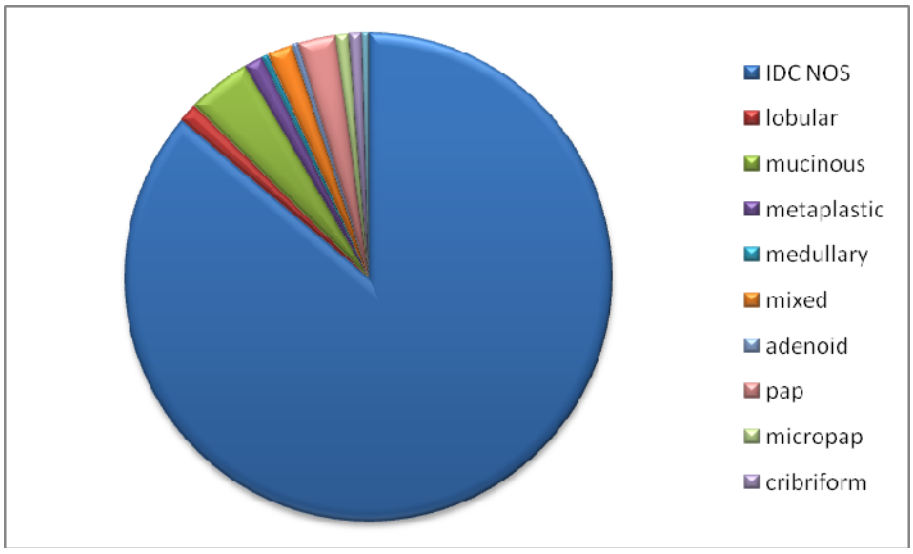


CHART 3 SEX DISTRIBUTION IN BREAST CANCER

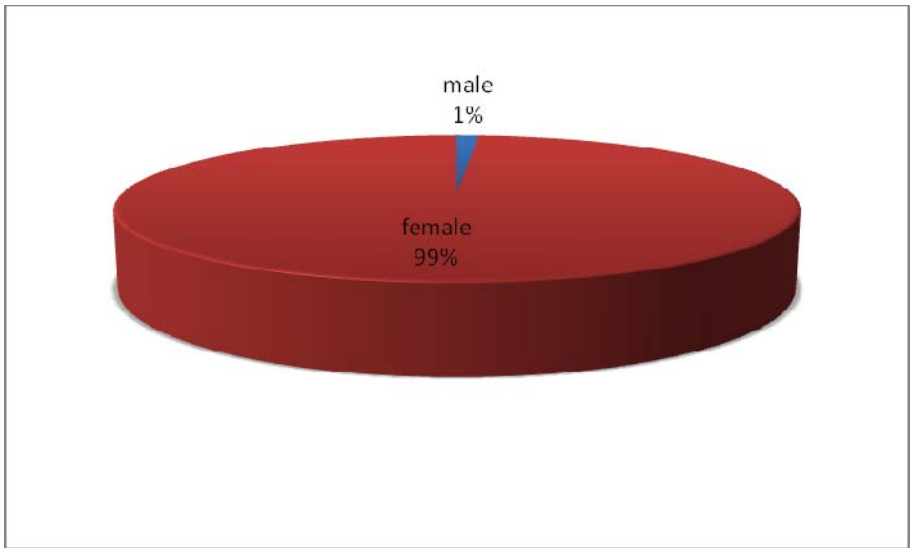


CHART 4

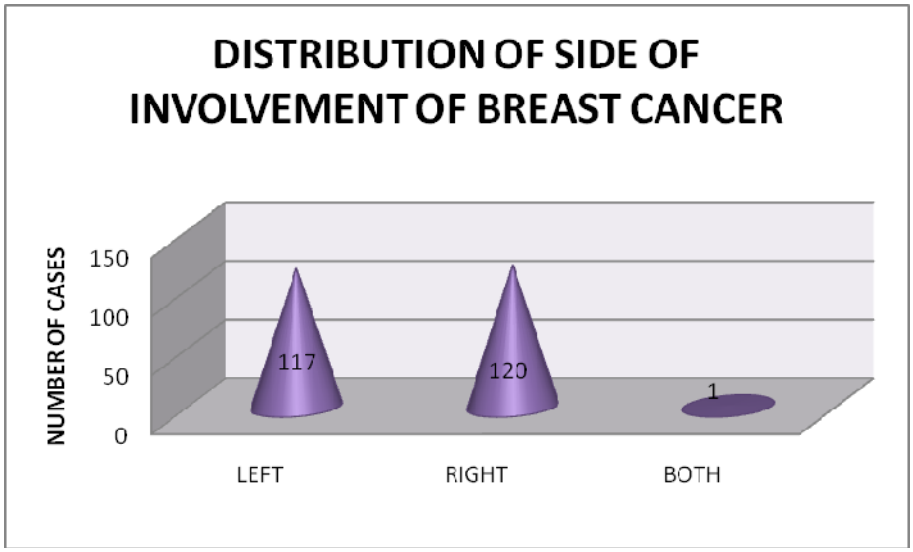


CHART 5

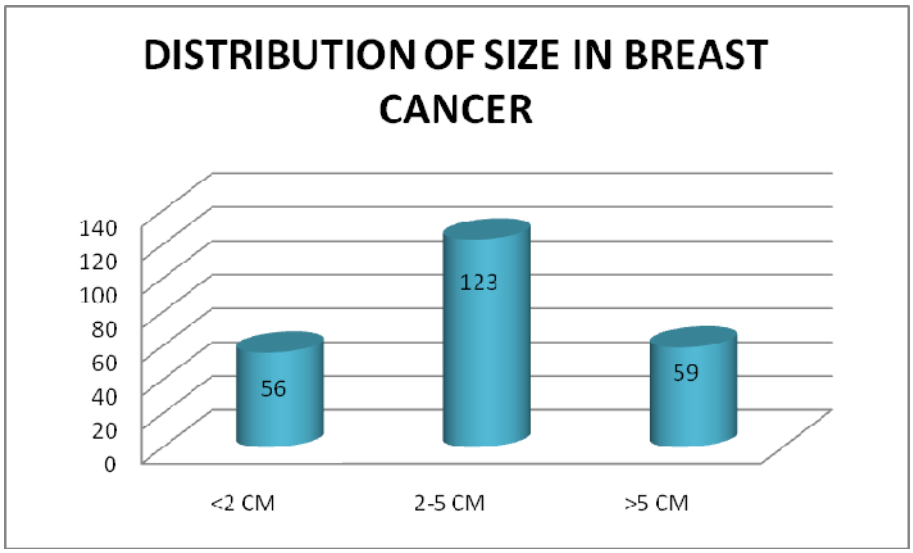


CHART 6

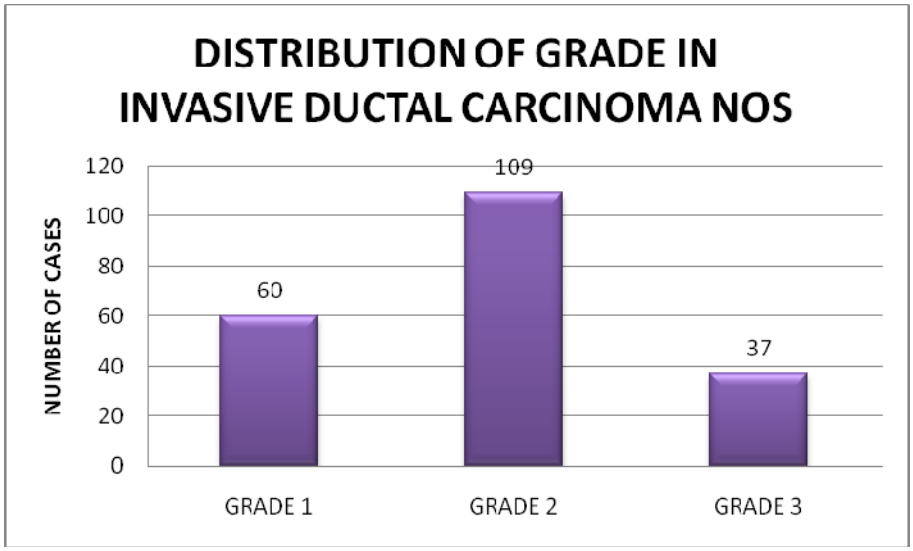


CHART 7

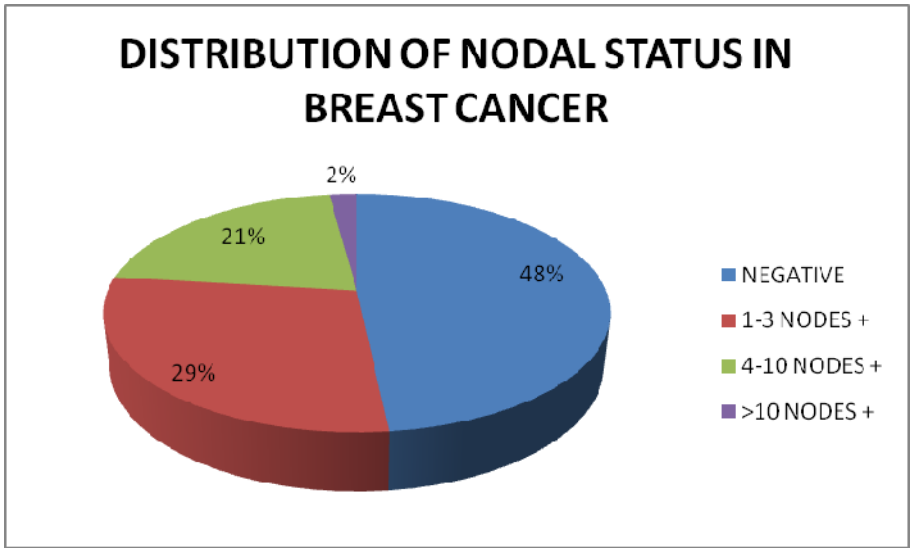


CHART 8

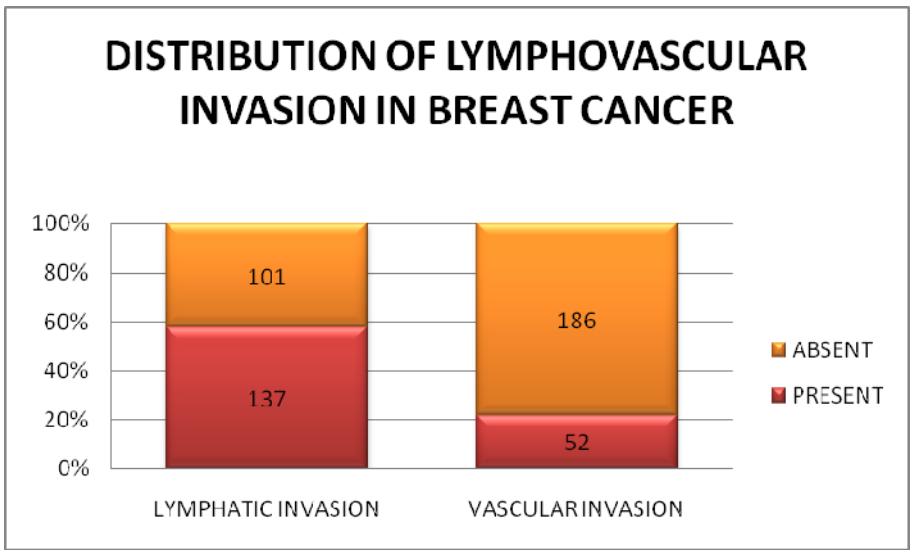


CHART 9 DISTRIBUTION OF SKIN INFILTRATION,

LYMPHOCYTIC INFILTRATION AND NECROSIS IN BREAST

CANCER

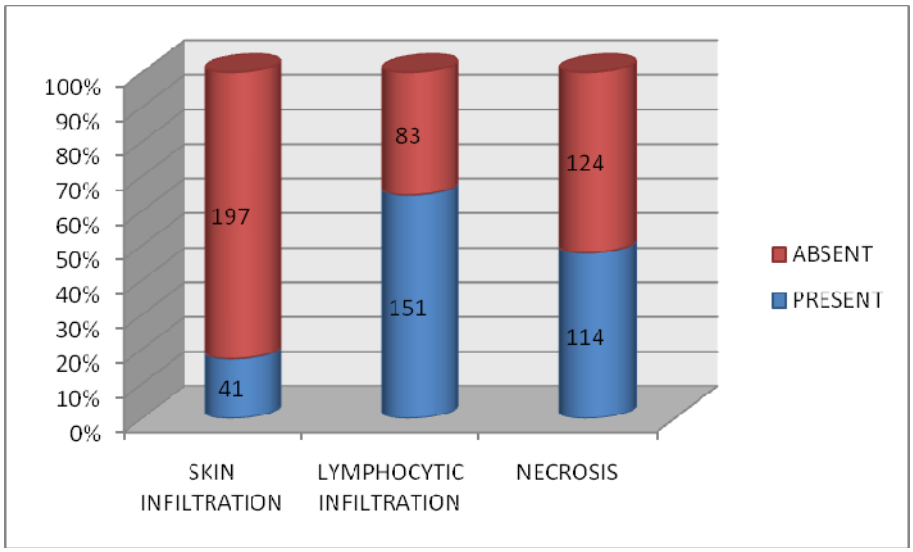


CHART 10

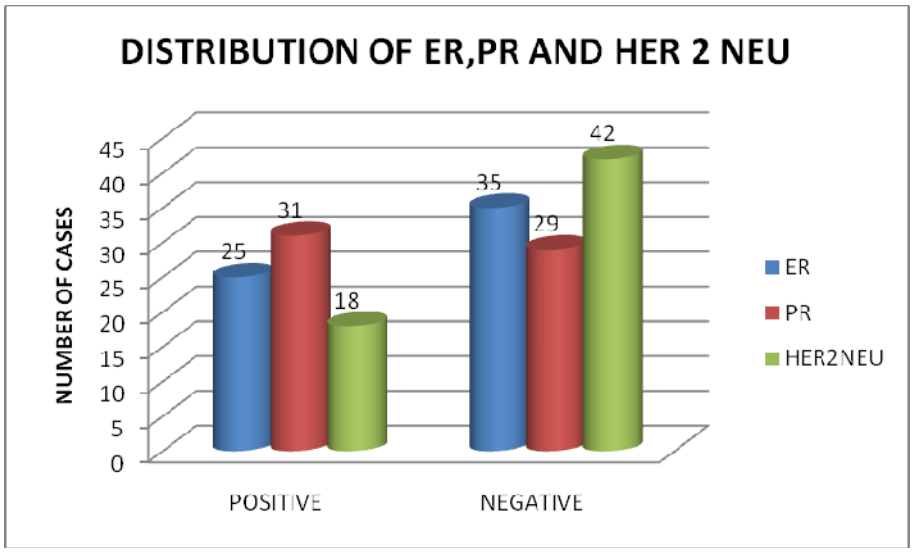


CHART 11

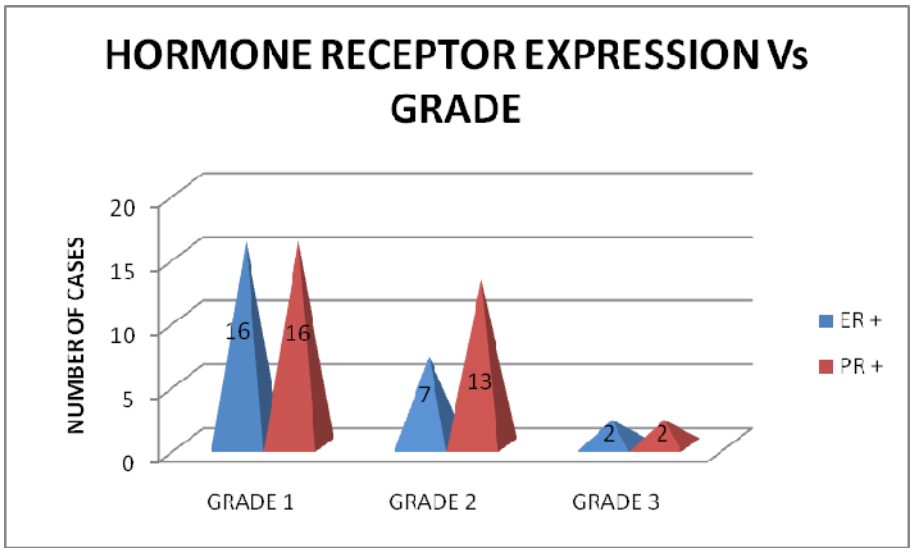


CHART 12

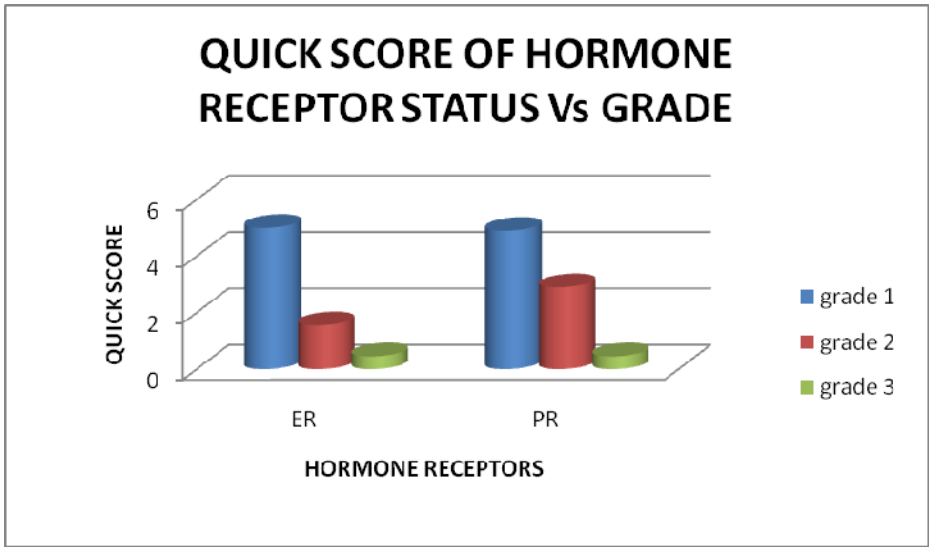


CHART 13

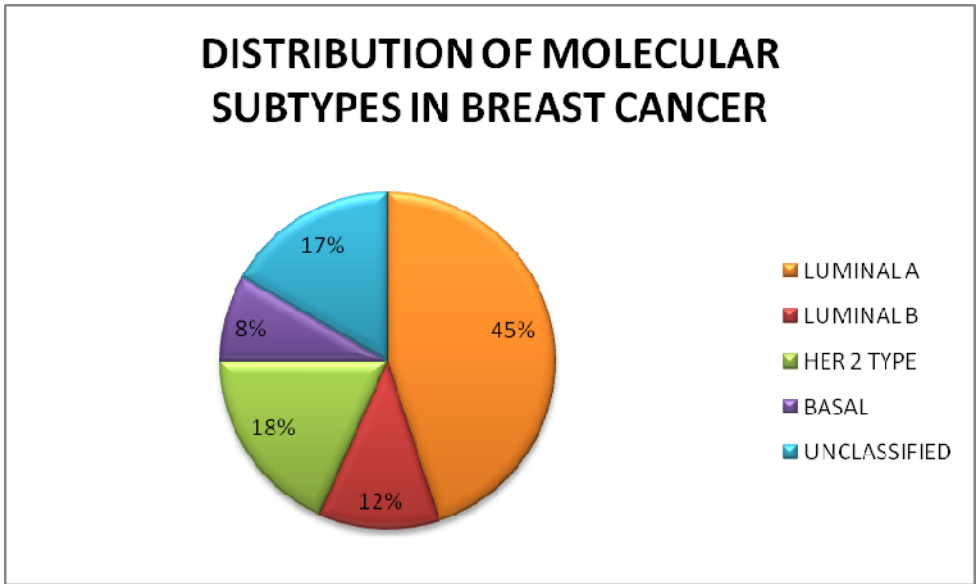


CHART 14

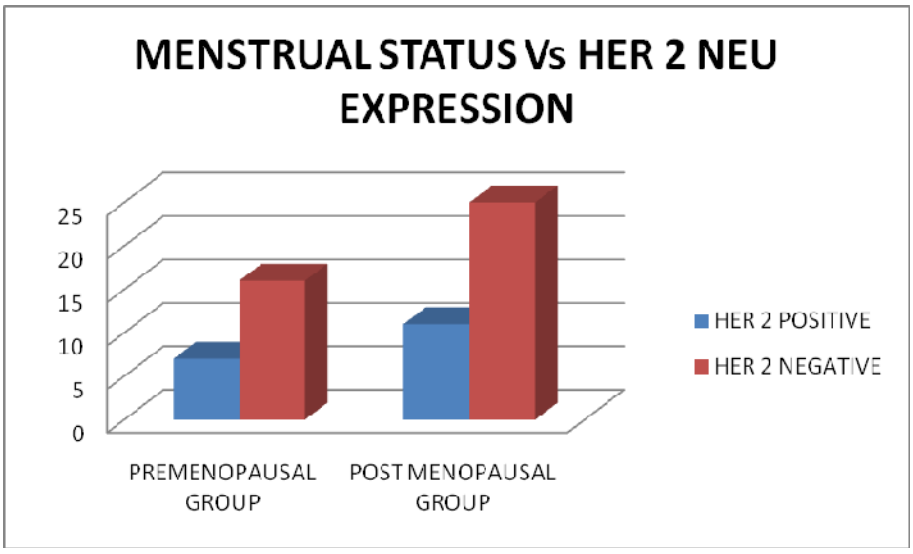


CHART 15

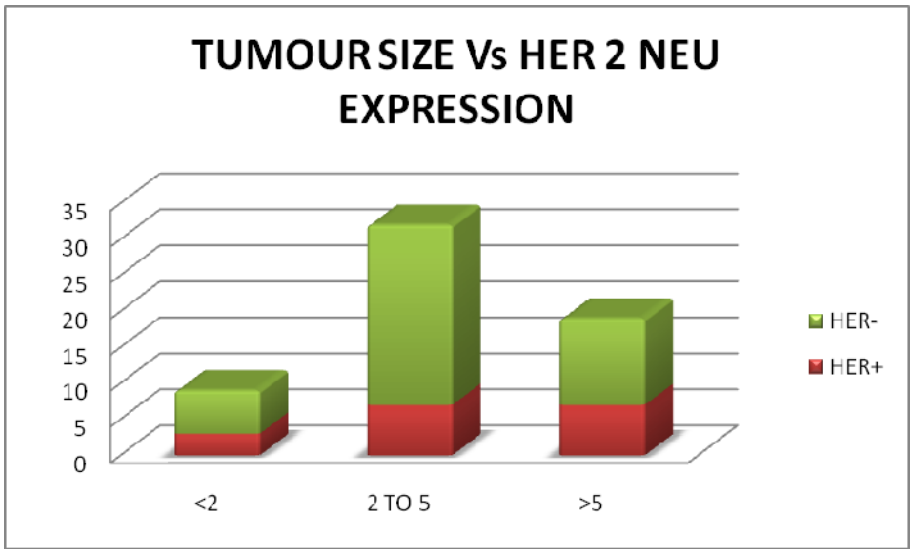


CHART 16

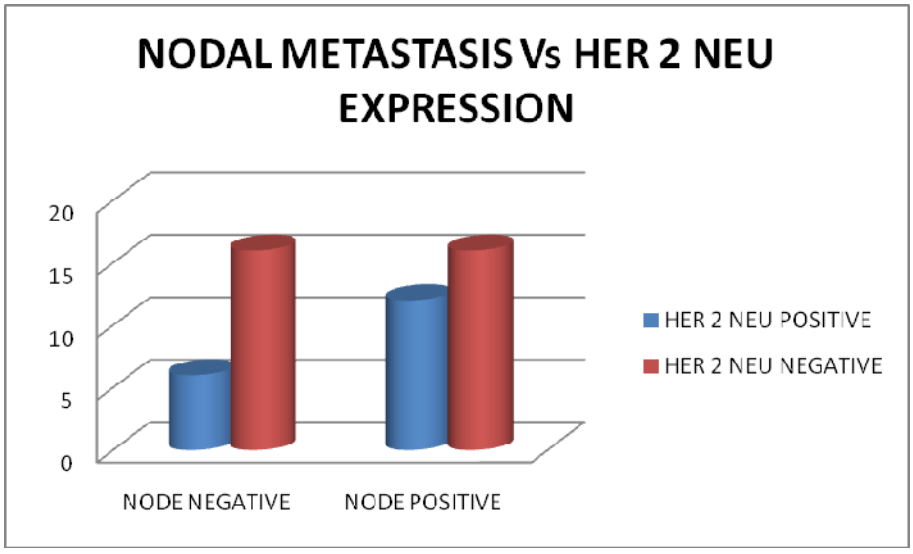


CHART 17

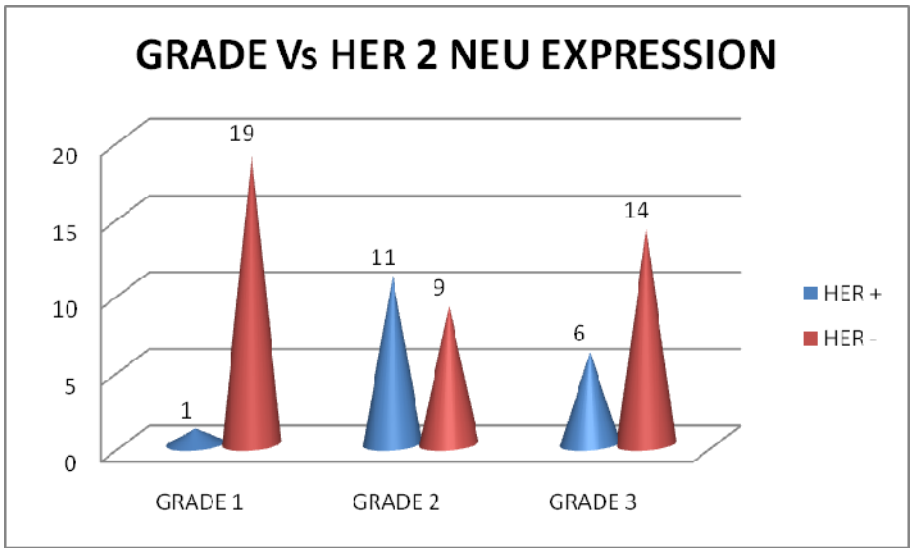


CHART 18

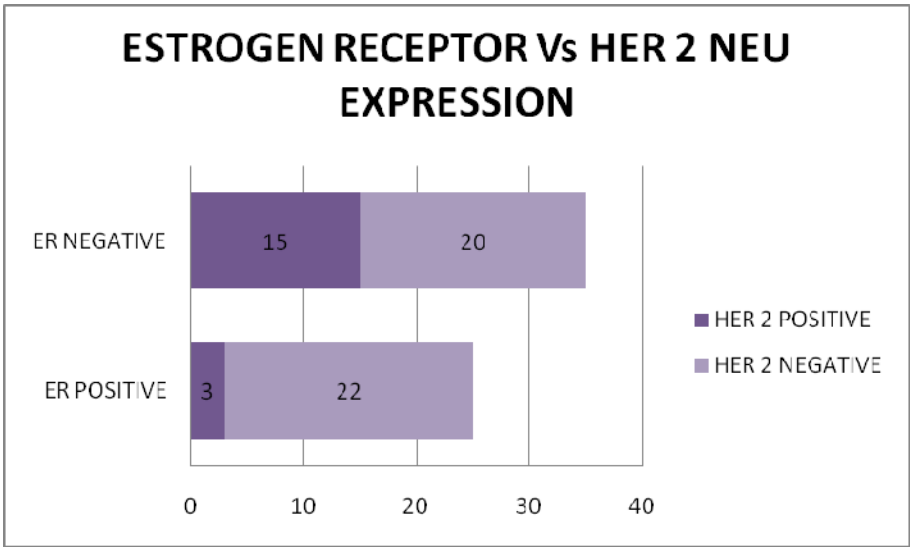


CHART 19

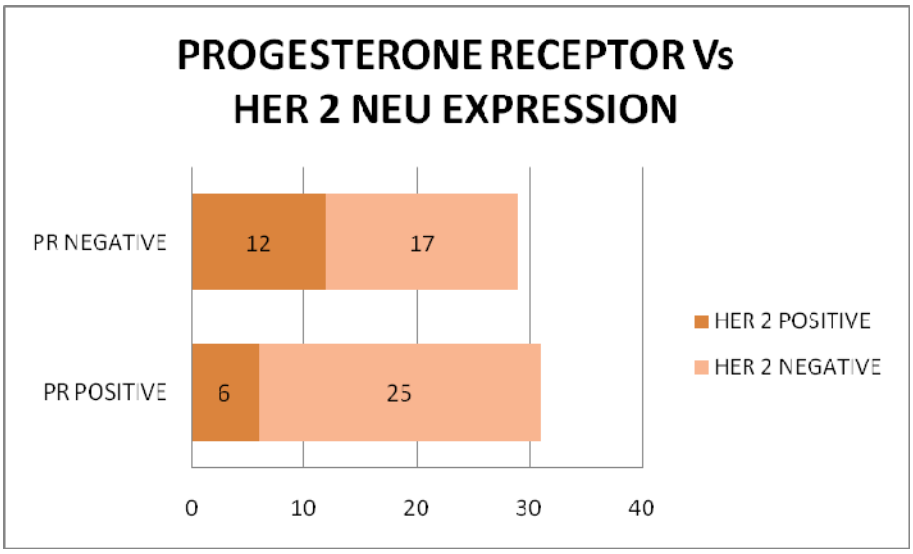


CHART 20

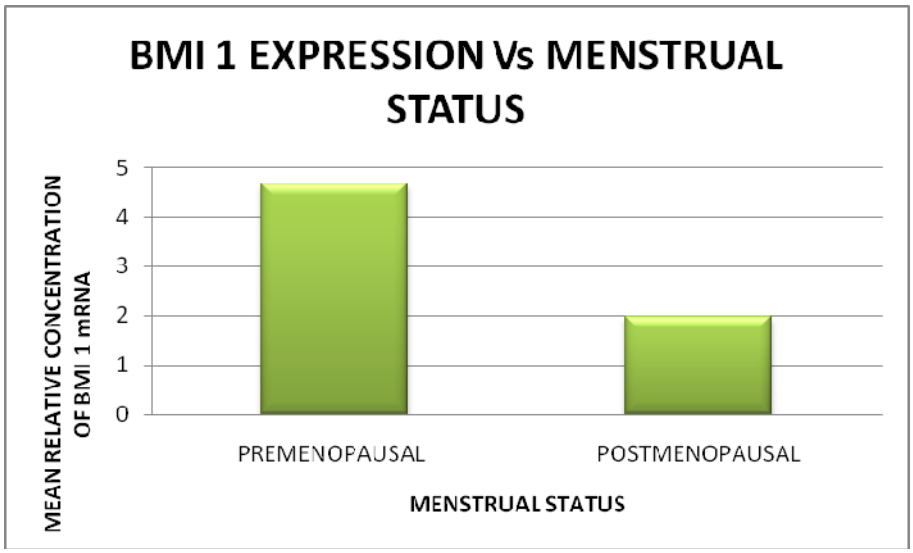


CHART 21

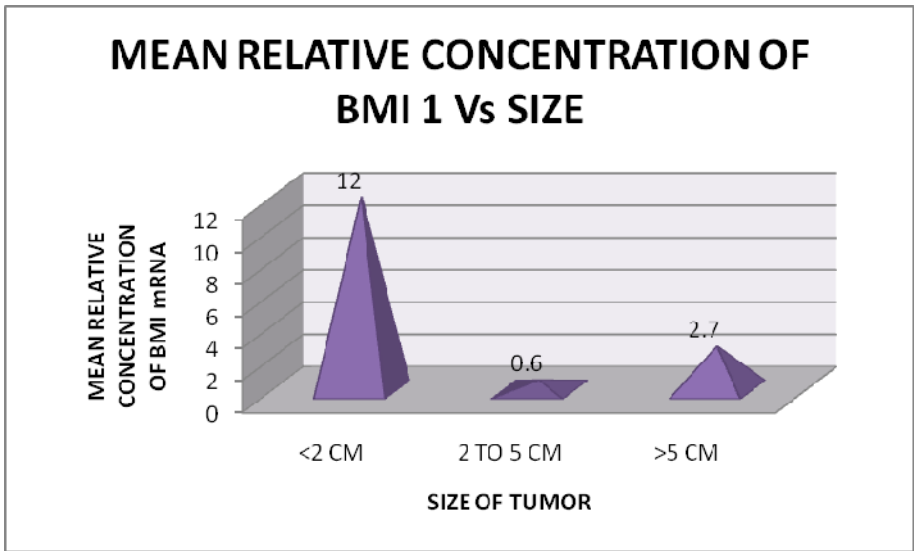


CHART 22

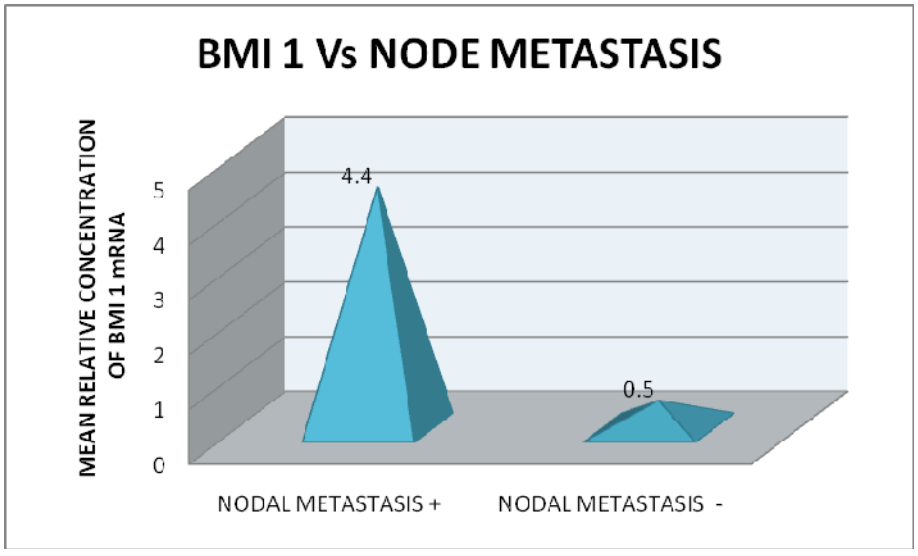


CHART 23

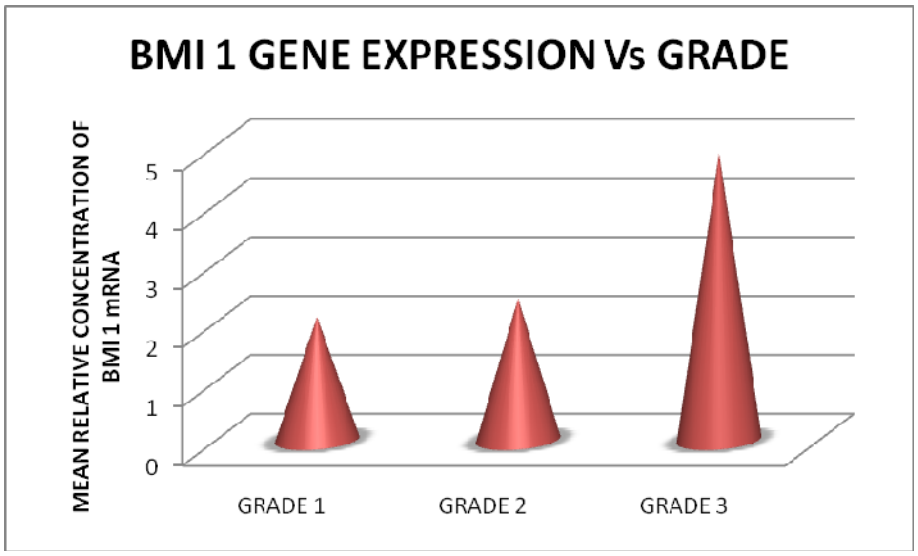


CHART 24

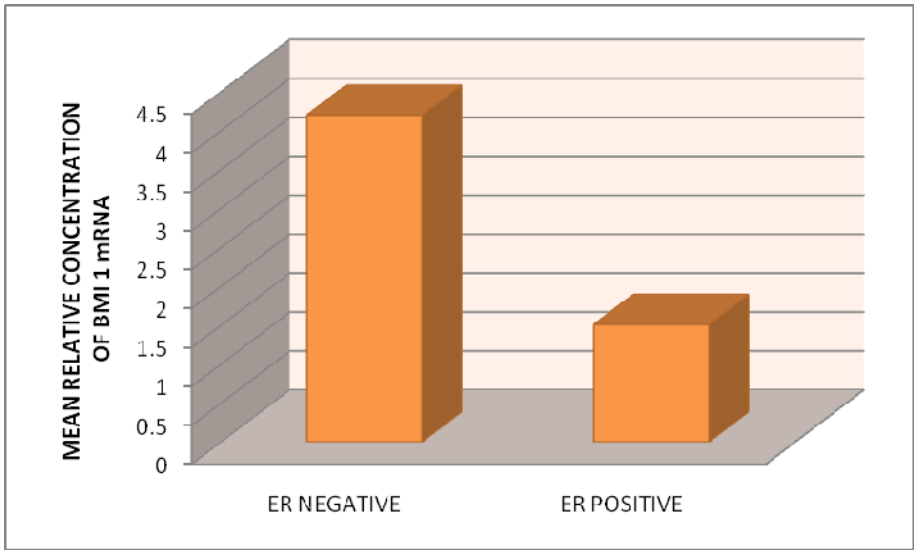


CHART 25

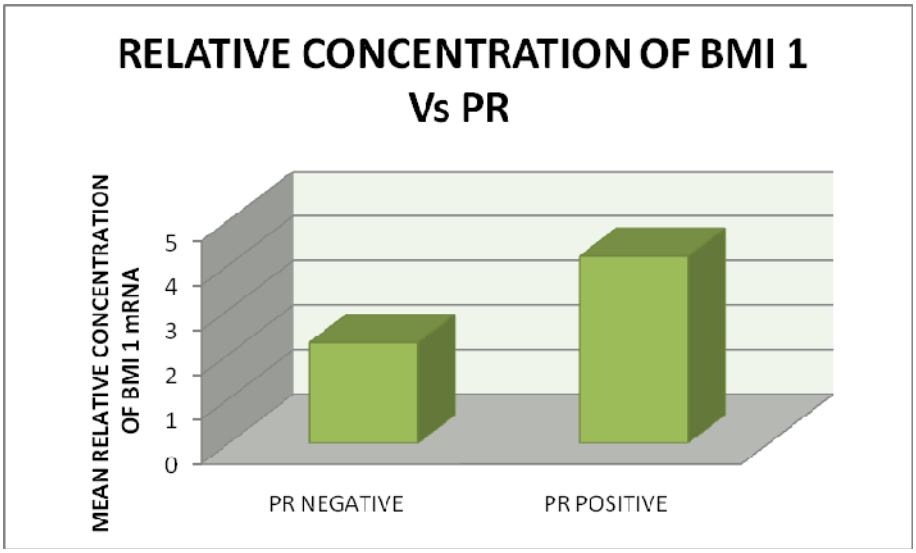


CHART 26

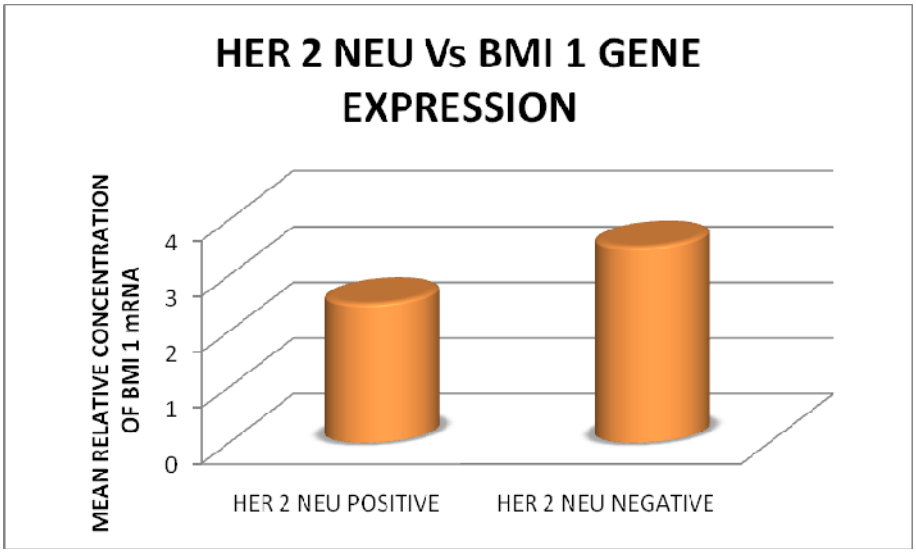


CHART 27

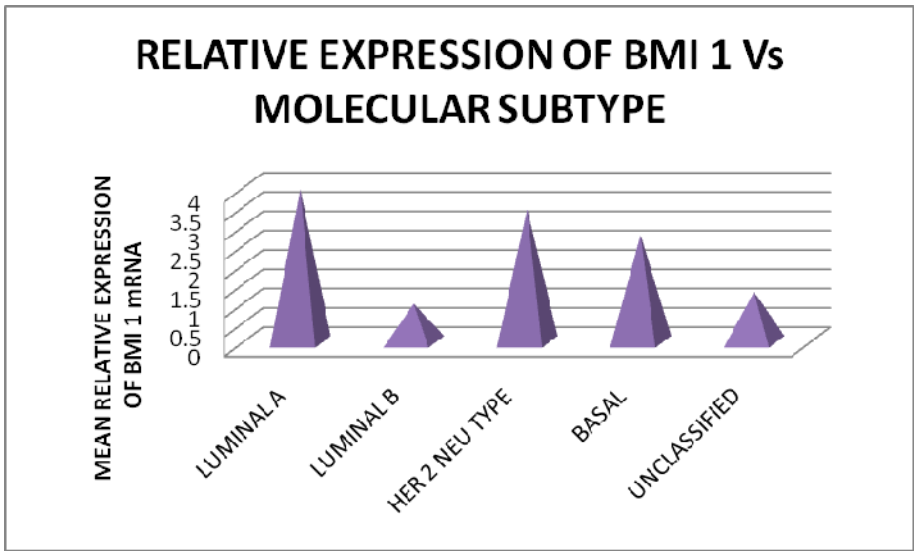
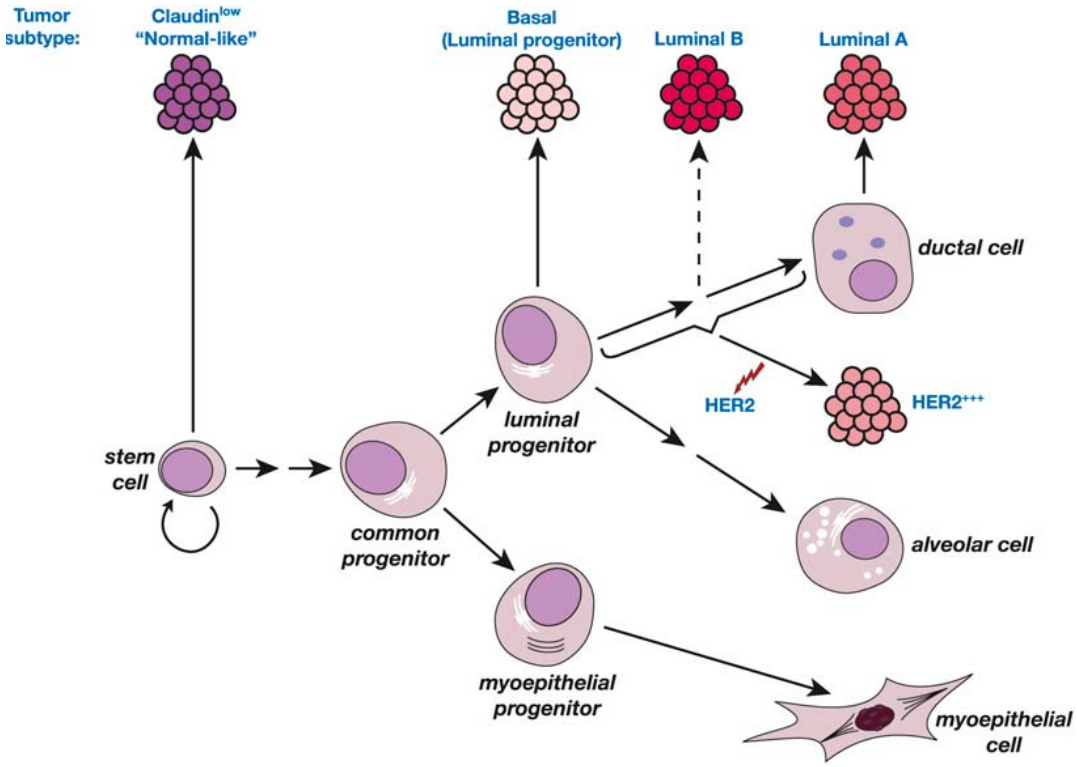

RELATIVE EXPRESSION OF BMI 1 Vs MOLECULAR SUBTYPE
MEAN RELATIVE EXPRESSION OF BMI 1 mRNA
4
3.5
3
2.5
2
1.5
1
0.5
0
LUMINAL A
LUMINAL B
HER 2 NEU TYPE
BASAL
UNCLASSIFIED

Figure 1- Schematic model of the human breast epithelial hierarchy and potential relationships with breast tumor subtypes[3]



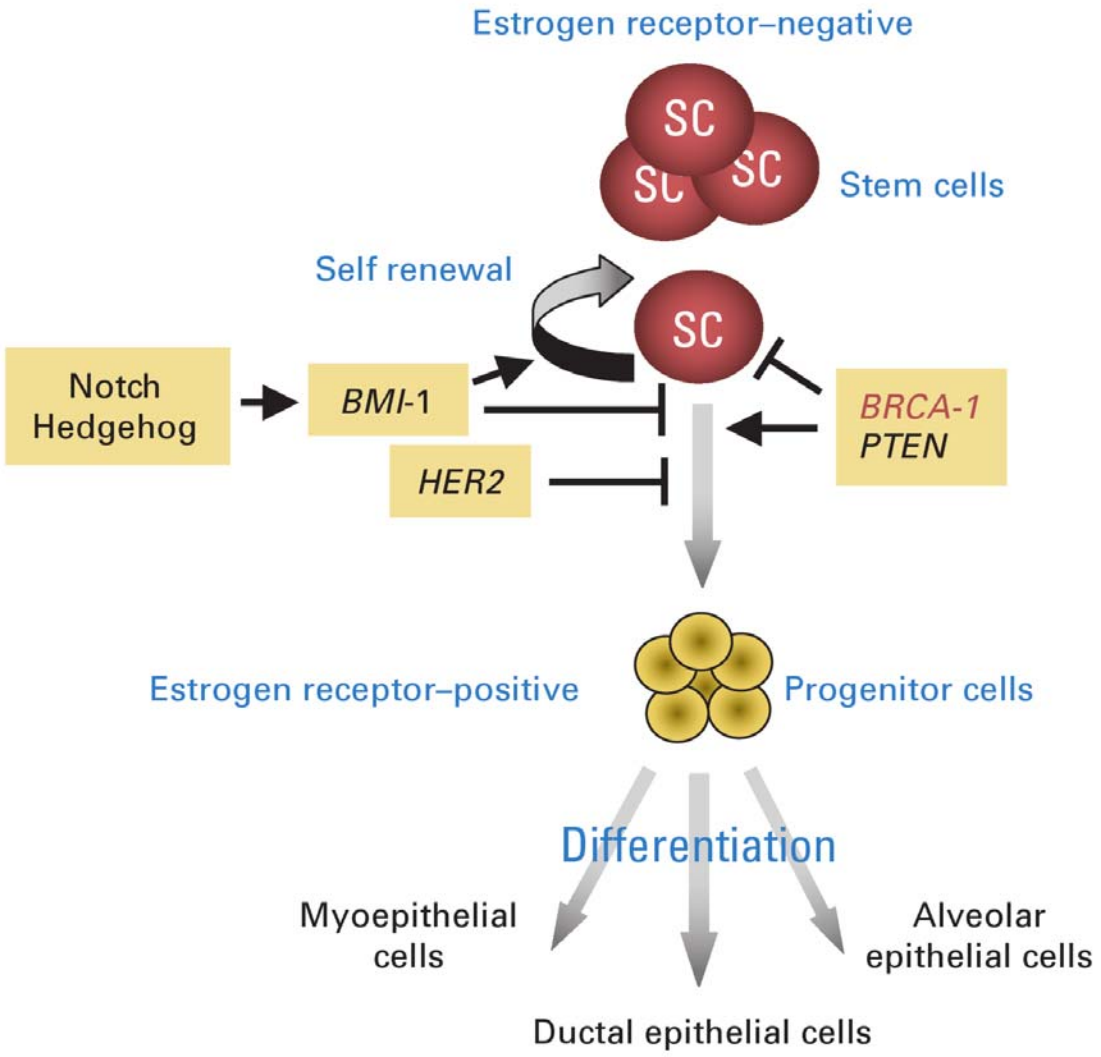


Figure 2: Self-renewal and differentiation pathways in breast stem cells.

Loss of BRCA1 &PTEN or activation of the human epidermal growth factor receptor 2, Notch, or Hedgehog pathways result in clonal expansion of stem cells providing targets for further carcinogenic events.[2]

FIGURE 3: IMMUNOHISTOCHEMISTRY KIT AND EQUIPMENTS

FIGURE 4: RNEASY KIT FOR TOTAL RNA EXTRACTION

FIGURE 5: EPPENDORF BIOPHOTOMETER TO MEASURE CONCENTRATION OF RNA

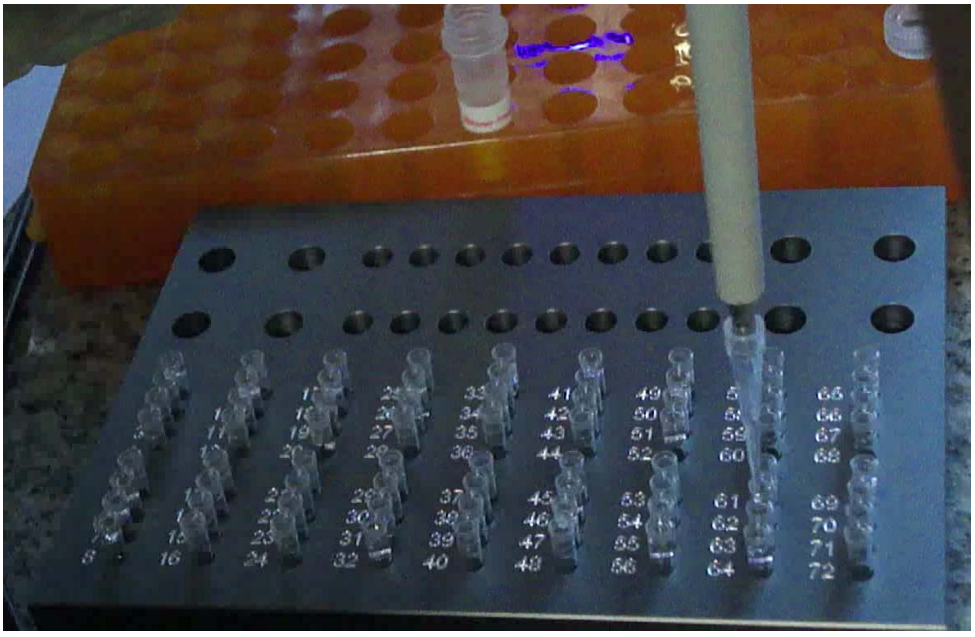


FIGURE 6: PCR TUBES LOADING WITH REACTION MIX

FIGURE 7: ROTOR GENE Q WITH ATTACHED SOFTWARE SYSTEM

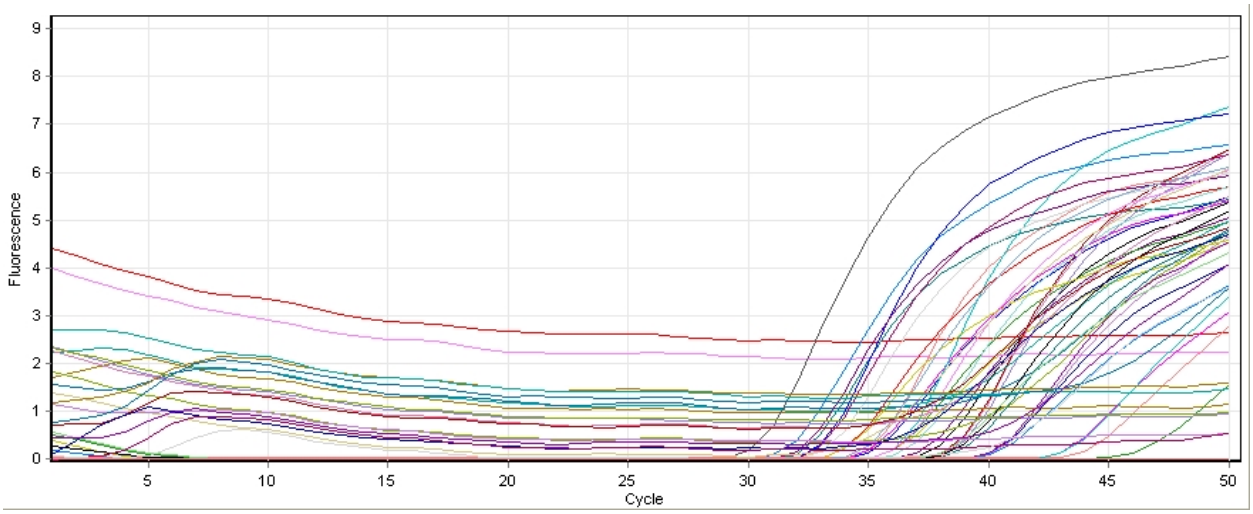


FIGURE 8: AMPLIFICATION PLOT OF FLUORESCENT SIGNALS (15 SAMPLES)
The amplitude of fluorescent signals increases with amplification. CT value is the cycle at which the amplification plot crosses the threshold. Lesser CT value refers to higher Bmi1 mRNA concentration in the sample.

DUCTAL CARCINOMA BREAST

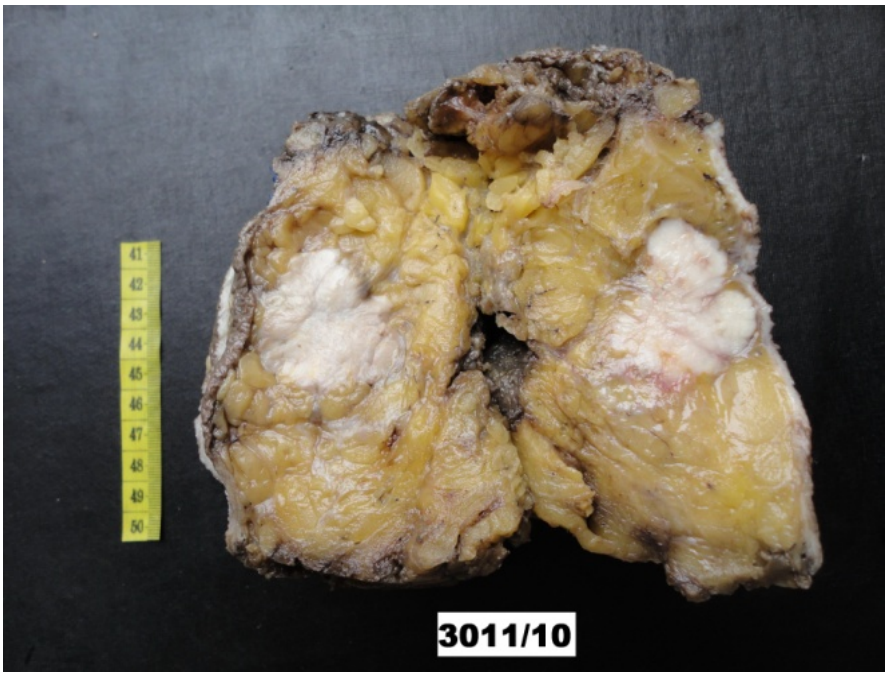


Figure 9: Ductal carcinoma – grey white growth with irregular margins

MUCINOUS CARCINOMA

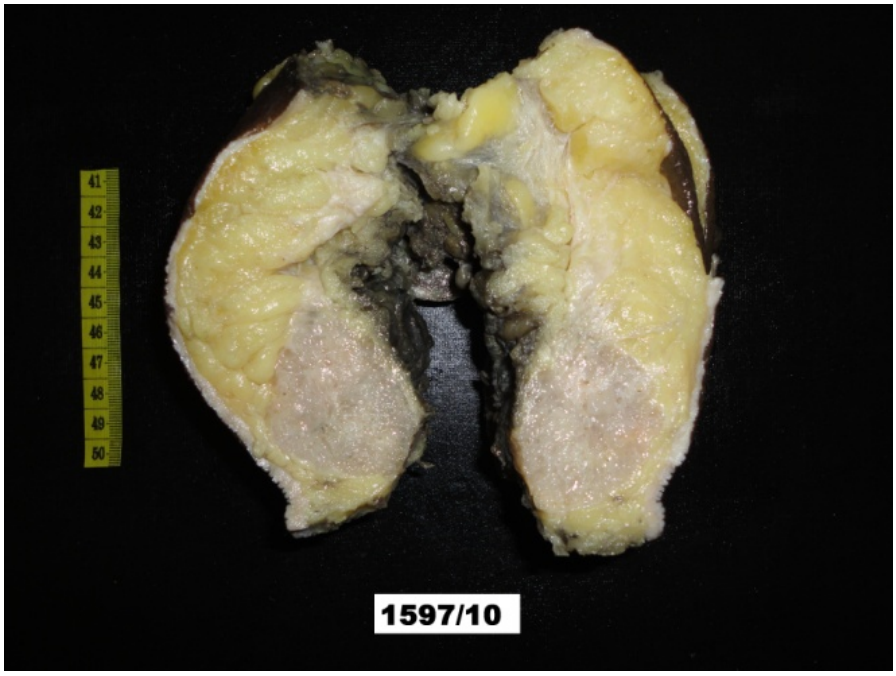


Figure 10: Mucinous carcinoma – glistening gelatinous growth

PAPILLARY CARCINOMA

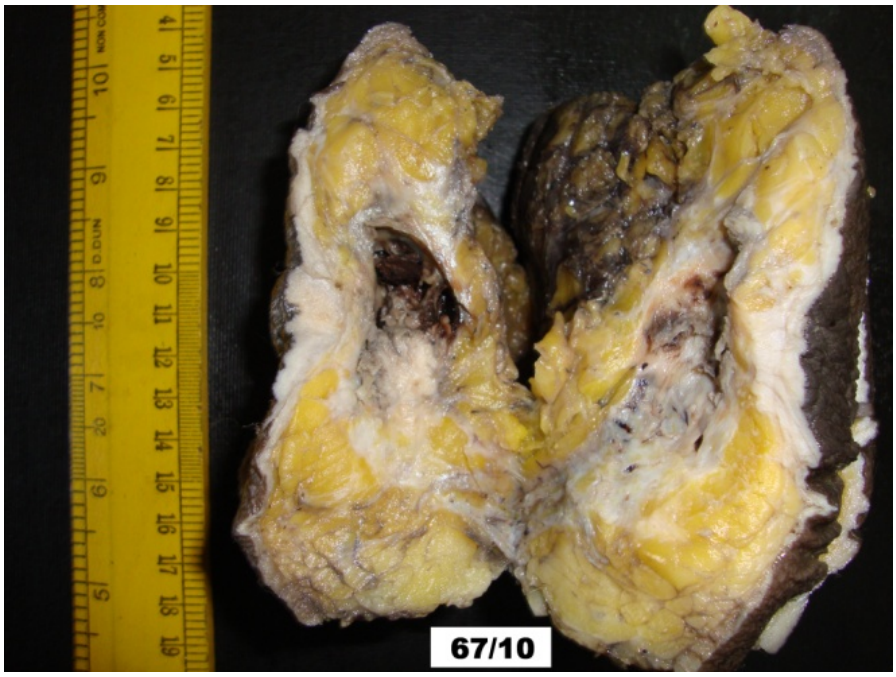


Figure 11: Cyst with a papillary growth in the wall presenting as mural nodule

MICROPAPILLARY CARCINOMA

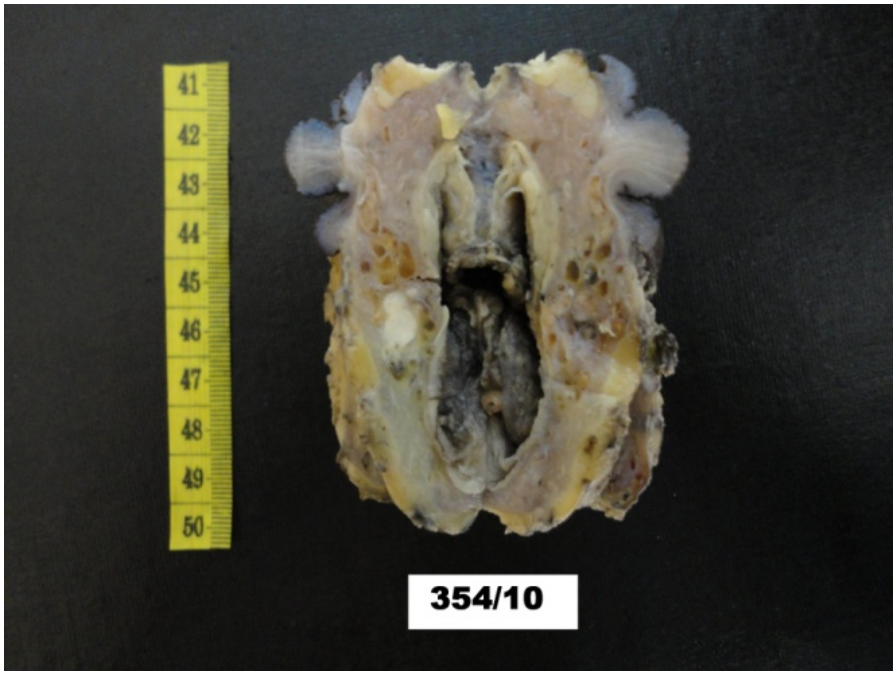


Figure 12: Small grey white growth with adjacent multiple tiny cystic spaces

MEDULLARY CARCINOMA

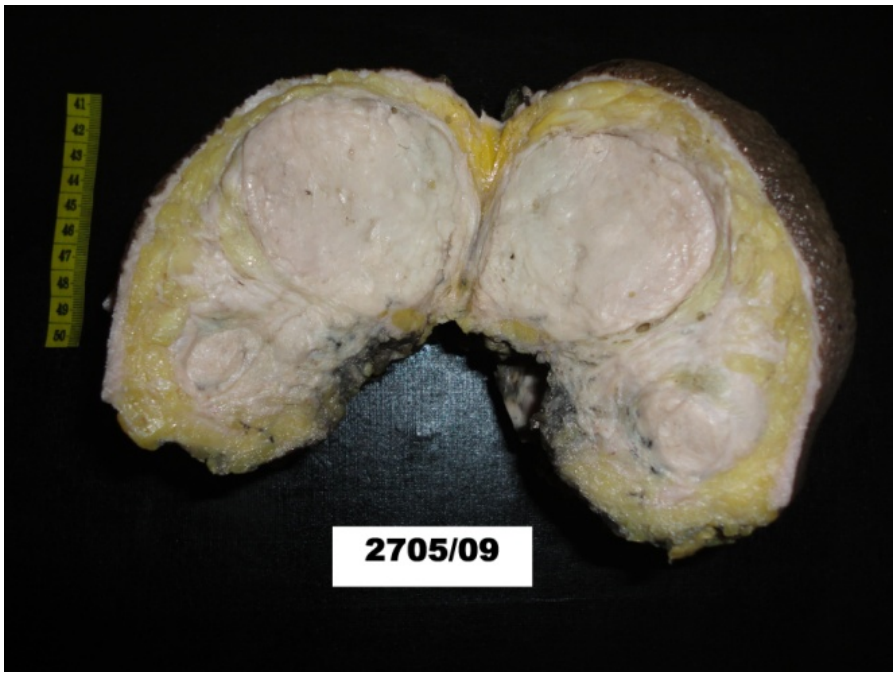


Figure13: Grey white fleshy growth with bulging cut surface

METAPLASTIC CARCINOMA

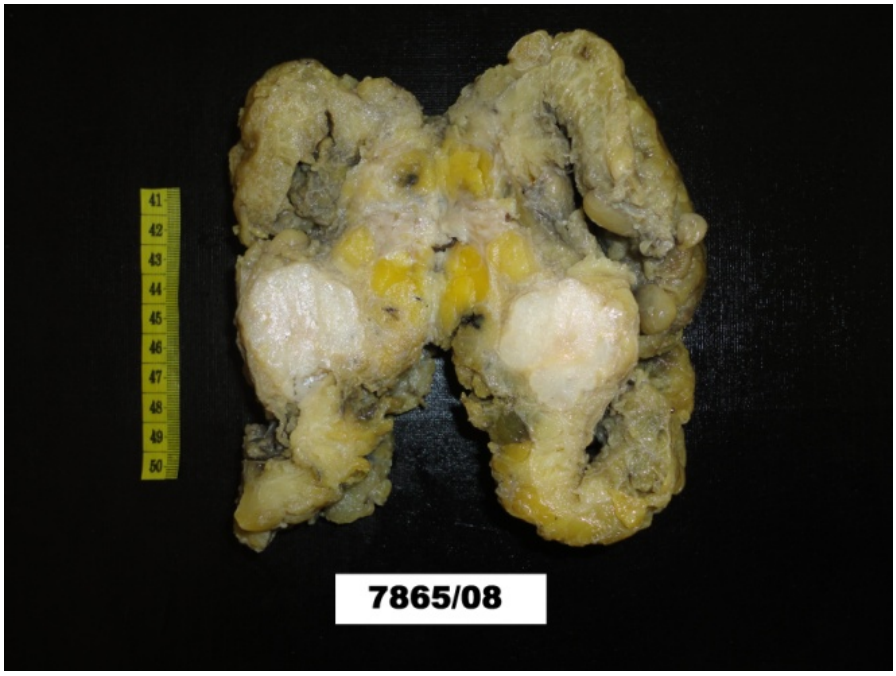


Figure 14: White glistening growth with grey blue cartilaginous areas

INVASIVE DUCTAL CARCINOMA NOS - GRADE 1

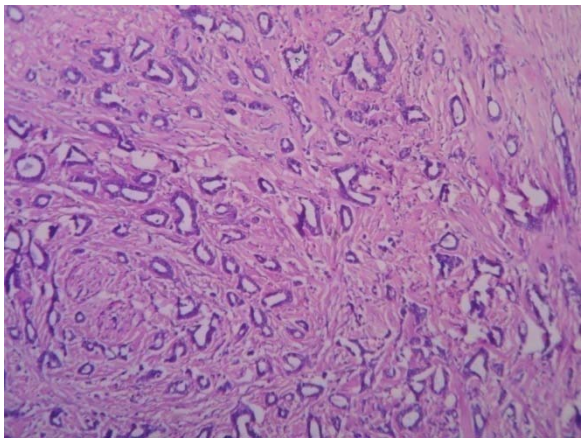


Figure15: Invasive ductal carcinoma NOS Tubule formations >90% tumor cells (100X) HPE 135/09

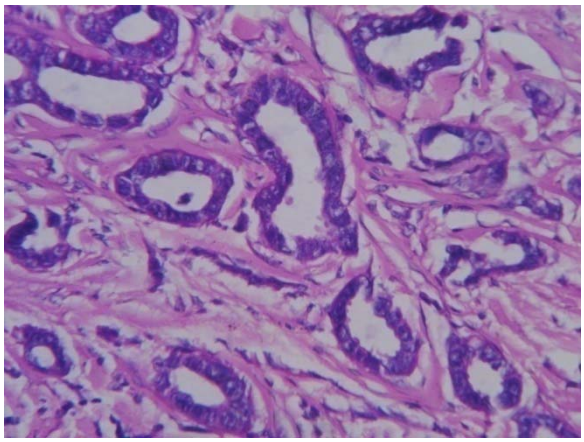


Figure16: Malignant ductal epithelial cells with mild nuclear pleomorphism & low mitosis HPE 135/09 (400X)

INVASIVE DUCTAL CARCINOMA NOS GRADE 2

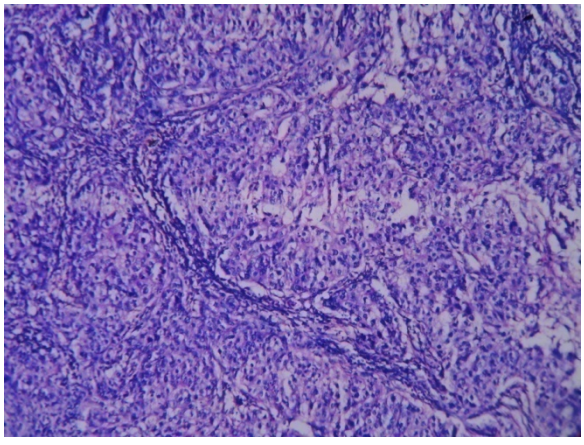


Figure 17: Sheets of malignant ductal epithelial cells, 10%tubule formation HPE 6029/09(100X)

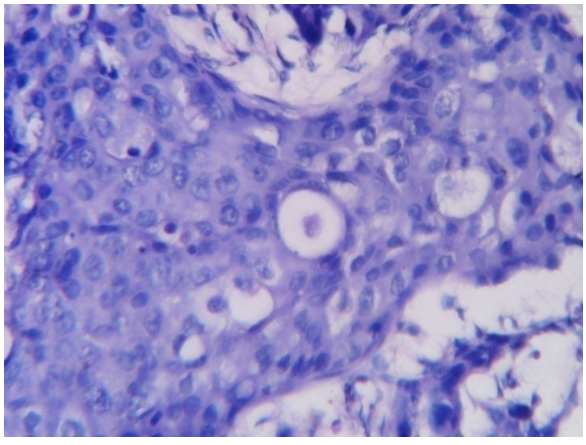


Figure 18: Malignant ductal epithelial cells in sheets, tubule 10%, mild nuclear pleomorphism and mitosis HPE 6029/09 (400X)

INVASIVE DUCTAL CARCINOMA NOS - GRADE 3

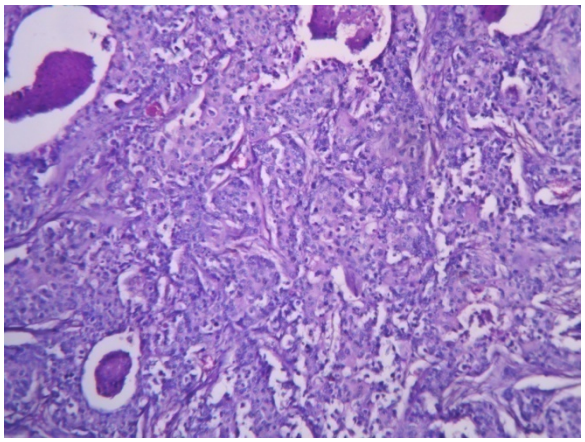


Figure 19: Malignant ductal epithelial cells in sheets with focal comedo necrosis HPE 453/09(100X)

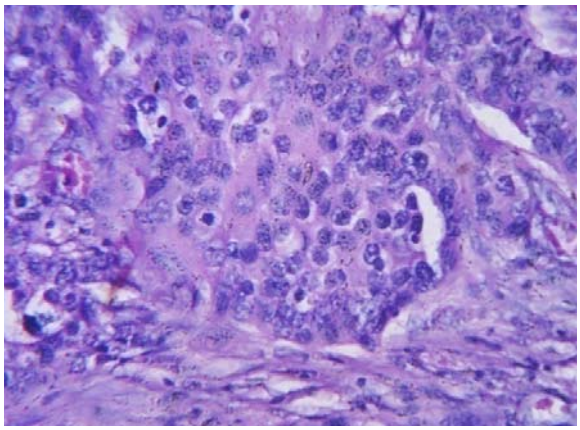


Figure 20: Malignant ductal epithelial cells with no tubules, marked nuclear pleomorphism, increased mitosis HPE 453/09(400X)

MUCINOUS CARCINOMA

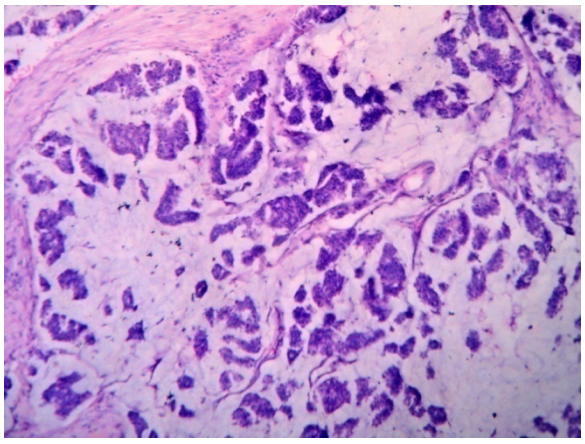


Figure 21: Tumor nests floating in mucin lakes HPE 1597/10(100X)

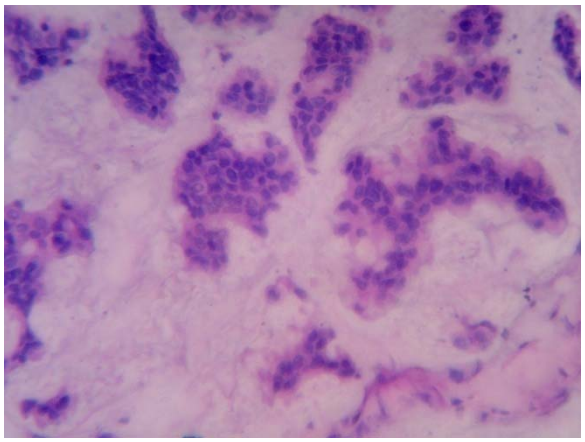


Figure 22: Malignant ductal epithelial cells with mild nuclear pleomorphism and no Mitosis HPE 1597/10 (400X)

LOBULAR CARCINOMA

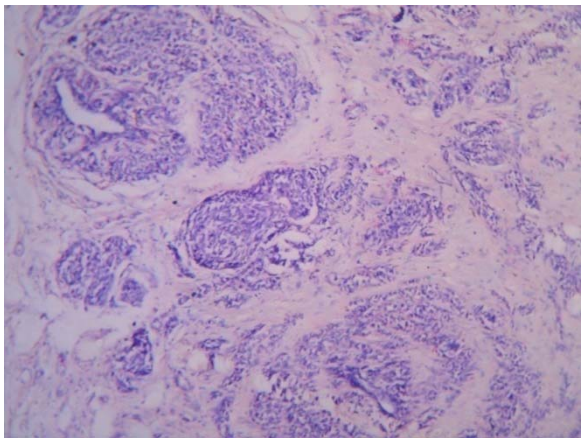


Figure 23: Tumor cells arranged in lobular pattern with pagetoid spread around ductal elements : HPE 291/10 (100X)

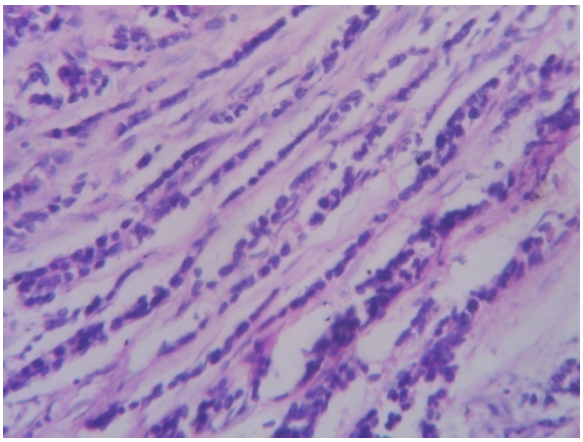


Figure 24: Tumor cells arranged in singles in Indian file pattern : HPE 291/10 (400X)

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MEDULLARY CARCINOMA

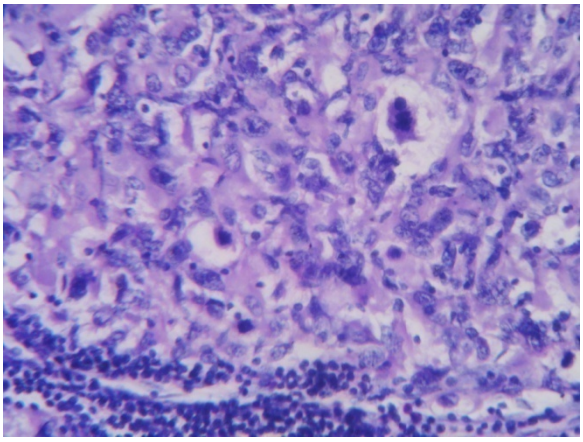

Figure 25: Tumor cells in sheets with lympho Plasmacytic infiltrate in periphery HPE 6156/09 (100X)

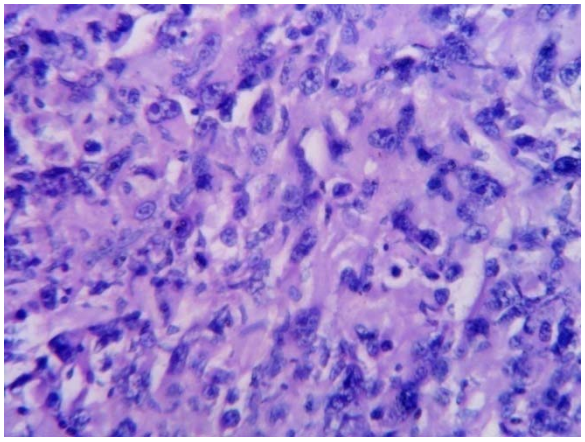

Figure 26: Tumor cells in syncytial pattern & lymphoplasmacytic infiltrate HPE 6156/09 (400X)

PAPILLARY CARCINOMA

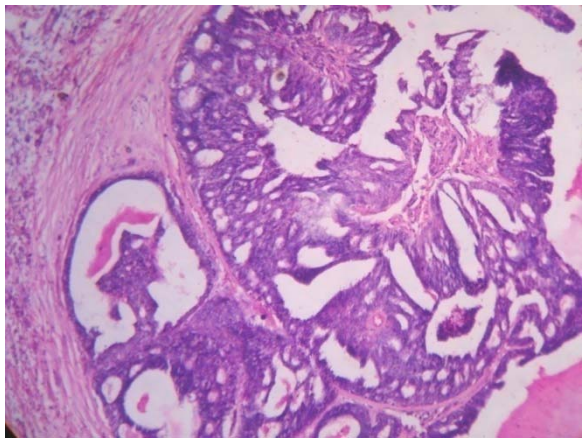

Figure 27: Tumor cells in papillary pattern and Infiltration : HPE 8635/08 (100X)

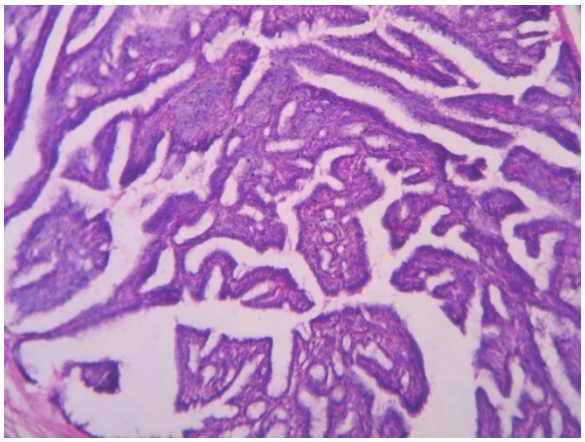

Figure 28: Tumor cells in delicate papillary pattern: HPE 8635/08 (400X)

INVASIVE CRIBRIFORM CARCINOMA

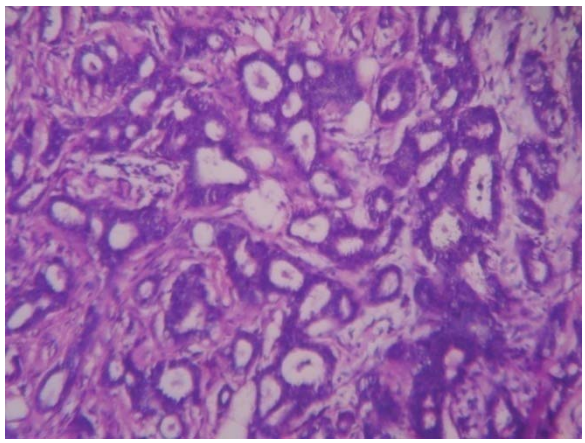

Figure 29: Tumor cells in cribriform pattern HPE 2640/10 (100X)

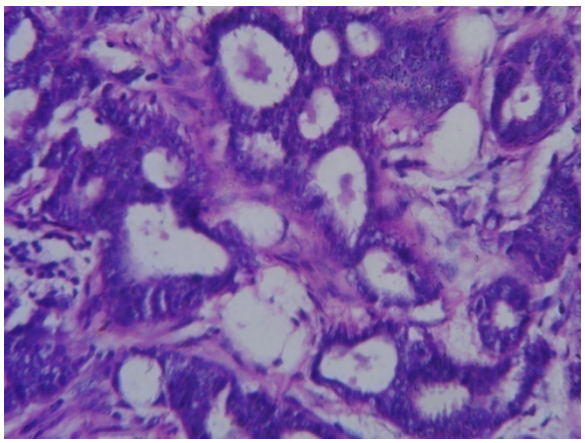

Figure 30: Malignant cells in cribriform pattern and infiltration: HPE 2640/10 (400X)

INVASIVE MICROPAPILLARY CARCINOMA

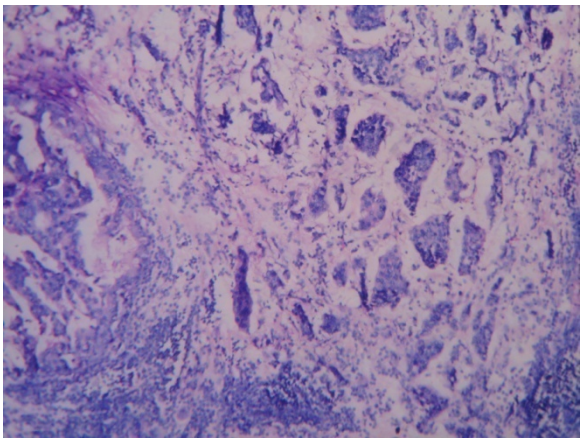

Figure31: Micropapillary DCIS with adjacent infiltrating neoplasm (100X) HPE 2683/10

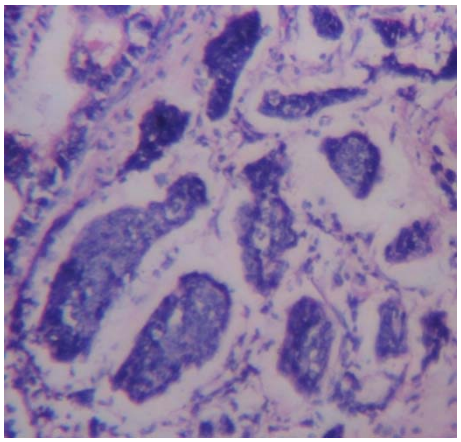

Figure 32: Clusters of tumor cells lying within clear stromal spaces HPE 2683/10 (400X)

ADENOID CYSTIC CARCINOMA

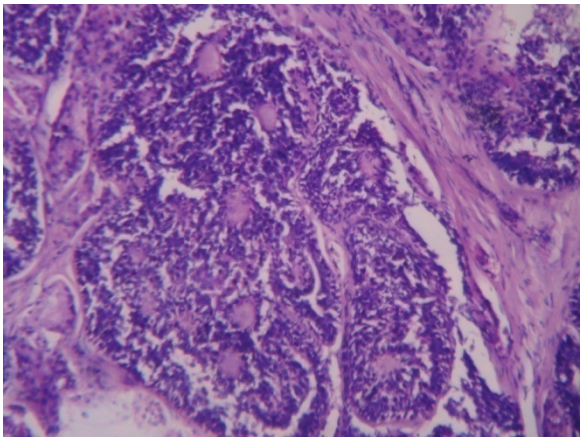

Figure 33: Tumor cells in cribriform pattern with eosinophilic material in spaces HPE 8343/08 (100X)

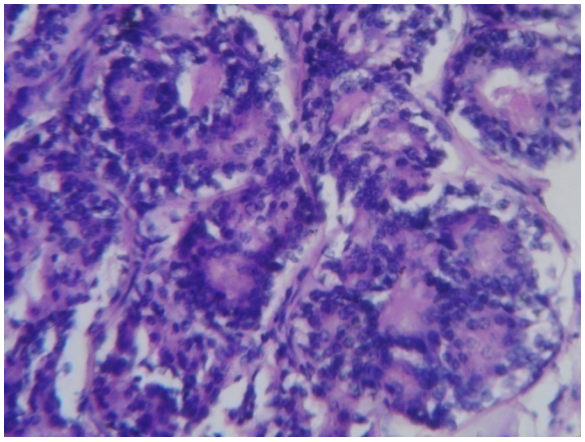

Figure 34: Basaloid tumor cells in cribriform pattern with eosinophilic basement membrane material in the lumen :HPE 8343/08 (400X)

APOCRINE CARCINOMA

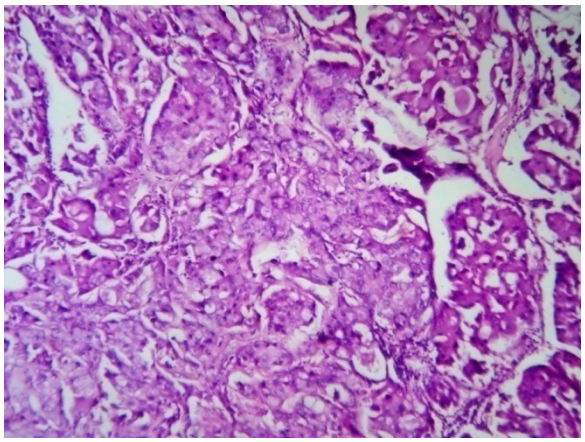

Figure 35: Apocrine cells in sheets and nests (100X) HPE 8385/08

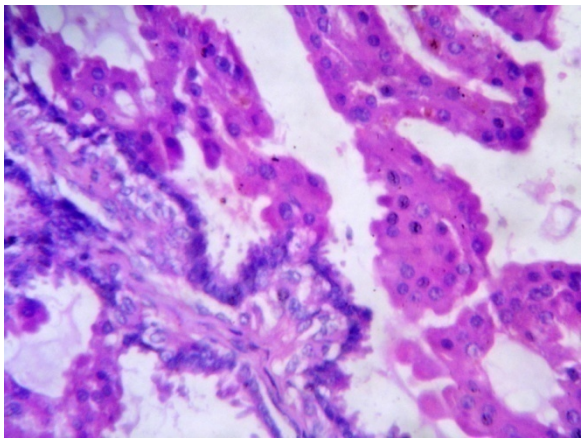

Figure 36: Apocrine cells with abundant granular eosinophilic cytoplasm (400X) HPE 8385/08

METAPLASTIC SARCOMATOID CARCINOMA

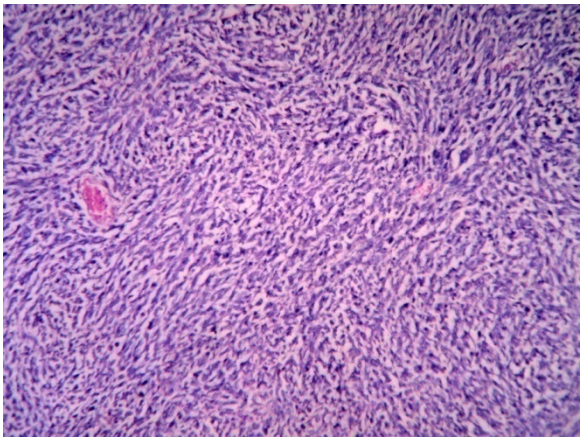

Figure 37: Spindle shaped tumor cells in sheets and fascicles :HPE 1301/09 (100X)

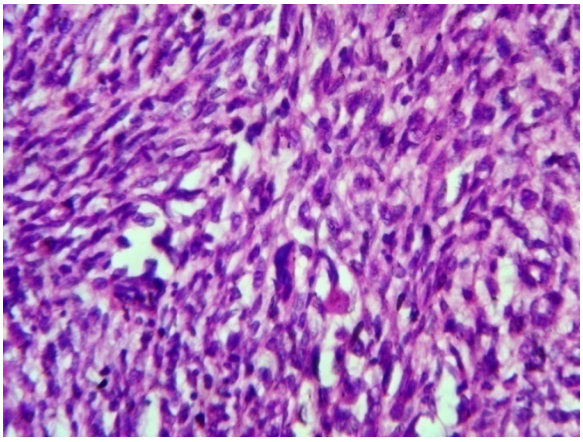

Figure 38: spindle shaped tumor cells with moderate nuclear pleomorphism and mitosis: HPE 1301/09 (400X)

METAPLASTIC CARCINOMA WITH SQUAMOUS DIFFERENTIATION

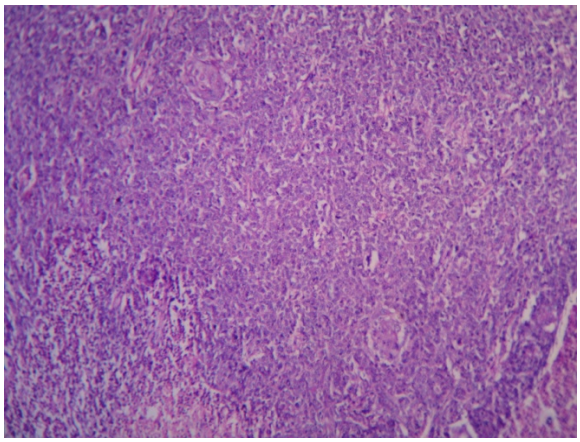

Figure 39: Tumor cells in sheets with two squamous cell nests : HPE 7719/08 (100X)

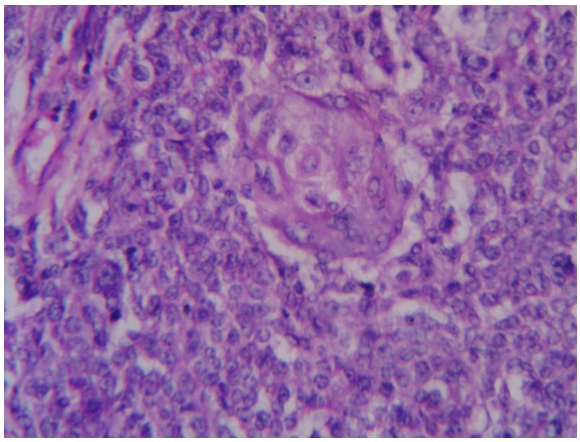

Figure 40: Squamous cell nest in between tumor cells: HPE 7719/08 (400X)

METAPLASTIC CARCINOMA – CHONDROID DIFFERENTIATION

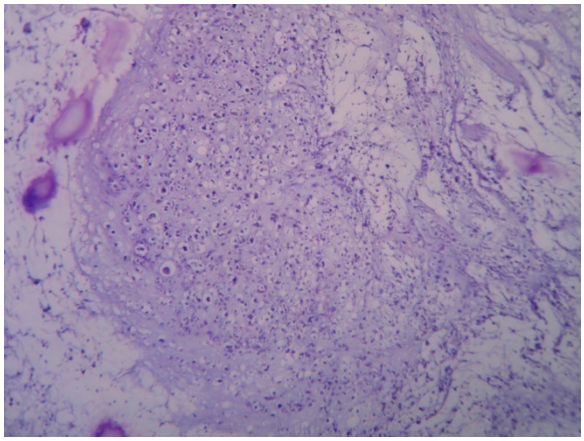

Figure 41: Tumor nest with cartilaginous differentiation : HPE 7865/09 (100X)

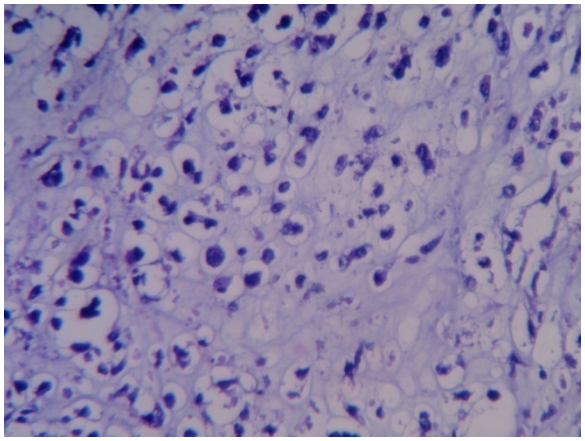

Figure 42:Tumor cells with chondrocytic differentiation within lacunae (400X) HPE 7865/09

OTHER PROGNOSTIC FACTORS

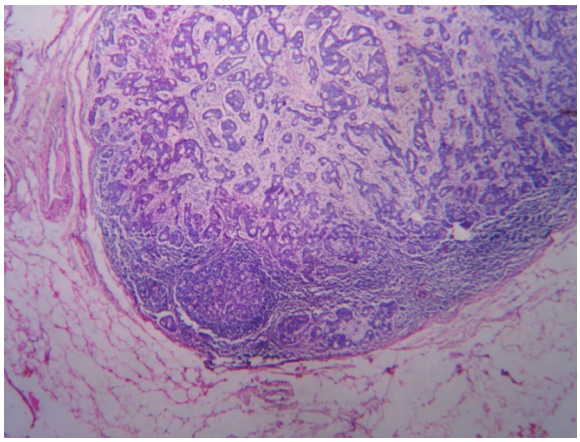

Figure 43:Metastatic deposit in node (40X)
HPE 7453/08

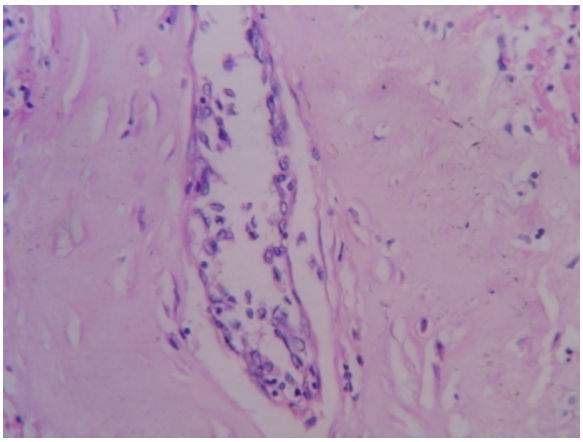

Figure44: Lymphatic invasion (400X)
HPE 7776/08

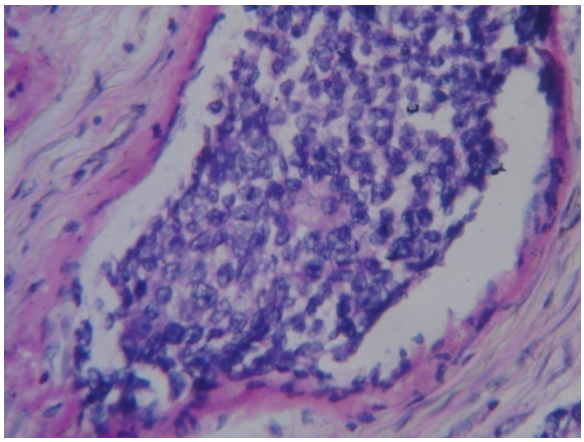

Figure 45: Vascular invasion (400X)
HPE 1857/09

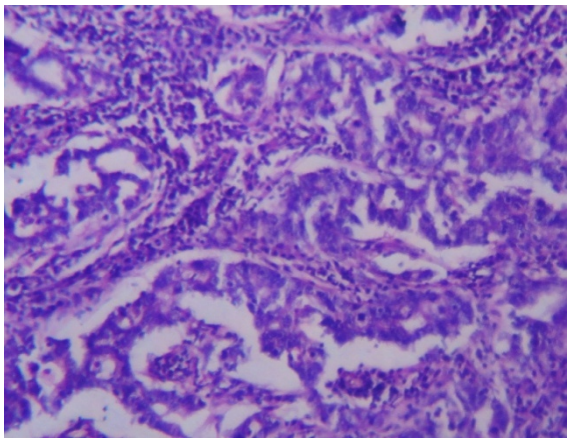

Figure 46: Lymphocytic infiltration (100X)
HPE 5990/09

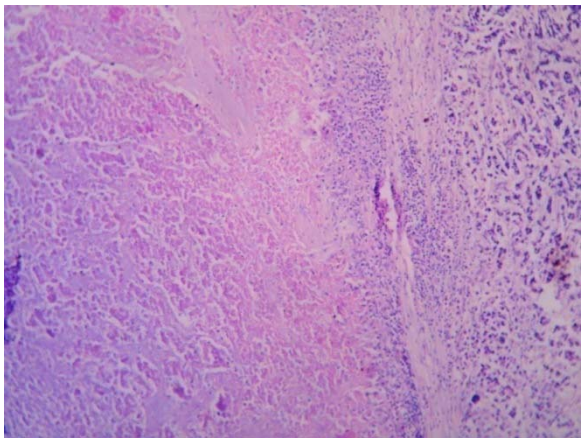

Figure 47: Necrosis : HPE 3984/09 (100X)

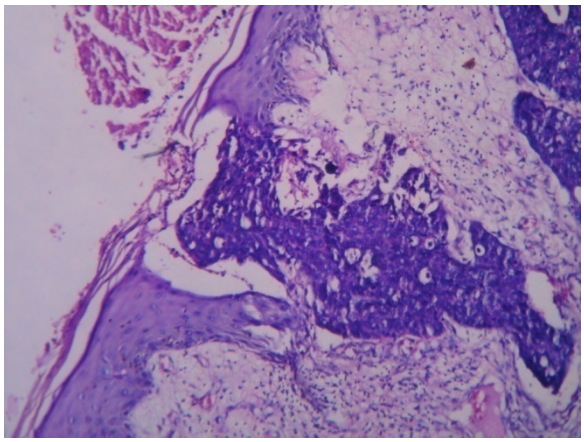

Figure 48: Skin infiltration: HPE 2720/09 (100X)

ER POSITIVE

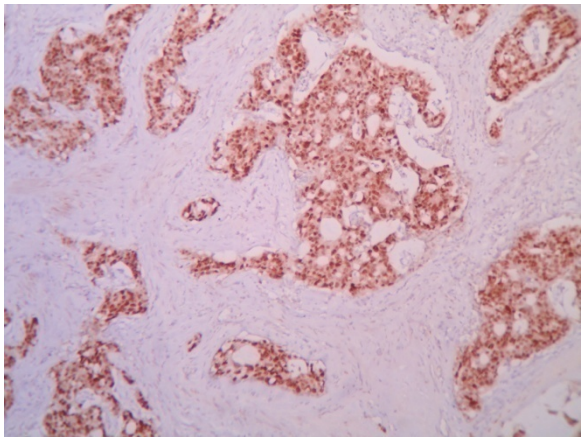

Figure 49: Invasive ductal carcinoma NOS
Strong staining in100% tumor cell nuclei
with ER antibody : HPE 3011/10 (100X)

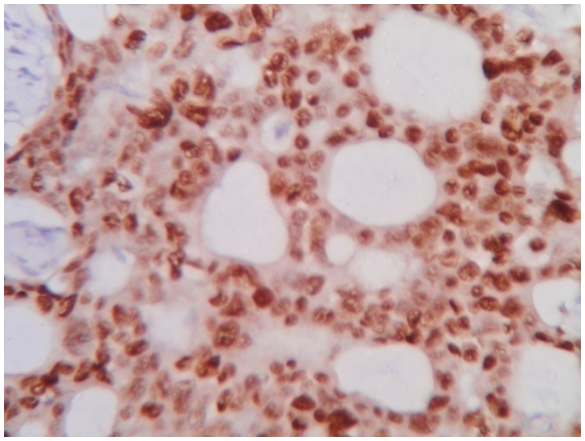

Figure 50: Invasive ductal carcinoma NOS
Strong staining in100% tumor cell nuclei
Quick score 8(5+3) (100X) HPE 3011/10

ER NEGATIVE

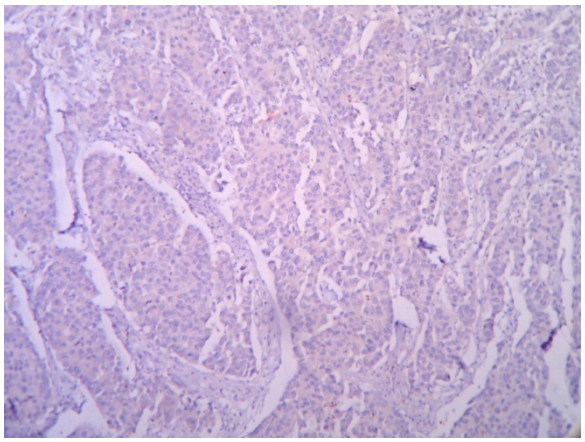

Figure 51: Invasive ductal carcinoma NOS
No staining with ER antibody
Quick score 0 : HPE 5580/09 (100X)

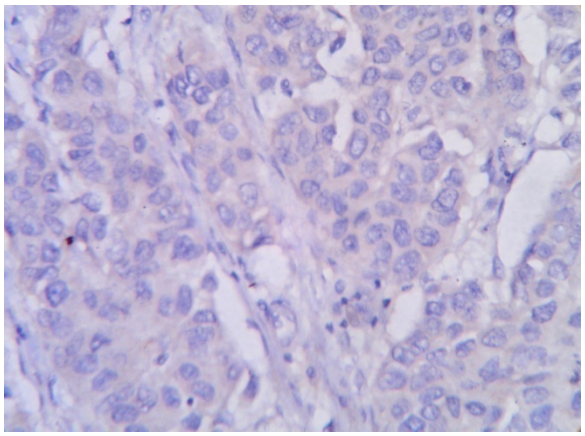

Figure 52: Invasive ductal carcinoma NOS
no staining with estrogen receptor antibody
Quick score 0 : HPE 5580/09 (400X)

PR POSITIVE

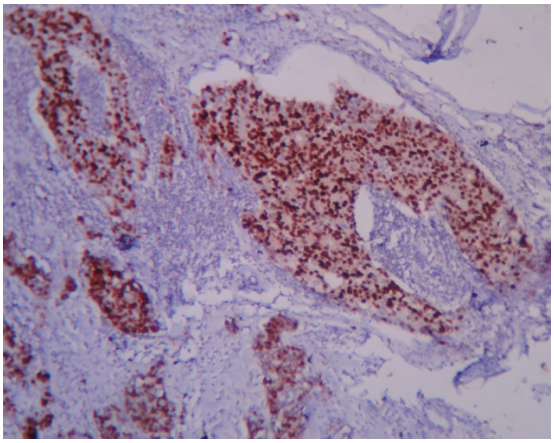

Figure53: Invasive ductal carcinoma NOS
Strong staining in100% tumor cells
With PR antibody :HPE 4333/09(100X)

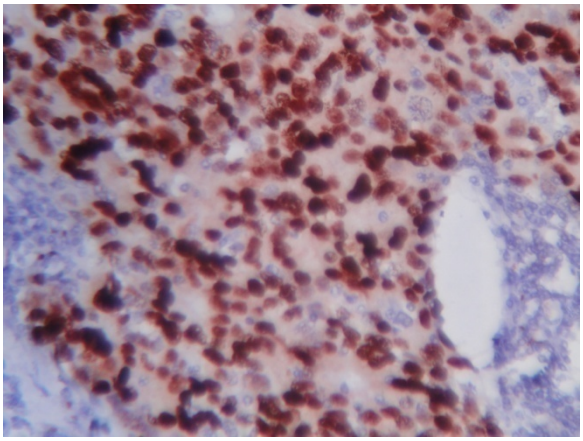

Figure 54: Invasive ductal carcinoma NOS
Strong staining in100% tumor cell nuclei
Quick score 8(5+3) (100X) HPE 4333/09

PR NEGATIVE

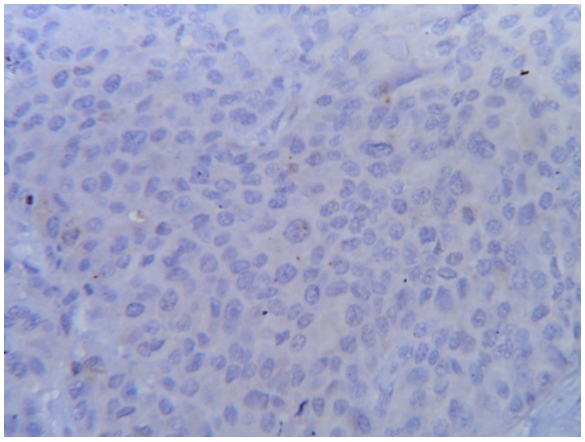

**Figure 55: Invasive ductal carcinoma NOS
No staining with PR antibody: HPE 3799/09(100X)**

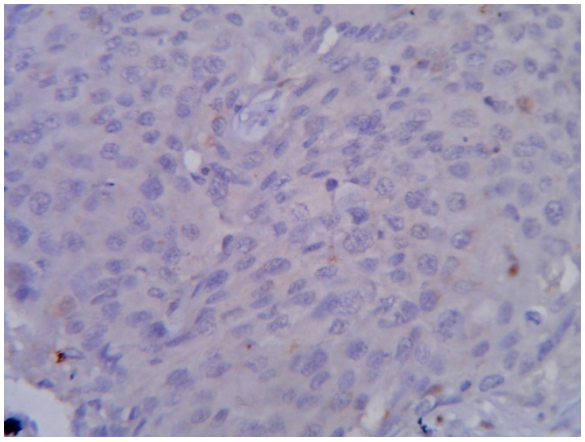

**Figure 56: Invasive ductal carcinoma NOS
no staining with progesterone receptor
antibody; Quick score 0: HPE 3799/09 (400X)**

HER 2 NEU NEGATIVE

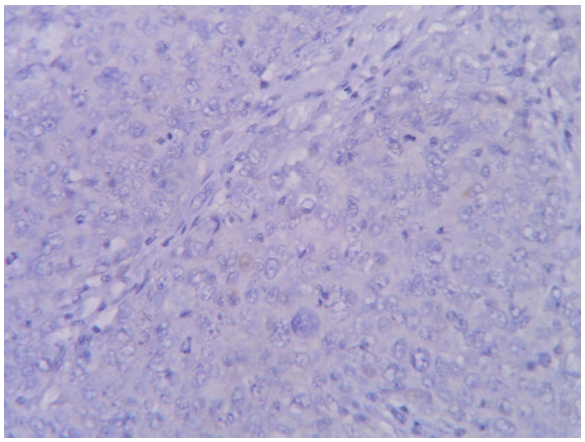

**Figure 57: No staining in tumour cells with
Her 2 neu antibody; Her 2 neu score 0
HPE 5200/08 (400X)**

HER 2 NEU SCORE 1

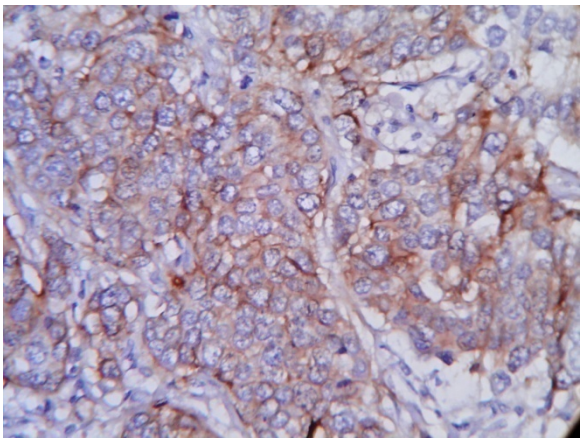

**Figure 58: Incomplete membrane staining
in tumor cells: Her 2 neu score 1+
HPE 4194/09 (400X)**

HER 2 NEU SCORE 2

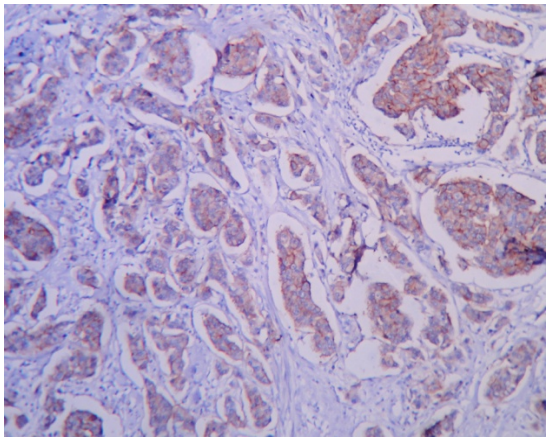

Figure 59: Complete membrane staining in Less than 30% tumor cells (100X) HPE 6576/09

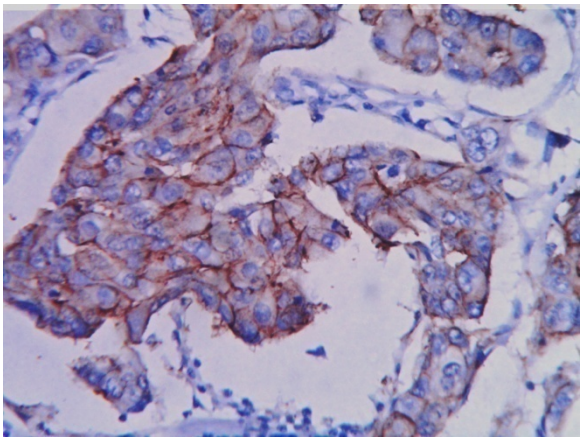

Figure 60: Complete intense membranous staining in less than 30% of tumor cells Her 2 neu score 2+: HPE 6576/09 (400X)

HER 2 NEU SCORE 3

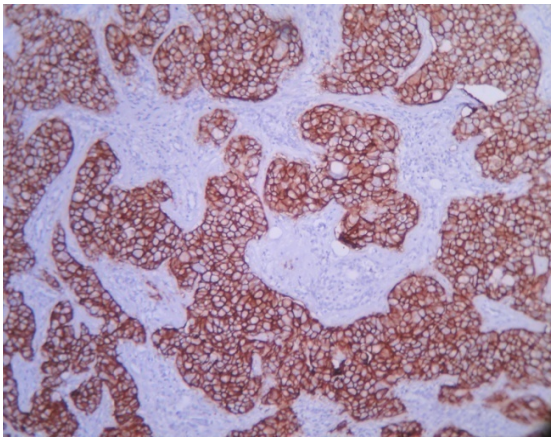

Figure 61: Complete intense membranous Staining in all the tumor cells: HPE 453/09 (40X)

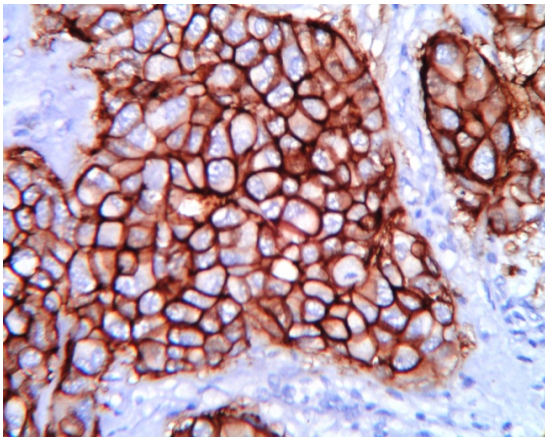

Figure 62: Complete intense membranous staining in all the tumor cells HPE 453/09 (400X)

CYTOKERATIN 5/6 POSITIVE

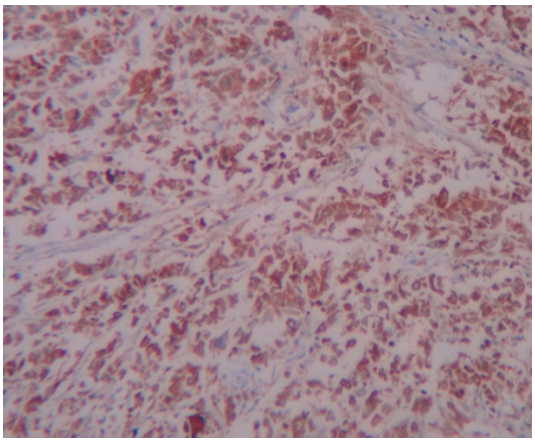

Figure 63: Cytoplasmic positive staining with Anti Cytokeratin antibody : HPE 6576/08(100X)

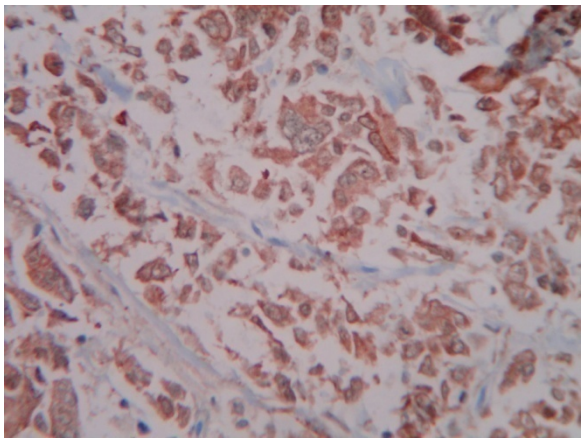

Figure 64: Cytoplasmic positivity with cytokeratin 5/6 antibody in all tumor cells HPE 6576/08 (400X)

CYTOKERATIN 5/6 NEGATIVE

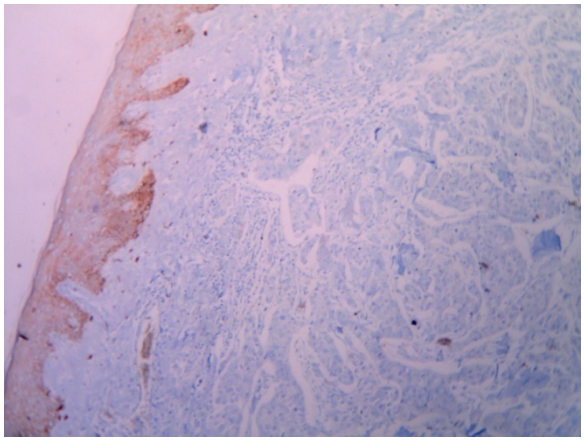

Figure 65: No staining in tumor cells while internal control(skin) –positive HPE 3929/09 (100X)

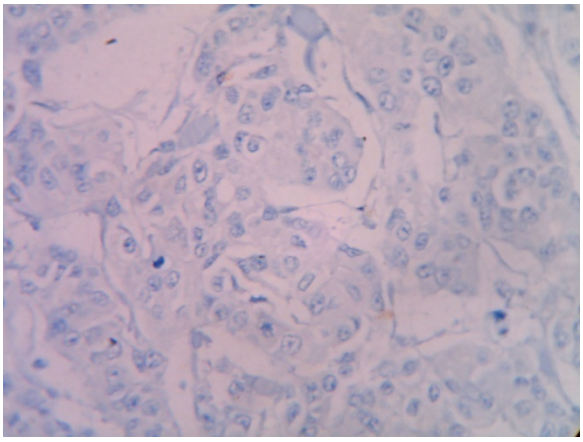

Figure 66: No staining in tumor cells with cytokeratin 5/6 antibody (400X) HPE 3929/09