HER-2/NEU RECEPTOR EXPRESSION IN BREAST CANCER AND ITS CORRELATION WITH THE OTHER ESTABLISHED PROGNOSTIC PARAMETERS AT KENYATTA NATIONAL HOSPITAL

DR NALYANYA WALTER WEKESA

2005

A dissertation Submitted in Partial Fulfillment of the Requirement for the Degree of Masters of Medicine in Pathology, University of Nairobi

DECLARATION

This thesis report is my original work and has not been presented for any award in any University

Name: NALYANYA W. W

Signature: [signature]

Date: 08/12/2005

This Thesis Report has been submitted for examination with my approval as the University Supervisor

Signature:---------- **Date:** 8/12/2005

Dr Muchiri LW

Senior Lecturer

Department of Human Pathology

University of Nairobi.

This Thesis Report has been submitted for examination with my approval as the University Supervisor

Signature:---------- **Date:** 8/12/05

Dr Khan MR

Consultant Surgeon

Kenyatta Nation Hospital

Acknowledgements

I thank my supervisors Dr. L. W Muchiri and Dr. M. R Khan who despite their busy schedule were always there to read through my work and make necessary corrections. I have learned greatly from their attention to detail and excellence.

I acknowledge the invaluable assistance I received from Mr. Kariuki and Mr.Nyagol in Nairobi Hospital Laboratory and Mr.Gitaka from KNH Laboratory.

I thank my professional colleagues, without whose support this work would not have been possible.

I thank my family, for they have walked with me each step of the way.

TABLE OF CONTENTS **Page**

LIST OF TABLES AND FIGURE **Page**

LIST OF ABBREVIATIONS

BRCA: Breast cancer gene

CC: Colloid carcinoma

CIS: Carcinoma In situ

DAB: Diaminobenzidine

DNA: Deoxyribonucleic acid

EGF: Epidermal growth factor

ELISA: Enzyme labelled immunosorbent assay

ER: Estrogen receptor

ERC: Ethical and research committee

FISH: Fluorescent in situ hybridization

FNA: Fine needle aspirate

HER: Human epidermal growth factor receptor

IDC: Invasive ductal carcinoma

IHC: Immunohistochemistry

ILC: Invasive lobular carcinoma

KNH: Kenyatta National Hospital

LSAB: Labelled streptavidin biotin

mRNA: Messenger ribonucleic acid

NNBC: Node-negative breast cancer

PBS: Phosphate buffered saline

PC: Papillary carcinoma

PCR: Polymerase chain reaction

PR: Progesterone receptor

PTK: Protein tyrosine kinase

SCC: Squamous cell carcinoma

TC: Tubular carcinoma

TNM: Describes the anatomic extent of the disease

T Extent of primary tumour

N Absence/presence and extent of regional lymph node metastasis

M Absence/presence of distant metastasis

ABSTRACT

Introduction

Breast cancer is quickly becoming a burden to the Kenyan healthcare system as the disease prevalence has been on an upward trend. It is the second commonest malignancy affecting Kenyan females. Some patients with breast cancer show overexpression of HER-2 oncoprotein and this affects the biological behaviour of the malignant cells and the tumour response to therapeutic agents. The pattern of this receptor expression and its utility have not been adequately explored in Kenyan breast cancer patients.

The objective of this study was to determine the prevalence of HER-2 receptor expression in breast cancers seen at KNH and correlate the results with the other parameters for poor prognosis.

Methodology

This was a descriptive cross-sectional study of breast cancer patients at KNH. A sample size of 133 (50 from the breast clinic/wards and 83 from information in the records department) cases of breast cancer diagnosed through histology had their malignant cells analyzed for HER-2 expression by immunohistochemical methods. Immunohistochemical findings were correlated with age, histological type, tumour grade, tumour size, axillary lymph node status and clinical stage.

Results

HER-2 was overexpressed in 33.8% of the cases. The mean age of breast cancer cases with HER-2 overexpression was 50.3 years while that of cases without overexpression was 48.9 years ($p = 0.578$). No significant correlation between HER-2 overexpression and histological type was established. HER-2 positive tumours tended to be larger than HER-2 negative tumours but this was not statistically significant. The proportion of tumours with HER-2 overexpression within each grade increased linearly with the grade but still no significant correlation was noted. No association was seen between clinical stage and HER-2 status but a strong correlation between HER-2 overexpression and axillary lymph node status was established ($p = 0.013$).

Conclusion

HER-2 overexpression was seen in 33.8% of breast cancer patients at KNH. HER-2 was significantly correlated with axillary lymph node status but there was no association between HER-2 positivity and age, histological type, tumour size, histological grade, and clinical stage.

LITERATURE REVIEW

Introduction

The female breast is under constant physiologic and anatomic alterations due to its extreme sensitivity to hormonal influences especially during the menstrual cycle, pregnancy, and menopause. This predisposes the organ to various pathologic conditions, most of which are fortunately benign. Breast cancer is one of such conditions and is the commonest malignancy among women in the world; it also carries a very high mortality worldwide. Because of this, research investment in breast cancer is quite high and continues to grow.

Prognostic factors such as TNM stage, tumour grade, hormone receptor status, age, histological type, DNA ploidy and proliferation index are used to assess the prognosis of breast cancer patients. Despite these prognostic markers, high risk patients cannot be identified with sufficient accuracy.

It has long been known that oncogenes like HER-2 play an important role in the etiology and behaviour of human breast cancers. Yet, it is only until recently that this knowledge has been utilized in the development of therapeutic agents. There is a trend towards less radical operations in patients with breast malignancy, therefore these new therapeutic agents are essential for post-operative management and improved outcome.

Breast cancer

A Epidemiology

Breast cancer is the commonest malignancy among women worldwide with an estimated world incidence of about 1.2 million cases. About 50% of these occur in the developed world. In the West, its incidence is 100 cases per 100 000 people. Globally, about 377,000 people die from breast cancer every year. Breast cancer annual mortality rates range from 27/100 000 people in northern Europe to 4/100 000 in Asia. It is estimated that 40,000 women and 400 men die from breast cancer in the United States each year.

The incidence in the developing countries is about less than 10% that of the developed world (1, 2).

There are about 59 000 new cases of breast cancer per year in Africa, with the annual mortality estimate of 27 000 people. Sub-Saharan Africa has an incidence of about 20 cases per 100 000 people (3). It has been known that breast cancer in blacks has a more aggressive clinical behaviour and therefore carry a relatively higher mortality. Despite the fact that poor health care may contribute to this, it is postulated that genetic factors also play a role. Breast cancer is most often diagnosed in black patients between 35 and 45 years of age, and more than fifteen years earlier than women in Europe and North America (4).

Cancer of the breast is the second commonest malignancy among women in Kenya after cervical cancer (5). Breast cancer incidence is said to be about 1.08 cases per 100 000 people (6). This may not be a true reflection of the situation as most of the rural population has difficulties accessing health facilities and therefore there is incomplete statistical data. Indeed some studies estimate breast cancer prevalence in this country to be about 25 cases per 100 000 people (5). In the year 2000, 180 new cases of breast cancer were recorded at KNH (5). Figures from the statistics section of the KNH records department show that in the year 2003, 183 breast cancer patients were admitted in the wards either for operation or management of complications related to the advanced disease. Mortality figures in this country are grossly inadequate due to low autopsy rate and poor reporting.

B Risk factors

Despite extensive studies on breast cancer, there is as yet no single precise cause that has been identified. However, a variety of risk factors have been identified.

The most important risk factors that have been established include the following;

1. Country of birth- Breast cancer incidence is highest in North America and Northern Europe, is intermediate in Southern Europe and Latin America and is lowest in Africa and Asia. Race and longevity of life may contribute to this

pattern but perhaps more crucial are the environmental factors and lifestyles. Some studies have linked dietary fat and heavy alcohol consumption to increased risk of the cancer (7, 8).

2. Gender is by far the greatest risk factor for breast cancer. Women are 100 times more likely to develop the cancer than men.
3. Older women are at a greater risk. Breast malignancy is rare before 25 years of age. Then there is a steady rise from age of 40 years to the time of menopause and thereafter, the incidence increases slowly throughout life with a peak at 64 years.
4. Ionizing radiation exposure is a significant risk factor for the development of breast cancer. Exposure may be accidental like Cherynobyl nuclear disaster or even therapeutic radiotherapy. Some studies have attributed a small risk to mammography (1).
5. A family history of breast cancer has for a long time been known to be a risk factor for breast cancer. Cancer in a first degree relative increases the risk 2-3 fold. The risk goes up higher if breast cancer comes at an early age and if it is bilateral.

   Upto 5-10 percent of all breast cancers are due to inheritance of autosomal dominant genes. Here the cancers occur at an earlier age. There are two tumour suppressor genes whose mutations account for about 80 percent of hereditary breast cancers. These are BRCA-1 (Breast Cancer gene 1) which is located on chromosome 17q and BRCA-2 located on chromosome 13q. Mutations in p53 oncosuppressor gene and the presence of ataxia telangiectasia gene also contribute to hereditary breast cancers (9, 10).

6. The most well known risk factors are those related to menstrual and reproductive history. The more the number of completed menstrual cycles the higher the risk, that is, risk increases with increased exposure to estrogen peaks. Hence there is increased risk of breast cancer development in the following groups of women;
   (a) those with early menarche or late menopause.
   (b) Nulliparous females are more at risk.

(c) Women who get their first pregnancy when they are more than 30 years old.

(d) Some risk has been associated with exogenous estrogens and oral contraceptives but the link appears weak.

C Classification and distribution

Breast cancer is divided into non-invasive (in- situ) carcinoma and invasive carcinoma. Carcinoma in-situ makes up about 15-30% of the cancers, this proportion depends on how often mammography is used.

The various invasive carcinoma histologic subtypes and their frequencies are (11):

1. Invasive ductal carcinoma
   (a) Invasive ductal carcinoma (no specific type)-79%
   (b) Tubular/cribriform carcinoma-6%
   (c) Colloid (mucinous) carcinoma-2%
   (d) Medullary carcinoma-2%
   (e) Papillary carcinoma-1%
2. Lobular carcinoma-10%

D Spread

Breast cancer spreads by local invasion, via the lymphatic route and metastasizes via the blood stream.

Local invasion involves the spread of cancer through the breast parenchyma itself, to the skin and chest wall. The axillary lymph nodes are the most common site of breast cancer spread by the lymphatic route, followed by the supraclavicular lymph nodes and the internal mammary chain of lymph nodes. Axillary lymph nodes are involved in 40-50% of breast cancer cases. About 40% of clinically negative axillary lymph nodes are microscopically involved. Distant metastases involve many organs but the commonest are lungs, liver and the bones especially the lumbar vertebra.

Some metastases are already present at the time of diagnosis, others become manifest clinically months, years and decades after initial therapy (12).

E Prognosis

A variety of clinical and pathological prognostic factors in breast cancer have exploded over the past several years. Pathologists have played a major role in identifying different histological and immunohistochemical markers that have a direct bearing on both the treatment and behaviour of breast cancer.

1. Breast cancer stage. The most important prognostic parameters, that is, tumour size, locally advanced disease, lymph node metastases, and distant metastases are used in clinical staging. The diameter of the primary tumour shows a good correlation with the incidence of nodal metastases and with survival rate. This easily determined parameter has been found to be one of the strongest predictors of dissemination and rate of relapse in node – negative breast carcinomas (13). In a study by Anan et al, it was found that those tumours with a diameter less than 1cm with negative nodes have 98 % survival at 5 years while tumours less than 2cm have 96 % survival (14). As axillary lymph node metastasis is one of the most important prognostic parameters, there is a sharp difference in survival rates between patients with positive and negative nodes. With no lymph node involvement, 10 year survival rate is about 80% , which falls to about 35% with even only one node involved (15).
   Staging refers to classification of cancer patients into clinically distinct prognostic categories. Manchester (stage 0 to IV) and international union against cancer TNM systems are commonly used (16, 17). (Appendix I)

   Prognosis worsens as one moves from stage 0 to stage IV.
   The staging of breast cancer at diagnosis helps in the choice of the appropriate method of management. This could be surgery, radiotherapy or systemic therapy. Carcinoma in-situ is usually treated with surgery alone. Stage I and II cancer is potentially curable with localized treatment modalities (surgery or radiation or both). However recent evidence shows that local treatment failure rate for stage I may be as high as 35% , so there is compelling need for effective systemic adjuvant therapy (18).

Studies have shown that breast cancer does not advance by a direct anatomic spread from breast, but rather there are haematogenous and lymphatic micrometastases by time of diagnosis (19). This is one of the reasons for the gradual shift of the surgical option from the extended radical mastectomy to that of modified mastectomy.

Stage III and IV breast cancer generally cannot be cured with currently available therapies. Combined therapy (systemic, radiation and surgery) appears to shrink tumours, relieve symptoms and prolong survival for some women (20),(21).

2. Histologic types. Invasiveness is one of the single most important prognostic parameter in breast carcinoma. Practically, non-invasive carcinomas are 100% curable with mastectomy. These include ductal carcinoma-in-situ, lobular carcinoma-in-situ and most cases of paget's disease (22). Morphologic variants of invasive ductal carcinoma with a more favourable prognosis are tubular carcinoma, colloid carcinoma and papillary carcinoma. These have a 5-year survival rate of 85%. Less favourable histologic types are medullary carcinoma, invasive lobular carcinoma and invasive ductal carcinoma. Signet ring carcinoma (a variant of lobular carcinoma) and inflammatory carcinomas associated with tumour-plugged subdermal lymphatics have a particularly bad prognosis with a 5-year survival rate of about 30% (23).
3. Tumour grade. Both the architecture and cytology of routinely stained sections of breast cancer tissue as determined by visual microscopic examination have been found to correlate with prognosis.

The Scarff-Bloom-Richardson scoring system is the most commonly used grading system (24).

Table 1: Tumour score and proportion of tubular tumour

% of tubular formation	Score
> 75	1
10 - 75	2
< 10	3

Table 2: Tumour score and nuclear pleomorphism

Nuclear pleomorphism	Score
Small and uniform cells	1
Moderate increase in size & variation	2
Marked pleomorphism	3

Table 3: Tumour score and mitotic index

Mitotic count / HPF	Score
Upto 7	1
8 to 14	2
15 or more	3

Final score is from 3-9

Score 3,4 or 5 –well differentiated(Grade1)

Score 6 or 7 -Moderately differentiated (Grade 2)

Score 8 or 9 -poorly differentiated (Grade 3)

Patients with grade 1 breast cancer have a 5-year survival rate of 95%, grade 2 have 75% survival rate while those with grade 3 have a 5-year survival rate of 50%. Cancers with a high grade and necrosis are more likely to recur after breast cancer treatment than other breast cancers (25).

4. Hormone receptor status. Patients whose cancers have estrogen receptors (ER) or progesterone receptors (PR) tend to have a better prognosis than patients whose cancers do not have these receptors. Cancers with ER or PR- positive receptors are also much more likely to respond to chemotherapy or hormone treatment. Breast cancer cells that express ER- positive receptors in their nuclei also tend to respond better to hormonal manipulation (26). Researchers know less about PR- positive receptors but have noticed that cells that contain ER- positive receptors often contain PR- positive receptors too. If a cell contains a PR- positive receptor but no ER- positive receptors, a patient's prognosis may be worsened (27).

5. High tumour proliferation rate is associated with poor prognosis. These can be measured by the usual mitotic count, determining amount of Ki-67 produced during cell cycle using immunohistochemistry or calculating S-phase fraction by flow cytometry (28). Ki-67 is a nuclear protein that is tightly linked to the cell cycle. It is a marker of cell proliferation and has been used to stratify good and poor prognostic categories in invasive breast cancer. In a study by Tan et al, it was demonstrated that increased Ki-67 protein expression correlated with high histologic grade, mitotic score and estrogen receptor immunonegativity (29). In addition, Ki-67 overexpression significantly correlated with the expression of genes that are associated with apoptosis and cell death.

6. DNA ploidy as measured by flow cytometry. Cancers with the same amount of DNA as normal cells are called diploid and those cancers with either more or less than that amount are called aneuploid. Several studies have shown that aneuploid cancers tend to be more aggressive than normal cancers (30).

7. The immunohistochemical demonstration of accumulated p53 (usually as a result of gene mutation)is usually not a reliable prognostic indicator in the breast cancer patients and it is not associated with major epidemiologic risk factors (31). The combined immunophenotypic expression of p53 and HER-2 was significantly associated with some histologic types of breast carcinoma and with prognosis in $T_1N_OM_O$. The detection of p53 may be particularly promising in clinical trials of new molecular therapies directed at the p53 tumour suppressor gene (32).

8. There has been an observation made that invasive breast carcinomas having a prominent vascular component in the surrounding stroma behave in a more aggressive fashion than the others. Angiogenesis is the propelling force for tumour growth and metastasis and antiangiogenic therapy represents one of the most promising modalities for cancer treatment. CD 105 is a proliferative-associated and hypoxia-inducible protein abundantly expressed in angiogenic endothelial cells. It is a receptor for transforming growth factor (TGF)-B1 and –B3.
Immunohistochemistry studies have revealed that
CD 105 is strongly expressed in blood vessels of tumour tissues. The degree of CD 105 expression in the tumour is used to quantify the degree of angiogenesis (33).

9. Protein kinase B (Akt) is a serine/ threonine kinase. Protein kinase B signaling is believed to promote proliferation and increased cell survival and thereby contributing to cancer progression. The determination of protein kinase B status may be of value in identifying high-risk patients, who would benefit from adjuvant therapy, and gives a rationale to investigate new therapy strategies by specific inhibition of the protein kinase B signaling pathway in breast cancer (34).

10. Bcl-2 is a protein that regulates programmed cell death and apoptosis. The expression of this gene can be regulated, at least in part, by the tumour suppressor p53, but the effects of p53 are highly tissue specific. Expression of Bcl-2 is determined using immunohistochemistry. Patients with tumours expressing high levels of Bcl-2 have a significantly longer disease-free and overall survival. High

levels of Bcl-2 are preferentially expressed in well-differentiated tumours and are associated with favourable prognosis. However, Bcl-2 expression is not an independent prognostic factor (35).

11. Women who are younger than 50 years of age at the time of diagnosis have the best prognosis. Relative survival declines after the age of 50 years and is particularly low in older women. Some studies have shown that very young women (< 35 years of age) have a significantly higher risk for recurrence and distant metastases (36).

HER-2

HER-2 (Human Epidermal growth factor Receptor), also known as *C-erbB-2* or *neu* is a growth factor which is the protein product of a proto-oncogene. Proto-oncogenes are a group of entirely normal cellular genes concerned with the regulation of cellular growth. Proto-oncogenes can be changed to oncogenes by activation and this involves changes in the nucleotide sequences of their DNA. Activation can result from mutations, translocations, or insertions. Tumourigenicity can also be from amplification of the proto-oncogene, resulting in excess production of transcripts (37).

HER-2 belongs to type 1 human epidermal growth factor (EGF) receptor family.

The 4 members of this family are ; HER-1(c-erbB-1); HER-2(c-erbB-2); HER-3(c-erbB-3); HER-4(c-erbB-4) receptors (38).

These proteins bear a close homology to each other and they effect the biological action through their respective transmembrane protein tyrosine kinases (39, 40).

HER-2 is a 185kDa transmembrane glycoprotein (41). See the illustration in figure 1.

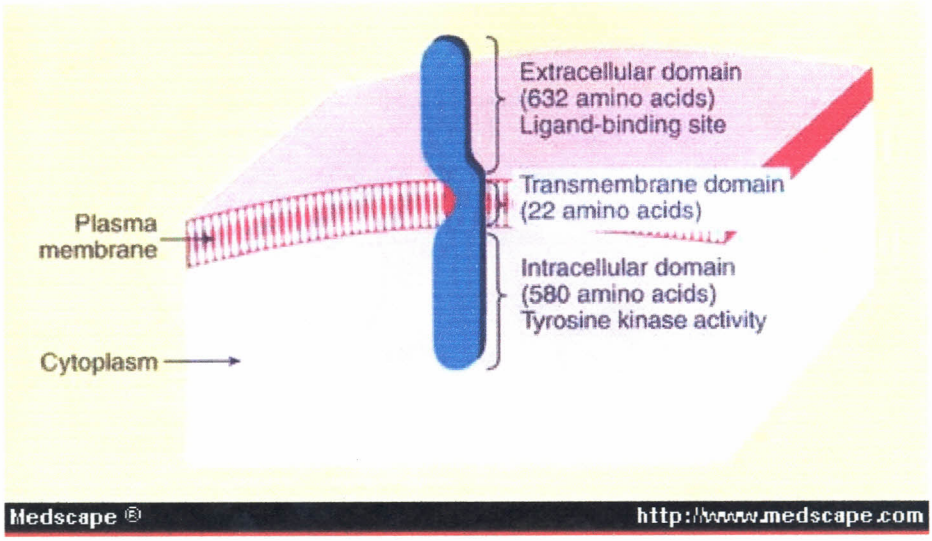


Figure 1: HER-2 transmembrane glycoprotein

A Biological activity of HER-2 gene

HER-2 receptor activation stimulates a signal transduction pathway leading to increased plasma membrane potential. Through mitogenic signal transduction, the HER-2 Protein tyrosine kinases (PTKS) play central roles in control of cell proliferation and differentiation of both normal and malignant cells (42,43).

In normal somatic cells, HER-2 proto-oncogenes are involved in embryological cell growth control. Postnatally these genes are repressed except in tumour cells where their gene products, either in a mutated or overexpressed form, are in part responsible for malignant transformation (44).

All normal epithelial cells contain two copies of c-erbB-2 gene on chromosome 17q21 and express low levels of HER-2 receptors on cell surface (45). In some cases of oncogenic transformation, the number of gene copies per cell is increased, leading to increase in mRNA transcription and a 10-100 fold in number of HER-2 protein receptors on the cell surface, this is called overexpression (41). See figure 2.

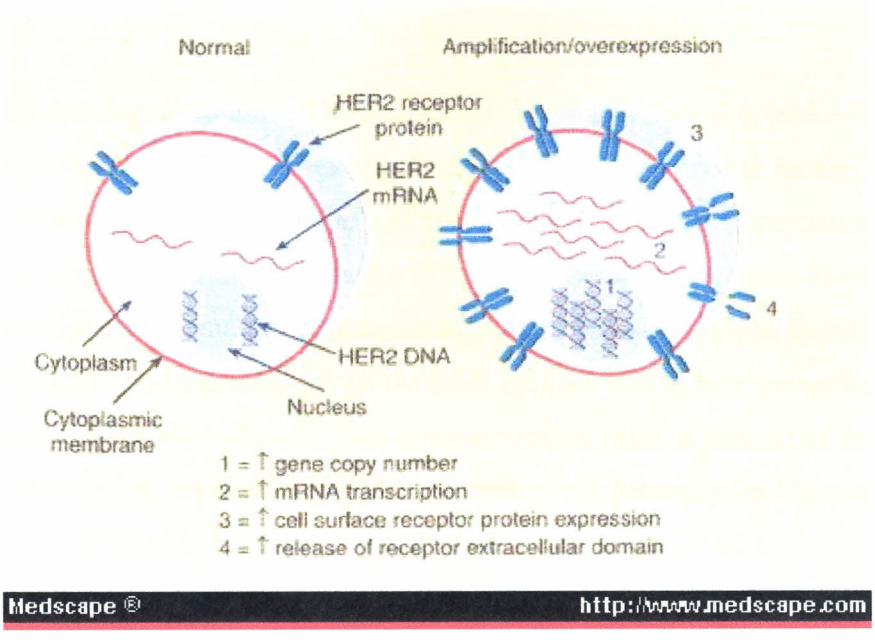


Figure 2: Illustration of HER-2 overexpression

B HER-2 role in carcinogenesis

In a study by Dr Cook, it was demonstrated that transfection (incorporation of exogenous DNA into a cell) of c-erbB-2 gene into human breast and ovarian tumour cell lines produces more aggressive growth characteristics such as increased DNA synthesis, cell growth, tumourigenicity and metastatic potential in nude mice (46).

The HER-2 receptor is overexpressed in a number of human malignancies especially breast cancer and ovarian carcinoma. It has also been demonstrated in the carcinomas of the bladder, stomach, pancreas and prostate (47).

HER-2 receptor overexpression is present in 10-25% of breast cancers in USA and is associated with poor prognosis (48,49). About 10% of breast cancers with HER-2 overexpression have been demonstrated to have no corresponding gene amplification. HER-2 overexpression in conjunction with amplification, and in another few percent of breast cancers without gene amplification, is further accentuated by transcriptional upregulation of c-erbB-2, which is mediated by the transcription factor AP-2 (50).

EGF family receptors are directly regulated by binding of diverse polypeptide hormones

that is homologous to EGF. The ligands consist of amphiregulin, betacellulin, EGF, epiregulin, various forms of neuregulin, and transforming growth factor (TGF)-α. They have different abilities to bind to and activate the ErbB family receptors when expressed singly. Ligand-dependent regulation of the ErbB family of receptors is expanded through the promiscuous formation of receptor heterodimers. For example, in cells that express EGF receptor and c-erbB2, any of the EGF agonists will induce formation of EGF receptor-ErbB2 heterodimers. This cross-activation extends to most of the receptor combinations, so that activation of one receptor will generally lead to some activation of other coexpressed ErbB family of receptor tyrosine kinases (42).

HER-2 is an orphan receptor, because none of the soluble ligands bind to HER-2 that is expressed independently. HER-2 is strongly activated through interactions with other EGF family receptors, however, and ligand-induced c-erbB-2 heteromers are favored over other heteromers or homomers. Because c-erbB-2 is jointly expressed with other c-erbB family receptors, it can be thought of as a common subunit that expands the signaling repertoire of the other c-erbB family of receptors (51).

Activation of c-erbB receptors induces tyrosine phosphorylation. The activation-induced phosphopeptides recruit many other different proteins, forming an enzyme cascade that conveys the signal further. This results in transmission of signals across cell membrane and across intracellular space to the nucleus, where gene activation occurs (52).
Once activated, signals are finally withdrawn through ligand-receptor dissociation, through phosphorylation (eg by protein kinase C), and by receptor-mediated endocytosis, which can be followed by recycling to the cell surface, or proteolytic destruction of the receptor. The activities of tyrosine phosphatases will further influence the intensity and duration of signaling (51).

C Clinical implications of HER-2

Understanding of tumour control growth factors and their signal transduction control pathways, together with the development of interventions that modulate their biologic activity, represent a major focus for current cancer research. This may change the practice of clinical oncology.

Immunotherapies aimed at HER-2 receptors are being developed. These include a wide variety of vaccine trials using purified HER-2 peptides or tumour cells that overexpress HER-2 receptors.

A humanized monoclonal antibody to HER-2 receptor has been developed. It is called trastuzumab (Herceptin^{R}) and has been shown to have significant antitumour properties on its own (53,54).

D Testing for HER-2 receptor status.

The amount of research currently focused on HER-2 has led to its wide acceptance as a strong independent breast cancer prognostic marker and predictor of therapeutic response.

HER-2 status is determined by measuring the amount of its DNA, mRNA, or protein receptors in the tumour. See appendix IV for the description of the following techniques in common use:

- Immunohistochemistry
- Fluorescent in situ hybridization
- Polymerase chain reaction
- Enzyme labelled immonosorbent assay

RATIONALE OF STUDY

The clinical stage of breast cancer does not always accurately predict its clinical course. For instance, axillary lymph node status is widely accepted as an important parameter for assessing prognosis in breast cancer patients, yet recurrence and death also occur in patients with node-negative breast cancer (NNBC). Other traditional prognostic factors such as morphological grade, hormone receptor status, histological type, DNA ploidy and proliferation index do not provide all answers about the behaviour of the tumour.

HER-2 receptor status has been proven in other geographical regions and populations to be a sensitive independent prognostic parameter. There are no published studies that have been done in Kenya to establish the prevalence of HER-2 status of breast cancer patients in our setting and how the receptor expression correlates with other prognostic parameters.

It is hoped that the findings in this study will encourage the routine testing for HER-2 status in the breast cancer patients. The traditional prognostic parameters along with HER-2 / neu status are all well established predictors of recurrence and or death from breast cancer and should be reported by pathologists for all the tumours. Therefore, better management decisions will be made given the recent trend towards less radical operations and the emergence of therapy based on HER-2/neu receptor antibody.

There is still a lot that is not yet known about the HER-2 status in our setting. Hence, we need to constantly research in this area in order to better understand how the knowledge in this oncogene can be harnessed in diagnosis, management and even vaccination.

HYPOTHESIS

There is overexpression of HER-2/neu transmembrane glycoprotein in breast cancers having other parameters for poor prognosis.

OBJECTIVE OF THE STUDY

A Broad objective

Aim of this study was to determine the status of HER-2 receptor expression in breast cancer patients seen at KNH and correlate the results with other prognostic parameters.

B Specific objectives

1. To determine the prevalence of HER-2/neu overexpression in breast cancer patients seen at KNH.
2. To establish whether there is a correlation between HER-2 receptor overexpression in breast cancer and its:

    (a) Histological subtype
    (b) Histological grade.
    (c) Tumour size i.e if less than 2cm or more than 2cm.
    (d) Clinical stage.
    (e) Axillary lymph node status.
3. Relate the HER-2 receptor expression score and the age of the patient.

RESEARCH QUESTIONS

1. What is the prevalence of HER-2 overexpression in breast cancer patients at KNH?
2. Does HER-2 overexpression in breast cancer correlate with other prognostic parameters at KNH?

METHODOLOGY

A Study design

This was a descriptive cross-sectional study.

B Study location

The Kenyatta National Hospital breast clinic and general surgical wards were the locations of study. The clinic was held on the afternoon of every Monday. The KNH breast cancer clinic register and the Records department were of assistance in obtaining clinical information concerning the retrospective cases.

The histopathology laboratory in KNH was used for the histological processing of the specimens. The histopathology laboratory in Nairobi Hospital was used for the determination of HER-2 receptor status using immunohistochemistry.

C Study duration

This study was conducted over a period of eight months from October 2004 to May 2005.

D Study population

Study participants were those with breast cancer seeking treatment at KNH , either attending breast clinic or admitted in the general surgical wards. This also included those patients who already had surgery, radiotherapy, chemotherapy and even those with recurrency of the disease. All patients had surgery and the histopathological diagnosis of

breast cancer made before being started on radiotherapy and chemotherapy. So the paraffin embedded tissue used in IHC was free from the effects of chemotherapy and radiotherapy.

E Inclusion criteria

All patients with breast cancer diagnosed by histology or FNA in the period 2003 to 2005. For the patients seen in the breast clinic or the wards, those who had a consent of participation in the study signed either by themselves or their close relatives.

F Exclusion criteria

Those patients in the clinic or ward who declined to give consent. Also excluded were those cases where axillary lymph node sampling was not done during surgery.

G Sample size

133 subjects, using the following formula for descriptive cross-sectional study (55):

$$n = \frac{(1.96)^2 p (1-p)}{d^2} = \frac{(1.96)^2 x 0.1(1-0.1)}{(0.05)^2}$$

where n= sample size

p=prevalence=10% have HER-2 overexpression

d=level of precision=0.05

H Sampling method

All patients with breast cancer consecutively seen at KNH and who fitted in the inclusion criteria were recruited.

I Recruitment

During the period of study, 50 patients who fitted the inclusion criteria for the study were identified by the principal investigator or his assistant in the breast clinic and the wards. The ward doctors and nurses were also very helpful in the identification of admitted cases. For the other 83 cases, the clinical information was gathered from files in the Medical records department.

DATA COLLECTION

A Consent

The identified patients were told about the nature and the likely benefits of the study. Those who were willing to participate signed the prepared consent form in appendix III. For the patients who were too sick, an informed consent was obtained from a close relative. Permission for records retrieval was obtained from ERC – KNH for the 83 cases where the clinical data came from the Medical records department..

B Questionnaire administration

The clinical data concerning the patient came from history taking and physical examination. Further information was obtained from the patient's clinical notes especially where the patient had had surgery. The data was entered into the questionnaire. A copy of the questionnaire is in appendix II.

C Specimen collection

Pre – surgical subjects

These were patients with breast cancer diagnosed by FNA. They were booked for lumpectomy or mastectomy. KNH histopathology laboratory was used for histological processing of the breast tissues and the lymph nodes.

Post – surgical subjects

For those patients who already had histology reports of the breast tissue and the accompanying lymph nodes at the time of first contact with the investigator, their paraffin-embedded archival specimens were retrieved and processed for histology.

The prepared Haematoxylin/Eosin histology slides were examined by the investigator and reviewed by the consultant pathologist supervisor to designate the breast cancers into various histological types and grades. In this study the sampled axillary lymph nodes were examined histologically to find out whether they were involved by metastases. The results were then entered in the questionnaire.

D Specimen analysis for HER-2 expression

The paraffin-embedded sections of malignant breast tissue of the recruited patients were analysed for HER-2 expression by the chief investigator with the assistance of the laboratory technical staff. Immunohistochemical method of staining was used in this study to measure HER-2 expression. . It employs the use of monoclonal antibodies to localize HER-2 receptor proteins on the cell surface membranes. The procedure used was the biotin-avidin immunoenzymatic technique (56). See illustration in figure 3.

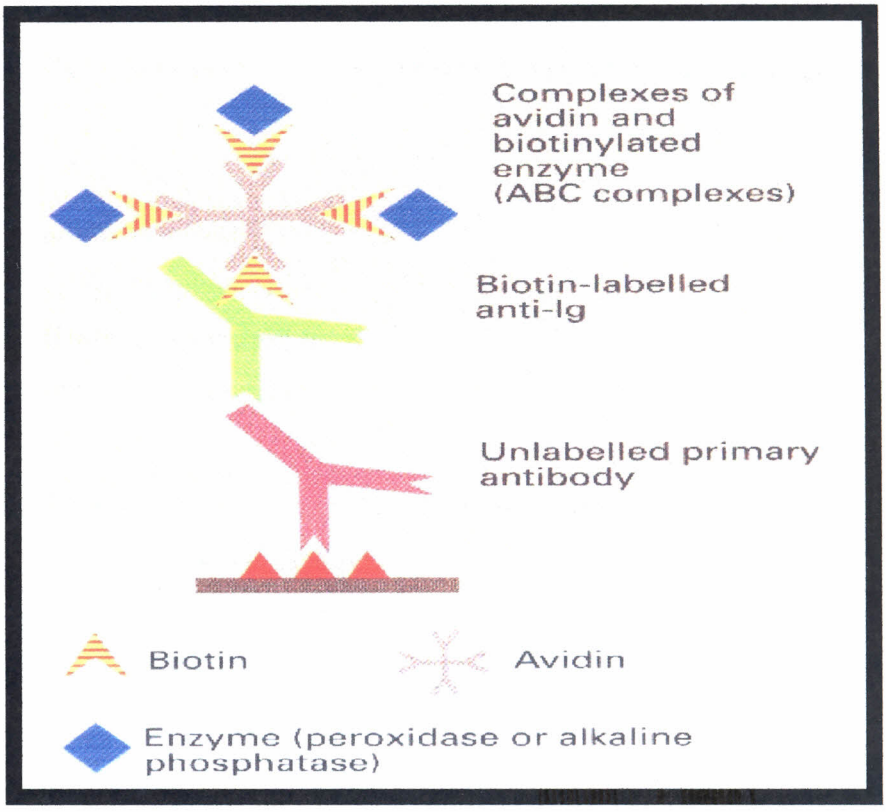


Figure 3. Antigen localization by avidin –biotin –peroxidase complex method.

The immunohistochemical method of analysis was adopted for this work because:

- It is rapid and highly sensitive.
- Relatively cheap and available locally.
- Can be used on paraffin-embedded archival tissue.

The shortcomings of this IHC technique are:

- Fixation procedure may denature epitopes giving false results.
- Lack of standardization in the performance of the test since the procedure is not automated.
- Interpretation of the results is subjective as it relies on the human eye/brain.

DAKO'S scoring system is the one that was used when interpreting HER-2 receptor status on tumour cells in this study. The score varies with the density of the receptors on the cell membrane.

score 0 (about 20,000 receptors/cell);
score 1+ (about 100,000 receptors/cell);
score 2+ (about 500,000 receptors/cell);
score 3+ (about 2,000 000 receptors/cell).

Following is the criteria used in scoring the cell membrane staining;

Score	Pattern of staining
0 (negative)	Less than 10 % of tumour cells with membrane staining.
1+ (negative)	More than 10% of tumour cells with punctate, faint and incomplete staining of the cell membrane.
2+ (positive)	More than 10 % of tumour cells with weak to moderate complete membrane staining.
3+ (positive)	More than 10 % of tumour cells with strong complete membrane staining. A fish net pattern is often observed.

Score 0 and 1+ are regarded as negative. Score 2+ and 3+ are positive for HER-2 receptor overexpression (52). (figure 4)

0 1+ 2+ 3+

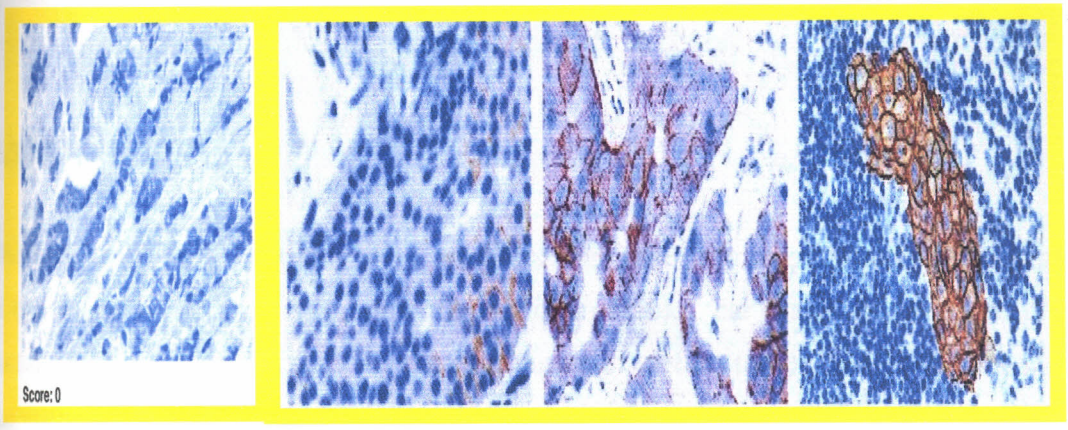


Figure 4:The illustrated possible scores of the immunostaining results

Appendix V shows the detailed laboratory procedure.

E Quality control

Internal quality control was included in the immunohistochemistry procedure in order to ascertain that everything was functioning properly.This was done with every staining run.

1. Positive control specimen

   A known positive control specimen was included to monitor the accurate performance of the reagents and the procedure.

2. Negative control specimen

   A negative control reagent instead of the prepared primary antibody was used for the negative control specimen.This was to verify the specificity of the primary antibody.

Nairobi Hospital usually participates in external quality assurancy programme in collaboration with WHO external quality control from South Africa.

DATA ANALYSIS

The results of HER-2 staining were correlated with other clinico-pathologic parameters of breast cancer prognosis and presented in form of tables, bar charts and pie charts. For instance, there are tables showing the distribution of various tumour parameters among the subjects. In addition every prognostic parameter has a table showing HER-2 expression score frequencies.

Comparison of results in any given breast cancer parameter was done using X^2 and t-tests. In all statistical calculations, a 5% level of significance was applied.

ETHICAL CONSIDERATIONS

The study was only undertaken after approval by the KNH ethical and research committee.

A detailed client information and consent form was administered in order to obtain informed consent from the participating patients in the clinic and the ward. Those consenting were assured of total confidentiality and voluntary nature of participation without coercion. It was not possible to get consent from those who participated in the study retrospectively.There was only minimal risks to patients taking part in the study.

For those breast cancer patients involved in the study, the results were communicated to their physicians in the usual manner that all results are communicated but were discussed with the attending physician where possible. Counselling of patients on the implication of the results was left to the clinician in-charge.

RESULTS

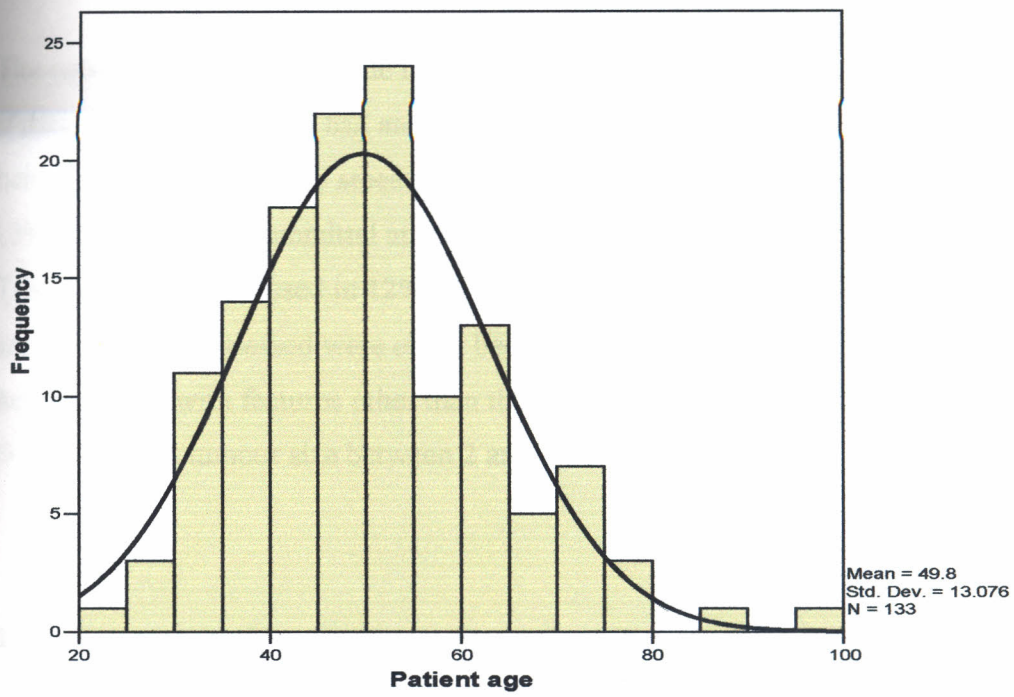
This study was carried out between October 2004 and May 2005. The total number of cases was 133.

Age / parity of the patients

The mean age of the study population was 49.8 years with the median age at 48.0 years. (figure 5) The youngest patient with breast cancer was 24 years and the oldest 100 years.

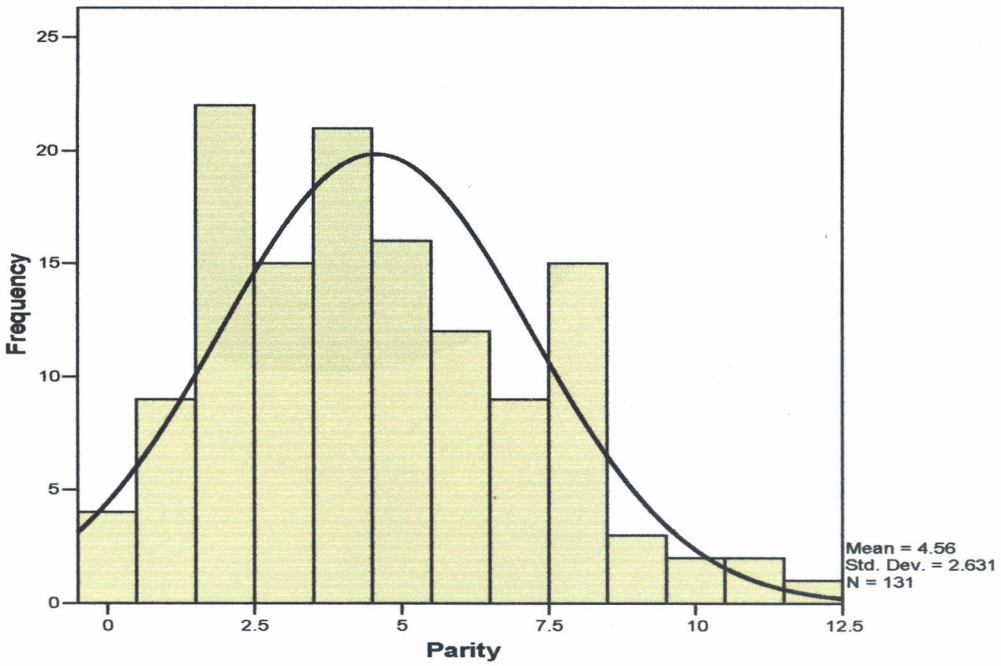
The majority of the patients were female, there being only two males. Of the 133 subjects studied, 95 (71%) were married. The patients who were single only numbered 6 (4.5%). The remaining patients were either divorced or widowed. Mean age of menarche among those studied was 14.9 years, with the earliest at 12 years and the latest at 20 years of age. The commonest clinical presentation among the breast cancer patients studied was breast lump which was present in 129 (97%) patients. Only 29 (21.8%) patients had nipple discharge.

Figure 5: Age distribution of patients with breast cancer



Female subjects had a mean parity of 4.6 children. (figure 6) The parity range was from four women who were nulliparous to the two with 11 children each.

Figure 6: Parity distribution of the patients with breast cancer



Correlation between FNA diagnosis and tumour size

Fine needle aspiration was done in 90 (67.7 %) of the 133 patients studied. The majority of these patients (76.7%) had an FNA diagnosis of malignancy, 20 % of the patients had their breast lesion cytology smears reported as benign and the diagnosis in the remaining 3.3% cases was non-committal at FNA.. (figure 7)

Tumour size was assessed in 129 subjects. The remaining four patients in whom tumour size could not be assessed were either because of cancer recurrency after mastectomy or they presented with features other than the breast lump. Majority of the patients, that is, 69 (51.9%) had tumour size between 2 and 5 centimeters.

Figure 7: Accuracy of breast cancer diagnosis at FNA

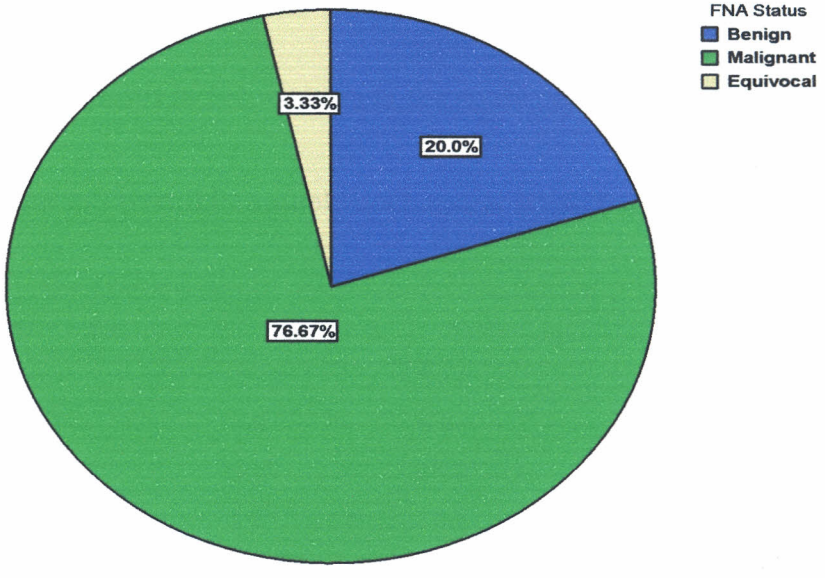


Table 4: The FNA diagnosis at various tumour sizes

		FNA Status			Total
		Benign	Malignant	Equivocal	
Tumour sizes	< 2cm	3	8	1	12
	2cm - 5cm	14	33	2	49
	> 5cm	1	28	0	29
Total		18	69	3	90

There is a significant correlation between the tumour size and FNA diagnosis of malignancy (p value = 0.039, pearson chi-square). The mean tumour size in patients with a nipple discharge was 5.9 cm and in those without discharge was 4.8 cm., giving a significant correlation (p = 0.050, 2-tailed). (Table 4)

HER-2 status of the patients in the study group

Table 5: Overall immunohistochemical HER-2 score results

Her – 2 status	Frequency	Percentage
0+	79	59.4
1+	9	6.8
2+	20	15.0
3+	25	18.8
Total	133	100.0

Scores 0+ and 1+ are taken as negative and scores 2+ and 3+ positive for HER-2 overexpression. There were 45 of the 133 patients with HER-2 overexpression giving a prevalence of 33.8%. (Table 5) (Figures 9, 10, 11 & 12)

Correlation between HER-2 overexpression and age

Table 6: HER-2 status versus age

HER-2 status	Frequency	Mean age (years)
Positive	45	50.3
Negative	88	48.9

The mean age of breast cancer cases with HER-2 overexpression was 50.3 years while that of cases without overexpression was 48.9 years. No significant correlation between age and HER-2 status was established (p value = 0.578, 2-tailed). (Table 6)
The number of the study subjects who were above 50 years of age was roughly equal to those who were below 50 years. HER-2 positivity in the elderly patients was 30.8% and in the young ones was 37.9%. Still no significant correlation was found between these two groups of the subjects. (p value = 0.251, Fisher`s exact test).

Correlation between histological type and HER-2 status

Table 7: HER-2 positivity and the breast cancer histological type

Tumour type	Number	Percentage	HER +	HER -
Carcinoma in situ	6	4.5	2	4
Invasive ductal carcinoma	98	73.7	33	65
Invasive lobular carcinoma	18	13.5	5	13
Tubular carcinoma	3	2.3	1	2
Colloid carcinoma	2	1.5	2	0
Papillary carcinoma	2	1.5	2	0
Anaplastic carcinoma	3	2.3	0	3
Sq cell carcinoma	1	0.8	0	1
Total	133	100	45	88

Of the 133 cases included in this study, infiltrating ductal carcinoma (IDC) was the largest group, accounting for 73.7% (98/133) of all the cases. The second largest group, composed of eighteen (13,5%) cases, was lobular carcinoma. The rest included six cases of ductal carcinoma *in situ*, three cases of tubular carcinoma, three cases of anaplastic carcinoma, cases of colloid and papillary carcinoma were two each. There was one case of metaplastic (squamous cell) carcinoma. (Table 7) (Figure 8, 13) There was no significant correlation between histological type and HER-2 overexpression of breast cancer (p value = 0.179, Pearson chi-square).

Figure 8: Metaplastic (squamous cell) carcinoma

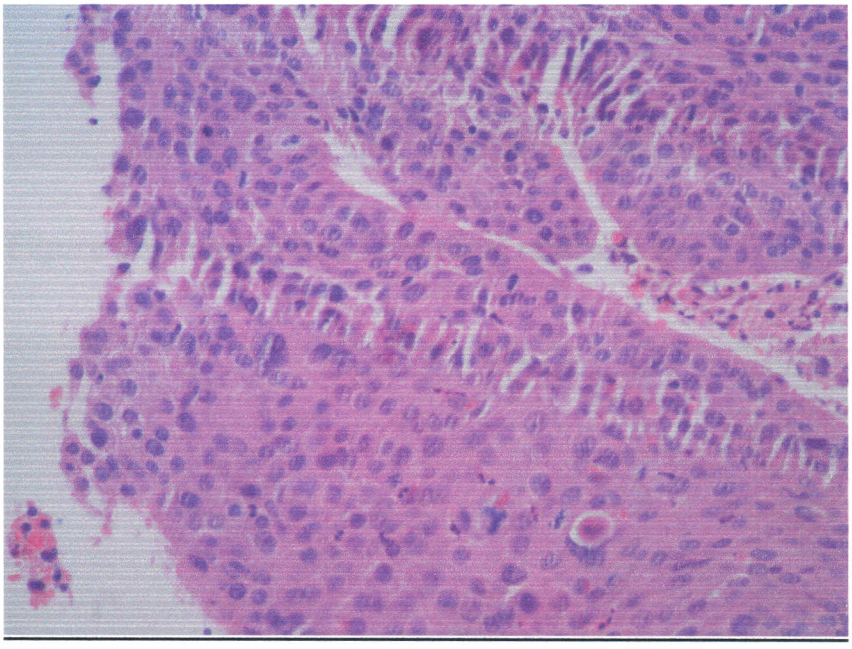


Figure 9: Immunohistochemical score of 0+

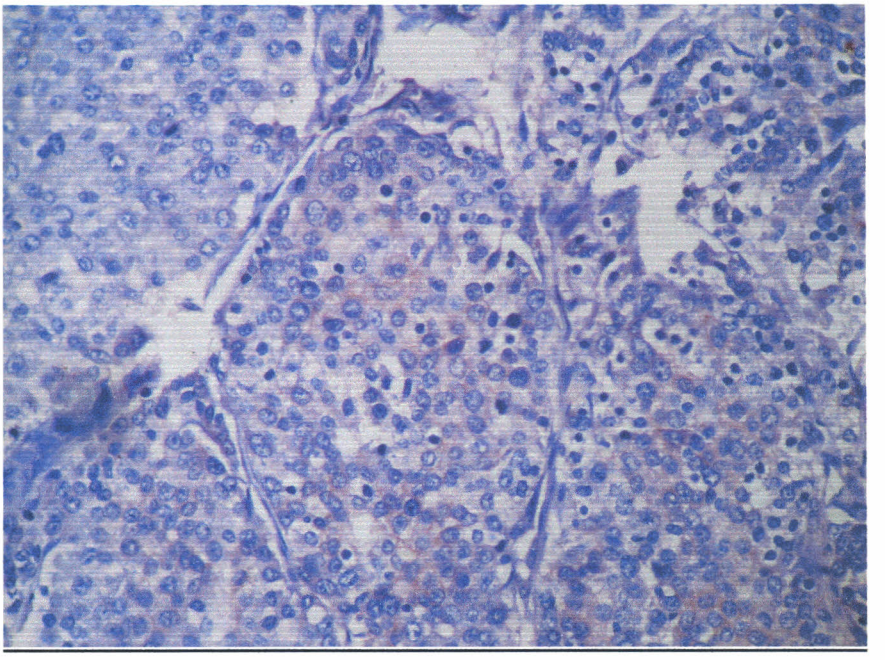


Figure 10: Immunohistochemical score of 1+

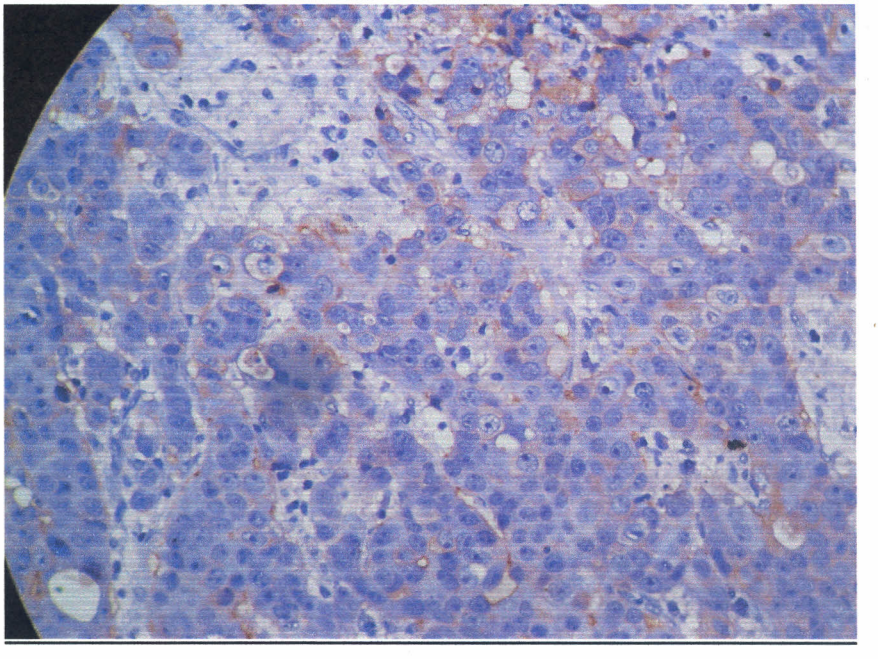


Figure 11: Immunohistochemical score of 2+

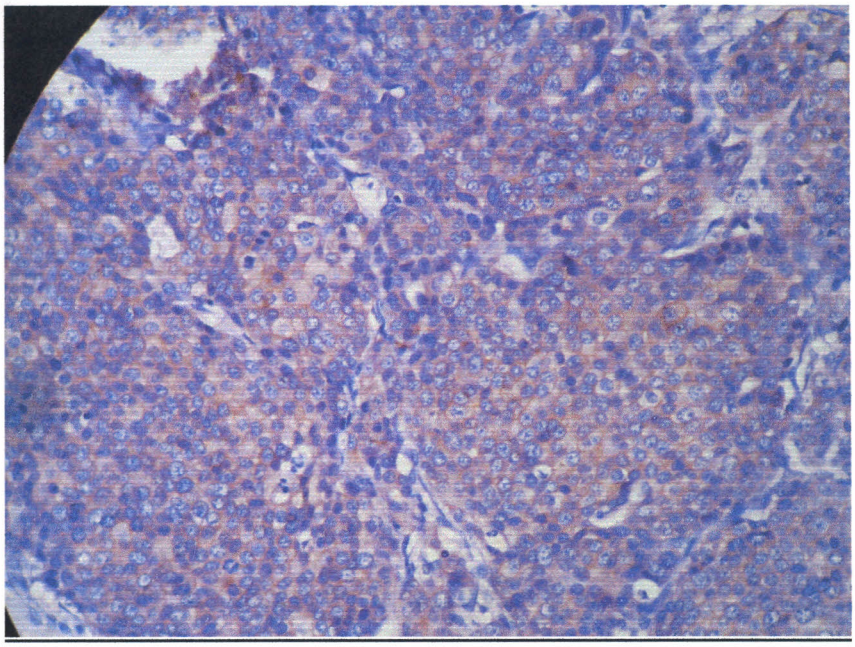


Figure 12: Immunohistochemical score of 3+

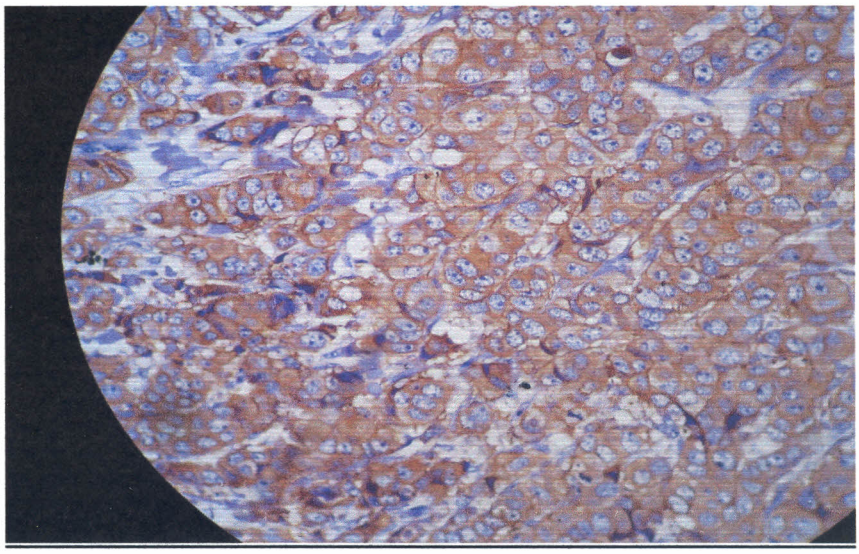
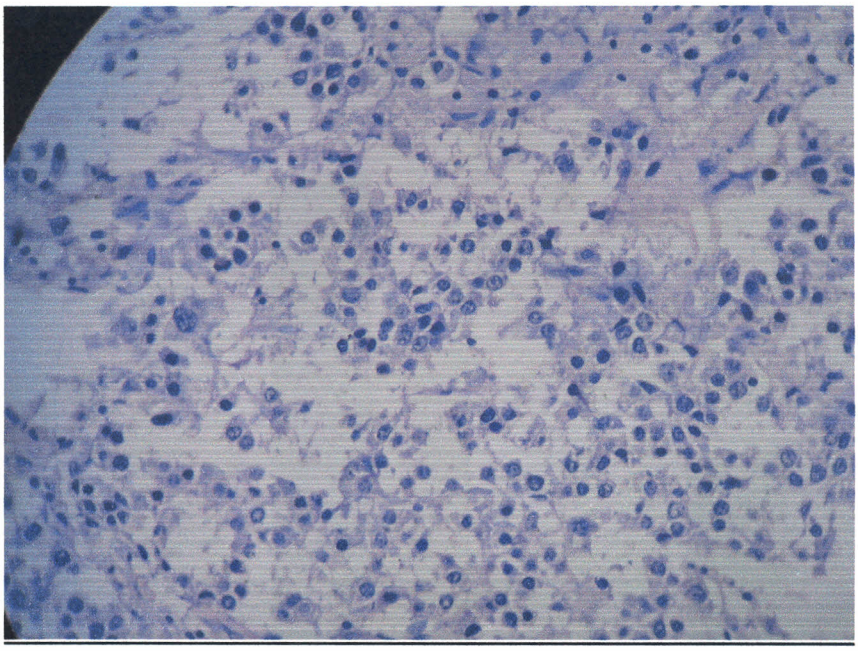


Figure 13: HER-2 score 0+ in anaplastic carcinoma



Correlation between tumour size and HER-2

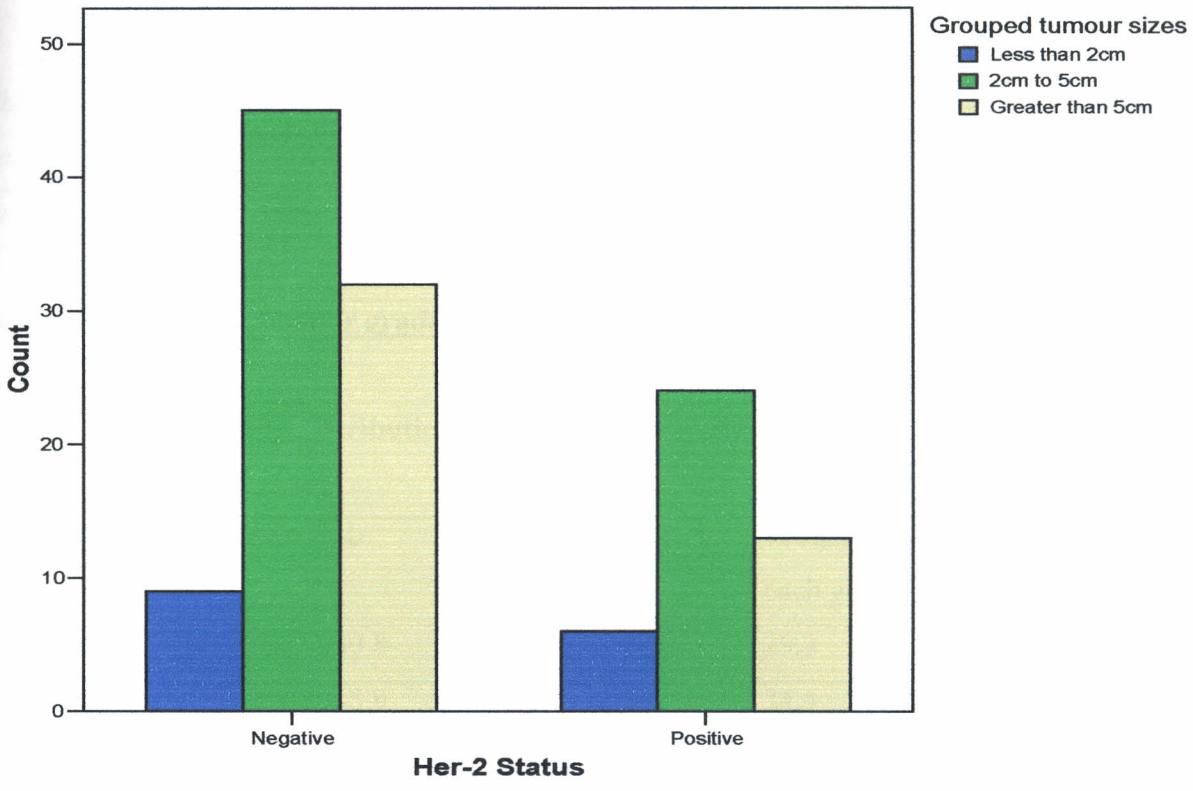


Figure 14: HER-2 status according to tumour size

No significant correlation was noted between tumour sizes and HER-2 overexpression (p value = 0.801, 2-tailed), however tumors with positive HER-2 expression tended to be larger than those lacking expression (scores 0+ and 1+), with mean sizes of 5.05 cm and 5.18 cm, respectively. (Figure 8) When tumours with HER score 0+, 1+ and 2+ were taken as a group, they had a mean size of 4.8 cm compared with a mean size of 6.3 for tumours with HER-2 score 3+, hence showing a significant positive correlation (p = 0.020, 2-tailed). (Table 8)

Table 8: Tumour sizes versus HER-2 status

HER-2 status	Number	Mean tumour size(cm)
Negative	106	4.832
Positive	23	6.283

Correlation between tumour grade and HER-2 overexpression

Table 9: Tumour grade distribution and HER-2 positivity

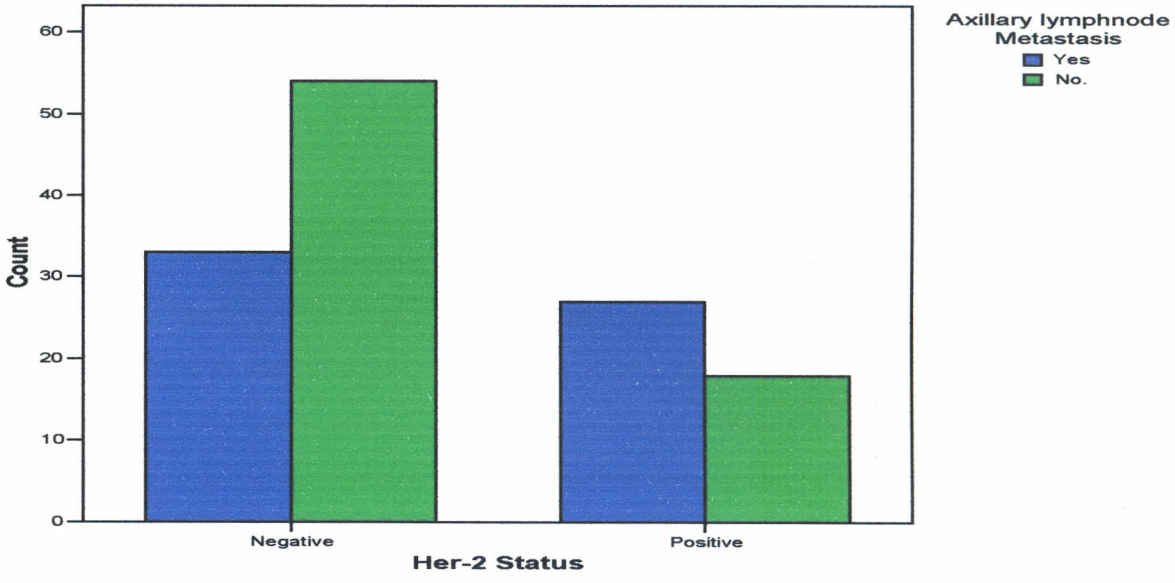
Tumour grade	Number	%	HER-2 +	HER-2-	% HER-2 positivity in each grade
1	41	30.8	12	29	29.3
2	61	45.9	21	40	34.4
3	30	22.6	12	18	40
Total	132	100	45	87	

Grading analysis was limited to 132 of the 133 cases of breast cancer as one case of squamous cell carcinoma was not graded. HER-2 overexpression was seen in 29.3% of grade 1, 34.4% of grade 2 and 40% of grade 3 breast carcinomas.There was no significant correlation between tumour grade and HER-2 positivity (p = 0.640, pearson chi-square). (Table 9) P value markedly reduced when only HER-2 score 3+ was regarded as positive (p value = 0.073).

Correlation between axillary lymph node metastasis and HER-2 overexpression

From the study population, only 60 of the 133 patients had axillary lymph node metastasis. HER-2 overexpression was present in 45% of those with axillary lymph node metastasis and in 24.7% of those without axillary lymph node metastasis. (Figure 9) Axillary lymph node metastasis had a significant positive correlation with HER-2 overexpression (p = 0.013, Fisher's exact test).

Figure 15: HER-2 status versus axillary lymph node metastasis



Correlation between breast cancer clinical stage and HER-2 overexpression

Table 10: HER-2 status by clinical stage stratification

Clinical *stage*	Number	(%)	Number of *HER + tumours*	Number of *HER- tumours*	% HER-2 overexpression *in each stage*
1	6	4.5	1	5	16.1
2	46	34.6	20	26	43.5
3	62	46.6	15	47	24.2
4	17	12.8	8	9	47.1
Total	131	98.5	44	87	

Only 6 (4.5%) of the patients sought treatment when breast cancer was in an early stage (stage 1). For the 17 (12.8%) patients who had distant metastasis (stage IV), 6 (35.3%) had involvement of the supraclavicular lymph nodes, 5 (29.4%) had metastasis to the lungs / pleural cavity and 3 (17.6%) had metastatic deposits to the bone (vertebra / femur). One case had involvement of both the liver and the lung. Other sites affected by tumour spread were; the peritoneum, eye, and contralateral lymph nodes. Only 16.1% of tumours in stage I had HER-2 overxpression as opposed to 47.1% of tumours in stage IV. (Table 10) No significant linear correlation was noted between clinical stage and HER-2 overexpression of breast cancer ($p = 0.085$, pearson chi-square).

DISCUSSION

In this cross – sectional study, the mean age of breast cancer patients was 49.8 years with a median age of 48 years. Only 2 of the 133 subjects were male. Garfinkel et al found the mean age of breast cancer patients in USA to be 64 years (7) while local studies show breast cancer patients with a mean age of about 50 years (6). The lower mean age for Kenyan patients may be due to the fact that our population is relatively young compared to that of the West or may be the tumours in black people have a different biological behaviour. The male : female ratio which is 1:67 in this study is compared to the ratio of 1:100 in the study by Garfinkel.

Majority of the female subjects were married, that is 71%. The single ones were 5% , 8% were divorced, and 16% were widowed. This seemed to reflect the general trend in the Kenyan population (76). The mean age of menarche was 14.9 years, and 30% of the subjects could not remember their age at menarche. These findings on menarche were in close agreement with those of the study by Bwibo et al in 1982 (57).

Of the patients studied, 97% had breast lump as the clinical presenting feature. Nipple discharge was present in only 22% of the patients. This results are fairly close to the findings by Farrow (74) who had 80% of breast cancers presenting as a lump and 22% of the patients had nipple discharge. There was a significant positive correlation between tumour size and nipple discharge ($p = 0.05$).This probably can be explained by the fact that the bigger the size of the tumour, the more likely it is to exert higher pressure within the breast, and hence the nipple discharge.

The mean parity of the study population was 4.6 + 0 for the female subjects. The range of parity was such that 4 patients were nulliparous and 2 had 11 children each. This parity

compares well with the findings by Macrae et al in a 2001 study on *fertility trends and population policy in Kenya*, where average parity was 5 chidren (58).

Fine needle aspiration was not done in 43 of the 133 cases studied even though the protocol in breast lump management is that FNA should come first. This was because some clinicians opted to go for incisional biopsy straight away, especially if diagnosis was made in a facility without FNA capability. Furthermore, only palliative mastectomy was done for those patients who came with advanced disease. Of the 90 cases diagnosed at FNA 77% were reported as malignant, 20% benign, and 3% had an equivocal diagnoses. So from this study, sensitivity was 77%. In a 1984 study by Norton et al in North America, FNA had a sensitivity of 87% (59). The FNA diagnosis of malignancy had a positive significant correlation with the tumour size ($p = 0.039$). This correlation can easily be explained by the fact that the smaller the lump the easier it is to be missed during fine needle aspiration and vice versa.

In this study, it was found that 45 (33.8%) of 133 cases were HER-2 positive. HER-2 positivity was defined as the tumour immunohistochemical score of 2+ or 3+ (52). Although there is a wide variation in HER-2 overexpression and amplification, this prevalence appears to be close to the commonly accepted rate of 10% to 25% (49). In a review of 97 studies involving 22,616 patients in France, Revillion et al found HER-2 gene amplification prevalence to lie between (5 – 55)% with a mean of 26% (60). From a recent unpublished study in Nairobi Hospital by Nyagol et al, prevalence of HER-2 positivity using immunohistochemical methods was found to be 27.85%. In some cases, it is difficult to differentiate IHC HER-2 score 1+ from 2+ or 2+ from 3+ because the analysis of the score is done semiquantitatively using a microscope. Score 3+ usually show amplification of the gene while score 1+ lack the amplification. Much attention has been focused on IHC HER-2 score 2+ cases. A substantial proportion of 2+ cases are not HER-2 gene amplified by FISH, for instance, more than 20% may turn out negative in most studies (61). For those tumours with a score of 2+, there is need to confirm gene

amplication by carrying out FISH. If FISH was done in this study, the prevalence of HER-2 gene amplification may have been lower. FISH has a much higher sensitivity in picking out tumours with overexpression as it detects the amplification of HER-2 gene.

The mean age of HER-2 positive patients was 1 year and 5 months less than those patients lacking HER-2 overexpression, a statistically not significant difference (p = 0.578). Similarly, the percentage HER-2 positivity in patients younger than 50 years of age was higher (38%) than in those who are 50 years or above (31%), but still no significant correlation was found. Nidal et al (62) found a clear negative correlation between HER-2 overexpression and age in Jordanian women. Most studies including that of Koeppen et al (52) find no significant correlation between HER-2 overexpression and age of the patient.

The distribution of histological types among the study population was as follows: Infitrating ductal carcinoma (IDC) – 73.7%; Infiltrating lobular carcinoma (ILC) – 13.5%; Carcinoma in situ (CIS) – 4.5%; Tubular carcinoma (TC) – 2.3%; Anaplastic carcinoma – 2.3%; Colloid carcinoma (CC) – 1.5%; Papillary carcinoma (PC) – 1%; Squamous cell carcinoma (SCC)– 0.8%. These results closely agree with the following findings by Dixon et al in the 1985 USA study (11): IDC – 79%; ILC – 13.5%; CIS – 15-30%; TC – 6%; CC – 2%; PC – 1%; Medullary carcinoma – 2%. The relatively low percentage of CIS in this study may be due to the fact that most of our patients come to the hospital late unlike in the West. In addition, breast cancers are diagnosed at an earlier stage in the developed countries due to the widespread use of mammography.

HER-2 was overexpressed in 33.7% of the infiltrating ductal carcinoma cases compared to 27.7% of the eighteen lobular carcinoma cases in this study. This pattern of low HER-2 expression in lobular carcinoma is in agreement with data reported in the literature (63).

Nevertheless, there was no significant correlation between HER-2 overexpression and histological type (p = 0.179).

The mean of tumour size in the study subjects was 5.1cm. Our results show a tendency of HER-2 overexpression to be more associated with larger tumor size. Tumors overexpressing HER-2 were on the average 0.13 cm larger than those lacking HER-2 overexpression, even though this difference was not statistically significant (p = 0.801). Nidal et al (62) did not also find a correlation (p = 0.13). In this study the tumours with IHC score (0+, 1+, 2+) had a mean size of 4.8 cm compared to a mean size of 6.3cm for score 3+ tumours, giving a significant correlation (p = 0.020). Most studies including one by Hoff et al (64) found a significant correlation. The significant correlation established when only IHC score 3+ was regarded as overexpressed may mean that if FISH was carried out in this study population, there might have been a positive significant correlation between the tumour size and HER-2 overexpression.

The percentage number of patients overexpressing HER-2 in grades 1, 2, and 3 was 29.3%, 34.4%, and 60% respectively. This means that the higher the tumour grade the more likely the patient is to be HER-2 positive. However, the study did not show a significant relationship between HER-2 expression and the histologic grade of breast carcinoma (p = 0.640). Most other studies concluded that HER-2 overexpression or amplification is associated with the grade (64,65). The findings in this study are similar to those of a Taiwanese study by Huang et al, where there was no significant correlation (66). It should be pointed out, however, that the many different breast cancer grading systems would not allow the investigator to evaluate this variable with any degree of confidence, hence making comparisons with other studies difficult. Furthermore, tumour grading often has poor reproducibility between pathologists (24).

The proportion of the patients who had axillary lymph node metastasis was 60 (45%) of the 133 subjects. Other studies showed that about (40 – 50)% of breast cancer patients had axillary metastasis (12). The data from this study reveal that HER-2 overexpression was 45% in tumors that had axillary lymph node metastases, as opposed to 24.7% in tumours without axillary lymph node metastasis, and this difference was statistically significant ($p = 0.013$). Other groups have shown a direct relationship between lymph node metastases and HER-2 overexpression (67).

Only 4.5% of the studied patients came to hospital early enough when the breast cancer was in stage 1. This was the group that would benefit most from local therapy alone i.e surgery. For the 17 patients who came with advanced breast cancer (stage IV), majority (82%) had distant metastasis to the supraclavicular lymph nodes, the lung and the bone. This was in agreement with other studies including one by Ghulam et al in Pakistan (68). HER-2 overexpression in stages I, II, III, and IV was 16.1%, 43.5%, 24.2%, and 47.1% respectively. Although a linear correlation between HER-2/neu overexpression and clinical stage cannot be established at this time ($p = 0.085$), it is interesting to note that when stages I and II, and when stages III and IV are combined, respectively, the latter category has a higher rate of overexpression than the former. It was also noted that stage IV, which is the most aggressive disease stage was associated with the highest frequency of overexpression. Mark et al in the study of 40 breast cancer patients in USA also found that stage III and IV tumours had a higher frequency of HER-2 overexpression than tumours with lower stages, despite lack of significant correlation (69). Unlike most studies, Seshadri et al in the study of 73 Australian breast cancer patients, found a positive significant correlation between HER-2 overexpression and advanced stage (70).

Breast carcinoma is a disease with a considerable heterogeneity in its clinical behavior. There are clinical and pathological variables that may help in predicting prognosis and the need for adjuvant therapy. These include tumour size, histologic grade, histologic type, lymph node metastases, vascular space invasion, tumor cell proliferation, tumor necrosis, extent of ductal carcinoma *in situ*, age, and pregnancy (71). Estrogen and

progesterone receptor status in combination have for long been used as prognostic factors and predictors of response to therapy. Newer parameters are needed to distinguish subgroups with different biological features within carcinomas that otherwise appear homogenous according to classic pathological and clinical criteria. HER-2 status along with the above tumour parameters has been shown to reasonably predict prognosis, response or resistance to treatment, and the potential use of newer drugs such as trastuzumab in specific subgroups of breast cancer (54).

The oncoprotein encorded by the HER-2 oncogene is the first successfully exploited target molecule in new biomolecular therapies of solid tumours. The association of HER-2 gene amplification with tumours, its extracellular accessibility and its involvement in tumour aggressiveness are the factors that make this receptor an appropriate target for tumour – specific therapy. From numerous studies (72), it has been proved that tumours with HER-2 gene amplification show good therapeutic response to specific treatment regimes incorporating trastuzumab. Also, HER-2 overexpression fosters the oncoprotein immunogenicity, as shown by the frequency of B and T cell-mediated responses against this oncoprotein in cancer patients, and it is being investigated as a promising molecule for either passive and active immunotherapy strategies (75).

LIMITATIONS OF THE STUDY

1. The investigator would have preferred a larger sample size.This was not possible as the time and financial resources for the study were limited.

2. The study population may not have been sampled properly, as many cases were excluded due to the lack of axillary lymph node sampling.

3. Fluorescence in situ hybridization would have been a useful further analytical method for those tumours with HER-2 IHC score 2+ but it could not be done in this study due to the unavailability of the facilities for the test locally.

4. This study was only correlating HER-2 overexpression with other factors for poor prognosis and may not be fully reflective of the prognostic value of HER-2 status. The right procedure may have been following up these patients to determine their mean survival years.

CONCLUSIONS

1. This study showed that the prevalence of HER-2 overexpression in breast cancer patients at KNH was 33.8 % in the cases analyzed.

2. There was no significant correlation between HER-2 overexpression and age.

3. No significant correlation was demonstrated between the histological type of the breast cancer and HER-2 positivity, just like in many other studies done else where.

4. Unlike the well known significant correlation between breast cancer histological grade and HER-2 overexpression, none was found in this study. This may be due to using different grading systems and lack of tumour grading reproducibility.

5. Tumour size failed to significantly correlate with HER-2 overexpression in this study, but when only those breast cancers with IHC score of 3+ were regarded as positive, there was a significant positive correlation between tumour size and HER-2 overexpression.

6. There was significant correlation between axillary lymph node metastasis and HER-2 overexpression. Other studies give conflicting results about the relationship between axillary lymph node status and HER-2 positivity.

7. No significant correlation between HER-2 overexpression and clinical stage of the breast cancer was established in this study. The studies that have so far been conducted in this area show no consistent relationship between HER-2 overexpression and the clinical stage.

RECOMMENDATIONS

1. HER-2 overexpression in human breast cancers may be of more significance as a therapeutic parameter rather than as a prognostic parameter.

2. There is significant HER-2 overexpression among breast cancer patients in KNH, therefore this oncoprotein should routinely be tested in breast cancer patients at KNH as the patients with HER-2 gene amplification can benefit from a monoclonal antibody based therapy.

3. Those tumours immunohistochemically staining for HER-2 with a score of 2+ should be further subjected to FISH analysis to determine their HER-2 gene amplification status. If this was done in the study, then HER-2 overexpression prevalence at KNH may have been less than 33.8%. In addition, significant correlation between HER-2 overexpression and other established prognostic factors may have been established and this might have been in agreement with most other studies.

4. Use of standard breast surgery protocols allows for comparison of this study with others done elsewhere.

5. A larger cohort of patients should be studied to find out how HER-2 overexpression relates with several other breast cancer clinico-pathological features. Follow up of these cases over a number of years is crucial for learning about prognosis in our local settings.

REFERENCES

1. Sondik EJ: Breast cancer trends. Incidence, mortality, and survival. Cancer 74: 995-999, 1994.
2. Sesco AJ: Breast cancer and environment. Horm Res 60 (suppl 3): 50, 2003.
3. Ferlay J, Bray F, Pisani P, Parkin DM: GLOBOCAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Version 1.0. AARC CancerBase No. 5. Lyon, IARCPress, 2001.
4. Alero Fregene, Lisa Newman: Breast cancer in Sub- Saharan Africa: How Does It Relate to Breast Cancer in African-American Women? http://www.Cancer. Org/docroot/MED.
5. Neondo Henry: Kenyan breast cancer survivor fights stigma, shame. http://www. Womensenews. Org/030331.cfm.
6. Bjerregaard B and Kung'u A: Breast cancer in Kenya. A histopathologic and epidemiologic study. E. Afr. Med. J. 69: 62, 1992.
7. Garfinkel L, Boring CC, Heath CW, et al: Changing trends. An overview of breast cancer incidence and mortality. Cancer 74: 222-227, 1994.
8. Kohlmeier L, Mendez M: Controversies surrounding diet and breast cancer. Proc. Nutr. Soc. 56: 369, 1997.
9. Radford DM, Zehnbauer BA: Inherited breast cancer. Surg. Clin. North AM 76: 205, 1996.
10. Zhang H, Tombline G, Weber B: BRCA 1, BRCA 2, and DNA damage response: Collision or collusion? Cell 92: 433, 1998.
11. Dixon JM, Page DL, Anderson TJ, et al: Long- term survivors after breast cancer. Br J Surg 72: 445, 1985.
12. Brinkley D, Haybittle JL: The curability of breast cancer. Lancet 2: 95- 97, 1975.

13. Quiet CA, Ferguson DJ, Weichselbaum RR, Hellman S: Natural history of node – negative breast cancer. A study of 826 patients with long – term follow – up. J Clin Oncol 13: 1144 – 1151, 1995.

14. Anan K, Mitsuyama S, Tamae K: Axillary lymph node metastases in patients with small carcinomas of the breast: is accurate prediction possible? Eur J Surg 166(8):610-5, 2000.

15. Goldstein NS: The significance of extracapsular axillary lymph node extension by metastatic breast cancer. Int. J. Surg Pathol 3: 65-66, 1995.

16. Kinne DW: Staging and follow-up of breast cancer patients. Cancer 67: 1196- 1198, 1991.

17. Beahrs OH, Henson DE, Hutter RVP, et al: American Joint Committee on Cancer.Philadelphia, 1992, J.B. Lippincott.

18. Erykber ER, Bland KI, Copeland EM: The detection and treatment of early breast cancer. Adv. Surg. (US) 23: 119-94, 1990.

19. Coccono G: The natural history of operable breast cancer after primary treatment. Ann. Oncol. (Netherlands) 6 Suppl. 2: 11-21, 1995

20. YeatmannTJ: The natural history of locally advanced primary breast carcinoma and metastatic disease. Surg. Oncol. Clin N. AM 4: 569-89,1995.

21. Eberlain TJ: Current management of carcinoma of breast. Ann. Surg. (US) 220: 212-36, 1994.

22. Lash RH, Bauer TW, Medendorp SV: Prognostic significance of the proportion of intraductal and infiltrating ductal carcinoma in women treated by partial mastectomy. Surg Pathol 3:47-58, 1990.

23. Fields JN, Kuske RR, Perez CA, Fineberg BB, Bartlett N: Prognostic factors in inflammatory breast cancer. Univariate and multivariate analysis. Cancer 63: 1225-1232, 1989.

24. American Medical Association: Breast cancer diagnosis. http:// imagines. com/breasthealth/histologic-grades.asp.

25. The American Cancer Society. http://www3. Cancer.org/cancerinfo/main cont.asp?

26. Hawkins RA, Roberts MM, Forrest APM,et al: Oestrogen receptors and breast cancer. Current status. Br J Surg 67: 162-165,1980.

27. Mohsin SK, Weiss H, Harighurst T: Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study: Mod Pathol 17 (12):1545-54, 2004.

28. Sahin AA, Ro J, Ro JY, et al: Ki-67 immunostaining in node-negative stage I/II breast carcinoma. Significant correlation with prognosis. Cancer 68: 549-557,1991.

29. Tan PH, Bay BH, Yip G, Selvarajan S: Immunohistochemical detection of Ki-67 in breast cancer correlates with transcription regulation of genes related to apoptosis and cell death: Mod Pathol 18 (3):374-81, 2005.

30. Wenger CR, Beardslee S, Owens MA: DNA ploidy, S-phase, and steroid receptors in more than 127 000 breast cancer patients: Breast Cancer Res Treat 28 (1): 9-20, 1993.

31. Barnes DM, Dublin EA, Fisher CJ, et al: Immunohistochemical detection of p53 protein in mammary carcinoma. An important new independent indicator of prognosis? Hum Pathol 24: 469-476, 1993.

32. Rossen PP, Lesser ML, Arroyo CD: P53 in node-negative breast carcinoma: an immunohistochemical study of an epidemiologic risk factors, histologic features, and prognosis: J Clin Oncol 13 (4): 821-30, 1995.

33. Duff SE, Chenggang LI, Garland JM, Kumar S: CD 105 is important for angiogenesis: evidence and potential applications: The FASEB Journal 17: 984-992, 2003.

34. Nicholson KM, Anderson NG: The protein kinase B /Akt signaling pathway in human malignancy: Cell Signal 14 (5): 381-95, 2002.

35. Krajewski M, Thor AD, Edgerton SM, Moore DH: Analysis of Bax and Bcl-2 expression in p53- immunopositive breast cancers: Clinical Cancer Research 3 (2): 199-208, 1997.

36. Nixon AJ, Neuberg, Hayes DF, Gelman R, Connolly JL: Relationship of patient age to pathologic features of the tumour and prognosis for patients with stage I and stage II breast cancer: J Clin Oncol 12: 888-894, 1994.

37. Wiedemann LM, McCarthy KP, Chan LC, et al: Chromosome rearrangement, oncogene activation, and other clonal events in cancer: their use in molecular diagnostics. Journal of pathology 163: 7-12, 1991.

38. Suo Z, Emilsen E, Tveit, et al: Type 1 protein tyrosine kinases in benign and malignant breast lesions. Histopathol 33: 514-521, 1998.

39. Carpenter G, King L, Cohen S, et al: Epidermal growth factor stimulates phosphorylation in membrane preparation in vitro. Nature 276: 409-410, 1978.

40. Conssens L, Yang-Feng TL, Liao YC, et al: Tyrosine kinase receptor with extensive homology to Epidermal growth factor (EGF) receptor shares chromosomal location with neu oncogene. Science 230: 1132, 1985.

41. Burstein HJ: Special introduction to integrated session on HER-2/neu. http://www.medscape. Com/viewarticle/427437?src=search.

42. Hynes NE, Stein DF: The biology of erb-2/neu/HER-2 and its role in cancer. Biochem. Biophys. Acta. 1198: 165-184, 1994.

43. Berger MS, Locher GW, Saurer S, et al: Correlation of C-erbB-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. Cancer Res. 48: 1238-1243, 1998.

44. Heim S,Mitelman F: Primary chromosome abnormalities in human neoplasia. Advances in cancer Research 52: 1-43, 1989.

45. Fukushige S, Matsubera K, Yoshida M, et al: Localization of a novel v-erbB-related gene, c-erbB-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol. Cell Biol. 6: 955-958, 1986.

46. Cook JC, Chan L, Grimm, et al: Trasduction of mammalian cells expressing the Her-2/neu receptor. Eur. J. Biochem. 270: 2287, 2003.

47. Ross JS, Nazeer T, Church K, et al: Contribution of HER-2/neu oncogene expression to tumour grade and DNA content analysis in the prediction of prostatic cancer metastasis. Cancer 72: 3020-3028, 1993.

48. Frolik D, Cardiff R, Verge Z, et al: Pleomorphic lobular carcinoma of the breast: its cell kinetics, expression of oncogenes and TS genes compared with invasive ductal carcinomas and classical infiltrating lobular carcinomas. Histopathology 39: 503-513, 2001.

49. Barnes DM: C-erbB-2 amplification in mammary carcinoma. J. Cell. Biochem. 17: 132-138, 1993.

50. Venter DJ, Tuzi N, Kumar S, et al: Overexpression of the c-erbB-2 oncoprotein in human breast carcinomas: immunohistochemical assessment correlates with gene amplification. Lancet 2: 69-72, 1987.

51. David FS: Tyrosine kinase signaling in breast cancer: ErbB family receptor tyrosine kinases. Breast Cancer Res 2:176-183, 2000.

52. Koeppen HKW, Wright BD, Burt AD,et al:Overexpression of HER-2/neu in solid tumours: an immunohistochemical survey. Histopathology 38 : 96-104, 2001.

53. Carter P, Presta L, Gormen C, et al. Humanization of an anti-P 185 HER-2 antibody for human cancer therapy. Proc. Natl. Acad. Sci. 89: 4285-89, 1992.

54. Slamon D, Leyland-Jones B, ShakS, et al: Addition of Herceptin™ (humanized anti-HER-2 antibody) to first line chemotherapy for HER-2 overexpressing metastatic breast cancer (HER2+/MBC) markedly increases anti-cancer activity: a randomized , multinational controlled phase III trial. Proc. AM. Soc. Clin. Oncol. 17: 98a, 1998.

55. Fisher AA, Laing JE, Shoeckel JE, Townsend JW: Handbook for Family Planning Operations Research Design. Population Council Publisher; 1998.

56. HSU SM ,Raine L,Fanger H:Use of avidin –biotin peroxidase complex (ABC) in immunoperoxidase techniques. A comparison between ABC and unlabelled antibody peroxidase antiperoxidase (PAP) Procedures.J Histochem Cytochem 29:557-580,1981.

57. Neuman C, Bwibo NO, Sigman M: Menarche in African secondary school girls in Kenya. E. Afri. Med. J. 64 (8): 511-515, 1992.

58. Macrae SM, Bauni and J Blacker (2001): Fertility trends and population policy in Kenya. In Brass Tacks. Essays in Medical Demography, Basia Zaba and John Blacker, eds. London.

59. Norton LW, Davis JR, Wiens JL: Accuracy of aspiration cytology in detecting breast cancer. Surgery 96: 806-811, 1984.

60. Revillion F, Bonneterre J, Peyrat JP: ERBB2 oncogene in human breast cancer and its clinical significance. *Eur J Cancer* **34:**791-808, 1998.

61. Ridoffi R, Mehdi R, Jamehdor MD et al: HER-2/neu testing in breast carcinoma: a combined immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) approach. Mod Pathol 13: 866-873, 2000.

62. Nidal MA and Mohammed AH: Immunohistochemical evaluation of human epidermal growth factor receptor 2 and estrogen and progesterone receptors in breast carcinoma in Jordan. Breast Cancer Research 7:R598-R604, 2005.

63. Arpino G, Bardou VJ, Clark GM, Elledge RM: Infiltrating lobular carcinoma of the breast: tumor characteristics and clinical outcome. *Breast Cancer Res* 6:R149-156, 2004.

64. Hoff ER, Tubbs RR, Myles JL, Procop GW: HER2/neu amplification in breast cancer: stratification by tumor type and grade. *Am J Clin Pathol* 2002, 117:916-921.

65. Taucher S, Rudas M, Mader RM, Gnant M, Dubsky P, Bachleitner T, Roka S, Fitzal F, Kandioler D, Sporn E, *et al*: Do we need HER-2/neu testing for all patients with primary breast carcinoma. *Cancer* 2003, 98:2547-2553.

66. Huang HJ, P Neven, M Driskoningen, et al: Association between tumour characteristics and HER-2 /neu by immunohistochemistry in 1362 women with primary operable breast cancer. J Clin Pathol 58 (6): 611-6, 2005.

67. Hartmann LC, Ingle JN, Wold LE, et al: Prognostic value of c-erbB2 overexpression in axillary lymph node positive breast cancer. Results from a randomized adjuvant treatment protocol. Cancer 74: 2956-2963, 1994.

68. Ghulam F and Imtiaz R: Locoregional Recurrence after Management of Carcinoma of Breast. JCPSP, Vol 15 (4); 218-228,2005.

69. Mark HF, Aswad B, Bassily N, et al: HER-2/neu gene amplification in stages I – IV breast cancer detected by FISH. Genet Med 1(3); 98-103, Mar-Apr 1999.

70. Seshadri R, Mathews, Dobrovic A, et al: The significance of oncogene amplification in primary breast cancer. Int J Cancer 43(2); 270-2, Feb 1989.

71. Tavassoli FA, Devilee P, Eds: *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Breast and Female Genital Organs*. Lyon: IARC Press; 2004.

72. Kearney N, Mcphail G: Herceptin: Implications for breast cancer management. Eur J Oncol Nurs: 4(5a): 37-41, 2000.

73. De Lellis RA:In situ hybridization techniques for the analysis of gene expression. Applications in tumour pathology. Hum pathol 25:580-585, 1994.

74. Farrow JH: Detection of early breast cancers. Cancer 25: 468, 1970.

75. Pupa SM and Tagliabue F: HER-2: A biomarker at the crossroads of breast cancer immunotherapy and molecular medicine. J cell physiol. May 10. 2005.

76. Agwa JO: Kenya Demographic and Health Survey; 2003.

APPENDIX I

Staging of breast cancer based on TNM system

Primary tumuor (T)

T0-No evidence of primary tumour

Tis- Carcinoma in situ, or Paget's disease of the nipple with no tumour

T1- Tumour 2 cm or less in greatest dimension

T2- Tumour more than 2 cm but not more than 5 cm

T3- Tumour more than 5 cm

T4- Tumour of any size invading chest wall or skin

Lymph node (N)

N0- No metastasis to regional lymphnodes

N1- Metastasis to movable ipsilateral axillary nodes

N2- Metastasis to fixed ipsilateral axillary nodes

N3- Metastasis to ipsilateral internal mammary nodes

Distant metastasis (M)

M0- No distant metastasis

M1-Distant metastasis (includes metastasis to ipsilateral supraclavicular lymph nodes)

Groups by stage:

0	Tis	N0	M0
I	T1	N0	M0
II	T0---T3	N1	M0
	T2---T3	N0	M0
III	T0----T3	N2	M0
	T4	Any N	M0
	Any T	N3	M0
IV	Any T	Any N	M1

APPENDIX 1I

Questionnaire

HER-2/neu receptor expression in breast cancer and its correlation with the other established prognostic parameters at Kenyatta National Hospital

1. Study number

2. Patient information
   - Name.....
   - Hospital No. .....
   - Age

   - Gender F/M
   - Married?

   - Single

   - Divorced

   - Widowed

3. Disease History.

(a) Presenting symptoms

(i) Breast lump? Yes/No

(ii) Nipple discharge? Yes/No

4. Menstrual history

(a) Menarche

(b) Last normal menstrual period.....

(c) Contraceptive use? Yes/No

(d) Parity

		+			

5. Examination

i. Breast mass size (cm)

ii Nipple discharge? Yes/No

ii. Skin changes? Yes/No

iii. Tenderness? Yes/No

iv. Axillary lymph node enlargement? Yes/No

If yes, lymph node mobility? Mobile/Fixed

6. Investigations

(a)FNA cytology

- Benign ☐
- Malignant ☐
- Equivocal ☐
- Not done ☐

(b) Histopathology (i) Type

- Carcinoma in situ ☐
- Invasive ductal carcinoma ☐
- Invasive lobular carcinoma ☐

- Tubular/cribriform carcinoma ☐
- Colloid (mucinous) carcinoma ☐
- Medullary carcinoma ☐
- Papillary carcinoma ☐
- Other types ----------

(ii) Morphological grade 1 to 3 ☐

(iii) Histology of sampled lymph node

Metastasis / No metastasis

(iv) Stage 0 to iv ☐

(c) HER-2/neu status 0 to 3+ ☐

APPENDIX 1II

Patient information and consent form

HER-2/neu receptor expression in breast cancer and its correlation with the other established prognostic parameters at kenyatta national hospital

My name is Dr Nalyanya. I am carrying out a study whose objective is to measure the levels of a certain protein in breast cancer tissue. If the amount of this protein in the diseased breast is excess, it helps the doctor to know the probable course of your disease and how it can be better managed.The specimen which was removed from your breast was positive for cancer of the breast and I request your permission so that I use use it in my study. The results from this test will be send to your doctor. There are no special risks involved as I will just use part of the tissue that had already been removed for the initial diagnosis of the disease.

Participation in this study is voluntary and you can withdraw at any time without losing your health benefits to which you are entitled.

I.....hereby do consent to take part in the study being carried out by Dr Nalyanya, the nature and effect of which has been explained to me by him. I have had opportunity to ask questions.

Date.....

Signature of patient / relative.....

Signature of questionnaire administrator.....

If you have any concerns about this study you can contact Prof. K. M. Bhatt, chairperson of the ethical and research committee at KNH, on telephone 202725452 at the university of Nairobi's department of medicine.

You may also contact my supervisors:

1.Dr. Muchiri LW-Tel.2022711055
Department of pathology
University of Nairobi

2. Dr. Khan MR-Tel.2027263000 ext 43772
Surgical out-patient clinic
Kenyatta National Hospital

TheContact of Dr. Nalianya, the principal investigator, is 0722368788

APPENDIX IV

Test methods used in determining HER-2 status

1. Immunohistochemistry (IHC) is a very widely used test for evaluating HER-2 receptor status and it can be used on fresh tissue specimens or old paraffin tissue blocks.

2. Fluorescent in situ hybridization (FISH) consists of the detection of HER-2 gene DNA or RNA sequences in malignant breast tissue sections or cell preparations using a labelled complementary nucleic acid sequence or probe.

   Advantages of FISH:

   - Test measures HER-2 overexpression by detecting the amplification of the gene.
   - Has higher sensitivity than IHC.

   Disadvantages:

   - Procedure is more complex and longer than IHC.
   - Needs nucleic acid probe and other specialized equipments.
   - It is expensive.

3. Polymerase chain reaction (PCR) allows millions of copies of any specific DNA sequence to be generated within a few hours. It only needs a small amount of fresh or archival specimen tissue. This test is expensive and therefore it is not routinely used in HER-2 testing.

4. Enzyme labelled immunosorbent assay (ELISA) can also be used to test HER-2 over-expression in a breast cancer patient. It measures the HER-2 proteins released into plasma from the malignant cells.

The best technique for testing HER-2 status is one that is simple to perform, sensitive, standardized, and stable over time. At the moment, the immunohistochemical method meets these criteria. Most laboratories use IHC and only carry out FISH if IHC score is 2+.

APPENDIX V

Laboratory methods

Determination of HER-2 receptor expression by immunohistochemistry

Materials required

Poly-L-lyzine adhesive solution for prepairing slides; Xylene; Ethanol - Absolute &95%; Citrate buffer PH 6.0; phosphate buffered saline (PBS) PH 7.6; Primary antibody. Detection kit is also needed, in this case DAKO^{R} LSAB + Kit. The kit has the following reagents:

1. 3% Hydrogen peroxide
2. Link; Biotinylated anti-rabbit,anti-mouse and anti-goat immunoglobulins containing carrier protein.
3. Streptavidin peroxidase with carrier protein.
4. Buffered substrate solution containing hydrogen peroxide
5. 3,3'-diaminobenzidine (DAB) chromogen solution

Other materials required will include; Microwave, Timer, Counterstain, Distilled water, Coverslips.

Preparation of citrate buffer PH 6.0

Stock Solution A: 2.1g Citric acid in 100ml H_2O

Stock Solution B: 2.94g Sodium citrate in 100ml H_2O

Working Solution

9ml Solution A

41ml Solution B

Make up to 500mls with d H_2O. Use soln B to increase the PH and soln A to lower it.

Preparation of PBS PH 7.6

16g NaCl

3.5g $N_a2HPO_4.2H2O$

0.4g KCl

0.4 KH_2PO_4

2000MLS d *H2O*

Once made , this buffer can be stored in the fridge for use the next day.

Preparation of primary antibody

Use 1: 100 dilution i.e 10ul of antibody and 990ul of PBS.

Preparation of Substrate-Chromogen solution

Depending on the number of slides to be stained, transfer enough 1 ml aliquots of Buffered Substrate solution into the calibrated test tube. For each 1ml of buffer, add one drop (approx. 20ul) of DAB Chromogen. Mix immediately.

Immunohistochemistry procedure for HER-2/neu receptors

1. Cut sections at 3-5 um and immobilize on adhesive slides. Incubate at 37°C overnight.
2. Dewax in xylene for 15 minutes and re-hydrate slides, in descending concentrations of ethanol, to water.
3. Put the slides in prepared citrate buffer and pretreat them through microwave at between (60-80)°C. Pretreat for 5 minutes, then for a further 5 minutes after a rest of 1 minute.
4. Rinse in the prepared phosphate buffered saline (PBS-PH 7.6).
5. Quench endogenous peroxidase activity by applying enough drops of 3% hydrogen peroxide to cover specimen. Incubate five minutes.
6. Rinse in buffer.

7. Block non-specific antigen activity in normal serum for 30 minutes. Use horse serum.
8. Drain slides and wipe around sections-do not rinse!.
9. Apply enough user prepared antibody to cover the specimen. Incubate 30 minutes.
10. Rinse well in buffer.
11. Apply enough secondary antibody to cover specimen. Incubate 15 minutes.
12. Rinse well in buffer.
13. Apply enough of streptavidin peroxidase to cover specimen. This is the tertiary antibody (detection kit) . Incubate 15 minutes.
14. Rinse well in buffer.
15. Apply enough of the prepared substrate-chromogen solution to cover specimen. Incubate 7 minutes.
16. Put in PBS and rinse in running tap water for 5 minutes.
17. Put slides in a bath of haematoxylin. Incubate for 5 minutes. Rinse in running hot tap water. Then dip slides in absolute alcohol and 3 more of descending concentrations. Put in xylene for about 2 minutes. Specimen is then coverslipped using DPX as a mountant.

Interpretation of staining

Both the positive and negative control specimens are to be examined first to check for the validity of test specimen results. Positive staining is indicated by the presence of a coloured end-product at the site of the target antigen. DAB chromogen yields a characteristic brown end-product.

The test specimens are then examined. Use only intact cells for interpretation since necrotic or degenerated cells often stain non-specifically.

The positive immunostaining results shall be analysed semiquantitatively by the investigator and confirmed later by one of my supervisors who is a consultant pathologist.

KENYATTA NATIONAL HOSPITAL
Hospital Rd. along, Ngong Rd.
P.O. Box 20723, Nairobi.

Tel: 726300-9
Fax: 725272
Telegrams: "MEDSUP", Nairobi.
Email: KNHplan@Ken.Healthnet.org

Ref: KNH-ERC/01/2355

Date: 22 July 2004

Dr. Nalyanya W W
Dept. of Human Pathology
Faculty of Medicine
University of Nairobi

Dear Dr. Nalyanya

RESEARCH PROPOSAL "HER-2/NEU RECEPTOR EXPRESSION IN BREAST CANCER AND ITS CORRELATION WITH THE OTHER ESTABLISHED PROGNOSTIC PARAMETERS AT KENYATTA NATIONAL HOSPITAL" (P79/6/2004)

This is to inform you that the Kenyatta National Hospital Ethics and Research Committee has reviewed and **approved** the revised version of your above cited research proposal for the period 22 July 2004 – 21 July 2005. You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given.

On behalf of the Committee, I wish you fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of database that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely,

PROF. A N GUANTAI
SECRETARY, KNH-ERC

Cc Prof. K M Bhatt, Chairperson, KNH-ERC
The Deputy Director (C/S), KNH
The Dean, Faculty of Medicine, UON
The Chairman, Dept. of Human Pathology, UON
CMRO
Supervisors: Dr. Muchiri L W, Dept of Human Pathology, UON
Dr. Khan M R, Dept. of Surgery, KNH