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Case Study on Metastatic Breast cancer

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ABSTRACT

In this case study, a women aged 40 was diagnosed with Metastatic breast cancer. Metastatic breast cancer is a complex multi-stage disease involving the expansion of cancerous cells from the breast to other parts of the body. It is a serious complication of breast cancer, with limited treatments avail, as metastatic disease in breast cancer is often fatal. Different molecular techniques were used to diagnosis the patient and observed, overexpression of HER2/neu Oncogene and vascular endothelial growth factor (VEGF), positive Estrogen (ER) and Progesterone receptors (PR) Expression, unusual occurrence rate of Bone micrometastases and abnormal Ras activation. Suppression of these genetically transformed cancers cell with minimal effect on normal body cells can be done with the combination of anti HER2, antiVEGF treatment, Farnesyltransferase inhibitors, together with Bisphosphonates (pamidronate or zoledronic acid). This procedure may contribute to the proliferation of cancerous cells into a fatal disease.

Keywords: Metastatic breast cancer, HER2/neu, vascular endothelial growth factor (VEGF), positive Estrogen (ER) and Progesterone receptors (PR) Expression.

INTRODUCTION

Metastatic breast cancer is an invasive form of breast cancer which involves spreading of cancer cells to other organs in the body. This can also be called secondary or Stage IV breast cancer [1]. In July 2006 the cancer research UK survey gave that Breast Cancer is 15% of all cancers diagnosis and the are estrogen receptor–negative (ER^-)/progesterone receptor–negative (PgR^-)/$HER2^-$ ("triple-negative") breast cancers, in which 99% of women are affect by the disease. This condition has been reported to be a major concern of public health; particularly this disease is increasing in the developed countries, which resulted in 91000 deaths annually in Europe alone [2].

Other aspects like environmental , therapeutic agents , food contaminants and oestrogenic endocrine disruptors may leads to cancer development. [71]

Many risk factors have been reported to be involved with breast cancer, such as age, estrogen treatment after menopause, breast feeding, age at the first pregnancy, age at menopause, environment, stress, and nutrition. History of breast cancer occurrence in the family which may involve genetic predisposition of the disease is another major risk factor [1].

At the molecular level there are more important genetical factors to be considered in order to have promising results in both diagnosis and treatment of breast cancer. Important genetical alterations which are involved in cancer as documented by Segawa, *et al* [3], are such as proto-oncogenes activation, tumor suppressor genes inactivation and DNA repair genes inactivation.

1.1 Case study of Mrs. X Age: 40

Mrs. X was diagnosed to have metastatic breast cancer, and her cancer was found to be resistant to conventional therapies. However she agreed to have her tumour analysed for biochemical and molecular abnormalities. Before she was exposed to these tests her case history was taken so as to help with the diagnostic and treatment decisions to be taken.

Screening:

Early screening of healthy women for breast cancer is an attempt to achieve an earlier diagnosis, which can help in better outcomes. A number of screening test have been employed including: clinical and self breast exams, mammography, genetic screening, ultrasound, and magnetic resonance imaging.

The cochrane collaboration in 2009 concluded that it is unclear that whether mammography screening will be helpful in breast cancer, even though it reduces mortality from breast cancer by 15% but ends up with avoidable surgery and anxiety, resulting in their view that mammography screening may do more harm than good [4].

Molecular diagnosis:

Molecular diagnosis involves investigation of specific gene amplifications which are common in different types of tumours. These gene amplifications are reported to provide prognostic, therapeutic and diagnostic information which may aid in patient management as reported by [1].
Using the clinical history which was assumed for Mrs. X as explained above, different Molecular techniques were suggested so as to have information on the kind of diagnostic markers which can be related to her tumour so as to help in decision making on the kind of treatment needed for her case. Techniques suggested for the diagnosis of Mrs. X tumour were as discussed bellow.

2.1 DNA Microarray technology

Microarray analysis is the technique that uses biology as well as computer technology, to produce a genetical profile for a sample that reflects the activity of thousands of genes. Two different expressed genes (BRCA1 and BRCA2), which are found in many cases of breast cancer can be identified using Microarray Technique. However these two genes mutations were not diagnosed in Mrs. X case.

Tissue Microarray technology has also been used in the analysis of different prognostic markers in Breast cancer such as Estrogen receptor expression (ER) (As shown in Figure 1 below)and the HER2/neu gene amplification. (See table 1 below)

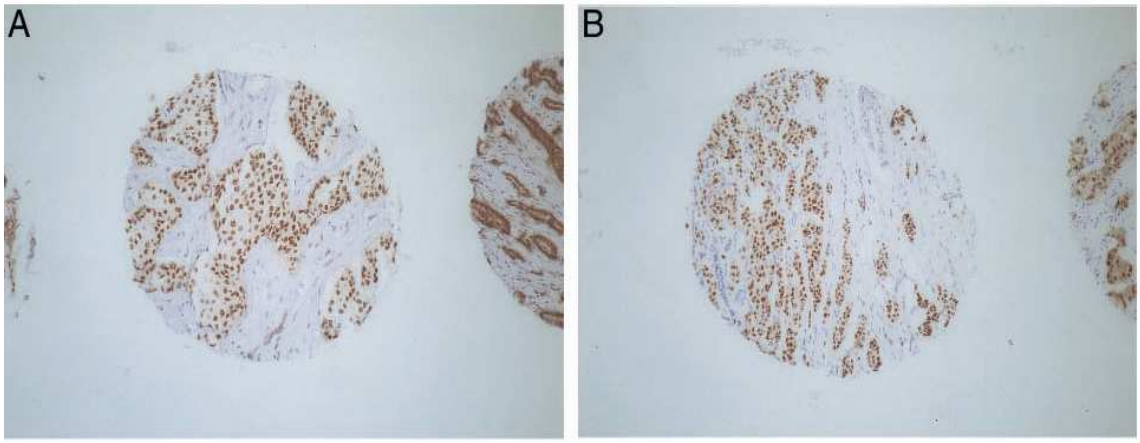


FIGURE 1. Examples of positive and negative tumor samples for ER (A) and PR (B). (Torhorst *et al*,2001)

Table 1. Breast cancer metastatic prognostic markers

Marker	Use in clinic	Metastatic determinant	Details	References
Upa/PAI1 Protein level	Newly established marker	High protein levels of Upa and PAI1 are associated with high metastatic risk	Independent prognostic marker	Foekens *et al*, 2000, Konecny,*et al*,2001 , Janicke, *et al*,2001 and Look *et al*, 2002
Steroid – Receptor Expression	Established for adjuvant therapy decision	Low steroid receptor levels are associated with metastatis	Short term predictor of metastasis risk(5 years)related to histological grade	Page, 1991
ERBB2 gene Amplification and protein expression	Established for adjuvant therapy decision	ERBB2 amplification/ overexpression is associated with metastasis	In patients with lymphnode positive tumors	Ross, *et al*, 2003

Apart from analysis of prognostic markers, DNA Microarray can be used in the analysis of Gene Expression profiling in breast cancer. Gene expression is measured at both mRNA levels (Transcriptional) and protein levels (Translational). This method of assay is carried out on biological samples by quantitative and qualitative analysis mRNA levels (Transcriptome) which is performed through the use of DNA Chips.

DNA Microarray is made up of solid surfaces consisting of multiple cDNA or oligonucleotide which acts as capture probes. These probes represent genes or parts of genes which are embedded on the surface (Oligonucleotide based array) whereas in cDNA- based arrays the probes are laid down through the use of specific device known as an arrayer.

This is a procedure in which cDNA is synthesized from RNA by reverse transcription thus RNA is extracted from serum and plasma samples of patient with breast cancer and a reference sample is noted, a labeled probe is hybridized against RNA in tissue sections [5]. Tissue sections are made from either paraffin-embedded or frozen tissue using a cryostat and then mounted on to glass slides. A hybridization mix including the probe is applied to the section on the slide and covered with glass cover slip .Typically the hybridization mix has formamide at a concentration of 50% in order to reduce hybridization temperature and minimize evaporation problems. Although double stranded cDNA have been used as probes, single stranded complementary RNA probes(riboprobes) are preferred; The sensitivity of initially single stranded probes is generally higher than that of double stranded probes, presumably because a proportion of the

denatured double –stranded probe renatures to form probe homoduplexes. This technique operates on a reverse nucleic acid hybridization approach.

The probe is set of unlabeled nucleic acid fixed to the Microarray. Once a Microarray has been constructed and the source (test sample) of nucleic acid to be investigated has been isolated, the hybridization reaction is then carried out. The target gene is labeled with a fluorophore and allowed to come into contact with the Microarray enabling probe –target heteroduplexes to form ,after which hybridization washes minimize non specifically bound label. Most Microarray hybridization uses two fluorophores, usually CY3 (green channel excitation) and CY5 (red channel excitation).

Following hybridization, bound Fluorescent label is detected using a high resolution laser scanner and the scanning process involves acquiring an image for both fluorophores to build a ratio image .The final hybridization pattern is obtained by analyzing the signal emitted from each spot on the array using digital imaging software which converts the signal into one of a palette of colors according to it's intensity.

Barlund et al, [6], detected the overexpression of ribosomal s6 kinase gene in breast cancer cell line by cDNA microarrays. Tissue Microarray was used in 9-15% of breast cancer cases there was amplification in s6 kinase gene thereby over-expressing the encoded oncoprotein. S6 kinase could be a significant prognostic indicator in breast cancer. cDNA allows expression of thousands of genes such as erbB2, S6 kinase, HER2, Keratin17 in the tumor specimen. This method therefore permits rapid molecular profiling. TMA also possess a number of advantages over other conventional techniques. The speed of molecular profiling is increased more than 100 folds and tumor cells are not destroyed during analyses Antibody staining is performed onto the TMA slides by using ISH or other molecular detection useful in detection of protein Antigens using IHC for identification of targets for drug discovery and therapeutic values.

Tissue Microarray can also be applied in DNA screening variation, due to its high potentials for assaying for mutations in known disease gene. There have been vigorous efforts to identify and catalog human single nucleotide polymorphism (SNP) marker.

A second approach in Gene Expression Profiling predicts clinical behavior of the tumor as seen in the molecular profiling of 70 genes thus indicating the likelihood of distant metastases in young patients(<55yrs of Age Premenopause women) having lymphnode negative tumors [7].

The third Approach to Gene Profiling of DNA Microarray is identification of metagenes linked with lymphnode status at diagnosis and a three years recurrence risk in breast cancer patients of all ages [8]. Ma et al, [9], identified expression of ratio of two metagenes (novel markers) H1XB13 and IL17BL indicating metastatic development.

Table 2. List of markers whose specific mRNA has shown to vary in breast cancer tissues or cells

Gene name	Gene product name	Cell function or tumor property	Locus	Pubmed Identifier
BRCA1	Breast cancer 1, Early onset; Breast cancer type 1 susceptibility protein		17q21	10371343
BRCA2	Breast cancer 2, early onset; Breast cancer type 11		13q12-13q13	10498873
EGF	Epidermal growth factor		4q25	9191980
EGFR	Epidermal growth factor receptor		7p12	10326794

ERBB2	c-erbB-2 Herstatin (HER2) Neu		17q11.2-17q12	10706127
ESR1	Estrogen receptor 1,alpha type	Hormone sensitivity	6q25.1	9816251
ESR2	Estrogen receptor 2,beta type	Hormone sensitivity	14q	10554009
FGF2	Fibroblast growth factor 2	Angiogenesis	4q26-4927	9041202
FGFR2	FGF RECEPTOR2	Angiogenesis	10q26	7705943
MAP3K8	Mitogen-activated protein kinase-kinase-kinase 8 Tumor Progression locus2 (TPL)		10P11.2	10490831
MET	Met proto-oncogene product Hepatocytes growth factor receptor	Angiogenesis	7q31	9221809
P8	P8 Protein candidate of metastasis1 protein		16p11.2	11034106
PGR	Progesterone receptors	Hormone sensitivity	11q22-11q23	7927940
PLAT	Plasminogen activator tissue type-(Tpa)	Proteolysis	8p12	2112958
PLAU	Plasminogen activator urokinase Upa	Proteolysis	10q24	10930085

http/www.gene.ucl.ac.uk/hugo

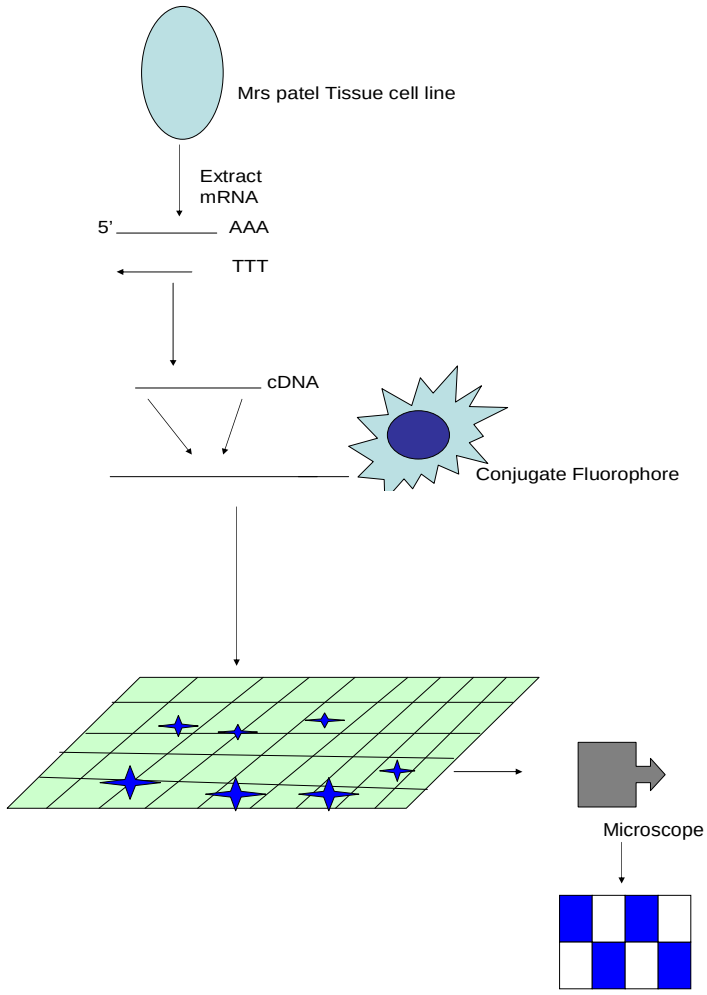


FIGURE 2: Hybridization of fluorescently labeled Probes to Oligonucleotide Array.

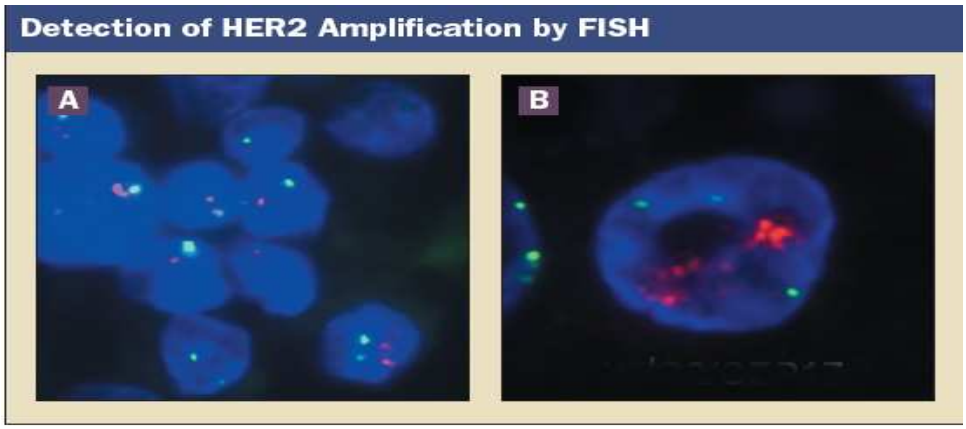
This can be observed and recorded in computer as hybridization of the individual cells [10].

2.2 Fluorescence in situ hybridization (FISH)

Fluorescence in situ Hybridization (FISH) technique is a recent alternative to Immunohistochemistry (IHC), used for the assessment of HER2/neu oncogene status in breast cancer. Bartlett, *et al*, [6] suggested FISH to be superior and more accurate in determining gene expression levels as compared to IHC. This was concluded in studies done to correlate determination of HER2 status in breast cancer samples using both techniques. Pauletti, *et al* [11] has also reported FISH to provide better prediction for trastuzumab therapy clinical benefits as compared to IHC.

FISH technique provides enumeration of gene copy number through fluorescent signal counting in the nuclei of the invasive tumor cells. This technique has also been reported to be used in detection of genetic abnormalities such as numerical abnormalities, translocations and inversions [12, 8]. Hicks, *et al*, [13] documented that, the importance of FISH technique in breast cancer is that, it is able to provide high correlation between protein overexpression and the HER2 gene expression of about 90-95%.

FISH is best performed during metaphase and/or interphase stages of cell cycle when the chromosomes are visible. The DNA sample has to be denatured by using heat and/or formamide so as to allow separation of individual DNA strands thus exposure of the base sequences. Then hybridization is done by incubating the DNA sample with the probe which has been attached to the colored fluoros. Fluorescence viewed through the microscope will prove the presence or absence of the DNA sequence of interest in the given DNA sample. (See figure 3)



This figure shows an example of FISH testing for the HER2 gene. The assay shown here utilizes 2 different color probes, one for the HER2 gene (red) and another for the centromeric region of chromosome 17 (green). The ratio of HER2 gene copies to chromosome 17 copies allows the distinction between true gene amplification (with a ratio > 2) and pseudoamplification due to polysomic state (multiple copies of chromosome 17). Breast cancer without HER2 gene amplification is seen in panel A, and breast cancer in which the HER2 gene is amplified is seen in panel B.
Abbreviation: FISH = fluorescence in situ hybridization

FIGURE 3. An example of fish testing for the her2 gene (Hicks, *et al*, 2004)

2.3 Serum Proteomics

Proteomics is a technique which involves separation, quantification and identification of different kinds of proteins in the given sample [14]. This technique is used for investigation of protein levels as well as post-translational modifications in different types of cells. This can be done under different physiological conditions. 2-DE and other pinpointed techniques can be used to compare total proteins extracts from both cancerous cells and normal cells, while mass spectrometry can be used in the characterization of proteins of interest. Proteomics therefore is a

powerful method which can be used to study any kinds of protein expression changes in relation to the pathogenesis of cancer [15].

The new technology Proteomics, where clinical proteomics should be more effective and efficient to treat the human tumor, especially research in biomarkers using cell lines, body fluids and animal models [16] with limited success. The basic approach involved in the identification and quantification of changes in protein expression in sample from diseased and normal models. The main use of proteomic analysis in cancer is to discover and identify serum biomarkers necessary for tumor profiling as well as to explore intracellular signaling pathways that are involved in the development of cancer cells [1, 17]. It can also be used in detection and quantification of both Proto-oncogenes and mutations in tumor suppressor genes. In this way therapeutic targets against the disease can be identified as well as discovery and development of the new strategies can be done [15].

Molecular profiling of cancers is nowadays possible by the use clinical proteomics in an efficient way, as this technique allow us to establish new noval biomarkers from complex proteomes. This approach is also used to correlate the information of all proteins with breast cancer from various samples such as tissue, serum, plasma, nipple aspirate fluid, interstitial cells, cell lines and so on. Generally in most cases the breast cancer cell lines are preferred for proteomics analysis because of its known consistent cellular composition and genetic background, which makes this cell line unique in characteristics from all other types [18].

Important biomarkers which can be analyzed using serum proteomics are as explained below;
Breast cancer cells sensitivity for estrogens and progesterone which is checked through investigation for the presence or absence of estrogen (ER) and progesterone receptors (PR). This helps in the identification of patients who will benefit from Hormonal therapy.

Investigation of the HER2/neu over expression helps to rule out patients who can be treated using Trastuzumab. Proteomic can also be used in the analysis of the angiogenesis signaling pathway occurring in breast cancer cells.

Finally, the detection of mutations in the tumor-suppressor genes BCRA1 and BCRA2 is used nowadays for early breast cancer detection in susceptible families. The status of the patient is known earlier enough to make decisions for treatment. Proteins of clinical interest as elucidated by Hondermarck, *et al*, [15], can be identified using the strategy summarized on a flow chart in Fig. 4.

2.4 Reverse transcription PCR

Reverse transcriptase

The technique which is used for the detection of the degree at which a specific gene is expressed is Polymerase Chain Reaction (RT-PCR). It involves the amplification of the DNA segment of interest which has been taken from its harmonizing copy of mRNA. This makes it possible to identify and determine expression of specific genes of interest.

The frequently used breast cancer test Oncotype DX which utilizes RT-PCR enables the examination of individual patient risk of reappearance of the disease, especially in patients which are node negative ER positive. This test is mainly practical in the evaluation of the expression of 21 genes which may occur in breast cancer used to establish prognosis of the disease.

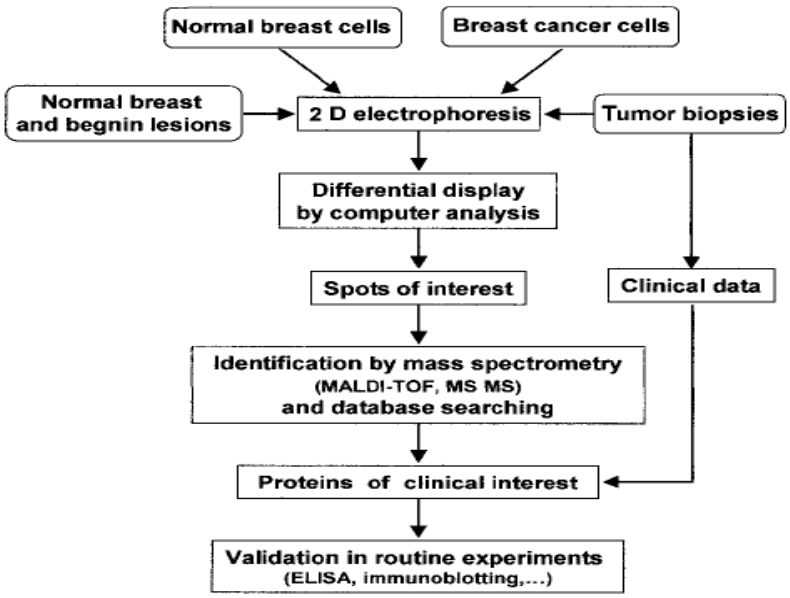


Figure 4. Strategy for the identification of proteins of clinical interest [Hondermarck, *et al,* 15].

The most regular use of RT-PCR in clinical oncology is the method concerning detection of both lymph node micrometastases and bone metastases in breast cancer, where, the detection of both micrometastases in the bone marrows and peripheral blood stem cells contamination by cancer cells can also be done [3].

Besides bone metastases, RT-PCR was reported to be used in the diagnosis of Ras activation. Von Linting *et al*, [19], documented that, when Ras is abnormally activated in breast cancer leads to EGF and/or HER2 receptors overexpression. Ras has for that reason been considered to be among the new targets for cancer treatment.

RESULTS

Different prognostic markers and oncogenes which were diagnosed in Mrs. X's case using the techniques explained above are as discussed below:

3.1 HER2/neu Oncogene

Amplified level of Her2/neu protein was obtained using DNA Microarray, Serum proteomics and FISH (Fluorescence in situ hybridization). However FISH is the method recommended and is commonly used to determine whether patients with breast cancer overexpress the HER2/neu oncogene [13].

HER2/neu oncogene is a gene which carries the genetic code of the HER2 receptor which has been found in some of the cancer cells. This HER2 receptor is a transmembrane tyrosine kinase receptor which is homologous to the epidermal growth factor receptor [20]. HER2 is reported to aid growth of cancer cells due to its ability to bind to growth factors in the blood, is therefore directly involved with the pathogenesis and also the biology of breast cancer, making it to be among the predictive and prognostic markers for the disease. HER2/neu oncogene amplification has been observed in almost 20 -30% of breast cancer patients [21].

HER2/neu amplification is utilized as a predictive marker for therapy response in cancer patients [7, 22, 23 and 5]. Gusterson , *et al*, [24], postulated that, patient with HER2/neu overexpression

were observed to have poor response to chemotherapy, such as cyclophosphamide, 5-fluorouracil, methotrexane and tamoxifen, as compared to patients having normal levels of this gene production.

However, HER2/neu amplification investigation is also useful in the identification of women with advanced level of breast cancer that may benefit from the monoclonal antibody therapy such as Trastuzumab [25], which is the anti-HER2 antibody [26]. Patients with the amplification of this gene may also benefit from doxorubin therapy but in high doses [9, 27 and 28].

3.2 Estrogen (ER) and Progesterone receptors (PR) Expression

Both DNA Microarray and Serum proteomics were used to detect ER and PR status of Mrs. X, diagnosing her to be ER positive. Both Estrogen receptor (ER) and progesterone receptor (PR) are reported to be good predictive markers for breast cancer patients in relation to the response of the disease to therapy. ER and PR positive patients are reported to respond well to hormonal therapy as compared to these receptor's negative patients [29]. However, resistance or adverse reactions to hormonal treatment has been reported in ER positive patients with HER2/neu overexpression.

3.3 Angiogenesis signaling pathway in breast cancer cells

Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are two of the major among many factors involved in angiogenesis. Serum proteomics was used to diagnose angiogenic signaling pathways status for Mrs. X, where amplified levels of vascular endothelial growth factor (VEGF) were observed.

Angiogenesis is the formation of new blood vessels from the existing ones. Under normal circumstances, this process occurs in regulated condition. Tumor cells take advantage and control of this normal process, causing deregulations to the whole process for their benefit [18]. Tumor progression depends on angiogenesis, due to the need for oxygen and nutrient supply from the vasculature for tumor cells expansion and growth. Metastasis and tumor progression has been reported to be seriously affected by alteration of its blood supply [30]. That is the reason as to why anti-angiogenic therapy has been recently considered to be among the anticancer drugs with promising success.

Donald [18], documented that, gene expression for the vascular endothelial growth factor (VEGF) which is the most important angiogenic factor, may be upregulated by different types of stimuli like nitric oxide, hypoxia, estrogens, growth factors, progestins, activation of Ras, loss of p53 and HER2/neu amplification. VEGF was also reported to cause stimulation of tboth proliferation and migration of endothelial cells as well as induction of the metalloproteinase and plasminogens activity.

Preclinical studies as reported by [31], showed that, Bevacizumab which is the humanized monoclonal antibody to the VEGF can cause reduction or inhibition of tumor angiogenesis as a result tumor growth as well. This can be achieved when Bevacizumab is used alone or in combination with chemotherapy.

However, HER2 and VEGF signaling pathways are postulated to be interconnected at the molecular level where both HER2 and VEGF overexpression have been reported to occur simultaneously in breast cancer. Therefore a combination of both antiVEGF and antiHER2 therapy can be recommended in advanced breast cancer with HER2 overexpression [31].

3.4 Bone micrometastases and Abnormal Ras activation

RT-PCR is used for the diagnosis of both bone micrometastases and abnormal Ras activation. Bone micrometastases are among the most common complications of both breast and prostate cancers. The documented incidence rate of their occurrence is between 68% and 75% [32]. These complications are reported to be affecting the patient's life quality as well as causing limitations to their survival by causing bone pain, need for radiotherapy, pathological fractures and surgery [33].

Mutation of Ras on the other hand is reported to be found in 30% of all cancers found in human [34]. Under normal circumstances, Ras activation and function depends on external stimulation from growth factors which is highly regulated (See figure 5 below). However, the mutated Ras doesn't respond to the normal regulatory mechanisms and persists on its activated GTP bound state even in the absence of external stimulation [34, 35]. Specifically, mutations which occurs at residues number 12, 13 and 61 in Ras, makes it unresponsive to GTPase activating protein (GAPs), which acts to terminate Ras activaty under normal situation [34].

Zarnestra *et al* postulated that tipifarnib or R115777, is a nonpeptidomimetic methyl quinolone derivative can selectively inhibit Ras FTase. [73]

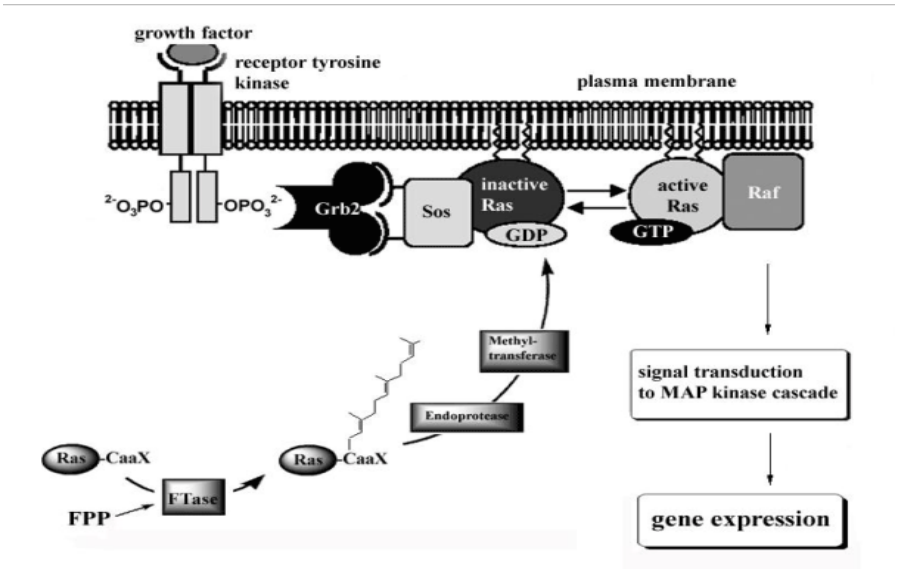


Figure 5 Shows normal signaling pathways which involves Ras (Nose, 2003)

4.0 TREATMENT

Gasparini, *et al*, [36] postulated that, the main purpose of breast cancer therapy at the molecular level is to suppress genetical transformations which occur in cancer cells with minimal effects to the normal body cells. Furthermore, the pathogenesis of cancer at the molecular level has been documented to involve alteration of normal signal transduction mechanisms into abnormal pathways [37].

Chemotherapy has whispered benefits to treat advanced stages of cancer. Researches denotes that the conventional chemotherapy is not effective treatment as the drug does not reach the target site in effective concentration. [72]

This report therefore focuses specifically on the molecular targeting compounds which are directed to the specific known molecular pathways which are involved in breast cancer, such as HER-2/neu and VEGF (Vascular endothelial growth factor). (As shown in Figure 6 below)

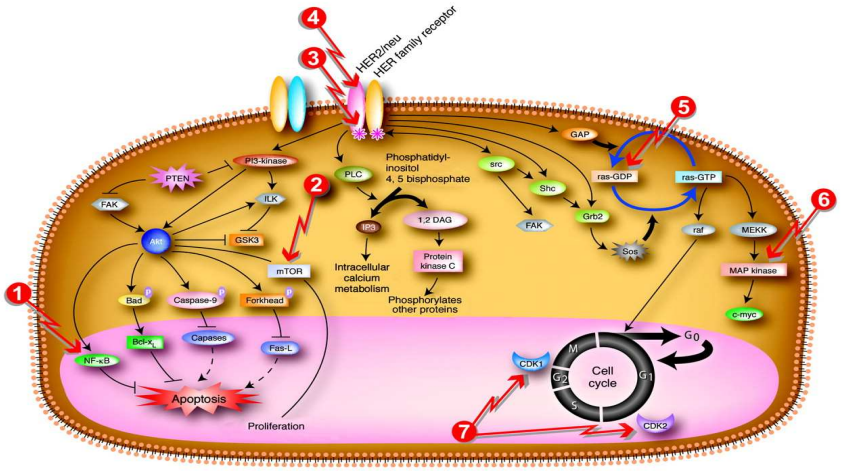


Figure 6. Multiple signaling pathways involved in cancer and action of targeted therapeutics. 1) Proteasome inhibitors (among other targets, NF-κB inhibitor IKB); 2) mTOR inhibitors; 3) Receptor tyrosine kinase inhibitors; 4) Growth factor receptor antibodies; 5) Farnesyl transferase inhibitors; 6) MEK inhibitors; 7) CDK inhibitors [38].

Donald *et al*, [35], has also reported different signaling pathways involved in cancer most of which are important in the action of targeted therapeutics (see Table 3 below*).*

Table 3. Cancer-associated genes implicated in angiogenesis (Donald *et al,* 2006)

Oncogene	*Growth Factors or Cytokine Levels*
H-/K-ras	VEGF ↑; TSP-1 ↓; bFGF ↑
src	VEGF ↑
erb2/HER-2	VEGF ↑; TSP-1 ↓
EGFR	VEGF ↑; IL-8 ↑; bFGF ↑
HPV16	VEGF ↑
bcr-abl	VEGF ↑
n-myc/c-myc	VEGF ↑; TSP-1 ↓
p53	VEGF ↑; TSP-1 ↓
C-jun	VEGF ↑; TSP-1 ↓

↑ = increased level; ↓ = decreased level. VEGF = vascular endothelial growth factor; TSP = thrombospondin; bFGF = basic fibroblast growth factor; EGFR = epidermal growth factor receptor; IL = interleukin.

Table 4. Humanized anti-erbb monoclonal antibodies in development for the treatment of various types of cancers [39]

Antibody	Specificity	Selected tumor types	Development phase
Trastuzumab (Herceptin®; Genentech, Inc.; South San Francisco, CA)	HER-2 (ErbB-2)	HER-2$^+$ metastatic breast	Approved
Cetuximab (Erbitux®; ImClone Systems, Inc.; New York, NY)	EGFR (ErbB-1)	Colorectal, NSCLC, pancreatic, breast, HNSCC	III
ABX-EGF (Amgen; Thousand Oaks, CA)	EGFR (ErbB-1)	NSCLC, colorectal, prostate, renal, HNSCC	II
EMD 72000 (EMD Pharma; Durham, NC)	EGFR (ErbB-1)	NSCLC, colorectal, ovarian, HNSCC	II
Pertuzumab (Omnitarg™; Genentech, Inc.; South San Francisco, CA)	HER-2 (ErbB-2), (ligand dependent signaling)	Prostate, ovarian, breast, NSCLC	I/II
Abbreviations: HNSCC = head and neck squamous cell carcinoma; NSCLC = non-small cell lung cancer			

4.1 Monoclonal antibodies

Monoclonal antibodies are reported to be highly specific therapies at the molecular level with the ability to induce a direct cell death specifically to cancer cells. They can also activate the immune system which results into direct tumor cells toxicity [39]. However, their function may be limited due to different factors such as size, heterogeneity in antigen expressions and sometimes expression of tumor antigens may occur in the normal cells. Their size has been reported to affect their ability in tumor cell penetration. Most antiHER2 monoclonal antibodies are still in different developmental stages for the treatment of various types of cancers, as shown in table 4 below.

4.1.1 Trastuzumab/Herceptin

Trastuzumab is a monoclonal antibody which has been synthesized through biotechnological engineering. It acts by targeting specifically breast cancer cells with high expression of the HER2 receptors on their surfaces. The regiment is given at 4mg/kg initially, followed by a weekly dose of 2mg/kg.

Three kinds of mechanism of actions of Trastuzumab have been reported as recently documented by Herceptin.com. The first is according to the observations done on In vitro studies that, binding of the drug to the cancer cells which are HER2 positive, prevents them from dividing and thus cancer growth is inhibited. Secondly, binding of Trastuzumab to the HER2 receptors prevents the ligand from binding to the same receptors. This leads to receptor internalization which prevents the ErbBs, at the same time stabilizes the cell membrane. All the processes in a nut shell inhibit the downstream signaling pathways leading to the inhibition of cancer cell growth.

The third mechanism of action is reported to involve the cells of the immune system. The HER2 – Trastuzumab complexes in the tumor cells, attracts the Natural killer cells (NK) which recognize them as foreign cells and kill them.

However, there are recent studies done with the aim of improving breast cancer therapy, with the approach in testing Trastuzumab in combination with other chemotherapies, and promising results have been obtained up to now (Herceptin.com). An example of different anticancer drugs combination aiming to block erbb signaling pathway is summarized on figure 7 below.

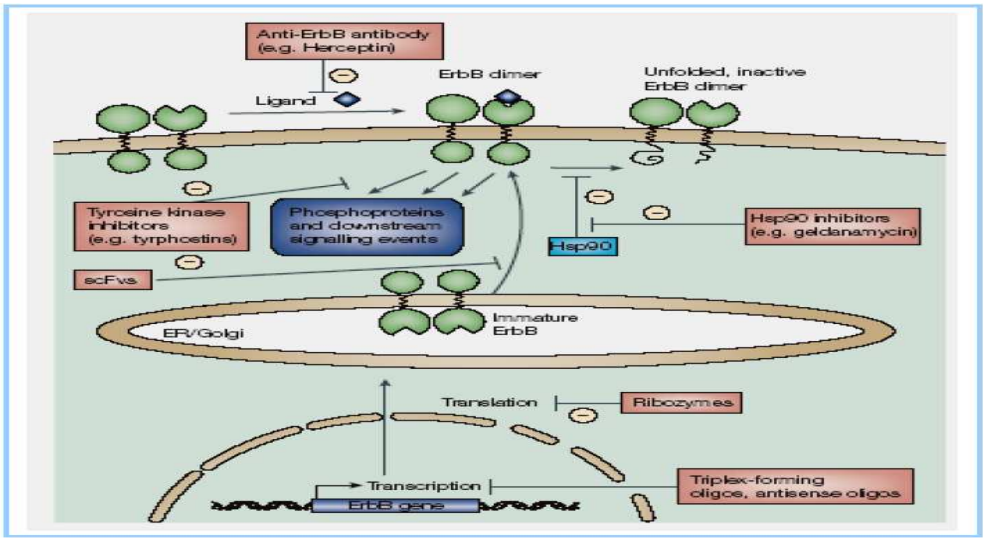


FIGURE 7. Proposed therapeutic strategies for blocking the erbb signaling network.[40]

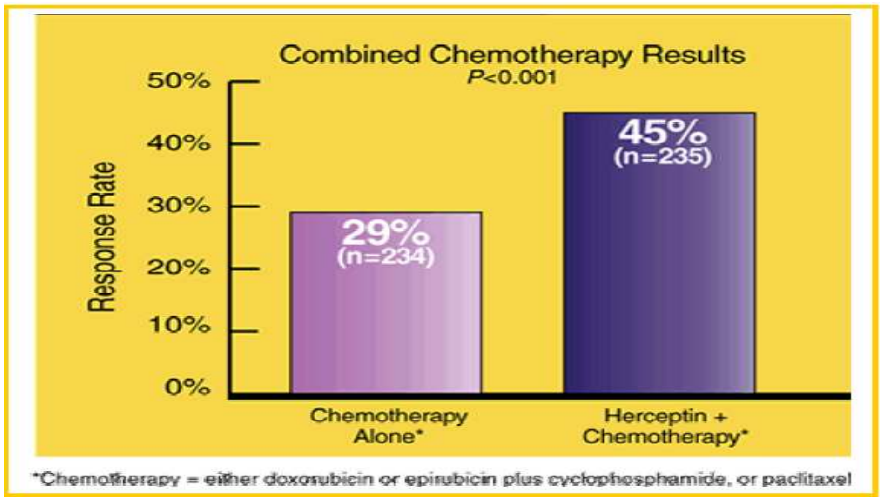


FIGURE 8. Response rates between chemotherapy treated. patients and chemotherapy combined with herceptin patients. (Herceptin.com)

Research as presented by Extra, *et al* [41] suggests that the combination of Taxotere with Trastuzumab produced promising results for management of metastatic breast cancer which have HER2 overexpression. They did a 24-month study which indicated that, patients who were treated with both Taxotere and Trastuzumab showed better responses to treatment than those who received Taxotere alone.

Apart from the combination of Taxotere and Trastuzumab, other drugs like paclitaxel, and Gemzar have also been tried in combination with Trastuzumab. However, they are still under clinical trials so as to find out which one among the treatment regiments will be ideal with fewer side effects.

4.1.1.1 Side Effects of Herceptin

As it's clearly known, majority of drugs have side effects, and Herceptin is one of them. Herceptin has been reported to have many side effects some of which are life threatening. Observations done in clinical studies showed that, almost 25% women given Herceptin alone or in combination with other drugs, showed common side effects such as fever, chills, nausea, weakness, diarrhea and coughing. However, 10% of them showed serious, life threatening and less common side effects such as:

Heart problems: This may include heart problems such as ventricular dysfunction and congestive heart failure. These side effects were specifically observed to be common in women received a combination of Herceptin, Anthracyclines and/or Cyclophosphamides. However, this kind of regiment has not been approved for treatment outside clinical trial setting. Thus, patients have to undergo heart function evaluation before and continuous heart function monitoring after they receive Herceptin treatment, so as to avoid occurrence of any kind of heart problem.

Fatigue and/or difficulty breathing has also been observed in some women treated with Herceptin. This is believed to occur as a result of reduction of red blood cells leading to anemia. Finally, herceptin has been reported to cause loss of white blood cells also known as leucopenia.

However, the United States Food and Drug Administration (FDA) have recently approved the use of Herceptin (Trastuzumab) in the management of metastatic breast cancer. This is because

Trastuzumab was observed to cause reduced growth and spread metastases in patients involved in a number of clinical trials.

4.1.2 Bevacizumab (Avastin)

Bevacizumab is a recombinant humanized monoclonal antibody against VEGF, which acts to inhibit new blood vessels formation that are needed for oxygen and nutrient supply to nourish tumor cells, thereby preventing both tumor growth and metastasis.

However, this drug has not been approved yet for metastasic breast cancer management, is currently under clinical trials. Recently documented clinical trial results showed that, when Bevacizumab was used in combination with chemotherapy in metastatic colorectal cancer treatment low toxicity and high survival rates were observed. It has been observed to bring promising results in metastatic breast cancer as well [31]. Apart from Bevacizumab there are other anti-angiogenic drugs which are under clinical trials and have shown to have promising results [42].

Donald, *et al* [35] postulated that, Bevacizumab may cause an increase in the risks of many side effects such as leucopenia, hypertension and diarrhea. They also reported other major risks such as thrombosis which may lead to myocardial infarction and/or stroke and fatal hemorrhage. However, Donald, *et al* [35], also recommended Bevacizumab to be used in combination with radiotherapy so has to have improved control of tumor metastasis.

Other suggestions such as a combination of Bevacizumab and Tarceva have been reported, though is still under clinical trials. Tarceva is a drug molecule which targets epidermal growth factor (EGF), it acts by causing inhibition of the tyrosine kinase epidermal growth factor receptors (EGFR) [43].

4.1.3 Other anti-angiogenic drugs

Apart from Bevacizumab, there are other different anti-angiogenic agents which are still under clinical trials, as listed in Table 3 below, most of which includes agents that have a specific effect on VEGF ligand-receptor system [44].

Table 5. Anti-angiogenic agents in clinical trials [44]

Agent	Company	Phase of trials	Mechanism of action
2C6	ImClone Systems	I	Antibody to VEGF-R2
ABT- 510	Abbott Lab's	II	TSP-1analog
ABX- IL8	Abgenix	II	Antibody to IL- 8
Angiostatin	EntreMed	II	Unknown
Angiozyme	Ribozyme	II	Ribozyme targeting VEGF- R1
Avastin	Genetech Biotech.	II- III	Antibody to VEGF
CEP- 7055	Cephalon	I	TKI to VEGF- R1,-R2,-R3
Combrestatin A- 4 prodrug	Oxigene	I	Tubulin- binding agent
CNTO 95	Centocor	I	Antibody to avβ3 and avβ5 integrin
EMD- 121974	Merck KGA	I- II	Integrin antagonist
Endostatin	EntreMed	II	Unknown
PTK 787/ZK222584	Novartis	II	TKI to VEGF- R2
SU11248	SUGEN	I	TKI to VEGF- R2 and PDGF- Rβ
Thalidomide	Celgene	II- III	Unknown
Vitaxin II	MedImmune	I- II	Antibody to avβ3 integrin
VEGF trap	Regeneron	I	Soluble receptor hybrid of VEGF
ZD6126	AstraZeneca	II	Tubulin binding agent
ZD6474	AstraZeneca	II	TKI to VEGF- R2, EGF- R

Anti-angiogenic drugs have got various advantages and disadvantages as compared to other anticancer drugs, as summarized on table 6 below.

Table 6. Advantages and disadvantages of antiangiogenic drugs (Schnitzer et al, 1998)

Anti-angiogenic Drugs	
Advantages	Disadvantages
Low toxicity	Chronic treatment that requires a large amount of drug
Effective at preventing growth of small tumors and metastases	Large tumors are less responsive
Applicable to wide range of tumor types	Tumor growth resumes upon termination of the treatment

4.1.4 Pertuzumab (Omnitarg)

Pertuzumab is a new drug among the HER2 targeted therapies. This group of drugs is also known as HER dimerization inhibitors [45]. Pertuzumab is a recombinant humanized monoclonal antibody that binds to the HER-2 receptors thereby preventing occurrence of ligand associated dimerization to other HER receptors such as HER1, HER3 and HER4, thus the expected intracellular signaling pathways which are important for cancer cell growth, proliferation and survival are inhibited [46,45].

The advantage of Pertuzumab from Trastuzumab is that, Pertuzumab can cause inhibition of tumor growth even in tumors which displays a low amount of HER/neu, unlike Trastuzumab which acts only on tumors with HER2/neu overexpression [47]. However, Agus *et al* [45] reported that, tumor cells which do not express the HER2 gene have no response to Pertuzumab.

Pertuzumab is still under phase II clinical studies, where it has been tested in breast, lung, ovarian and prostate cancers. The most common side effects observed with this drug were rash and diarrhea, and has been reported to be well tolerated right from its phase I trials [46]. This may be among the anticancer therapies which are thought to be effective and safe [45].

4.2 Bisphosphonates

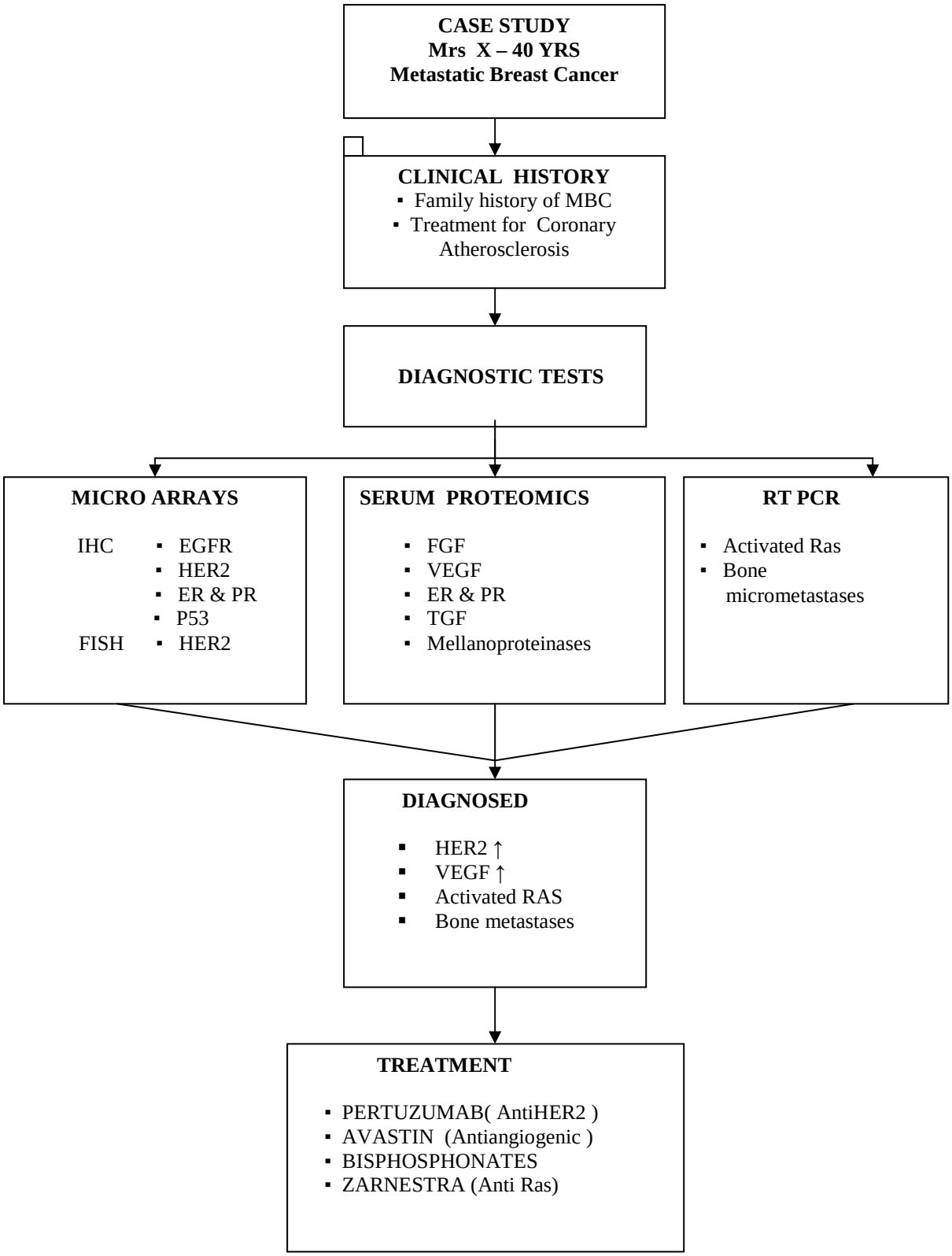
Bisphosphonates are drugs which are given as a secondary treatment to breast cancer which acts to prevent complications that may occur as a result of breast cancer metastases to bones [48, 49 & 33].The main use of these drugs in breast cancer is to decrease both fracture incidences, bone metastases associated pain and the need for palliative radiation therapy, and also they cause bone strengthening [48].

These drugs are reported to be equally effective and helpful, as they are given to many patients as a standard measure of care [50].They are normally given intravenously once per day. However, longer time for intravenous infusion is needed for Pamidronate than zoledronic acid. American Society of Clinical Oncology (ASCO) recommended that, this drug has to be continuously used for the whole life time from the beginning of the dose, as this may also decrease the progress of bone disease.

Bisphoshonates are reported to induce obliteration of the local blood vessels which supplies bones, thus leading to the occurrence of Osteonecrosis, and specifically jaw osteonecrosis in many patients [48], however, when necrosis of the jaw occurs withdrawal from Bisphosphonates is not recommended [50].

4.3 Farnesyltransferase inhibitors (FTIs)

Farnesyltransferase inhibitors are the drugs that inhibit Farnesyltransferase (FTase), which is a heterodimeric enzyme, needed for the binding process of the oncogenic Ras proteins to the plasma membrane leading to suppression of tumor growth and progression [51,52].



FLOW DIAGRAM OF MRS.X CASE STUDY

Most of Farnesyltransferase inhibitors are reported to be under clinical and Preclinical development stage, showing promising results for the preclinical activity against both xenografts

as well as in breast cancer cell lines. Zarnestra was reported to be the furthest in development as compared to the others [53].

5.0 Recommendations

Having amplified Her-2/neu protein levels, node positive ER, abnormal Ras activation, bone metastases and amplified VEGF levels, the combination of anti HER2, antiVEGF treatment, Farnesyltransferase inhibitors, together with Bisphosphonates (pamidronate or zoledronic acid) can be suggested for the treatment.

Selection of drugs is based on the fact that, resistance of Mrs. X breast cancer to conventional therapy may be due to overexpression of the HER2/neu oncogene as explained above. HER2/neu oncogene overexpression also is accompanied by high response to treatment with Monoclonal antibodies such as Trastuzumab and Pertuzumab.

Although Trastuzumab is the fist line drug for breast cancer with HER2 overexpression, it has shown to have a lot of serious side effects especially when compared with Pertuzumab. More serious side effects may be observed to patients with cardiovascular diseases as the case with Mrs. X, putting into considerations the patient clinical history obtained above. However, Pertuzumab outweighs Trastuzumab in that it can inhibit tumor growth even in cancer cells which have low HER2/neu expression. Therefore Pertuzumab is suggested in this case in combination with Bevacizumab (as antiVEGF) so as to increase the chances of successful treatment.

Zarnestra which is among the Farnesyltransferase inhibitors (FTIs) can be used so as to inhibit abnormal Ras activation.

Finally, Bisphosphonates can be used here specifically to help in strengthening the bones and decrease the incidence of pathological fractures; especially in this case where we assumed that metastasis for Mrs. X cancer has extended to the bones. Considering the fact that, these drugs are also capable of decreasing the pain associated with bone metastasis and may decrease the need for palliative radiation therapy to the bone.

REFERENCES

[1] Huang, X., Wei, Y., Li, L., Wen, Y., Yang, J., Liu, B., Song, X. and Zhao, J. *Oral Oncology*., **2006**, 42, 25-30.
[2] Alldridge L, Metodieva G, Greenwood C, et al. Journal of Proteome Research. **2008**, 7(4):1458-1469.
[3] Vannucchi, M.A., Bosi, A., Glinz, S., Pacini, P., LinarI, S., Saccardi, R., Alterini, R., Rigacci, L., Guidi, S., Lombardini, L., Longo, G., Mariani, P.M. & Rossi-Ferrini, P. *British Journal of Haematology.*, **1998**, 103, 610–617.
[4] Gøtzsche PC, Nielsen M, *Cochrane Database Syst Rev.*, **2009**, (4): CD001877. doi:10.1002/14651858.CD001877.pub3. PMID 19821284.
[5] Pegram, M.D., Pauletti, G., Slamon, D.J. *Breast Cancer Research and Treatment*., **1998**, 52 (1 -3)65-77.
[6] Bartlett, J., Mallon, E., Cooke, T. *Journal of Pathology*., **2003**, 199(4); 411-417.
[7] Press, M.F., Slamon, D.J., Flom, K.J., Park, J., Zhou, J.Y. and Bernstein, L. *Journal of clinical Oncology.*, **2002**, 20, 3095-3105.
[8] Spurbeck, J.L., Adams, S.A., Stupca, P.J., Dewald, G.W. *Mayo Clinic Proceedings*., **2004**, **79**, 58-75.

[9] Muss, H.B., Thor, A.D., Berry, D.A., Kute, T., Liu, E.T., Koerner, F., Cirrincione, C.T., Budman, D.R., Wood ,W.C. & Barcos, M. *The New England journal of Medicine*., **1994**, 331(3)211.
[10] Segawa, T., Sasagawa, T., Saijoh, K. and Inoue, M. *Clinical Cancer Research.*, **2004**, 6, 2341-2348.
[11] Pauletti G, Dandekar S, Rong H, et al. *Journal of Clinical Oncology*., **2000**, 18, 3651-64.
[12] Spagnolo, S.D., Ellis, D.W., Juneja, S., Leong, A.S., et al., **2004**, *Journal of Pathology*. **36** (1)19-44.
[13] Hicks, G. D., Tubbs, R. R., Fountzilas, G.,You, F. and Harris, N. L. *Physicians Education Resourse*., **2004**, 5 (2). 4-6.
[14] Quero1, C., Colomé, N., Prieto, M.R., Carrascal, M., Posada, M., Gelpí, E. & Abian, J. *Proteomics*., **2004**, 4, 303–315.
[15] Hondermark, H., Vercoutter-Edouart, A.S., Revillion, F., Lemoine, J., Nurcombe, V. and Peyrat, P.J. *Proteomics*., **2001**, 1, 1216–1232.
[16] Dwek MV, Alaiya AA. *Br J Cancer*., **2003**, **89:** 305-307.
[17] Rosenblatt, K.P., Bryant-Greenwood, P., Killian, J.K., Mehta, A., Geho, D., Espina, V., Petricoin, E.F. and Liotta1, L.A. *Annu. Rev. Med.*, **2004**, 55, 97–112.
[18] Donald, R., Yance, Jr, M. H. and Stephen M. S. *Intergrative cancer Therapies*., **2006**, 5(1)9-29.
[19] Von Linting, F.C., Dreillinger, A.D., Varki, N.M., Wallace, A.M., Casteel, D.E. and Boss,G. R. *Breast cancer Res. Treat*., **2000**, 62(1)51 – 62.
[20] Penichet, M.L., Challita, P.M., Shin, S.U., Sampogna, S.L., Rosenblatt, J.D. & Morrison, S.L. *Lab Anim Sci*., **1999**, 49(2)179-188.
[21] Duffy, M.J. *Clinical Chemistry*., **2005**, .51, 494-503.
[22] Carlomagno, C., Perrone, F., Gallo, C., De Laurentiis, M., Lauria, R., Morabito, A., Pettinato, G., Panico, L., D'Antonio, A., Bianco, A.R. & De Placido, S. *Journal of Clinical Oncology*., **1996**, 14, 2702-2708.
[23] Wright, C., Nicholson, S., Angus, B., Sainsbury, J.R., Farndon, J., Cairns, J., Harris, A.L. & Horne, C.H. *British Journal of Cancer*., **1992**, 65, 118-121.
[24] Gusterson BA, Gelber RD, Goldhirsch A, Price KN, Save-Soderborgh J, Anbazhagan R, Styles J, Rudenstam CM, Golouh R, Reed R. *Journal of Clinical Oncology*., **1992**, 10, 1049-1056.
[25] Ross, J. S., Fletcher, J.A., Linette, P.G., Stec, J., Clark, E., Ayers, M., Symmans, F.W., Pusztai, L. & Bloom, J.K. *The Oncologist*., **2003**, 8, 307–325.
[26] Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J. & Norton, L. *N Engl J Med*., **2001**, 344, 783-792.
[27] Paik, S., Bryant, J., Park, C., Fisher, B., Tan-Chiu, E., Hyams, D., Fisher, E.R., Lippman, M.E., Wickerham, D.L., Wolmark, N. *Journal of the National cancer Institute*., **1998**, 90,1361-1370.
[28] Clark, G.M . *Journal of National Cancer Institute*., **1998**, 90, 1320-1321.
[29] Kleer, G. C. *et al. Medical sciences*., **2003**, **100**(20)11606 – 11611.
[30] Severin, S.E. et al. Biochemistry., **2002**, 68, 286- 300.
[31] Rugo, S.H. *The Oncologist*., **2004**, 9, 43-49.
[32] Chow, E., Hoskin, P., van Der linden, Y., Bottomley, A. & Velikova, G. *Clinical Oncology*., **2005**, 18(1)67-69.
[33] Saad, F. *European Urology supplements*., **2006**, 5(7)547-550.
[34] Cox, D.A. & Der, J.C. *Cancer Biology and Therapy*., **2002**, 6,599-606.
[35] Dolinsky,C. Role of Ras Inhibitors in the Management of Breast Cancer. *Oncolink.*, **2005**, http://www.oncolink.org/conferences/article.cfm?c=3&s=35&ss=205&id=1351.

[36] Gasparini, G., Longo, R., Torino, F. and Morabito, A. *Annals of oncology*., **2005**, 16(4)28-36.
[37] Sausville, A.E., Elsayed, Y., Monga, M. and Kim, G. *Annu. Rev. Pharmacol. Toxicol*., **2002**, 43,199–231.
[38] Osborne, C., Wilson, P. and Tripathy, D. *The oncologist*., **2004**, 9(4)361 – 377.
[39] Esteva, J. F. *The oncologist*., **2004**, 9(3). 4 -9.
[40] Yarden, Y. et al. *Nature Reviews/Molecular Cell Biology* 2., **2002**, 127- 137.
[41] Extra, J., Cognetti, F., Maraninchi, D. *Proceedings from the 41st Annual Meeting of the American Society of Clinical Oncology*., **2005**, Orlando FL; Abstract #555t.
[42] Jain, R.K. et al *Nature Medicine*., **2004**, 10, 145- 147.
[43] Goldman, B. *Journal of the National Cancer Institute*., **2003**, 95, 1744- 1746.
[44] McCarty, M.F., Liu,W., Fan,F., Parikh, A., Reimuth,N., Stoeltzing, O. & Ellis, L.M. *Trends in Molecular Medicine*., **2003**, 9,53- 58.
[45] Agus, D. B. , Gordon M. S.,Taylor C., Natale, B.R., Karlan,B., Mendelson,S.D., Press, F.M., Allison, D.E., Sliwkowski, X.M., Lieberman,G. Kelsey,M.S. and Fyfe, G. *Journal of Clinical Oncology*., **2005**, 23(11)2534-2543.
[46] Allison, D.E., Derynk, K.M.C.Ng., Lutzker, G.S., Jmenez,V. and Kelsey. Pharmacokinetics (PK) of Pertuzumab (rhuMAb 2C4) in Phase II Studies of Ovarian, Breast, Prostate, and Lung Cancers *ASCO Annual Meeting*., **2005**, Abstract No.2532.
[47] Badache, A. & Hynes,E.N. *Journal of Cancer cell*., **2005**, 5(4)299-301.
[48] Hansen,T., Kunkel, M., Weber, A. & Kirkpatrick, J.C. *Journal of Oral Pathology and Medicine*., **2006**, 35(3)155.
[49] Hortobagyi, N.G. *Cancer Treatment Reviews*., **2005**, 31,9-18.
[50] Lenz, J.H., Steiner-Krammer, B., Schmidt, W., Fietkau, R., Mueller, C.P. and Gundlach, K.K.H. *Journal of Cranio-Maxillofacial surgery*., **2005**, 33(6),395-403.
[51] Nosse, B. *Asian Student Medical Journal*., **2003**.
[52] Rokosz, L.L., Huang, C.Y., Reader, J.C., Stauffer, M.T., Chelsky, D., Sigal, N.H., Ganguly, A.K. and Baldwin, J.J. *Bioorganic* & *Medicinal Chemistry Letters*., **2005**, 15(24) 5537-5543.
[53] Hobday, J.T. and Perez, A.E. *Cancer Control*., **2005**, 12(2)7.
[54] Bärlund, M., Forozan, F., Kononen, J., Bubendorf, L., Chen, Y., Bittner, M. L., Torhorst, J., Haas, P., Bucher, C., Sauter, G., Kallioniemi, O. and Kallioniemi, A. *Journal of the National Cancer Institute* ., **2000**, 92(15).1252 -1259.
[55] Cognetti, F., Extra, J., Maraninchi, D. Proceedings from the 41st Annual Meeting of the American Society of Clinical Oncology., **2005**.
[56] Foekens, J. A., Peters, H.A., Look, P. M., Portengen, H., Schmitt, M., Kramer, D.M., Brunner, N., Janicke, F., Meijer-van Gelder, E.M., Henzen-Logmans, S.C., van Putten, W.L.J. and Klijn, M.G.J. *Cancer Research*., **2000**, 60, 636-643.
[57] Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., Norton, L. *The New England journal of Medicine*., **2001**, 344, 783-792.
[58] Harbeck, N., Kates, R. E. & Schmitt, M. *Journal of Clinical Oncology*., **2002**, 20, 1000–1007.
[59] Janicke, F., Prechtl, A., Thomssen, C., Harbeck, N., Meisner, C., Untch, M., Sweep, C.G.J.F., Selbmann, H., Graeff, H. and Schmitt, M. *Journal of the National cancer Institute*., **2001**, 93 (12)913–920.
[60] Konecny, G., Untch, M., Arboleda, J., Wilson, C., Kahlert, S., Boettcher, B., Felber, M., Beryt, M., Lude, S., Hepp, H., Slamon, D. and Pegram, M. *Clinical Cancer Research*., **2001**, 7, 2448–2457.
[61] Look, M. P., et al. *Journal of the National cancer Institute*., **2002**, 94 (2)116–128.
[62] Ma, X. J. *et al. Cancer Cell*., **2004**, 5(6)607–616.
[63] Nathanson, K.N., Wooster, R. & Weber, B. L. *Nature Medicine*., **2001**, 7,552-556.

[64] Page, D. L. *Am. J. Surg. Pathol*., **1991**, 15, 334–349.
[65] Perou, C. M. *et al. Nature*., **2000**, 406, 747–752.
[66] Posadas, E.M., Simpkins, F., Liotta, L.A., McDonald, C. and Kohn, E.C. *Annals of oncology*., **2005**, 16, 16-22.
[67] Schnitzer, J.E. et al. *New England Journal of Medicine.*, **1998**, 339, 472- 474.
[68] Sorlie, T. *et al. Proc.Natl Acad. Sci*., **2001**, *USA* 98, 10869–10874.
[69] Sudbery, P. Human Molecular Genetics. 2nd ed, Dorchester, Dorset press, **2002**, 123.
[70] Torhorst, J., Bucher, C., Kononen, J., Haas, P., Zuber, M., Kochli, R.O., Mross, F., Dieterich, H., Moch, H., Mihatsch, M., Kallioniemi, O.P. and Sauter, G. *American journal of pathology*., **2001**, 159 (6)2249 – 2256.
[71] Govind Pandey., Madhuri S. *J. Chem. Pharm. Res.*, **2010**, 2(4):687-695.
[72] Mukesh R., Patel *et al J. Chem. Pharm. Res.*, **2011**, 3(2):786-791.
[73] Dholakia S.P., Suhagia B. N., *J. Chem. Pharm. Res.*, **2011**, 3(4):315-332.