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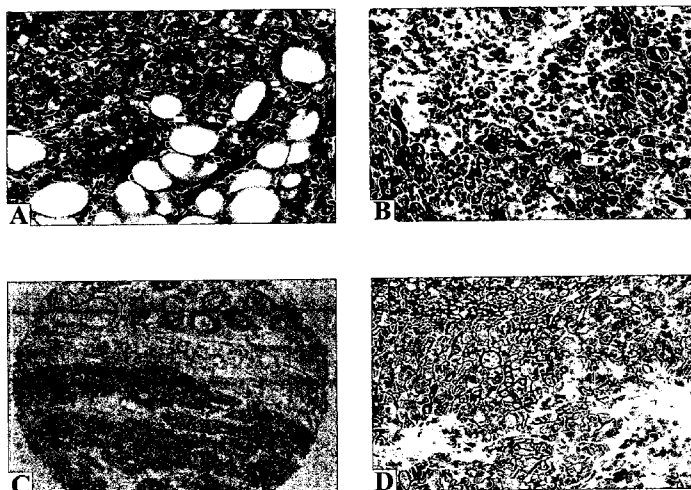


FIGURE 1

(57) Abstract: A method of treating a treating a metastatic breast cancer in a human is provided which includes the step of administering to an individual with an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain, a therapeutic agent effective in inhibiting at least one ErbB receptor. Also provided are methods of identifying a therapeutic agent for treating a metastatic breast as well as methods of diagnosing a metastatic breast cancer which utilise detecting elevated levels of Her-3.

TITLE

CANCER THERAPY AND/OR DIAGNOSIS

FIELD OF THE INVENTION

THIS invention relates to cancer therapy and/or diagnosis. More particularly,
5 this invention relates to therapeutic targets and diagnostic markers for metastatic
breast cancer and preferably metastatic breast cancer in the brain.

BACKGROUND TO THE INVENTION

Breast cancer is one of the most common malignant neoplasias and a leading
cause of female cancer mortality (1, 2). Among women with breast cancer in western
10 countries, 30%-40% will develop metastatic disease (3).

The natural history of metastatic breast cancer to the brain demonstrates that
symptomatic brain metastases develop in 10% to 20% of patients with metastatic
breast cancer and most of these patients will have a mean survival of 6 months
following the diagnosis of the brain disease (4-6). Some studies have found
15 associations between younger age, p53 positive tumours (7) and enrichment for
estrogen receptor negative and epidermal growth factor receptor positive cancers (8)
and the development of brain metastases.

Presently, the epidermal growth factor receptor family comprises four
receptors, HER1, HER2, HER3 and HER4. Such receptors are triggered by several
20 ligands. Upon activation, hetero or homo-dimerization occurs, followed by
phosphorylation of specific tyrosine residues in the intracellular region. Such
activation, leads to regulation of a variety of cellular processes including cell
proliferation and survival. Much evidence suggests that the kinases Akt and Erk
mediate a substantial part of the HER signaling (70).

25 Basal-like tumours are a molecular subgroup of breast cancer (13, 14) that
generally are high grade, negative for estrogen and progesterone receptors and do not
overexpress HER2. Hence, these tumours are believed to be unlikely to respond to
trastuzumab-based therapy and no other targeted therapy for this group is currently
available, although clinical trials are ongoing (15). Trastuzumab (Herceptin®;
30 Genentech Inc, South San Francisco, CA) treatment aims to block HER2 and its
downstream pathways. Other studies showed a trend for primary tumours of patients

who developed brain metastases during disease progression to express markers related to the basal-like tumours (8, 16-18).

Palmieri et al (2007) Cancer Research, 67: 4190 to 4198 examined Her-2 expression profiles of breast cancer cells in the brain and demonstrated that Her-2 overexpression increases the outgrowth of metastatic tumour cells in the brain. Moreover, expression levels of Her-2, Her-3 and Her-4 proteins were examined in MDA-MB-231BR brain-seeking cell line and found to be comparable and at the lower limit of detection.

SUMMARY OF THE INVENTION

The invention is broadly directed to targets and methods for the diagnosis and/or treatment of metastatic outgrowth of breast cancer cells, particularly to the brain.

In another broad form, the invention is directed to targeting one or more members of the epidermal growth factor receptor family for treatment of metastatic outgrowth of breast cancer cells, particularly located in the brain, and more particularly in basal-like breast cancer tumours in the brain.

In a first aspect, the invention provides a method of treating a metastatic breast cancer in a human, said method including the step of administering to an individual with an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain, a therapeutic agent effective in inhibiting at least one ErbB receptor.

In preferred embodiments, the method further includes the step of determining whether said individual has an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain.

In a second aspect, the invention provides a method of designing, engineering, screening or otherwise producing a therapeutic agent for treating a metastatic breast cancer, said method including the step of identifying a candidate agent that is suitable for use in treatment of a metastatic breast cancer, by the presence of an elevated expression level of Her-3 in one or more metastatic breast cancer cells of the brain.

In a third aspect, the invention provides a therapeutic agent for treating a

metastatic breast cancer designed, engineered, screened or otherwise produced according to a method of the second aspect.

In a fourth aspect, the invention provides a pharmaceutical composition comprising a therapeutic agent for treating a metastatic breast cancer of the third aspect, together with a pharmaceutically-acceptable carrier, diluent or excipient.

In a fifth aspect, the invention provides a method of treating a metastatic breast cancer, said method including the step of administering a therapeutic agent for treating a metastatic breast cancer according to the third aspect or a pharmaceutical composition according to the fourth aspect, to an individual with an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain.

In a sixth aspect, the invention provides a method of determining whether a human with a metastatic breast cancer is potentially responsive to treatment with a therapeutic agent effective in inhibiting at least one ErbB receptor and/or a therapeutic agent for treating a metastatic breast cancer designed, engineered, screened or otherwise produced according the method of the second aspect, said method including the step of detecting an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain.

In a seventh aspect, the invention provides a method of determining whether a human is predisposed to a metastatic breast cancer or is suffering from a metastatic breast cancer, said method including the step of detecting an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain.

In preferred embodiments of any one of the aforementioned aspects, the metastatic breast cancer has one or more cells with normal expression levels of Her-2.

More preferably, the metastatic breast cancer has one or more breast cancer cells with normal expression levels of Her-2.

Preferably, the metastatic breast cancer is located in the brain.

In preferred embodiments, the metastatic breast cancer is derived from one or more breast cancer cells.

Preferably, the at least one ErbB receptor is selected from the group consisting Her-1, Her-2, Her-3, Her-4, and combinations thereof.

More preferably, the at least one ErbB receptor is selected from Her-1 and Her-2.

Even more preferably, the at least one ErbB receptor is Her-2.

5 In preferred embodiments, the candidate agent inhibits at least one ErbB receptor.

In preferred embodiments, the therapeutic agent and/or the candidate agent inhibits at least one ErbB receptor by directly inhibiting at least one ErbB receptor.

10 In other preferred embodiments, the therapeutic agent and/or the candidate agent inhibits at least one ErbB receptor by indirectly inhibiting at least one ErbB receptor.

According to any of the aforementioned aspects, the therapeutic agent and/or the candidate agent selectively inhibits at least one ErbB receptor.

15 In preferred embodiments, the one or more cells with normal levels of Her-2 have levels of an estrogen receptor and a progesterone receptor which are below a threshold level of detection.

Preferably, the therapeutic agent and/or the candidate agent is selected from the group consisting of an isolated protein, an isolated nucleic acid, a small-molecule compound, and combinations thereof.

20 More preferably, the therapeutic agent or the candidate agent is an antibody, or a fragment thereof and even more preferably, a monoclonal antibody.

According to preferred embodiments of the aforementioned aspects, the therapeutic agent and/or the candidate agent is selected from the group consisting of trastuzumab, pertuzumab, scFvFRP5, CAB051, ertumaxomab, lapatinib, CI-1033, HKI-272, AEE-788, BIBW-2992, TAK165, BMS-599626, matuzumab, cetuximab, 25 panitumumab, gefitinib, erlotinib, ICR62, nimotuzumab, Ch806, L8A4, MDX-447, zalutumumab, IMC-11F8, AZD4769, PF299804, EGFR501, ZD6474, EKB-569, EXEL 7647, EXEL 0999, AZD8931, MP412, herstatin, pelitinib, CP724714, XL647, PD-169414, and combinations thereof.

30 Even more preferably, the therapeutic agent and/or candidate agent is trastuzumab.

Throughout this specification, unless the context requires otherwise, the

words “comprise”, “comprises” and “comprising” will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF FIGURES

5 In order that the invention may be readily understood and put into practical effect, preferred embodiments will now be described by way of example with reference to the accompanying:

FIGURE 1 - A- Grade III invasive ductal carcinoma; B- Same tumour as in “A” showing CK14 expression; C- Primary breast carcinoma negative for CD44; D-
10 Matched metastasis of “C” showing positivity for CD44.

FIGURE 2 - A –Unsupervised hierarchical clustering of DASL gene expression data from 22 unmatched (black bar) and 15 matched primary and brain metastases (shades of grey bars). Thirteen out of 15 matched samples are clustering together. B -
15 Heatmap and dendogram showing clustering of the samples based on the 20 genes differentially expressed between primary tumors (light grey bar) and brain metastases (dark grey bar).

FIGURE 3 - Her-3 expression. Summary of RT-PCR (first bar at each point) and DASL assay (second bar at each point) for 12 matched samples – Positive values indicate increased fold change in metastases and negative values increased fold change in primary. X-axis are 12 matched primary breast cancers and brain metastases; Y-axis is ration of fold-change-Brain metastasis/Primary cancer
20

FIGURE 4 - A-Brain metastasis; B- Nuclear expression of HER3 for the tumour shown in “A”; C- Negativity for pAKT in the matched primary of “B”; D- Positivity for ERK1/2 in the brain metastasis shown in “B”. E - Positivity for ERK5 in the brain metastasis shown in “B”; F - Positivity for EGFR in the brain metastasis from a non-
25 breast cancer.

FIGURE 5 - Growth curves of the cell line SKBR-3 grown on its own (BC only; vertical line) and on co-culture with ReNcell CX neural cells (NC; circle). Treatment with trastuzumab (square), anti-neuregulin 1 antibody (Neu-Ab; diamond), lapatinib (asterisk) and their combinations (trastuzumab plus Neu-Ab is triangle; lapatinib plus
30 trastuzumab is cross) was also added to the co-culture system. The double-headed

arrow highlights statistical significant ($p < 0.05$, ANOVA) between the curves above and below the blue arrow. X-axis is time in hours while Y axis is EpCam positive particles per 250 μ l of buffer.

FIGURE 6 - MRI images from week 10 showing brain tumours in the left hemisphere from an animal of (A) the control group, (B) the trastuzumab treated group, (C) the anti-neuregulin 1 treated group. Average tumor size was smaller in B and C compared to control A ($p < 0.05$, unpaired T test). D – Relative levels of HER3 transcripts in the parental cell line and in the four groups of mice. *Highlights statistical significance ($p < 0.05$, unpaired T test) between the parental group and the other groups; Y-axis is HER3 expression relative to GAPDH and X-axis the subgroups of treatment.

FIGURE 7 - Colony forming assay. Effects of trasztuzumab and neuregulin on the number of colonies of MB-MDA-231 breast cancer cell line. Y –axis is average number of colonies and X-axis the subgroups of treatment.

FIGURE 8 - Colony forming assay. Effects of trasztuzumab and neuregulin on the size of colonies of MB-MDA-231 breast cancer cell line. Y –axis is average number of colonies and X-axis the subgroups of treatment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, at least in part, on the finding that Her-3 is overexpressed in tumours located in the brain which result from metastatic outgrowth of breast cancer tumours that exhibit a basal-like phenotype. Furthermore, even in the absence of HER2 amplification/overexpression in such tumours, an anti (α)-Her-2 monoclonal antibody is useful in reducing a tumour growth located in the brain resulting from metastatic breast cancer outgrowth *in vitro* and *in vivo*. Moreover, the association of Her-3 with other members of the ErbB receptor family suggests that therapies directed to members of the ErbB receptor family could be useful for treatment of a subset of metastatic breast cancers in the brain which normally do not respond well to, at least, α -Her-2 therapies such as trastuzumab.

In general aspects, the invention provides methods of treatment and/or diagnosis of a metastatic breast cancer in a human with elevated levels of Her-3 in one or more metastatic breast cancer cells of the brain. In preferred embodiments, the

metastatic breast cancer is located in the brain. In other preferred embodiments, the metastatic breast cancer is derived from one or more cells, and more preferably breast cancer cells, with normal expression levels of Her-2.

By "*breast cancer*" is meant a malignant tumour of the breast tissue.
5 Typically, breast cancer forms in tissues of the breast, usually the ducts (tubes that carry milk to the nipple) and lobules (glands that make milk).

By "*metastatic breast cancer*" is meant one or more primary breast cancer cells which have undergone metastases and spread to one or more parts of the body to form secondary tumours which are otherwise referred to as metastatic tumours. A
10 "*metastatic breast cancer*" may be derived from one or more cancer cells which originate from a breast cancer, or alternatively the "*metastatic breast cancer*" may derived from a non-breast cancer cell source.

As used herein, by "*one or more cells with normal expression levels of Her-2*" is meant that said one or more cells are considered to have, exhibit or display a
15 lack of expression or amplification of Her-2, and preferably either gene amplification from the *HER-2* gene locus or a *HER-2* amplicon or protein expression, when compared to one or more cells which overexpress Her-2. In particular embodiments, the "*one or more cells with normal expression levels of Her-2*" have expression levels of Her-2 which are below a threshold level of detection and are thus
20 considered not to be Her-2 positive. Although not wishing to be bound by any particular theory, a positive HER2 result may be considered as immunohistochemistry (IHC) staining of 3+ (uniform, intense membrane staining of >30% of invasive tumour cells), a fluorescent in situ hybridization (FISH) result of more than six *HER2* gene copies per nucleus or a FISH ratio (*HER2* gene signals to
25 chromosome 17 signals) of more than 2.2. By way of example, a negative result for Her-2 may be an IHC staining value of 0 or 1+, a fluorescence *in situ* hybridization (FISH) result of less than 4.0 *HER2* gene copies per nucleus, or FISH ratio of less than 1.8. Reference is made to Wolff et al (2007) J Clin Oncol 25: 118-145 which provides non-limiting examples of assays and criteria for use of Her-2 as a marker in
30 breast cancer and is incorporated herein by reference. In light of the foregoing it will be appreciated that normal Her-2 expression levels may result from basal or normal

expression of the *Her-2* gene locus located on genomic DNA, and in particular, two chromosomal copies of the *Her-2* gene locus or alternatively expression of *Her-2* which results from one or more cancer cells that are polysomic.

Although not wishing to be bound by any particular theory, “*one or more cells with normal expression levels of Her-2*” are considered not amenable to conventional targeted therapies for breast cancer such as α -*Her-2* monoclonal antibodies (eg. trastuzumab). It will be appreciated that the “*one or more cells with normal expression levels of Her-2*” may originate from a variety of sources such as *in vitro* culture (such as cultured breast cancer cell lines), *ex vivo* or alternatively, *in vivo*. In preferred embodiments, the “*one or more cells with normal expression levels of Her-2*” are one or more cancer cells and more preferably, one or more breast cancer cells.

In determining whether “*one or more cells with normal expression levels of Her-2*” have *Her-2* expression levels that are below the threshold level of detection, applicable detection or assay methods include immunohistochemistry, histopathology, genomic analysis (such as fluorescence *in situ* hybridization) and gene expression profiling (such as DNA microarray-based techniques), although without limitation thereto. Reference is made to Sorlie et al Proc Natl Acad Sci USA 2001;98(19):10869-74; and Perou et al Nature 2000;406(6797):747-52 which provide non-limiting examples of suitable molecular methods, and in particular gene expression profiling methods, to determine or evaluate various markers in breast cancer whilst Lal et al *Am J Clin Pathol* 2004, 121:631-636 provides non-limiting examples of *Her-2* testing in breast cancer using immunohistochemical analysis and fluorescence *in situ* hybridization.

In particular embodiments, the “*one or more cells with normal expression levels of Her-2*” may have also have expression levels of an estrogen receptor and progesterone receptor which are below a threshold level of detection. Detection methods include those and for example as described in Korsching et al *J Clin Pathol*. 2008 May;61(5):553-60; Kusińska et al *Pol J Pathol*. 2005;56(3):107-10; and Mullan and Millikan, *Cell Mol Life Sci*. 2007 Dec;64(24):3219-32.

The one or more cells which exhibit a phenotype of normal *Her-2* levels as

defined hereinabove and have expression levels of an estrogen receptor and a progesterone receptor which are below a threshold level of detection are also known in the art as “triple-negative tumour”, “basal-like tumour” or “basal-like phenotype” and may be used interchangeably herein.

5 In the context of the present invention, by “*ErbB receptor*” is meant any member of the epidermal growth factor receptor family of receptor tyrosine kinase or Her receptors. The ErbB (or otherwise referred to as Her receptors or epidermal growth factor receptors) family of receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. Her-1 is specific for epidermal growth
10 factor and EGF related peptides including transforming growth factor- α , amphiregulin, and heparin-binding EGF-like growth factor. The members of the ErbB family and particularly Her-1, Her-2, Her-3 and Her-4 share extensive homology and are able to homodimerise and heterodimerise with other members of the family. Reference is made to Zhang *et al* (2007) *J. Clin. Invest.* **117**:2051–2058,
15 which provides a review of ErbB receptor biology.

The term “*Her-3*” as used herein refers to a Her-3 protein, and preferably a human Her-3 protein, as for example described in US Patent No. 5,480,968; Plowman *et al*, Proc Natl Acad Sci U S A. 1990 Jul;87(13):4905-9 and Kraus *et al* Proc Natl Acad Sci U S A. 1989 Dec;86(23):9193-7, which are incorporated herein
20 by reference. “*Her-3*” may also referred to as “*ErbB3*”, “*Her-3 receptor*” and “*ErbB3 receptor*” and other terms as are known in the art such as “*LCSS2*”, “*c-erbB3*”, “*erbB3-S*”, “*MDA-BF-1*”, “*MGC88033*”, “*c-erbB-3*”, “*p180-ErbB3*”, “*p45-sErbB3*” and “*p85-sErbB3*” and may be used interchangeably herein.

The term “*Her-2*” as used herein refers to a Her-2 protein, and preferably a
25 human Her-2 protein, as for example described in King *et al* 1985 Science, Sep 6;229(4717):974-6. “*Her-2*” may also be referred to as “*ErbB2*”, “*neu*”, “*Her-2 receptor*”, “*ErbB2 receptor*” and other terms as are known in the art such as “*ngl*”, “*TKR1*”, “*CD340*”, “*HER-2/neu*” and “*p185^{her2/neu}*” and may be used interchangeably herein.

30 The term “*Her-1*” as used herein refers to a Her-1 protein, and preferably a human Her-1 protein which is the mature, tyrosine kinase cell surface receptor, as for

example described in Lin *et al* (1984) Science 224: 843 - 848 and Ullrich *et al.*, (1984) Nature, 309:418-425. "*Her-1*" is also referred to "*epidermal growth factor receptor (EGFR)*", "*ErbB1*", "*c-erbB-1*", "*ErbB*" and "*PIG61*" and may be used interchangeably herein.

5 The term "*Her-4*" as used herein refers to a Her-4 protein, and preferably a human Her-4 protein, as for example described in Plowman *et al*, Proc Natl Acad Sci U S A. 1993 Mar 1;90(5):1746-50. "*Her-4*" may also be referred to as "*MGC138404*", "*p180^{erbB4}*", "*tyro2*" and "*ErbB4*" and may be used interchangeably herein.

10 General aspects of the present invention contemplate detecting, determining or otherwise measuring an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain, either as part of diagnostic methods or in methods of treatment of humans (preferably as a selection step), according to the present invention.

15 In certain broad aspects, the present invention contemplates methods of treating a metastatic breast cancer in a human by administering to an individual with an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain, a therapeutic agent designed, screened, engineered or otherwise produced according to methods of the present invention as described herein.

20 In other general aspects, the present invention contemplates methods of treating one or more breast cancer cells with normal expression of Her-2 in a human which includes the step of administering to an individual with an elevated level of Her-3, a therapeutic agent effective in inhibiting at least one ErbB receptor.

25 In other aspects, the invention relates to methods of diagnosis of a metastatic breast cancer, preferably located in the brain, derived from one or more breast cancer cells with normal expression levels of Her-2. Such methods may assist in determining whether a human suffers from said metastatic breast cancer, is predisposed to said metastatic breast cancer and/or is more or less responsive to therapies directed to metastatic breast cancer and in particular metastatic breast
30 cancer located in the brain.

Thus "*predisposed*" and "*predisposition*" are used in the context of a

probability that an individual may display clinical symptoms of a metastatic breast cancer, preferably located in the brain, derived from one or more breast cancer cells with normal expression levels of Her-2, or that any existing, manifest clinical symptoms of said metastatic breast cancer are the result of an underlying biochemical cause.

It will also be appreciated that the diagnostic methods of the invention may be used alone or combined with other forms of molecular and/or clinical diagnosis to improve the accuracy of diagnosis.

In the context of the present invention, by “*an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain*” is meant an amplified, overexpressed, up-regulated, enhanced or otherwise increased amount of Her-3 when compared to breast cancer cells which have not metastasized to the brain.

It will be appreciated that in particular embodiments, Her-3 *per se* may be assayed or measured to determine an elevated level of Her-3. The present invention also contemplates detecting or assaying alternative forms of Her-3, such as post-translationally modified Her-3 (for example, phosphorylated Her-3) or variants such as splice variants.

It is also contemplated that in other embodiments that molecules associated with Her-3 or its function, such as downstream signalling proteins of the mitogen-activated protein kinase pathway which Her-3 activates, may also be assayed. In particularly preferred embodiments, a ligand of Her-3 such as, but not limited to, neuregulin-1 may also be assayed in order to determine whether there is an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain.

In light of the foregoing, it will be appreciated that a variety of methods as are known in the art may be utilised to determine an expression level of Her-3 in one or more metastatic breast cancer cells of the brain in methods of the present invention.

In particular embodiments, the invention contemplates protein-based detection techniques and in particular for detection of Her-3, or a fragment thereof.

By “*protein*” is also meant “*polypeptide*”, “*peptide*” or fragments thereof, referring to an amino acid polymer, comprising natural and/or non-natural amino acids, including L- and D-isomeric forms as are well understood in the art. For

example, Her-3 or any other ErbB receptor or ErbB receptor ligand may be referred to as either a protein or polypeptide. In particular embodiments, the protein or polypeptide is an isolated protein or an isolated polypeptide.

A “*peptide*” is a protein having no more than fifty (50) amino acids.

5 A “*polypeptide*” is a protein having fifty (50) or more amino acids.

Proteins and peptides may be useful in native, chemical synthetic or recombinant synthetic form.

For the purposes of this invention, by “*isolated*” is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native, chemical synthetic or recombinant form.

15 A “*fragment*” is a segment, domain, portion or region of an ErbB receptor, and in particular embodiments Her-3, which constitutes less than 100% of the full-length protein.

A fragment preferably comprises less than 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 85%, 80%, 75%, 70%, 60%, 50%, 40%, 30%, 20% or as little as even 10%, 5% or 3% of the entire protein.

20 In particular aspects, a protein fragment may comprise, for example, at least 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 150, 200, 250, 300, 350, 400, 450 or 500 contiguous amino acids of an ErbB receptor, and in particular Her-3.

In particular embodiments, a fragment is a “*biologically-active fragment*”. By “*biologically-active fragment*” is meant a segment, portion or fragment of a biological active molecule which has at least about 0.1%, preferably at least about 10%, more preferably at least about 25% and even more preferably at least 50% of the activity of the molecule and even more preferably at least 70%, 80% or 90% of the biological activity of the entire or full length protein.

30 By way of example only, fluorescence activated cell sorting (FACS) analysis using labelled antibodies is readily amenable to quantitative measurement of cell

surface expression of proteins and in particular receptor proteins such as Her-3. Immunofluorescence and other fluorescence microscopy methods can also be used to stain metastatic breast cancer outgrowth in the brain to detect levels of Her-3 as well as other conventional immunohistochemistry techniques.

5 Alternatively, relative protein expression levels may be determined by other protein-based methods which include immunoassays, for example ELISA and immunoblotting (such as western blotting using α -Her-3 antibodies) to detect relative expression levels of Her-3.

10 The invention also contemplates techniques which detect nucleic acid-protein interactions such as, but not limited to, northwestern blotting and electrophoretic mobility shift assay

15 The invention further contemplates use of protein-based microarray technology, preferably to determine the expression pattern profile of metastatic breast cancer in order to analyse whether Her-3 expression is up-regulated or overexpressed in patients with metastatic breast cancer, preferably a metastatic breast cancer located in the brain.

20 Proteomic pattern analysis provides an alternative diagnostic method which is particularly useful for global expression pattern analysis of proteins. Methods of cancer diagnosis using proteomic patterns are provided in Conrads *et al* Expert Rev Mol Diagn. 2003 Jul;3(4):411-20 and is incorporated herein by reference.

25 In particular embodiments, a plurality of proteins may be used in a protein library displayed in a number of ways, *e.g.*, in phage display or cell display systems or by two-dimensional gel electrophoresis, or more specifically, differential two-dimensional gel electrophoresis (2D-DIGE). These particular embodiments may generally be referred to as "*proteomic*" or "*protein profiling*" methods, such as described in Chapters 3.9.1 and 22 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, John Wiley & Sons NY USA (1996-2002).

 In embodiments relating to protein arrays, preferably Her-3 is located at an identifiable address on the array.

30 Preferably, the protein array comprises a substrate to which is immobilized, impregnated, bound or otherwise coupled ErbB protein, or a fragment thereof.

The substrate may be a chemically-derivatized aluminium chip, a synthetic membrane such as PVDF or nitrocellulose, a glass slide or microtiter plates.

Detection of substrate-bound proteins may be performed using mass spectrometry, ELISA, immunohistochemistry, fluorescence microscopy or by colorimetric detection but is not limited thereto.

In alternative or additional embodiments, the invention provides methods to detect elevated levels of Her-3 indirectly by measurement of Her-3 activity and/or function.

A person of skill in the art will appreciate that the methods of the invention, and in particular the diagnostic methods of the invention, could employ nucleic acid detection techniques as are well known in the art to measure levels of a nucleic acid encoding a Her-3 in order to ascertain expression levels of Her-3.

In principle, any nucleic acid sequence detection technique may be applicable, such as nucleic acid sequencing, northern hybridization, nucleic acid sequence amplification, nucleic acid arrays and methods that detect melting temperature differences to identify whether a nucleic acid harbours one or more polymorphisms compared to a "wild type" reference nucleic acid.

The term "*nucleic acid*" as used herein designates single or double stranded mRNA, RNA, cRNA and DNA, said DNA inclusive of cDNA and genomic DNA. A nucleic acid may be native or recombinant and may comprise one or more artificial nucleotides, e.g. nucleotides not normally found in nature. RNA includes single-stranded and double-stranded unprocessed RNA, mRNA, siRNA, miRNA, RNAi and tRNA. Nucleic acid also encompasses modified purines (for example, inosine, methylinosine and methyladenosine) and modified pyrimidines (thiouridine and methylcytosine).

The term "*isolated nucleic acid*" as used herein refers to a nucleic acid subjected to *in vitro* manipulation into a form not normally found in nature. Isolated nucleic acid includes both native and recombinant (non-native) nucleic acids. For example, a nucleic acid may be isolated from human.

The terms "*mRNA*", "*RNA*" and "*transcript*" are used interchangeably when referring to a transcribed copy of a transcribable nucleic acid.

The present invention also contemplates detection of variant nucleic acids.

“*Variants*” include within their scope naturally-occurring variants such as allelic variants, orthologs and homologs and mutants, for example.

5 The terms “*mutant*”, “*mutation*” and “*mutated*” are used herein generally to encompass conservative or non-conservative amino acid substitutions, deletions and/or insertions present in a protein, or fragment thereof or introduced into an isolated protein or fragment thereof.

10 In particular embodiments, nucleic acid detection techniques may include detection of variants such as alternate transcriptional splice variants encoding different isoforms of Her-3. By way of example only, one particular Her-3 isoform represents a longer mRNA transcript and thereby encodes a longer isoform.

15 Suitable nucleic acid amplification techniques are well known to the skilled addressee, and include polymerase chain reaction (PCR) as for example described in Ausubel *et al.* (*supra*) which is incorporated herein by reference; strand displacement amplification (SDA) as for example described in U.S. Patent No 5,422,252 which is incorporated herein by reference; rolling circle replication (RCR) as for example described in Liu *et al.*, (1996, *J. Am. Chem. Soc.* **118**:1587-1594 and International application WO 92/01813) and Lizardi *et al.* (International Application WO 97/19193) which are incorporated herein by reference; nucleic acid sequence-based
20 amplification (NASBA) as for example described by Sooknanan *et al.*, (1994, *Biotechniques* **17**:1077-1080) which is incorporated herein by reference; and Q- β replicase amplification as for example described by Tyagi *et al.*, (1996, *Proc. Natl. Acad. Sci. USA* **93**:5395-5400) which is incorporated herein by reference.

25 The abovementioned are examples of nucleic acid sequence amplification techniques but are not presented as an exhaustive list of techniques. Persons skilled in the art will be well aware of a variety of other applicable techniques as well as variations and modifications to the techniques described herein.

As used herein, an “*amplification product*” is a nucleic acid generated by a nucleic acid sequence amplification technique as hereinbefore described.

30 Detection of amplification products may be achieved by detection of a probe hybridized to an amplification product, by direct visualization of amplification

products by way of agarose gel electrophoresis, nucleotide sequencing of amplification products or by detection of fluorescently-labelled amplification products.

As used herein, a "*probe*" is a single- or double-stranded oligonucleotide or polynucleotide, one and/or the other strand of which is capable of hybridizing to another nucleic acid, to thereby form a "hybrid" nucleic acid.

Probes and/or primers of the invention may be labelled, for example, with biotin or digoxigenin, with fluorochromes or donor fluorophores such as FITC, TRITC, Texas Red, TET, FAM6, HEX, ROX or Oregon Green, acceptor fluorophores such as LC-Red640, enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) or with radionuclides such as ^{125}I , ^{32}P , ^{33}P or ^{35}S to assist detection of amplification products by techniques as are well known in the art.

As used herein, an "*oligonucleotide*" is a single- or double-stranded nucleic acid having no more than one hundred (100) nucleotides (bases) or nucleotide pairs (base pairs). A "*polynucleotide*" has more than one hundred (100) nucleotides or nucleotide pairs.

In the particular context of nucleic acid sequence amplification, an oligonucleotide of the invention may be in the form of a primer.

As used herein, a "*primer*" is a single-stranded oligonucleotide which is capable of hybridizing to a nucleic acid "template" and being extended in a template-dependent fashion by the action of a suitable DNA polymerase such as *Taq* polymerase, RNA-dependent DNA polymerase or SequenaseTM.

Typically, a primer may have at least twelve, fifteen, twenty, twenty-five, thirty, thirty five, forty or fifty contiguous nucleotide bases.

As used herein, "*hybridization*", "*hybridize*" and "*hybridizing*" refers to formation of a hybrid nucleic acid through base-pairing between complementary or at least partially complementary nucleotide sequences under defined conditions, as is well known in the art. Normal base-pairing occurs through formation of hydrogen bonds between complementary A and T or U bases, and between G and C bases. It will also be appreciated that base-pairing may occur between various derivatives of purines (G and A) and pyrimidines (C, T and U). Purine derivatives include inosine,

methylinosine and methyladenosines. Pyrimidine derivatives include sulfur-containing pyrimidines such as thiouridine and methylated pyrimidines such as methylcytosine. For a detailed discussion of the factors that generally affect nucleic acid hybridization, the skilled addressee is directed to Chapter 2 of CURRENT
5 PROTOCOLS IN MOLECULAR BIOLOGY, *supra*.

More specifically, the terms "*anneal*" and "*annealing*" are used in the context of primer hybridization to a nucleic acid template for a subsequent primer extension reaction, such as occurs during nucleic acid sequence amplification or nucleotide sequencing, as for example described in Chapter 15 of CURRENT PROTOCOLS IN
10 MOLECULAR BIOLOGY Eds Ausubel et al. (John Wiley & Sons NY 1995-1999).

In another embodiment, detection may be performed by melting curve analysis using probes incorporating fluorescent labels that hybridize to amplification products in a sequence amplification reaction. A particular example is the use of Fluorescent Resonance Energy Transfer (FRET) probes to hybridize with
15 amplification products in "real time" as amplification products are produced with each cycle of amplification.

In yet another embodiment, the invention contemplates use of melting curve analysis whereby nucleic acid-intercalating dyes such as ethidium bromide (EtBr) or SYBR Green I bind amplification products and fluorescence emission by the
20 intercalated complexes is detected.

In light of the foregoing, it will be appreciated that the invention contemplates in particular embodiments detection of variant nucleic acid with polymorphisms. It will also be well understood by the skilled person that detection of nucleotide sequence polymorphisms may be performed using any of a variety of techniques such
25 as PCR-RFLP analysis, fluorescence-based melt curve analysis, SSCP analysis, denaturing gradient gel electrophoresis (DGGE) or direct sequencing of amplification products.

Measurement of relative levels of Her-3 nucleic acid levels compared to an expressed level of a reference nucleic acid may be conveniently performed using a
30 nucleic acid array.

Nucleic acid array technology has become well known in the art and

examples of methods applicable to array technology are provided in Chapter 22 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.* (John Wiley & Sons NY USA 1995-2001).

5 An array can be generated by various methods, e.g., by photolithographic methods (see, *e.g.*, U.S. Patent Nos. 5,143,854; 5,510,270; and 5,527,681), mechanical methods (*e.g.*, directed-flow methods as described in U.S. Patent No. 5,384,261), pin-based methods (*e.g.*, as described in U.S. Pat. No. 5,288,514), and bead-based techniques (*e.g.*, as described in PCT US/93/04145).

10 Reference is also made to Affymetrix nucleic acid array systems such as described in United States Patent 5,858,659 and United States Patent 6,300,063 which provide specific teaching in relation to nucleic acid array-based detection of disease-related polymorphisms but may provide useful methodology applicable to the present invention.

15 A particular advantage of nucleic acid arrays is that they are useful with a variety of different techniques such as primer extension, allele-specific hybridization, allele-specific ligation and allele-specific cleavage of a flap probe, as for example described in Kwok, 2000, *supra*, Pastinen *et al.*, 2000, Genome Res. **10** 1031.

20 Nucleic acid or gene expression levels may also be measured by a variety of other gene expression profiling methods as are known in the art such as, but not limited to, the DASL assay (cDNA-mediated annealing, selection extension and ligation) as described hereinafter.

25 *In situ* hybridisation techniques are also particularly useful for the detection of expression levels and includes chromogenic *in situ* hybridisation and fluorescence *in situ* hybridisation as are known in the art. Reference is made to Chapter 14 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.* (John Wiley & Sons NY USA 1995-2008) for non-limiting examples of applicable *in situ* hybridisation technology.

30 In another particular form of this embodiment, quantitative or semi-quantitative PCR using primers corresponding to Her3-encoding nucleic acids may be used to quantify relative expression levels Her-3 nucleic acid to thereby determine whether an individual is predisposed to or suffering from breast cancer.

PCR amplification is not linear and hence end point analysis does not always allow for the accurate determination of nucleic acid expression levels.

Real-time PCR analysis provides a high throughput means of measuring gene expression levels. It uses specific primers, and fluorescence detection to measure the amount of product after each cycle. Hybridization probes utilise either quencher dyes or fluorescence directly to generate a signal. This method may be used to validate and quantify nucleic acid expression differences in cells and/or tissues obtained from metastatic breast cancer sufferers compared to cells and/or tissues obtained from non-sufferers or alternatively, comparison of the expression levels between the primary tumour and the metastases. In preferred embodiments, the metastatic breast cancer is located in the brain of a sufferer.

With regard to the above and any method of the present invention, samples for diagnostic or analysis may be isolated from any cell and/or tissue source, inclusive of neural tissue, although more easily obtained cells and tissues such as blood, skin and the like may also be advantageous.

For the purposes of diagnostic methods or selection methods as described herein, the invention contemplates particular embodiments of such methods which utilise the aforementioned techniques either alone or in combination.

It will be appreciated that the aforementioned techniques for detection of either nucleic acid or protein expression levels of Her-3 are also applicable for detection of expression levels of other ErbB receptors, and in particular Her-2, in methods of the present invention, as may be required.

In further aspects, the invention relates to methods of treatment of a human which includes the step of administering to an individual with an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain, a therapeutic agent effective in inhibiting at least one ErbB receptor.

Accordingly, suitable therapeutic agents of the invention may be peptides, proteins such as antibodies or other organic molecules, preferably small organic molecules, with a desired biological activity and half-life.

In particular embodiments, the invention relates to methods of treatment of a human which includes the step of administering to an individual with an elevated

level of Her-3 in one or more metastatic breast cancer cells of the brain, a therapeutic agent effective in directly inhibiting at least one ErbB receptor.

In other particular embodiments, the invention relates to methods of treatment of a human which includes the step of administering to an individual with an elevated
5 level of Her-3 in one or more metastatic breast cancer cells of the brain, a therapeutic agent effective in indirectly inhibiting at least one ErbB receptor. Non-limiting examples of suitable indirect inhibitors include molecules which disrupt an interaction between at least one ErbB receptor and its respective ligand such as an antibody directed to an ErbB ligand.

10 In the context of the present invention, by "*inhibit*", "*inhibition*", "*inhibited*", "*inhibitory*" or "*inhibitor*" is meant a therapeutic agent and/or a candidate agent which interferes with, inhibits, blocks or hinders at least one ErbB receptor, either by a direct or an indirect mechanism. In particular embodiments, the inhibitory effect may be of a function or functional activity of at least one ErbB
15 receptor, such as inhibition of tyrosine kinase activity of said ErbB receptor. In other embodiments, an inhibitor may block dimerisation between two different types of ErbB receptors, which in turn may block signaling by other ErbB family receptors.

In particular embodiments, the therapeutic agent is an antagonist. In yet other embodiments, the therapeutic agent is an agonist. In other particular embodiments,
20 the therapeutic agent inhibits or disrupts the heterodimeric interaction between different ErbB receptors such as, but not limited to, Her-2 and Her-3.

According to certain embodiments, the therapeutic agent is a direct inhibitor of at least one ErbB receptor which binds or interacts directly with at least one ErbB receptor. A non-limiting example of a direct inhibitor is an antibody which binds to
25 or has been raised against the at least one ErbB receptor or a fragment thereof, although without limitation thereto. In other non-limiting embodiments, a direct inhibitor is a small-molecule which binds to at least one ErbB receptor or a fragment thereof.

According to other embodiments, the therapeutic agent is an indirect inhibitor
30 of at least one ErbB receptor which does not bind to or interact with directly with the at least one ErbB receptor but inhibit the activity or function of the at least one ErbB

receptor. In particular, indirect inhibitors target or are directed to a modulator of at least one ErbB receptor. In particular embodiments, an indirect inhibitor of at least one ErbB receptor targets or is directed to one or more ligands of at least one ErbB receptor. Non-limiting examples of suitable ligands of ErbB receptors which may be
5 a target for an indirect inhibitor include epidermal growth factor (EGF), transforming growth factor (TGF)-alpha, amphiregulin, betacellulin, heparin-binding EGF, epiregulin and members of the neuregulin family such as neuregulin 1, 2, 3 and 4, although without limitation thereto.

In other preferred embodiments, an indirect inhibitor of at least one ErbB
10 receptor targets or is directed to a modulator, and in particular an activator, of ligands of ErbB receptors. According to these embodiments, a modulator of the ErbB receptor ligand may be an enzyme which cleaves an ErbB receptor ligand from the cell surface, which in turn activates the ErbB receptor ligand. A non-limiting example of a suitable enzyme are any one of the members of the ADAM (A
15 Disintegrin And Metalloprotease) family such as ADAM10, ADAM12, ADAM17 and ADAM19, although without limitation thereto.

In particular preferred embodiments, the therapeutic agent selectively inhibits at least one ErbB receptor.

By "*selective*" or "*selectively*" is meant a therapeutic agent that primarily
20 affects the function of an ErbB receptor but may also have an effect upon other ErbB receptors. Therefore in one embodiment, "*selective*" or "*selectively*" encompasses a situation where at least 50%, 55%, 60%, 65%, preferably at least 70%, 75%, 80%, 85% and more preferably 90%, 95%, 96%, 98%, 99% and 100% of the inhibitory activity of a therapeutic agent can be attributable to a single ErbB receptor.

25 In another embodiment, "*selective*" or "*selectively*" includes a therapeutic agent that can bind to and inhibit an ErbB receptor. A non-limiting example of such a therapeutic agent or candidate agent is a suitable binding and/interaction partner, such as a protein. In particular embodiments, the protein is an antibody.

In yet another embodiment, "*selective*" or "*selectively*" includes a
30 therapeutic agent that specifically regulates an ErbB receptor such as, but not limited to, nucleic acid molecules.

In yet a further embodiment, "*selective*" or "*selectively*" includes a therapeutic agent that specifically regulates post-translational forms of an ErbB receptor.

5 In preferred embodiments, the methods of the present invention contemplate a therapeutic agent that is an antibody, or an antibody fragment, which binds and/or has been raised against at least one ErbB receptor or alternatively, an antibody (or an antibody fragment) which binds and/or has been raised against a ligand of at least one ErbB receptor. It is envisaged that both polyclonal and monoclonal antibodies directed to either the entire protein or a biologically-active fragment thereof are
10 suitable therapeutic agents.

In preferred embodiments, the therapeutic agent is an α -Her-3 antibody. Reference is made to United States Patent Nos 5,480,968 and 5,968,511 and International Publication No. 2008/100624 which provides non-limiting examples of suitable α -Her-3 antibodies.

15 In other preferred embodiments, the therapeutic agent is an α -Her-2 antibody. For non-limiting examples of α -Her2 antibodies, see, for example, United States Patent No 6,165,464; 5,677,171; and 6,054,561. In particularly preferred embodiments the α -Her-2 antibody is trastuzumab which is sold under the name Herceptin® by Genentech.

20 In yet other preferred embodiments, the therapeutic agent is an α -Her-1 antibody. Suitable non-limiting examples of α -Her-1 antibodies are provided in US Patent Nos. 6,235,883 and 5,844,093 and International Publication No. WO/2007/058823.

25 In further preferred embodiments, the therapeutic agent is an α -Her-4 antibody. Reference is made to United States Patent Nos. 5,811,098 and 7,332,579 which provide non-limiting examples of suitable α -Her-4 antibodies.

As mentioned above, antibodies may be monoclonal or polyclonal, obtained for example by immunizing a suitable production animal (*e.g.* a mouse, rat, rabbit, sheep, chicken or goat). Serum or spleen cells may be then isolated from the immunized animal according to whether polyclonal or monoclonal antibodies are
30 required.

Monoclonal antibodies may be produced by standard methods such as described in CURRENT PROTOCOLS IN IMMUNOLOGY (Eds. Coligan et al. John Wiley & Sons. 1995-2000) and Harlow, E. & Lane, D. Antibodies: A Laboratory Manual (Cold Spring Harbour, Cold Spring Harbour Laboratory, 1988).

5 Such methods generally involve obtaining antibody-producing cells, such as spleen cells, from an animal immunized as described above, and fusing spleen cells with an immortalized fusion partner cell.

Recombinant antibodies are also contemplated. Selection of appropriate recombinant antibodies can be achieved by any of a number of methods including
10 phage display, microarray or ribosome display, such as discussed in Hoogenboom, 2005, Nature Biotechnol. **23** 1105, by way of example only.

Also contemplated are antibody fragments such as Fab, F(ab)₂, Fv, scFv and Fc fragments as well understood in the art.

As is also well understood in the art, in order to assist detection of antibody-
15 antigen complexes, antibodies may be conjugated with labels including but not limited to a chromogen, a catalyst, an enzyme, a fluorophore, a chemiluminescent molecule, biotin and/or a radioisotope.

As used herein, the terms "*antibody*" or "*immunoglobulin*", as used interchangeably herein, includes whole antibodies and any antigen binding fragment
20 or single chains thereof. The terms "*antibody*" or "*immunoglobulin*" includes any antigen-binding protein product of the immunoglobulin gene complex, including immunoglobulin isotypes IgA, IgD, IgM, IgG and IgE and antigen-binding fragments thereof. Examples of antigen-binding fragments include, but are not limited to, Fab, F(ab)₂, Fv, scFv, *etc.*

25 It will be appreciated by a person of skill in the art that antibodies employed for therapeutic applications in humans must have specific properties which make these antibodies suitable for use in humans. Typically, therapeutic antibodies are "*humanised*", wherein the antibody or at least one chain thereof, typically comprises a variable framework region substantially from a human antibody and the
30 complementary determining regions substantially from an antibody derived from a non-human (such as, but not limited to, rodent antibodies and shark antibodies).

Humanised antibodies are particularly advantageous for medical applications due to the decrease likelihood of eliciting a foreign body immune reaction.

In other embodiments, pharmaceutical compositions and treatment methods may utilize nucleic acid constructs (including but not limited to inhibitory RNA constructs) for treatment of a metastatic breast cancer derived from one or more breast cancer cells with normal expression levels of Her-2. In preferred embodiments, the metastatic breast cancer is located in the brain.

Generally, a nucleic acid construct may be any recombinant nucleic acid that facilitates delivery, expression, propagation or manipulation of a desired nucleic acid component of the construct.

By way of example only, a construct may be a plasmid, a cosmid, a modified virus or containing virus-derived elements, an artificial chromosome, a phagemid, an anti-sense oligonucleotide, RNA (*e.g.* siRNA or shRNA) or the like.

Particular virus-derived expression constructs suitable for human delivery include constructs comprising adenovirus-, adeno-associated virus-, lentivirus-, flavivirus- and/or vaccinia virus-derived elements.

In light of the foregoing it will be appreciated that RNA-based methods may be employed for inhibition of at least one ErbB receptor. RNA interference, and in particular (but not limited thereto) siRNA and shRNA, provides an attractive method for silencing of potential therapeutic gene targets by sequence-specific cleavage of cognate mRNA. Takeshita and Ochiya (*Cancer Sci*, 2006, 97: 689-696) provides numerous examples of the therapeutic potential of RNA interference against cancer and is incorporated herein by reference.

The term "*gene*" is used herein to describe a discrete nucleic acid locus, unit or region within a genome that may comprise one or more of introns, exons, splice sites, open reading frames and 5' and/or 3' non-coding regulatory sequences such as a promoter and/or a polyadenylation sequence.

Therefore a person of skill in the art will readily appreciate that the invention contemplates a genetic construct which comprises one or more nucleotide sequences capable of directing synthesis of an RNA molecule, said nucleotide sequence selected from the list comprising:-

- (i) a nucleotide sequence transcribable to an RNA molecule comprising an RNA sequence which is substantially homologous to an RNA sequence encoded by a nucleotide sequence of interest;
- (ii) a reverse complement of the nucleotide sequence of (i);
- 5 (iii) a combination of the nucleotide sequences of (i) and (ii),
- (iv) multiple copies of nucleotide sequences of (i), (ii) or (iii), optionally separated by a spacer sequence;
- (v) a combination of the nucleotide sequences of (i) and (ii), wherein the nucleotide sequence of (ii) represents an inverted repeat of the nucleotide sequence of
- 10 (i), separated by a spacer sequence; and
- (vi) a combination as described in (v), wherein the spacer sequence comprises an intron sequence spliceable from said combination.

Where the nucleotide sequence comprises an inverted repeat separated by a non-intron spacer sequence, upon transcription, the presence of the non-intron spacer

15 sequence facilitates the formation of a stem-loop structure by virtue of the binding of the inverted repeat sequences to each other. The presence of the non-intron spacer sequence causes the transcribed RNA sequence (also referred to herein as a “transcript”) so formed to remain substantially in one piece, in a form that may be referred to herein as a “hairpin”. Alternatively, where the nucleotide sequence

20 comprises an inverted repeat wherein the spacer sequence comprises an intron sequence, upon transcription, the presence of intron/exon splice junction sequences on either side of the intron sequence facilitates the removal of what would otherwise form into a loop structure. The resulting transcript comprises a double-stranded RNA (dsRNA) molecule, optionally with overhanging 3' sequences at one or both ends.

25 Such a dsRNA transcript is referred to herein as a “perfect hairpin”. The RNA molecules may comprise a single hairpin or multiple hairpins including “bulges” of single-stranded DNA occurring in regions of double-stranded DNA sequences.

Depending upon the application, the RNA molecule may directed to a single target or alternatively, a plurality of targets.

30 In particular embodiments, the RNA molecule encodes at least one ErbB receptor selected from the group consisting of Her-1, Her-2, Her-3 and Her-4.

In other particular embodiments, the RNA molecule encodes a ligand to at least one ErbB receptor.

In particular embodiments, inhibitory RNA constructs include siRNA or shRNA construct that down-regulate expression of at least one ErbB receptor and
5 more preferably at least one ErbB receptor selected from group consisting of Her-1, Her-2, Her-3 and Her-4.

Non-limiting examples of suitable siRNA molecules, and in particular ErbB receptor-specific siRNA, according to the present invention are provided in Sergina et al Nature. 2007 445(7126):437-41 and Sithanandam et al Cancer Gene Ther. 2008
10 15(7):413-48.

The present invention also contemplates peptide mimetics as therapeutic agents, and more particularly antibody-like peptidomimetics such as described in Zhang et al (2007) *J. Clin. Invest.* 117:2051–2058 in relation to ErbB receptors, although without limitation thereto.

15 Particular embodiments contemplate use of small-molecule inhibitors which directly or indirectly inhibit one or a plurality of ErbB receptors. Such inhibitors may be reversible or irreversible. It is also envisaged that such inhibitors are selective against a particular ErbB receptor protein or in other embodiments, inhibit more than one ErbB receptor protein. A non-limiting example of a multi-target inhibitor is
20 BIBW-2992, which inhibits Her-1 and Her-2. Spector et al (2007) *Breast Cancer Research* 9:205 provides other examples of multi-target inhibitors. It is also appreciated that according to these embodiments that small-molecule inhibitors may inhibit a particular function of the at least one ErbB receptor and in preferred embodiments, is a tyrosine kinase inhibitor.

25 Non-limiting examples of a suitable small-molecule inhibitors includes derivatives of quinazolines and more particularly, 4-anilinoquinazoline-based inhibitors such as those inhibitors described in United States Patent No's 6391874 and 6713485 and International Publication No. WO/1999/035146. Reference is also made to
30 N-[3-chloro-4-[(3-fluorophenyl)methoxy]phenyl]-6-[5-[(2-methylsulfonyl)ethylamino)methyl]furan-2-yl]quinazolin-4-amine (otherwise known as "lapatinib" and sold under the name Tykerb® by GlaxoSmithKline; PubChem

Substance Compound ID: 208908) which is dual inhibitor of Her-1 and Her-2 and provides a further non-limiting example of a suitable small molecule inhibitor of ErbB inhibitors. Non-limiting examples of selective inhibitors include N-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine

5 (otherwise known as “gefitinib” which is sold under the name Iressa™ by Astra Zeneca; PubChem Substance Compound ID: 123631) and N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine (otherwise known as “erlotinib” which is sold under the name Tarceva® by Genentech; PubChem Substance Compound ID: 176870).

10 Non-limiting examples of suitable irreversible inhibitors include CI-1033, EKB-569 (PubChem Substance Compound ID: 6445562) and HKI-272 as provided in Zhang et al (2007) *J. Clin. Invest.* 117:2051–2058.

In preferred embodiments, a therapeutic agent of the invention is a small-molecule inhibitor of Her-2. Reference is made to Spector et al (2007) *Breast*
15 *Cancer Research* 9:205 which provides non-limiting examples of suitable small molecule Her-2 inhibitors and is incorporated herein by reference.

Reference is made to Table 5 which provides non-limiting examples of suitable ErbB inhibitors which may be applied or used according to preferred embodiments of the present invention.

20 In further aspects, the invention relates to methods of designing, engineering, screening or otherwise producing a therapeutic agent for treating a metastatic breast cancer derived from one or more metastatic breast cancer cells with normal expression levels of Her-2, which includes the step of identifying a candidate agent that is suitable for use in treatment of a metastatic breast cancer derived from one or
25 more breast cancer cells with normal expression levels of Her-2, by the presence of an elevated expression level of Her-3 in one or more metastatic breast cancer cells of the brain.

Accordingly, suitable therapeutic agents and/or candidate agents of the invention may be peptides, proteins such as antibodies or other organic molecules,
30 preferably small organic molecules, with a desired biological activity and half-life.

In preferred embodiments, a therapeutic agent for treating a metastatic breast

cancer derived from one or more metastatic breast cancer cells with normal expression levels of Her-2 designed, screened, engineered or other produced according to the present invention inhibits at least one ErbB receptor. More preferably, said therapeutic agent selectively inhibits at least one ErbB receptor.

5 Persons skilled in the art will be aware that the therapeutic agents of the invention may be identified by any number of methods.

 In one embodiment, suitable therapeutic agents may be identified by way of screening libraries of molecules such as synthetic chemical libraries, including combinatorial libraries, by methods such as described in Nestler & Liu, 1998, Comb. Chem. High Throughput Screen. **1** 113 and Kirkpatrick *et al.*, 1999, Comb. Chem. High Throughput Screen **2** 211.

 It is also contemplated that libraries of naturally-occurring molecules may be screened by methodology such as reviewed in Kolb, 1998, Prog. Drug. Res. **51** 185.

 Screening methods of the present invention also contemplate use of *in vitro* assays which mimic and/or replicate the natural biological environment of a metastatic breast cancer, in order to test the potential therapeutic efficacy of one or more candidate agents. Particularly preferred embodiments contemplate an *in vitro* assay which mimics a metastatic breast cancer located in the brain, wherein differentiated neural cells are co-cultured with a breast cancer cell line, with the result being a cell-based matrix derived from the co-culture neural cells and breast cancer cells. Differentiated neural cells may be derived from differentiation of neural progenitor cells (and preferably neural stem cells) such as, but not limited to ReNcell CX cell line (Chemicon International). Examples of suitable breast cancer cells for use in the aforementioned *in vitro* assays include SKBR-3 and basal-like breast cancer cell lines such as, but not limited to, MDA-MB-231.

 More rational approaches to designing therapeutic agents of the present invention may employ X-ray crystallography, NMR spectroscopy, computer assisted screening of structural databases, computer-assisted modelling, or more traditional biophysical techniques which detect molecular binding interactions, as are well known in the art.

 A review of structural bioinformatics approaches to drug discovery is

provided in Fauman *et al*, 2003, Meth. Biochem. Anal. **44**: 477.

Computer-assisted structural database searching and bioinformatic approaches are becoming increasingly utilized as a procedure for identifying and/or engineering agonists and antagonist molecules. Examples of database searching methods may be found in United States Patent No. 5,752,019 and International Publication WO 97/41526 (directed to identifying EPO mimetics) and United States Patents 7,158,891 and 5,680,331 which are directed to more general computational approaches to protein modelling and structural mimicry of protein activity.

Generally, other applicable methods include any of a variety of biophysical techniques which identify molecular interactions. Methods applicable to potentially useful techniques such as competitive radioligand binding assays, electrophysiology, analytical ultracentrifugation, microcalorimetry, surface plasmon resonance and optical biosensor-based methods are provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997) which is incorporated herein by reference.

A person skilled in the art will appreciate that suitable therapeutic agents may be in the form of a binding partner and as such, identified by interaction assays such as yeast two-hybrid approaches and the like, but without limitation thereto. Two-hybrid screening methods are provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997) which is incorporated herein by reference.

For the purpose of methods of treatment described herein, the present invention also contemplates particular embodiments where the therapeutic agents of the invention may be used in combination with other therapeutic agents such as chemotherapeutic agents including cytotoxic drugs (for example capecitabine, doxorubicin (including its liposomal formulation), fluorouracil, cisplatin, gemcitabine, the taxanes paclitaxel and docetaxel, and vinorelbine) and hormone therapies (such as endocrine therapies) for individuals that are estrogen or progesterone positive and in particular modulators of a hormone receptor such as estrogen and/or progesterone (eg: tamoxifen, luteinising hormone releasing hormone analogues and aromatase inhibitors).

In light of the foregoing, it will be appreciated that the invention provides a pharmaceutical composition comprising a therapeutic agent effective for treatment of a metastatic breast cancer, preferably located in the brain, derived from one or more breast cancer cells with normal expression levels of Her-2 designed, screened, engineered or otherwise produced according as hereinbefore described, together with
5 a pharmaceutically acceptable carrier, diluent, or excipient.

In other aspects, the invention provides a method of treating a metastatic breast cancer derived from one or more breast cancer cells with normal expression levels of Her-2 in a human including the step of administering to an individual with
10 an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain, a therapeutic agent effective for treatment of said metastatic breast cancer designed, screened, engineered or otherwise produced as hereinbefore described. According to these aspects, the metastatic breast cancer is located in the brain.

In light of the foregoing, it will be appreciated that preferably the therapeutic agents of the present invention may be in the form of a small organic molecule, peptide or the like formulated with a pharmaceutically-acceptable carrier, diluent or excipient suitable for oral administration, as a transdermal patch or other non-invasive route of administration.

By "*pharmaceutically-acceptable carrier, diluent or excipient*" is meant a
20 solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate
25 buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J.
30 USA, 1991) which is incorporated herein by reference.

Any safe route of administration may be employed for providing a patient

with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, intracranial, inhalational, intraocular, intraperitoneal, intracerebroventricular and transdermal administration may be employed.

5 Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be
10 effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

15 Pharmaceutical compositions of the present invention suitable for oral or parenteral administration may be presented as discrete units such as capsules, sachets or tablets each containing a pre-determined amount of one or more therapeutic agents of the invention, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil
20 liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the agents of the invention with liquid carriers or finely divided solid
25 carriers or both, and then, if necessary, shaping the product into the desired presentation.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as is pharmaceutically-effective. The dose administered to a patient, in the context of the present invention, should be
30 sufficient to effect a beneficial response in a patient over an appropriate period of time. The quantity of agent(s) to be administered may depend on the subject to be

treated inclusive of the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

So that the invention may be readily understood and put into practical effect, the following non-limiting Examples are provided

5

EXAMPLES

EXAMPLE 1

Metastatic breast cancer to the brain: implications of the local microenvironment for colonization and treatment

10 In the present study, the present inventors used a set of 29 matched pairs of human primary breast cancer and brain metastases, 22 unmatched brain metastases of breast cancer and a second set for validation comprised of 10 matched pairs of primary breast cancer and brain metastases and 11 non-breast metastatic brain cancers to explore the metastatic breast cancer colonization of the brain. A combination of morphology, immunohistochemistry, differential gene expression, co-
15 culture of human neural cells and breast cancer cell lines and in vivo brain injections of tumour cells in mice (treated with trastuzumab and neutralizing anti-neuregulin antibody) was used in order to elucidate possible mechanisms that could enhance the colonization of the brain parenchyma by tumour cells.

Herein, the present inventors provide *in vitro* and *in vivo* evidence that
20 suggests that the inhibition of HER family receptors or the neutralization of local secreted growth factors like neuregulin could play a role in preventing tumour progression and colonization in the brain microenvironment and also may shed light on the paradigm that basal-like tumours, for not overexpressing HER2, cannot have response to trastuzumab.

25 Material and Methods:

Clinical samples:

Formalin fixed-paraffin embedded (FFPE) tumour blocks were retrieved from the archives of the Pathology Queensland: The Royal Brisbane & Women's Hospital, Brisbane, Australia - Medical Faculty of Charles University in Plzen, Czech Republic
30 - Instituto Nacional do Câncer, Brazil - Laboratório Salomão e Zoppi, Brazil and Institute of Clinical Pathology and Medical Research, Westmead Hospital, University

of Sydney, Australia. An initial set of 29 matched pairs of primary breast cancer and their brain metastases and 22 unmatched brain metastases of breast cancer was available. A second set of samples comprised of 10 matched pairs of primary breast cancer and their brain metastases and 11 non-breast metastatic brain cancers was also available. Clinical data regarding the timeframe from the day of the primary diagnosis to the day of the removal of the brain metastases and patient age was obtained for the initial set of samples. The study was approved by the local research ethics committees. Haematoxylin & eosin sections were reviewed by two pathologists (LDS, SRL) to confirm diagnosis, assess morphology and grade the tumours according to the World Health Organization criteria (48). For the initial set of samples two tissue arrays containing replicates of primary female breast cancer and their metastases to the brain were built. Both tissue microarrays were comprised of 51 cores of metastases from female breast cancer to the brain and 29 cores of primary female breast cancer. Haematoxylin and eosin slides were dotted by one pathologist in order to take cores to the recipient block. Both arrays were built using the tissue arrayer, model MTAI (Beecher Instruments, Inc, Sun Prairie, WI 53590 USA). The tissue cores had 0,6 mm in diameter and were 1,0mm away from each other. For the second set of samples whole sections were used for haematoxylin & eosin staining. Another TMA was built for the second set of samples following the same descriptions as for the initial set.

Immunohistochemistry and Chromogenic in Situ hybridization:

For the immunohistochemistry assay, sections of the paraffin blocks of the tissue arrays and whole sections of the second set of samples were cut to generate 4µm sections that were mounted on silane-coated slides. Immunohistochemistry was performed using the Envision® dual link system (Dakocytomation, Denmark) according to the manufacturer's recommendations. Table 1 shows all antibodies used, along with the antibody clone and dilution. Antigenic retrieval for all antibodies (except smooth muscle actin (SMA) which did not require any antigen retrieval) required two minutes pressure cooking (105°C) in EDTA (pH8.0) buffer. Positive and negative controls were included in all runs and all slides were analysed by at least two pathologists (LDS, PP or SRL) under a double headed optical light

microscope. Cellular localization, intensity and percentage/number of positive and negative tumours for each antibody were recorded on primary tumours and brain metastases. Intensity was based on a three tier-system – weak, moderate, strong. Samples were considered positive if more than 10% of cells were stained. HER2 staining and scoring was according to the Herceptest® (Dakocytomation, Denmark) protocol. Chromogenic in situ hybridization was performed following instructions of the Zymed Spot-Light®HER2 CISH™ Kit (Zymed, California, USA) on the first set of samples. Briefly, the paraffin embedded sections were dewaxed and subjected to heat and enzyme digestion treatments followed by denaturation and hybridization to labelled nucleic acid probes. Immunodetection was performed and 3, 3'-Diaminobenzidine tetrachloride was used for visualization. Slides were counterstained with haematoxylin. One slide with a HER2 amplified – formalin fixed cell line was included as a positive control. Signals were counted under a brightfield microscope and results were classified into diploid, polysomy, low amplification and high amplification according to the manufacture's recommendation.

Gene expression profiling:

Gene expression profiling was performed using the DASL assay (cDNA-mediated annealing, selection extension and ligation, Illumina Inc., California, USA) interrogating the DASL Cancer Panel which contained 512 cancer related genes (49, 50). The above-mentioned first and second sets of samples were analyzed in two separated experiments. The RNA derived from the animal experiment was analyzed in conjunction with the second set of samples. All protocols were as specified by Illumina Inc. Briefly, RNA (250ng) was converted to cDNA through a reverse transcription reaction with biotinylated oligo-d(T)₁₈ and random nonamers. Oligonucleotides (three unique pairs for each of the 512 genes) were annealed to the biotinylated cDNA, the duplexes were bound to streptavidin conjugated paramagnetic particles to remove non-hybridized oligos. The annealed oligos were then extended and ligated (to incorporate an address sequence and primer site) to generate amplifiable products. These products were subjected to PCR amplification using fluorescently labeled (Cy3 and Cy5) primers. The labeled PCR products were hybridized to a Sentrix array or Beadchip. Following hybridization, the arrays were

scanned with a BeadArray Reader (Illumina) and data was extracted using BeadStudio software (Illumina). Samples exhibiting a median hybridization intensity across all probes of <600 (background corrected) were excluded from analysis. Probes with a BeadStudio detection score of <0.99 were also excluded. Array data transformation and normalization (per chip normalized to 50th percentile and per gene normalized to median) was done in Genespring® version 7.0 software (Agilent Technologies, Santa Clara, USA). Hierarchical clustering was performed using: Similarity measure; Pearson Correlation and clustering algorithm; Average Linkage. Genes with no data in half the starting conditions were discarded. Genes differentially expressed between primary tumours and brain metastases were identified using analysis of variance (ANOVA, $p < 0.01$) or parametric test, variances not assumed equal (Welch t-test, $p < 0.005$). Up and down regulation was considered for normalized data values that were greater or less than a factor of 2 fold for the conditions compared.

15 **RNA extraction and Real-Time RT-PCR**

Three 5µm thick FFPE sections of clinical samples were cut and de-waxed in two lots of xylene and two lots of 100% ethanol, 10 minutes each. RNA was extracted using the High Pure RNA Paraffin Kit® (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. RNA was quantified using the NanoDrop® ND-1000 (NanoDrop Technologies, DE, USA). Relative expression levels of *HER3*, *HER2*, *EGFR*, *HER4*, *HIF1-alfa* and *CCNH* was assessed using RT-PCR. Complementary DNA (cDNA) was synthesized from 240ng of total RNA using random hexamers and the SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carsbald, CA, USA). TaqMan One-Step UniversalMaster Mix (Applied Biosystems) was used for all reactions. TaqMan reaction was done in a standard 96-well plate format with ABI 7500 OneStepPlus PCR system. For data analysis, raw deltaCt (dCt) was first normalized to an endogenous control gene (RPL13a) for each sample to generate normalized dCt. The normalized dCt was then calibrated to a cell line pool reference (MCF-7, SKBR-3 and MDA-MB-231) to generate a ddCt. In the final step of data analysis, the ddCt was converted to fold change (2^{-ddCt}) relative to the reference allowing comparison between samples.

Cell lines, co-culture and flow cytometry assays:

MCF-7, MDA-MB-231 and SKBR-3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, and 1% penicillin G-streptomycin solution. ReNcell CX (CHEMICON International, Inc. Catalogue SCC007) is an immortalized human neural progenitor cell line with the ability to differentiate into neurons and glial cells. Resuspended ReNcells were allowed to grow in a total volume of 5 mL of serum-free DMEM/F12 (NSA) medium containing freshly added 20 ng/ml rhEGF (R&D Systems), 10 ng/ml rhFGF (R&D Systems), 4 ug/ml heparin, 10% proliferation supplement (NeuroCult®, Stem Cell Technologies Inc.), 2% BSA (Sigma). After formation of neurospheres, the cells were differentiated into neurons and glial cells in order to create a neural cell layer following the replacement of the NSA media by DMEM/F12 with 10% FBS, 2 mmol/L glutamine, and 1% penicillin G-streptomycin solution. Co-culture was established after plating in 24 wells dishes 15,000 cells in triplicates of the MCF-7 and SKBR-3 cancer cell lines with 10,000 differentiated human neural cells. Triplicates of the cell lines without neural cells were cultured for comparison of the growth and the co-culture was also performed in the presence of trastuzumab 20ng/μl, lapatinib 5nmol/mL, anti-neuregulin antibody 50μg/mL and a combination of trastuzumab plus lapatinib. A growth curve was generated after counting the cells in a flow cytometer. The cells were washed once with phosphate-buffered saline (PBS) and then harvested with 0.05% trypsin/0.025% EDTA. Detached cells were washed with PBS. Combinations of fluorochrome-conjugated monoclonal antibodies against human CD56 and Epcam (BD Biosciences, San Diego, CA, USA) were used to distinguish between neural and epithelial cells, were added to the cell suspension at concentrations recommended by the manufacturer and incubated at 4°C in the dark for 30 minutes. The labeled cells were washed with FACS buffer (PBS supplemented with 1%BSA) and then analyzed on a LSRII (BD Biosciences). Comparisons between cell lines growth curves were done using one way ANOVA. This experiment was repeated twice.

Soft agar colonization assay

Two hundred thousand MDA-MB-231 cells were cultured in a six well plate

containing 1 ml of RPMI-10% FBS, with the addition of agarose (final 0.5% ,w/v), over a 1 ml layer of bottom agar (final 0.35%, w/v). Cells were cultured with the addition of trastuzumab (20ng/ μ l), recombinant beta-1-neuregulin (50ng/ml) and combination of recombinant neuregulin and trastuzumab. Cells without the addition of trasztuzumab and neuregulin were used for control. Trastuzumab and neuregulin were replenished every four days. Three replicates per combination were used. After 20 days in culture, colonies were counted and measured using a CKX41 Olympus inverted microscope. Images were captured using QCapture Pro 6.0 (Qimaging, Canada). Results are representative of three independent wells. A total of 30 colonies (only size bigger than 10 μ m were counted) were measured per well and the average number of colonies was derived from twenty 0,196 mm² random optical fields per well. One way ANOVA was used to determine statistical significance between experiments.

Intracerebral injections of breast cancer cell line in mice and drug treatment:

Severe combined immunodeficiency (SCID)-beige female mice (6 weeks old) were obtained from a colony housed by the University of Queensland Animal House and were maintained according to the University of Queensland Animal House guidelines. The research complied with national legislation and was authorized by the local animal ethical committees. The animals were divided into four groups; neuregulin-group which had anti-neuregulin antibody mixed with the cells at the time of injection, trastuzumab-group which was treated with intraperitoneal trastuzumab on the day of the injection of the breast cancer cells in the brain, one control group not treated and a fourth group designed to be treated once tumour was detected. Each group was comprised of three animals. The mice were inoculated directly in the right brain hemisphere sub ventricular zone with 200,000 MB-MDA-231 cells. The concentrations of the anti-neuregulin antibody and trastuzumab were 50 μ g/mL and 30mg/Kg respectively. Mice were weighted before injections. Statistical significance of the differences across all four groups was analyzed by t-Student test. Cells were allowed to grow in the brain for approximately 10 weeks. At this point, the mice in the neuregulin, trastuzumab and controls group were sacrificed and the treatment of the fourth group started.

Magnetic resonance imaging of the mice and measurement of the tumours:

The tumour progression was followed up using MRI at weeks 4, 6 and 10. MRI scans were performed using Bruker 16.4T vertical wide-bore scanner, equipped with a micro-mouse AHS probe and a head coil (20 mm SAW linear coil, M2M imaging). The animal was kept under anesthesia with isoflurane (1% at flow rate of 0.5-0.7L/min), its respiration rate was monitored using the BIOTRIG, and the animal body temperature is maintained at 30 degree Celsius. The animal heads are scanned at 78 x 78 micron in-plane resolution and 1 mm slice thickness. The differentiation of the tumour and normal tissues were achieved using 2D T1-multislice spin echo sequence with TR/TE=600/9 ms, NEX = 8; and 2D diffusion-weighted imaging with TR/TE = 2400/21.6 ms, diffusion encoding was applied in the read direction, with the parameters $d/D = 2/14\text{ms}$, and $b = 1200 \text{ s/mm}^2$, with 50 kHz bandwidth. Images were processed and the tumour sizes were measured by manual ROI segmentation using the software Paravision 4.0. Final sizes were reconfirmed in the post-mortem examination when haematoxinilin & eosin stained sections were cut at 4 μm and scanned using a scanscope® (Aperio Technologies, Vista, CA, USA). Sizes were achieved after analysing the images with Image scope (Aperio Technologies, Vista, CA, USA).

Results**20 Clinical features and morphological-immunohistochemical profiling of primary and metastatic breast cancer to the brain:**

Clinical data regarding age and time of relapse of the disease in the brain was available for 29 matched pairs of primaries and brain metastases. The median age was 48.5 years old at the initial diagnosis and the median time for the development of the brain metastasis was 3.5 years. All but one of the initial set of 29 matched pairs of primary breast cancer and their brain metastases and 22 unmatched brain metastases of breast cancer were grade III invasive ductal carcinomas not-otherwise-specified (48). The one which was not grade III was a mucinous carcinoma. In the second set of samples, 10 matched pairs of primary breast cancer and their brain metastases were also grade III invasive ductal carcinomas not-otherwise-specified and the 11 non-breast metastatic brain cancers were comprised of metastases from

one melanoma, six lung adenocarcinomas, one prostate carcinoma and two kidney carcinomas. Table 2 shows the summary of the results for all of the antibodies applied. Noteworthy, 68% of the metastases and 61% of the primary tumours of the initial set of samples displayed positivity for at least one of the basal-like markers (CK14, CK5/6, p63, S100 and SMA) confirming an enrichment of metastatic breast cancer to brain by tumours with the basal-like phenotype. 37% and 30% of the matched pairs brain metastases showed positivity for ER and PR respectively. The unmatched brain metastasis had 15% of positivity for both receptors. Regarding HER2 status, 24% of the primaries and metastases demonstrated overexpression of the protein and the same percentage of amplification of the gene was confirmed by CISH. Interestingly, the percentage of CD44 positive tumours was higher amongst the metastases (56%) in comparison to the primaries (30%). Six matched brain metastases and 3 unmatched brain metastases displayed positivity for EGFR, in contrast to the non-brain metastases where 9 out of 11 tumours were positive. Figure 1 shows an example of a high grade tumour, positive for CK 14 and the matched pair immunohistochemistry staining for CD44.

Gene expression profiling of human primary and metastatic breast cancer to the brain

The availability of good quality RNA and stringent filtering of the DASL data yielded gene expression profiling data on 37/61 brain metastases from breast cancer (15/39 from matched pairs and 22/22 from unmatched metastases) and 15 matched primaries. Unsupervised analysis highlighted a strong similarity between primary tumors and their matched metastases (Figure 2A). Only 20 genes were, differentially expressed genes between the matched primaries and metastases (Figure 2B). This may be a consequence of the overall strong similarity between primaries and metastases coupled with the sample size (n=30) and number of genes analyzed (n=512 cancer genes in DASL panel). The comparison between primaries and all metastases (matched and unmatched) identified 27 statistically significant differentially expressed genes. All twenty genes identified in the matched pair analysis were part of this 27-gene set. Among this 20-gene set, were *HER3* and one of its downstream target molecules *GRB2*, hypoxia related molecule *HIF1-alfa*,

MAPKinase cascade related protein *CREBBP*, cell cycle regulator RB1 and proliferation related genes *CCNH*, *CDK7* and *CDC25B*. Since the brain is rich in neuregulin 1 and this is a ligand for HER3, we hypothesized that the neuregulin-HER3 activation was important in allowing breast cancer cells to colonize the brain.

5 **HER family receptors and downstream molecules expression**

HER3, *EGFR*, *HER2*, *HER4* and *HIF1-alfa* expression was assessed using quantitative RT-PCR in 12 matched breast/brain samples for which DASL data and RNA were available. Similar to the DASL data, ten cases showed increased fold change by RT-PCR of *HER3* gene expression relative to their matched primaries ranging from 1.12 to 5.8 and with an average of 2.4. Immunohistochemistry for HER3 was similar, showing positivity in 11/37 (29.7%) of the primary tumors, 22/37 (59%) of the matched metastases and 13/21 (62%) of the unmatched brain metastases. In agreement, phosphorylated HER3 confirmed more frequent activation in the brain metastases, with positivity in 15/37 (40%) of the primary tumors, 24/37 (64%) of the matched metastases and 18/21 (85%) of the unmatched brain metastases.

Immunohistochemistry for GRB2, HIF1-alfa (p value check , remove if not significant) and phosphorylated ERK1/2, JNK1/2, ERK5 and p38 also demonstrated increased activation in the metastases compared to the primary tumors. In contrast, phosphorylated AKT was equally high in both the primaries and metastases. Interestingly, the non-breast derived brain metastases showed similarly high activation of the MAPK pathway together with over-expression (3+ stain) of EGFR (in 9/11 (81%) metastases (a prostate and one colon carcinoma did not) but in the absence of HER3 activation.

25 **Co-culture of MCF-7 and SKBR-3 cell lines and human differentiated neural cells**

In order to investigate if the neuronal cells would enhance the growth of breast cancer cell lines, we co-cultured MCF-7 and SKBR-3 cell lines with ReNcell CX (CHEMICON International, Inc. Catalogue SCC007). ReNcell CX cell line was grown as neurosphere and differentiated into adult type neuronal cells. The cells were co-cultured and the number of cell was counted using flow cytometry and antibodies

to discriminate between epithelial phenotype (EpCam) and neuronal phenotype (CD56) every 24 hours for four days. The total number of cells in the wells was generated in triplicates so that a growth curve was created. The results revealed an enhancement of growth in the group of cells which was co-cultured when compared to the group where neuronal cells were not added to the plate. In parallel, the same co-culture experiment was performed with addition of trastuzumab and a neutralizing anti-neuregulin antibody to the cell culture media. This aimed to test if neuregulin could be playing a role in the growth enhancement. The results showed that both the trastuzumab and the anti-neuregulin antibody reduced the rate of growth of these cell lines. However, resistance to the trastuzumab was seen after day 3 of the cell culture and surprisingly the same pattern of growth was observed for the anti-neuregulin antibody. As described previously, resistance could be due to activation of other member of the HER family of receptors. Therefore, the experiment was repeated with addition of lapatinib plus trastuzumab and lapatinib and anti-neuregulin antibody to another group two groups of co-culture cells. This overcame the resistance found on day 3. Figure 5 shows the growth curves summary for this experiment.

Soft agar colonization assay

The colony forming assay demonstrated that the average number of colonies was higher among the control group compared to the group treated with trastuzumab ($p = 0.049$). When compared to the group of cells treated with trastuzumab and neuregulin, the number of colonies in the control group was still higher although not statistically significant ($p = 0.275$). The use neuregulin showed enhancement of cell growth in the group treated with neuregulin only but this did not reach statistical significance ($p = 0.184$) when compared to the control group. In terms of size, the group of cells treated with neuregulin had an average colony size of $29.8 \mu\text{m}$, the control $27.3 \mu\text{m}$, the trastuzumab group $21.3 \mu\text{m}$ and the neuregulin + trastuzumab $18.7 \mu\text{m}$. The summary of results is shown in Figures 7 and 8.

Intracerebral injections of a basal-like breast cancer cell line (MDA-MB-231) and treatment with trastuzumab and anti-neuregulin antibody and differential gene expression

The present inventors aimed to investigate if trastuzumab would have any

effect on the cells in the brain microenvironment and also if the blocking of the local factor growth neuregulin with an anti-neuregulin antibody would interfere in the rate of growth of the injected cells. Four groups of mice, each containing 3 animals, were used. Remarkably, the groups of mice that were treated on the day of injection with trastuzumab and anti-neuregulin antibody had smaller average tumours sizes compared to the control group ($p < 0.01$). The difference in size for the control group and the group of mice treated after detection of the tumour masses by MRI was not statistically significant. The tumour sizes are available in Table 3. Differential gene expression by ANOVA of the parental cells and the cells injected in the brain of the mice revealed several genes expressed (Table 4). Interestingly, HER3 and CD44 were some of such genes. RT-PCR confirmed higher levels of HER3 compared to the parental cells and immunohistochemistry showed higher percentage of positive cell among the cells injected in the brain compared to the parental. Immunohistochemistry also revealed higher percentage of CD44 positive cells among the injected cells compared to the parental. Figure 6 illustrates MRI images at week 10, patterns of expression of CD44 and HER3 in the parental cell line and in the cells that colonized the brain as well as the RT-PCR for HER3 in the parental cell line and the injected cells.

Discussion

Breast cancer is one of the most common cancers and a leading cause of mortality in women (1, 2). Because there are few long-term survivors among patients with systemic metastatic breast cancer, the main objectives of therapy are to increase the time lapse before progressive disease is established and to improve symptom relief without increasing toxicity and comprising quality of life. As systemic therapy of metastatic breast cancer improves, central nervous system involvement is becoming a more widespread problem. (5) It is predicted that brain metastases will become increasingly prevalent as greater control over systemic metastases is achieved, particularly with regard to HER2-positive tumours (6). Brain metastases from breast cancer may become a serious problem that oncologists may encounter frequently (10) and it is considered one of the most feared complications of cancer because even small tumours may cause incapacitating neurologic symptoms (4). Over

the last five decades, autopsies series have reported brain metastases in 6,7%-36% of selected patients (57, 58).

In the present study, the median age of presentation of the brain metastases in the group of patients where follow-up data was available was 48.5 years old, most of the tumours showed negativity for estrogens and progesterone receptors and around 23% showed overexpression of HER2, confirmed by CISH. The percentage of HER2 overexpression tumours was in accordance with previous studies (25, 26). We assessed the expression of basal-like tumour markers such as CK14, CK17, CK5/6, SMA and p63 and found that 68% of the metastases and 61% of the primary tumours of the initial set had expression of at least one of these markers. This is consistent with other studies where patients with expression of basal-like and negativity for estrogens and progesterone receptors had a trend to develop brain metastases (8, 16-18, 59).

Interestingly, the percentage of CD44⁺CD24⁻ positive tumours was higher among the brain metastases compared to the primary tumours. This is noteworthy because CD44⁺CD24⁻ cells have been shown to express higher levels of proinvasive genes and have highly invasive properties (29). In addition, this phenotype is also believed to enrich for cells with stem cell properties (27). Hyaluronic acid stimulates CD44s-associated p185 (HER2) tyrosine kinase activity, leading to an increase in the ovarian carcinoma cell growth (35). The brain matrix and is rich in hyaluronan (60). Interaction between CD44 and hyaluronan has been shown to provide resistance to shear under physiologic conditions and to support the initial steps of lymphocyte extravasation (34). Hence, it is logical to hypothesize that CD44 could be implicated in the homing and local facilitation of growth of breast cancer cell in the brain.

The HER/erbb family of tyrosine kinase receptors consists of four members: HER1, HER2, HER3, and HER4 (38). On one hand HER1, HER2, and HER3 have been shown to contribute to aggressive tumour formation (61), on the other hand HER4 signaling may impair cellular proliferation of human breast cells and promote differentiation (62). The differential gene expression between the primary tumours and the brain metastases showed that, in the first set of samples, HER3 was differentially expressed with statistical significance and in the second set a twofold

up-regulation was detected. This differential expression was confirmed by RT-PCR and immunohistochemistry. Interestingly, the samples stained with antibody against phosphorylated HER3 showed nuclear positivity. ErbB receptors have been reported to translocate to the nucleus. HER1, HER2, HER3 and HER4, may translocate to the nucleus as full-length receptors (HER1-3) or as a proteolytically derived intracellular domain (HER4) (52-55, 63).

In the brain microenvironment, induced HER2 overexpression has been shown to enhance the outgrowth of a specialized brain-seeking breast cancer cell line in animal model (65). In addition, reactive glia was shown to enhance growth of this same cell line (66). Furthermore, the 231-Br1 cell line, which was derived from a brain metastases in a nude mouse, showed increased adhesion to astrocytes and enhanced growth in vitro in the presence of an astrocyte conditioned media when compared to the parental MDA-MB-231 and the lung metastasis-derived variant 231-Lung2 (67). The ligand for HER3 is neuregulin-1, a transmembrane epidermal growth factor-like molecule which is abundant in the brain microenvironment (44, 64). We aimed to test if the growth of the of breast cancer cell lines (MCF-7 and SKBR-3) would be enhanced when co-cultured with adult neural cells and to verify what growth response would be seen when trastuzumab, lapatinib and a neutralizing anti-neuregulin antibody were added to the cell co-culture media. The cells were counted using flow cytometry and the results demonstrated an enhancement of growth compared to the breast cancer cell lines which were not co-cultured. In keeping with what has been shown before, trastuzumab and lapatinib reduced the growth rate but drug resistance was seen from day 3 to 4 (42, 68). The same result was seen when the neutralizing anti-neuregulin antibody was added suggesting that the neuregulin of the neural cells could also be playing a role in the growth enhancement. The combination of trastuzumab and lapatinib overcame the resistance from day 3 to 4, suggesting that the resistance could be via activation of the other members of the HER family of receptors as indicated previously (42, 43). The mechanisms of action of lapatinib and trastuzumab are different and their combination was shown to lead to synergism (69). The mechanism of action of trastuzumab is distinct from tyrosine kinase inhibitors such as gefitinib or erlotinib,

which bind competitively to the intracellular adenosine triphosphate binding site of erbb receptors (70, 71) and which is the same site of action for lapatinib. (72)

Despite the fact that the MDA-MB-231 cell line has low levels of HER2 and HER3, the treatment of the mice with trastuzumab and anti-neuregulin antibody
5 resulted in smaller tumours when compared to the control group. This may reflect a potential for some tumours with low expression of HER2 to be responsive to trastuzumab and also suggests that the brain local environment may be playing a role in the enhancement of growth in vivo. The colony forming assay supports this view since less and smaller colonies were detected in the group treated with trasztuzumab
10 compared to the control group and the group treated with neuregulin. These values were statistical significant. Furthermore, the combination of neuregulin and trasztuzumab resulted in a difference not statistical significant when compared to the control group, suggesting that neuregulin was interfering in the action of trastuzumab on the cells.

15 Recently, it has been shown that subgroups of HER2 negative patients can benefit of the treatment with trastuzumab. One of these studies was a retrospective analysis of the phase III Cancer and Leukemia Group B (CALGB) 9840 trial and it demonstrated that HER2-negative metastatic breast cancer patients with multiple copies of the chromosome carrying HER2 had significantly better response rates
20 (63% vs. 26%, $P = .048$) when they were treated with trastuzumab in addition to paclitaxel (73). The second study showed that a small group of HER2-negative patients in the phase III National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31 trial had significantly better disease-free survival with a relative risk of 0.40 ($P = .026$) when given trastuzumab after completing treatment for early breast
25 cancer (74). Furthermore, another study suggested that the spectrum of patients who may benefit from trastuzumab-based therapies could be expanded to include patients with metastatic breast cancer without HER-2 amplification but who express transmembrane neuregulin, the ligand of HER3 (75). Pertuzumab, is a recombinant humanized monoclonal antibody and binds to extracellular domain II of the HER-2
30 receptor and blocks its ability to dimerize with other HER receptors. The pertuzumab binding site within domain II does not overlap with the epitope on HER-2 (76) that is

recognized by trastuzumab. Hence, this monoclonal antibody could play a major role in cases where a tumour is also dependent on other members of the HER family of receptors.

5 In conclusion, the present study provides evidence that local factor neuregulin may enhance tumour growth in the brain microenvironment whether in vitro or in vivo and shed some light on the paradigm that HER2 negative cannot have response to trastuzumab. We suggest that there might be cohorts of HER-negative patients that may benefit of trastuzumab or combinations of HER family receptors inhibitors. Specially, in the cases where tumours are dependent on members of the erbB family
10 receptors that are fueled by local factors, autocrine loops or even autophosphorylated.

Example 2

We have already replicated the 3 members of the HER-family of receptors in plasmids (pLHCX-EGFR; hygromycin resistant, pLPCX-HER2; puromycin resistant and pLNCX2-HER3; G418 resistant). The transduction will be performed using an
15 amphotropic producer cell line such as Phoenix amphotropic cells. Within 72 hours the medium will be collected filtered and used for infecting the breast cancer cells. Luminal and basal-like breast cancer cell lines (with and without the insertion of the HER-family of receptor genes) will be co-cultured with adult CNS cells in order to mimicry an artificial brain matrix. We will utilize 96-well plates that can be analysed
20 by the BD FACSArray™ bioanalyzer from BD Biosciences. The epithelial luminal and basal-like breast cancer cell lines will be place onto a matrix of adult CNS cells. The epithelial and CNS cells will be differentially labelled with epithelial and neuronal florescent antibodies so that number of viable cells can be assessed by this device. Human fibroblasts and cells which are not in co-culture will be used as
25 controls. Growth curve for each experiment will be then generated and the influence of the co-culture conditions analysed. We are currently using EpCAM and CD56 for differential fluorescent labelling the epithelial and neuronal cells respectively. A baseline characterization of the expression of these two markers in the chosen cell lines will necessary as to whether they are reliable in distinguishing the epithelial and
30 neuronal cells. This will be done using equal number of both types of cells in the flow cytometer with an expectation of obtaining even figures at the end.

Neurosphere assay²⁶ is a serum-free culture system whereby the isolation and propagation of CNS-derived stem cells is feasible. Adult precursors are dissociated and plated in a liquid growth medium that contains the stem-cell mitogens epidermal growth factor and/or fibroblast growth factor. Because of the lack of serum and the low plating density, most cells die, except those that divide in response to the stem-cell mitogens. The growth-factor responsive cells proliferate to form floating clusters of cells that are referred to as neurospheres. These can be further dissociated into a single-cell suspension and then re-plated in fresh medium to produce secondary neurospheres. After removal of mitogenic factors, the progeny of the proliferating precursors can be differentiated into neurons, astrocytes and oligodendrocytes, which are the three primary cell types that are found in the adult human CNS. This will be our source of adult CNS cells to create the artificial brain matrix.

Concurrently, the addition of modulators of the HER-family of receptor into the 96-well plates will be performed. These modulators will comprise drugs currently used in clinical practice to target the HER-family of receptor, for example trastuzumab and lapatinib but not limited thereto. Non-limiting examples of other drugs currently used in clinical practice to target the HER-family of receptor are given in this text, particularly at Table 5. The wells will have quadruplicates samples and the statistical difference between the growth curves will be established using one-way Analysis of variance. The growth curves will use time points at 12, 36, 60 and 84 hours and the drugs will be titrated.

The selective and robust effect of RNA interference (RNAi) on gene expression makes it a valuable research tool, both in cell culture and in living organisms because synthetic dsRNA introduced into cells can induce suppression of specific genes of interest. So, the above mentioned experiment will be also performed with the use of RNAi targeting the HER-family of receptor and also using an antibody targeting the local growth factor neuregulin 1, the ligand of HER3 (the adult CNS cells express this molecule). RNAi targeting HER-family of receptors and scrambles for negative controls are available for purchasing from several companies. We expect this second experimental phase to mirror at some extend the findings of the experimental phase where drugs were used.

The BD FACSAArray™ bioanalyzer from BD Biosciences is capable fast and sensitive high-content measurements of cells and proteins in cell biology, supporting analysis of apoptosis, cytokine and chemokine profiling, and phosphorylation of key signal transduction proteins. This flow cytometer rapidly detects and quantifies concentrations of secreted proteins, proteins in cell lysates, and cell-associated proteins using small sample volumes directly from a 96-well microtiter plate. This device will be used for the above mentioned experiments.

The same co-culture system will also be used to assess activation of molecules which are downstream of the HER-family of receptors. The molecules targeted will be the ones assessed in AIM 1(RAS, SOS, RAF1, ERK1/2, MEK1 and ELK-1 for the MAPK pathway and p21, p27, MDM2, BAD, CASP9 and mTOR for the AKT pathway). The BD FACSAArray™ bioanalyzer will again be used. We expect that the results under non-co-culture conditions should mirror the results of the baseline assessment with western blots across the cell lines. This experimental phase will also show the changes the will occur in the AKT and MAPK related molecules under co-culture conditions, allowing a better understanding of the behaviour of breast cancer cells in a brain environment. In addition the assessment of apoptotic indexes can also be generated by the BD FACSAArray™ bioanalyzer using markers such as annexine and caspases.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.

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TABLES**Table 1 - Details of Antibodies Used in Immunohistochemistry**

Antibody	Company	Clone - Dilution
PR	Novocastra	1A6 - 1:500
ER	Novocastra	6F11 - 1:100
HER2	DAKO	Herceptest
p63	DAKO	4A4 - 1:400
SMA	DAKO	1A4 - 1:100
p53	DAKO	D07- 1:100
CD44	DAKO	DF1485 - 1:100
CD24	Serotec	SN3 - 1:200
CK5/6	DAKO	D5/16B4 - 1:50
CK14	Neomarkers	LL002 - 1:50
E-cadherin	Zymed	HECD-1 1:20
CK19	DAKO	RCK108 – 1:25
CK7	DAKO	OV-TL12/30 – 1:500
CK17	DAKO	E3 -1:100
CK8/18	Novocastra	5D3- 1:100
Phospho HER3	Cell Signalling	Tyr ¹²⁸⁹ (21D3) -1:300
Phospho AKT	Invitrogen	pT ³⁰⁸ -1:300
Phospho ERK1/ERK2	Invitrogen	pTpY ^{185/187} -1:300
Phospho JNK1/2	Invitrogen	pTpY ^{185/187} -1:300
Phospho ERK5	Invitrogen	pTpY ^{185/187} -1:300
p38	Invitrogen	pTpY ^{185/187} -1:300

Table 2 - Antibodies positivity across the samples

Antibody	Primary breast tumour N/T (%)	Matched brain metastases N/T (%)	Unmatched brain metastases N/T (%)	Non-breast brain metastases N/T (%)
PR	9/27 (30%)	9/27 (30%)	5/21 (23%)	NP
ER	10/27 (37%)	10/27 (37%)	4/21 (19%)	NP
HER2	6/25 (24%)	6/25 (24%)	5/24 (20%)	NP
p63	0/26	0/26	1/15 (5%)	NP
SMA	6/26 (23%)	6/26 (23%)	2/15 (13%)	NP
p53	16/26 (61%)	19/26 (73%)	5/15 (33%)	NP
CD44	5/20 (25%)	13/20 (65%)	10/18 (55%)	NP
CD24	5/22 (22%)	2/22 (9%)	3/22 (14%)	NP
CK5/6	9/26 (34%)	9/26 (34%)	2/16 (12%)	NP
CK14	12/26 (46%)	12/26 (46%)	8/16 (50%)	NP
CK17	5/26 (19%)	5/26 (19%)	5/16 (30%)	NP
E-cadherin	19/26 (73%)	19/23 (82%)	18/18 (100%)	NP
CK19	24/26 (92%)	24/26 (92%)	15/15 (100%)	NP
CK8/18	14/26 (53%)	14/26 (53%)	15/15 (100%)	NP
Phospho HER3	9/27 (30%)	16/27 (59%)	18/21(85%)	0/9
Phospho AKT	23/27 (85%)	23/27 (85%)	15/21 (71%)	9/9 (100%)
Phospho ERK1/2	18/27 (66%)	26/27 (97%)	20/21(95%)	9/9 (100%)
Phospho JNK1/2	17/27 (66%)	24/27 (88%)	19/21 (90%)	9/9 (100%)
Phospho ERK5	19/27 (71%)	26/27 (96%)	20/21 (95%)	9/9 (100%)
p38	21/27(77%)	27/27 (100%)	21/21 (100%)	9/9 (100%)
EGFR	6/26 (23%)	6/26 (23%)	3/16 (18%)	9/11 (81%)

Legend: N = number of tumour cores showing positivity; T = total number of cores assessable for the antibody specified; % = percentage of cores showing positivity; NP = not performed

Table 3 - Summary of brain injections experiment

Control Group – not treated	Tumour size- mm³	Days to death	Average of days to death
Animal 1	544	80	
Animal 2	250	77	
Animal 3	187	77	74
Average tumour size mm ³	327		
Mice treated on the day of injection of tumour cells with Trastuzumab	Tumour size- mm³	Days to death	Average of days to death
Animal 1	78	77	
Animal 2	65	90	
Animal 3	74	95	88
Average tumour size mm ³	72		
Mice treated on the day of injection of tumour cells with anti-neuregulin antibody	Tumour size- mm³	Days to death	Average of days to death
Animal 1	99	77	
Animal 2	100	90	
Animal 3	64	95	88
Average tumour size mm ³	87		
Mice treated at Day 84 with Trastuzumab	MRI Tumour Size at day of injection-mm³	Tumour Size at autopsy-mm³	Days to death after treatment
Animal 1	144	142	0
Animal 2	193	199	11
Animal 3	239	250	14
Average tumour size mm ³	210	197	

Table 4 - Genes differentially expressed between parental and tumour cells injected in the brain

JUND	CREBBP	LTA	TIMP3	TK1	CDK9	PRKR	DEK	MADH4
VHL	SH3BP2	PPARG	FAT	STAT3	JUN	FGF1	PCNA	APC
ELL	PTCH	CDKN2B	WNT8B	ITGB4	PTK2	COL18A1	ING1	CDKN1A
ARHI	TIMP3	CTSD	GAS7	SMARCA4	MSH3	CDK10	ARHA	TGFB1
CD59	IL1RN	PTGS1	VEGF	RELA	BIRC5	WEE1	FZD7	PCTK1
COL18A1	AREG	AREG	MCL1	FLT4	RELA	CAV1	XPA	SERPINE1
CTSD	BIRC3	QARS	NOTCH4	CD59	EV1	E2F1	BARD1	RAN
FANCG	FGF8	RBBP2	GLI3	EV12A	BIRC2	CDK7	GADD45A	ICAM1
TFF1	MPL	MAS1	ETS1	TIMP2	MLLT3	ELK3	AHR	LYN
CEACAM1	AKT2	PDGFB	FES	SOD1	IRF1	BIRC3	BAG1	CTNNA1
MCAM	MDS1	EPS8	PTPRF	CYP11A1	EPO	CD44	VIL2	CTSL
KRAS2	IL11	PML	MCC	IL12A	DEK	RAD23A	RAP2A	TGFB2
BAG1	VAV2	APAF1	LCN2	JAK2	COMT	MMP14	ZNF146	MDM4
CDH11	YY1	IGF1	NBS1	TCF7L2	DVL3	XPA	XRCC1	GADD45A
ARHA	ARAF1	COL18A1	PTK2	VBP1	NFKBIA	CD9	TFE3	IGFBP1
YES1	CRK	PDGFRA	CDH13	MAPK10	VEGFB	IL12B	CCND1	NOTCH1

RAP1GDS1	WNT10B	MMP1	VAV1	JUND	EXT2	CDKN1A	CDK7	FOLR1
NFKB1	DCC	PGF	FHIT	TIMP1	APC	MCL1	PTEN	MBD2
PRCC	ARHGDIB	ICAM1	PRKR	FVT1	FANCG	ARHGDIB	PCTK1	LYN
MPL	CDKN2C	PTPRH	CTNNA1	CDK10	MLH1	WEE1	ERG	TPR
FGF7	CBFA2T1	TFDP1	AREG	MLF1	ZNFN1A1	TFG	CSF2	KRAS2
MYCL1	MLF2	AHR	RARA	TYMS	PLG	LAMB1	RAP1GDS1	CTGF
CSF1R	MYC	ETV6	STAT5B	CCNH	DCN	MYC	IL6	TSG101
RAD51	RAD51	PRKR	CDK4	TGFB3	SKIL	CD34	EPS15	SKI
CASP3	VBP1	VIL2	DVL3	MSH2	TNFRSF1A	HDAC1	COPEB	PTEN
MRE11A								

Table 5 A sample of ErbB inhibitors which are either used for treatment or in pre-clinical and clinical trials

Compound	Type	Target	Reference
Cetuximab (Erbix TM ; Merck Serono)	mAb = monoclonal antibody		
Matuzumab	mAb	EGFR	Hynes N & Lane H (2005) Nature Reviews Cancer
Panitumumab (Vectibix TM ; Amgen)	mAb	EGFR	Hynes N & Lane H (2005) Nature Reviews Cancer
Gefitinib (Iressa TM ; Astra Zeneca)	Tyrosine kinase inhibitor	EGFR	Hynes N & Lane H (2005) Nature Reviews Cancer
Erlotinib (Tarceva®; Genentech)	Tyrosine kinase inhibitor	EGFR	Hynes N & Lane H (2005) Nature Reviews Cancer
ICR62	mAb	EGFR	Friedlander E et al (2008) Immunology Letters
Nimotuzumab (TheraCIM TM ; YM Biosciences Inc)	mAb	EGFR	Friedlander E et al (2008) Immunology Letters
Ch806	mAb	EGFR	Friedlander E et al (2008) Immunology Letters
L8A4	mAb	EGFR	Friedlander E et al (2008) Immunology Letters
MDX-447	mAb	EGFR	Friedlander E et al (2008) Immunology Letters
Zalutumumab	mAb	EGFR	Peipp M et al (2008) Current Opinion in Immunology
IMC-11F8	mAb	EGFR	Peipp M et al (2008) Current Opinion in Immunology
Ch806	mAb	EGFR	Peipp M et al (2008) Current Opinion in Immunology
AZD4769	Tyrosine kinase inhibitor	EGFR	www.medtrack.com
PF299804	Tyrosine kinase inhibitor	EGFR	www.medtrack.com
EGFR501	Tyrosine kinase inhibitor	EGFR	www.medtrack.com
Vandetanib/ZD6474 (Zactima TM ; Astra Zeneca)	Tyrosine kinase inhibitor	EGFR	www.medtrack.com
Trastuzumab (Herceptin®; Genentech)	mAb	ErbB2	Hynes N & Lane H (2005) Nature Reviews Cancer

Pertuzumab (Omnitarg™; Omnitarg)	mAb	ErbB2	Hynes N & Lane H (2005) Nature Reviews Cancer
scFvFRP5	scFv fragment	ErbB2	Friedlander E et al (2008) Immunology Letters
CAB051	mAb	ErbB2	www.medtrack.com
Ertumaxomab (Rexomun®; Fresenius)	mAb	ErbB2	www.medtrack.com
Lapatinib (Tykerb®; GlaxoSmithKline)	Tyrosine kinase inhibitor	EGFR/ErbB2	Hynes N & Lane H (2005) Nature Reviews Cancer
AEE788	Tyrosine kinase inhibitor	EGFR/ErbB2	Hynes N & Lane H (2005) Nature Reviews Cancer
CI-1033	Tyrosine kinase inhibitor	EGFR/ErbB2	Hynes N & Lane H (2005) Nature Reviews Cancer
EKB-569	Tyrosine kinase inhibitor	EGFR/ErbB2	Hynes N & Lane H (2005) Nature Reviews Cancer
EXEL 7647/EXEL 0999	Tyrosine kinase inhibitor	EGFR/ErbB2	Hynes N & Lane H (2005) Nature Reviews Cancer
BIBW 2992 (Tovok™; Boehringer Ingelheim)	Tyrosine kinase inhibitor	EGFR/ErbB2	www.medtrack.com
AZD8931	Tyrosine kinase inhibitor	EGFR/ErbB2	www.medtrack.com
MP412	Tyrosine kinase inhibitor	EGFR/ErbB2	www.medtrack.com
herstatin (Dimercept™)	Tyrosine kinase inhibitor	EGFR/ErbB2	www.medtrack.com
Pelitinib	Tyrosine kinase inhibitor	EGFR/ErbB2	www.medtrack.com
CP724714	Tyrosine kinase inhibitor	EGFR/ErbB2	www.medtrack.com
HKI-272 (Neratinib; Wyeth)	Tyrosine kinase inhibitor	EGFR/ErbB2	www.medtrack.com
XL647	Tyrosine kinase inhibitor	EGFR/ErbB2	www.medtrack.com
AEE788	Tyrosine kinase inhibitor	EGFR/ErbB2	www.medtrack.com
PD183805 (Canertinib/CI-1033; Pfizer)	Tyrosine kinase inhibitor	EGFR/ErbB2/ErbB4	www.medtrack.com
PD-169414	Tyrosine kinase inhibitor	EGFR/ErbB2/ErbB4	www.medtrack.com

Table 5 continued

CLAIMS

1. A method of treating a metastatic breast cancer in a human, said method including the step of administering to an individual with an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain, a therapeutic agent effective
5 in inhibiting at least one ErbB receptor.
2. The method of Claim 1, wherein the metastatic breast cancer has one or more cells with normal expression levels of Her-2.
3. The method according to any one of the preceding claims, wherein the metastatic breast cancer has one or more breast cancer cells with normal expression
10 levels of Her-2.
4. The method according to any one of the preceding claims, wherein the metastatic breast cancer is located in the brain.
5. The method according to any one of the preceding claims, wherein the at least one ErbB receptor is selected from the group consisting of Her-1, Her-2, Her-3, Her-
15 4, and combinations thereof.
6. The method according to any one of the preceding claims, wherein the at least one ErbB receptor is selected from the group consisting of Her-1, Her-2 and Her-3.
7. The method according to any one of the preceding claims, wherein the at least one ErbB receptor is Her-2.
- 20 8. The method according to any one of the preceding claims, wherein the therapeutic agent directly inhibits at least one ErbB receptor or indirectly inhibits at least one ErbB receptor.
9. The method according to any one of the preceding claims, wherein the therapeutic agent selectively inhibits at least one ErbB receptor.
- 25 10. The method according to any one of the preceding claims, wherein the therapeutic agent is selected from the group consisting of an isolated protein or a fragment thereof, an isolated nucleic acid or a fragment thereof, a small-molecule compound, and combinations thereof.
11. The method according to any one of the preceding claims, wherein the
30 therapeutic agent is an antibody, or a fragment thereof.
12. The method according to any one of the preceding claims, wherein the

antibody is a monoclonal antibody.

13. The method according to any one of the preceding claims, wherein the antibody binds to and/or has been raised against Her-2.

14. The method according to any one of the preceding claims, wherein the
5 therapeutic agent is selected from the group consisting of trastuzumab, pertuzumab, scFvFRP5, CAB051, ertumaxomab, lapatinib, CI-1033, HKI-272, AEE-788, BIBW-2992, TAK165, BMS-599626, matuzumab, cetuximab, panitumumab, gefitinib, erlotinib, ICR62, nimotuzumab, Ch806, L8A4, MDX-447, zalutumumab, IMC-11F8, AZD4769, PF299804, EGFR501, ZD6474, EKB-569, EXEL 7647, EXEL 0999,
10 AZD8931, MP412, herstatin, pelitinib, CP724714, XL647, PD-169414, and combinations thereof.

15. The method according to any one of the preceding claims, wherein the therapeutic agent is trastuzumab.

16. The method according to any one of the preceding claims, which further
15 includes the step of administering to an individual, one or more therapeutic agents selected from the group consisting of a chemotherapeutic agent and a modulator of a hormone receptor.

17. The method according to any one of the preceding claims, which further includes the step of determining whether said individual has an elevated level of Her-
20 3 in one or more metastatic breast cancer cells of the brain.

18. A method of designing, engineering, screening or otherwise producing a therapeutic agent for treating a metastatic breast cancer, said method including the step of identifying a candidate agent that is suitable for use in treatment of a metastatic breast cancer by the presence of an elevated expression level of Her-3 in
25 one or more metastatic breast cancer cells of the brain.

19. The method of Claim 18, wherein the candidate agent inhibits at least one ErbB receptor.

20. The method of Claim 18 or Claim 19, wherein the candidate agent directly inhibits at least one ErbB receptor or indirectly inhibits at least one ErbB receptor.

30 21. The method according to any one of Claims 18 to Claim 20, wherein the candidate agent selectively inhibits at least one ErbB receptor.

22. The method according to any one of Claims 18 to 21, wherein the at least one ErbB receptor is selected from the group consisting of Her-1, Her-2, Her-3, Her-4, and combinations thereof.
23. The method according to any one of Claims 18 to 22, wherein the at least one
5 ErbB receptor is selected from the group consisting of Her-1, Her-2 and Her-3.
24. The method according to any one of Claims 18 to 23, wherein the at least one ErbB receptor is Her-2.
25. The method according to any one of Claims 18 to 24, wherein the candidate agent is selected from the group consisting of an isolated protein or a fragment
10 thereof, an isolated nucleic acid or a fragment thereof, a small-molecule compound, and combinations thereof.
26. The method according to any of Claims 18 to 25, wherein the candidate agent is an antibody, or a fragment thereof.
27. The method according to any one of Claims 18 to 26, wherein the antibody is
15 a monoclonal antibody.
28. The method according to any one of Claims 18 to 27, wherein the antibody binds to and/or has been raised against Her-2.
29. The method according to any one of Claims 18 to 28, wherein the metastatic breast cancer has one or more cells with normal expression levels of Her-2.
- 20 30. The method according to any one of Claims 18 to 29, wherein the metastatic breast cancer has one or more breast cancer cells with normal expression levels of Her-2.
31. The method according to any one of Claims 18 to 30, wherein the one or more cells with normal levels of Her-2 have levels of an estrogen receptor and a
25 progesterone receptor which are below a threshold level of detection.
32. The method according to any one of Claims 18 to 31, wherein the metastatic breast cancer is located in the brain.
33. A therapeutic agent for treating a metastatic breast cancer designed, engineered, screened or otherwise produced according to a method of any one of
30 Claims 18 to 32.
34. A pharmaceutical composition comprising a therapeutic agent for treating a

metastatic breast cancer according to Claim 33, together with a pharmaceutically-acceptable carrier, diluent or excipient.

35. The pharmaceutical composition of Claim 34, which further comprises one or more therapeutic agents selected from the group consisting of a chemotherapeutic agent and a modulator of a hormone receptor.

36. A method of treating a metastatic breast cancer, said method including the step of administering a therapeutic agent for treating a metastatic breast cancer according to Claim 33 or a pharmaceutical composition according to Claim 34 or Claim 35, to an individual with an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain.

37. The method according to Claim 36, wherein the metastatic breast cancer has one or more cells with normal expression levels of Her-2.

38. The method according to Claim 36 or Claim 37, wherein the metastatic breast cancer has one or more breast cancer cells with normal expression levels of Her-2.

39. The method according to any one of Claims 36 to 38, wherein the one or more cells with normal levels of Her-2 have levels of an estrogen receptor and a progesterone receptor which are below a threshold level of detection.

40. The method according to any one of Claims 36 to 39, wherein the metastatic breast cancer is located in the brain.

41. A method of determining whether a human with a metastatic breast cancer is potentially responsive to treatment with a therapeutic agent effective in inhibiting at least one ErbB receptor and/or a therapeutic agent for treating a metastatic breast cancer designed, engineered, screened or otherwise produced according to any one of Claims 18 to 32, said method including the step of detecting elevated levels of Her-3 in one or more metastatic breast cancer cells of the brain.

42. The method of Claim 41, wherein the at least one ErbB receptor is selected from the group consisting Her-1, Her-2, Her-3, Her-4, and combinations thereof.

43. The method according to Claim 41 or Claim 42, wherein the at least one ErbB receptor is selected from the group consisting of Her-1, Her-2 and Her-3.

44. The method according to any one of Claims 41 to 43, wherein the at least one ErbB receptor is Her-2.

45. The method according to any one of Claims 41 to 44, wherein the metastatic breast cancer has one or more cells with normal expression levels of Her-2.
46. The method according to any one of Claims 41 to 45, wherein the metastatic breast cancer has one or more breast cancer cells with normal expression levels of
- 5 Her-2.
47. The method according to any one of Claims 41 to 46, wherein the one or more cells with normal levels of Her-2 have levels of an estrogen receptor and a progesterone receptor which are below a threshold level of detection.
48. The method according to any one of Claims 41 to 47, wherein the metastatic
- 10 breast cancer is located in the brain.
49. A method of determining whether a human is predisposed to a metastatic breast cancer or is suffering from a metastatic breast cancer, said method including the step of detecting an elevated level of Her-3 in one or more metastatic breast cancer cells of the brain.
- 15 50. The method according to Claim 49, wherein the metastatic breast cancer has one or more cells with normal expression levels of Her-2.
51. The method according to Claim 49 or Claim 50, wherein the metastatic breast cancer has one or more breast cancer cells with normal expression levels of Her-2.
52. The method according to any one of Claims 49 to 51, wherein the one or
- 20 more cells with normal levels of Her-2 have levels of an estrogen receptor and a progesterone receptor which are below a threshold level of detection.
53. The method according to any one of Claims 49 to 52, wherein the metastatic breast cancer is located in the brain.

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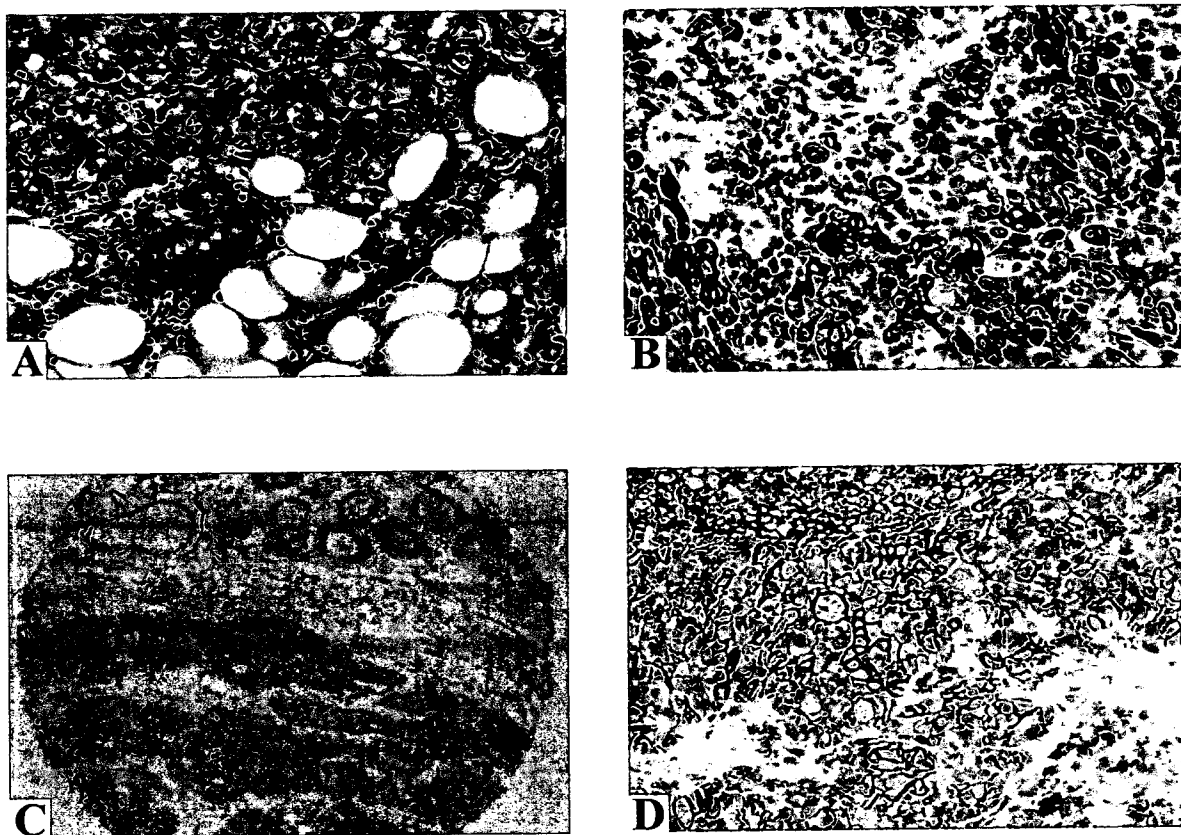


FIGURE 1

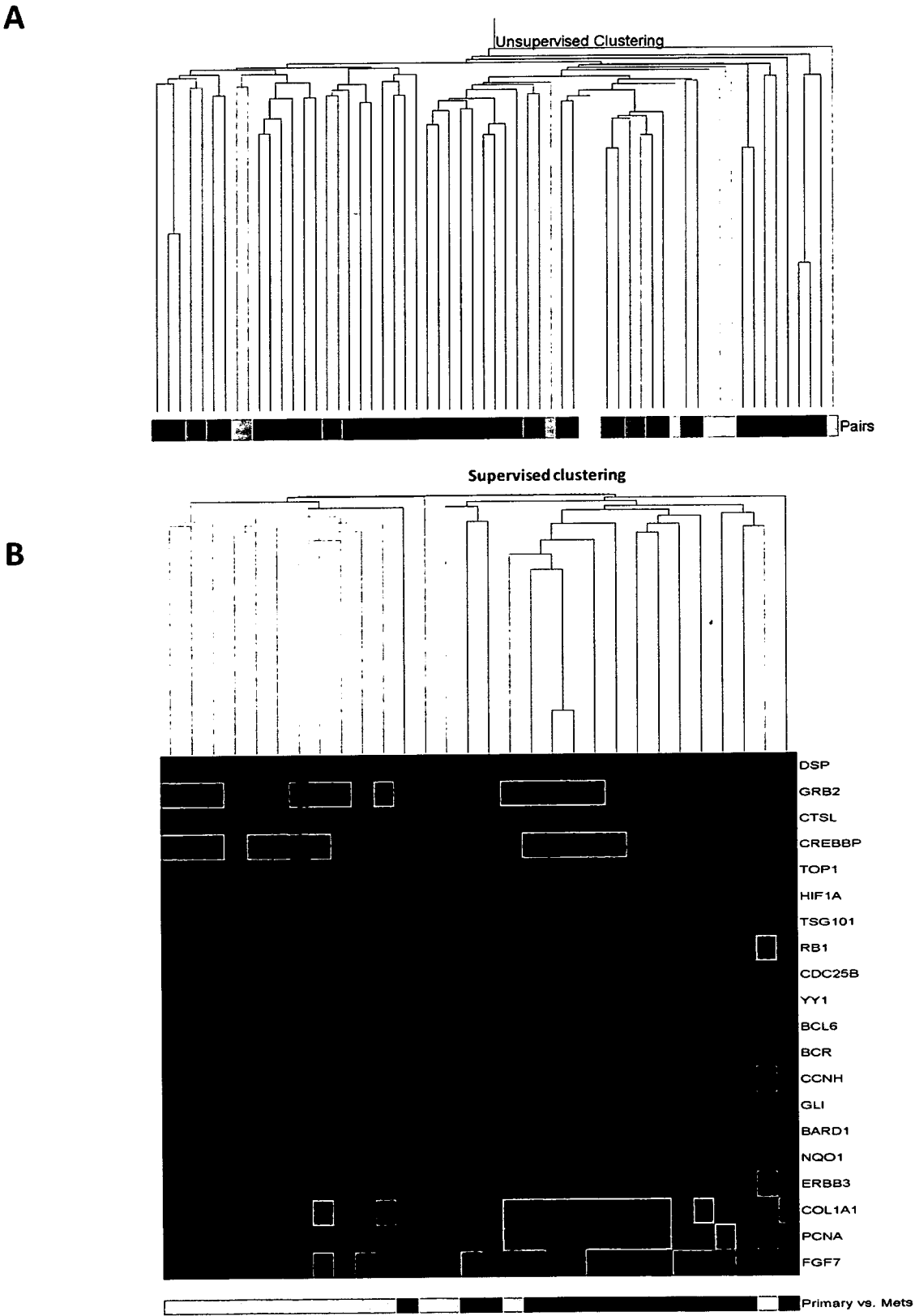


FIGURE 2

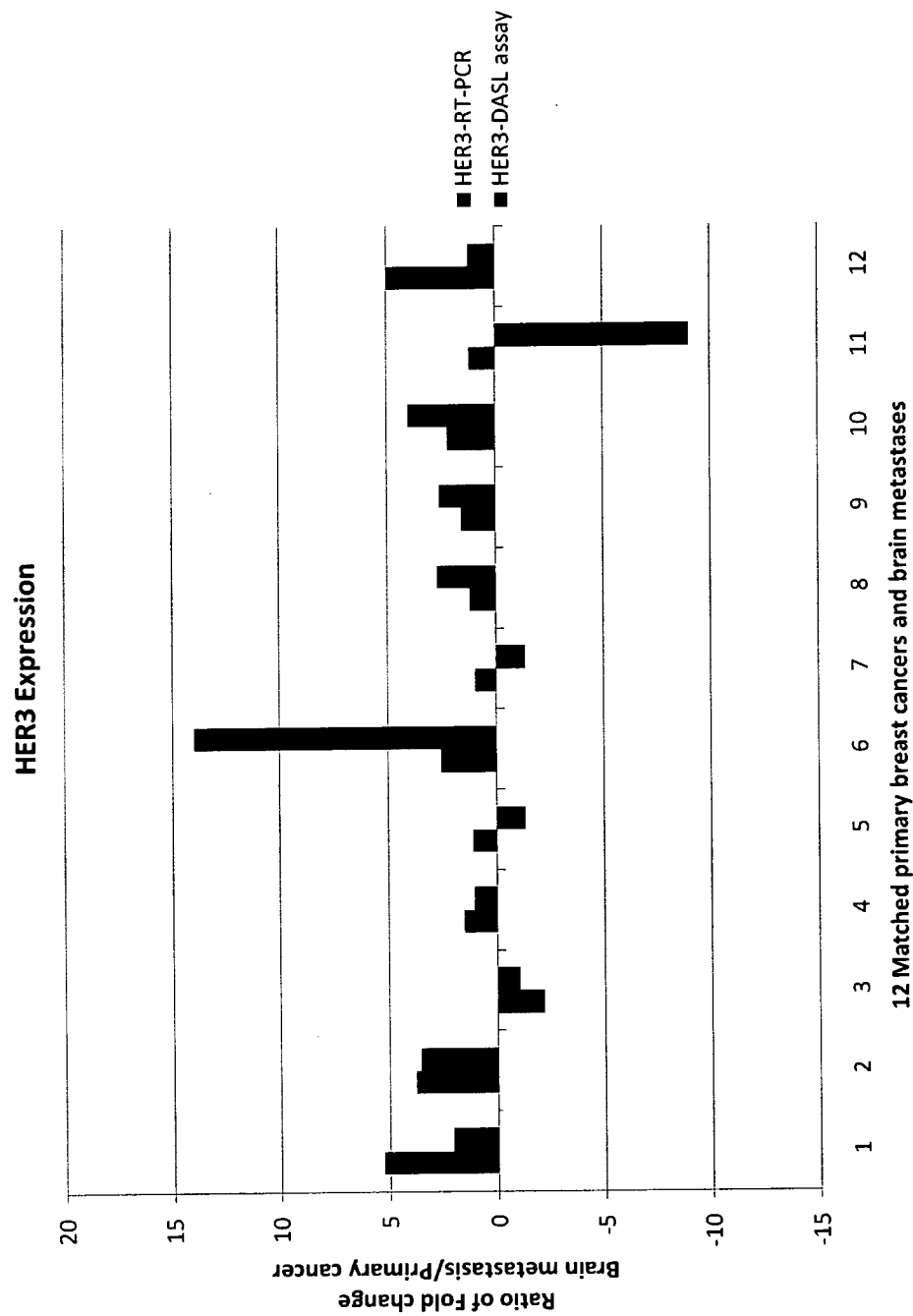


FIGURE 3

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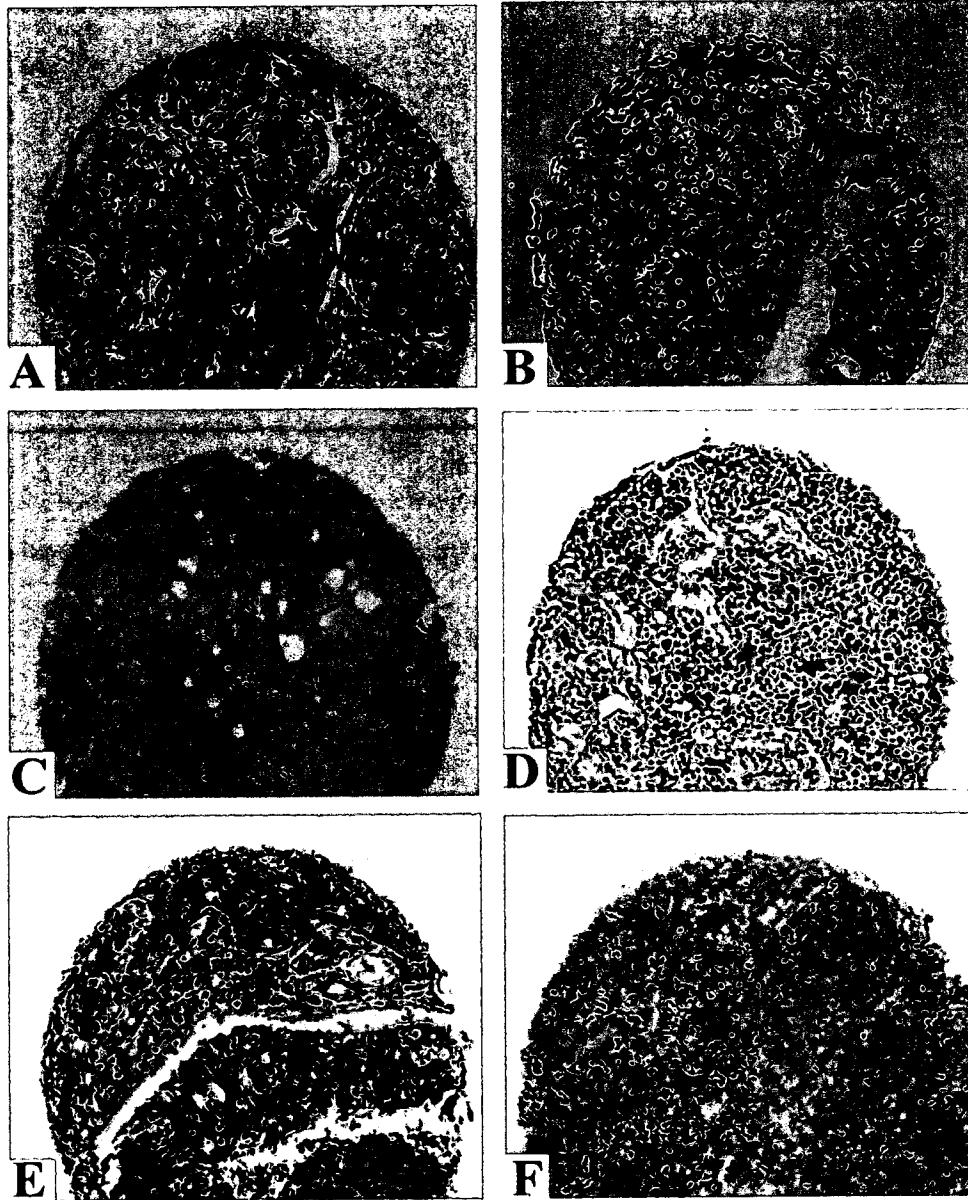


FIGURE 4

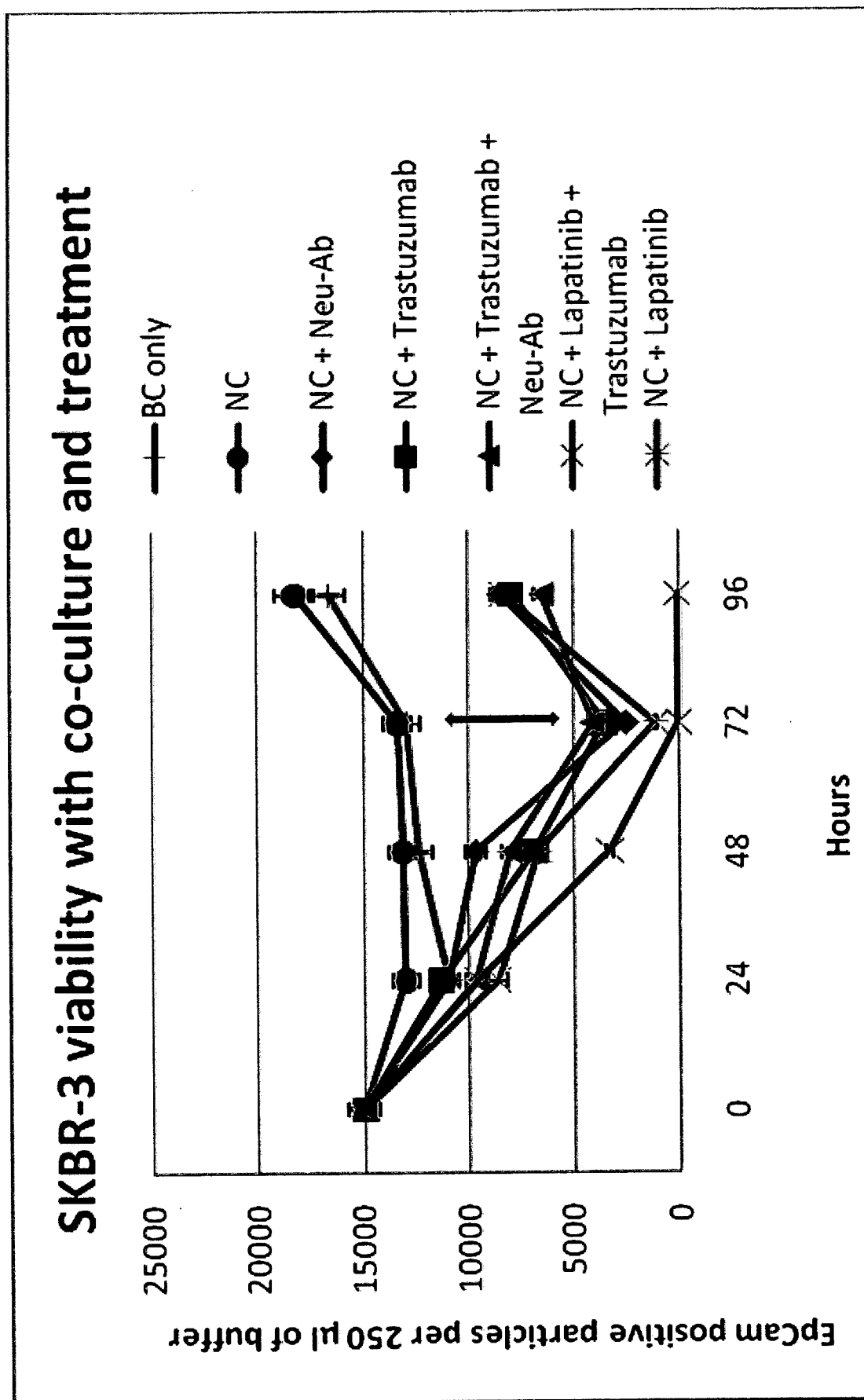


FIGURE 5



HER3 Expression in MDAMB231 cell mouse brain xenografts

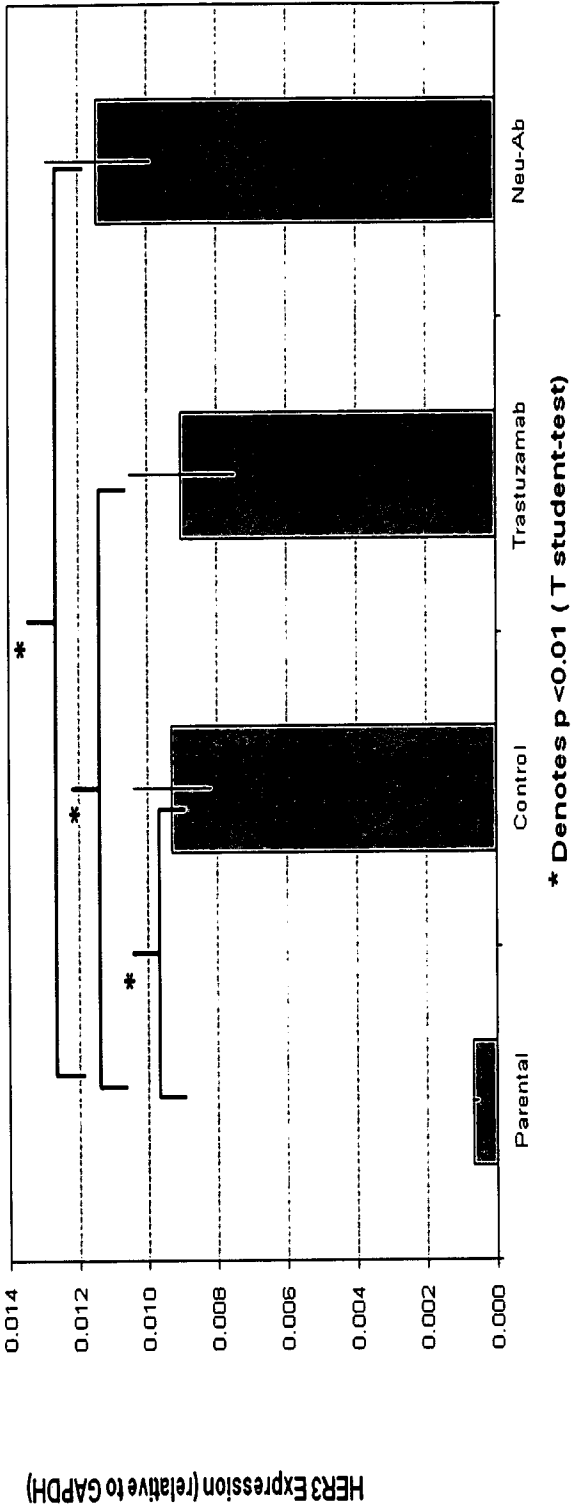


FIGURE 6

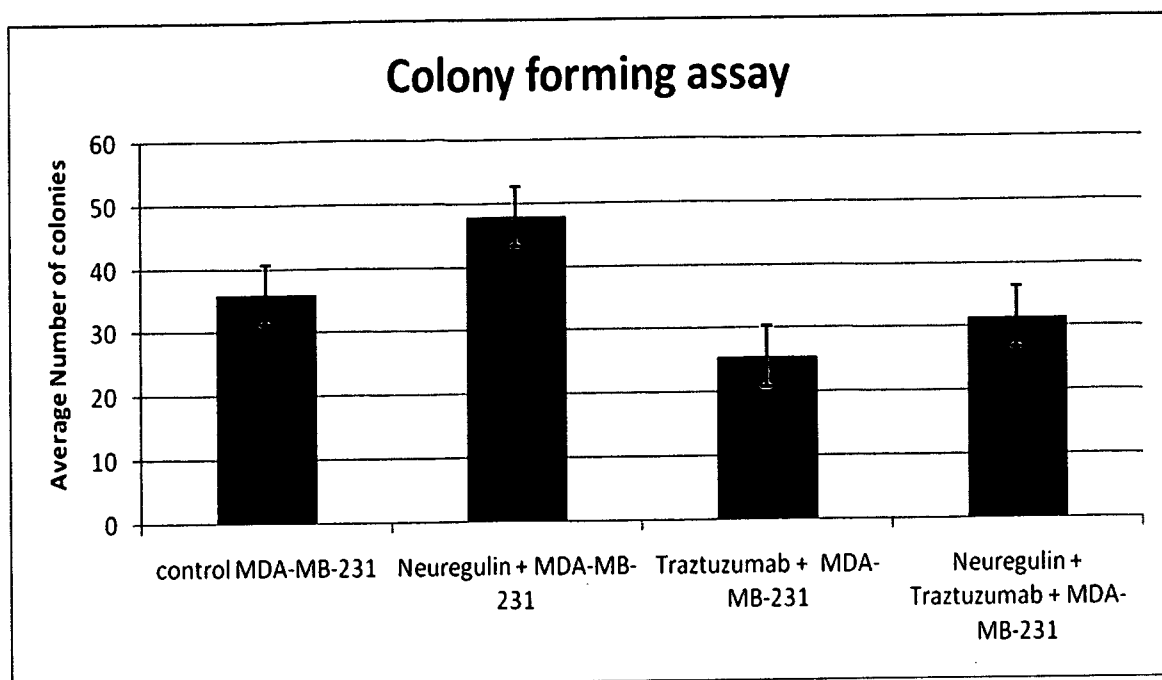


FIGURE 7

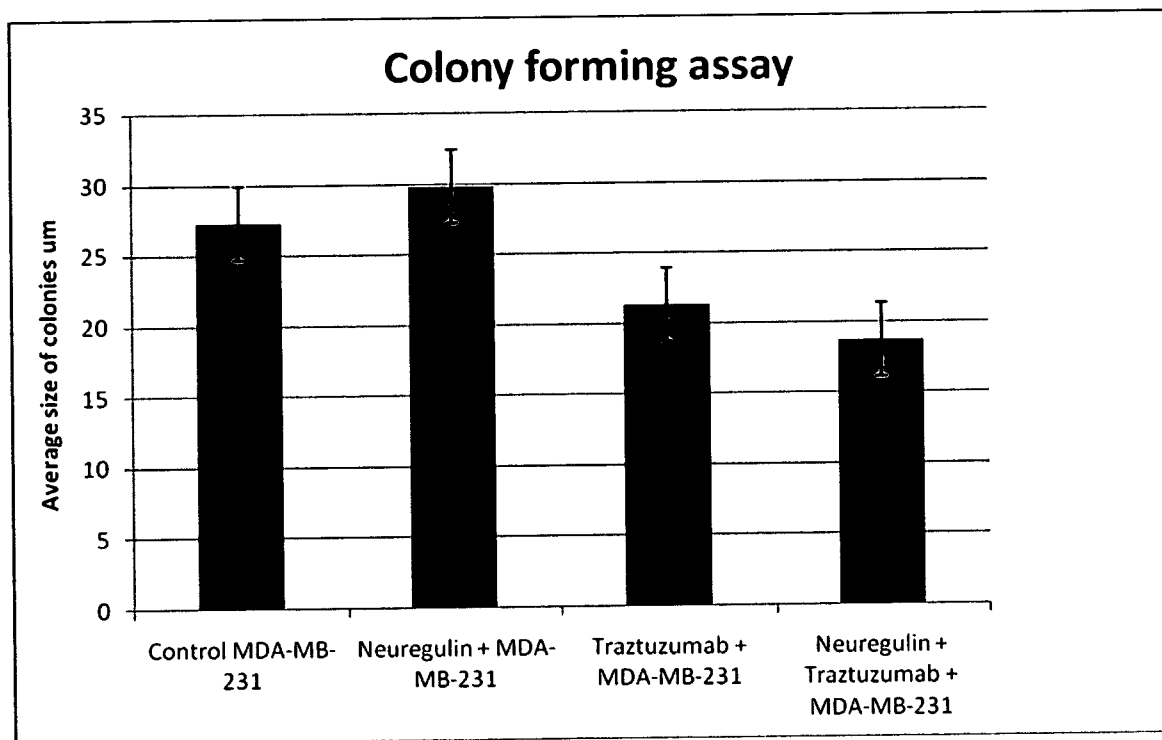


FIGURE 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2010/000081

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. <i>G01N 33/574</i> (2006.01) <i>A61P 35/04</i> (2006.01) <i>A61K 39/395</i> (2006.01) <i>C12Q 1/68</i> (2006.01) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) MEDLINE, CAPLUS, BIOSIS, WPI, EPODOC, TXTEN; KEYWORDS: breast, metast?, tumour, tumor, cancer, neoplasm, malignan?, carcinoma, brain, nervous system, cns, her-3, her3, erbB3, erbB-3, c-erbB-3, cerbB3		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2008/0124345 A1 (ROTHER et al.) 29 May 2008 See especially: abstract, paragraphs [0083] and [0095] and example 19	18-27 & 33-35
X	XUE, C. et al., "ErbB3-dependent motility and intravasation in breast cancer metastasis", Cancer Research, 2006, Vol. 66, No. 3, pages 1418-1426 See especially: page 1418, 1423 and Figure 3	18-23, 25 & 33-3
X	US 2004/0197332 A1 (ULLRICH et al.) 7 October 2004 See especially: abstract, paragraphs [0003] and [0053]	18-27 & 33-35
A	RÉVILLION, F. et al., "Real-time reverse-transcription PCR to quantify a panel of 19 genes in breast cancer: relationships with sentinel lymph node invasion", The International Journal of Biological Markers, 2008, Vol. 23, No. 1, pages 10-17 See especially: abstract and page 1425	
☒ Further documents are listed in the continuation of Box C ☒ See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search 02 March 2010/15 March 2010	Date of mailing of the international search report 16 MAR 2010	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE, PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer ANITA COCHRANE AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2438	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2010/000081

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, A	HERNANDEZ, L. et al., "The EGF-CSF-1 paracrine invasion loop can be triggered by heregulin β 1 and CXCL12", Cancer Research, 1 April 2009, Vol 69, No. 7, pages 3221-3227 See especially: abstract	
P, A	WO 2009/108637 A1 (PROMETHEUS LABORATORIES, INC.) 3 September 2009 See especially: paragraph [0048] and page 135	
P, A	WO 2009/145924 A2 (INDIANA UNIVERSITY RESEARCH & TECHNOLOGY CORPORATION) 3 December 2009 See especially: abstract	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2010/000081

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
US	2008/0124345	AR	056857	AU	2006332065	CA	2633222
		CN	101365723	EA	200801621	EP	1984402
		KR	20080099235	MX	2008008248	NO	20083268
		WO	2007/077028	ZA	200805474		
US	2004/0197332	CA	2456723	CN	1541109	EP	1283053
		EP	1414494	EP	2067792	WO	2003/013602
WO	2009/108637	NONE					
WO	2009/145924	NONE					
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.							
END OF ANNEX							