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(54) **Title:** DUSP22 AS A PROGNOSTIC MARKER IN HUMAN BREAST CANCER

(57) **Abstract:** The present invention provides a prognostic marker in human breast cancer, high copy number of 6p25 chromosome region or copy number and/or expression level of a gene included in this region being associated with a good prognosis. The present invention also provides a method for selecting a subject affected with a breast cancer for a therapy.



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DUSP22 as a prognostic marker in human breast cancer

FIELD OF THE INVENTION

The present invention relates to the field of medicine, in particular of oncology. It provides a new prognostic marker in human breast cancer.

5 BACKGROUND OF THE INVENTION

Cancer occurs when cell division gets out of control and results from impairment of a DNA repair pathway, the transformation of a normal gene into an oncogene or the malfunction of a tumor suppressor gene. Many different forms of cancer exist. While different forms of cancer have different properties, one factor which many cancers share is the ability
10 to metastasize. Distant metastasis of all malignant tumors remains the primary cause of death in patients with the disease.

Breast cancer is the most common cancer in women in Western countries. While germ line mutations in BRCA1 or BRCA2 genes predispose women with the mutations to breast cancer, only about 5-10% of breast cancers are associated with these breast cancer
15 susceptibility genes. Currently employed clinical indicators of breast cancer prognosis are not accurate in identifying patients likely to have a favorable outcome. As a result, many more patients are subjected to adjuvant chemotherapy than will benefit from such treatment. Due to the increased morbidity and mortality if breast cancer is not detected early in its progression, considerable effort has been devoted to early detection of breast tumor development and it
20 remains a need in the art for better and more specific clinical predictors of breast cancer prognosis.

It is established that specific genetic aberrations are often associated with clinical characteristics. Examples include the association of 1p/19q deletions in breast cancers with improved response to chemotherapy, and the association of 8q gain with poor prognosis in
25 prostate cancer. Such aberrations have been detected with comparative genomic hybridization (CGH). Several studies have demonstrated the association of genetic aberrations with gene expression changes. In independent studies, Hyman et al (Cancer Res. 2002) and Pollack et al (PNAS 2002) have found a strong relationship between high amplification and high expression in breast tumors. Crawley et al (Genome Biol. 2002) have reported on a data
30 analysis method that accurately predicts regions of copy number aberrations in hepatocellular carcinomas using only gene expression data. These investigations support the notion that gene expression data can be used as a window to the underlying genetic defects, and thus the idea

that a combined analysis of gene expression data and CGH copy number data with the aim of identifying DNA markers is viable.

The therapeutic care of the patients having cancer is primarily based on surgery, radiotherapy and chemotherapy and the practitioner has to choose the most adapted therapeutic strategy for the patient. In the majority of the cases, the choice of the therapeutic protocol is based on the anatomo-pathological and clinical data. Currently, the methods to determine breast cancer prognosis and select patients for adjuvant therapy is mainly established based upon parameters such as tumor size, tumor grade, the age of the patient, and lymph node metastasis. Accurate prognosis or determination of distant metastasis-free survival in breast cancer patients would permit selective administration of adjuvant therapy, with women having poorer prognosis being given the most aggressive treatment. However, it is very difficult to predict which localized tumor will eventuate in distant metastasis.

Therefore, there is a great need for the identification of prognostic markers that can accurately distinguish tumors associated with good prognosis including low probability of metastasis, late disease progression, decreased disease recurrence or increased patient survival, from the others. Using such markers, the practitioner can predict the patient's prognosis and can effectively target the individuals who would most likely benefit from therapy or who need a more intensive monitoring.

20 SUMMARY OF THE INVENTION

The inventors demonstrate that a high copy number of 6p25 chromosome region or copy number and/or expression level of a gene included in this region, in particular DUSP22 gene, in a subject having a breast cancer is correlated with a good prognosis including late disease progression, decreased metastasis formation, decreased disease recurrence and/or increased patient survival.

Accordingly, in a first aspect, the present invention concerns a method for predicting the clinical outcome of a subject affected with breast cancer, wherein the method comprises the step of determining the copy number of the 6p25 chromosome region or the copy number and/or expression level of a gene included in said region in a sample from said subject, a high copy number of said 6p25 chromosome region or of said gene thereof and/or a high expression level of said gene being indicative of a good prognosis. Preferably, a good prognosis is an increased patient survival and/or a late disease progression and/or a decreased disease recurrence and/or a decreased metastasis formation.

In a second aspect, the present invention concerns a method for selecting a subject affected with breast cancer for a therapy, preferably an adjuvant therapy, or determining whether a subject affected with breast cancer is susceptible to benefit from a therapy, preferably an adjuvant therapy, wherein the method comprises the step of determining the
5 copy number of the 6p25 chromosome region or the copy number and/or expression level of a gene included in said region in a sample from said subject, a low copy number of said 6p25 chromosome region or of said gene thereof and/or a low expression level of said gene indicating that a therapy, preferably an adjuvant therapy, is required.

In a preferred embodiment, the gene included in 6p25 chromosome region is DUSP22.

10 In another embodiment, the copy number of the 6p25 chromosome region or of a gene included in this region, preferably DUSP22 gene, is determined by quantitative or semi-quantitative PCR, or by real time quantitative or semi-quantitative PCR, in situ hybridization (such as fluorescent in situ hybridization (FISH)), Southern blotting, array-based methods and/or comparative genomic hybridization (CGH). More preferably, detection is performed
15 by comparative genomic hybridization (CGH).

In a further embodiment, the expression level of a gene included in 6p25 chromosome region, preferably DUSP22 gene, is determined by measuring the quantity of protein or mRNA encoded by said gene, preferably DUSP22 protein or DUSP22 mRNA, more preferably DUSP22 protein.

20 In particular, the quantity of protein may be measured by immunohistochemistry, semi-quantitative Western-blot or by protein or antibody arrays, and the quantity of mRNA may be measured by quantitative or semi-quantitative RT-PCR, or by real time quantitative or semi-quantitative RT-PCR, or by Northern blot or by transcriptome approaches.

In a preferred embodiment, the sample is a breast cancer sample.

25 In a further embodiment, the reference copy number and/or the reference expression level is the copy number and /or expression level of in a normal sample or frequently present in a control group.

In still another embodiment, the breast cancer is an early stage breast cancer without local or systemic invasion, preferably a small-size breast cancer.

30 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Proportion of tumors exhibiting a genomic gain at the 6p25.3 locus according to the metastatic status in the bone marrow (MO) and the axillary sentinel node (GS). Metastatic status was classified as positive (1) (presence of metastatic cells) or

negative (0) (no metastatic cells). Numbers in histogram bars correspond to the number of cases with a genomic gain reported to the total number of cases in each class.

Figure 2: SNP6 analysis of six cases of invasive breast carcinoma (T1-T6) with that of the corresponding non tumor tissues (N1-N6) for each case. The copy number variation of the DNA observed at the 6p25 locus in tumor tissues either as gains (dotted lines) (T2, T3, T6) or losses (full lines) (T1, T5) is also observed in non tumor tissues (gains in N2, N3, N6 and losses in N1, N5). This indicates that the variation observed corresponds to a copy number polymorphism. A very slight increase in tumor T4 is not clearly detected in the corresponding normal tissue (N4). The locus of the *DUSP22* gene, indicated at the top, is located at the medium part of the region.

Figure 3: Invasive ductal carcinoma of the breast: significant expression level of DUSP22 in the nucleus of the tumor cells.

DETAILED DESCRIPTION OF THE INVENTION

Genetic variation in the human genome takes many forms, ranging from large, microscopically visible chromosome anomalies to single-nucleotide changes. The genomic sequence within populations is not identical when individuals are compared. Rather, the genome exhibits sequence variability between individuals at many locations in the genome. Such variations in sequence are commonly referred to as polymorphisms, and there are many such polymorphism sites within each genome. Beside single nucleotide polymorphisms (SNPs), many other types of sequence variants are found in the human genome, including mini- and microsatellites, and insertions, deletions, inversions, duplications and complex multi-site variants collectively termed copy number variations (CNVs) or copy number polymorphisms (CNPs). CNVs are receiving increased attention. These large-scale polymorphisms (typically 1 kb or larger) account for polymorphic variation affecting a substantial proportion of the assembled human genome; known CNVs cover over 15 percent of the human genome sequence (Estivill et al, 2007). Most of these polymorphisms are however very rare, and, on average, affect only a fraction of the genomic sequence of each individual. CNVs are known to affect gene expression, phenotypic variation and adaptation by disrupting gene dosage, and are also known to cause disease (microdeletion and microduplication disorders) and confer risk of common complex diseases, including HIV-I infection and glomerulonephritis (Redon et al., 2006). Methods for detecting CNVs include comparative genomic hybridization (CGH) and genotyping, including use of genotyping

arrays, as described by Carter (Nature 2007). The Database of Genomic Variants (<http://projects.tcag.ca/variation/>) contains updated information about the location, type and size of described CNVs. The database currently contains data for over 15,000 CNVs.

Recurrent chromosomal alterations are a hallmark of cancer cells and represent critical events in tumor development. In particular, oncogene activation through increased gene copy number resulting in overexpression contributes to the malignant transformation of various human solid cancers, including breast cancer (Collins et al, 2001). In breast cancer, major recurrent amplicons include 17q12 (*ERBB2/HER-2*), 8q24 (*MYC*), 11q13 (*CCND1*), 20q13, and 8p11-12 (Ethier et al., 2003).

10 The inventors surprisingly demonstrated the role of another amplicon in breast cancer. They show that a high copy number of a part of the short arm of chromosome 6, more specifically of locus 6p25, more particularly 6p25.3, is significantly correlated to decreased occurrence of metastasis, particularly medullary and/or sentinel lymph node metastasis.

Inventors herein also demonstrate that the copy number and/or expression level of a gene included in 6p25 chromosome region, in particular DUSP22 gene, are correlated with good prognosis. Copy number and/or expression level of this gene included in 6p25 chromosome region thus constitutes a marker for the prognosis of breast cancer. In particular, they show that high copy number and/or expression levels of a gene included in this chromosome region, in particular DUSP22, in breast carcinomas correlate with increased patient survival, late disease progression, decreased disease recurrence and/or decreased occurrence of metastasis over time, i.e. a good prognosis.

DUSP22 (or Dual specificity phosphatase 22) is also designated as LMW-DSP2 (low molecular weight dual specificity phosphatase 2); JKAP (JNK pathway associated phosphatase); JSP1 (JNK-stimulatory phosphatase 1); MKP-x (mitogen-activated protein kinase phosphatase x); VHX (VHR related MKPX) or FLJ35864. DUSP22 is a Dual specificity phosphatase/MAPK-phosphatase known to regulate MAPK-mediated signaling pathways. More specifically, DUSP22 have been shown to positively regulate JNK or p38 MAPK pathways. DUSP22 contained a single DSP (Dual Specific Phosphatase) catalytic domain but lacked the cdc25 homology domain, which is conserved in most known MKPs.

25
30 DUSP22 contains a Protein Tyrosine Phosphatase (PTP) signature motif HCxxGxxR and is expressed in various tissues and cells.

DUSP22 has been shown to regulate T-cell antigen receptor signaling through ERK2 (Alonso et al, 2002). Sekine and al have also demonstrated that DUSP22 is a negative regulator of interleukin-6 (IL-6)/leukemia inhibitory factor (LIF)-mediated signaling by

dephosphorylating STAT-3 (Sekine et al, 2006). Lewintre et al validate in 2009 a set of putative prognostic markers of B cell chronic lymphocytic leukemia (CLL) which are differentially expressed genes according to IgV_H gene status, i.e unmutated or mutated. DUSP22 is found overexpressed in CLL cells in this study.

5 Definitions

As used herein, the term “DUSP22” refers to the Dual specificity phosphatase 22. Accession number corresponding to the human DUSP22 gene in Genbank is NM_020185, and accession number corresponding to the human DUSP22 protein is NP_064570. This protein is encoded by the gene *DUSP22* (GeneID: 56940).

10 The term “cancer” or “tumor”, as used herein, refers to the presence of cells possessing characteristics typical of cancer-causing cells, such as uncontrolled proliferation, immortality, metastatic potential, rapid growth and proliferation rate, and certain characteristic morphological features. Preferably the cancer is a breast cancer, more preferably a breast cancer in an early stage without local or systemic invasion, still more
15 preferably a small-size breast cancer.

The terms “CNV” or “copy number of a chromosome region or of a gene”, as used herein, is defined as a DNA segment ranging from kilobases (kb) to megabase (Mb) in size and present at variable copy number in comparison with a reference genome (Feuk et al, Nature Rev. Genet., 2006). A CNV can be simple in structure, such as tandem duplication, or
20 may involve complex gains or losses of homologous sequences at multiple sites in the genome. The high or low copy number of a chromosome region or of a gene is, respectively, more or less frequently present in an individual affected for breast cancer, compared to the frequency of its presence in a comparison group (control). The control group may in one embodiment be a population sample, i.e. a random sample from the general population. In
25 another embodiment, the control group is represented by a group of individuals who are breast cancer-free. Such breast cancer -free controls are those that have not been diagnosed with the breast cancer. In another embodiment, the breast cancer -free control group is characterized by the absence of one or more breast cancer -poor prognosis markers or characterized by the presence of one or more breast cancer -good prognosis markers.

30 As used herein, the term “treatment”, “treat” or “treating” refers to any act intended to ameliorate the health status of patients such as therapy, prevention, prophylaxis and retardation of the disease. In certain embodiments, such term refers to the amelioration or eradication of a disease or symptoms associated with a disease. In other embodiments, this

term refers to minimizing the spread or worsening of the disease resulting from the administration of one or more therapeutic agents to a subject with such a disease.

The term “therapy”, as used herein, refers to any type of treatment of cancer (i.e., antitumoral therapy), including an adjuvant therapy and a neoadjuvant therapy. Therapy
5 comprises radiotherapy and therapies, preferably systemic therapies such as hormone therapy, chemotherapy, immunotherapy and monoclonal antibody therapy.

The term “adjuvant therapy”, as used herein, refers to any type of treatment of cancer given as additional treatment, usually after surgical resection of the primary tumor, in a patient affected with a cancer that is at risk of metastasizing and/or likely to recur. The aim of
10 such an adjuvant treatment is to improve the prognosis. Adjuvant therapies comprise radiotherapy and therapy, preferably systemic therapy, such as hormone therapy, chemotherapy, immunotherapy and monoclonal antibody therapy.

The term “hormone therapy” or “hormonal therapy” refers to a cancer treatment having for purpose to block, add or remove hormones. For instance, in breast cancer, the
15 female hormones estrogen and progesterone can promote the growth of some breast cancer cells. So in these patients, hormone therapy is given to block estrogen and a non-exhaustive list commonly used drugs includes: Tamoxifen, Toremifene, Anastrozole, Exemestane, Letrozole, Goserelin/Leuprolide, Megestrol acetate, and Flouxymesterone.

As used herein, the term “chemotherapeutic treatment” or “chemotherapy” refers to a
20 cancer therapeutic treatment using chemical or biochemical substances, in particular using one or several antineoplastic agents.

The term “radiotherapeutic treatment” or “radiotherapy” is a term commonly used in the art to refer to multiple types of radiation therapy including internal and external radiation therapies or radioimmunotherapy, and the use of various types of radiations including X-rays,
25 gamma rays, alpha particles, beta particles, photons, electrons, neutrons, radioisotopes, and other forms of ionizing radiations.

As used herein, the term “good prognosis” refers to an increased patient survival and/or a late disease progression and/or a decreased disease recurrence and/or a decreased metastasis formation. The term “poor prognosis” indicates a decreased patient survival and/or
30 an early disease progression and/or an increase disease recurrence and/or an increase metastasis formation.

As used herein, the term “subject” or “patient” refers to an animal, preferably to a mammal, even more preferably to a human, including adult and child. However, the term “subject” can also refer to non-human animals, in particular mammals such as dogs, cats,

horses, cows, pigs, sheeps and non-human primates, among others, that are in need of treatment.

The term “sample”, as used herein, means any sample containing cells derived from a subject, preferably a sample which nucleic acids and/or proteins. Examples of such samples include fluids such as blood, plasma, saliva, and urine as well as biopsies, organs, tissues or cell samples. The sample may be treated prior to its use. The term “breast cancer sample” refers to any sample containing breast tumoral cells derived from a patient, preferably a sample which contains nucleic acids and/or proteins. Preferably, the sample contains only tumoral cells. The term “normal sample” refers to any sample which does not contain any
10 tumoral cells.

The term “reference copy number” refers to copy number of a chromosome region or of a gene thereof which is frequently present in a control group or to copy number of chromosome region or gene thereof in a normal sample. The normal sample is a non-tumoral sample. The normal sample may be obtained from the subject affected with the breast cancer
15 or from another subject, preferably a normal or healthy subject, i.e. a subject who does not suffer from a breast cancer. The copy number of a chromosome region or of a gene of a control group, e.g. a group of individuals characterized by the absence of breast cancer or by the presence of breast cancer with good prognosis, can be compared to copy number of a chromosome region or of a gene presents in an individual characterized by the presence of
20 breast cancer with poor prognosis. The reference copy number may be established based on retrospective studies, in particular from samples thereof.

The term “reference expression level” refers to expression level of a gene which is frequently present in a control group or to expression level of the gene in a normal sample. The normal sample is a non-tumoral sample. The normal sample may be obtained from the
25 subject affected with the cancer or from another subject, preferably a normal or healthy subject, i.e. a subject who does not suffer from a breast cancer. The expression level of a chromosome region or of a gene of a control group, e.g. a group of individuals characterized by the absence of breast cancer or by the presence of breast cancer with good prognosis, can be compared to expression level of a gene presents in an individual characterized by the
30 presence of breast cancer with poor prognosis. The reference copy number may be established based on retrospective studies, in particular from samples thereof.

The methods of the invention as disclosed below may be *in vivo*, *ex vivo* or *in vitro* methods, preferably *in vitro* methods.

In a previous study, an increased expression level of DUSP22 has been monitored in lymphocytes derived from radiation-exposed individuals (Fachin et al, 2009). It is to be noted that ionizing radiation (IR) at low doses and low dose rates has the potency to initiate carcinogenesis. Inventors herein surprisingly demonstrate that high copy numbers of 6p25 chromosome region or high copy numbers and/or expression levels of a gene included in this region, in particular DUSP22, are on the contrary associated with a good prognosis and thus with a decrease metastasis occurrence and risk of death.

Accordingly, the present invention concerns a method for predicting clinical outcome of a subject affected with a breast cancer, wherein the method comprises the step of determining copy number of 6p25 chromosome region, in particular 6p25.3, or copy number and/or expression level of a gene included in this region, in particular DUSP22, in a breast cancer sample from said subject, a high copy number of 6p25 chromosome region or a high copy number and/or expression level of a gene thereof being indicative of a good prognosis.

In another embodiment, the present invention concerns a method for predicting clinical outcome of a subject affected with a breast cancer, wherein the method comprises the step of determining copy number of 6p25 chromosome region, in particular 6p25.3, or copy number and/or expression level of a gene included in this region, in particular DUSP22, in a breast cancer sample from said subject, a low copy number of 6p25 chromosome region or a low copy number and/or expression level of a gene thereof being indicative of a poor prognosis.

In a preferred embodiment, the gene included in 6p25 chromosome region is DUSP22.

In an embodiment, the method further comprises the step of providing a sample from the subject. In a first embodiment, the sample is a breast cancer sample. This sample may be provided from a biopsy or from a surgical resection. In an alternative embodiment, the sample is a blood or serum sample from the subject. Indeed, it is hypothesized that the copy number variation is constitutional and not acquired. Therefore, a blood or serum sample may contain the same information than a breast cancer sample.

The copy number of the 6p25 chromosome region or of a gene included in this region is determined by quantitative or semi-quantitative PCR, or by real time quantitative or semi-quantitative PCR, in situ hybridization (such as fluorescent in situ hybridization (FISH)), Southern blotting, array-based methods and/or comparative genomic hybridization (CGH). In embodiments wherein DNA is being analyzed, the DNA may be genomic fragmented (e.g., sonicated, nebulized, restriction enzyme digested, sheared), or whole (e.g., not intentionally fragmented). For example, in some embodiments, a microarray assay is a nucleic acid assay

for CGH for identification of insertions and/or deletions in a genome wherein both a reference genomic DNA sample and a test genomic DNA sample are compared.

The expression level of a gene included in 6p25 chromosome region, preferably DUSP22, can be determined from a sample by a variety of techniques. In an embodiment, the expression level of a gene included in 6p25 chromosome region is determined by measuring the quantity of protein or mRNA encoded by said gene, preferably DUSP22 protein or DUSP22 mRNA, more preferably DUSP22 protein.

In a particular embodiment, the expression level of a gene included in 6p25 chromosome region, preferably DUSP22 gene, is determined by measuring the quantity of protein, preferably DUSP22 protein. The quantity of protein may be measured by any methods known by the skilled person. Usually, these methods comprise contacting the sample with a binding partner capable of selectively interacting with the protein present in the sample. The binding partner is generally a polyclonal or monoclonal antibody, preferably monoclonal. Polyclonal and monoclonal antibodies anti-DUSP22 are commercially available. Examples of these marketed antibodies are the rabbit polyclonal anti-DUSP22 from GenWay Biotech (Ref N° 18-461-10260), the rabbit polyclonal anti-human DUSP22 from ProteinTech Group (Ref N° 51005-2-AP), and the mouse polyclonal anti-DUSP22 from Sigma-Aldrich (Ref N° SAB1401777). The other antibodies used in the different methods to quantify the DUSP22 protein are well known by the skilled person and are commercially available. The quantity of protein may be measured by semi-quantitative Western blots, enzyme-labeled and mediated immunoassays, such as ELISAs, biotin/avidin type assays, radioimmunoassay, immunoelectrophoresis or immunoprecipitation or by protein or antibody arrays. The protein expression level may be assessed by immunohistochemistry on a tissue section of the sample (e.g. frozen or formalin-fixed paraffin embedded material). The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith. Preferably, the quantity of protein is measured by immunohistochemistry or semi-quantitative western-blot.

In another embodiment, of a gene included in 6p25 chromosome region, preferably DUSP22 gene, is determined by measuring the quantity of mRNA, preferably DUSP22 mRNA. Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the sample (e.g., cell or tissue prepared from the patient) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's

instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis) and/or amplification (e.g., RT-PCR). Preferably quantitative or semi-quantitative RT-PCR is preferred. Real-time quantitative or semi-quantitative RT-PCR is particularly advantageous. Preferably, primer pairs were designed in order to overlap an intron, so as to distinguish cDNA amplification from putative genomic contamination. An example of primer pair which may be used in this method is presented in the experimental section and is constituted by the primers couple Hs00169616_ml or Hs00414885_ml commercialized by TaqManR Gene Expression Assays Applied Biosystems. Other primers may be easily designed by the skilled person. Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA). Preferably, the quantity of mRNA is measured by quantitative or semi-quantitative RT-PCR or by real-time quantitative or semi-quantitative RT-PCR or by transcriptome approaches.

In an embodiment, the method further comprises the step of comparing the copy number of 6p25 chromosome region or copy number and/or expression level of a gene included in this region to a reference copy number and/or to a reference expression level.

In a particular embodiment, the reference copy number is the copy number of a chromosome region or of a gene thereof which is frequently present in a control group or to copy number of chromosome region or gene thereof in a normal sample. Preferably, the normal sample is a non-tumoral sample. The copy number of a chromosome region or of a gene of a control group, e.g. a group of individuals characterized by breast cancer or by the presence of breast cancer with good prognosis, can be compared to copy number of a chromosome region or of a gene presents in an individual characterized by the presence of breast cancer with poor prognosis. The reference copy number may be established based on retrospective studies, in particular from samples thereof.

In another particular embodiment, the reference expression level is expression level of the gene in a normal sample. The normal sample is a non-tumoral sample, preferably from the same tissue than the cancer sample. The normal sample may be obtained from the subject affected with the cancer or from another subject, preferably a normal or healthy subject, i.e. a subject who does not suffer from a cancer. Expression levels obtained from cancer and normal samples may be normalized by using expression levels of proteins which are known to have stable expression such as RPLPO (acidic ribosomal phosphoprotein PO), TBP (TATA box binding protein), GAPDH (glyceraldehyde 3-phosphate dehydrogenase) or β -actin.

In another embodiment, the reference expression level is the expression level of a gene having a stable expression in different cancer samples. Such genes include for example, RPLPO, TBP, GAPDH or β -actin. Preferably, the reference expression level is the expression level of the TBP gene. In a preferred embodiment, the quantity of mRNA, preferably DUSP22 mRNA, is normalized according to the quantity of TBP mRNA. The quantity of TBP mRNA is used as reference quantity (i.e. 100%). The quantity of mRNA, preferably DUSP22 mRNA, is expressed as a relative quantity with respect to the quantity of TBP mRNA.

If the reference expression level is the expression level of a gene included in 6p25 chromosome region (i.e., DUSP22) in a normal sample, the expression level of a gene included in 6p25 chromosome region, in the breast cancer sample is considered as high if, after normalization, the level is at least 2-fold higher than the expression level in the normal sample. Preferably, the expression level of a gene included in 6p25 chromosome region, preferably DUSP22 gene, in the breast cancer sample is considered as high if the level is at least 3-fold higher, or 5 or 10-fold higher, than the expression level in the normal sample.

In an embodiment, the present method further comprises assessing at least one another cancer or prognosis marker such as tumor grade, hormone receptor status, mitotic index, tumor size, HJURP expression level or expression of proliferation markers such as Ki67, MCM2, CAF-1 p60, CAF-1 p150, HP1alpha and ASF1b. These markers are commonly used and the results obtained with these markers may be combined with the results obtained with the present method in order to confirm the prognosis. The use of these markers is well-known by the skilled person. As example, the tumor grade may be determined according the Elston & Ellis method (Elston & Ellis, 2002), the hormone receptor status (estrogen and progesterone) may be determined at the protein or at the mRNA level, the mitotic index may be determined by counting mitotic cells in ten microscopic fields of a representative tissue section and the tumor size can be detected by imaging techniques (e.g. mammography), by palpation or after surgery in the excised tissue. Expression of Ki67 and CAF-1 can be assessed at the protein level or at the mRNA level. High grade, high mitotic index, large size and/or high Ki67 or CAF-1 expression are indicative for a worse prognosis. The HJURP expression level may be assessed as described in Hu et al., 2010. High HJURP expression is associated with poor clinical outcomes. HP1alpha and ASF1b have also been identified as prognosis marker in breast cancer (EP 10 164 424.3 and WO 2010/122137, respectively).

Using multivariate statistical analyses, the inventors have demonstrated, in the experimental section below, that copy number of 6p25 chromosome region or copy numbers

and/or expression levels of a gene included in this region, preferably DUSP22, predict disease outcome better than these standard prognostic markers.

In a further aspect, the present invention concerns a method for selecting a subject affected with a breast cancer for a therapy, preferably an adjuvant therapy, or determining whether a subject affected with a breast cancer is susceptible to benefit from a therapy, preferably an adjuvant therapy, wherein the method comprises the step of determining copy number of 6p25 chromosome region or copy number and/or expression level of a gene included in this region in a breast cancer sample from said subject, a low copy number of 6p25 chromosome region or a low copy number and/or expression level of a gene thereof indicating that a therapy, preferably an adjuvant therapy, is required.

In an embodiment, the method further comprises the step of providing a sample or breast cancer sample from the subject.

The copy number of 6p25 chromosome region or copy number and/or expression level of a gene included in this region, preferably DUSP22 gene, in the cancer sample is determined as described above, preferably by measuring the quantity of protein or mRNA encoded by said gene, preferably DUSP22 protein or DUSP22 mRNA.

In an embodiment, the method comprises the step of comparing the copy number of 6p25 chromosome region or copy number and/or expression level of a gene included in this region to a reference copy number and/or to a reference expression level as described above in relation with the method for predicting clinical outcome. The reference copy number is the copy number of a chromosome region or of a gene thereof which is frequently present in a control group or to copy number of chromosome region or gene thereof in a normal sample. The reference expression level may be the expression level of a gene present in a control group or in a normal sample or the expression level of the gene having a stable expression in different cancer samples, such as the TBP gene. When the copy number or expression level is compared to a reference copy number or expression level in a normal or control sample, by low is intended that the copy number or expression level is of the same order or magnitude than the reference copy number or expression level.

Accordingly, the method further comprises the step of determining whether the expression level of gene included in 6p25 chromosome region (i.e., DUSP22) is low compared to said reference expression level. In a particular embodiment, the expression level of the gene included in 6p25 chromosome region (i.e., DUSP22) in the sample is considered as low if, after normalization, the level is of the same order or magnitude than the expression level in the normal sample.

A low copy number of 6p25 chromosome region or a low copy number and/or expression level of a gene thereof indicates a decreased patient survival and/or an early disease progression and/or an increase disease recurrence and/or an increase metastasis formation, i.e a poor prognosis. Accordingly, this type of cancer associated with poor prognosis has to be treated with a therapy, preferably an adjuvant therapy, in order to improve the patient's chance for survival. The type of adjuvant therapy is chosen by the practitioner. In addition, this type of cancer also requires a closer monitor of the patient.

In an embodiment, the present method further comprises assessing at least one another cancer and prognosis marker such as tumor grade, hormone receptor status, mitotic index, tumor size, HJURP expression level or expression of proliferation markers such as Ki67, MCM2, CAF-1 p60, CAF-1 p150, HP1alpha and ASF1b. The results obtained with these markers may be used to confirm the result obtained with the method according to the invention and/or to orientate the choice of the adjuvant therapy.

The present invention further concerns the use of a gene included in 6p25 chromosome region as a marker or prognosis marker in breast cancer. In a preferred embodiment, the gene included in 6p25 chromosome region (i.e., DUSP22) is used as a prognosis marker in an early stage breast cancer without local or systemic invasion. As used herein, the term "prognosis marker" refers to a compound, i.e. a gene included in 6p25 chromosome region or protein thereof (i.e., DUSP22), used to predict or monitor clinical outcome of a subject affected with a breast cancer.

The present invention also concerns the use of a the 6p25 chromosome region of a gene included in 6p25 chromosome region (i.e., DUSP22) as a marker for selecting a subject affected with a breast cancer for a therapy, preferably an adjuvant therapy, or determining whether a subject affected with a cancer is susceptible to benefit from for a therapy, preferably an adjuvant therapy. Preferably, the breast cancer is a human breast cancer.

Gene included in 6p25 chromosome region or protein thereof (i.e., DUSP22) may be used as marker in any one of the methods of the invention as described above.

In another aspect, the present invention further concerns a kit

- (a) for predicting clinical outcome of a subject affected with a breast cancer; and/or
- (b) for selecting a subject affected with a breast cancer for a therapy or determining whether a subject affected with a breast cancer is susceptible to benefit from a therapy, wherein the kit comprises

(i) at least one antibody specific to a protein encoded by a gene included in 6p25 chromosome region (i.e., DUSP22) and, optionally, means for detecting the formation of the complex between said protein and said at least one antibody; and/or

(ii) at least one probe specific to the genomic DNA, mRNA or cDNA of a gene included in 6p25 chromosome region (i.e., DUSP22) and, optionally, means for detecting the hybridization of said at least one probe on said genomic DNA, mRNA or cDNA; and/or

(iii) at least one nucleic acid primer pair specific to said genomic DNA, mRNA or cDNA (i.e., DUSP22) and, optionally, means for amplifying and/or detecting said genomic DNA, mRNA or cDNA ; and,

10 (iv) optionally, a leaflet providing guidelines to use such a kit.

Further aspects and advantages of the present invention will be described in the following example, which should be regarded as illustrative and not limiting.

15 **EXAMPLES**

EXAMPLE 1

MATERIALS AND METHODS

Patients and tumours

Patients (n=126) were treated between February 2001 and april 2004 at the Institut
20 Curie for an invasive breast carcinoma. The first step of treatment was surgery associated with sentinel node (SN) procedure and bone marrow aspiration for the search of occult tumour cells (OTC). Tumours were analysed according to standard histological procedures. Histological analysis included the histological type and grade, the presence of vascular invasion and the Immunohistochemical analysis of estrogen and progesterone receptors and of
25 ERBB2 expression level.

Sentinel node procedure

All SN were identified separately and fixed in AFA (5% acetic acid, 75% pure ethanol, 18% demineralized water, and 2% formalin). They were cut in two, three, or four parts of equal thickness. Nodes of <0.3 cm were not cut. Each lymph node was separately
30 embedded in paraffin. One 3-µm-thick section was removed from each block and stained with HES (hematein, eosin, saffron). Cases with all SN free of metastasis (>2 mm) or of micrometastasis (0.2-2 mm) after standard microscopic examination were further analysed

using IHC to disclose occult metastasis. Three histologic sections taken at 150- μ m intervals were prepared. IHC was performed using pan-anti-cytokeratin antibody (K11, Immunotech, Marseille, France). After rehydration and antigen retrieval in citrate buffer (10 mn, pH 6.1), tissue sections were incubated with the primary monoclonal antibody, for 1 hour, at 1/100 dilution. The revelation of the staining was performed using the Vectastain Elite ABC peroxidase mouse IgG kit (Vector Burlingame, CA) and the diamino-benzidine (DAKO A/S, Glostrup, Denmark), as chromogen. Immunoreactions were done with an automatic immunostainer (TechMate Horizon, LJI Biosystems Inc.). After microscopic examination, the number and the type (isolated cells or clusters) of stained cells were registered.

10 Morphological analysis allowed exclusion of irrelevant staining of macrophages or of endothelial cells in some cases. Only cells with morphology compatible with that of epithelial cells and showing unambiguous cytoplasmic labelling were considered as positive events.

Preparation of the BM and ICC Staining

BM sampling, processing and mononuclear cells (MNC) staining have been described previously, along with the sensibility and the specificity of our protocol. Briefly, BM aspirates was performed at diagnosis from sternum or during primary surgery from both anterior iliac crests, under local or general anaesthesia respectively. After separation by density centrifugation, MNC were collected, and cytospins were prepared (1×10^6 MNC/slide). Three slides were incubated with the primary pan-cytokeratin monoclonal antibody A45-B/B3 (Micromet, Germany and Chromavision, USA), which recognizes several cytokeratin epitopes CK 8, CK 18 and CK 19. Negative controls, stained with anti-FITC IgG1 mouse antibody (Sigma Immuno Chemicals), were performed on an equivalent number of cells (i.e. 3 slides, 3×10^6 MNC) for each patient. Immune complexes formed by secondary anti-mouse antibody were revealed by the alkaline phosphatase/antialkaline phosphatase reaction, and the slides were counterstained with hematoxylin to study nuclear morphology. Bone marrows were classified according into three categories: absence of detected cytokeratin positive cell (BM-), presence of CK+ cells (Ock+), and presence of CK+ cells with atypical cytology (ITC). Atypical cytology was defined as large cell size (larger than surrounding haematopoietic cells), a high nuclear/cytoplasm ratio for isolated cells, or the presence of

30 clusters of cohesive cells with a large size.

Genomic profiles

A sufficient amount of tumour DNA was available for genomic profiling in 99 of the 126 patients. Genomic profiles were performed using Pac Bac Arrays. (IntegraChip genome-wide BAC arrays V7), NCBI Accession number: GPL9715 (see site

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL9715>). This technology makes it possible to characterise DNA copy number alterations. The microarrays have been analysed using the classical methods (GLAD, MANOR, VAMP) developed in the bioinformatics unit at Institut Curie. Minimal regions of alteration (both gained and lost) have been identified over the complete set of samples. These minimal regions have been compared with clinical annotations. More specifically, we have considered the sentinel lymph node status (no metastasis, "occult" metastasis, micro-metastasis, or metastasis) and bone metastasis status (yes or no).

Protein expression level of DUSP22 in breast carcinoma cells

The level of expression of the DUSP22 gene was assessed on tumours using immunohistochemistry. Experiments were performed using the rabbit polyclonal anti-DUSP22 antibody (GenWay Biotech San Diego CA; catalogue number 18-461-10260). The antibody was used at the dilution of 1/600, during 20 min, after antigen retrieval procedure at pH9. Experiments were performed in the whole series of 126 cases. Nucleic and cytoplasmic immunolabeling were recorded. The percentage of labelled cells and the staining intensity (+ or ++) were recorded.

RESULTS

Genomic profiles

The results of the genomic profiles have shown that, a minimal region located at 6p25 locus was strongly associated with both sentinel lymph node status and bone metastasis status (figure 1).

Genotyping of 6p25.3 region using SNP6 Affymetrix

We have analysed 6p25 minimal region using SNP6 Affymetrix in non tumor DNA extracted from tissue specimens of a series of patient to confirm that the variations in copy number observed corresponded to polymorphism and not to genetic rearrangement in tumor cells. To determine whether the DNA copy number variations observed in the 6p25.3 region corresponded to DNA polymorphism, and to more accurately determine the boundaries of this polymorphism, these further experiments were performed using Affymetrix Genome-Wide Human SNP Array 6.0. Six of the 99 cases were analysed on DNA extracted from tumors and from non tumor tissues. Three cases corresponded to a gain, two cases to a loss and one case to a normal status.

This analysis showed that the DNA copy number variations observed in tumor DNA were also found in non tumour DNA, confirming thus it corresponded to a polymorphism (figure 2). This polymorphism encompasses the DUSP22 locus. Its boundaries are variable.

Protein expression level of DUSP22 in breast carcinoma cells

5 Results were classified as: 0: non staining; 1: faint staining (+) in any proportion of the cells; 2 staining (++) in more than 40% of the cells (figure 3). No case with staining intensity ++ in less than 40% of the cells has been observed.

Results showed that no nuclear staining was observed in 38 cases, faint staining (+) in 47 cases and significant staining (++) in 41 cases. For statistical analyses, the bone marrow
10 status of the 41 cases with strong staining was compared with that of the cases with no or low staining. This comparison with yielded the following results

Table 1 Comparison between DUSP22 protein level and medullary status

DUSP22	Nb of cases	medullary status	
		positive	negative
0/+	85	30	55
++	41	7	34
	126	37	89

15 Using a chi2 test, there is a statistically significant association ($p=0.035$) between the expression level of the DUSP22 protein in tumor cells and the metastatic status of the bone marrow at the time of the diagnosis. A high expression level of DUSP22 is more likely to be associated with a non metastatic (negative) status of the bone marrow than with a metastatic status. This is in accordance with the data on genomic status which show a higher copy
20 number in non metastatic than in metastatic tumors.

Conclusion

Three main facts are reported in the present invention:

1) There is a significant association between genomic status of the 6p25
25 chromosome region and the sub-clinical metastatic status of breast cancer at the time of the diagnosis.

2) The genomic status of this locus is not related to genomic changes in tumor cells but is related to a copy number polymorphism. This polymorphism includes the DUSP22 gene

3) The expression level of the DUSP22 in carcinoma cells is related to the bone marrow status of the patients.

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CLAIMS

1. An *in vitro* method for predicting clinical outcome of a subject affected with breast cancer, wherein the method comprises the step of determining the copy number of the 6p25
5 chromosome region or the copy number and/or expression level of a gene included in said region in a sample from said subject, a high copy number of said 6p25 chromosome region or a high copy number and/or expression level of said gene thereof being indicative of a good prognosis.

2. The method according to claim 1, wherein the method comprises the step of
10 determining copy number of Dual specificity phosphatase 22 (DUSP22) and/or expression level of DUSP22 in a sample from said subject, a high copy number and/or expression level of DUSP22 being indicative of a good prognosis.

3. An *in vitro* method for selecting a subject affected with breast cancer for a therapy, preferably an adjuvant therapy, or determining whether a subject affected with breast cancer
15 is susceptible to benefit from a therapy, preferably an adjuvant therapy, wherein the method comprises the step of determining the copy number of the 6p25 chromosome region or the copy number and/or expression level of a gene included in said region in a sample from said subject, a low copy number of said 6p25 chromosome region or a low copy number and/or expression level of said gene thereof indicating that a therapy, preferably an adjuvant therapy,
20 is required.

4. The method according to claim 3, wherein the method comprises the step of determining the copy number of DUSP22 and/or expression level of DUSP22 in a sample from said subject, a low copy number and/or expression level of DUSP22 indicating that a therapy, preferably an adjuvant therapy, is required.

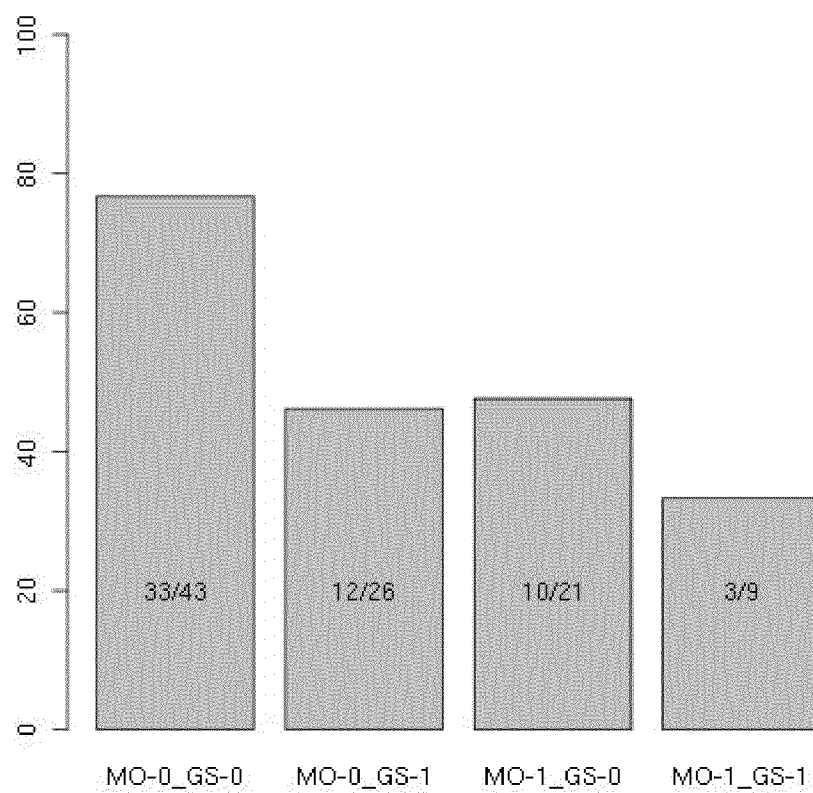
25 5. The method according to any one of claims 1 to 4, wherein the copy number of said 6p25 chromosome region or the copy number of said gene thereof, preferably DUSP22 gene, is determined by quantitative or semi-quantitative PCR, or by real time quantitative or semi-quantitative PCR, in situ hybridization (such as fluorescent in situ hybridization (FISH)), Southern blotting, array-based methods and/or comparative genomic hybridization (CGH).

6. The method according to any one of claims 1 to 4, wherein the expression level of said gene included in said 6p25 chromosome region, preferably DUSP22 gene, is determined by measuring the quantity of protein or mRNA encoded by said gene, preferably DUSP22 protein or DUSP22 mRNA, more preferably DUSP22 protein.
- 5 7. The method according to claim 6, wherein the quantity of protein, preferably DUSP22 protein, is measured by immunohistochemistry, semi-quantitative Western-Blot, or protein or antibody arrays.
8. The method according to claim 6, wherein the quantity of mRNA, preferably DUSP22 mRNA, is measured by quantitative or semi-quantitative RT-PCR, or by real time quantitative
10 or semi-quantitative RT-PCR or by Northern blot or by transcriptome approaches.
9. The method according to any one of the preceding claims, wherein said sample is a breast cancer sample.
10. The method according to any one of the preceding claims, wherein the method further comprises the step of comparing the copy number of said 6p25 chromosome region or the
15 copy number and/or expression level of said gene included in said region to a reference copy number and/or to a reference expression level.
11. The method according to any one of claims 1, 2 and 5 to 10, wherein a good prognosis is an increased patient survival and/or a late disease progression and/or a decreased disease recurrence and/or a decreased metastasis formation.
- 20 12. A use of the 6p25 chromosome region or of a gene included in said 6p25 chromosome region, preferably DUSP22, as a marker or prognosis marker in a subject affected with a breast cancer, in particular for determining whether a subject affected with a breast cancer is susceptible to benefit from a therapy, preferably an adjuvant therapy.
13. The method according to any one of claims 1 to 11 or the use according to claim 12,
25 wherein the breast cancer is an early stage breast cancer without local or systemic invasion, preferably a small-size breast cancer.
14. A kit (a) for predicting clinical outcome of a subject affected with a breast cancer; and/or (b) for selecting a subject affected with a breast cancer for a therapy or determining whether a subject affected with a breast cancer is susceptible to benefit from a therapy;

wherein the kit comprises (i) at least one antibody specific to a protein encoded by a gene included in 6p25 chromosome region; and/or (ii) at least one probe specific to the mRNA, cDNA or genomic DNA of a gene included in 6p25 chromosome region; and/or (iii) at least one nucleic acid primer pair specific to said genomic DNA, mRNA or cDNA; and optionally,
5 a leaflet providing guidelines to use such a kit.

15. Kit according to claim 14, wherein the gene is DUSP22.

1/3

**FIGURE 1**

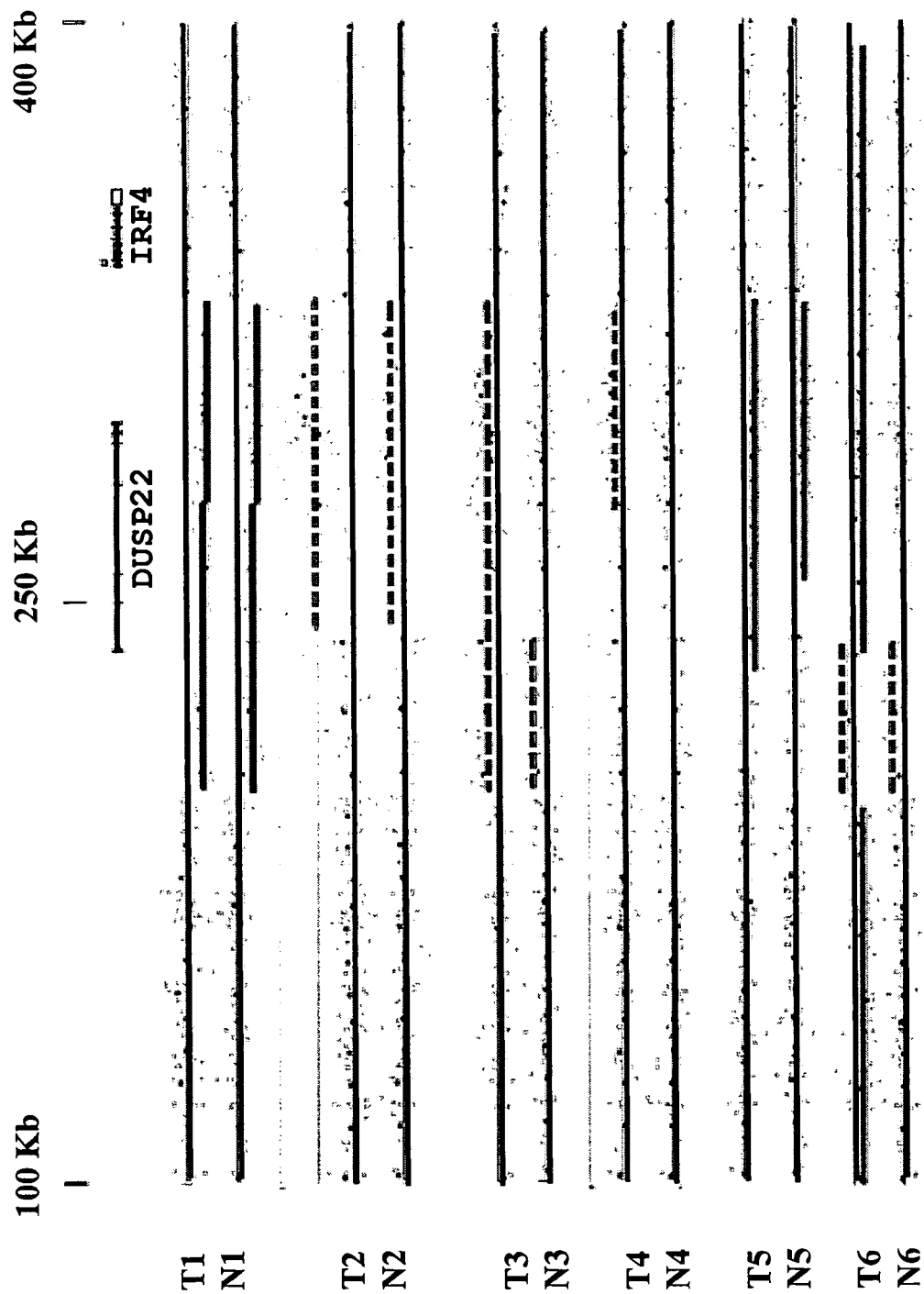


FIGURE 2

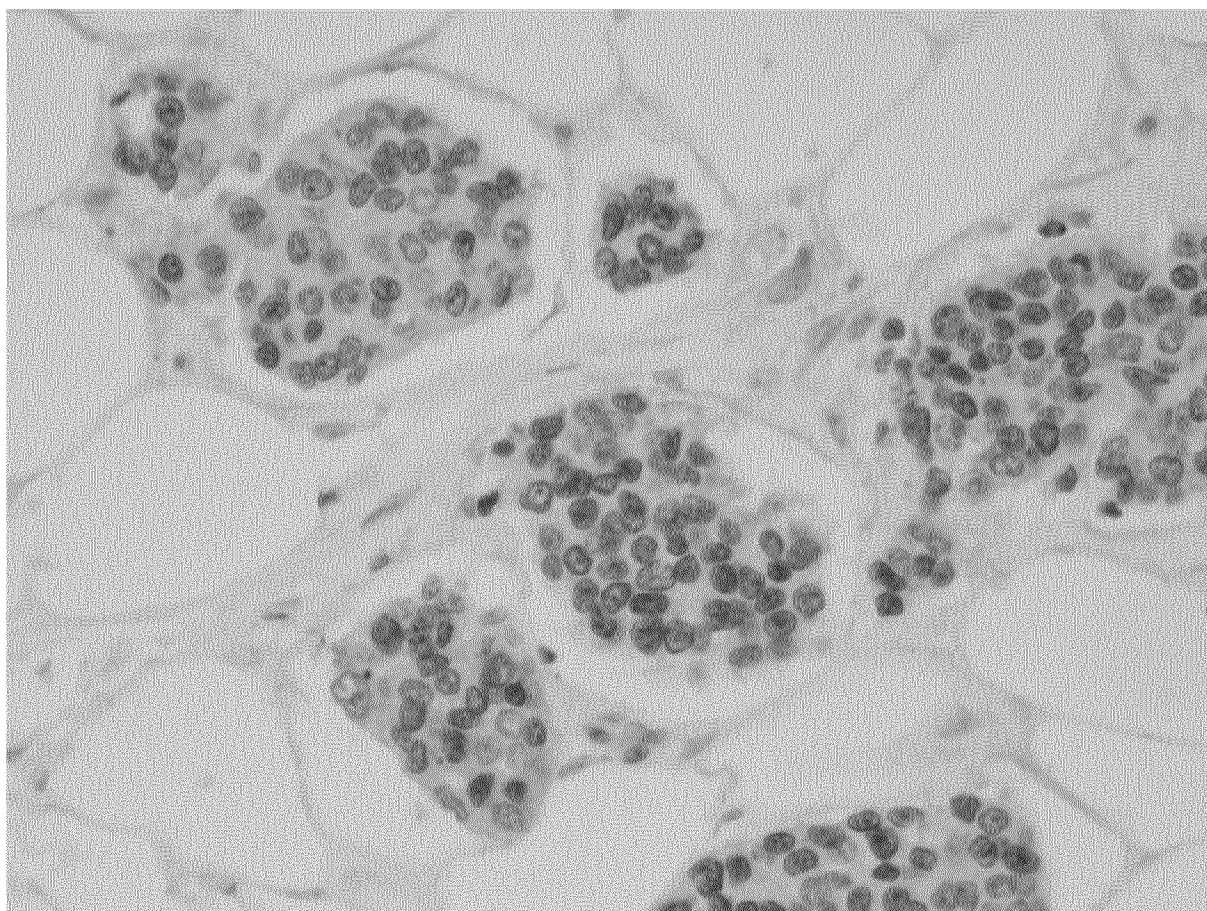


FIGURE 3

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2011/073905

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C12Q1/68 G01N33/574
 ADD.

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)

C12Q G01N

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 practical, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JENSSEN T-K ET AL: "Associations between gene expressions in breast cancer and patient survival", HUMAN GENETICS, SPRINGER, BERLIN, DE, vol. 111, 23 August 2002 (2002-08-23), pages 411-420, XP002963067, ISSN: 0340-6717, DOI: DOI:10.1007/S00439-002-0804-5 table 1 abstract page 412, left-hand column, paragraph 3rd full ----- -/--	1,3,5-13



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 document but published on or after the international filing date

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"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.

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Date of the actual completion of the international search

30 March 2012

Date of mailing of the international search report

12/04/2012

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2011/073905

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BERNARD-PIERROT ISABELLE ET AL: "Characterization of the recurrent 8p11-12 amplicon identifies PPAPDC1B, a phosphatase protein, as a new therapeutic target in breast cancer", CANCER RESEARCH, vol. 68, no. 17, September 2008 (2008-09), pages 7165-7175, XP002632510, ISSN: 0008-5472 figure 5d abstract page 7165, right-hand column, paragraph 2nd full page 7166, left-hand column, paragraph 1st full page 7173, left-hand column -----	1-13
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Y	US 2009/203051 A1 (GRAY JOE W [US] ET AL) 13 August 2009 (2009-08-13) paragraphs [0010], [0061], [0070] ----- -/--	1-15

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2011/073905

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2011/073905

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