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(54) Title: DNA SEQUENCES DIFFERENTIALLY EXPRESSED IN TUMOUR CELL LINES

(57) Abstract: A method for the diagnosis of cancer, or a predisposition thereto, in a patient, comprising the steps of: (1) establishing the level of expression of a gene selected from the group consisting of cytochrome B-245, alpha polypeptide (CYBA), cadherin 15 (CDH15), dipeptidase 1 (DPEP1), solute carrier family 7, member 5 (SLC7A5), BNO8, BNO205 and BNO221; and (2) comparing expression of the gene to a baseline established from expression in normal tissue controls; wherein substantial variants from the baseline indicates that the patient is susceptible to cancer.



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DNA SEQUENCES DIFFERENTIALLY EXPRESSED IN TUMOUR CELL  
LINES

Technical Field

5       The present invention is concerned with DNA sequences from the 16q24.3 region which have been shown to be differentially expressed in breast cancer cell lines, and are therefore implicated in the development of carcinomas.

Background Art

10       The development of human carcinomas has been shown to arise from the accumulation of genetic changes involving both positive regulators of cell function (oncogenes) and negative regulators (tumour suppressor genes). For a normal somatic cell to evolve into a metastatic tumour it  
15       requires changes at the cellular level, such as immortalisation, loss of contact inhibition and invasive growth capacity, and changes at the tissue level, such as evasion of host immune responses and growth restraints imposed by surrounding cells, and the formation of a blood  
20       supply for the growing tumour.

      Molecular genetic studies of colorectal carcinoma have provided substantial evidence that the generation of malignancy requires the sequential accumulation of a number of genetic changes within the same epithelial stem  
25       cell of the colon. For a normal colonic epithelial cell to become a benign adenoma, progress to intermediate and late adenomas, and finally become a malignant cell, inactivating mutations in tumour suppressor genes and activating mutations in proto-oncogenes are required  
30       (Fearon and Vogelstein, 1990).

      The employment of a number of techniques, such as loss of heterozygosity (LOH), comparative genomic hybridisation (CGH) and cytogenetic studies of cancerous tissue, all of which exploit chromosomal abnormalities  
35       associated with the affected cell, has aided in the identification of a number of tumour suppressor genes and oncogenes associated with a range of tumour types.

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In one aspect, studies of cancers such as retinoblastoma and colon carcinoma have supported the model that LOH is a specific event in the pathogenesis of cancer and has provided a mechanism in which to identify the cancer causing genes. For instance in colorectal carcinoma, inherited forms of the disease have been mapped to the long arm of chromosome 5 while LOH at 5q has been reported in both the familial and sporadic versions of the disease. The APC tumour suppressor gene, mapping to this region, was subsequently shown to be involved (Grodin et al., 1991). The model is further highlighted in Von Hippel-Lindau (VHL) syndrome, a rare disorder that predisposes individuals to a variety of tumours including clear cell carcinomas of the kidneys and islet cell tumours of the pancreas. Both sporadic and inherited cases of the syndrome show LOH for the short arm of chromosome 3 and somatic translocations involving 3p in sporadic tumours, and genetic linkage to the same region in affected families has also been observed. The VHL tumour suppressor gene has since been identified from this region of chromosome 3 and mutations in it have been detected in 100% of patients who carry a clinical diagnosis of VHL disease. In addition, the VHL gene is inactivated in approximately 50-80% of the more common sporadic form of renal clear cell carcinoma.

The genetic determinants involved in breast cancer are not as well defined as that of colon cancer due in part to the histological stages of breast cancer development being less well characterised. However, as with colon carcinoma, it is believed that a number of genes need to become involved in a stepwise progression during breast tumourigenesis.

Certain women appear to be at an increased risk of developing breast cancer. Genetic linkage analysis has shown that 5 to 10% of all breast cancers are due to at least two autosomal dominant susceptibility genes. Generally, women carrying a mutation in a susceptibility

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gene develop breast cancer at a younger age compared to the general population, often have bilateral breast tumours, and are at an increased risk of developing cancers in other organs, particularly carcinoma of the ovary.

Genetic linkage analysis on families showing a high incidence of early-onset breast cancer (before the age of 46) was successful in mapping the first susceptibility gene, *BRCA1*, to chromosome 17q21 (Hall et al., 1990). Subsequent to this, the *BRCA2* gene was mapped to chromosome 13q12-q13 (Wooster et al., 1994) with this gene conferring a higher incidence of male breast cancer and a lower incidence of ovarian cancer when compared to *BRCA1*.

Both *BRCA1* and *BRCA2* have since been cloned (Miki et al., 1994; Wooster et al., 1995) and numerous mutations have been identified in these genes in susceptible individuals with familial cases of breast cancer.

Additional inherited breast cancer syndromes exist, however they are rare. Inherited mutations in the *TP53* gene have been identified in individuals with Li-Fraumeni syndrome, a familial cancer resulting in epithelial neoplasms occurring at multiple sites including the breast. Similarly, germline mutations in the *MMAC1/PTEN* gene involved in Cowden's disease and the ataxia telangiectasia (*AT*) gene have been shown to confer an increased risk of developing breast cancer, among other clinical manifestations, but together account for only a small percentage of families with an inherited predisposition to breast cancer.

Somatic mutations in the *TP53* gene have been shown to occur in a high percentage of individuals with sporadic breast cancer. However, although LOH has been observed at the *BRCA1* and *BRCA2* loci at a frequency of 30 to 40% in sporadic cases (Cleton-Jansen et al., 1995; Saito et al., 1993), there is virtually no sign of somatic mutations in the retained allele of these two genes in sporadic cancers (Futreal et al., 1994; Miki et al., 1996). Recent data

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suggests that DNA methylation of the promoter sequence of these genes may be an important mechanism of down-regulation. The use of both restriction fragment length polymorphisms and small tandem repeat polymorphic markers has identified numerous regions of allelic imbalance in breast cancer suggesting the presence of additional genes, which may be implicated in breast cancer. Data compiled from more than 30 studies reveals the loss of DNA from at least 11 chromosome arms at a frequency of more than 25%, with regions such as 16q and 17p affected in more than 50% of tumours (Devilee and Cornelisse, 1994; Brenner and Aldaz, 1995). However only some of these regions are known to harbour tumour suppressor genes shown to be mutated in individuals with both sporadic (*TP53* and *RB* genes) and familial (*TP53*, *RB*, *BRCA1*, and *BRCA2* genes) forms of breast cancer.

Cytogenetic studies have implicated loss of the long arm of chromosome 16 as an early event in breast carcinogenesis since it is found in tumours with few or no other cytogenetic abnormalities. Alterations in chromosome 1 and 16 have also been seen in several cases of ductal carcinoma *in situ* (DCIS), the preinvasive stage of ductal breast carcinoma. In addition, LOH studies on DCIS samples identified loss of 16q markers in 29 to 89% of the cases tested (Chen et al., 1996; Radford et al., 1995). In addition, examination of tumours from other tissue types have indicated that 16q LOH is also frequently seen in prostate, lung, hepatocellular, ovarian, primitive neuroectodermal and Wilms' tumours.

Together, these findings suggest the presence of a gene mapping to the long arm of chromosome 16 that is critically involved in the early development of a large proportion of breast cancers as well as cancers from other tissue types, but to date no such gene has been identified.

Disclosure of the Invention

The present invention provides nucleic acid and protein sequences that are differentially expressed in breast cancer when compared to normal tissue controls, here-in termed "breast cancer sequences". As outlined below, breast cancer sequences that are differentially expressed include those that are down-regulated in breast cancer (tumour suppressor genes) as well as those that are up-regulated in breast cancer (oncogenes). The differential expression of these sequences in breast cancer combined with the fact they have been identified from a region of LOH seen in breast cancer as well as other carcinomas including prostate tumours suggests they are contributory factors in cancer. The breast cancer sequences of the invention are described in Table 1 and are represented by SEQ ID Numbers: 1-11.

"Down-regulation" as used herein means at least about a 15 to 49 fold decrease in expression, preferably at least about a 50 to 79 fold decrease in expression, with at least about an 80 fold or higher decrease in expression being preferred (assuming a relative fold variability index of 50 or higher).

"Up-regulation" as used herein means at least about a 15 to 49 fold increase in expression, preferably at least about a 50 to 79 fold increase in expression, with at least about an 80 fold or higher increase in expression being preferred (assuming a relative fold variability index of 50 or higher).

The present invention also encompasses isolated nucleic acid and/or amino acid sequences which are homologous to the breast cancer sequences described above. Such homology is based on the overall nucleic acid or amino acid sequence of the group described in Table 1 and represented by the SEQ ID Numbers: 1-11 and is determined using either homology programs or hybridisation conditions as outlined below.

A nucleic acid or protein is a breast cancer nucleic

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acid or protein if the overall homology of the nucleic acid or protein sequence to one of the sequences described in Table 1 and represented by the SEQ ID Numbers: 1-11is at least 70%, preferably 85% and most preferably 95%.

5 Homology in this context means sequence similarity or identity, with identity being preferred.

In a preferred embodiment, the sequences which are used to determine sequence identity or similarity are selected from the sequences described in Table 1 and  
10 represented by the SEQ ID Numbers: 1-11or are naturally occurring allelic variants, sequence variants or splice variants of these sequences.

Sequence identity is typically calculated using the BLAST algorithm, described in Altschul et al *Nucleic Acids*  
15 *Res.* 25, 3389-3402 (1997) with the BLOSUM62 default matrix.

In one embodiment, nucleic acid homology can be determined through hybridisation studies. Nucleic acids which hybridise under stringent conditions to the nucleic  
20 acids of the invention are considered breast cancer sequences. Under stringent conditions, hybridisation will most preferably occur at 42°C in 750 mM NaCl, 75 mM trisodium citrate, 2% SDS, 50% formamide, 1X Denhart's, 10% (w/v) dextran sulphate and 100 µg/ml denatured salmon  
25 sperm DNA. Useful variations on these conditions will be readily apparent to those skilled in the art. The washing steps which follow hybridization most preferably occur at 65°C in 15 mM NaCl, 1.5 mM trisodium citrate, and 1% SDS. Additional variations on these conditions will be readily  
30 apparent to those skilled in the art.

In a further aspect, the invention provides breast cancer sequences as described in Table 1 and represented by the SEQ ID Numbers: 1-11, or the nucleotide sequence of a nucleic acid which hybridises thereto as described  
35 above, and appropriate control elements of the breast cancer sequences.

Preferably the control elements are those which

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mediate expression in breast tissue, but may also mediate expression in other tissues including, but not restricted to, prostate, liver and ovary. The breast cancer nucleic acid sequences of the present invention can be engineered using methods accepted in the art so as to alter the sequences for a variety of purposes. These include, but are not limited to, modification of the cloning, processing, and/or expression of the gene product. PCR reassembly of gene fragments and the use of synthetic oligonucleotides allow the engineering of breast cancer sequences of the invention. For example, oligonucleotide-mediated site-directed mutagenesis can introduce mutations that create new restriction sites, alter glycosylation patterns and produce splice variants etc.

As a result of the degeneracy of the genetic code, a number of polynucleotide sequences encoding breast cancer proteins of the invention, some that may have minimal similarity to the polynucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention includes each and every possible variation of polynucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the polynucleotide sequence of naturally occurring breast cancer sequences, and all such variations are to be considered as being specifically disclosed.

The polynucleotides of this invention include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified, or may contain non-natural or derivatised nucleotide bases as will be appreciated by those skilled in the art. Such modifications include labels, methylation, intercalators, alkylators and modified linkages. In some instances it may be advantageous to produce nucleotide sequences encoding breast cancer sequences of the invention, or their



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derivatives, possessing a substantially different codon usage than that of the naturally occurring gene. For example, codons may be selected to increase the rate of expression of the peptide in a particular prokaryotic or eukaryotic host corresponding with the frequency that particular codons are utilized by the host. Other reasons to alter the nucleotide sequence encoding breast cancer sequences of the invention, or their derivatives, without altering the encoded amino acid sequences include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

In some instances the breast cancer nucleic acid sequences of the invention are fragments of larger genes and may be used to identify and obtain corresponding full-length genes. Full-length sequences of the breast cancer genes can be obtained using the partial gene sequences, such as BNO8, BNO205 and BNO221 described in Table 1, by methods known *per se* to those skilled in the art. For example, "restriction-site PCR" may be used to retrieve unknown sequence adjacent to a portion of DNA whose sequence is known. In this technique universal primers are used to retrieve unknown sequence. Inverse PCR may also be used, in which primers based on the known sequence are designed to amplify adjacent unknown sequences. These upstream sequences may include promoters and regulatory elements. In addition, various other PCR-based techniques may be used, for example a kit available from Clontech (Palo Alto, California) allows for a walking PCR technique, the 5'RACE kit (Gibco-BRL) allows isolation of additional 5' gene sequence while additional 3' sequence can be obtained using practised techniques (for eg see Gecz et al., 1997).

The invention also encompasses production of breast cancer sequences of the invention entirely by synthetic chemistry. Synthetic sequences may be inserted into expression vectors and cell systems that contain the

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necessary elements for transcriptional and translational control of the inserted coding sequence in a suitable host. Numerous types of appropriate expression vectors and suitable regulatory elements are known in the art for a variety of host cells. Regulatory elements may include regulatory sequences, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, 5' and 3' untranslated regions and specific translational start and stop signals (such as an ATG initiation codon and Kozak consensus sequence). Regulatory elements will allow more efficient translation of sequences encoding breast cancer genes of the invention. In cases where the complete coding sequence including the initiation codon and upstream regulatory sequences are inserted into the appropriate expression vector, additional control signals may not be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals as described above should be provided by the vector. Such signals may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate for the particular host cell system used (Scharf et al., 1994).

The present invention allows for the preparation of purified breast cancer polypeptide or protein, from the polynucleotides of the present invention or variants thereof. In order to do this, host cells may be transfected with a nucleic acid molecule as described above. Typically said host cells are transfected with an expression vector comprising a nucleic acid encoding a breast cancer protein according to the invention. Cells are cultured under the appropriate conditions to induce or cause expression of the breast cancer protein. The conditions appropriate for breast cancer protein expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art.

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A variety of expression vector/host systems may be utilized to contain and express the breast cancer sequences of the invention and are well known in the art. These include, but are not limited to, microorganisms such as bacteria transformed with plasmid or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with viral expression vectors (e.g., baculovirus); or mouse or other animal or human tissue cell systems. In a preferred embodiment the breast cancer proteins of the invention are expressed in mammalian cells using various expression vectors including plasmid, cosmid and viral systems such as adenoviral, retroviral or vaccinia virus expression systems. The invention is not limited by the host cell employed.

The polynucleotide sequences, or variants thereof, of the present invention can be stably expressed in cell lines to allow long term production of recombinant proteins in mammalian systems. These sequences can be transformed into cell lines using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. The selectable marker confers resistance to a selective agent, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be propagated using tissue culture techniques appropriate to the cell type.

The protein produced by a transformed cell may be secreted or retained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode a protein of the invention may be designed to contain signal sequences which direct secretion of the protein through a prokaryotic or eukaryotic cell membrane.

In addition, a host cell strain may be chosen for its

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ability to modulate expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, glycosylation, phosphorylation, and acylation. Post-translational cleavage of a "prepro" form of the protein may also be used to specify protein targeting, folding, and/or activity. Different host cells having specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO or HeLa cells), are available from the American Type Culture Collection (ATCC) and may be chosen to ensure the correct modification and processing of the foreign protein.

When large quantities of protein are needed such as for antibody production, vectors which direct high levels of breast cancer gene expression may be used such as those containing the T5 or T7 inducible bacteriophage promoter. The present invention also includes the use of the expression systems described above in generating and isolating fusion proteins which contain important functional domains of the protein. These fusion proteins are used for binding, structural and functional studies as well as for the generation of appropriate antibodies.

In order to express and purify the protein as a fusion protein, the appropriate cDNA sequence is inserted into a vector which contains a nucleotide sequence encoding another peptide (for example, glutathionine succinyl transferase). The fusion protein is expressed and recovered from prokaryotic or eukaryotic cells. The fusion protein can then be purified by affinity chromatography based upon the fusion vector sequence. The relevant protein can subsequently be obtained by enzymatic cleavage of the fusion protein.

In one embodiment, a fusion protein may be generated by the fusion of a breast cancer polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is

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generally placed at the amino- or carboxy-terminus of the breast cancer polypeptide. The presence of such epitope-tagged forms of a breast cancer polypeptide can be detected using an antibody against the tag polypeptide.

5 Also, provision of the epitope tag enables the breast cancer polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

10 Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine or poly-histidine-glycine tags and the c-myc tag and antibodies thereto.

15 Fragments of breast cancer polypeptide may also be produced by direct peptide synthesis using solid-phase techniques. Automated synthesis may be achieved by using the ABI 431A Peptide Synthesizer (Perkin-Elmer). Various fragments of breast cancer polypeptide may be synthesized separately and then combined to produce the full-length molecule.

20 In a further aspect of the invention there is provided a method of preparing a polypeptide as described above, comprising the steps of:

(1) culturing the host cells under conditions effective for production of the polypeptide; and

25 (2) harvesting the polypeptide.

Substantially purified breast cancer proteins or fragments thereof can then be used in further biochemical analyses to establish secondary and tertiary structure for example by x-ray crystallography of the protein or by  
30 nuclear magnetic resonance (NMR). Determination of structure allows for the rational design of pharmaceuticals to interact with the protein, alter protein charge configuration or charge interaction with other proteins, or to alter its function in the cell.

35 The breast cancer sequences of the present invention have been identified from a region of restricted LOH seen in breast cancer. In addition, these breast cancer genes

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have been shown to be differentially expressed in breast cancer samples compared with normal tissue controls. As LOH is suggestive of the presence of a tumour suppressor gene, those breast cancer genes of the invention that are down-regulated in their expression in cancerous tissue, as highlighted in Figures 2 and 3 and listed in SEQ ID Numbers: 1-9 represent tumour suppressor genes in the 16q24.3 region. As many of these genes are expressed in a wide variety of tissues and LOH of 16q has been found in cancers of other tissue types, including prostate, liver, ovary, primitive neuroectodermal and Wilms' tumours, they may represent tumour suppressor genes involved in a range of cancers. Such cancers may include, but are not limited to adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, teratocarcinoma, and, in particular, cancers of the breast, prostate, blood, germ cells, liver, ovary, adrenal gland, cervix, heart, brain, lung, placenta, skeletal muscle, synovial membrane, tonsil, lymph tissue, kidney, colon, uterus, skin and testis. Other cancers may include those of the head and neck, bladder, bone, bone marrow, gall bladder, ganglia, gastrointestinal tract, pancreas, parathyroid, penis, salivary glands, spleen, stomach, thymus and thyroid gland.

In addition, the identification of BNO223 (SEQ ID Numbers: 10 and 11) to be up-regulated in its expression in breast cancer samples suggests a role as an oncogene. This gene is also expressed in many tissue types and as such may be a causative factor in other cancers such as those listed above.

With the identification of the breast cancer nucleotide and protein sequences of the invention, probes and antibodies raised to the genes can be used in a variety of hybridisation and immunological assays to screen for and detect the presence of either a normal or mutated gene or gene product.

In addition the nucleotide and protein sequences of the breast cancer genes provided in this invention enable

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therapeutic methods for the treatment of cancers associated with one or more of these genes, enable screening of compounds for therapeutic intervention, and also enable methods for the diagnosis or prognosis of all  
5 cancers associated with the these genes. Examples of such cancers include, but are not limited to, those listed above.

In the treatment of cancers associated with down-regulated gene expression and/or activity, it is desirable  
10 to increase the expression and/or activity of the relevant gene. In the treatment of disorders associated with up-regulated gene expression and/or activity, it is desirable to decrease the expression and/or activity of the relevant gene.

15

Enhancing breast cancer gene or protein function

Enhancing, stimulating or re-activating the function of those breast cancer genes or proteins that are down-regulated in cancer can be achieved in a variety of ways  
20 as would be appreciated by those skilled in the art.

In a preferred embodiment a breast cancer gene of the invention is administered to a subject to treat or prevent a cancer associated with decreased activity and/or expression of the gene.

25

In a further aspect, there is provided the use of a nucleic acid molecule of the invention, as described above, in the manufacture of a medicament for the treatment of a cancer associated with decreased activity and/or expression of the corresponding gene.

30

Typically, a vector capable of expressing a breast cancer gene of the invention, or fragment or derivative thereof, may be administered to a subject to treat or prevent a cancer associated with decreased activity and/or expression of the gene, including but not limited to,  
35 those described above.

Transducing retroviral vectors are often used for somatic cell gene therapy because of their high efficiency

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of infection and stable integration and expression. The full-length breast cancer gene, or portions thereof, can be cloned into a retroviral vector and expression can be driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest. Other viral vectors can be used and include, as is known in the art, adenoviruses, adeno-associated virus, vaccinia virus, papovaviruses, lentiviruses and retroviruses of avian, murine and human origin.

Gene therapy would be carried out according to established methods (Friedman, 1991; Culver, 1996). A vector containing a copy of a breast cancer gene linked to expression control elements and capable of replicating inside the cells is prepared. Alternatively the vector may be replication deficient and may require helper cells or helper virus for replication and virus production and use in gene therapy.

Gene transfer using non-viral methods of infection can also be used. These methods include direct injection of DNA, uptake of naked DNA in the presence of calcium phosphate, electroporation, protoplast fusion or liposome delivery. Gene transfer can also be achieved by delivery as a part of a human artificial chromosome or receptor-mediated gene transfer. This involves linking the DNA to a targeting molecule that will bind to specific cell-surface receptors to induce endocytosis and transfer of the DNA into mammalian cells. One such technique uses poly-L-lysine to link asialoglycoprotein to DNA. An adenovirus is also added to the complex to disrupt the lysosomes and thus allow the DNA to avoid degradation and move to the nucleus. Infusion of these particles intravenously has resulted in gene transfer into hepatocytes.

In affected subjects that express a mutated form of a breast cancer gene of the invention, it may be possible to prevent the cancer by introducing into the affected cells



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a wild-type copy of the gene such that it recombines with the mutant gene. This requires a double recombination event for the correction of the gene mutation. Vectors for the introduction of genes in these ways are known in the art, and any suitable vector may be used. Alternatively, introducing another copy of the gene bearing a second mutation in that gene may be employed so as to negate the original gene mutation and block any negative effect.

In a still further aspect the invention provides a method for the treatment of a cancer associated with decreased activity and/or expression of a breast cancer gene of the invention, comprising administering a polypeptide as described above, or an agonist thereof, to a subject in need of such treatment.

In another aspect the invention provides the use of a polypeptide as described above, or an agonist thereof, in the manufacture of a medicament for the treatment of a cancer associated with decreased activity and/or expression of a breast cancer gene.

In affected subjects that have decreased expression of a breast cancer gene, a mechanism of down-regulation may be abnormal methylation of a CpG island if present in the 5' end of the gene. Therefore, in an alternative approach to therapy, administration of agents that remove breast cancer gene promoter methylation will reactivate its expression which may suppress the associated cancer phenotype.

#### Inhibiting breast cancer gene or protein function

Inhibiting the function of those breast cancer genes or proteins of the invention that are up-regulated in cancer can be achieved in a variety of ways as would be appreciated by those skilled in the art.

In one aspect of the invention there is provided a method of treating a cancer associated with increased activity and/or expression of a breast cancer gene, comprising administering an antagonist of the gene to a

subject in need of such treatment.

In still another aspect of the invention there is provided the use of an antagonist of a breast cancer gene in the manufacture of a medicament for the treatment of a cancer associated with increased activity and/or expression of the gene.

In one aspect of the invention an isolated DNA molecule, which is the complement of any one of the DNA molecules described above and which encodes an RNA molecule that hybridises with the mRNA encoded by a breast cancer gene of the invention, may be administered to a subject in need of such treatment.

In a still further aspect of the invention there is provided the use of an isolated DNA molecule which is the complement of a nucleic acid molecule of the invention and which encodes an RNA molecule that hybridises with the mRNA encoded by a breast cancer gene, in the manufacture of a medicament for the treatment of a disorder associated with increased activity and/or expression of the gene.

Typically, a vector expressing the complement of a polynucleotide encoding a breast cancer gene of the invention may be administered to a subject to treat or prevent a disorder associated with increased activity and/or expression of the gene including, but not limited to, those described above. Antisense strategies may use a variety of approaches including the use of antisense oligonucleotides, ribozymes, DNazymes, injection of antisense RNA and transfection of antisense RNA expression vectors. Many methods for introducing vectors into cells or tissues are available and equally suitable for use *in vivo*, *in vitro*, and *ex vivo*. For *ex vivo* therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection, by liposome injections, or by polycationic amino polymers may be achieved using methods which are well known in the art. (For example, see Goldman et al., 1997).

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According to still another aspect of the invention, there is provided a method of treating a cancer associated with increased activity and/or expression of a breast cancer gene of the invention comprising administering an  
5 antagonist of the gene to a subject in need of such treatment.

In still another aspect of the invention there is provided the use of an antagonist of a breast cancer gene of the invention in the manufacture of a medicament for  
10 the treatment of a cancer associated with increased activity and/or expression of the gene.

Such disorders may include, but are not limited to, those discussed above. In one aspect purified protein according to the invention may be used to produce  
15 antibodies which specifically bind the breast cancer protein. These antibodies may be used directly as an antagonist or indirectly as a targeting or delivery mechanism for bringing a pharmaceutical agent to cells or tissues that express the protein. Such antibodies may  
20 include, but are not limited to, polyclonal, monoclonal, chimeric and single chain antibodies as would be understood by the person skilled in the art.

For the production of antibodies, various hosts including rabbits, rats, goats, mice, humans, and others  
25 may be immunized by injection with a protein of the invention or with any fragment or oligopeptide thereof, which has immunogenic properties. Various adjuvants may be used to increase immunological response and include, but are not limited to, Freund's, mineral gels such as  
30 aluminum hydroxide, and surface-active substances such as lysolecithin. Adjuvants used in humans include BCG (bacilli Calmette-Guerin) and Corynebacterium parvum.

It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to the breast cancer  
35 proteins of the invention have an amino acid sequence consisting of at least about 5 amino acids, and, more preferably, of at least about 10 amino acids. It is also

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preferable that these oligopeptides, peptides, or fragments are identical to a portion of the amino acid sequence of the natural protein and contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of amino acids from these proteins may be fused with those of another protein, such as KLH, and antibodies to the chimeric molecule may be produced.

Monoclonal antibodies to breast cancer proteins of the invention may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique. (For example, see Kohler et al., 1975; Kozbor et al., 1985; Cote et al., 1983; Cole et al., 1984).

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature. (For example, see Orlandi et al., 1989; Winter et al., 1991).

Antibody fragments which contain specific binding sites for the breast cancer proteins may also be generated. For example, such fragments include, F(ab')<sub>2</sub> fragments produced by pepsin digestion of the antibody molecule and Fab fragments generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. (For example, see Huse et al., 1989).

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve

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the measurement of complex formation between a protein and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes is preferred, but a competitive binding assay may also be employed.

#### Drug screening

According to still another aspect of the invention, the breast cancer nucleic acids and proteins of the invention, and cells expressing these, are useful for screening of candidate pharmaceutical agents or compounds in a variety of techniques for the treatment of cancers associated with their dysfunction.

Candidate pharmaceutical agents or compounds encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having molecular weight of more than 100 and less than about 2,500 daltons. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids and steroids. Particularly preferred are peptides.

Agent screening techniques include, but are not limited to, utilising eukaryotic or prokaryotic host cells that are stably transformed with recombinant molecules expressing a particular breast cancer polypeptide of the invention, or fragment thereof, preferably in competitive binding assays. Binding assays will measure for the formation of complexes between the breast cancer polypeptide, or fragments thereof, and the agent being tested, or will measure the degree to which an agent being tested will interfere with the formation of a complex between the breast cancer polypeptide, or fragment thereof, and a known ligand.

Another technique for drug screening provides high-throughput screening for compounds having suitable binding affinity to a breast cancer polypeptide (see PCT published application WO84/03564). In this stated technique, large numbers of small peptide test compounds can be synthesised

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on a solid substrate and can be assayed through breast cancer polypeptide binding and washing. Bound breast cancer polypeptide is then detected by methods well known in the art. In a variation of this technique, purified  
5 polypeptides can be coated directly onto plates to identify interacting test compounds.

An additional method for drug screening involves the use of host eukaryotic cell lines which carry mutations in a particular breast cancer gene. The host cell lines are  
10 also defective at the polypeptide level. Other cell lines may be used where the gene expression of the breast cancer gene can be switched off or up-regulated. The host cell lines or cells are grown in the presence of various drug compounds and the rate of growth of the host cells is  
15 measured to determine if the compound is capable of regulating the growth of defective cells.

Breast cancer polypeptide may also be used for screening compounds developed as a result of combinatorial library technology. This provides a way to test a large  
20 number of different substances for their ability to modulate activity of a polypeptide. The use of peptide libraries is preferred (see patent WO97/02048) with such libraries and their use known in the art.

A substance identified as a modulator of polypeptide  
25 function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many in vivo pharmaceutical applications. In addition, a mimic or mimetic of the substance may be designed for pharmaceutical use. The design of mimetics based on a  
30 known pharmaceutically active compound ("lead" compound) is a common approach to the development of novel pharmaceuticals. This is often desirable where the original active compound is difficult or expensive to synthesise or where it provides an unsuitable method of  
35 administration. In the design of a mimetic, particular parts of the original active compound that are important in determining the target property are identified. These

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parts or residues constituting the active region of the compound are known as its pharmacophore. Once found, the pharmacophore structure is modelled according to its physical properties using data from a range of sources including x-ray diffraction data and NMR. A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be added. The selection can be made such that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, does not degrade *in vivo* and retains the biological activity of the lead compound. Further optimisation or modification can be carried out to select one or more final mimetics useful for *in vivo* or clinical testing.

It is also possible to isolate a target-specific antibody and then solve its crystal structure. In principle, this approach yields a pharmacophore upon which subsequent drug design can be based as described above. It may be possible to avoid protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analogue of the original binding site. The anti-id could then be used to isolate peptides from chemically or biologically produced peptide banks.

In further embodiments, any of the genes, proteins, antagonists, antibodies, complementary sequences, or vectors of the invention may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents may be made by those skilled in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, therapeutic efficacy with lower dosages of each agent may be possible, thus reducing the potential for adverse side effects.

In a further aspect a pharmaceutical composition and a pharmaceutically acceptable carrier may be administered. The pharmaceutical composition may comprise any one or more of a polypeptide as described above, typically a  
5 substantially purified breast cancer polypeptide, an antibody to a breast cancer polypeptide, a vector capable of expressing a breast cancer polypeptide, a compound which increases or decreases expression of a breast cancer gene, a candidate drug that restores wild-type activity to  
10 a breast cancer gene or an antagonist of a breast cancer gene.

The pharmaceutical composition may be administered to a subject to treat or prevent a cancer associated with decreased activity and/or expression of a breast cancer  
15 gene including, but not limited to, those provided above. Pharmaceutical compositions in accordance with the present invention are prepared by mixing a polypeptide of the invention, or active fragments or variants thereof, having the desired degree of purity, with acceptable carriers,  
20 excipients, or stabilizers which are well known. Acceptable carriers, excipients or stabilizers are nontoxic at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low  
25 molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine;  
30 monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or  
35 polyethylene glycol (PEG).

Any of the therapeutic methods described above may be applied to any subject in need of such therapy, including,



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for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

#### Diagnostic and prognostic applications

5 Polynucleotide sequences encoding the breast cancer genes of the invention may be used for the diagnosis or prognosis of cancers associated with their dysfunction, or a predisposition to such cancers. Examples of such cancers include, but are not limited to, adenocarcinoma, leukemia, 10 lymphoma, melanoma, myeloma, sarcoma, teratocarcinoma, and, in particular, cancers of the breast, prostate, blood, germ cells, liver, ovary, adrenal gland, cervix, heart, brain, lung, placenta, skeletal muscle, synovial membrane, tonsil, lymph tissue, kidney, colon, uterus, 15 skin and testis. Other cancers may include those of the head and neck, bladder, bone, bone marrow, gall bladder, ganglia, gastrointestinal tract, pancreas, parathyroid, penis, salivary glands, spleen, stomach, thymus and thyroid gland.

20 Diagnosis or prognosis may be used to determine the severity, type or stage of the disease state in order to initiate an appropriate therapeutic intervention.

In another embodiment of the invention, the polynucleotides that may be used for diagnostic or 25 prognostic purposes include oligonucleotide sequences, genomic DNA and complementary RNA and DNA molecules. The polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which mutations or abnormal expression of the relevant breast cancer gene may 30 be correlated with disease. Genomic DNA used for the diagnosis or prognosis may be obtained from body cells, such as those present in the blood, tissue biopsy, surgical specimen, or autopsy material. The DNA may be isolated and used directly for detection of a specific 35 sequence or may be amplified by the polymerase chain reaction (PCR) prior to analysis. Similarly, RNA or cDNA may also be used, with or without PCR amplification. To

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detect a specific nucleic acid sequence, direct nucleotide sequencing, reverse transcriptase PCR (RT-PCR), hybridization using specific oligonucleotides, restriction enzyme digest and mapping, PCR mapping, RNase protection, and various other methods may be employed. Oligonucleotides specific to particular sequences can be chemically synthesized and labelled radioactively or non-radioactively and hybridised to individual samples immobilized on membranes or other solid-supports or in solution. The presence, absence or excess expression of a particular breast cancer gene may then be visualized using methods such as autoradiography, fluorometry, or colorimetry.

In a particular aspect, the nucleotide sequences encoding a breast cancer gene of the invention may be useful in assays that detect the presence of associated disorders, particularly those mentioned previously. The nucleotide sequences encoding the relevant breast cancer gene may be labelled by standard methods and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and compared with a standard value. If the amount of signal in the patient sample is significantly altered in comparison to a control sample then the presence of altered levels of nucleotide sequences encoding the breast cancer gene in the sample indicates the presence of the associated disorder. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or to monitor the treatment of an individual patient.

In order to provide a basis for the diagnosis or prognosis of a disorder associated with a mutation in a particular breast cancer gene of the invention, the nucleotide sequence of the relevant gene can be compared between normal tissue and diseased tissue in order to

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establish whether the patient expresses a mutant gene.

In order to provide a basis for the diagnosis or prognosis of a disorder associated with abnormal expression of a particular breast cancer gene of the invention, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, encoding the relevant breast cancer gene, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with values from an experiment in which a known amount of a substantially purified polynucleotide is used. Another method to identify a normal or standard profile for expression of a particular breast cancer gene is through quantitative RT-PCR studies. RNA isolated from body cells of a normal individual, particularly RNA isolated from tumour cells, is reverse transcribed and real-time PCR using oligonucleotides specific for the relevant breast cancer gene is conducted to establish a normal level of expression of the gene.

Standard values obtained in both these examples may be compared with values obtained from samples from patients who are symptomatic for a disorder. Deviation from standard values is used to establish the presence of a disorder.

Once the presence of a disorder is established and a treatment protocol is initiated, hybridization assays or quantitative RT-PCR studies may be repeated on a regular basis to determine if the level of expression in the patient begins to approximate that which is observed in the normal subject. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences,

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including genomic sequences, encoding a particular breast cancer gene, or closely related molecules, may be used to identify nucleic acid sequences which encode the gene. The specificity of the probe, whether it is made from a highly specific region, e.g., the 5' regulatory region, or from a less specific region, e.g., a conserved motif, and the stringency of the hybridization or amplification will determine whether the probe identifies only naturally occurring sequences encoding the breast cancer gene, allelic variants, or related sequences.

Probes may also be used for the detection of related sequences, and should preferably have at least 50% sequence identity to any of the breast cancer encoding sequences. The hybridization probes of the subject invention may be DNA or RNA and may be derived from the sequence of SEQ ID Numbers: 1-11 or from genomic sequences including promoters, enhancers, and introns of the genes.

Means for producing specific hybridization probes for DNAs encoding the breast cancer genes of the invention include the cloning of polynucleotide sequences encoding these genes or their derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, and are commercially available. Hybridization probes may be labelled by radionuclides such as <sup>32</sup>P or <sup>35</sup>S, or by enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, or other methods known in the art.

According to a further aspect of the invention there is provided the use of a polypeptide as described above in the diagnosis or prognosis of a cancer associated with a breast cancer gene of the invention, or a predisposition to such cancers.

When a diagnostic or prognostic assay is to be based upon a breast cancer protein, a variety of approaches are possible. For example, diagnosis or prognosis can be achieved by monitoring differences in the electrophoretic mobility of normal and mutant proteins. Such an approach

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will be particularly useful in identifying mutants in which charge substitutions are present, or in which insertions, deletions or substitutions have resulted in a significant change in the electrophoretic migration of the resultant protein. Alternatively, diagnosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the various amino acid residues, or by functional assays demonstrating altered function of the gene products.

10 In another aspect, antibodies that specifically bind a breast cancer gene of the invention may be used for the diagnosis or prognosis of cancers characterized by abnormal expression of the gene, or in assays to monitor patients being treated with the gene or agonists, antagonists, or inhibitors of the gene. Antibodies useful for diagnostic purposes may be prepared in the same manner as described above for therapeutics. Diagnostic or prognostic assays include methods that utilize the antibody and a label to detect a breast cancer gene of the invention in human body fluids or in extracts of cells or tissues. The antibodies may be used with or without modification, and may be labelled by covalent or non-covalent attachment of a reporter molecule.

25 A variety of protocols for measuring a breast cancer gene of the invention, including ELISAs, RIAs, and FACS, are known in the art and provide a basis for diagnosing altered or abnormal levels of their expression. Normal or standard values for their expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to the breast cancer protein under conditions suitable for complex formation. The amount of standard complex formation may be quantitated by various methods, preferably by photometric means. Quantities of any of the breast cancer genes expressed in subject, control, and disease samples from biopsied tissues are compared with the standard values. Deviation between standard and

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subject values establishes the parameters for diagnosing disease.

Once an individual has been diagnosed with a cancer, effective treatments can be initiated. These may include  
5 administering a selective agonist to the relevant mutant breast cancer gene so as to restore its function to a normal level or introduction of the wild-type gene, particularly through gene therapy approaches as described above. Typically, a vector capable of expressing the  
10 appropriate full-length breast cancer gene or a fragment or derivative thereof may be administered. In an alternative approach to therapy, a substantially purified breast cancer polypeptide and a pharmaceutically acceptable carrier may be administered, as described  
15 above, or drugs which can replace the function of or mimic the action of the relevant breast cancer gene may be administered.

In the treatment of cancers associated with increased breast cancer gene expression and/or activity, the  
20 affected individual may be treated with a selective antagonist such as an antibody to the relevant protein or an antisense (complement) probe to the corresponding gene as described above, or through the use of drugs which may block the action of the relevant breast cancer gene.

25

#### Microarray

In further embodiments, complete cDNAs, oligonucleotides or longer fragments derived from any of the polynucleotide sequences described herein may be used  
30 as targets in a microarray. The microarray can be used to monitor the expression level of large numbers of genes simultaneously and to identify genetic variants, mutations, and polymorphisms. This information may be used to determine gene function, to understand the genetic  
35 basis of a disorder, to diagnose or prognose a disorder, and to develop and monitor the activities of therapeutic agents. Microarrays may be prepared, used, and analyzed

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using methods known in the art. (For example, see Skena et al., 1996; Heller et al., 1997).

#### Transformed hosts

5       The present invention also provides for the production of genetically modified (knock-out, knock-in and transgenic), non-human animal models transformed with the DNA molecules of the invention. These animals are useful for the study of breast cancer gene function, to  
10 study the mechanisms of cancer as related to the breast cancer genes, for the screening of candidate pharmaceutical compounds, for the creation of explanted mammalian cell cultures which express the protein or mutant protein and for the evaluation of potential  
15 therapeutic interventions.

One of the breast cancer genes of the invention may have been inactivated by knock-out deletion, and knock-out genetically modified non-human animals are therefore provided.

20       Animal species which are suitable for use in the animal models of the present invention include, but are not limited to, rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates such as monkeys and chimpanzees. For initial  
25 studies, genetically modified mice and rats are highly desirable due to their relative ease of maintenance and shorter life spans. For certain studies, transgenic yeast or invertebrates may be suitable and preferred because they allow for rapid screening and provide for much easier  
30 handling. For longer term studies, non-human primates may be desired due to their similarity with humans.

To create an animal model for a mutated breast cancer gene of the invention several methods can be employed. These include generation of a specific mutation in a  
35 homologous animal gene, insertion of a wild type human gene and/or a humanized animal gene by homologous recombination, insertion of a mutant (single or multiple)

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human gene as genomic or minigene cDNA constructs using wild type or mutant or artificial promoter elements or insertion of artificially modified fragments of the endogenous gene by homologous recombination. The  
5 modifications include insertion of mutant stop codons, the deletion of DNA sequences, or the inclusion of recombination elements (lox p sites) recognized by enzymes such as Cre recombinase.

To create a transgenic mouse, which is preferred, a  
10 mutant version of a particular breast cancer gene of the invention can be inserted into a mouse germ line using standard techniques of oocyte microinjection or transfection or microinjection into embryonic stem cells. Alternatively, if it is desired to inactivate or replace  
15 the endogenous breast cancer gene, homologous recombination using embryonic stem cells may be applied.

For oocyte injection, one or more copies of the mutant or wild type breast cancer gene can be inserted into the pronucleus of a just-fertilized mouse oocyte.  
20 This oocyte is then reimplanted into a pseudo-pregnant foster mother. The liveborn mice can then be screened for integrants using analysis of tail DNA for the presence of human breast cancer gene sequences. The transgene can be either a complete genomic sequence injected as a YAC, BAC,  
25 PAC or other chromosome DNA fragment, a cDNA with either the natural promoter or a heterologous promoter, or a minigene containing all of the coding region and other elements found to be necessary for optimum expression.

According to still another aspect of the invention  
30 there is provided the use of genetically modified non-human animals as described above for the screening of candidate pharmaceutical compounds.

It will be clearly understood that, although a number of prior art publications are referred to herein, this  
35 reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.



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Throughout this specification and the claims, the words "comprise", "comprises" and "comprising" are used in a non-exclusive sense, except where the context requires otherwise.

5

#### Brief Description of the Drawings

Figure 1. Schematic representation of tumours with interstitial and terminal allelic loss on chromosome arm 16q in the two series of tumour samples. Polymorphic markers are listed according to their order on 16q from centromere to telomere and the markers used for each series are indicated by X. Tumour identification numbers are shown at the top of each column. At the right of the figure, the three smallest regions of loss of heterozygosity are indicated.

Figure 2. Relative fold expression variability index (RFVI) for genes mapping to the 16q24.3 LOH region. Genes exhibiting an RFVI greater than 50 (Represented by hatched bars) were considered to be significantly differentially expressed in breast cancer cell line samples compared to normal breast tissue. The control tumour suppressor expression profiles for SYK and INK4A/ARF are also shown (Spotted bars).

Figure 3. Fold change of expression for genes mapping to the 16q24.3 LOH region in breast cancer cell line mRNA relative to normal breast tissue expression. For each gene, the percentage of cell lines exhibiting various fold differences in expression is indicated.

#### Modes for performing the invention

##### EXAMPLE 1: Collection of breast cancer patient material

Two series of breast cancer patients were analysed for this study. Histopathological classification of each tumour specimen was carried out by our collaborators according to World Health Organisation criteria (WHO, 1981). Patients were graded histopathologically according to the modified Bloom and Richardson method (Elston and

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Ellis, 1990) and patient material was obtained upon approval of local Medical Ethics Committees. Tumour tissue DNA and peripheral blood DNA from the same individual was isolated as previously described (Devilee et al., 1991) using standard laboratory protocols.

Series 1 consisted of 189 patients operated on between 1986 and 1993 in three Dutch hospitals, a Dutch University and two peripheral centres. Tumour tissue was snap frozen within a few hours of resection. For DNA isolation, a tissue block was selected only if it contained at least 50% of tumour cells following examination of haematoxylin and eosin stained tissue sections by a pathologist. Tissue blocks that contained fewer than 50% of tumour cells were omitted from further analysis.

Series 2 consisted of 123 patients operated on between 1987 and 1997 at the Flinders Medical Centre in Adelaide, Australia. Of these, 87 were collected as fresh specimens within a few hours of surgical resection, confirmed as malignant tissue by pathological analysis, snap frozen in liquid nitrogen, and stored at -70°C. The remaining 36 tumour tissue samples were obtained from archival paraffin embedded tumour blocks. Prior to DNA isolation, tumour cells were microdissected from tissue sections mounted on glass slides so as to yield at least 80% tumour cells. In some instances, no peripheral blood was available such that pathologically identified paraffin embedded non-malignant lymph node tissue was used instead.

**EXAMPLE 2: LOH analysis of chromosome 16q markers in breast cancer samples.**

In order to identify the location of genes associated with breast cancer, LOH analysis of tumour samples was conducted. A total of 45 genetic markers mapping to chromosome 16 were used for the LOH analysis of the breast tumour and matched normal DNA samples collected for this study. Figure 1 indicates for which tumour series they

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were used and their cytogenetic location. Details regarding all markers can be obtained from the Genome Database (GDB) at <http://www.gdb.org>. The physical order of markers with respect to each other was determined from a combination of information in GDB, by mapping on a chromosome 16 somatic cell hybrid map (Callen et al., 1995) and by genomic sequence information.

Four alternative methods were used for the LOH analysis:

1) For RFLP and VNTR markers, Southern blotting was used to test for allelic imbalance. These markers were used on only a subset of samples. Methods used were as previously described (Devilee et al., 1991).

2) Microsatellite markers were amplified from tumour and normal DNA using the polymerase chain reaction (PCR) incorporating standard methodologies (Weber and May, 1989; Sambrook et al., 1989). A typical reaction consisted of 12  $\mu$ l and contained 100 ng of template, 5 pmol of both primers, 0.2 mM of each dNTP, 1  $\mu$ Curie [ $\alpha$ -<sup>32</sup>P]dCTP, 1.5 mM MgCl<sub>2</sub>, 1.2  $\mu$ l Supertaq buffer and 0.06 units of Supertaq (HT biotechnologies). A Phosphor Imager type 445 SI (Molecular Dynamics, Sunnyvale, CA) was used to quantify ambiguous results. In these cases, the Allelic Imbalance Factor (AIF) was determined as the quotient of the peak height ratios from the normal and tumour DNA pair. The threshold for allelic imbalance was defined as a 40% reduction of one allele, agreeing with an AIF of  $\geq 1.7$  or  $\leq 0.59$ . This threshold is in accordance with the selection of tumour tissue blocks containing at least 50% tumour cells with a 10% error-range. The threshold for retention has been previously determined to range from 0.76 to 1.3 (Devilee et al., 1994). This leaves a range of AIFs (0.58 - 0.75 and 1.31 - 1.69) for which no definite decision has been made. This "grey area" is indicated by grey boxes in Figure 1 and tumours with only "grey area" values were discarded completely from the analysis.

3) The third method for determining allelic

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imbalance was similar to the second method above, however radioactively labelled dCTP was omitted. Instead, PCR of polymorphic microsatellite markers was done with one of the PCR primers labelled fluorescently with FAM, TET or HEX. Analysis of PCR products generated was on an ABI 377 automatic sequencer (PE Biosystems) using 6% polyacrylamide gels containing 8M urea. Peak height values and peak sizes were analysed with the GeneScan programme (PE Biosystems). The same thresholds for allelic imbalance, retention and grey areas were used as for the radioactive analysis.

4) An alternative fluorescent based system was also used. In this instance PCR primers were labelled with fluorescein or hexachlorofluorescein. PCR reaction volumes were 20  $\mu$ l and included 100 ng of template, 100 ng of each primer, 0.2 mM of each dNTP, 1-2 mM  $MgCl_2$ , 1X AmpliTaq Gold buffer and 0.8 units AmpliTaq Gold enzyme (Perkin Elmer). Cycling conditions were 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, followed by 25 cycles of 94°C 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, with a final extension of 72°C for 10 minutes. PCR amplimers were analysed on an ABI 373 automated sequencer (PE Biosystems) using the GeneScan programme (PE Biosystems). The threshold range of AIF for allele retention was defined as 0.61 - 1.69, allelic loss as  $\leq 0.5$  or  $\geq 2.0$ , or the "grey area" as 0.51 - 0.6 or 1.7 - 1.99.

The first three methods were applied to the first tumour series while the last method was adopted for the second series of tumour samples. For statistical analysis, a comparison of allelic imbalance data for validation of the different detection methods and of the different tumour series was done using the Chi-square test.

The identification of the smallest region of overlap (SRO) involved in LOH is instrumental for narrowing down the location of the gene targeted by LOH. Figure 1 shows the LOH results for tumour samples, which displayed small regions of loss (ie interstitial and telomeric LOH) and

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does not include samples that showed complex LOH (alternating loss and retention of markers). When comparing the two sample sets at least three consistent regions emerge with two being at the telomere in band 5 16q24.3 and one at 16q22.1. The region at 16q22.1 is defined by the markers D16S398 and D16S301 and is based on the interstitial LOH events seen in three tumours from series 1 (239/335/478) and one tumour from series 2 (237). At the telomere (16q24.2 - 16q24.3), the first region is 10 defined by the markers D16S498 and D16S3407 and is based on four tumours from series 2 (443/75/631/408) while the second region (16q24.3) extends from D16S3407 to the telomere and is based on one tumour from series 1 (559) and three from series 2 (97/240/466). LOH limited to the 15 telomere but involving both of the regions identified at this site could be found in an additional 17 tumour samples.

Other studies have shown that the long arm of chromosome 16 is also a target for LOH in prostate, lung, 20 hepatocellular, ovarian, rhabdomyosarcoma and Wilms' tumours. Detailed analysis of prostate carcinomas has revealed an overlap in the smallest regions of LOH seen in this cancer to that seen with breast cancer which suggests that 16q harbours a gene implicated in many tumour types.

25

#### EXAMPLE 3: Construction of a physical map of 16q24.3

To identify novel candidate breast cancer genes mapping to the smallest regions of overlap at 16q24.3, a clone based physical map contig covering this region was 30 needed. At the start of this phase of the project the most commonly used and readily accessible cloned genomic DNA fragments were contained in lambda, cosmid or YAC vectors. During the construction of whole chromosome 16 physical maps, clones from a number of YAC libraries were 35 incorporated into the map (Doggett et al., 1995). These included clones from a flow-sorted chromosome 16-specific YAC library (McCormick et al., 1993), from the CEPH Mark I

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and MegaYAC libraries and from a half-telomere YAC library (Riethman et al., 1989). Detailed STS and Southern analysis of YAC clones mapping at 16q24.3 established that very few were localised between the CY2/CY3 somatic cell  
5 hybrid breakpoint and the long arm telomere. However, those that were located in this region gave inconsistent mapping results and were suspected to be rearranged or deleted. Coupled with the fact that YAC clones make poor sequencing substrates, and the difficulty in isolating the  
10 cloned human DNA, a physical map based on cosmid clones was the initial preferred option.

A flow-sorted chromosome 16 specific cosmid library had previously been constructed (Longmire et al., 1993), with individual cosmid clones gridded in high-density  
15 arrays onto nylon membranes. These filters collectively contained ~15,000 clones representing an approximately 5.5 fold coverage of chromosome 16. Individual cosmids mapping to the critical regions at 16q24.3 were identified by the hybridisation of these membranes with markers identified  
20 by this and previous studies to map to the region. The strategy to align overlapping cosmid clones was based on their STS content and restriction endonuclease digestion pattern. Those clones extending furthest within each initial contig were then used to walk along the chromosome  
25 by the hybridisation of the ends of these cosmids back to the high-density cosmid grids. This process continued until all initial contigs were linked and therefore the region defining the location of the breast cancer tumour suppressor genes would be contained within the map.  
30 Individual cosmid clones representing a minimum tiling path in the contig were then used for the identification of transcribed sequences by exon trapping, and for genomic sequencing.

Chromosome 16 was sorted from the mouse/human somatic  
35 cell hybrid CY18, which contains this chromosome as the only human DNA, and *Sau3A* partially digested CY18 DNA was ligated into the *Bam*HI cloning site of the cosmid sCOS-1

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vector. All grids were hybridised and washed using methods described in Longmire et al. (1993). Briefly, the 10 filters were pre-hybridised in 2 large bottles for at least 2 hours in 20 ml of a solution containing 6X SSC; 10 mM EDTA (pH8.0); 10X Denhardt's; 1% SDS and 100 µg/ml denatured fragmented salmon sperm DNA at 65°C. Overnight hybridisations with [ $\alpha$ -<sup>32</sup>P]dCTP labelled probes were performed in 20 ml of fresh hybridisation solution at 65°C. Filters were washed sequentially in solutions of 2X SSC; 0.1% SDS (rinse at room temperature), 2X SSC; 0.1% SDS (room temperature for 15 minutes), 0.1X SSC; 0.1% SDS (room temperature for 15 minutes), and 0.1X SSC; 0.1% SDS (twice for 30 minutes at 50°C if needed). Membranes were exposed at -70°C for between 1 to 7 days.

Initial markers used for cosmid grid screening were those known to be located below the somatic cell hybrid breakpoints CY2/CY3 and the long arm telomere (Callen et al., 1995). These included three genes, *CMAR*, *DPEP1*, and *MC1R*; the microsatellite marker D16S303; an end fragment from the cosmid 317E5, which contains the *BBC1* gene; and four cDNA clones, yc81e09, yh09a04, D16S532E, and ScDNA-C113. The IMAGE consortium cDNA clone, yc81e09, was obtained through screening an arrayed normalised infant brain oligo-dT primed cDNA library (Soares et al., 1994), with the insert from cDNA clone ScDNA-A55. Both the ScDNA-A55 and ScDNA-C113 clones were originally isolated from a hexamer primed heteronuclear cDNA library constructed from the mouse/human somatic cell hybrid CY18 (Whitmore et al., 1994). The IMAGE cDNA clone yh09a04 was identified from direct cDNA selection of the cosmid 37B2 which was previously shown to map between the CY18A(D2) breakpoint and the 16q telomere. The EST, D16S532E, was also mapped to the same region. Subsequent to these initial screenings, restriction fragments representing the ends of cosmids were used to identify additional overlapping clones.

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Contig assembly was based on methods previously described (Whitmore et al., 1998). Later during the physical map construction, genomic libraries cloned into BAC or PAC vectors (Genome Systems or Rosewell Park Cancer Institute) became available. These libraries were screened to aid in chromosome walking or when gaps that could not be bridged by using the cosmid filters were encountered. All BAC and PAC filters were hybridised and washed according to manufacturers recommendations. Initially, membranes were individually pre-hybridised in large glass bottles for at least 2 hours in 20 ml of 6X SSC; 0.5% SDS; 5X Denhardt's; 100 µg/ml denatured salmon sperm DNA at 65°C. Overnight hybridisations with [ $\alpha$ -<sup>32</sup>P]dCTP labelled probes were performed at 65°C in 20 ml of a solution containing 6X SSC; 0.5% SDS; 100 µg/ml denatured salmon sperm DNA. Filters were washed sequentially in solutions of 2X SSC; 0.5% SDS (room temperature 5 minutes), 2X SSC; 0.1% SDS (room temperature 15 minutes) and 0.1X SSC; 0.5% SDS (37°C 1 hour if needed). PAC or BAC clones identified were aligned to the existing contig based on their restriction enzyme pattern or formed unique contigs which were extended by additional filter screens.

A high-density physical map consisting of cosmid, BAC and PAC clones has been established, which extends approximately 3 Mb from the telomere of the long arm of chromosome 16. This contig extends beyond the CY2/CY3 somatic cell hybrid breakpoint and includes the 2 regions of minimal LOH identified at the 16q24.3 region in breast cancer samples. To date, a single gap of unknown size exists in the contig and will be closed by additional contig extension experiments. The depth of coverage has allowed the identification of a minimal tiling path of clones which were subsequently used as templates for gene identification methods such as exon trapping and genomic DNA sequencing.



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**EXAMPLE 4: Identification of candidate breast cancer genes by analysis of genomic DNA sequence**

Selected minimal overlapping BAC and PAC clones from the physical map contig were sequenced in order to aid in the identification of candidate breast cancer genes. DNA was prepared from selected clones using a large scale DNA isolation kit (Qiagen). Approximately 25-50 ug of DNA was then sheared by nebulisation (10psi for 45 seconds) and blunt ended using standard methodologies (Sambrook et al., 1989). Samples were then run on an agarose gel in order to isolate DNA in the 2-4 Kb size range. These fragments were cleaned from the agarose using QIAquick columns (Qiagen), ligated into puc18 and used to transform competent DH10B or DH5a *E. coli* cells. DNA was isolated from transformed clones and was sequenced using vector specific primers on an ABI377 sequencer.

Analysis of genomic sequence was performed using PHRED, PHRAP and GAP4 software on a SUN workstation. To assist in the generation of large contigs of genomic sequence, information present in the high-throughput genomic sequence (htgs) database at NCBI was incorporated into the assembly phase of the sequence analysis. The resultant genomic sequence contigs were masked for repeats and analysed using the BLAST algorithm (Altschul et al., 1997) to identify nucleotide and protein homology to sequences in the GenBank non-redundant and EST databases at NCBI. The genomic sequence was also analysed for predicted gene structure using the GENSCAN program and specific screening of the mouse EST dataset was utilised to identify potential human orthologues that have poor representation in the human EST dataset.

Following the identification of homologous EST sequences, *in silico* cDNA walking experiments were initiated through further dbEST database screening. This was to identify overlapping cDNA sequences present in dbEST that would allow extension of the originally identified partial gene sequence. Overlapping EST

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sequences were assembled using the DNASTar LaserGene sequence assembly software. Homologous IMAGE cDNA clones in some instances were also purchased and sequenced. These longer stretches of sequence were then compared to known genes by nucleotide and amino acid sequence comparisons using the above procedures.

From *in silico* analysis of the dbEST database at NCBI using all genomic sequence obtained for the 16q24.3 critical LOH region, a total of 55 gene fragments or gene "signatures" were identified. In the majority of cases each novel gene fragment was represented by a distinct UniGene cluster composed of one or a number of overlapping cDNA clones. The majority of these UniGene clusters appeared to represent the 3' untranslated regions of their representative gene as their sequence was continuous with the genomic sequence and further *in silico* manipulation failed to identify open reading frames representing amino acid coding regions.

As well as the 55 gene signatures that were identified in the 16q24.3 region analysed, a total of 48 partial or full-length genes were also present based on *in silico* analysis of the genomic DNA generated.

Those sequences that are expressed in the breast were considered to be the most likely candidate breast cancer genes. Those genes whose function could implicate it in the tumourigenic process, as predicted from homology searches with known proteins, were treated with the highest priority. Further evidence that a particular candidate is the responsible gene comes from the identification of defective alleles of the gene in affected individuals or from analysis of the expression levels of a particular candidate gene in breast cancer samples compared with normal control tissues.

#### EXAMPLE 5: Examination of the expression level of breast cancer gene candidates

To investigate a potential role in breast cancer of

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the genes identified from the 16q24.3 LOH region, the level of expression of these genes, in a set of breast cancer cell lines, was compared with their expression in normal tissue controls. Differential expression (observed as either a down-regulation or up-regulation of gene expression) of a particular gene in a cancer cell line compared to normal controls provides evidence that the gene may be implicated in the cancer. The differential expression may be due to point mutations in the gene, which can decrease the stability of the mRNA of the gene (viewed as a down-regulation of expression) or may lead to enhanced expression of the gene (viewed as up-regulation of expression). In addition, epigenetic mechanisms such as abnormal promoter methylation may have the effect of switching off gene expression which will also be observed as a down-regulation in expression of the associated gene. Recent studies have shown that this latter mechanism has been responsible for the inactivation of other tumour suppressor genes such as RB1 (Ohtani-Fujita et al., 1997), VHL (Prowse et al., 1997), MLH1 (Herman et al., 1998) and BRCA1 (Esteller et al., 2000).

To detect the level of expression of the genes identified in the 16q24.3 region in cancer samples compared with normal controls, quantitative RT-PCR using individual gene specific primers was done. This initially involved the isolation of RNA from cancer cell lines along with appropriate cell line and normal tissue controls.

#### Breast Cancer Cell Lines and RNA Extraction

Breast cancer cell lines were purchased from ATCC (USA) and grown in the recommended tissue culture medium. The breast cancer cell lines that were chosen for RT-PCR analysis demonstrated homozygosity for a set of markers mapping to chromosome 16q indicating potential LOH for this chromosomal arm. Cells were harvested from confluent cultures and total RNA was extracted using the RNAeasy kit (Qiagen) or the TRIzol<sup>TM</sup> reagent (Gibco BRL) according to

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manufacturers recommendations. PolyA<sup>+</sup> mRNA was subsequently isolated from all sources using the Oligotex bead system (Qiagen) according to recommended procedures. Total RNA derived from 21 human tissues (18 adult and 3 fetal) was purchased commercially (Clontech, Stratagene, Ambion). DNA contamination was removed from all RNA preparations using DNasefree (Ambion) according to manufacturers protocols.

#### Reverse Transcription

Total RNA and PolyA<sup>+</sup> mRNA was primed with oligo-dT primers and reverse transcribed using the Omniscript RT kit (Qiagen) according to manufacturers conditions or using Superscript<sup>TM</sup> RNaseH<sup>-</sup> reverse transcriptase (Gibco BRL). In the latter method, 1 ug of total RNA sample was mixed with 500 ng of oligo (dT)<sub>16</sub> and made up to a volume of 10 ul with DEPC treated water. Following a 10 minute incubation at 70°C, 4 ul of 5X first strand buffer, 2 ul of 0.1 M DTT, 1 ul of 10 mM dNTP, 20 units of RNasin<sup>TM</sup> (Promega) and 100 units of Superscript reverse transcriptase were added and the reaction incubated at 42°C for 2 hours. Reactions were terminated at 95°C for 5 minutes and cDNA:RNA hybrids were removed from samples by addition of 2 units of RNase H (Promega) and incubation at 37°C for 30 minutes. Control reactions were included for each RNA template, which omitted reverse transcriptase from the cDNA synthesis step. This was to determine the presence of any genomic DNA contamination in the RNA samples. All samples were stored at -20°C.

#### cDNA Normalisation

Internal standard curve amplicons were generated from a mixed pool of normal tissue cDNA using the HotStarTaq<sup>TM</sup> DNA Polymerase kit (Qiagen). A reaction mix sufficient to generate >1 ug of amplicon cDNA contained 10 ul of 10X PCR buffer (containing 15 mM MgCl<sub>2</sub>), 2 ul of 10 mM dNTP mix, 0.5 uM of each primer, 0.5 ul of 2.5 units HotStarTaq polymerase (Qiagen), 100 ng of cDNA template and DEPC

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treated water to 100 ul. Amplification cycling was performed as follows: 94°C for 10 minutes followed by 35 cycles at 93°C for 20 seconds, 60°C for 30 seconds and 70°C for 30 seconds with a final extension at 72°C for 4 minutes. Amplicons were purified using the QIAquick gel extraction kit (Qiagen) according to manufacturers conditions and concentrations were measured at A<sub>260</sub>. Purified amplicons were serially diluted 10-fold from 10 ng/ul to 1 fg/ul. These dilutions served as internal standards of known concentration for real-time analysis of each gene specific amplicon as described below.

#### Real-time PCR

All cDNA templates were amplified using the SYBR Green I PCR Master Mix kit (PE Biosystems, USA). Primer sets for the amplification of each gene were selected using the Lasergene Primer Select<sup>TM</sup> software (DNASTAR). PCR reactions were in a volume of 25 ul and included 12.5 ul of SYBR Green I PCR Master mix, 0.5 uM of each primer, 2 ul normalised cDNA template (see below) and 9.5 ul of water. Real-time PCR analysis was performed using the Rotor-Gene<sup>TM</sup>2000 (Corbett Research, AUS) with the following amplification cycling conditions: 94°C for 10 minutes followed by 45 cycles of 93°C for 20 sec, 60°C for 30 sec and 70°C for 30 sec. Fluorescence data was acquired at 510 nm during the 72°C extension phase. Melt curve analyses were performed with an initial 99-50°C cycling followed by fluorescence monitoring during heating at 0.2°C/second to 99°C. Prior to real-time quantification, product size and specificity was confirmed by ethidium bromide staining of 2.5% agarose gels following electrophoresis of completed PCRs.

#### Real-time PCR Quantification

Quantification analyses were performed on the Rotor-Gene<sup>TM</sup> DNA sample analysis system (Version 4.2, Build 96). Standard curves were generated by amplifying 10-fold

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serial dilutions (1 ul of 10  $\mu$ g/ul down to 1 ul of 1 fg/ul in triplicate) of the internal standard amplicon during real-time PCR of gene specific amplicons from normal tissues and breast cancer cell lines. Internal standard amplicon concentrations were arbitrarily set to 1.0e+12 copies for 10  $\mu$ g standards to 1.0e+08 copies for 1 fg standards.  $C_T$  (cycle threshold) coefficients of variation for all internal standard dilutions averaged 2% between triplicate samples within the same and different runs. The Rotor-Gene™ quantification software generated a line of best-fit at the parameter  $C_T$  and determined unknown normal tissue and breast cancer cell line amplicon copy numbers by interpolating the noise-band intercept of each amplicon against the internal standards with known copy numbers.

15

#### Normalization and relative expression of data

Using the expression value of each gene in normal breast tissue as a baseline, the relative fold-difference between the cell line exhibiting the highest expression and the cell line exhibiting the lowest expression was calculated. This value was termed the "Relative Fold Variability Index" (RFVI).

In order to establish an RFVI baseline range, five house-keeping genes were first examined. These included Cyclophilin, APRT, RNA Polymerase subunit II, ATP synthase and GAPDH. This baseline range reflects mRNA expression differences that are due to normal population variations or experimental reproducibility.

The degree of variation in mRNA expression levels for the housekeeping genes was relatively uniform between the normal tissues and cancer cell lines examined. Three-way combinations for normalization between Cyclophilin, RNA polymerase II subunit and APRT demonstrated a mean 7-fold and maximum 50-fold variance in mRNA expression level between samples. The significance of variable mRNA expression levels within a gene of interest may therefore reasonably be evaluated based on these normalization

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results. A predicted aberrant alteration in gene of interest mRNA copy number of >50 fold in breast cancer cell lines relative to a 'baseline' normal breast expression level was therefore considered to be significantly abnormal.

Following establishment of a baseline RFVI value of 50, the RFVI was determined for the SYK and p16INK4a genes. These represent known tumour suppressor genes that have been previously shown to exhibit aberrant expression in breast cancer cells (Coopman et al., 2000; Bisogna et al., 2001). Both genes had significant RFVI values with the observed RFVI for SYK being almost 10 times greater than the baseline range (baseline=50; SYK=460).

Results from the examination of the RFVI values for the genes identified in the 16q24.3 region are shown in Figure 2. A total of 7 genes were identified to have an RFVI of greater than 50 indicating significant differential expression in breast cancer cell lines compared with normal tissue controls. A description of these genes is summarised in Table 1 and their sequences are represented by the SEQ ID Numbers: 1-11.

The data generated from the expression studies is also represented in Figure 3. In this instance, the percent fold change in expression of each gene across the entire panel of breast cancer cell lines examined is displayed. Results indicate that the differential expression in 6 of the 7 genes described above (BNO227, BNO8, BNO205, BNO221, BNO225, BNO226) was due to a down-regulation in gene expression, while the remaining gene (BNO223) showed an up-regulation in gene expression specific for a number of breast cancer cell lines.

BNO227 was identified to display the most significant differential expression and corresponds to the CYBA gene. CYBA associates with CYBB to form cytochrome b-558, which is the membrane component of NADPH oxidase and functions as the final electron transporter in the oxidation of NADPH, resulting in the generation of Reactive Oxygen

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Species (ROS) such as  $O_2$  and  $H_2O_2$ . The levels of ROS appear to be critical in the regulation of a number of genes involved in diverse pathways delineating transcription, proliferation and apoptosis (Burdon et al, 1996; Arnold et al, 2001; Jacobson, 1996). This regulatory role is thought to encompass influences on gene expression and protein function (Burdon, 1995). It has been suggested that alteration of protein function is effected by modifications on redox-sensitive amino acids such as cystein and histidine. The role of CYBA as a NADPH oxidase in the microbicidal function of phagocytes has been studied extensively. Mutations in this gene are causative to the chronic granulomatous disease characterized by recurrent bacterial and fungal infections (Rae et al, 2000). More recently, CYBA has been implicated in oxidases involved in epithelial and muscle cell gene regulation and function with demonstrated implications in atherosclerosis (Sorescu et al, 2001). Hitherto, there has been no direct demonstration of a CYBA NADPH oxidase role in breast cancer, however, several studies have shown the involvement of reactive oxygen species ( $O_2^{*-}$ , hypochlorite, hydroxyl radical, hydrogen peroxide) in carcinogenesis and tumour progression (Gupta et al, 2001; Brown and Bicknell, 2001). The relative concentration of ROS is critical to their function. ROS levels are under a tight regulatory control involving the interplay of NADPH oxidases and antioxidant ROS scavengers (Griendling and Ushio-Fukai, 2000). Any disruption to these control mechanisms is likely to result in aberrant cell behaviour as that seen in cancer. Consequently, the expression profiles of the molecules involved in ROS production and/or removal are of central importance. This example demonstrates that CYBA is expressed in normal breast tissue and in breast cancer cell lines. In addition, we have found that this gene is differentially expressed, with some cell lines expressing very low levels of this gene. This finding implicates CYBA as a potential tumour



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suppressor and suggests the possible involvement in carcinogenesis of the other membrane subunit of cytochrome-b558 as well as the cytoplasmic components of NADPH oxidase.

5

**EXAMPLE 6: Analysis of tumours and cell lines for breast cancer gene mutations**

Any one of the genes that have been shown to be differentially expressed in this study can be screened by single strand conformation polymorphism (SSCP) analysis in DNA isolated from tumours which display restricted LOH for the 16q24.3 region. This can be done to identify those samples where mutations in the gene are causative for the cancer rather than dysregulation of gene expression being the causative factor. In this instance DNA isolated from series 1 and series 2 tumours can be used. A number of breast cancer cell lines, or cell lines from other cancer types, may also be screened. Likewise, tissues from other cancer types can be screened by SSCP for disease causing mutations. Cell lines can be purchased from ATCC, grown according to manufacturers conditions, and DNA isolated from cultured cells using standard protocols (Wyman and White, 1980; Sambrook et al., 1989).

To perform mutation analysis of the candidate breast cancer genes using the SSCP technique, a number of variations can be employed. For example, breast cancer gene exons can be amplified by PCR using flanking intronic primers, which are labeled at their 5' ends with HEX. Typical PCR reactions are performed in 96-well plates in a volume of 10  $\mu$ l using 30 ng of template DNA. Cycling conditions involve an initial denaturation step at 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds, 60°C for 1<sup>1/2</sup> minutes and 72°C for 1<sup>1/2</sup> minutes. A final extension step of 72°C for 10 minutes follows. Twenty  $\mu$ l of loading dye comprising 50% (v/v) formamide, 12.5 mM EDTA and 0.02% (w/v) bromophenol blue is added to completed reactions which are subsequently run on 4% polyacrylamide

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gels and analysed on the GelScan 2000 system (Corbett Research, AUS) according to manufacturers specifications.

Those samples that display a bandshift compared with normal controls are considered to have a different  
5 nucleotide composition in the amplicon being analysed compared to that of normal controls. The amplicon can be sequenced in this sample and compared to wild-type sequence to determine the nucleotide differences. Any base changes that are present in a tumour sample but not  
10 present in the corresponding normal control sample from the same individual or in other normal individuals most likely represents a deleterious mutation. This is further confirmed if the base change also leads to an amino acid change or the generation of a truncated form of the  
15 protein.

#### EXAMPLE 7: Analysis of the breast cancer genes

The following methods are used to determine the structure and function of any one of the breast cancer  
20 genes.

#### Biological studies

Mammalian expression vectors containing breast cancer gene cDNA can be transfected into breast, prostate or  
25 other carcinoma cell lines that have lesions in the gene. Phenotypic reversion in cultures (eg cell morphology, growth of transformants in soft-agar, growth rate) and in animals (eg tumourigenicity in nude mice) is examined. These studies can utilise wild-type or mutant forms of the  
30 breast cancer genes. Deletion and missense mutants of these genes can be constructed by *in vitro* mutagenesis.

#### Molecular biological studies

The ability of any one of the breast cancer proteins  
35 to bind known and unknown proteins can be examined. These proteins may give an insight as to the biological pathways in which the breast cancer proteins participate. In turn,

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proteins within these pathways may provide suitable targets for therapeutic applications such as gene therapy, screening for small molecule interactors, as well as antisense and antibody-based therapies directed at these interactors.

Procedures such as the yeast two-hybrid system are used to discover and identify any functional partners. The principle behind the yeast two-hybrid procedure is that many eukaryotic transcriptional activators, including those in yeast, consist of two discrete modular domains. The first is a DNA-binding domain that binds to a specific promoter sequence and the second is an activation domain that directs the RNA polymerase II complex to transcribe the gene downstream of the DNA binding site. Both domains are required for transcriptional activation as neither domain can activate transcription on its own. In the yeast two-hybrid procedure, the gene of interest or parts thereof (BAIT), is cloned in such a way that it is expressed as a fusion to a peptide that has a DNA binding domain. A second gene, or number of genes, such as those from a cDNA library (TARGET), is cloned so that it is expressed as a fusion to an activation domain. Interaction of the protein of interest with its binding partner brings the DNA-binding peptide together with the activation domain and initiates transcription of the reporter genes. The first reporter gene will select for yeast cells that contain interacting proteins (this reporter is usually a nutritional gene required for growth on selective media). The second reporter is used for confirmation and while being expressed in response to interacting proteins it is usually not required for growth.

#### Structural studies

Breast cancer recombinant proteins can be produced in bacterial, yeast, insect and/or mammalian cells and used in crystallographical and NMR studies. Together with

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molecular modeling of the proteins, structure-driven drug design can be facilitated.

5      **EXAMPLE 8: Generation of polyclonal antibodies against the breast cancer proteins**

        The knowledge of the nucleotide and amino acid sequence of the breast cancer genes and associated proteins allows for the production of antibodies, which selectively bind to these proteins or fragments thereof.  
10      Following the identification of mutations in these breast cancer genes, antibodies can also be made to selectively bind and distinguish mutant from normal protein. Antibodies specific for mutagenised epitopes are especially useful in cell culture assays to screen for  
15      malignant cells at different stages of malignant development. These antibodies may also be used to screen malignant cells, which have been treated with pharmaceutical agents to evaluate the therapeutic potential of the agent.

20      To prepare polyclonal antibodies, short peptides can be designed homologous to any one of the breast cancer amino acid sequences. Such peptides are typically 10 to 15 amino acids in length. These peptides should be designed in regions of least homology to the mouse orthologue to  
25      avoid cross species interactions in further down-stream experiments such as monoclonal antibody production. Synthetic peptides can then be conjugated to biotin (Sulfo-NHS-LC Biotin) using standard protocols supplied with commercially available kits such as the PIERCE<sup>TM</sup> kit  
30      (PIERCE). Biotinylated peptides are subsequently complexed with avidin in solution and for each peptide complex, 2 rabbits are immunized with 4 doses of antigen (200 ug per dose) in intervals of three weeks between doses. The initial dose is mixed with Freund's Complete adjuvant  
35      while subsequent doses are combined with Freund's Immuno-adjuvant. After completion of the immunization, rabbits are test bled and reactivity of sera assayed by dot blot

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with serial dilutions of the original peptides. If rabbits show significant reactivity compared with pre-immune sera, they are then sacrificed and the blood collected such that immune sera can be separated for further experiments.

5

EXAMPLE 9: Generation of monoclonal antibodies specific for the breast cancer proteins

Monoclonal antibodies can be prepared for any one of the breast cancer proteins in the following manner.

10 Immunogen comprising an intact breast cancer protein or peptide (wild type or mutant) is injected in Freund's adjuvant into mice with each mouse receiving four injections of 10 to 100 ug of immunogen. After the fourth injection blood samples taken from the mice are examined

15 for the presence of antibody to the immunogen. Immune mice are sacrificed, their spleens removed and single cell suspensions are prepared (Harlow and Lane, 1988). The spleen cells serve as a source of lymphocytes, which are then fused with a permanently growing myeloma partner cell

20 (Kohler and Milstein, 1975). Cells are plated at a density of  $2 \times 10^5$  cells/well in 96 well plates and individual wells are examined for growth. These wells are then tested for the presence of specific antibodies by ELISA or RIA using wild type or mutant breast cancer target protein. Cells in

25 positive wells are expanded and subcloned to establish and confirm monoclonality. Clones with the desired specificity are expanded and grown as ascites in mice followed by purification using affinity chromatography using Protein A Sepharose, ion-exchange chromatography or variations and

30 combinations of these techniques.

#### Industrial Applicability

The DNA sequences of the present invention are useful in the diagnosis of cancer, or a pre-disposition thereto

35 and, where they are not full-length gene sequences, they may be used to identify full-length genes involved in

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carcinogenesis. Methods of treatment of cancer and methods of screening for drugs are also made available.

TABLE 1

Differentially Expressed Breast Cancer Genes Identified at Chromosome 16q24.3

Gene	Associated UniGene Cluster	Description	SEQ ID Numbers
BNO227 <sup>1</sup>	Hs.68877	Cytochrome b-245, alpha polypeptide (CYBA)	1, 2
BNO8 <sup>1</sup>	Hs.189419	ESTs	3
BNO205 <sup>1</sup>	Hs.174074	ESTs	4
BNO221 <sup>1</sup>	None	EST	5
BNO225 <sup>1</sup>	Hs.148090	Cadherin 15 (CDH15)	6, 7
BNO226 <sup>1</sup>	Hs.109	Dipeptidase 1 (DPEP1)	8, 9
BNO223 <sup>2</sup>	Hs.184601	Solute carrier family 7 (cationic amino acid transporter), member 5 (SLC7A5)	10, 11

*Note:* <sup>1</sup> Down-regulation of expression in breast cancer cell lines compared to normal tissue controls. <sup>2</sup> Up-regulation of expression in breast cancer cell lines compared to normal tissue controls.

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20

Claims

1. A method for the diagnosis of cancer, or a predisposition thereto, in a patient, comprising the steps of:

5 (1) establishing the level of expression of a gene selected from the group consisting of cytochrome B-245, alpha polypeptide (CYBA), cadherin 15 (CDH15), dipeptidase 1 (DPEP1), solute carrier family 7, member 5 (SLC7A5), BNO8, BNO205 and  
10 BNO221; and

(2) comparing expression of the gene to a baseline established from expression in normal tissue controls;

15 wherein substantial variance from the baseline indicates that the patient has, or is susceptible to, cancer.

2. A method as claimed in claim 1 wherein down-regulation of CYBA, CDH15, DPEP1, BNO8, BNO205 or BNO221  
20 is observed.

3. A method as claimed in claim 1 wherein up-regulation of SLC7A5 is observed.

25 4. A method for determining whether a human tissue is predisposed to a neo-plastic transformation, comprising determining whether in a cell from the tissue a nucleic acid molecule selected from the group consisting of CYBA, CDH15, DPEP1, BNO8, BNO205 or BNO221 is absent, present in  
30 a mutant form or down-regulated through epigenetic mechanisms.

5. A method according to claim 4 wherein the human tissue is human breast tissue.

35

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6. A method as claimed in claim 4 comprising determining whether the encoded polypeptide is absent or expressed at reduced levels.

5 7. A method as claimed in claim 6 comprising contacting the cell with an antibody for binding the peptide under conditions which permit the antibody to bind the peptide if it is present.

10 8. A method for determining whether a human tissue has undergone a neoplastic transformation, comprising, determining whether in a cell from the tissue the SLC7A5 gene is up-regulated and/or present in mutant form.

15 9. A method as claimed in claim 8 wherein the human tissue is human breast tissue.

10. A method as claimed in claim 8 comprising determining whether the encoded polypeptide is over-expressed and/or a  
20 mutant peptide is expressed.

11. A method as claimed in claim 10 comprising contacting the cell with an antibody specific for the mutant peptide under conditions which permit the antibody to bind to the  
25 mutant peptide, if present.

12. A mutant form of CYBA, CDH15, DPEP1, BNO8, BNO205 or BNO221 in which the tumour suppressor activity of the gene is compromised.

30

13. A mutant form of SLC7A5 which renders it active as an oncogene.

14. A polypeptide encoded by a mutant form of a gene as  
35 defined in claim 12.

- 60 -

15. A polypeptide encoded by a mutant form of a gene as defined in claim 13.

16. An antibody to a mutant form of a gene as defined in  
5 claim 12.

17. An antibody to a mutant form of a gene as defined in claim 13.

10 18. Use of a DNA molecule selected from the group consisting of DNA molecules having the sequence set forth in SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5 to identify and/or obtain full-length human genes involved in the tumourigenic process.

15 19. Use as claimed in claim 18 wherein a full-length human gene is identified by hybridisation with one or more of said nucleotide molecules.

20 20. Use as claimed in either one of claims 18 or 19 wherein additional sequence is obtained using inverse PCR, restriction site PCR, PCR walking techniques or RACE.

21. Use as claimed in claim 18 wherein PCR probes are  
25 designed based upon the nucleotide sequence of said DNA molecule.

22. Use as claimed in claim 21 wherein the probes comprise 5 to 20 nucleotides from said DNA molecule.

30 23. Use as claimed in claim 22 wherein the probes are labelled with radionuclides or enzymatic labels.

24. Use as claimed in any one of claims 18 to 23  
35 wherein the full-length human gene is a tumour suppressor gene.

- 61 -

25. A method of identifying a gene involved in the tumourigenic process, comprising the steps of:

5 (1) providing a nucleic acid probe derived from or comprising a DNA molecule selected from the group consisting of DNA molecules having the sequence set forth in SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5, or a fragment thereof;

10 (2) assembling a hybridisation mixture containing said nucleic acid probe in single stranded form and a plurality of single stranded nucleic acid molecules derived from a region of loss of heterozygosity at 16q24.3; and

15 (3) isolating a double stranded hybrid of said nucleic acid probe and a complementary nucleic acid molecule derived from a region of loss of heterozygosity at 16q24.3, the DNA molecule from which the complementary nucleic acid molecule is derived representing at least a portion of a candidate gene.

20 26. A gene when identified by the method of claim 25.

27. A gene incorporating the sequence set forth in any of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5.

25 28. A gene when identified through the use as claimed in any one of claims 18 to 24.

29. A gene located at 16q24.3 which hybridises under stringent conditions to one or more nucleotide molecules  
30 selected from the group consisting of nucleotide molecules derived from DNA molecules having the sequence set forth in SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5.

30. A gene as claimed in any one of claims 26 to 29  
35 which is a tumour suppressor gene.

31. A polypeptide encoded by a gene as claimed in any

- 62 -

one of claims 26 to 30.

32. An antibody to a polypeptide as defined in claim 31.

5

33. An antibody to a polypeptide having an amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:7, SEQ ID NO:9 and SEQ ID NO:11.

10 34. Use of a gene as claimed in claims 26 to 30 in the diagnosis of cancer or in establishing the prognosis of a patient diagnosed with cancer.

15 35. Use of a nucleotide molecule having the nucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10 in the diagnosis of cancer or in establishing the prognosis of a patient diagnosed with cancer.

20 36. Use as claimed in claims 34 and 35 wherein the cancer is breast cancer.

25 37. Use of a polypeptide as defined in claim 31 in the diagnosis of cancer or in establishing the prognosis of a patient diagnosed with cancer.

30 38. Use of a polypeptide having the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:7, SEQ ID NO:9 and SEQ ID NO:11 in the diagnosis of cancer or in establishing the prognosis of a patient diagnosed with cancer.

35 39. Use as claimed in claims 37 and 38 wherein the cancer is breast cancer.

40. Use of an antibody as claimed in claim 32 or 33 in the diagnosis of cancer or in establishing the

- 63 -

prognosis of a patient diagnosed with cancer.

41. Use as claimed in claim 40 wherein the cancer is breast cancer.

5

42. A microarray comprising oligonucleotides or longer fragments derived from any one or more of the genes CYBA, CDH15, DPEP1, SLC7A5 or a gene as claimed in any one of claims 26 to 30.

10

43. A method of treating or inhibiting cancer in a patient in need of such treatment, said method comprising administering to said patient a vector capable of expressing CYBA, CDH15, DPEP1 or a gene as claimed in any one of claims 26 to 30.

15

44. A method of treating or inhibiting cancer in a patient in need of such treatment, said method comprising administering to said patient a compound which increases expression or activity of CYBA, CDH15, DPEP1 or a gene as claimed in any one of claims 26 to 30.

20

45. A method of treating or inhibiting cancer in a patient in need of such treatment, said method comprising administering to said patient an antagonist of SLC7A5.

25

46. A method as claimed in claim 45 wherein the antagonist of SLC7A5 is an antibody thereto, or an antisense thereto, or a nucleic acid complementary in sequence to at least a portion of SLC7A5.

30

47. A method as claimed in any of claims 43 to 46 wherein the cancer is breast cancer.

35

48. Use of a gene as claimed in any of claims 26 to 30 in the treatment of cancer through gene therapy.



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49. Use of a nucleotide molecule having the nucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10 in the treatment of cancer through gene therapy.
50. Use of a polypeptide as defined in claim 31 in the treatment of cancer.
51. Use of a polypeptide having the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:7, SEQ ID NO:9 and SEQ ID NO:11 in the treatment of cancer.
52. Use of an antibody as claimed in either one of claims 32 or 33 in the treatment of cancer.
53. Use as claimed in any one of claims 48 to 52 wherein the cancer is breast cancer.
54. A genetically modified non-human animal in which a gene selected from the group consisting of CYBA, CDH15, DPEP1, BNO8, BNO205, BNO221, or a gene as claimed in any one of claims 26 to 30 has been inactivated by knockout deletion.
55. A genetically modified non-human animal in which the SLC7A5 gene is over-expressed and/or expressed in mutant form as an oncogene.
56. A genetically modified animal as claimed in either of claims 54 or 55 wherein the animal is selected from the group consisting of rats, mice, hamsters, guineas pigs, rabbits, dogs, cats, goats, sheep, pigs and non-human primates such as monkeys and chimpanzees.
57. A method of screening for candidate drugs which restore tumour suppressor activity, comprising the steps of:

- 65 -

- 5 (1) contacting a cell, in which the functioning of a gene selected from the group consisting of CYBA, CDH15, DPEP1, BNO8, BNO205, BNO221, or a gene as claimed in any one of claims 26 to 30 is compromised, with a candidate drug; and
- (2) assaying for the amount of tumour suppressor activity in the cell.

10 58. A method of screening for candidate drugs which reverse oncogene activity, comprising the steps of:

- (1) contacting a cell in which SLC7A5 is over-expressed and/or expressed in a mutant form with a candidate drug; and
- 15 (2) assaying for the level of expression and/or activity of SLC7A5 in the cell.

59. A method of screening for a drug capable of binding SLC7A5 and modulating its activity, said method

20 comprising combining the SLC7A5 protein and a candidate drug and determining the binding of said candidate drug to the SLC7A5 peptide.

60. Use of a drug identified by the method of any one

25 of claims 57 to 58 in the treatment of cancer.

61. Use of a drug identified by the method any one of claims 57 to 59 in the manufacture of a medicament for the treatment of cancer.

30 62. Use as claimed in any one of claims 60 or 61 wherein the cancer is breast cancer.

63. An anti-cancer drug when identified by the method

35 of any one of claims 57, 58 or 59.

64. A compound which increases the expression of or

- 66 -

activity of CYBA, CDH15, DPEP1, BNO8, BNO205, BNO221 or a gene as claimed in claim 26.

65. An antagonist of SLC7A5.

5

66. A cell comprising an expression vector capable of expressing CYBA, CDH15, DPEP1, BNO8, BNO205, BNO221 or a gene as claimed in any one of claims 26 to 30.

10 67. A cell transformed with a nucleotide molecule having the nucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10.

15 68. A pharmaceutical composition comprising any one or more of the genes selected from the group consisting of CYBA, CDH15, DPEP1, SLC7A5, BNO8, BNO205, BNO221 or genes as claimed in any one of claims 26 to 30, or active fragments thereof, their expression products and  
20 antibodies to their expression products, or any one or more isolated nucleotides selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10, and an inert carrier.

25

69. A pharmaceutical composition comprising any one or more of a polypeptide as claimed in claim 29 or 30 or having the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:7, SEQ ID NO:9 and SEQ ID NO:11, an antibody as  
30 claimed in claim 32 or 33, an expression vector according to claim 43, a compound according to claim 64, an anti-cancer drug according to claim 63, or an antagonist of SLC7A5 as claimed in claim 65, and an inert carrier.

35

SUBSTITUTE SHEET (RULE 26) RO/AU

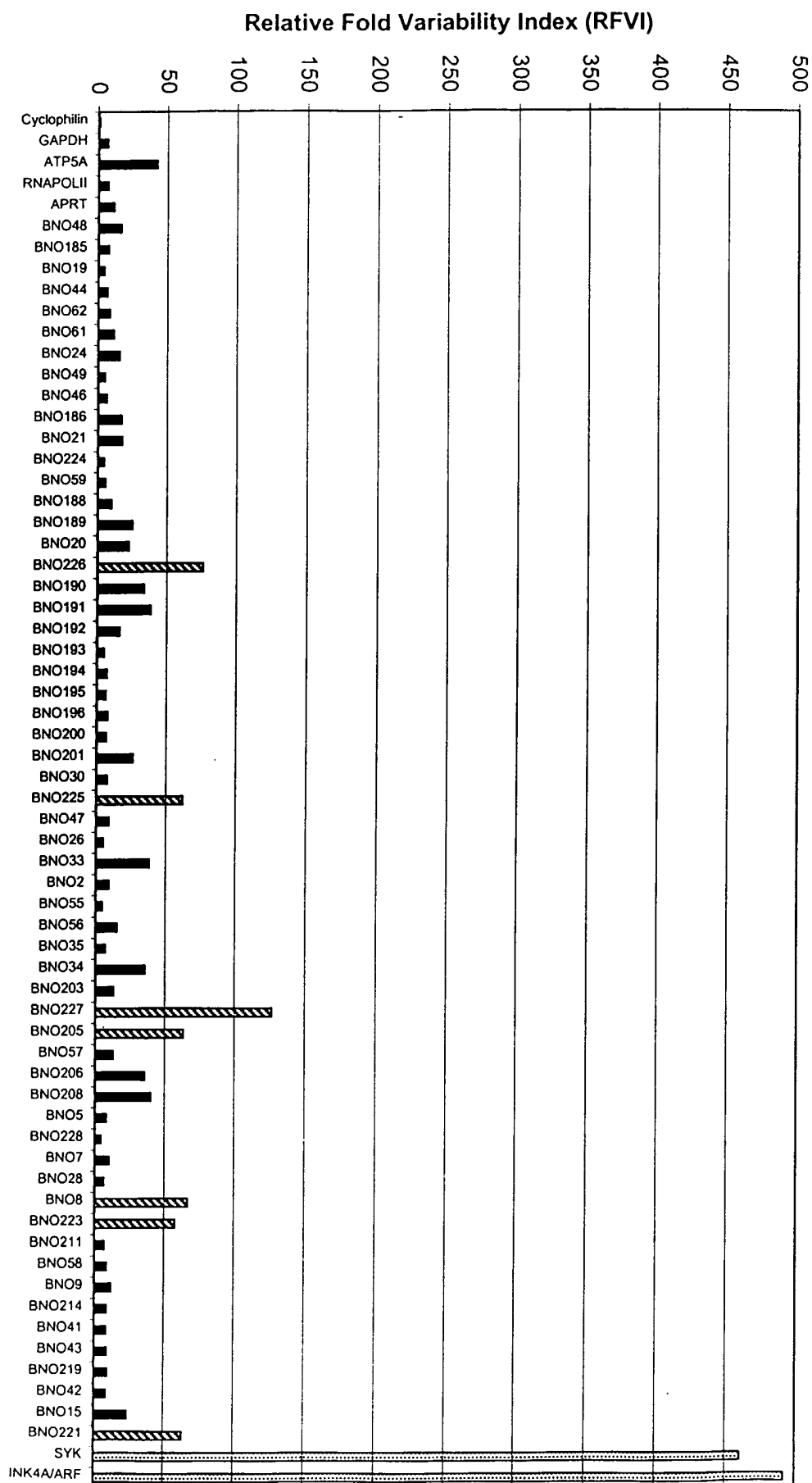


Figure 2

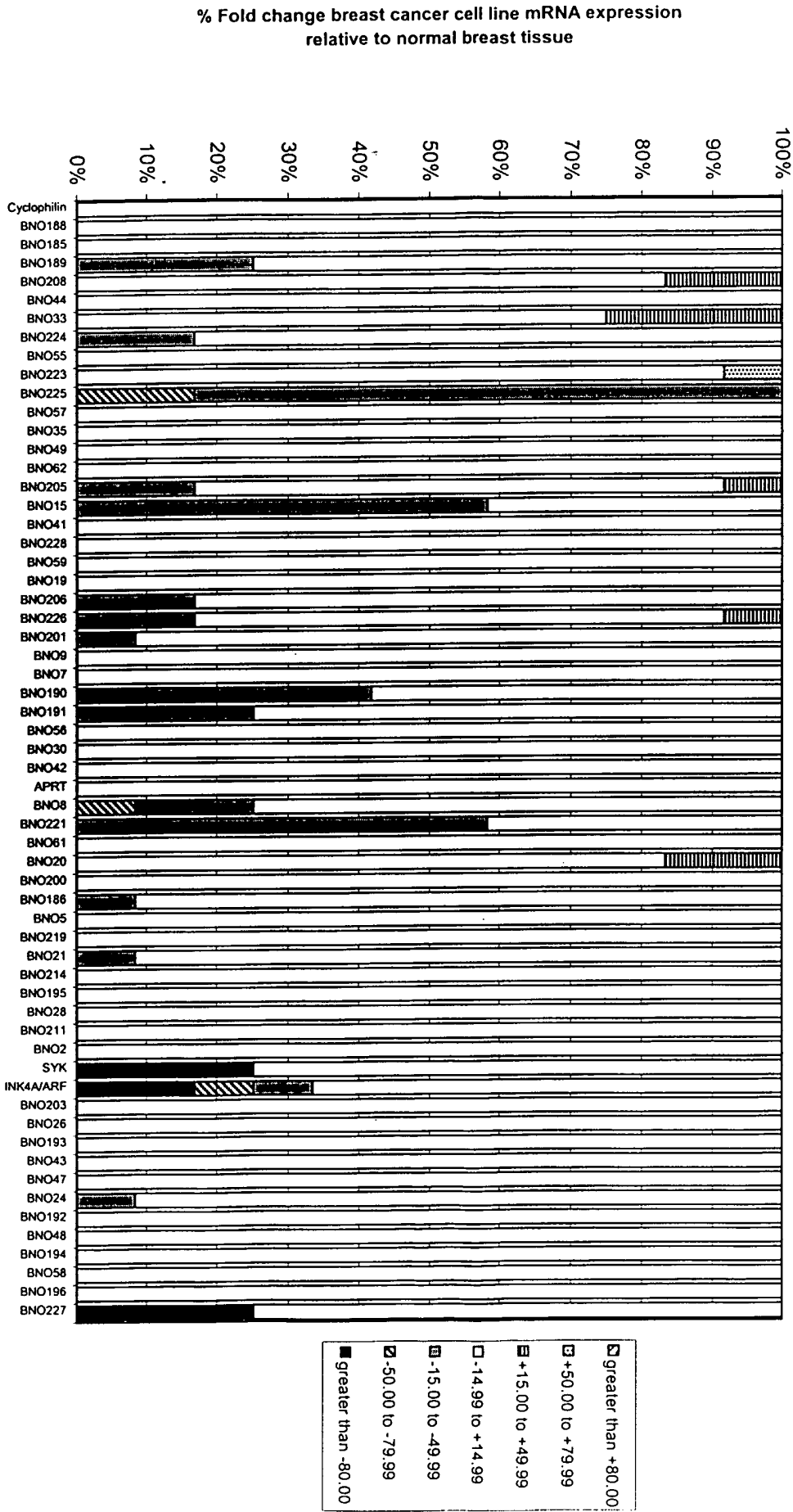


Figure 3

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 195 200 205

Gly Glu Ile Arg Thr Val Gln Val Gly Leu Asp Arg Glu Val Val Ala  
 210 215 220

Val Tyr Asn Leu Thr Leu Gln Val Ala Asp Met Ser Gly Asp Gly Leu  
 225 230 235 240

Thr Ala Thr Ala Ser Ala Ile Ile Thr Leu Asp Asp Ile Asn Asp Asn  
 245 250 255

Ala Pro Glu Phe Thr Arg Asp Glu Phe Phe Met Glu Ala Ile Glu Ala  
 260 265 270

Val Ser Gly Val Asp Val Gly Arg Leu Glu Val Glu Asp Arg Asp Leu  
 275 280 285

Pro Gly Ser Pro Asn Trp Val Ala Arg Phe Thr Ile Leu Glu Gly Asp  
 290 295 300

Pro Asp Gly Gln Phe Thr Ile Arg Thr Asp Pro Lys Thr Asn Glu Gly  
 305 310 315 320

Val Leu Ser Ile Val Lys Ala Leu Asp Tyr Glu Ser Cys Glu His Tyr  
 325 330 335

Glu Leu Lys Val Ser Val Gln Asn Glu Ala Pro Leu Gln Ala Ala Ala



## DiffExpr.ST25.txt

340

345

350

Leu Arg Ala Glu Arg Gly Gln Ala Lys Val Arg Val His Val Gln Asp  
 355 360 365

Thr Asn Glu Pro Pro Val Phe Gln Glu Asn Pro Leu Arg Thr Ser Leu  
 370 375 380

Ala Glu Gly Ala Pro Pro Gly Thr Leu Val Ala Thr Phe Ser Ala Arg  
 385 390 395 400

Asp Pro Asp Thr Glu Gln Leu Gln Arg Leu Ser Tyr Ser Lys Asp Tyr  
 405 410 415

Asp Pro Glu Asp Trp Leu Gln Val Asp Ala Ala Thr Gly Arg Ile Gln  
 420 425 430

Thr Gln His Val Leu Ser Pro Ala Ser Pro Phe Leu Lys Gly Gly Trp  
 435 440 445

Tyr Arg Ala Ile Val Leu Ala Gln Asp Asp Ala Ser Gln Pro Arg Thr  
 450 455 460

Ala Thr Gly Thr Leu Ser Ile Glu Ile Leu Glu Val Asn Asp His Ala  
 465 470 475 480

Pro Val Leu Ala Pro Pro Pro Gly Ser Leu Cys Ser Glu Pro His  
 485 490 495

Gln Gly Pro Gly Leu Leu Leu Gly Ala Thr Asp Glu Asp Leu Pro Pro  
 500 505 510

His Gly Ala Pro Phe His Phe Gln Leu Ser Pro Arg Leu Pro Glu Leu  
 515 520 525

Gly Arg Asn Trp Ser Leu Ser Gln Val Asn Val Ser His Ala Arg Leu  
 530 535 540

Arg Pro Arg His Gln Val Pro Glu Gly Leu His Arg Leu Ser Leu Leu  
 545 550 555 560

## DiffExpr.ST25.txt

Leu	Arg	Asp	Ser	Gly	Gln	Pro	Pro	Gln	Gln	Arg	Glu	Gln	Pro	Leu	Asn	
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Val	Thr	Val	Cys	Arg	Cys	Gly	Lys	Asp	Gly	Val	Cys	Leu	Pro	Gly	Ala	
			580					585					590			
Ala	Ala	Leu	Leu	Ala	Gly	Gly	Thr	Gly	Leu	Ser	Leu	Gly	Ala	Leu	Val	
		595					600					605				
Ile	Val	Leu	Ala	Ser	Ala	Leu	Leu	Leu	Leu	Val	Leu	Val	Leu	Leu	Val	
	610					615					620					
Ala	Leu	Arg	Ala	Arg	Phe	Trp	Lys	Gln	Ser	Arg	Gly	Lys	Gly	Leu	Leu	
625					630					635					640	
His	Gly	Pro	Gln	Asp	Asp	Leu	Arg	Asp	Asn	Val	Leu	Asn	Tyr	Asp	Glu	
				645					650					655		
Gln	Gly	Gly	Gly	Glu	Glu	Asp	Gln	Asp	Ala	Tyr	Asp	Ile	Ser	Gln	Leu	
			660					665					670			
Arg	His	Pro	Thr	Ala	Leu	Ser	Leu	Pro	Leu	Gly	Pro	Pro	Pro	Leu	Arg	
		675					680					685				
Arg	Asp	Ala	Pro	Gln	Gly	Arg	Leu	His	Pro	Gln	Pro	Pro	Arg	Val	Leu	
	690					695					700					
Pro	Thr	Ser	Pro	Leu	Asp	Ile	Ala	Asp	Phe	Ile	Asn	Asp	Gly	Leu	Glu	
705					710				715						720	
Ala	Ala	Asp	Ser	Asp	Pro	Ser	Val	Pro	Pro	Tyr	Asp	Thr	Ala	Leu	Ile	
				725				730						735		
Tyr	Asp	Tyr	Glu	Gly	Asp	Gly	Ser	Val	Ala	Gly	Thr	Leu	Ser	Ser	Ile	
			740					745					750			
Leu	Ser	Ser	Gln	Gly	Asp	Glu	Asp	Gln	Asp	Tyr	Asp	Tyr	Leu	Arg	Asp	
		755					760					765				
Trp	Gly	Pro	Arg	Phe	Ala	Arg	Leu	Ala	Asp	Met	Tyr	Gly	His	Pro	Cys	
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## DiffExpr.ST25.txt

Gly Leu Glu Tyr Gly Ala Arg Trp Asp His Gln Ala Arg Glu Gly Leu  
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Ser Pro Gly Ala Leu Leu Pro Arg His Arg Gly Arg Thr Ala  
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<212> DNA  
<213> Homo sapiens

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120

ccatgtggag cggatggtgg ctgtggcccc ttgtggccgt ctgcactgca gacttctttc  
180

gggacgaggc agagaggatc atgagggact cccctgtcat tgatgggcac aatgacctcc  
240

cctggcagct gctggatatg ttcaacaacc ggctgcagga cgagaggggc aacctgacca  
300

ccttggccgg cacacacacc aacatcccca agctgagggc cggctttgtg ggaggccagt  
360

tctggtccgt gtacacgccc tgcgacaccc agaacaaaga cgccgtgcgg aggacgctgg  
420

agcagatgga cgtgggtccac cgcatgtgcc ggatgtaccc ggagaccttc ctgtatgtca  
480

ccagcagtgc aggcattcgg caggccttcc ggggaagggaa ggtggccagc ctgatcggcg  
540

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600

## DiffExpr.ST25.txt

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720  
tgaaggagct gaaccgtctg ggggtcctca tcgacttggc tcacgtgtct gtggccacca  
780  
tgaaggccac cctgcagctg tccagagccc cggtcactct cagccactcc tcggcctaca  
840  
gcgtgtgcgc aagccggcgc aacgtgcctg acgacgtcct gaggctgggtg aaacagacag  
900  
acagcctggg gatgggtgaac ttctacaaca attacatttc ctgcaccaac aaggccaacc  
960  
tgtcccaagt ggccgaccat ctggatcaca tcaaggaggt ggcaggagcc agagccgtgg  
1020  
gttttggtgg ggactttgat ggtgttccaa gggtcctga ggggctggag gacgtctcca  
1080  
agtatccaga cctgatcgct gagctgctca ggaggaactg gacggaggcg gaggtcaagg  
1140  
gcgcactggc tgacaacctg ctgaggggtct tcgaggtgtt ggaacaggcc agcaacctca  
1200  
cacaggctcc cgaggaggag cccatcccgc tggaccagct ggggtggctcc tgcaggacct  
1260  
attacggcta ctctcttggg gcttccagcc tccatcgcca ctgggggctc ctgctggcct  
1320  
ccctcgctcc cctgggtcctc tgtctgtctc tcctgtgaaa cctgggagac cagagtcccc  
1380  
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1440

## DiffExpr.ST25.txt

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gcaggatgcc tggggacagt tcaggacaca cacacagtag gcccgcaata aaagcaacac  
1560

cccttc

1566

<210> 9  
<211> 411  
<212> PRT  
<213> Homo sapiens

<400> 9

Met Trp Ser Gly Trp Trp Leu Trp Pro Leu Val Ala Val Cys Thr Ala  
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Asp Phe Phe Arg Asp Glu Ala Glu Arg Ile Met Arg Asp Ser Pro Val  
20 25 30

Ile Asp Gly His Asn Asp Leu Pro Trp Gln Leu Leu Asp Met Phe Asn  
35 40 45

Asn Arg Leu Gln Asp Glu Arg Ala Asn Leu Thr Thr Leu Ala Gly Thr  
50 55 60

His Thr Asn Ile Pro Lys Leu Arg Ala Gly Phe Val Gly Gly Gln Phe  
65 70 75 80

Trp Ser Val Tyr Thr Pro Cys Asp Thr Gln Asn Lys Asp Ala Val Arg  
85 90 95

Arg Thr Leu Glu Gln Met Asp Val Val His Arg Met Cys Arg Met Tyr  
100 105 110

Pro Glu Thr Phe Leu Tyr Val Thr Ser Ser Ala Gly Ile Arg Gln Ala  
115 120 125

Phe Arg Glu Gly Lys Val Ala Ser Leu Ile Gly Val Glu Gly Gly His  
130 135 140

## DiffExpr.ST25.txt

```

Ser Ile Asp Ser Ser Leu Gly Val Leu Arg Ala Leu Tyr Gln Leu Gly
145                      150                      155                      160

Met Arg Tyr Leu Thr Leu Thr His Ser Cys Asn Thr Pro Trp Ala Asp
                      165                      170                      175

Asn Trp Leu Val Asp Thr Gly Asp Ser Glu Pro Gln Ser Gln Gly Leu
                      180                      185                      190

Ser Pro Phe Gly Gln Arg Val Val Lys Glu Leu Asn Arg Leu Gly Val
                      195                      200                      205

Leu Ile Asp Leu Ala His Val Ser Val Ala Thr Met Lys Ala Thr Leu
210                      215                      220

Gln Leu Ser Arg Ala Pro Val Ile Phe Ser His Ser Ser Ala Tyr Ser
225                      230                      235                      240

Val Cys Ala Ser Arg Arg Asn Val Pro Asp Asp Val Leu Arg Leu Val
                      245                      250                      255

Lys Gln Thr Asp Ser Leu Val Met Val Asn Phe Tyr Asn Asn Tyr Ile
260                      265                      270

Ser Cys Thr Asn Lys Ala Asn Leu Ser Gln Val Ala Asp His Leu Asp
275                      280                      285

His Ile Lys Glu Val Ala Gly Ala Arg Ala Val Gly Phe Gly Gly Asp
290                      295                      300

Phe Asp Gly Val Pro Arg Val Pro Glu Gly Leu Glu Asp Val Ser Lys
305                      310                      315                      320

Tyr Pro Asp Leu Ile Ala Glu Leu Leu Arg Arg Asn Trp Thr Glu Ala
                      325                      330                      335

Glu Val Lys Gly Ala Leu Ala Asp Asn Leu Leu Arg Val Phe Glu Ala
340                      345                      350

Val Glu Gln Ala Ser Asn Leu Thr Gln Ala Pro Glu Glu Glu Pro Ile

```

## DiffExpr.ST25.txt

355

360

365

Pro Leu Asp Gln Leu Gly Gly Ser Cys Arg Thr His Tyr Gly Tyr Ser  
 370 375 380

Ser Gly Ala Ser Ser Leu His Arg His Trp Gly Leu Leu Leu Ala Ser  
 385 390 395 400

Leu Ala Pro Leu Val Leu Cys Leu Ser Leu Leu  
 405 410

&lt;210&gt; 10

&lt;211&gt; 4539

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 10

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120

aggaagaggc gcgggagaag atgctggccg ccaagagcgc ggacggctcg gcgccggcag

180

gcgagggcga gggcgtgacc ctgcagcggc acatcacgct gctcaacggc gtggccatca

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360

gcgcgctctg ctacgcggag ctcggcacca ccatctccaa atcgggcggc gactacgcct

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480

tcatccggcc ttcacgcgag tacatcgtgg ccttggtctt cgccacctac ctgctcaagc

## DiffExpr.ST25.txt

540

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600

tgctgctgct cacggccgtg aactgctaca gcgtgaaggc cgccaccggt gtccaggatg

660

cctttgccgc cgccaagctc ctggccctgg ccctgatcat cctgctgggc ttcgtccaga

720

tcgggaaggg tgatgtgtcc aatctagatc ccaacttctc atttgaaggc accaaactgg

780

atgtggggaa cattgtgctg gcattataca gcggcctctt tgcctatgga ggatggaatt

840

acttgaattt cgtcacagag gaaatgatca acccctacag aaacctgccc ctggccatca

900

tcctctccct gcccatcgtg acgctggtgt acgtgctgac caacctggcc tacttcacca

960

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1320

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## DiffExpr.ST25.txt

1380

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1440

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1620

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1920

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2040

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2100

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2160

## DiffExpr.ST25.txt

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2400

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2460

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2520

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2580

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2760

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2880

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3000

## DiffExpr.ST25.txt

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3060

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3120

tgcccagtgg agcctgccgc cccacactgg gcaccgggac cccctcacca tccagtgggc  
3180

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3240

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3300

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3360

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3420

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3480

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3540

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3600

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3660

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3720

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3780

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## DiffExpr.ST25.txt

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3900

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4539

&lt;210&gt; 11

&lt;211&gt; 507

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

## DiffExpr.ST25.txt

&lt;400&gt; 11

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Asp	Gly	Ser	Ala	Pro	Ala	Gly	Glu	Gly	Glu	Gly	Val	Thr	Leu	Gln	Arg	
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Asn	Ile	Thr	Leu	Leu	Asn	Gly	Val	Ala	Ile	Ile	Val	Gly	Thr	Ile	Ile	
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Gly	Ser	Gly	Ile	Phe	Val	Thr	Pro	Thr	Gly	Val	Leu	Lys	Glu	Ala	Gly	
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Ser	Pro	Gly	Leu	Ala	Leu	Val	Val	Trp	Ala	Ala	Cys	Gly	Val	Phe	Ser	
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Ala	Phe	Leu	Lys	Leu	Trp	Ile	Glu	Leu	Leu	Ile	Ile	Arg	Pro	Ser	Ser	
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Gln	Tyr	Ile	Val	Ala	Leu	Val	Phe	Ala	Thr	Tyr	Leu	Leu	Lys	Pro	Leu	
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Phe	Pro	Thr	Cys	Pro	Val	Pro	Glu	Glu	Ala	Ala	Lys	Leu	Val	Ala	Cys	
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Leu	Cys	Val	Leu	Leu	Leu	Thr	Ala	Val	Asn	Cys	Tyr	Ser	Val	Lys	Ala	
			180					185					190			
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## DiffExpr.ST25.txt

Ala	Leu	Ile	Ile	Leu	Leu	Gly	Phe	Val	Gln	Ile	Gly	Lys	Gly	Asp	Val
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Ser	Asn	Leu	Asp	Pro	Asn	Phe	Ser	Phe	Glu	Gly	Thr	Lys	Leu	Asp	Val
225					230					235					240
Gly	Asn	Ile	Val	Leu	Ala	Leu	Tyr	Ser	Gly	Leu	Phe	Ala	Tyr	Gly	Gly
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Trp	Asn	Tyr	Leu	Asn	Phe	Val	Thr	Glu	Glu	Met	Ile	Asn	Pro	Tyr	Arg
			260					265					270		
Asn	Leu	Pro	Leu	Ala	Ile	Ile	Ile	Ser	Leu	Pro	Ile	Val	Thr	Leu	Val
		275					280					285			
Tyr	Val	Leu	Thr	Asn	Leu	Ala	Tyr	Phe	Thr	Thr	Leu	Ser	Thr	Glu	Gln
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Met	Leu	Ser	Ser	Glu	Ala	Val	Ala	Val	Asp	Phe	Gly	Asn	Tyr	His	Leu
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Gly	Val	Met	Ser	Trp	Ile	Ile	Pro	Val	Phe	Val	Gly	Leu	Ser	Cys	Phe
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Gly	Ser	Val	Asn	Gly	Ser	Leu	Phe	Thr	Ser	Ser	Arg	Leu	Phe	Phe	Val
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Gly	Ser	Arg	Glu	Gly	His	Leu	Pro	Ser	Ile	Leu	Ser	Met	Ile	His	Pro
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Gln	Leu	Leu	Thr	Pro	Val	Pro	Ser	Leu	Val	Phe	Thr	Cys	Val	Met	Thr
	370					375					380				
Leu	Leu	Tyr	Ala	Phe	Ser	Lys	Asp	Ile	Phe	Ser	Val	Ile	Asn	Phe	Phe
385					390					395					400
Ser	Phe	Phe	Asn	Trp	Leu	Cys	Val	Ala	Leu	Ala	Ile	Ile	Gly	Met	Ile
				405					410					415	
Trp	Leu	Arg	His	Arg	Lys	Pro	Glu	Leu	Glu	Arg	Pro	Ile	Lys	Val	Asn
			420					425					430		

## DiffExpr.ST25.txt

Leu Ala Leu Pro Val Phe Phe Ile Leu Ala Cys Leu Phe Leu Ile Ala  
435 440 445

Val Ser Phe Trp Lys Thr Pro Val Glu Cys Gly Ile Gly Phe Thr Ile  
450 455 460

Ile Leu Ser Gly Leu Pro Val Tyr Phe Phe Gly Val Trp Trp Lys Asn  
465 470 475 480

Lys Pro Lys Trp Leu Leu Gln Gly Ile Phe Ser Thr Thr Val Leu Cys  
485 490 495

Gln Lys Leu Met Gln Val Val Pro Gln Glu Thr  
500 505

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/00136

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>																						
Int. Cl. <sup>7</sup> : C12N 15/63 15/12, A61K 39/395 48/00, C07K 16/18 14/435																						
According to International Patent Classification (IPC) or to both national classification and IPC																						
<b>B. FIELDS SEARCHED</b>																						
Minimum documentation searched (classification system followed by classification symbols) SEE ELECTRONIC DATABASE BELOW																						
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched SEE ELECTRONIC DATABASE BOX BELOW																						
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EMBL, SWISS-PROT, PIR, EMBL, GENE BANK: SEQ ID Nos: 1-11																						
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
X	GenPept database Accession Number: AAA90925. <i>Parkos, C.A et al.</i> human neutrophil cytochrome b light chain p22 phagocyte b-cytochrome mRNA. 30 June 1988. & Swiss-Prot database Accession Number C24A Human. 1 January 1990. See whole document. Amino acid sequence is 100% identical to SEQ ID No: 2 (CYBA) over 195 & 194 amino acids respectively.	12, 14, 16, 33, 42 & 54																				
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input type="checkbox"/> See patent family annex																						
<p>* Special categories of cited documents:</p> <table border="0"> <tr> <td>"A"</td> <td>Document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>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</td> </tr> <tr> <td>"E"</td> <td>Earlier application or patent but published on or after the international filing date</td> <td>"X"</td> <td>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</td> </tr> <tr> <td>"L"</td> <td>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)</td> <td>"Y"</td> <td>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</td> </tr> <tr> <td>"O"</td> <td>Document referring to an oral disclosure, use, exhibition or other means</td> <td>"&amp;"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>Document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	Document defining the general state of the art which is not considered to be of particular relevance	"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	"E"	Earlier application or patent but published on or after the international filing date	"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	"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)	"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	"O"	Document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	Document published prior to the international filing date but later than the priority date claimed		
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"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)	"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																			
"O"	Document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																			
"P"	Document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 15 March 2002		Date of mailing of the international search report - 8 APR 2002																				
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer  <b>PHILIPPA WYRDEMAN</b> Telephone No : (02) 6283 2554																				



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/00136

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Genomics (1999) 60(3): 295-308. <i>Loftus, B.J et al.</i> "Genome duplications and other features in 12 mb of DNA sequence from human chromosome 16p and 16q". & EMBL database Accession No: AC003007. 29 October 1997. See whole document. Nucleic acid sequence is 100% identical to SEQ ID No: 3 over 327 nucleic acids.	12, 14, 16, 18-32, 42, 54, 66 & 67
X	EMBL database Accession No: AC005632. <i>Sulston, J.E et al.</i> Homo sapiens BAC clone CTB-31N19 from 16. 9 September 1998. See whole document. Nucleic acid sequence is 100% identical to SEQ ID No: 3 over 327 nucleic acids.	12, 14, 16, 18-32, 42, 54, 66 & 67
X	EMBL database Accession No: AC009086. DOE Joint Genome Institute. Homo sapiens chromosome 16 clone RP11-368N21. 4 August 1999. See whole document. Nucleic acid sequence is 99% identical to SEQ ID No: 3 over 327 nucleic acids.	12, 14, 16, 18-32, 42, 54, 66 & 67
X	J. Biol. Chem. (1998) 273: 10011-10018. <i>Shimoyama, Y et al.</i> "Molecular cloning and characterisation of a novel human classic cadherin with mouse nucle cadherin". & EMBL database Accession No: D83542, 29 February 1996. See whole document. Nucleic acid sequence is 99.6% identical to SEQ ID No: 6 (CDH15) over 2817 nucleic acids.	1, 2, 4-7, 12, 14, 16, 33, 54, 66 & 67
X	Biochem Biophys Res Commun (1999) 255(2):283-8. <i>Prasad, P.D et al.</i> "Human LAT1, a subunit of system L amino acid transporter: molecular cloning and transport function". & EMBL database Accession No: AF104032, 2 November 1998. See whole document. Nucleic acid sequence is 99% identical to SEQ ID No: 10 (SLC7A5) over 4539 nucleic acids.	13, 15, 17 & 33
X	EMBL database Accession No: AB018009. Cloning and characterisation of a human system transporter. 26 September 1998. See whole document. Nucleic acid sequence is 100% identical to SEQ ID No: 10 (SLC7A5) over 4539 nucleic acids.	1, 3, 8-11, 35-42, 51, 53, 55, 56, 58, 59 & 67-69
X	Nature (1998) 395(6699): 288-291. <i>Mastroberardino, L et al.</i> "Amino acid transport by heterodimers of 4F2hc/CD98 and members of permease family". See whole document. Amino acid sequence is 99% identical to SEQ ID No: 11 (SLC7A5) over 507 amino acids. & EMBL database Accession Nos: Q01650 & AAC61479.	13, 15, 17 & 33
X	EMBL database Accession No: BAA75746. <i>Minato, N et al.</i> 4F2 light chain [Homo sapiens] 9 March 1999. See whole document. Amino acid sequence is 98% identical to SEQ ID No: 11 (SLC7A5) over 507 amino acids.	13, 15, 17 & 33

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**Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos : **60-65**  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
See supplemental box
  
3. ☐ Claims Nos :  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

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**Supplemental Box**

(To be used when the space in any of Boxes I to VIII is not sufficient)

**Continuation of Box No: 1**

The scope of claims 60-65 is so inadequately supported by the specification that no meaningful search could be carried out.

These claims refer to the use of an anti-cancer drug or an anti-cancer drug when identified by the method of the invention or a compound for the increased activity/expression of a gene or an antagonist of a gene. However the claims are not restricted to "compounds" (including drugs and antagonists) produced using the applicant invention because the process of identifying "compounds" does not produce a product, it simply further characterises pre-existing "compounds". As such, the claims include within their scope "compounds", including pre-existing known compounds, whose engineering or isolation owes nothing to the teachings of the patent application. Therefore the only association between the applicant's invention and the compounds is that the invention has been used to further characterise the inherent properties of pre-existing compounds.