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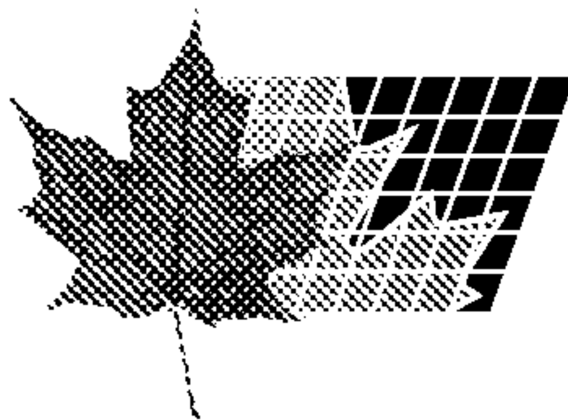

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(54) **INHIBITEUR DU BRN-3B ET SON UTILISATION DANS LE TRAITEMENT CONTRE LE CANCER DU SEIN ET LE CANCER DES OVAIRES**

(54) **INHIBITOR OF BRN-3B AND ITS USE FOR THE TREATMENT OF BREAST AND OVARIAN CANCER**

(57) An inhibitor of Brn-3b expression and/or activity is useful in the treatment of breast or ovarian cancer. A method for identifying an inhibitor of Brn-3b expression comprises: (a) providing a test construct comprising a Brn-3b promoter operably linked to a coding sequence; (b) contacting a substance to be tested with the test construct under conditions that would permit the polypeptide encoded by the said coding sequence to be expressed in the absence of the said substance; and (c) determining whether the said substance inhibits the expression of Brn-3b.

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(54) Title: INHIBITOR OF BRN-3B AND ITS USE FOR THE TREATMENT OF BREAST AND OVARIAN CANCER

(57) Abstract

An inhibitor of Brn-3b expression and/or activity is useful in the treatment of breast or ovarian cancer. A method for identifying an inhibitor of Brn-3b expression comprises: (a) providing a test construct comprising a Brn-3b promoter operably linked to a coding sequence; (b) contacting a substance to be tested with the test construct under conditions that would permit the polypeptide encoded by the said coding sequence to be expressed in the absence of the said substance; and (c) determining whether the said substance inhibits the expression of Brn-3b.

INHIBITOR OF BRN-3B AND ITS USE FOR THE TREATMENT OF BREAST AND OVARIAN CANCERS

Field of the invention

This invention relates to inhibitors of Brn-3b expression and/or activity and screening methods for the identification of such inhibitors. It further relates to the use of said 5 inhibitors in the treatment of cancer.

Background to the invention

The POU (Pit-Oct-Unc) family of transcription factors was originally defined on the basis of a common 150-160 amino acid domain which was found in found in the mammalian transcription factor Pit-1, Oct-1 and Oct-2, and the nematode regulatory 10 protein Unc-86 (for review see 1, 2). The common POU domain constitutes the DNA binding domain of these proteins and consists of two portions, a POU-specific domain which is unique to the POU factors and a POU-homeodomain which is related to the homeobox found in a number of other transcription factors.

Following the identification of the original POU family transcription factors, a number 15 of other members of this family have been defined (1-3) and shown to play critical roles in the regulation of gene expression. Moreover, mutations in the genes encoding several of these factors have been shown to be responsible for particular human diseases. Thus, for example, mutation in the gene encoding Pit-1 has been shown to result in a failure of pituitary gland development and consequent dwarfism in both mice and humans (for 20 review see 4) whilst a mutation in the gene encoding Brn-4 has been shown to result in X-linked deafness (5). More recently, the gene encoding the POU factor Brn-3c has been shown to be mutated in a family with progressive late onset deafness (6).

Brn-3a, Brn-3b and Brn-3c are closely related members of the POU family which are encoded by different genes (7) and are expressed in distinct but overlapping patterns in the 25 developing and adult nervous system (3, 8-11). In addition however, expression of Brn-3a and Brn-3b has also been detected in some non-neuronal cells such as cervical epithelium (9, 12).

Brn-3a has been shown to be over-expressed in aggressive neuroendocrine tumours (14), whilst both Brn-3a and Brn-3b are expressed at high levels in human neuroblastomas

(15, 16). Brn-3a and Brn-3b have previously been detected in the human breast cancer cell line MCF-7 (13). We have recently shown that mean Brn-3a levels are increased over 300 fold in human samples exhibiting cervical intra-epithelial neoplasia grade 3 (CIN3) compared to normal human cervical samples but Brn-3b levels are similar in the two
5 groups of samples (12).

Summary of the invention

The present invention is based on the finding that mammary tumour tissue which has reduced expression levels of the BRCA-1 gene shows elevated expression of the Brn-3b POU family transcription factor. The BRCA-1 gene was identified on the basis of its
10 mutation in a number of cases of familial breast cancer indicating that its inactivation can cause this disease. Athough BRCA-1 does not appear to be mutated in cases of sporadic breast cancer, its expression has been shown to be reduced in a number of cases.

The elevated expression of Brn-3b is not found in normal mammary cells, benign tumours or malignant tumour samples which do not exhibit reduced levels of the BRCA-1
15 gene. In contrast no correlation between the level of BRCA-1 expression and the expression of the related POU family transcription factor Brn-3a. Moreover, Brn-3b but not Brn-3a can strongly repress the BRCA-1 promoter approximately 20 fold in transfections carried out in mammary tumour cells.

Thus, Brn-3b may play an important role in regulating expression of BRCA-1 in
20 mammary tumours with enhanced expression of Brn-3b resulting in reduced BRCA-1 expression and thereby being potentially involved in tumour development. The repression of Brn-3b expression by either pharmacological or by gene therapy procedures represents a potential method for treating breast cancers.

Thus, the invention provides an inhibitor of Brn-3b expression and/or activity for use
25 in a method of treatment of the human of animal body. Such inhibitors are useful, in particular, in the treatment of breast cancer and/or ovarian cancer.

Screens may be carried out to identify inhibitors of Brn-3b expression and/or activity. Accordingly, the invention also provides:

- a method for identifying an inhibitor of Brn-3b expression comprising:

30 (a) providing a test construct comprising a Brn-3b promoter operably linked to a coding sequence;

(b) contacting a substance to be tested with the test construct under conditions that would permit the polypeptide encoded by the said coding sequence to be expressed in the absence of the said substance; and

(c) determining whether the said substance inhibits the expression of Brn-3b; and

5 - a method for identifying an inhibitor of Brn-3b activity comprising:

(a) providing a Brn-3b polypeptide or a homologue thereof, or a fragment thereof;

(b) contacting a substance to be tested with the Brn-3b polypeptide under conditions that would permit activity of the polypeptide in the absence of said substance; and

(c) determining whether the said substance inhibits the activity of Brn-3b.

10 The invention further provides a pharmaceutical composition comprising an inhibitor of Brn-3b expression and/or activity and a therapeutically acceptable carrier or diluent.

As noted above, the inhibitors of Brn-3b expression and/or activity may be used in treating breast or ovarian cancer and therefore the invention provides a method of treatment of treating a host suffering from breast cancer or ovarian cancer, which method comprises 15 administering to the host a therapeutically effective amount of an inhibitor of Brn-3a expression and/or activity.

Brief description of the Figures

Figure 1 shows levels of Brn-3a mRNA as determined by RT/PCR assay in normal/benign mammary material or in malignant mammary tumours from pre- 20 menopausal or post-menopausal women. Bar designates the mean with standard deviation (SD) and population size 'n'.

Figure 2 shows levels of Brn-3b mRNA as determined by RT/PCR assay in normal/benign mammary material or in malignant mammary tumours from pre-menopausal or post-menopausal women. Bar designates the mean with standard deviation 25 (SD) and population size 'n'.

Figure 3 shows comparison of the mRNA levels of Brn-3b and BRCA-1 in malignant mammary tumour material obtained from pre (panel a) or post (panel b) menopausal women or from benign mammary material (panel c). Brn-3b levels are shown as solid bars, BRCA-1 levels as open bars.

30 Figure 4 shows comparison of the mRNA levels of Brn-3a and BRCA-1 in malignant mammary tumour material obtained from pre (panel a) or post (panel b) menopausal

women or from benign mammary material (panel c). Brn-3a levels are shown as solid bars, BRCA-1 levels as open bars.

Figure 5 shows comparison of BRCA-1 levels in mammary tumours with low or high levels of Brn-3b. Bar designates the mean with standard deviation (SD) and population
5 size 'n'.

Figure 6 shows a luciferase reporter assay of MCF7 cells co-transfected with a promoter/reporter construct containing the full length BRCA-1 promoter together with expression vector lacking any insert (V) or the same vector expressing either Brn-3a or Brn-3b. Values have been equalized relative to the activity obtained in the co-transfection
10 with the reporter construct and empty expression vector (set at 100%) and are the mean of five determinations whose standard deviation is shown by the bars.

Detailed description of the invention

Any suitable inhibitor of Brn-3b expression or activity may be employed in the present invention. For example, the expression of Brn-3b in a cell may be reduced by
15 presence in that cell of a polynucleotide which can hybridize to the Brn-3b mRNA. Therefore a polynucleotide which is capable of hybridizing to Brn-3b mRNA can constitute an appropriate inhibitor of Brn-3b expression. In this regard, two approaches are as follows:

(1) Antisense RNA

20 The delivery of a nucleic acid construct which allows the expression of an RNA which can hybridize to the Brn-3b mRNA. This results in the formation of an RNA-RNA duplex which may result in the direct inhibition of translation and/or the destabilization of the target message, for example, rendering susceptibility to nucleases. Therefore, the nucleic acid construct will typically lead to the expression of a polynucleotide which hybridizes to
25 the ribosome binding region or the coding region of the Brn-3b mRNA.

(2) Antisense oligonucleotides

An oligonucleotide is delivered which hybridizes to the Brn-3b mRNA. Antisense oligonucleotides are postulated to inhibit target gene expression by interfering with one or more aspects of RNA metabolism including processing, translation and metabolic turnover.
30 Chemically modified oligonucleotides may be used and may enhance resistance to nucleases and/or cell permeability.

Antisense

The coding sequence of Brn-3b is given in SEQ ID NO:1. An inhibitor of Brn-3b comprises a polynucleotide which can hybridize to the Brn-3b mRNA. Typically such a polynucleotide will be an RNA molecule. Such a polynucleotide may hybridize to all or part of the Brn-3b mRNA. Typically the polynucleotide will be complementary to all of or a region of the Brn-3b mRNA. For example, the polynucleotide may be the exact complement of all or a part of Brn-3b mRNA. However, absolute complementarity is not required and preferred polynucleotides which have sufficient complementarity to form a duplex having a melting temperature of greater than 40°C under physiological conditions are particularly suitable for use in the present invention. The polynucleotide may be a polynucleotide which hybridises to the Brn-3b mRNA under conditions of medium to high stringency such as 0.03M sodium chloride and 0.03M sodium citrate at from about 50 to about 60 degrees centigrade.

It is preferred that the polynucleotide hybridizes to the region of the mRNA corresponding to the coding sequence defined by nucleotides 213 to 392 of SEQ ID NO:1. The polynucleotide may hybridize to all or part of this region. However, a polynucleotide may be employed which hybridises to all or part of the 5'- or 3'-untranslated region of the mRNA. These regions correspond to nucleotides 1 to 212 and 1446 to 3110 of SEQ ID NO:1.

The polynucleotide will typically be at least 40, for example at least 60 or at least 80, nucleotides in length and up to 100, 200, 300, 400, 500, 600 or 700 nucleotides in length or even up to a few nucleotides, such as five or ten nucleotides, shorter than SEQ ID NO: 1.

When the polynucleotide is an antisense RNA it may be expressed in a cell from a recombinant replicable vector. Such a replicable vector comprises a polynucleotide which when transcribed gives rise to antisense RNA. Preferably the polynucleotide giving rise to the antisense RNA is operably linked to a control sequence which is capable of providing for the transcription of the polynucleotide giving rise to the antisense RNA. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a sequence giving rise to an antisense RNA is ligated in such a way that transcription of the sequence is achieved under conditions compatible with the control sequences.

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for transcription to occur and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used in vitro, for example for the production of antisense RNA or used to transfect or transform a host cell. The vector may also be adapted to be used in vivo, for example in a method of gene therapy.

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, mammalian promoters, such as b-actin promoters, may be used. Tissues-specific promoters, in particular neuronal cell specific promoters (for example the tyrosine hydroxylase (TH), L7, or neuron specific enolase (NSE) promoters), are especially preferred. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the promoter rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are readily available in the art. Preferred promoters are tissue specific promoters such as the casein gene promoter.

Vectors may further include sequences flanking the polynucleotide giving rise to antisense RNA which comprise sequences homologous to eukaryotic genomic sequences, preferably mammalian genomic sequences, or viral genomic sequences. This will allow the introduction of the polynucleotides of the invention into the genome of eukaryotic cells or viruses by homologous recombination. In particular, a plasmid vector comprising the expression cassette flanked by viral sequences, for example HSV1 or HSV2 sequences, can be used to prepare a viral vector, fpr example an HSV vector, suitable for delivering the polynucleotides of the invention to a mammalian cell. Other examples of suitable viral vectors include retroviruses, including lentiviruses, adenoviruses and adeno-associated viruses. Gene transfer techniques using these viruses are will known to those skilled in the art. Retrovirus vectors for example may be used to stably integrate the polynucleotide giving rise to the antisense RNA into the host genome. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression.

Antisense oligonucleotides

An antisense oligonucleotide will typically have a sequence such that it will bind to the Brn-3b mRNA. Therefore it will typically have a sequence which is the complement of a region of the sequence shown in SEQ ID NO: 1. An antisense oligonucleotide will generally be from 6 to 40 nucleotides in length. Preferably it will be from 12 to 20 nucleotides in length.

5 Generally the oligonucleotide used will have a sequence that is absolutely complementary to the target sequence. However, absolute complementarity may not be required and in general any oligonucleotide having sufficient complementarity to form a stable duplex (or triple helix as the case may be) with the target nucleic acid is considered to be suitable. The stability of a duplex (or triplex) will depend *inter alia* on the sequence and length of the hybridizing 10 oligonucleotide and the degree of complementarity between the antisense oligonucleotide and the target sequence. The system can tolerate less complementarity when longer oligonucleotides are used. However oligonucleotides, especially oligonucleotides of from 6 to 40 nucleotides in length, which have sufficient complementarity to from a duplex having a melting temperature of greater than 40°C under physiological conditions are particularly 15 suitable for use in the present invention. The polynucleotide may be a polynucleotide which hybridises to under conditions of medium to high stringency such as 0.03M sodium chloride and 0.03M sodium citrate at from about 50 to about 60 degrees centigrade.

Antisense oligonucleotides may be chemically modified. For example, phosphorothioate oligonucleotides may be used. Other deoxynucleotide analogs include methylphosphonates, 20 phosphoramidates, phosphorodithioates, N3'P5'-phosphoramidates and oligoribonucleotide phosphorothioates and their 2'-O-alkyl analogs and 2'-O-methylribonucleotide methylphosphonates.

Alternatively mixed backbone oligonucleotides (MBOs) may be used. MBOs contain segments of phosphothioate oligodeoxynucleotides and appropriately placed segments of 25 modified oligodeoxy- or oligoribonucleotides. MBOs have segments of phosphorothioate linkages and other segments of other modified oligonucleotides, such as methylphosphonate, which is non-ionic, and very resistant to nucleases or 2'-O-alkyloligoribonucleotides.

Administration

The vectors and antisense oligonucleotides of the invention, optionally with an additional 30 therapeutic polypeptide or nucleic acid/vector encoding said therapeutic polypeptide, may thus be administered to a human or animal in need of treatment. Cancers which may be treated using the vectors, viral strains, antisense oligonucleotides and compositions of the invention

include breast or ovarian cancer and, in particular, breast or ovarian cancer in which Brn-3b expression is up-regulated such as non-familial breast cancer. The condition of a patient suffering from such a cancer can thus be improved.

The antisense oligonucleotides and compositions comprising antisense oligonucleotides of the invention together may be administered by direct injection into the site to be treated, for example mammary tissue. Preferably the antisense oligonucleotides are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

The dose at which an antisense oligonucleotide is administered to a patient will depend upon a variety of factors such as the age, weight and general condition of the patient, the cancer that is being treated and the stage which the cancer has reached, and the particular antisense oligonucleotide that is being administered. A suitable dose may however be from 0.1 to 100 mg/kg body weight such as 1 to 40 mg/kg body weight.

The polynucleotides giving rise to antisense RNA of the invention may be administered directly as a naked nucleic acid construct. Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™).

Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition.

Preferably the naked nucleic acid construct, viral vector comprising the polynucleotide or composition is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

The pharmaceutical composition is administered in such a way that the polynucleotide of the invention, viral vector for gene therapy, can be incorporated into cells at an appropriate area. When the polynucleotide of the invention is delivered to cells by a viral vector, the amount of virus administered is in the range of from 10^4 to 10^8 pfu, preferably from 10^5 to 10^7 pfu, more

preferably about 10^6 pfu for herpes viral vectors and from 10^6 to 10^{10} pfu, preferably from 10^7 to 10^9 pfu, more preferably about 10^8 pfu for adenoviral vectors. When injected, typically 1-2 ml of virus in a pharmaceutically acceptable suitable carrier or diluent is administered. When the polynucleotide of the invention is administered as a naked nucleic acid, the amount of
5 nucleic acid administered is typically in the range of from 1 μg to 10 mg.

Where the polynucleotide giving rise to the antisense RNA is under the control of an inducible promoter, it may only be necessary to induce gene expression for the duration of the treatment. Once the condition has been treated, the inducer is removed and expression of the polypeptide of the invention ceases. This will clearly have clinical advantages. Such a system
10 may, for example, involve administering the antibiotic tetracycline, to activate gene expression via its effect on the tet repressor/VP16 fusion protein.

The use of tissue-specific promoters will be of assistance in the treatment of disease using the polypeptides, polynucleotide and vectors of the invention. For example, several neurological disorders are due to aberrant expression of particular gene products in only a small
15 subset of cells. It will be advantageous to be able express therapeutic genes in only the relevant affected cell types, especially where such genes are toxic when expressed in other cell types.

The routes of administration and dosages described above are intended only as a guide since a skilled physician will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

20 Screening for inhibitors of Brn-3b expression and/or activity

The invention provides a method for identifying an inhibitor of Brn-3b expression comprising:

(i) providing a test construct comprising a Brn-3b promoter operably linked to a coding sequence;
25 (ii) contacting a substance to be tested with the test construct under conditions that would permit expression of the polypeptide encoded by the said coding sequence to be expressed in the absence of the said substance; and
(iii) determining whether the said substance inhibits the expression of Brn-3b.

Any suitable assay format may be used for identifying an inhibitor of Brn-3b expression.
30 Typically, however, the assay is carried out using a cell harbouring a promoter:reporter polypeptide construct. A typical assay is as follows:

- a defined number of cells are inoculated, in for example 100μl of growth medium, into the

wells of a plastics micro-titre plate in the presence of a substance to be tested.

- optical density (OD) at 590nm may be measured as may expression of the reporter polypeptide according to any method appropriate for the reporter polypeptide being used.
- the micro-titre plates are covered and incubated at 37°C in the dark.
- the OD is read again and expression of the reporter polypeptide assayed at convenient time intervals. The change in OD is a measure of cell proliferation. Control experiments can be carried out, in which the substance to be tested is omitted.

Also the substance may be tested with any other known promoter to exclude the possibility that the test substance is a general inhibitor of gene expression.

Any reporter polypeptide may be used, for example GUS or GFP are used. GUS is assayed by measuring the hydrolysis of a suitable substrate, for example 5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid (X-gluc)or 4-methylumbelliferyl-β-glucuronide (MUG). The hydrolysis of MUG yields a product which can be measured fluorometrically. GFP is quantified by measuring fluorescence at 590nm after excitation at 494nm. These methods are well known to those skilled in the art.

Alternatively the coding sequence may be the Brn-3b coding sequence itself. In such an experiment a mammary cancer cell line which exhibits Brn-3b overexpression could be used. The expression of Brn-3b may be followed by for example, Northern/RNA blotting, Western/antibody blotting, RNA in situ hybridization or immunolocalisation.

The invention further provides a method for identifying an inhibitor of Brn-3b activity comprising:

(i) providing a Brn-3b polypeptide or a homologue thereof, or a fragment thereof;
(ii) contacting a substance to be tested with the Brn-3b polypeptide under conditions that would permit activity of the polypeptide in the absence of said substance; and
(ii) determining whether the said substance inhibits the activity of Brn-3b.

Suitable Brn-3b for the assay can be obtained, for example, recombinantly by any method known to those skilled in the art. Any suitable format may be used for identifying an inhibitor of Brn-3b.

Also the substance may be tested with any other known transcription factor to exclude the possibility that the test substance is a general inhibitor of transcription factors activity.

In addition to the Brn-3b polypeptide, the reaction mixture can contain a suitable buffer. A suitable buffer includes any suitable biological buffer that can provide buffering capability at

a pH conducive to the reaction requirements of the Brn-3b polypeptide.

Test substances

A substance which inhibits the expression of Brn-3b may do so by binding directly to the promoter, thus preventing the initiation of transcription. Alternatively a substance could bind to
5 a protein which is associated with the promoter and is required for transcription. This may result in reduced levels of transcription.

The Brn-3b promoter:reporter polypeptide constructs of the invention may include the untranslated region of the Brn-3b gene. Therefore a substance may reduce Brn-3b expression by binding to the untranslated region of the Brn-3b gene. This could prevent the initiation of
10 translation. Alternatively a substance could bind to a protein associated with the untranslated region and prevent the protein associating with the untranslated region.

A substance which inhibits the activity of Brn-3b may do so by binding to one or both of the enzymes. Such enzyme inhibition may be reversible or irreversible. An irreversible inhibitor dissociates very slowly from its target enzyme because it becomes very tightly bound
15 to the enzyme, either covalently or non-covalently. Reversible inhibition, in contrast with irreversible inhibition, is characterised by a rapid dissociation of the enzyme-inhibitor complex.

The test substance may be a competitive inhibitor. In competitive inhibition, the enzyme can bind substrate (forming an enzyme-substrate complex) or inhibitor (enzyme-inhibitor complex) but not both. Many competitive inhibitors resemble the substrate and bind the active
20 site of the enzyme. The substrate is therefore prevented from binding to the same active site. A competitive inhibitor diminishes the rate of catalysis by reducing the proportion of enzyme molecules bound to a substrate.

The inhibitor may also be a non-competitive inhibitor. In non-competitive inhibition, which is also reversible, the inhibitor and substrate can bind simultaneously to an enzyme
25 molecule. This means that their binding sites do not overlap. A non-competitive inhibitor acts by decreasing the turnover number of an enzyme rather than by diminishing the proportion of enzyme molecules that are bound to substrate.

The inhibitor can also be a mixed inhibitor. Mixed inhibition occurs when an inhibitor both effects the binding of substrate and alters the turnover number of the enzyme.

30 A substance which inhibits the activity of Brn-3b may also do so by binding to the substrate. The substance may itself catalyze a reaction of the substrate, so that the substrate is

not available to the enzyme. Alternatively the inhibitor may simply prevent the substrate binding to the enzyme.

Suitable candidate substances include antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimaeric antibodies and CDR-grafted
5 antibodies) which are specific for Brn-3b Furthermore, combinatorial libraries, defined chemical entities, peptide and peptide mimetics, oligonucleotides and natural product libraries may be screened for activity as inhibitors of Brn-3b in assays such as those described below. The candidate substances may be chemical compounds. The candidate substances may be used in an initial screen of, for example, ten substances per reaction, and the substance of these
10 batches which show inhibition tested individually.

Inhibitors of Brn-3b expression and/or activity

An inhibitor of Brn-3b expression and/or activity is one which produces a measurable reduction in Brn-3b expression and/or activity in the assays described above. Preferred substances are those which inhibit Brn-3b expression and/or activity by at least 10%, at least
15 20%, at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% at a concentration of the inhibitor of $1\mu g\ ml^{-1}$, $10\mu g\ ml^{-1}$, $100\mu g\ ml^{-1}$, $500\mu g\ ml^{-1}$, $1mg\ ml^{-1}$, $10mg\ ml^{-1}$, $100mg\ ml^{-1}$. The percentage inhibition represents the percentage decrease in expression/activity in a comparison of assays in the presence and absence of the test substance. Any combination of the above mentioned degrees of percentage
20 inhibition and concentration of inhibitor may be used to define an inhibitor of the invention, with greater inhibition at lower concentrations being preferred.

Candidate substances which show activity in assays such as those described below can then be tested on mammary cancer cell lines for example. Candidate inhibitors could be tested for their ability to attenuate Brn-3b expression in mammary cancer cell lines in which Brn-3b is
25 up-regulated and also for the effect on BRCA-1 expression in mammary cancer cell lines in which BRCA-1 is down-regulated.

Human use

Inhibitors of Brn-3b expression and/or activity identified by the screening procedures described above may be used to treat breast or ovarian cancer and, in particular, breast or
30 ovarian cancer in which Brn-3b expression is up-regulated such as non-familial breast cancer. The condition of a patient suffering from a cancer can therefore be improved by administration of such an inhibitor. A therapeutically effective amount of such an inhibitor may be given to a

human patient in need thereof.

The formulation of an inhibitor for use in preventing or treating breast or ovarian cancer will depend upon factors such as the nature of the substance identified, whether a pharmaceutical or veterinary use is intended, etc. Typically an inhibitor is formulated for use with a pharmaceutically acceptable carrier or diluent. For example it may be formulated for topical, parenteral, intravenous, intramuscular, subcutaneous, intraocular, transdermal or oral administration. A physician will be able to determine the required route of administration for each particular patient. The pharmaceutical carrier or diluent may be, for example, an isotonic solution.

The dose of inhibitor may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient.

The following Example illustrates the invention.

EXAMPLE

Materials and Methods

Unless indicated otherwise, the methods used are standard biochemistry and molecular biology techniques. Examples of suitable general methodology textbooks include Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley and Sons, Inc.

Normal and breast cancer samples

Total RNA from normal mammary gland and from malignant breast tumours (from pre and post menopausal women) was obtained from the Candis Tissue Bank Research Centre Liverpool University.

Reverse transcriptase/polymerase chain reaction assay

About 0.1μg of RNA from each sample was used as a template for cDNA synthesis. Th synthesized cDNA was used in RT-PCR assays as previously described (17) using the following oligonucleotide primers: Brn-3a: 5' GTCGACATGGACTCGGACACG-3', 3'-ACGGTGAATGACTCCCCCGA-5'; Brn-3b: 5'GGAGAAGAAGCGCAAGC-3', 3'CTGAGAACCGGAGAGGTCT-5'. Amplification of the invariantly expressed human cyclophilin mRNA used as a control was carried out in parallel using the following primers: 5'-TTGGGCCGCGGTACTCCTTTCA-3', and 3'-TTTCGTATGGCCCAGGACCG-5'.

In all cases, 20μl of each PCR product was fractionated on a 2% agarose gel and blotted onto Hybond-N+ nylon membrane (Amersham International, Little Chalfont, United Kingdom) and hybridized with homologous complimentary ^{32}P-lablled probes. Membranes were exposed to films, (Eastman Kodak Co., Rochester, NY) and the subsequent autoradiographs were then analyzed using a densitometer (Bio-Rad Laboratories, Hercules, CA). We have previously shown that this blotting procedure, in conjunction with the RT-PCR conditions used, allows accurate quantification of the Brn-3a and Brn-3b mRNAs relative to the constitutively expressed cyclophilin mRNA (9, 12, 17).

Plasmid constructs

The Brn-3a and Brn-3b expression vectors contain full length cDNA clones under the control of the moloney murine leukaemia virus promoter and have previously been described (7, 17). The BRCA-1 promoter/reporter constructs contain 4 kilo-bases or 400 bases of upstream sequence containing the BRCA-1 α and β promoters cloned into the pGL2 luciferase vector.

Transient transfection

MCF7 cells were routinely grown in Dulbecco's modified Eagle's medium containing L-glutamate and phenol red which was supplemented with 10% foetal calf serum and 10ng of insulin per ml. Before experiments were carried out subconfluent cells were maintained in phenol red-free Dulbecco's modified Eagle's medium containing 10% dextran coated charcoal-striped foetal calf serum prepared according to the method described by Migliaccio *et al.*, (18) and 10ng of insulin per ml for 72 hours. The medium was replaced by 5 ml of fresh medium 12 hours prior to transfection. Transfection of plasmid DNA was carried out according to the method of Gorman (19). Routinely 5μg of reporter DNA and 5μg of each expression vector were transfected into 5×10^5 cells and the cells harvested after 72 hours. The amount of DNA taken up by the cells in each case was measured by slot blotting of 15μl of the extract and hybridization with a probe derived from the ampicillin resistance gene in the plasmid vector (20). Differences in the intensity of the bands were measured by densitometry and used to equalize the volumes of extracts used for subsequent assay. All transfections also included an internal reporter gene encoding the renilla luciferase with a dual luciferase assay being carried out to control for transfection efficiency. Luciferase assays were done as described by the manufacturers (Promega) protocol with results measured on a Turner 20-E luminometer.

Results

To quantitate the level of Brn-3a and Brn-3b mRNAs we used a reverse transcriptase/polymerase chain reaction (RT/PCR) assay which we have previously used to reliably quantitate these mRNAs in small amounts of clinical and other material (12, 15). This assay was used to compare the levels of Brn-3a and Brn-3b mRNAs in normal breast tissue or benign breast tumour material with that obtained from malignant breast tumours from pre- or post- menopausal women. The mean levels of Brn-3a (figure 1) and Brn-3b (figure 2) both showed some increase in the material prepared from the pre-menopausal malignant tumours compared to that obtained from normal/benign tumour material and a further increase was observed in both cases in the post-menopausal tumour material but this was not statistically significant ($p>0.05$ in all comparisons of normal versus tumour levels).

Interestingly, however, clear differences were noticed in the distribution pattern of Brn-3a and Brn-3b expression levels in the different tumour samples. Thus, Brn-3a levels appeared to show a continuous distribution between the different samples. In contrast, Brn-3b levels exhibited a bipartite distribution in the different tumour samples with approximately 40% of the samples showing low levels of Brn-3b within the range observed for the normal samples whilst the remaining samples showed much higher levels outside the normal range. This bipartite distribution was observed for tumour samples obtained from both pre-and post-menopausal women.

To determine whether this bipartite distribution had any functional significance, we searched for differences between the tumour samples with respectively low or high levels of Brn-3b expression. In particular, we examined the level in the different samples of the mRNA encoding BRCA-1 protein which is mutated in many cases of familial breast cancer (21, 22) and which has also been reported to exhibit reduced expression in non-hereditary (sporadic) breast cancer (23). Most interestingly, the levels of BRCA-1 in the different samples showed an inverse expression pattern to that observed with Brn-3b. Thus, the samples with low levels of Brn-3b exhibited high levels of BRCA-1 which was observed in the samples derived from both pre-menopausal (figure 3a) and post-menopausal (figure 3b) women, although the effect was particularly marked in the samples from pre-menopausal women. In contrast no significant variation in BRCA-1 mRNA levels was noted in different samples of normal mammary material or material from benign tumours which also had similar levels of Brn-3b (figure 3c).

In contrast to the results with Brn-3b, the level of BRCA-1 mRNA in malignant material from pre-menopausal (figure 4a) or post-menopausal (figure 4b) women showed no correlation with the level of Brn-3a mRNA in the different samples. Moreover, similar levels of BRCA-1 mRNA were observed in the normal or benign material despite the different Brn-3a levels in 5 the different samples (figure 4c). The specific association of high Brn-3b levels with low BRCA-1 expression was confirmed by dividing the samples into those with high Brn-3b and low Brn-3b. As illustrated in figure 5, BRCA-1 levels were highly elevated in the sample group with low Brn-3b compared to the level in those with high Brn-3b. This effect was statistically significant ($p<0.001$ comparing BRCA-1 levels in the high and low Brn-3b 10 expressing groups).

These findings therefore raise the possibility that the Brn-3b transcription factor may have an inhibitory effect on expression of BRCA-1, with the high levels of Brn-3b observed in some malignant mammary tumour samples being associated with a low level of BRCA-1. To investigate this possibility further, we co-transfected MCF7 cells with expression vectors 15 encoding Brn-3a or Brn-3b and a construct in which a four kilo-base fragment of the BRCA-1 promoter drives expression of a luciferase reporter gene.

In this experiment (figure 6) the activity of the promoter was reduced by approximately 50% in the sample co-transfected with Brn-3a compared to the level observed in the sample co-transfected with the corresponding expression vector lacking any insert. However, a much 20 more dramatic inhibitory effect was observed in the sample co-transfected with the Brn-3b expression vector where promoter activity was reduced to approximately 5% of that observed in the sample transfected with empty expression vector. Similar results were also obtained with a reporter construct containing only 400 base pairs of the BRCA-1 promoter indicating that this effect was dependent on this short promoter region (data not shown). This effect was specific 25 to the BRCA-1 promoter, since transfection of the same Brn-3b expression vector with reporters containing an oestrogen response element resulted in a strong stimulatory effect on the promoter in accordance with our previous results (13) (data not shown). Hence Brn-3b can indeed directly inhibit the BRCA-1 promoter in co-transfections into breast cancer cells.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: UNIVERSITY COLLEGE LONDON
5 (B) STREET: Gower Street
(C) CITY: London
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): WC1E 6BT

(ii) TITLE OF INVENTION: TREATMENT OF CANCER

10 (iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
15 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3110 base pairs
20 (B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
25 (A) NAME/KEY: CDS
(B) LOCATION:213..1445

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Arg Ser
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ATG CGG AGA GCC TGT CTT CCA ACC CCA CCG AGC AAT ATA TTC GGC GGG       521
Met Arg Arg Ala Cys Leu Pro Thr Pro Pro Ser Asn Ile Phe Gly Gly       10
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CTG GAT GAG AGT CTG CTG GCC CGC GCC GAG GCT CTG GCA GCC GTG GAC       569
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Ile Val Ser Gln Ser Lys Ser His His His His Pro Pro His His Ser
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Pro Phe Lys Pro Asp Ala Thr Tyr His Thr Met Asn Thr Ile Pro Cys
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ACG TCG GCC GCC TCT TCT TCA TCG GTG CCC ATC TCG CAC CCT TGC GCG       713
Thr Ser Ala Ala Ser Ser Ser Ser Val Pro Ile Ser His Pro Cys Ala
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20 TTTTA 3110

CLAIMS

1. Use of an inhibitor of Brn-3b expression and/or activity for the manufacture of a medicament for use in the treatment of breast cancer and/or ovarian cancer.

2. Use according to claim 1, wherein the inhibitor comprises a polynucleotide which is capable of hybridizing to the Brn-3b mRNA.

3. Use according to claim 1, wherein the inhibitor comprises a nucleic acid vector which is capable of expressing a polynucleotide as defined in claim 2 in a host cell.

4. Use according to claim 3, wherein the vector is a viral vector.

5. Use according to claim 4, wherein the viral vector is a an adenovirus vector, a retroviral vector, an adeno-associated virus vector or herpes simplex virus strain.

6. An inhibitor of Brn-3b expression and/or activity for use in a method of treatment of the human or animal body by therapy.

7. An inhibitor according to claim 6 for use in a method of treatment of breast cancer or ovarian cancer.

8. An inhibitor according to claim 6 or 7 comprising a polynucleotide which is capable of hybridizing to the Brn-3b mRNA.

9. An inhibitor according to claim 6 or 7 comprising a nucleic acid vector which is capable of expressing a polynucleotide as defined in claim 8 in a host cell.

10. An inhibitor according to claim 9, wherein the vector is a viral vector.

11. An inhibitor according to claim 10, wherein the viral vector is a an adenovirus vector, a retroviral vector, an adeno-associated virus vector or herpes simplex virus strain.

12. A method for identifying an inhibitor of Brn-3b expression for use in a method of treatment of breast and/or ovarian cancer comprising:

(a) providing a test construct comprising a Brn-3b promoter operably linked to a coding sequence;

(b) contacting a substance to be tested with the test construct under conditions that would permit the polypeptide encoded by the said coding sequence to be expressed in the absence of the said substance; and

(c) determining whether the said substance inhibits the expression of Brn-3b.

13. A method according to claim 12 wherein the coding sequence encodes a Brn-3b polypeptide.

14. A method according to claim 12, wherein the coding sequence encodes a reporter polypeptide.

15. A method according to any one of claims 12 to 14, wherein a host cell harbours the test construct.

5 16. A method according to any one of claims 15, wherein the host cell is a mammalian cell.

for use in a method of treatment of breast and/or ovarian cancer,

17. A method for identifying an inhibitor of Brn-3b activity comprising:

(a) providing a Brn-3b polypeptide or a homologue thereof, or a fragment thereof;

(b) contacting a substance to be tested with the Brn-3b polypeptide under conditions that

10 would permit activity of the polypeptide in the absence of said substance; and

(c) determining whether the said substance inhibits the activity of Brn-3b.

18. A method according to any one of claims 12 to 17 further comprising:

(d_1) administering a said substance which has been determined to inhibit Brn-3b to mammalian cells; and

15 (e_1) determining the effect of the said substance on the expression of BRCA-1.

19. A method according to claim 18, wherein the mammalian cells are breast cancer cells or a ovarian cancer cells.

20. A method according to claim 19, wherein the ability of the said substance to inhibit the proliferation of the cell is determined.

20 21. An inhibitor identified by a method according to any one of claims 12 to 20.

22. An inhibitor according to claim 21 for use in a method of treatment of the human or animal body by therapy.

23. An inhibitor according to claim 22 for use in a method of treatment of breast cancer or ovarian cancer.

25 24. Use of an inhibitor as defined in claim 21 for the manufacture of a medicament for use the treatment of breast cancer and/or ovarian cancer.

25. A pharmaceutical composition comprising an inhibitor according to any one of claims 1 to 6 and 21 and a pharmaceutically acceptable carrier or diluent.

26. A method of treatment of treating a host suffering from breast cancer or ovarian

30 cancer, which method comprises administering to the host a therapeutically effective amount of an inhibitor of Brn-3b expression and/or activity.

27. A method of treatment according to claim 26, wherein the inhibitor is as defined in any one of claims 1 to 6 and 21.

Fig.1.

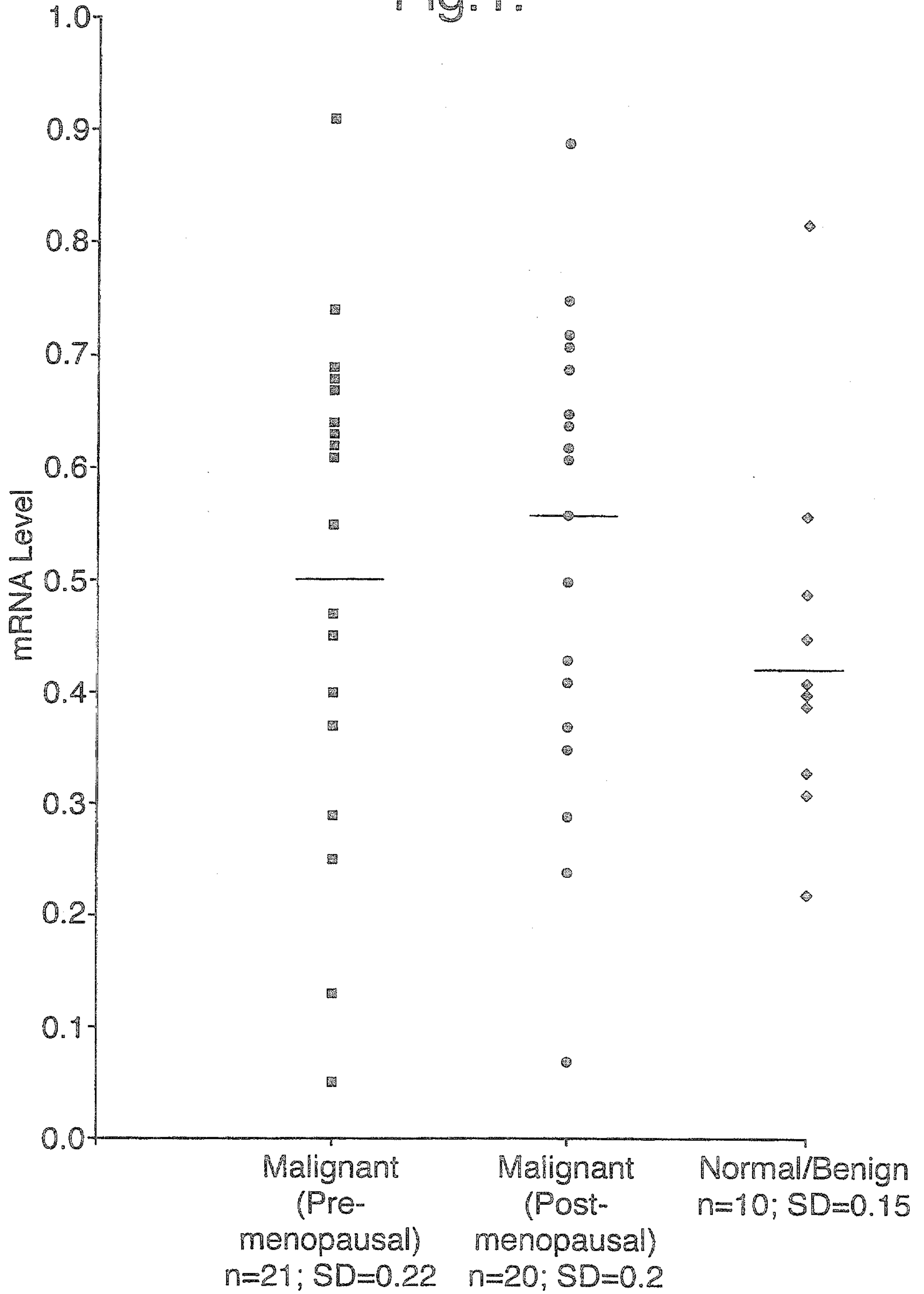
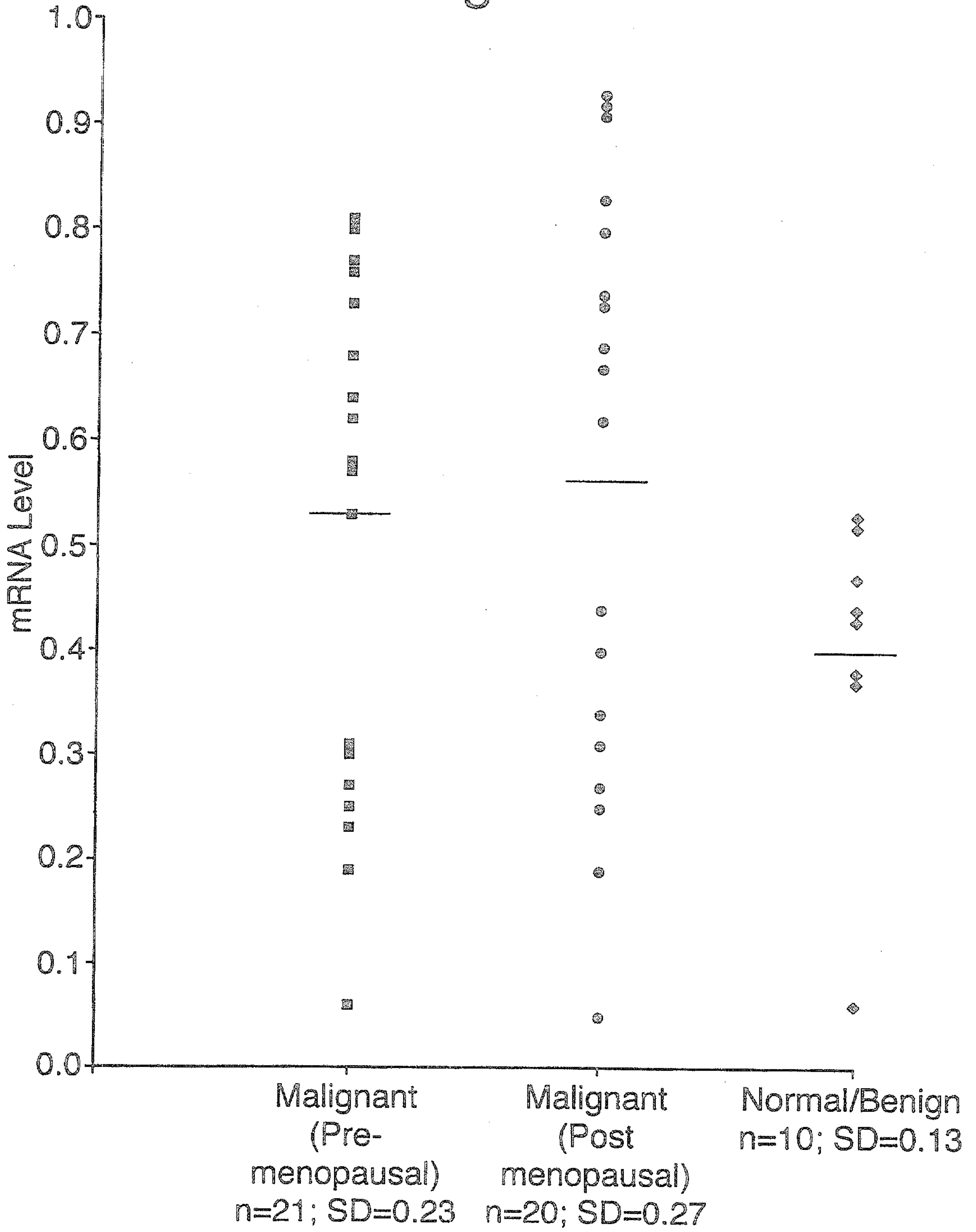


Fig.2.



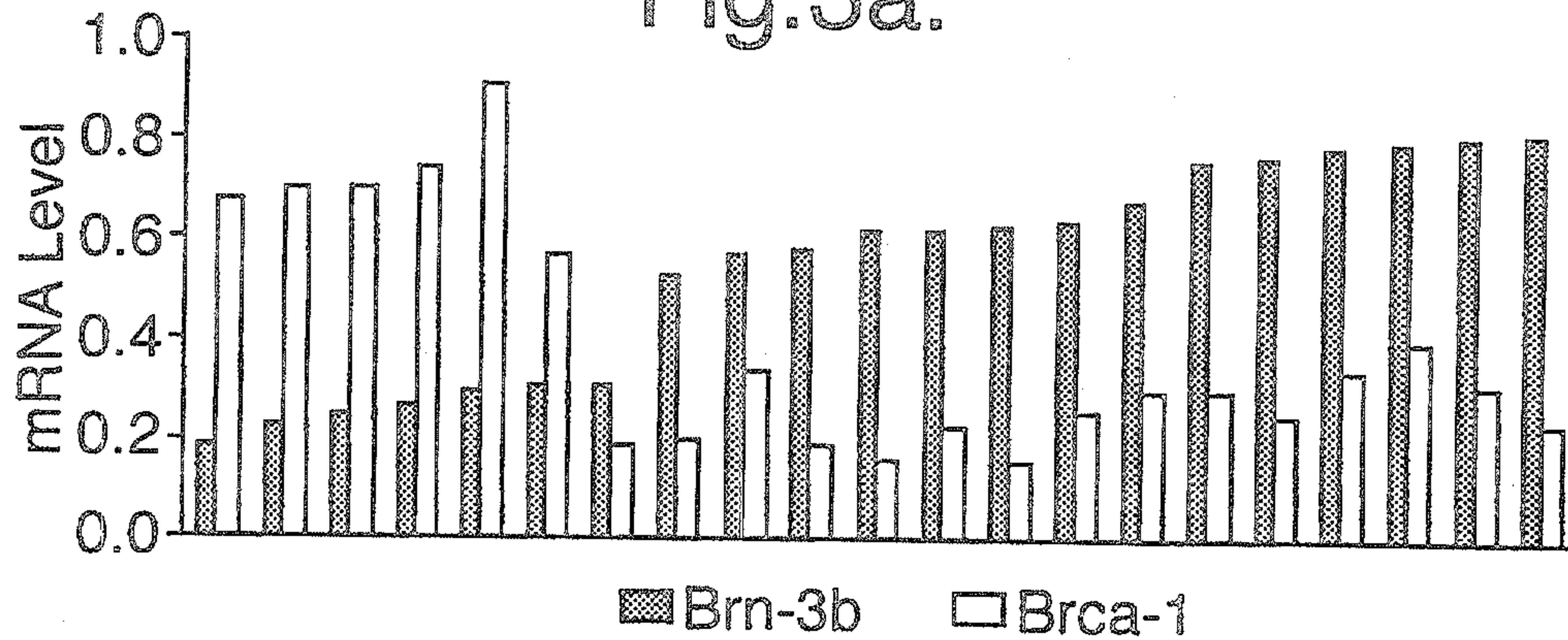


Fig.3a.
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0.6
0.4
0.2
0.0
Brn-3b
Brca-1

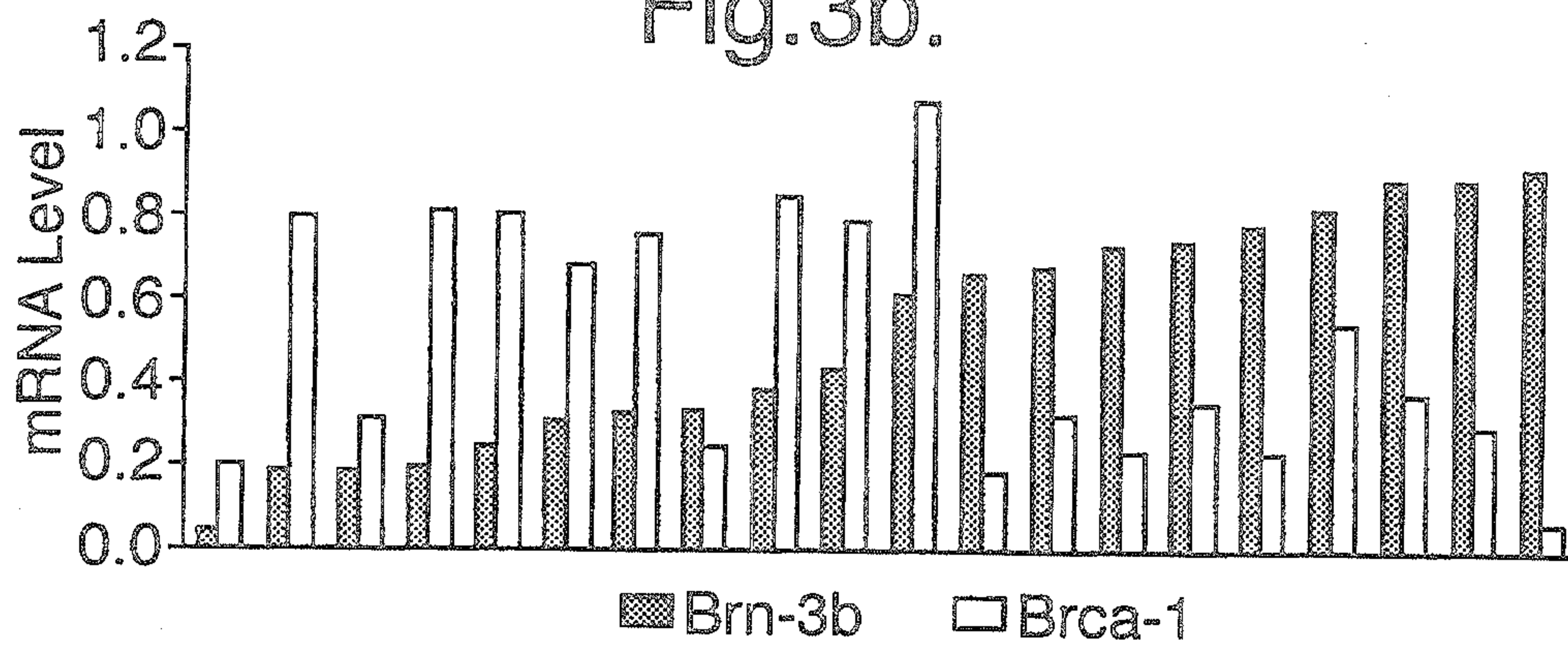
Fig.3b.
mRNA Level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Brn-3b
Brca-1

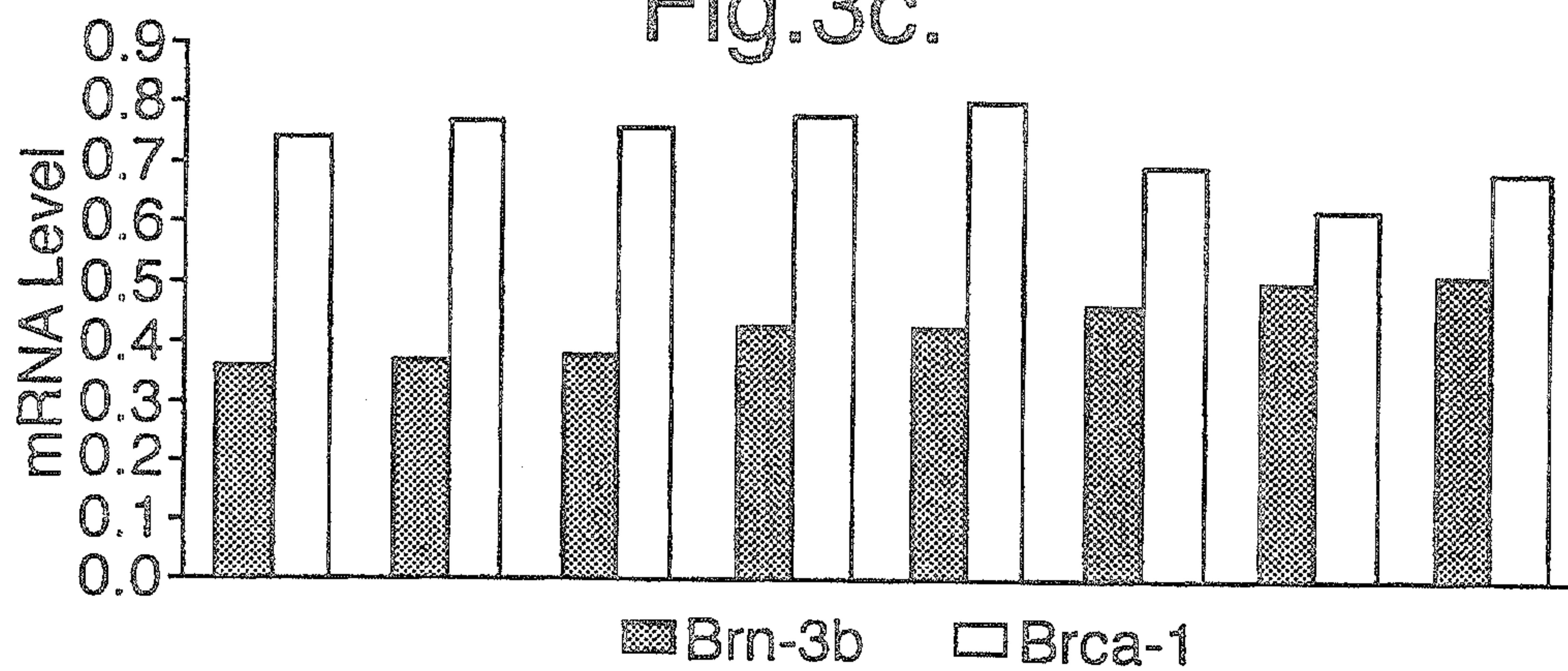
Fig.3c.
mRNA Level
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Brn-3b
Brca-1

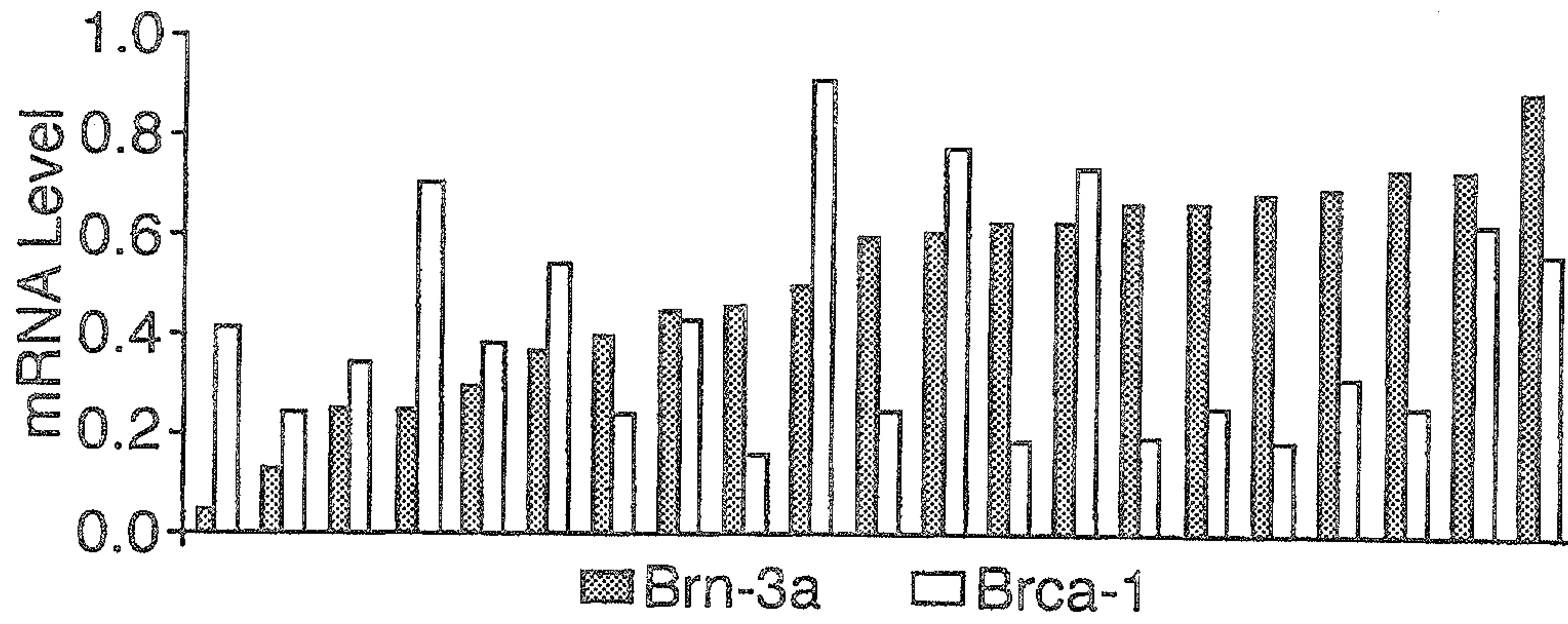
Fig.4a.
mRNA Level
1.0
0.8
0.6
0.4
0.2
0.0
Brn-3a
Brca-1

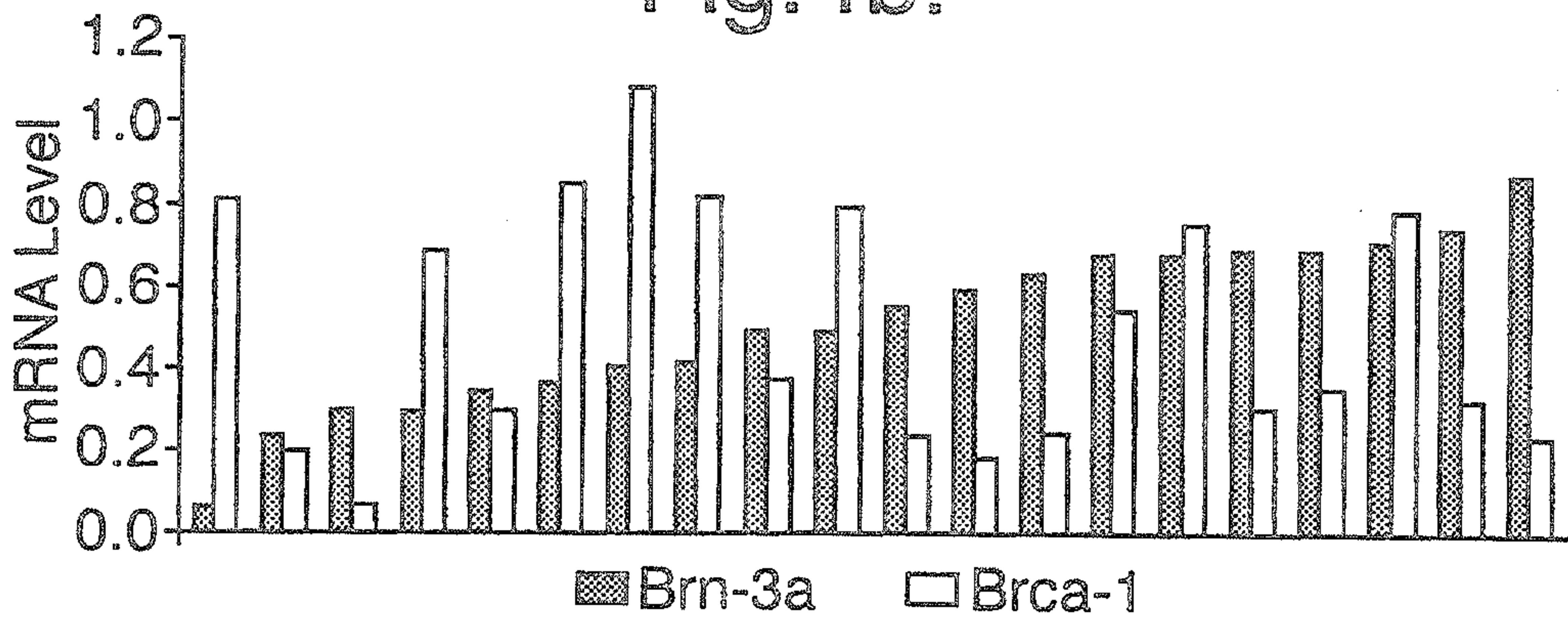
Fig.4b.
mRNA Level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Brn-3a
Brca-1

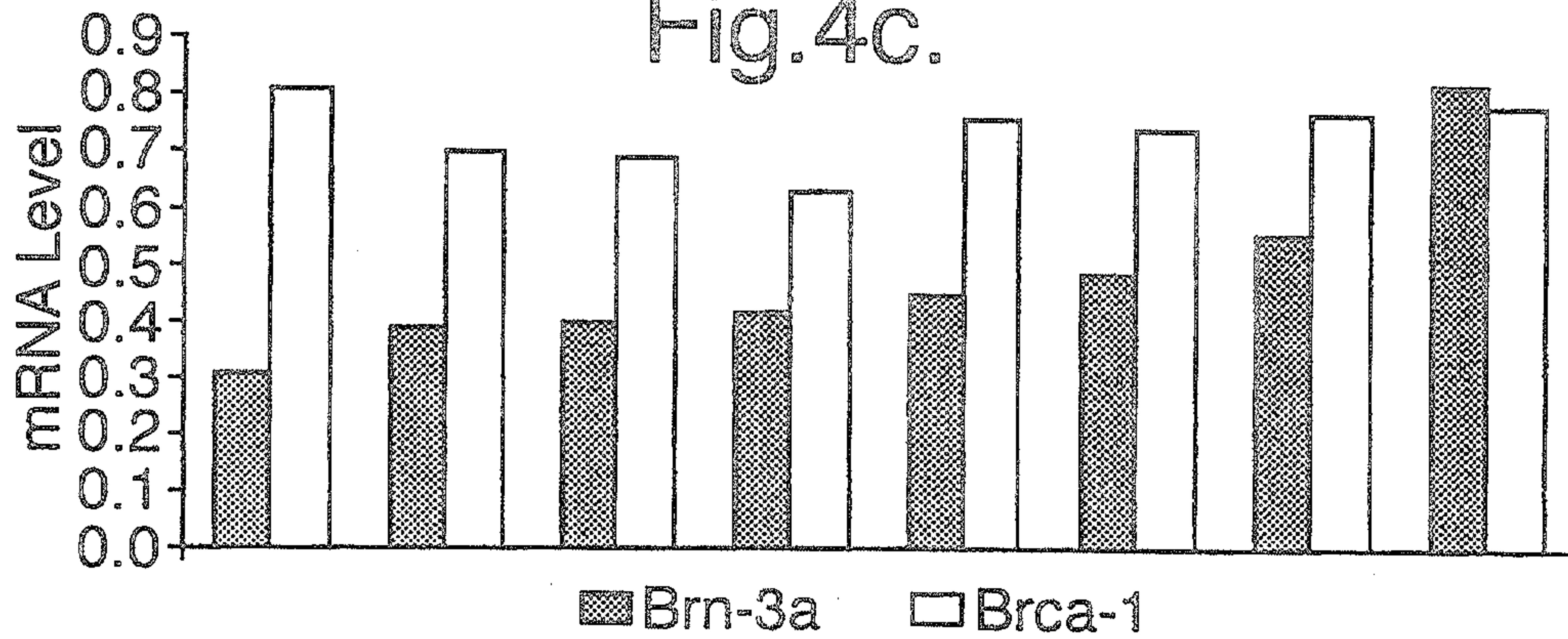
Fig.4c.
mRNA Level
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Brn-3a
Brca-1

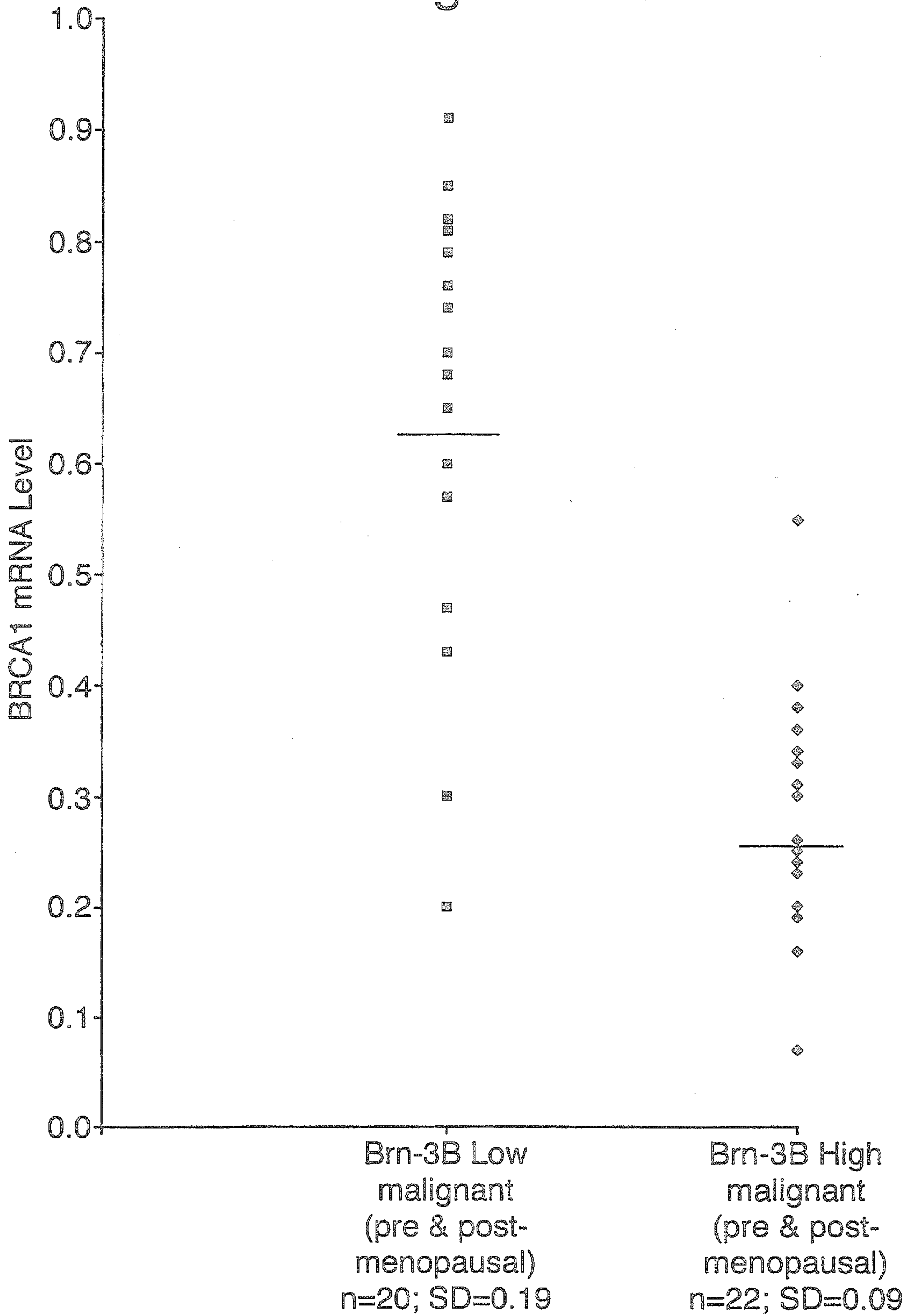
Fig.5.
BRCA1 mRNA Level
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Brn-3B Low
malignant
(pre & post-
menopausal)
n=20; SD=0.19
Brn-3B High
malignant
(pre & post-
menopausal)
n=22; SD=0.09

Fig.6.