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Serrero

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(54) **METHODS AND KITS FOR DIAGNOSING TUMORIGENICITY AND DETERMINING RESISTANCE TO THE ANTINEOPLASTIC EFFECTS OF ANTIESTROGEN THERAPY**

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(51) **Int. Cl.**⁷ **G01N 33/543**; G01N 33/577; G01N 33/53

(52) **U.S. Cl.** **435/7.23**; 435/7.1; 436/501; 436/518

(58) **Field of Search** 436/501, 518; 530/350; 438/7.1; 435/7.23

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(57) **ABSTRACT**

Methods and kits for diagnosing tumorigenicity and for determining whether a cancer patient is resistant to the pharmacological effects of antiestrogen therapy. Increased levels of the PCDGF (GP88) growth factor are indicative of tumorigenicity and resistance to the pharmacological effects of antiestrogen therapy. The methods and kits of the invention are useful for assessing the tumorigenicity of a biological sample from a patient and determining whether the patient is a candidate for antiestrogen, including tamoxifen, therapy.

35 Claims, 30 Drawing Sheets

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PRODUCTION OF GP88 BY TUMORIGENIC AND NON-TUMORIGENIC CELLS

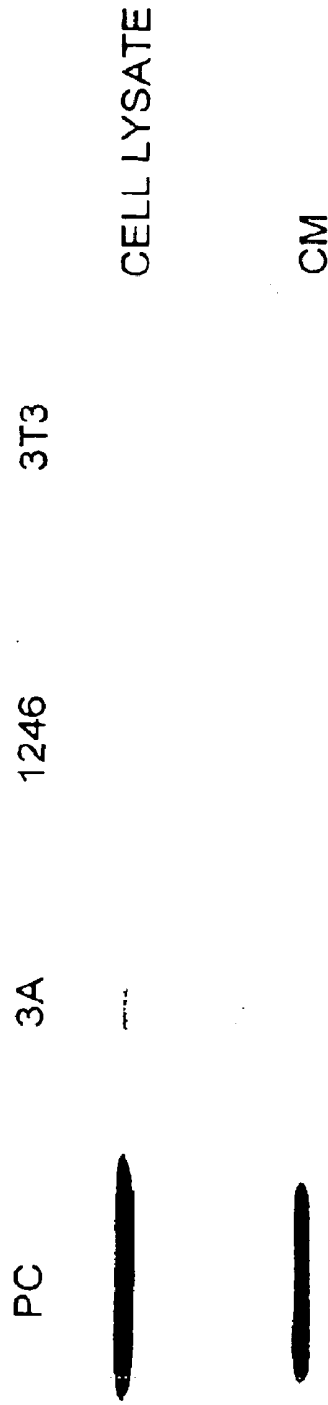


FIG. 1A

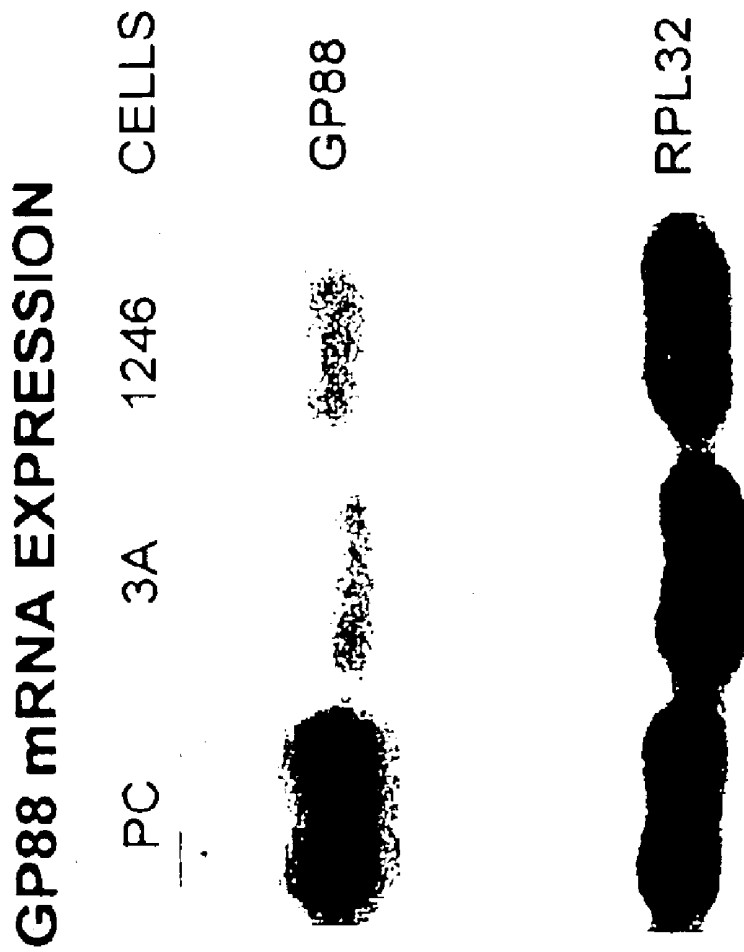


FIG. 1B

GP88 mRNA EXPRESSION IN VARIOUS CULTURE CONDITIONS

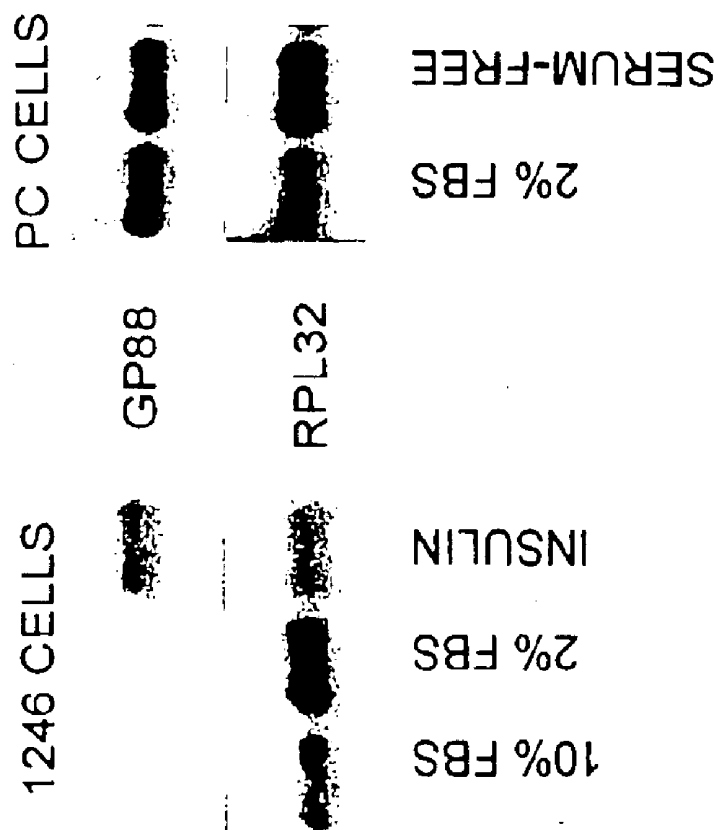


FIG.1C

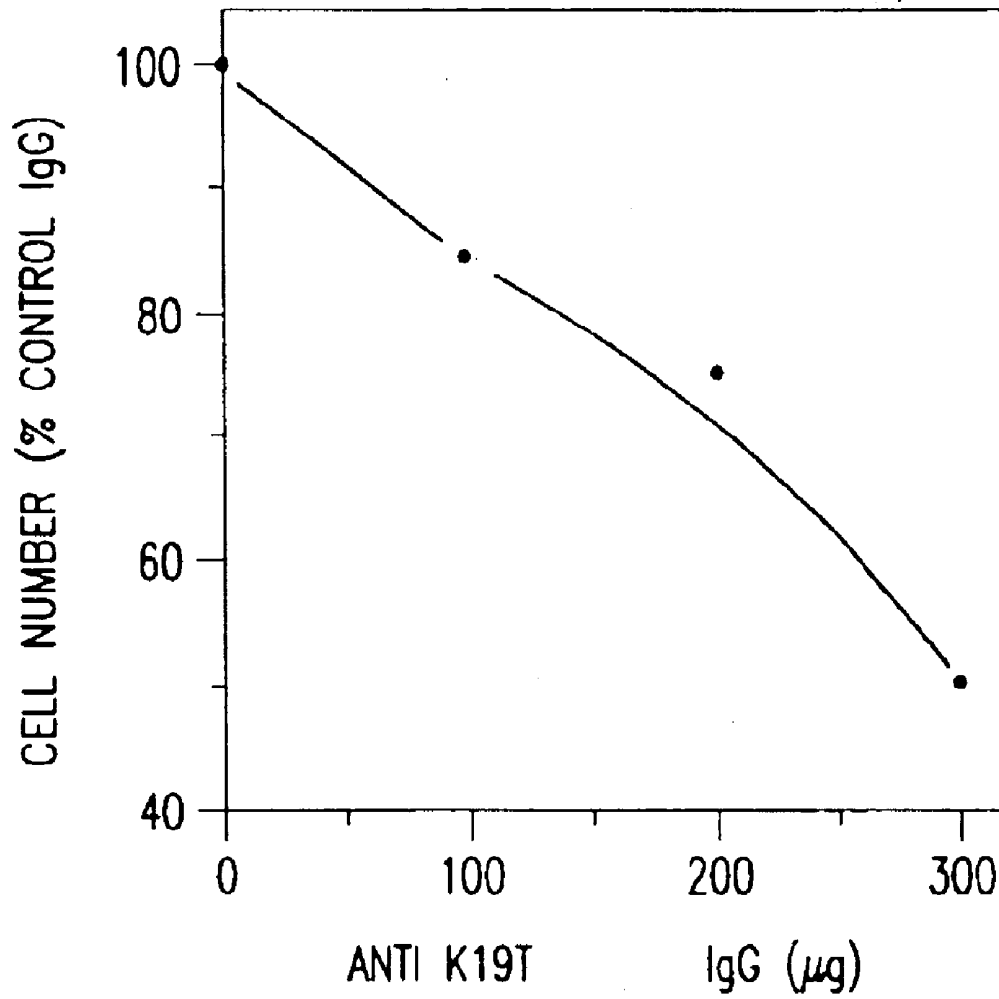
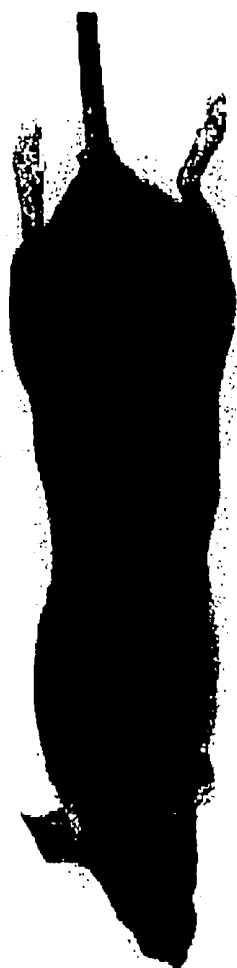


FIG.2

**ABSENCE OF TUMOR FORMATION IN C3H MICE BY INHIBITION OF GP88
EXPRESSION**



GP88 ANTISENSE TRANSFECTED PC CELLS



CONTROL TRANSFECTED PC CELLS

FIG. 3

GP88 PROTEIN EXPRESSION IN TUMOR AND SURROUNDING TISSUES

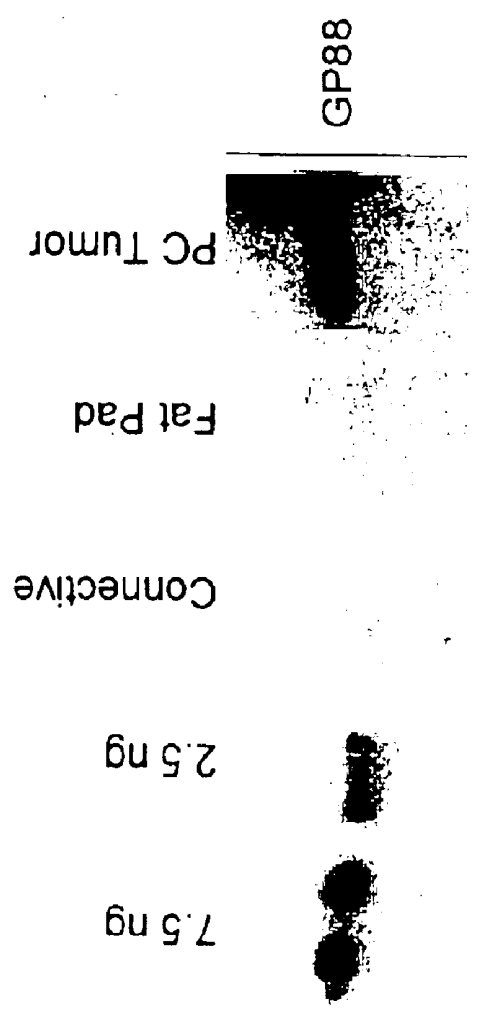


FIG.4

**GP88 mRNA EXPRESSION IN ESTROGEN-DEPENDENT AND
INDEPENDENT HUMAN MAMMARY CARCINOMA CELLS**

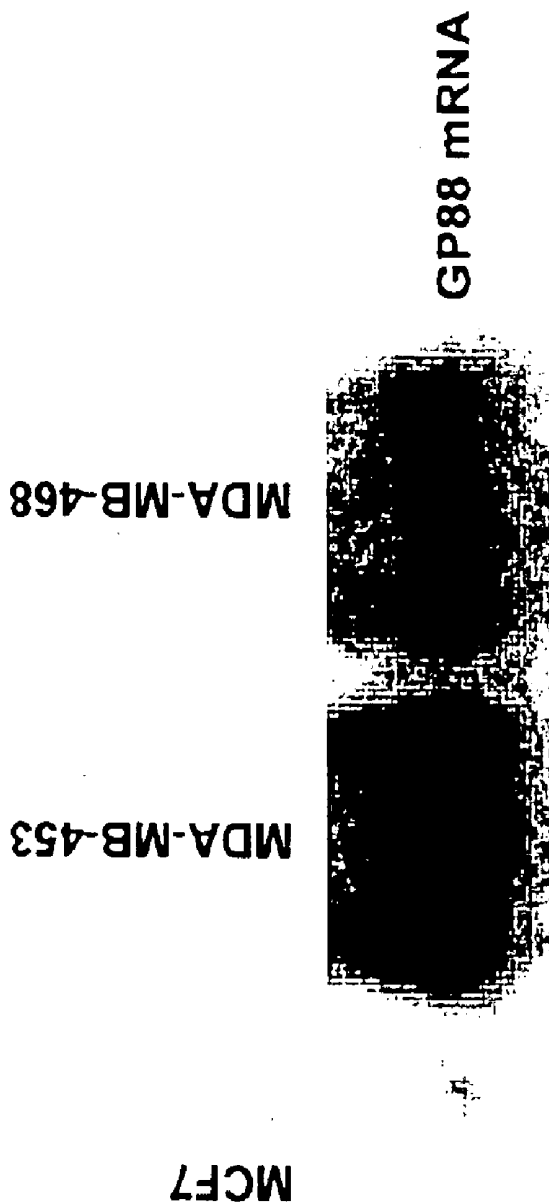


FIG. 5

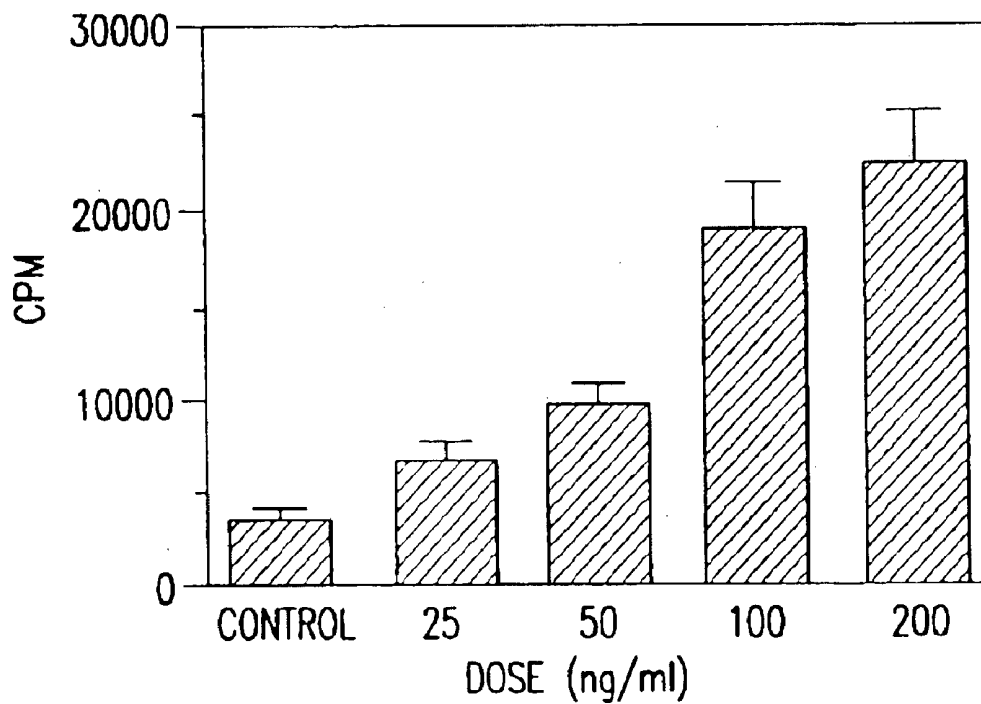


FIG. 6A

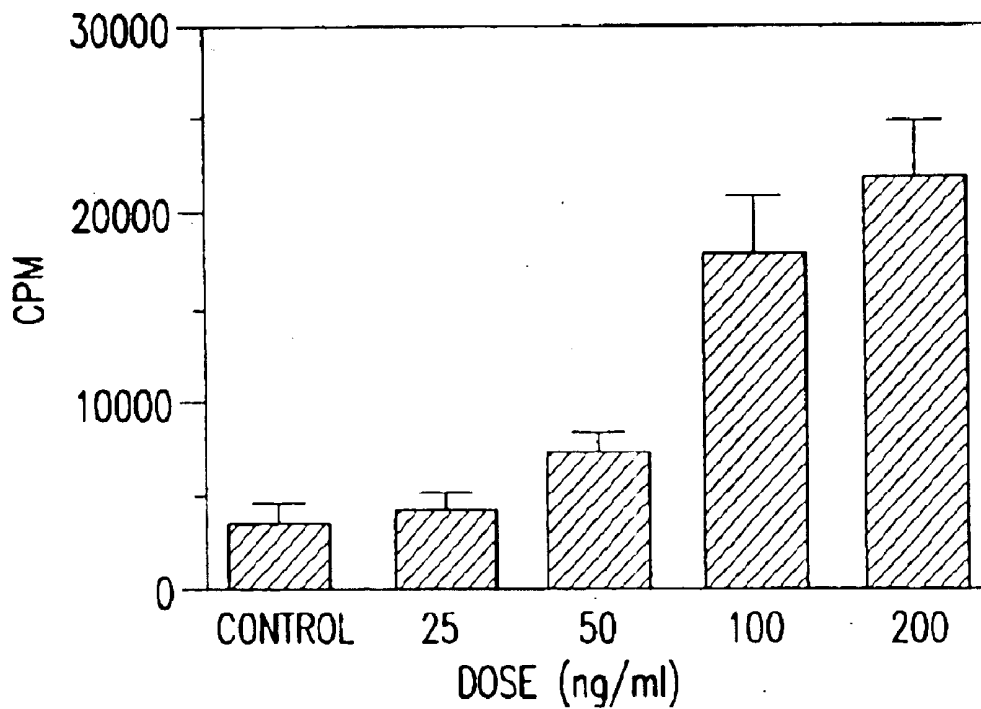


FIG. 6B

**EXPRESSION OF GP88 IN ANTISENSE AND CONTROL
TRANSFECTED PC CELLS**

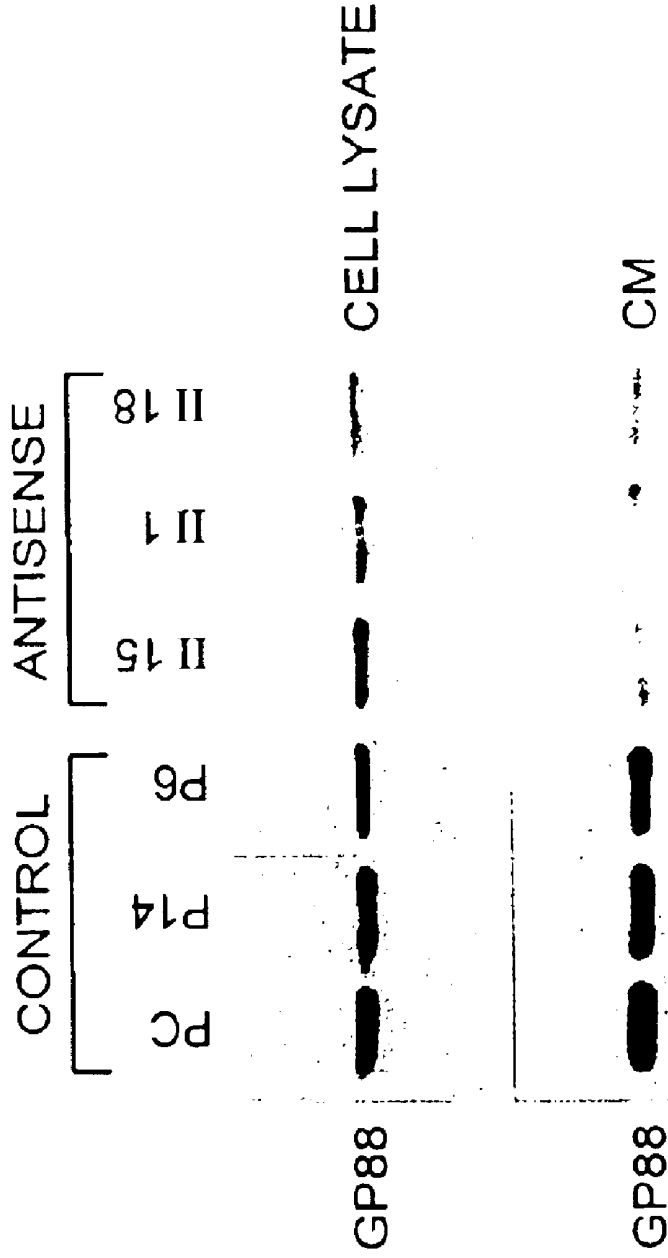


FIG. 7

Mouse GP88 cDNA

CGGA CCC CGA CGC AGA CAG ACC ATG TGG GTC CTG ATG AGC TGG CTG	46
M W V L M S W L	8
GCC TTC GCG GCA GGG CTG GTA GCC GGA ACA CAG TGT CCA GAT GGG CAG	94
A F A A G L V A G T Q C P D G Q	24
TTC TGC CCT GTT GCC TGC TGC CTT GAC CAG GGA GGA GCC AAC TAC AGC	142
F C P V A C C L D Q G G A N Y S	40
TGC TGT AAC CCT CTT CTG GAC ACA TGG CCT AGA ATA ACG AGC CAT CAT	190
C C N P L L D T W P R I T S H H	56
CTA GAT GGC TCC TGC CAG ACC CAT GGC CAC TGT CCT GCT GGC TAT TCT	238
L D G S C Q T H G H C P A G Y S	72
TGT CTT CTC ACT GTG TCT GGG ACT TCC AGC TGC TGC CCG TTC TCT AAG	286
C L L T V S G T S S C C P F S K	88
GGT GTG TCT TGT GGT GAT GGC TAC CAC TGC TGC CCC CAG GGC TTC CAC	334
G V S C G D G Y H C C P Q G F H	104
TGT AGT GCA GAT GGG AAA TCC TGC TTC CAG ATG TCA GAT AAC CCC TTG	382
C S A D G K S C F Q M S D N P L	120
GGT GCT GTC CAG TGT CCT GGG AGC CAG TTT GAA TGT CCT GAC TCT GCC	430
G A V Q C P G S Q F E C P D S A	136
ACC TGC TGC ATT ATG GTT GAT GGT TCG TGG GGA TGT TGT CCC ATG CCC	478
T C C I M V D G S W G C C P M P	152
CAG GCC TCT TGC TGT GAA GAC AGA GTG CAT TGC TGT CCC CAT GGG GCC	526
Q A S C C E D R V H C C P H G A	168
TCC TGT GAC CTG GTT CAC ACA CGA TGC GTT TCA CCC ACG GGC ACC CAC	574
S C D L V H T R C V S P T G T H	184
ACC CTA CTA AAG AAG TTC CCT GCA CAA AAG ACC AAC AGG GCA GTG TCT	622
T L L K K F P A Q K T N R A V S	200
TTG CCT TTT TCT GTC GTG TGC CCT GAT GCT AAG ACC CAG TGT CCC GAT	670
L P F S V V C P D A K T Q C P D	216

FIG.8A

Mouse GP88 cDNA (continued)

GAT TCT ACC TGC TGT GAG CTA CCC ACT GGG AAG TAT GGC TGC TGT CCA	718
D S T C C E L P T G K Y G C C P	232
ATG CCC AAT GCC ATC TGC TGT TCC GAC CAC CTG CAC TGC TGC CCC CAG	766
M P N A I C C S D H L H C C P Q	248
GAC ACT GTA TGT GAC CTG ATC CAG AGT AAG TGC CTA TCC AAG AAC TAC	814
D T V C D L I Q S K C L S K N Y	264
ACC ACG GAT CTC CTG ACC AAG CTG CCT GGA TAC CCA GTG AAG GAG GTG	862
T T D L L T K L P G Y P V K E V	280
AAG TGC GAC ATG GAG GTG AGC TGC CCT GAA GGA TAT ACC TGC TGC CGC	910
K C D M E V S C P E G Y T C C R	296
CTC AAC ACT GGG GCC TGG GGC TGC TGT CCA TTT GCC AAG GCC GTG TGT	958
L N T G A W G C C P F A K A V C	312
TGT GAG GAT CAC ATT CAT TGC TGC CCG GCA GGG TTT CAG TGT CAC ACA	1006
C E D H I H C C P A G F Q C H T	328
GAG AAA GGA ACC TGC GAA ATG GGT ATC CTC CAA GTA CCC TGG ATG AAG	1054
E K G T C E X G I L Q V P W M <u>K</u>	344
AAG GTC ATA GCC CCC CTC CGC CTG CCA GAC CCA CAG ATC TTG AAG AGT	1102
<u>K V I A P L R L P D P Q I L K S</u>	360
GAT ACA CCT TGT GAT GAC TTC ACT AGG TGT CCT ACA AAC AAT ACC TGC	1150
<u>D T P C D D F T R C P T N N T C</u>	376
TGC AAA CTC AAT TCT GGG GAC TGG GGC TGC TGT CCC ATC CCA GAG GCT	1198
C K L N S G D W G C C P I P E A	392
GTC TGC TGC TCA GAC AAC CAG CAT TGC TGC CCT CAG GGC TTC ACA TGT	1246
V C C S D N Q H C C P Q G F T C	408
CTG GCT CAG GGG TAC TGT CAG AAG GGA GAC ACA ATG GTG GCT GGC CTG	1294
L A Q G Y C Q K G D T M V A G L	424
GAG AAG ATA CCT GCC CGC CAG ACA ACC CCG CTC CAA ATT GGA GAT ATC	1342
E K I P A R Q T T P L Q I G D I	440

FIG.8B

Mouse GP88 cDNA (continued)

GGT TGT GAC CAG CAT ACC AGC TGC CCA GTA GGG CAA ACC TGC TGC CCA	1390
G C D Q H T S C P V G Q T C C P	456
AGC CTC AAG GGA AGT TGG GCC TGC TGC CAG CTG CCC CAT GCT GTG TGC	1438
S L K G S W A C C Q L P H A V C	472
TGT GAG GAC CGG CAG CAC TGT TGC CCG GCC GGG TAC ACC TGC AAC GTG	1486
C E D R Q H C C P A G Y T C N V	488
AAG GCG AGG ACC TGT GAG AAG GAT GTC GAT TTT ATC CAG CCT CCC GTG	1534
K A R T C E K D V D F I Q P P V	504
CTC CTG ACC CTC GGC CCT AAG GTT GGG AAT GTG GAG TGT GGA GAA GGG	1582
L L T L G P K V G N V E C G E G	520
CAT TTC TGC CAT GAT AAC CAG ACC TGT TGT AAA GAC AGT GCA GGA GTC	1630
H F C H D N Q T C C K D S A G V	536
TGG GCC TGC TGT CCC TAC CTA AAG GGT GTC TGC TGT AGA GAT GGA CGT	1678
W A C C P Y L K G V C C R D G R	552
CAC TGT TGC CCC GGT GGC TTC CAC TGT TCA GCC AGG GGA ACC AAG TGT	1726
H C C P G G F H C <u>S A R G T K C</u>	568
TTG CGA AAG AAG ATT CCT CGC TGG GAC ATG TTT TTG AGG GAT CCG GTC	1774
<u>L R K K I P R</u> W D M F L R D P V	584
CCA ACA CCG CTA CTG TAA GGA AGG GCT ACA GAC TTA AGG AAC TCC ACA	1822
P R P L L *	589
GTC CTG GGA ACC CTG TTC CGA GGG TAC CCA CTA CTC AGG CCT CCC TAG	1870
CGC CTC CTC CCC TAA CGT CTC CCC GGC CTA CTC ATC CTG AGI CAC CCT	1918
ATC ACC ATG GGA GGT GGA GCC TCA AAC TAA AAC CTT CTT TTA TGG AAA	1966
GAA GGC TGT GGC CAA AAG CCC CGT ATC AAA CTG CCA TTT CTT CCG GTT	2014
TCT GTG GAC CTT GTG GCC AGG TGC TCT TCC CGA GCC ACA GGT GTT CTG	2062
TGA GCT TGC TTG TGT GTG TGT GCG CGT GTG CGT GTG TTG CTC <u>GAA TAA</u>	2110
<u>AGT</u> TTG TAC GCT TTC TGA AAA AAA AAA	2137

FIG.8C

Nucleotide sequence of human granulin/epithelin precursor (human GP88).
Human Granulin Genbank M75161\$

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[cgcaggcaga ccattgtggac cttggtgagc tgggtggcct taacagcagg gctggtggct
ggaacgcggt gccagatgg tcagttctgc cctgtggcct gctgcctgga ccccgaggga
gccagctaca gctgctgccg tcccctctg gacaaatggc ccacaacact gaggcaggcat
ctgggtggcc cctgccagggt tgatgcccac tgctctgccg gccactcctg catctttacc
gtctcagggg cttccagttg ctgccccttc ccagagggcg tggcatgcgg ggatggccat
cactgctgcc cacggggcct ccactgcagt gcagacggga gatcctgctt ccaaagatca
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ccacaagcct tgaagagaga tgtcccctgt gataatgca gcagctgtcc ctctccgat
acctgctgcc aactcacgtc tggggagtgg ggctgctgtc caatcccaga ggctgtctgc
tgctcggacc accagcaactg ctgccccag cgatacacgt gtgtagctga ggggcagtgt
cagcgaggaa gcgagatcgt ggctggactg gagaagatgc ctgcccgcg cggttctta
tcccaccca gagacatcgg ctgtgaccag cacaccagct gcccggtgg cgaacctgc
tgcccagacc aggggtgggag ctgggcctgc tgccagttgc cccatgctgt gtgctgcgag
gatcgccagc actgctgccc ggctggctac acctgcaacg tgaaggctcg atcctgcgag
aaggaagtgg tctctgccc gctgcccacc ttctgccc gtagecctca cgtgggtgtg
aaggacgtgg agtgtgggga aggacacttc tgccatgata accagacctg ctgccgagac
aaccgacagg gctgggcctg ctgtccctac gccagggcg tctgttgtgc tgatcgggc
cactgctgtc ctgctggctt ccgctgcgca cgcaggggta ccaagtgtt gcgcaggag
gccccgcgt gggacgcccc ttgagggac ccagcctga gacagctgct gtgagggaca
gtactgaaga ctctgcagcc ctcgggacc cactcggagg gtgcccctctg ctcaggcctc
gtactgaaga ctctgcagcc ctcgggacc cactcggagg gtgcccctctg ctcaggcctc
cctagcacct ccccctaacc aaattctccc tggacccat tctgagctcc ccateccat
gggaggtggg gctcaatct aaggcccttc cctgtcagaa gggggttgag gcaaaagccc
attacaagct gccatcccct ccccgttca gtggacctg tgccagggtg ctttcccta
tccacagggg tgtttgtgtg ttgggtgtgc tttcaataaa gtttgcact ttctt*
```

FIG. 9A

Amino-acid sequence of human granulin/epithelin precursor (human GP88).

MWTLVSWVALTAGLVAGTRCPDGQFCPVACCLDPGGASYSCCRP
LLDKWPTTLSRHLGGPCQVDAHCSAGHSCIFTVSGTSSCCPFPEAVACGDGHHCCPRG
FHCSADGRSCFQRSGNNSVGAIQCPSQFECPDFSTCCVMVDGWSGCCPMPQASCCED
RVHCCPHGAFCDLVHTRCITPTGTHPLAKKLPARTNRAVALSSSVMCPDARSRCPDG
STCCLEPSGKYGCCPMPNATCCSDHLHCCPQDTVCDLIQSKCLSKENATDILLTYLPA
HTVGDVKCDMEVSCPDPGYTCCRLQSGAWGCCPFTQAVCCEDHIHCCPAGFTCDTQKGT
CEQGPHQVPWMEKAPAHLSLPDPQALKRDVPCDNVSSCPSSDTCCQLTSGEWGCCPIP
EAVCCSDHQHCCPQRYTCVAEGQCQRGSEIVAGLEKMPARRGSLSHPRDIGCDQHTSC
PVGGTCCPSQGGSWACCQLPHAVCCEDRQHCCPAGYTCNVKARSCEKEVSAQPATFL
ARSPHVGKDVCEGEGHFCHDNQTCRDNRQGWACCPYAQGVCCADRRHCCPAGFRCA
RRGTKCLRREAPRWDAPLRDPALRQLL*

FIG.9B

se GP88 protein sequence

WVLM^{sw}LFAAGLVAG 17

TQCPDQGF-CPVA--CCLDQG-GANYSCCNPLLDTWPRITSHHL 57

DGSC-QTHGHCPAGY-SCLLTVSGTS-SCCPFSKGVSCGDGYHCCPQGFHCSADGKSCFQMSDNPL 120

GAVQCPGSQFEC^{PD}SATCCIMVD-G-SWGCCPMPQASCCEDRVHCCPHGASCDLVHTRCVSPTGHTLLKKFPAQKTNAAVSLPFS 204

VVCPDAKTQCPDDSTCC^{EL}P-TGK-YGCCPMPNAICCS^{DL}HCCPQDTVCDLIQSKCLSKNYTTDLLTKLPGYPVK 278

EVKC-DMEVSCPEGYTCCALN-TGA-WGCCPF^{AK}AVCCEDHIHCCPAGFOCHTEKIGICEMGILOVPW^{MKKV}IAPRRLPDPQILKS 360

DTPCDD^{FT}R-CPTNNTCC^{KL}N-SGD-WGCCPIPEAVCCSDNQHCCPQGF^TCLAQGY-CQKGDTMVAGLEKIPARQTTPLQIG 438

DIGCDuHT-SCPVGOTCCPSLK-G-SWACCQLPHAVCCEDRQHCCPAGYTCNVKARTCEKDVDFIQPPVLLTLGPKVG 513

NVEGEGHF-CHDNQTCC^{KD}SA-GV-WACCPYLKGVCCRRGRHCCPGGF^{HCS}ARGTKCLAKKI^{PRWDM}FLADPVPRPLL 589

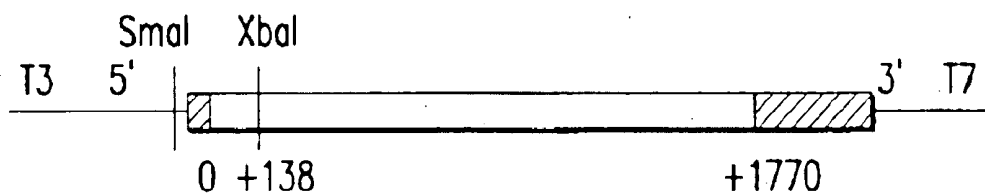
onsensus sequence:

C.....C.....G.....CC.....CC.D..HCCP....C.....C

.2:mouse epithelin 1,2.
.B,C,D,e,f,g: granulin A,B,C,D,E,F,G;N-terminus of granulin A,B,C,D have been sequenced.
ouse epithelin precursor sequence is from Plowman et al.(1992).

FIG.10

GP88 cDNA CLONE in SK



STRUCTURE OF pCMV₄ EXPRESSION VECTOR

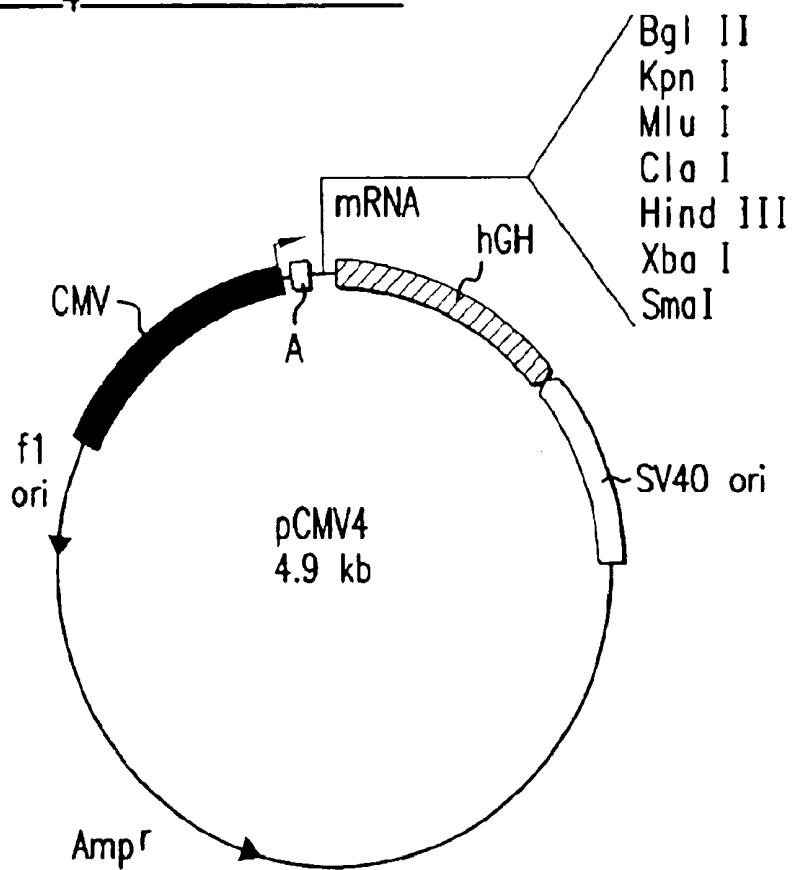


FIG.11

CROSS-LINKING OF ¹²⁵I-rGP88 TO CCL64 CELLS

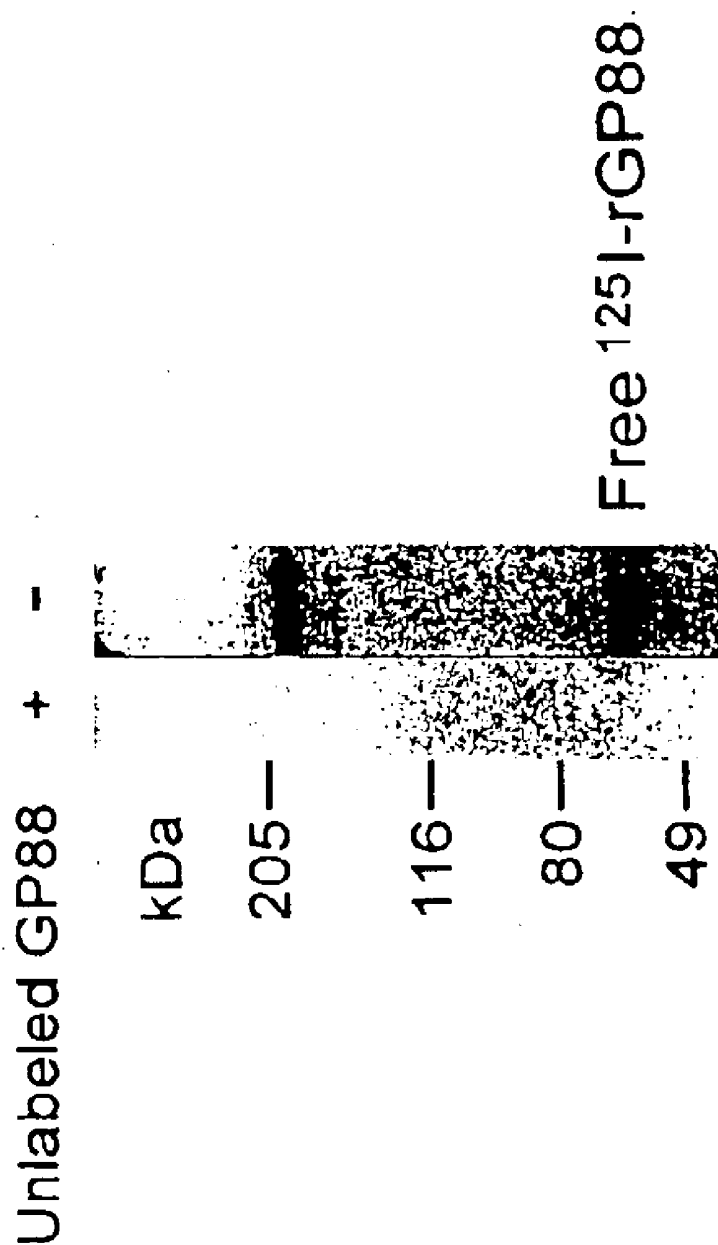


FIG. 12

CROSS-LINKING OF ¹²⁵I-rGP88

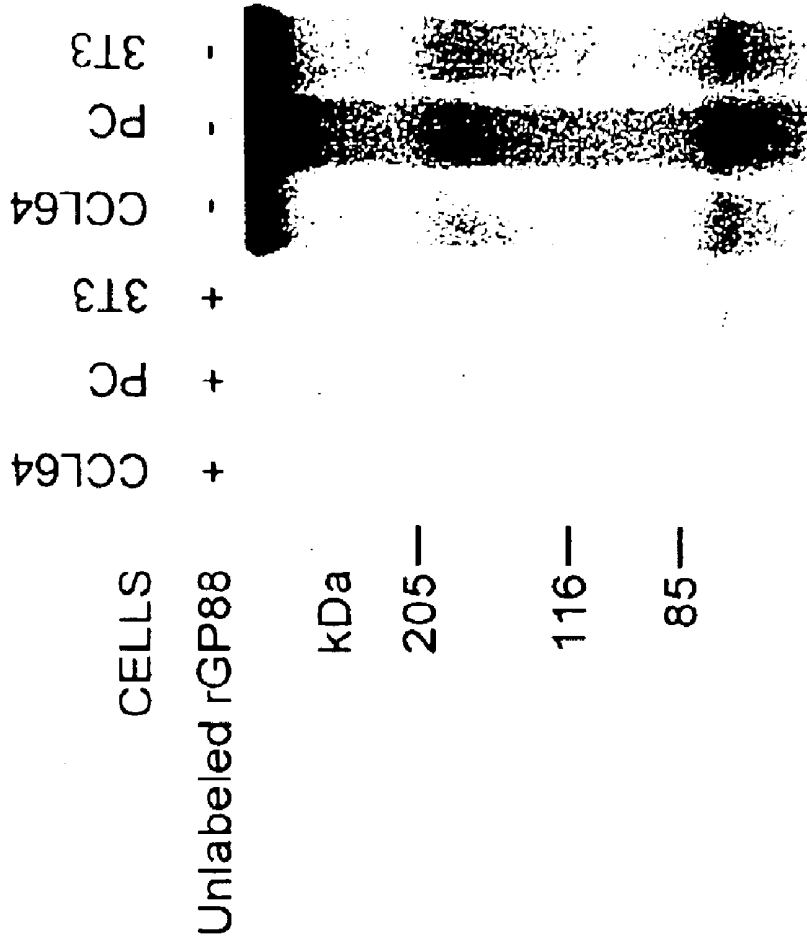


FIG.13

**GP88 EXPRESSION IN NON TUMORIGENIC (MCF 10A)
AND MALIGNANT (MCF 7, MDA-468) HUMAN
MAMMARY EPITHELIAL CELLS**

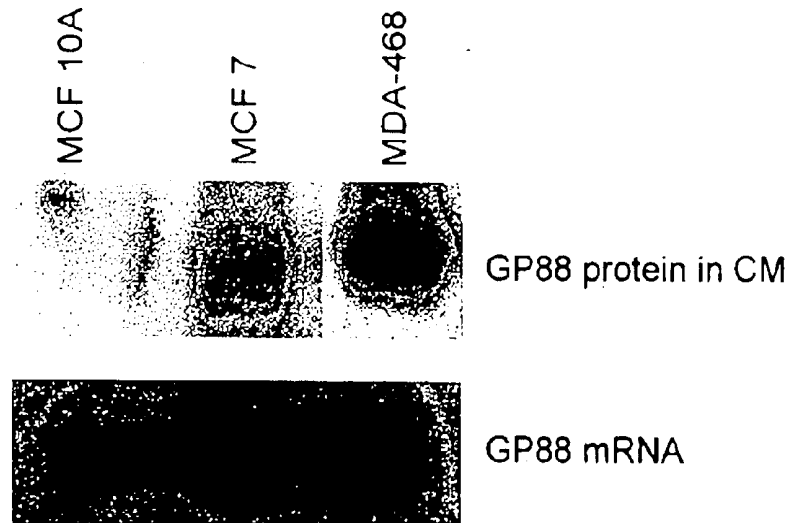


FIG. 14

**GP88 EXPRESSION IS INHIBITED BY ANTISENSE GP88
cDNA TRANSFECTION IN HUMAN BREAST
CARCINOMA MDA-468**

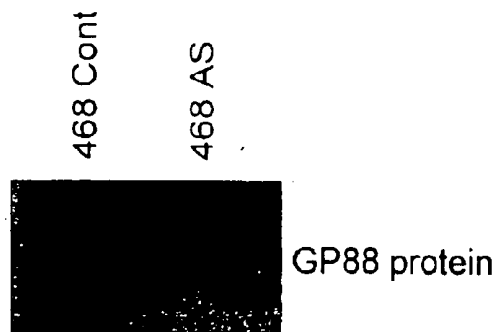


FIG. 15

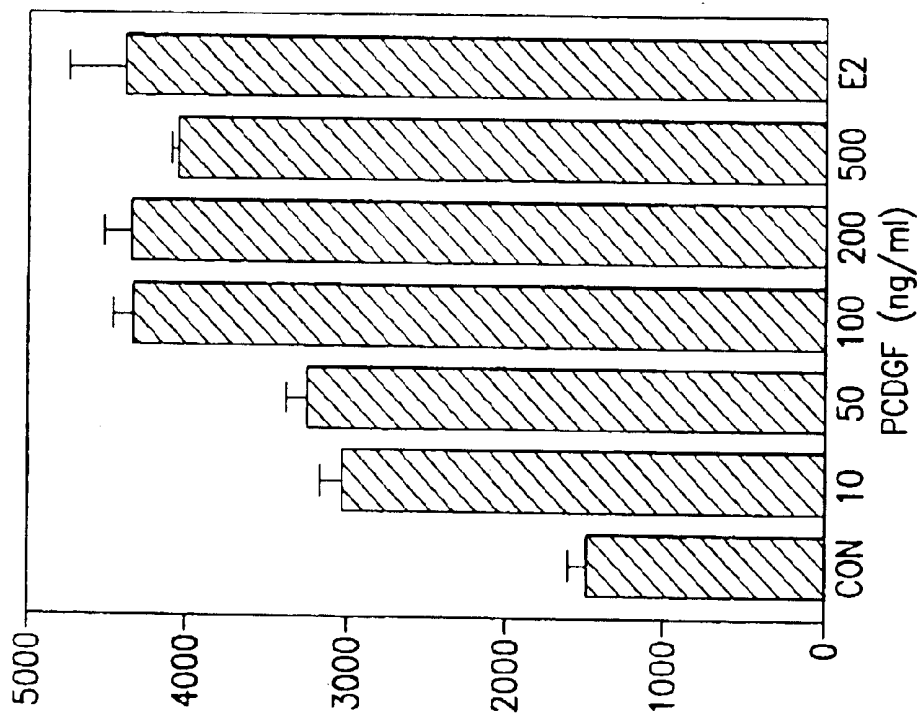


FIG. 16A

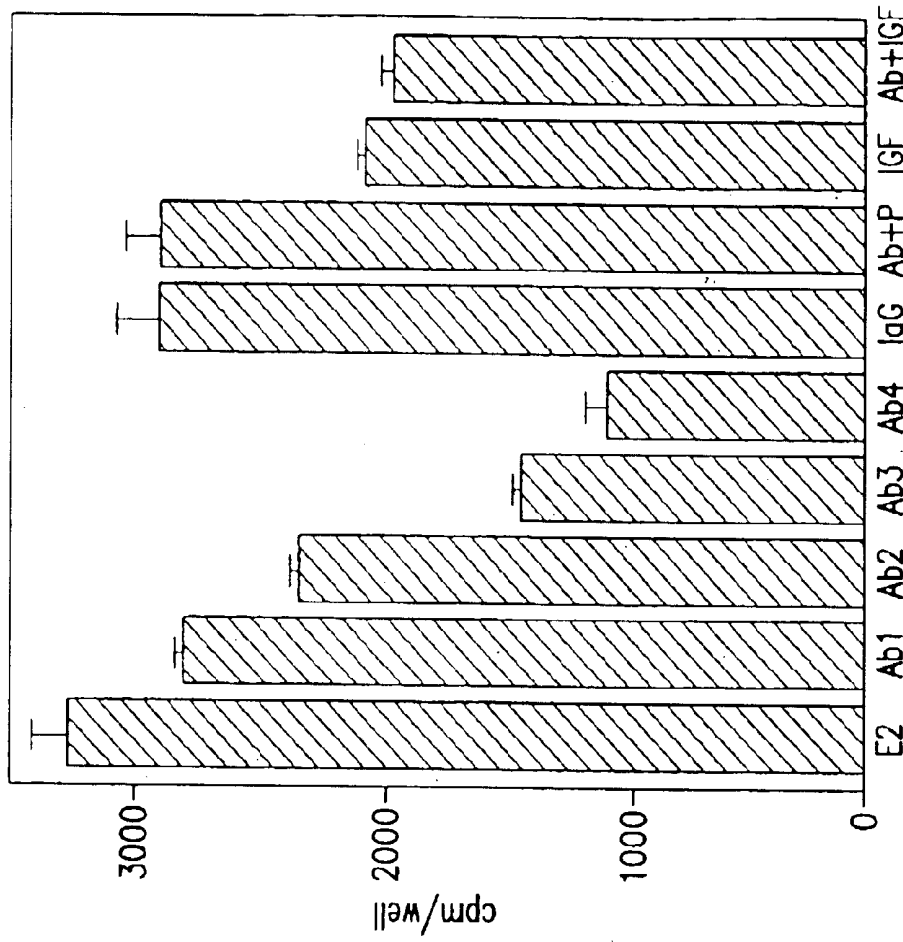


FIG. 16B

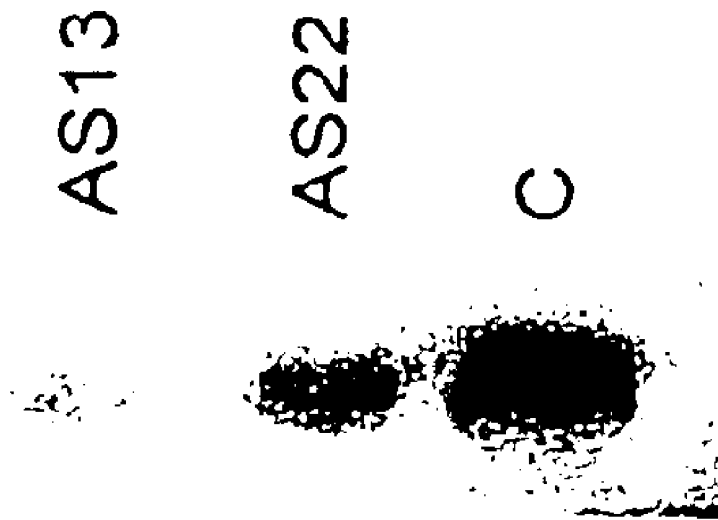


FIG. 17A

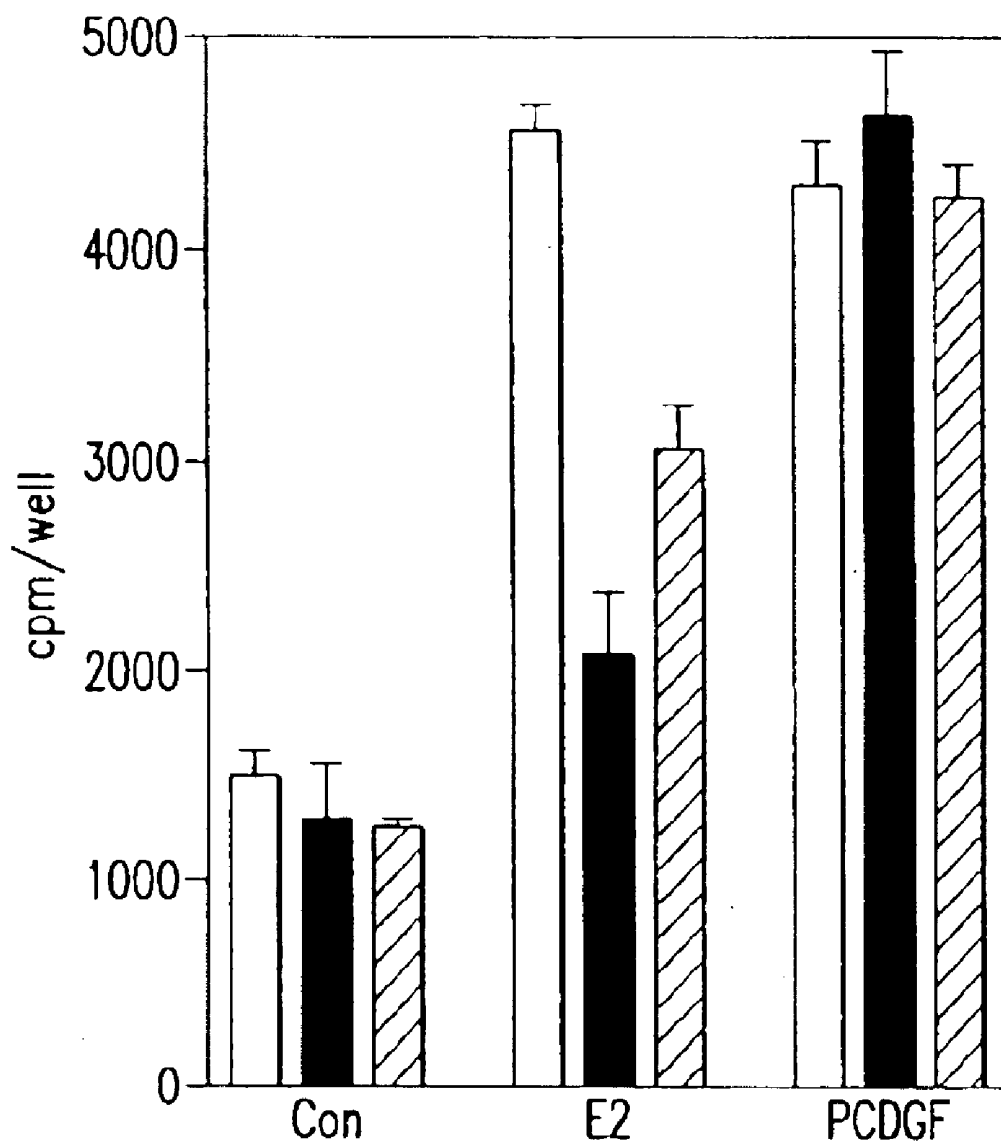


FIG.17B

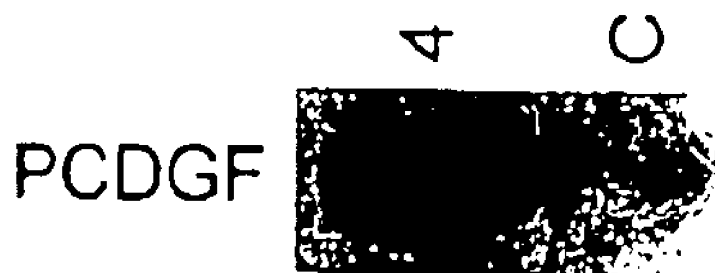


FIG. 18A

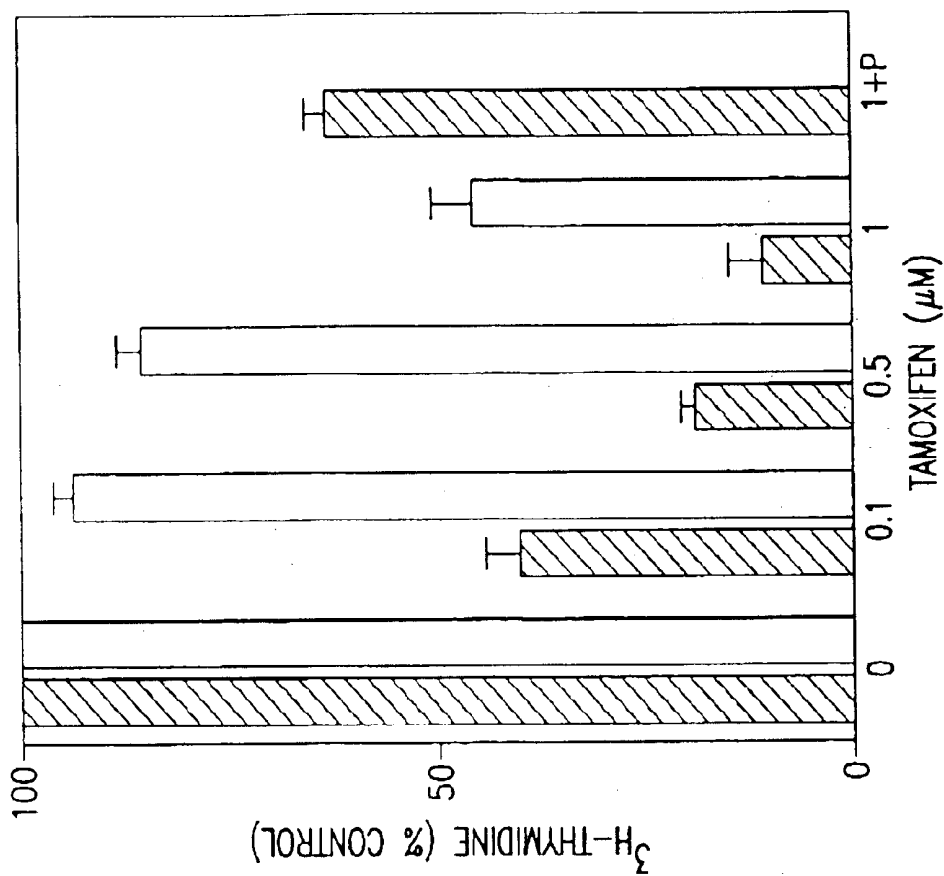


FIG. 18C

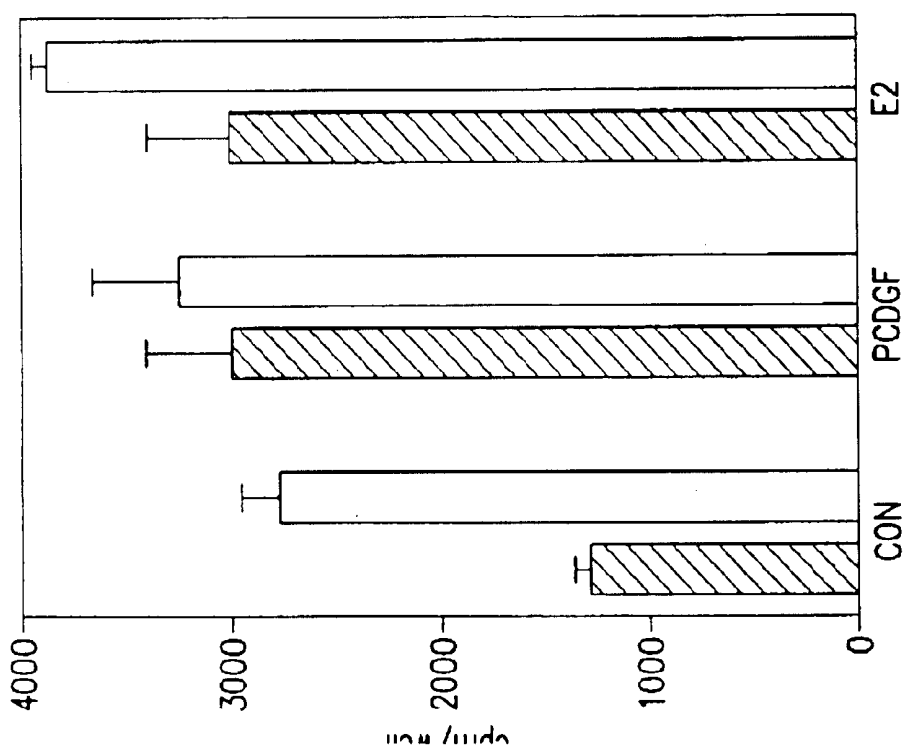


FIG. 18B

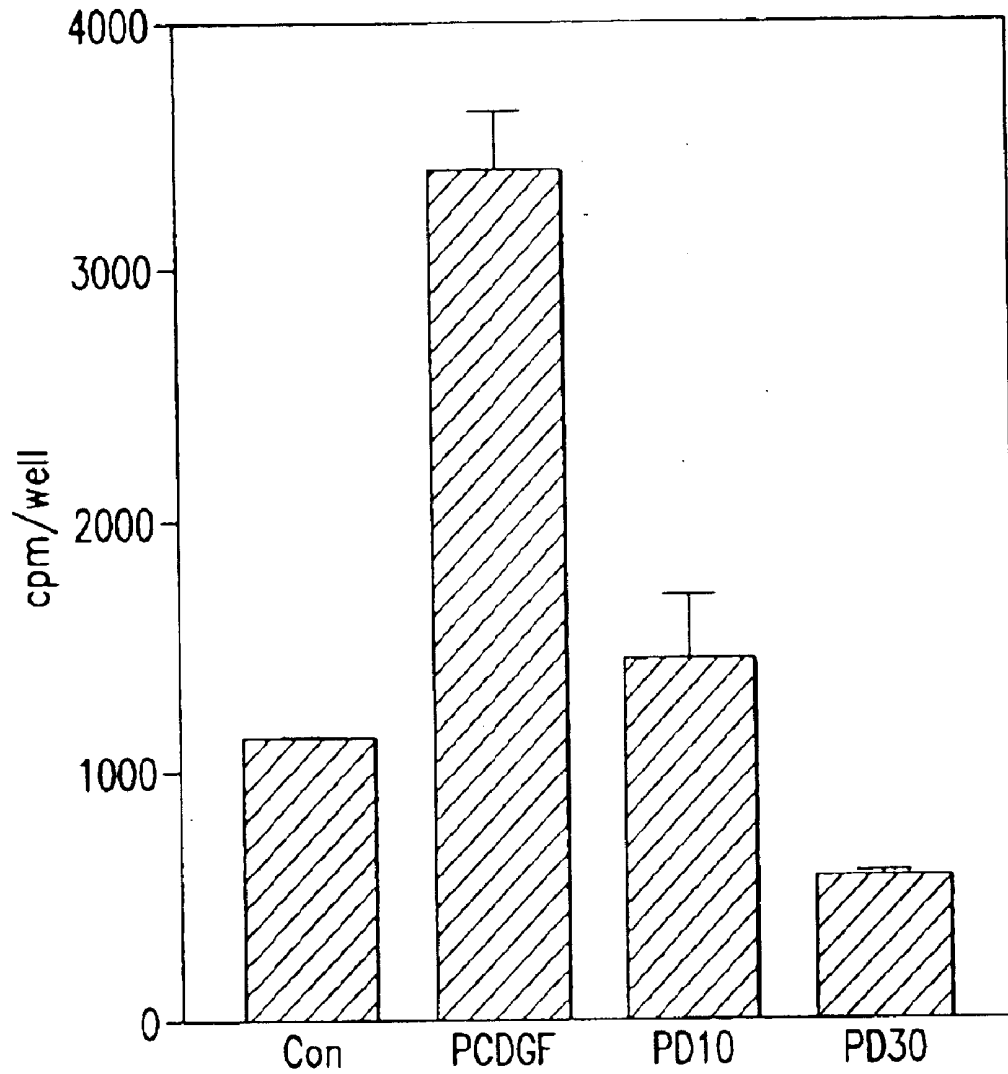


FIG.19A

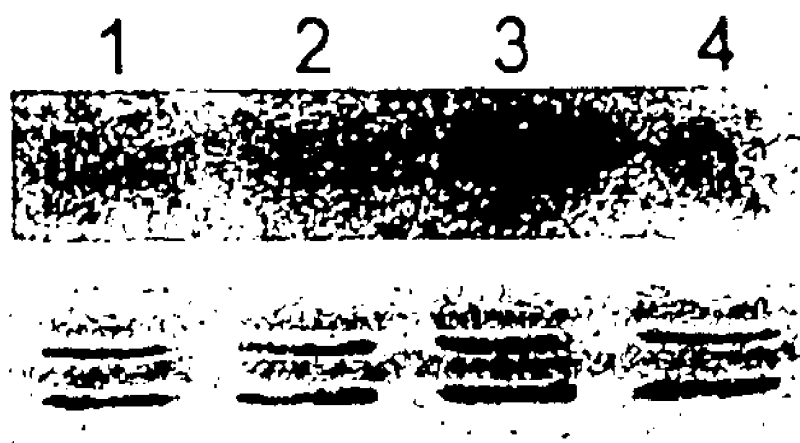


FIG. 19B

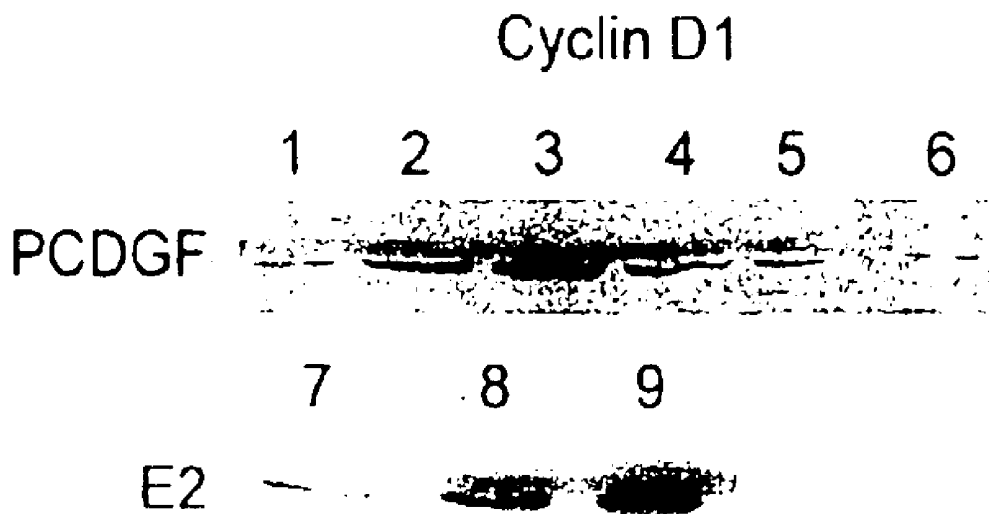


FIG.20A

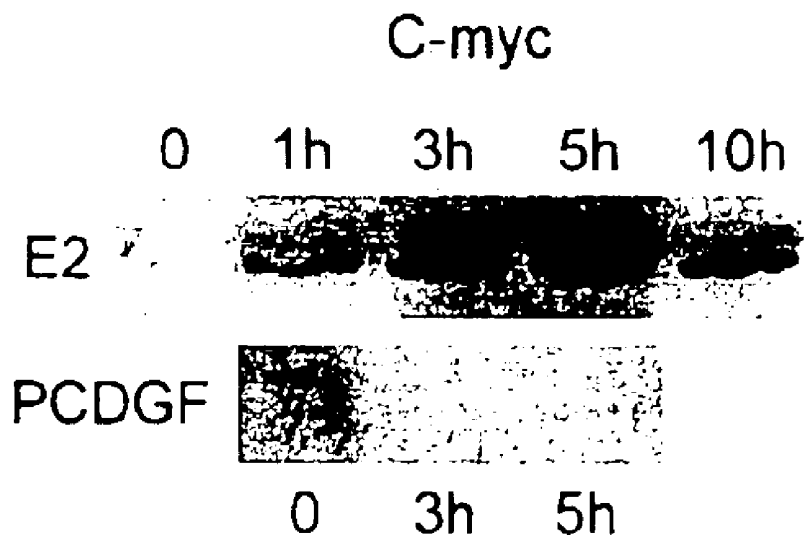


FIG.20B



DUCTAL INVASIVE CARCINOMA



BENIGN LESION

GP88 STAINING WITH ANTI-GP88 ANTIBODY
ON PARAFFIN EMBEDDED BREAST CANCER BIOPSIES
BY IMMUNOHISTOCHEMISTRY (IHC)

FIG. 21

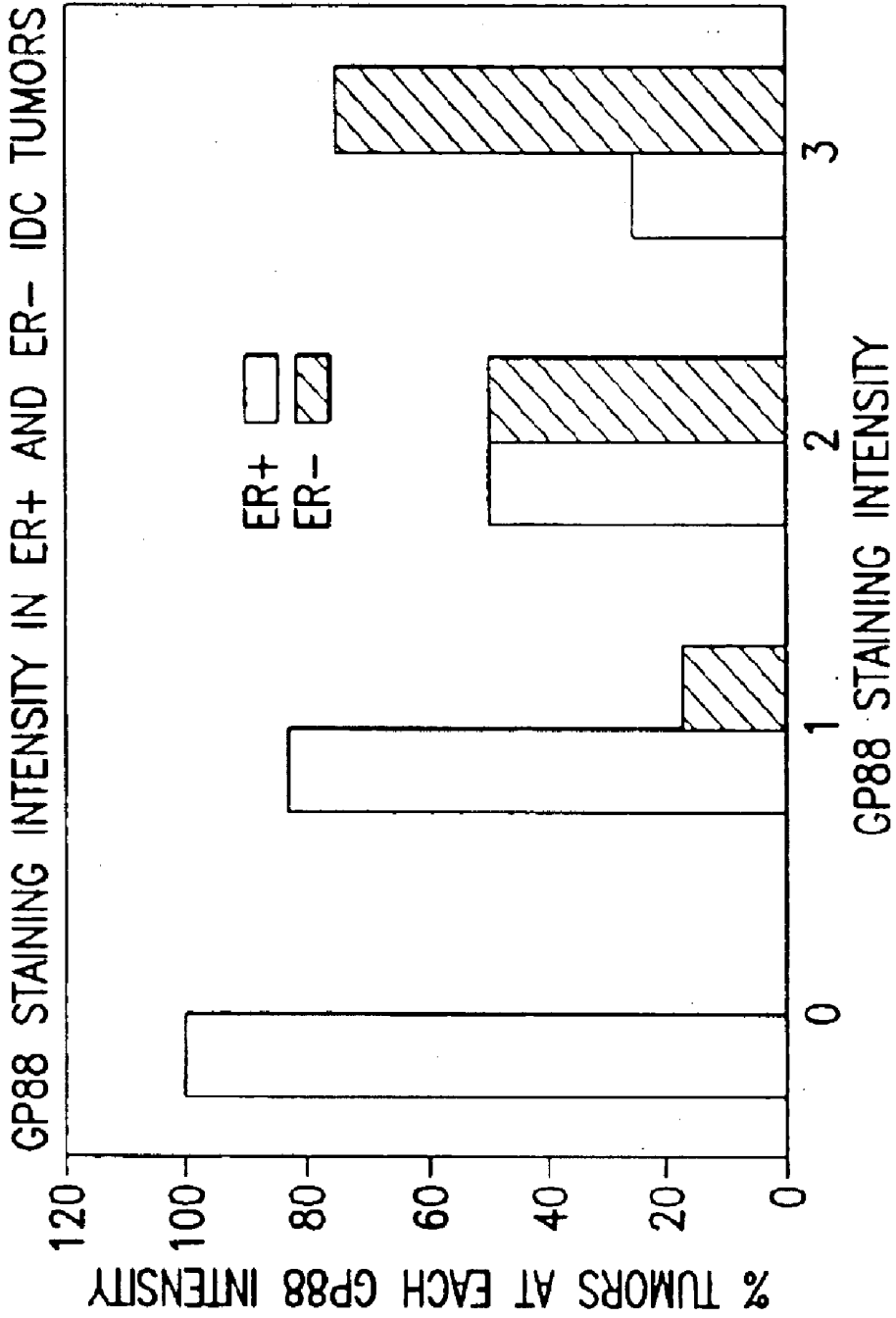


FIG.22

TAMOXIFEN RESPONSIVENESS OF ER+ IDC TUMORS CLASSIFIED BY GP88 STAINING INTENSITY

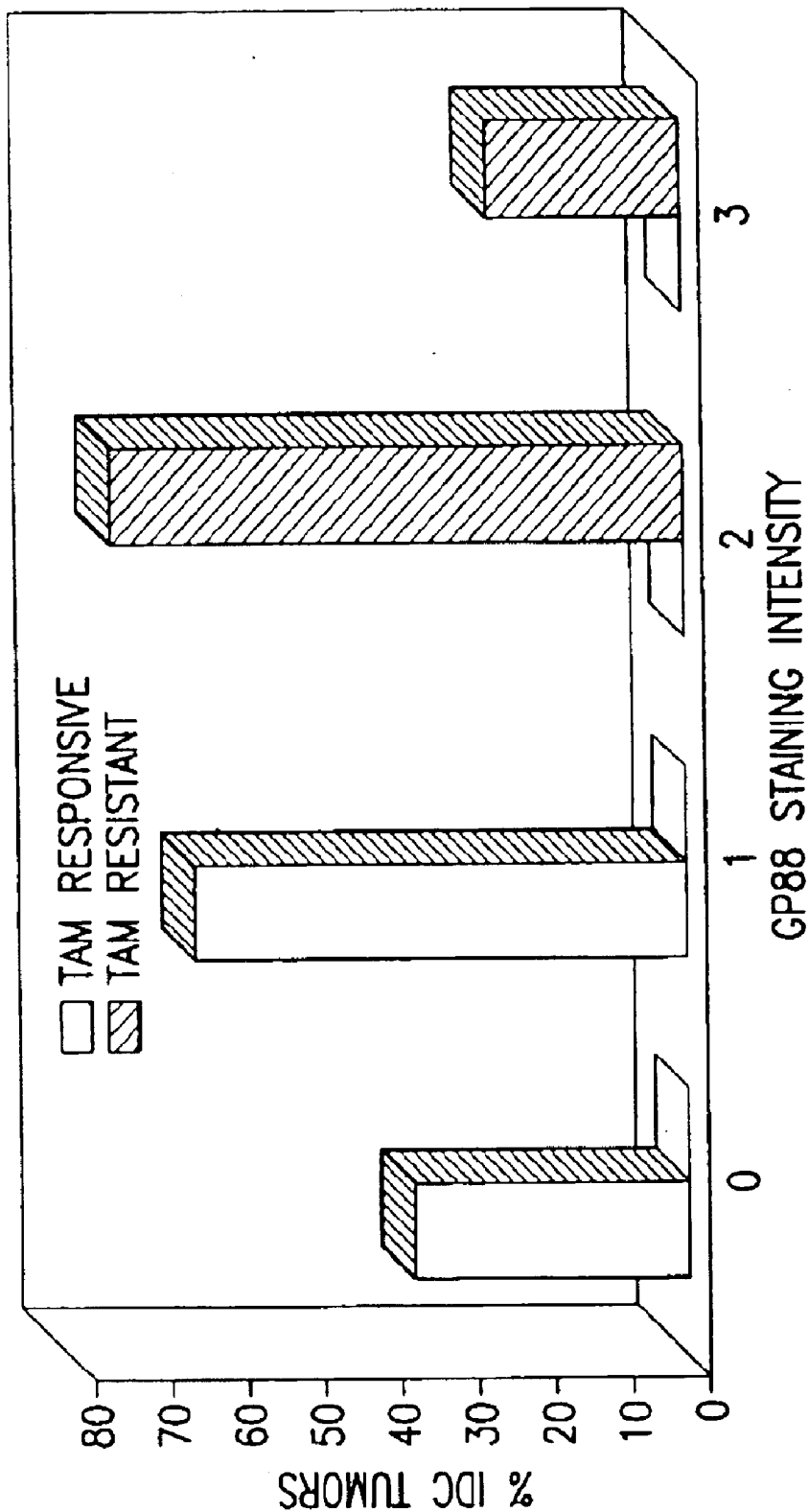


FIG. 23

**METHODS AND KITS FOR DIAGNOSING
TUMORIGENICITY AND DETERMINING
RESISTANCE TO THE ANTINEOPLASTIC
EFFECTS OF ANTIESTROGEN THERAPY**

This application is a continuation-in-part of U.S. application Ser. No. 09/456,886, filed Dec. 8, 1999, now U.S. Pat. No. 6,720,159 which is a divisional of U.S. application Ser. No. 08/863,079, filed May 23, 1997, now abandoned.

BACKGROUND OF THE INVENTION

The proliferation and differentiation of cells in multicellular organisms is subject to a highly regulated process. A distinguishing feature of cancer cells is the absence of control over this process; proliferation and differentiation become deregulated resulting in uncontrolled growth. Significant research efforts have been directed toward better understanding this difference between normal and tumor cells. One area of research focus is growth factors and, more specifically, autocrine growth stimulation.

Growth factors are polypeptides which carry messages to cells concerning growth, differentiation, migration and gene expression. Typically, growth factors are produced in one cell and act on another cell to stimulate proliferation. However, certain malignant cells, in culture, demonstrate a greater or absolute reliance on an autocrine growth mechanism. Malignant cells which observe this autocrine behavior circumvent the regulation of growth factor production by other cells and are therefore unregulated in their growth.

Study of autocrine growth control advances understanding of cell growth mechanisms and leads to important advances in the diagnosis and treatment of cancer. Toward this end, a number of growth factors have been studied, including insulin-like growth factors ("IGF1" and "IGF2"), gastrin-releasing peptide ("GRP"), transforming growth factors alpha and beta ("TGF- α " and "TGF"), and epidermal growth factor ("EGF").

The present invention is directed to a recently discovered growth factor. This growth factor was first discovered in the culture medium of highly tumorigenic "PC cells," an insulin-independent variant isolated from the teratoma derived adipogenic cell line 1246. This growth factor is referred to herein as "GP88." GP88 has been purified and structurally characterized. Amino acid sequencing of GP88 indicates that GP88 has amino acid sequence similarities with the mouse granulins/epithelin precursor.

Granulins/epithelins ("grn/epi") are 6 kDa polypeptides and belong to a novel family of double cysteine rich polypeptides. U.S. Pat. No. 5,416,192 (Shoyab et al.) is directed to 6 kDa epithelins, particularly epithelin 1 and epithelin 2. According to Shoyab, both epithelins are encoded by a common 63.5 kDa precursor, which is processed into smaller forms as soon as it is synthesized, so that the only natural products found in biological samples are the 6 kDa forms. Shoyab et al. teaches that the epithelin precursor is biologically inactive.

Contrary to the teachings of Shoyab et al., the present inventor's laboratory has demonstrated that the precursor is not always processed as soon as it is synthesized. Studies, conducted in part by this inventor, have demonstrated that the precursor (i.e., GP88) is in fact secreted as an 88 kDa glycoprotein with an N-linked carbohydrate moiety of 20 kDa. Analysis of the N-terminal sequence of GP88 indicates that GP88 starts at amino acid 17 of the grn/epi precursor, demonstrating that the first 17 amino acids from the protein sequence deduced from the precursor cDNA correspond to

a signal peptide compatible with targeting for membrane localization or for secretion. In contrast to the teachings of Shoyab et al., GP88 is biologically active and has growth promoting activity, particularly as an autocrine growth factor for the producer cells.

Breast cancer is a major worldwide cause of morbidity and mortality among women. Estrogen is known to be a primary stimulator for estrogen receptor positive (ER⁺) human breast cancer cell growth in vivo and in vitro. Although estrogen is initially required for establishment and proliferation of breast tumors, the development of estrogen-independent tumors during the course of breast cancer is indicative of poor prognosis. It has been postulated that the mitogenic effect of estrogen in breast cancer cells is mediated, at least partially, by autocrine growth factors, including growth factors regulated by estrogen. Thus, the identification and characterization of estrogen-responsive genes, particularly genes encoding growth factors, contributes to the understanding of the effects of estrogen in breast cancer cells.

Tamoxifen citrate ("tamoxifen") is a nonsteroidal antiestrogen commonly prescribed to patients suffering from breast cancer that has demonstrated potent antiestrogenic and antineoplastic properties. See U.S. Pat. No. 4,536,516. Tamoxifen is an estrogen receptor antagonist that competes with estrogen for binding to estrogen receptors. Other antiestrogens include, raloxifene, aromatase inhibitors (e.g., Arimidex® (anastrozole), Femera®), and estrogen receptor down-regulators (e.g., Faslodex®). The antiestrogenic effects of tamoxifen may be related to its ability to compete with estrogen for binding sites in target tissues. Other antiestrogens, such as aromatase inhibitors, inhibit or reduce the amount of estrogen available. For example, aromatase inhibitors prevent the conversion of androgen into estrogen thereby reducing the amount of estrogen available. Estrogen receptor down regulators inhibit or reduce the number of estrogen receptors on the cell.

Tamoxifen is currently available in 10 and 20 mg tablets from AstraZeneca and Barr Laboratories under the brand name Nolvadex® and is indicated for metastatic breast cancer, adjuvant therapy of breast cancer, ductal carcinoma in situ, and reduction of breast cancer incidence in women at high risk for breast cancer. Thus, tamoxifen can be used to treat and prevent various cancers and particularly breast cancer.

Tamoxifen administration, however, carries with it potentially serious risks for the patient. For example, tamoxifen administration has been associated with an increased risk of ovarian cancer. Moreover, some patients exhibit resistance to the intended beneficial effects of tamoxifen. Therefore, administration of tamoxifen to a patient who is resistant to its benefits causes unnecessary harm to the patient by increasing the risk of ovarian cancer and at the same time delaying or causing the patient to forego more effective treatments.

Accordingly, tamoxifen administration should be limited to those patients who are most likely to benefit from treatment with tamoxifen. An accurate determination of whether a patient will be susceptible or resistant to the antineoplastic effects of tamoxifen administration, before embarking on such a treatment course, would be a valuable diagnostic tool.

The art currently teaches that the absence of estrogen receptor in a patient corresponds with tamoxifen resistance. Cancer patients are routinely tested for the presence or absence of the estrogen receptor in an attempt to predict whether the patient's will be resistant or responsive to

tamoxifen therapy. Based on the current test, cancer patients who tests positive for the presence of estrogen receptors (“ER₊” or “estrogen receptor positive patients”) are typically prescribed tamoxifen.

However, a significant number of ER₊ patients are in fact resistant to tamoxifen. Thus, routinely prescribing tamoxifen for ER₊ patients may be harmful, and increase the patient’s risk of developing ovarian or other forms of cancer.

SUMMARY OF INVENTION

The inventor has now unexpectedly discovered that a glycoprotein (GP88), which is expressed in a tightly regulated fashion in normal cells, is overexpressed and unregulated in highly tumorigenic cells derived from the normal cells, that GP88 acts as a stringently required growth stimulator for the tumorigenic cells and that inhibition of GP88 expression or action in the tumorigenic cells results in an inhibition of the tumorigenic properties of the overproducing cells. Furthermore, the level of GP88 in a cell directly correlates with the tumorigenicity of the cell.

The inventor has further unexpectedly discovered that the level of GP88 expression indicates whether a patient will be resistant to the pharmacological effects of antiestrogenic compounds such as tamoxifen. The estrogen receptor status of tumor cells in breast cancer patients does not provide sufficient information to determine whether tamoxifen is an appropriate or preferable treatment course in breast cancer patients. The instant invention provides a more accurate tool for determining whether a subject is tamoxifen resistant and therefore represents a valuable advancement in the treatment of cancer.

Preferred embodiments of the invention provide methods for diagnosing tumorigenicity comprising obtaining a biological sample containing cells from a patient, detecting GP88 in the cells of the sample, determining the number of GP88 positive or stained cells in the sample, and determining the ratio of GP88 positive or stained cells to the total number of cells in said biological sample. The ratio is indicative of tumorigenicity.

The present invention also provides methods of determining whether a subject is resistant to the anti-neoplastic effects of antiestrogens. According to a particularly preferred embodiment, GP88 is detected in a biological sample containing cells from a patient and the amount of GP88 in the sample is determined. The amount of GP88 in the biological sample is indicative of resistance to the antineoplastic effects of antiestrogen therapy. In another preferred embodiment, the number of GP88 positive or stained cells in a biological sample is determined, and the ratio of GP88 positive or stained cells to the total number of cells in the biological sample is determined. The ratio is indicative of resistance to the antineoplastic effects of antiestrogen therapy.

Preferred embodiments of the invention also provide kits for diagnosing tumorigenicity and determining whether a patient is resistant to the anti-neoplastic effects of antiestrogens. Such kits preferably comprise a container and a compound or compounds for detecting GP88 (e.g., anti-GP88 antibodies, and anti-GP88 nucleotide probes).

Another preferred embodiment of the invention provides methods of treating or preventing cancer. According to one preferred embodiment, the amount of GP88 in a biological sample obtained from a patient is determined and antiestrogen therapy is administered to the patient if the amount of GP88 in the sample is less than about 5%. Alternatively, antiestrogen therapy is administered to an estrogen receptor

positive patient if the amount of GP88 in the biological samples is less than about 10%. In another preferred embodiment, the percentage of GP88 positive cells in a biological sample obtained from a patient is determined and antiestrogen therapy is administered to the patient in an amount sufficient to treat or prevent the cancer if the percentage of GP88 positive or stained cells in the biological sample is less than about 5%. Alternatively, antiestrogen therapy is administered to an estrogen receptor positive patient if the percentage of GP88 positive or stained cells in the biological sample is less than about 10%.

The present invention provides compositions for diagnosis and treatment of diseases such as, but not limited to, cancer in which cells exhibit an altered expression of GP88 or altered response to GP88. Use of the term “altered expression” herein means increased expression or overexpression of GP88 by a factor of at least two-fold, and at times by a factor of 10 or more, based on the level of mRNA or protein as compared to corresponding normal cells or surrounding peripheral cells. The term “altered expression” also means expression which became unregulated or constitutive without being necessarily elevated. Use of the terms increased or altered “response” to GP88 means a condition wherein increase in any of the biological functions (e.g., growth, differentiation, viral infectivity) conferred by GP88 results in the same or equivalent condition as altered expression of GP88.

“Neoplasia” refers to abnormal cell or tumor cell growth that persists in the absence of the original growth stimulus. The term “anti-neoplastic effect” refers to the property of reversing or inhibiting neoplasia. The term “cancer” refers to any disease caused by abnormal cell growth and includes, but is not limited to, breast, ovarian, kidney, bone, pancreatic, testicular, liver, brain, and skin cancer. The term “tumorigenicity” refers to the degree to which a cell or tissue exhibits the characteristics of neoplasia.

As used herein, “ER” refers to estrogen receptor, unless otherwise stated. “PR” refers to progesterone receptor, unless otherwise stated.

“GP88 positive cells” or “GP88 stained cells” refers to those cells in which GP88 is detected. As used herein, “GP88 negative cells” refers to those cells in which GP88 is not detected. Whether a cell is GP88 positive or negative may depend, in part, on the sensitivity of the detection method (i.e., immunostaining, in situ hybridization, imaging). “Total number of cells” refers to the total number of cells analyzed in a biological sample and may include all or some of the cells present in a particular portion of a biological sample.

The term “biological sample” refers to material derived from the body of a vertebrate animal, including, but not limited to, blood, serum, plasma, urine, nipple aspirate, cerebrospinal fluid, liver, kidney, breast, bone, bone marrow, testes or ovaries and brain, colon, and lung.

As used herein, a subject who is “resistant to” antiestrogen therapy is a patient that is nonresponsive to or has reduced or limited response to the antineoplastic effects of antiestrogen therapy.

The term “antiestrogen therapy” refers to administration of compounds that prevent the formation or interfere with the function of estrogen or estrogen analogs. Examples of compounds used for antiestrogen therapy are referred to herein as “antiestrogens” and include, but are not limited to, tamoxifen, raloxifene, aromatase inhibitors (e.g., Arimidex®, Femera®), and estrogen receptor down-regulators (e.g., Faslodex®).

Use of the term "GP88" herein means epithelin/granulin precursor in cell extracts and extracellular fluids, and is intended to include not only GP88 according to the amino acid sequences included in FIGS. 8 or 9, which are of mouse and human origins, but also GP88 of other species. In addition, the term also includes functional derivatives thereof having additional components such as a carbohydrate moiety including a glycoprotein or other modified structures. The terms "GP88" and "PCDGF" are used interchangeably herein. Also intended by the term GP88 is any polypeptide fragment having at least 10 amino-acids present in the above mentioned sequences. Sequences of this length are useful as antigens and for making immunogenic conjugates with carriers for the production of antibodies specific for various epitopes of the entire protein. Such polypeptides are useful in screening such antibodies and in the methods directed to detection of GP88 in biological fluids. It is well known in the art that peptides are useful in generation of antibodies to larger proteins. In one embodiment of this invention, it is shown that peptides from 12-19 amino-acids in length have been successfully used to develop antibodies that recognize the full length GP88.

The polypeptide of this invention may exist covalently or non-covalently bound to another molecule. For example, it may be fused to one or more other polypeptides via one or more peptide bonds such as glutathione transferase, polyhistidine, green fluorescent protein, myc tag, or other tagged compounds (e.g., biotin).

The polypeptide is sufficiently large to comprise an antigenetically distinct determinant or epitope which can be used as an immunogen to reproduce or test antibodies against GP88 or a functional derivative thereof.

One embodiment includes the polypeptide substantially free of other mammalian peptides. GP88 of the present invention can be biochemically or immunochemically purified from cells, tissues or a biological fluid. Alternatively, the polypeptide can be produced by recombinant means in a prokaryotic or eukaryotic expression system and host cells.

A "fragment" of GP88 refers to any subset of the molecule that is a shorter peptide. This corresponds for example but is not limited to regions such as K19T and S14R for mouse GP88, and E19V and A14R (equivalent to murine K19T and S14R, respectively) for human GP88.

"variant" of GP88 refers to a molecule substantially similar to either the entire peptide or a fragment thereof. Variant peptides may be prepared by direct chemical synthesis of the variant peptide using methods known in the art.

Alternatively, amino acid sequence variants of the peptide can be prepared by modifying the DNA which encodes the synthesized protein or peptide. Such variants include, for example, deletions, insertions, or substitutions of residues within the amino-acid sequence of GP88. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided the final construct possesses the desired activity. The mutation that will be made in the DNA encoding the variant peptide must not alter the reading frame and preferably will not create complementary regions that could produce secondary mRNA structures. At the genetic level these variants are prepared by site directed mutagenesis (8) of nucleotides in the DNA encoding the peptide molecule thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. The variant typically exhibits the same qualitative biological activity as the nonvariant peptide.

An "analog" of GP88 protein refers to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

The antibodies of the invention (neutralizing and others) are preferably used as a treatment for cancer or other diseases in cells which exhibit an increased expression of GP88. By the term "neutralizing" it shall be understood that the antibody has the ability to inhibit or block the normal biological activity of GP88, including, but not limited to, GP88's ability to stimulate cell proliferation, increase cell survival, block apoptosis, or to induce tumor growth in experimental animals and in humans. An effective amount of anti-GP88 antibody is administered to an animal, including humans, by various routes. In an alternative embodiment, the anti-GP88 antibody is used as a diagnostic to detect cells which exhibit an altered (increased) expression of GP88 as occurring in diseases such as but not limited to cancers, and to identify diseased cells whose growth is dependent on GP88 and which will respond to GP88 antagonizing therapy. In yet another embodiment, the anti-GP88 antibody is used to deliver compounds such as cytotoxic factors or antisense oligonucleotides to cells expressing or responsive to GP88.

It is to be understood that the application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and, together with the description, serve to explain the principles of the invention. Examples of the products of the present invention and processes for their use appear in the following examples.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A compares the level of expression of GP88 protein in the 1246, 1246-3A and PC cell lines. Cells were cultured in DME-F12 medium supplemented with 2% fetal bovine serum (FBS). GP88 expression levels were measured by immunoprecipitation and Western blot analysis with anti-K19T antibody.

FIG. 1B compares the level of GP88 mRNA expression in the 1246, 1246-3A and PC cell lines. mRNA for RPL32 is used as an internal control for equal amounts of RNA loading.

FIG. 1C compares the expression of GP88 mRNA in 1246 cells (left panel) and in PC cells (right panel) in serum-free and serum containing medium. The results show that GP88 expression in 1246 cells is inhibited by the addition of fetal bovine serum whereas such inhibition is not observed in the highly tumorigenic PC cells.

FIG. 2 illustrates the effect of treatment of the highly tumorigenic PC cells with increasing concentrations of anti-GP88 neutralizing antibody.

FIG. 3 shows C3H mice injected subcutaneously with 10^6 antisense GP88 transfected PC cells (bottom) and with empty vector transfected control PC cells (top).

FIG. 4 shows in vivo GP88 expression levels in C3H mice tumor tissues and in surrounding normal tissues.

FIG. 5 shows GP88 mRNA expression levels in estrogen receptor positive and estrogen receptor negative human mammary carcinoma cell lines.

FIG. 6 shows the effect of increasing concentrations of GP88 on the growth of the mouse mammary epithelial cell line C57.

FIG. 7 shows the growth properties and tumorigenic ability of PC cells transfected with a cytomegalovirus promoter controlled expression vector containing GP88 in antisense orientation and PC cells transfected with an empty vector.

FIG. 8 shows the nucleotide and deduced amino-acid sequence of mouse GP88. (SEQ ID NOS 1 & 2 respectively) Peptide regions used as antigens to raise anti-GP88 antibodies K19T and S14R are underlined. The region cloned in the antisense orientation in the pCMV4 mammalian expression vector is indicated between brackets.

FIG. 9A shows the nucleotide sequence of human GP88 cDNA (SEQ ID NOS 16 & 17, respectively). Indicated between brackets is the region cloned in the antisense orientation into the pcDNA3 mammalian expression system; and

FIG. 9B shows the deduced amino-acid sequence of human GP88. The E19V region used as antigen to develop anti-human GP88 neutralizing antibody is underlined. It also indicates the region A14R equivalent to the mouse S14R region.

FIG. 10 shows the amino-acid sequence of mouse GP88 (SEQ ID NO: 2) arranged to show the 7 and one-half repeats defined as granulins g, f, B, A, C, D and e (right side). This representation shows that the region K19T and S14R used to raise GP88 antibodies for developing anti-GP88 neutralizing antibodies is found between two epithelin/granulin repeats in what is considered a variant region. Indicated on the right hand side is the granulin classification of the repeats according to Bateman et al (6) Granulin B and granulin A are also defined as epithelin 2 and epithelin 1 respectively according to Plowman et al., 1992 (5).

FIG. 11 shows a schematic representation of pCMV4 and a GP88 cDNA clone indicating the restriction sites used to clone GP88 antisense cDNA into the expression vector.

FIG. 12 shows the cross-linking of ^{125}I -rGP88 to GP88 cell surface receptors on CCL-64 cells. The cross-linking reaction was carried out with disuccinimidyl suberate (DSS). Reaction products were analyzed by SDS-PAGE on a 7% polyacrylamide gel.

FIG. 13 shows the cross-linking of ^{125}I -rGP88 to GP88 cell surface receptors on 3T3 fibroblasts, PC cells and C57MG mammary epithelial cells. The results show that these various cell lines display GP88 cell surface receptors of similar molecular weight as the ones on CCL64 cells (FIG. 12).

FIG. 14 shows GP88 expression levels in non-tumorigenic MCF 10A and in malignant (MCF 7, MDA-MB-468) human mammary epithelial cells.

FIG. 15 shows that GP88 expression is inhibited by antisense GP88 cDNA transfection in human breast carcinoma, estrogen receptor negative MDA-MB-468 cells.

FIG. 16 shows the effect of PCDGF on the proliferation of MCF-7 cells. FIG. 16(A) illustrates that PCDGF stimulates DNA synthesis of MCF-7 cells in the absence of 17β -estradiol (E2). FIG. 16(B) illustrates that anti-PCDGF antibody specifically inhibits the E2 mitogenic effect. The results are expressed as means \pm SD of triplicate determinations.

FIG. 17 shows the effect of inhibiting PCDGF expression on the E2 mitogenic effect. FIG. 17(A) illustrates inhibition of PCDGF expression by antisense PCDGF cDNA transfection in MCF-7 cells. FIG. 17(B) illustrates that inhibition of PCDGF expression in MCF-7 cells inhibits the E2 mitogenic activity.

FIG. 18 shows that overexpression of PCDGF in MCF-7 cells results in cells that are able to proliferate in the absence of E2 and are resistant to tamoxifen. FIG. 18(A) shows PCDGF expression in O4 cells and control MCF-7C cells. FIG. 18(B) shows proliferation of O4 and control MCF-7

cells in estrogen-depleted PFMEM medium. FIG. 18(C) shows a comparison of the response of MCF-7 and O4 cells to tamoxifen. The results are expressed as means \pm SD.

FIG. 19 shows the determination of PCDGF signaling pathway in MCF-7 cells. FIG. 19(A) illustrates the effect of MAP kinase inhibitor on the mitogenic effect of PCDGF. FIG. 19(B) illustrates activation of MAP kinase by PCDGF.

FIG. 20 shows the effect of PCDGF on cyclin D1 and c-myc expression in MCF-7 cells. FIG. 20(A) shows stimulation of cyclin D1 expression by PCDGF. FIG. 20(B) shows the effect of E2 and PCDGF on c-myc expression.

FIG. 21 shows GP88 staining intensity with anti-GP88 antibody on paraffin embedded breast cancer biopsies by immunohistochemistry (IHC).

FIG. 22 shows the correlation between estrogen receptor status and GP88 staining intensity in Invasive Ductal Carcinoma (IHC) tumors.

FIG. 23 shows the correlation between tamoxifen responsiveness of ER₊ IDC tumors and GP88 staining intensity. As GP88 staining intensity increases, estrogen receptor positive tumors become resistant to antiestrogen therapy.

DETAILED DESCRIPTION OF THE INVENTION

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the following examples, serve to explain the principles of the invention.

Biological Activity of GP88

The invention relates to GP88 and antitumor and antiviral compositions useful for treating and diagnosing diseases linked to altered (increased) expression of GP88. Alternatively this invention is used for treating and diagnosing diseases linked to increased responsiveness to GP88. Using a murine model system consisting of three cell lines, the inventor has shown that cells which overexpress GP88 form tumors. The parent cell line, 1246, is a C3H mouse adipogenic cell line which proliferates and differentiates into adipocytes in a defined medium under stringent regulation by insulin. The 1246 cells cannot form tumors in a syngeneic animal (C3H mouse) even when injected at a high cell density. An insulin independent cell line, 1246-3A, was isolated from 1246 cells maintained in insulin-free medium. The 1246-3A cells lost the ability to differentiate and form tumors when 10^6 are injected subcutaneously in syngeneic mice. A highly tumorigenic cell line, PC, was developed from 1246-3A cells by an in vitro-in vivo shuttle technique. The PC cells formed tumors when 10^4 cells were injected into syngeneic mice.

GP88 is overexpressed in the insulin-independent tumorigenic cell lines relative to the parent non-tumorigenic insulin-dependent cell line. Moreover, the degree of overexpression of GP88 positively correlates with the degree of tumorigenicity of these cells, demonstrating for the first time that GP88 is important in tumorigenesis (FIG. 1). With reference to FIG. 1, since GP88 is synthesized by cells but also secreted in culture medium, the level of GP88 was determined in cell lysates and in culture medium (CM). All cells were cultivated in DME/F12 nutrient medium supplemented with 2% fetal bovine serum. When cells reached confluency, culture medium (CM) was collected and cell lysates were prepared by incubation in buffer containing detergent followed by a 10,000 \times g centrifugation. Cell lysate and conditioned medium were normalized by cell number.

Samples from cell lysate and conditioned medium were analyzed by Western blot analysis using an anti-GP88 antibody, as explained below.

The development of a neutralizing antibody confirmed GP88's key role in tumorigenesis. When an anti-GP88 antibody directed to the K19T region of mouse GP88 was added to the culture medium, the growth of highly tumorigenic PC cells was inhibited in a dose dependent fashion (FIG. 2). With reference to FIG. 2, PC cells were cultivated in 96 well plates at a density 2×10^4 cells/well in DME/F12 medium supplemented with human fibronectin (2 μ g/ml) and human transferrin (10 μ g/ml). Increasing concentrations of anti-GP88 IgG fraction were added to the wells after the cells were attached. Control cells were treated with equivalent concentrations of non-immune IgG. Two days later, 0.25 mCi of 3 H-thymidine was added per well for 6 hrs. Cells were then harvested to count 3 H-thymidine incorporated into DNA as a measure for cell proliferation.

Moreover, when the expression of GP88 was specifically inhibited by antisense GP88 cDNA in PC cells, the production of GP88 was reduced and these PC cells could no longer form tumors in syngeneic C3H mouse. In addition, these PC cells regained responsiveness to insulin. With reference to FIG. 3 and Tables 1 and 2, C3H female mice were injected subcutaneously with 10^6 antisense GP88 transfected PC cells (as explained below) or 10^6 empty vector transfected PC cells. Mice were monitored daily for tumor appearance. Photographs were taken 45 days after injection of the cells. The results show that mice injected with antisense GP88 PC cells do not develop tumors, in contrast to the mice injected with empty vector transfected PC cells used as control.

TABLE 1

COMPARISON OF TUMORIGENIC PROPERTIES OF GP88 ANTISENSE TRANSFECTED CELLS, CONTROL TRKNSFECTED CELLS AND PC CELLS			
CELLS INJECTED	AVERAGE DAY OF TUMOR DETECTION	NUMBER OF MICE WITH TUMORS	AVERAGE TUMOR WEIGHT (g)
PC	15 \pm 3.0	5/5	9.0 \pm 3.2
P14	15 \pm 3.7	5/5	7.8 \pm 2.7
ASGP88	—	0/5	—

PC: Control non-transfected cells
 P-14: Empty vector control transfected PC cells
 ASGP88: PC cells transfected with expression vector containing GP88 antisense cDNA

Tumors were excised and weighed at 45 days. —indicates no tumor formation.

TABLE 2

COMPARISON OF PROPERTIES OF 1246, PC CELLS AND GP88 ANTISENSE CELLS		
1246 cells	PC cells	Antisense GP 88 cells
Insulin responsive for growth and differentiation	insulin-independent for growth differentiation deficient	Recovery of insulin responsiveness for growth (differentiation?)
Cell surface insulin receptor expression high	Autocrine production of insulin-related factor cell surface insulin receptor expression very low	cell surface insulin receptor expression elevated
GP88 expression low	GP88 expression constitutively high	GP88 expression inhibited by antisense

TABLE 2-continued

COMPARISON OF PROPERTIES OF 1246, PC CELLS AND GP88 ANTISENSE CELLS		
1246 cells	PC cells	Antisense GP 88 cells
GP88 expression inhibited by serum	No inhibition by serum	
GP88 expression regulated by insulin	GP88 expression constitutive	Recovery of insulin regulation for endogenous GP88 expression
Non-tumorigenic	highly tumorigenic	non-tumorigenic

Comparison of the expression of GP88 indicates that in vivo GP88 levels in tumors is dramatically higher than in normal tissues (FIG. 4). C3H mice were injected with 10^6 PC cells. Tumor bearing mice were euthanized. Tumors, fat pads and connective tissue were collected. Cell lysates were prepared by incubation in buffer containing detergent as described above for FIG. 1. Protein concentration of tissue extracts was determined, and equivalent amounts of proteins for each sample were analyzed by SDS-PAGE followed by Western blot analysis using anti-GP88 antibody to measure the content of GP88 in tissue extracts. The results showed that the level of GP88 in tumor extracts is at least 10-fold higher than in surrounding connective and fat tissues.

In normal cells (1246 cells, fibroblasts), the expression of GP88 is regulated, in particular by insulin, and inhibited by fetal bovine serum. In tumorigenic cells, a loss of regulation of normal growth leads to the increased expression of GP88 and the acquisition of GP88 dependence for growth. Therefore, inhibition of GP88 expression and/or action is an effective approach to suppression of tumorigenesis. Detection of an elevated GP88 expression in biopsies provides diagnostic analysis of tumors that are responsive to GP88 inhibition therapy.

GP88 is also a tumor inducing factor in human cancers. As seen in the 1246-3A cell line, a loss of responsiveness to insulin (or to IGF-I) and a concurrent increase in malignancy has been well documented (13, 14) in several human cancers including but not limited to breast cancers. Specifically, breast carcinoma is accompanied by the acquisition of an insulin/IGF-I autocrine loop, which is also the starting point of the development of tumorigenic properties in the mouse model system discussed above. Furthermore, GP88 expression is elevated in human breast carcinomas. More specifically, with reference to FIG. 5, human GP88 was highly expressed in estrogen receptor positive and also in estrogen receptor negative insulin/IGF-I independent highly malignant cells. Also, GP88 is a potent growth factor for mammary epithelial cells (FIG. 6). The data in FIG. 5 was obtained by cultivating MCF7, MDA-MB-453 and MDA-MB-468 cells in DME/F12 medium supplemented with 10% fetal bovine serum (FBS). RNA was extracted from each cell line by the RNazol method and poly-A⁺ RNA prepared. GP88 mRNA expression was examined by Northern blot analysis with 3 μ g of poly-A⁺ RNA for each cell line using a 32 P-labeled GP88 cDNA probe.

For Northern blot analysis of GP88 mRNA expression in rodent cells or tissues (mouse and rats), we used a mouse GP88 cDNA probe 311 bp in length starting at nucleotide 551 to 862 (corresponding to amino-acid sequence 160 to 270). RNA can be extracted by a variety of methods (Sambrook, Molecular Biology manual: 35) well known to people of ordinary skill in the art. The method of choice was to extract RNA using RNazol (Cinnabiotech) or Trizol (Gibco-BRL) solutions which consists of a single step extraction by guanidinium isothiocyanate and phenol-chloroform.

For Northern blot analysis of GP88 mRNA expression in human cell lines, a 672 bp human GP88 cDNA probe was

developed corresponding to nucleotide 1002 to 1674 (corresponding to amino-acid sequence 334–558) of human GP88.

With respect to FIG. 6, C57MG cells were cultivated in the presence of increasing concentrations of GP88 purified from PC cells conditioned medium (top panel), and recombinant GP88 expressed in insect cells (bottom panel), to demonstrate the growth stimulating effect of increasing concentrations of GP88 on the growth of the mouse mammary epithelial cell line C57MG.

A correlation between IGF-1 autocrine production and increased malignancy has also been well established for glioblastomas, teratocarcinomas and breast carcinomas. In these cancers, GP88 expression is also elevated in human tumors when compared to non-tumorigenic human fibroblasts and other human cell lines. GP88 promotes the growth of mammary carcinoma cells.

Anti-GP88 Antibodies

The invention provides compositions for treating and diagnosing diseases linked to increased expression of GP88. This also will apply to treatment and diagnosis of diseases linked to increased responsiveness to GP88. The compositions of this invention include anti-GP88 antibodies which neutralize the biological activity of GP88.

The present invention is also directed to an antibody specific for an epitope of GP88 and the use of such antibody to detect the presence or measure the quantity or concentration of GP88 molecule, a functional derivative thereof or a homologue from different animal species in a cell, a cell or tissue extract, culture medium or biological fluid. Moreover, antibody can be used to target cytotoxic molecules to a specific site.

For use as antigen for development of antibodies, the GP88 protein naturally produced or expressed in recombinant form or functional derivative thereof, preferably having at least 9 amino-acids, is obtained and used to immunize an animal for production of polyclonal or monoclonal antibody. An antibody is said to be capable of binding a molecule if it is capable of reacting with the molecule to thereby bind the molecule to the antibody. The specific reaction is meant to indicate that the antigen will react in a highly selective manner with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The term antibody herein includes but is not limited to human and non-human polyclonal antibodies, human and non-human monoclonal antibodies (mAbs), chimeric antibodies, anti-idiotypic antibodies (anti-IdAb) and humanized antibodies. Polyclonal antibodies are heterogeneous populations of antibody molecules derived either from sera of animals immunized with an antigen or from chicken eggs. Monoclonal antibodies (“mAbs”) are substantially homogeneous populations of antibodies to specific antigens. mAbs may be obtained by methods known to those skilled in the art (e.g., U.S. Pat. No. 4,376,110). Such antibodies may be of any immunological class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing human and non-human antibodies to GP88 may be cultivated *in vitro* or *in vivo*. For production of a large amount of InAbs, *in vivo* is the presently preferred method of production. Briefly, cells from the individual hybridomas are injected intraperitoneally into pristane primed Balb/c mice or Nude mice to produce ascites fluid containing high concentrations of the desired mAbs. mAbs may be purified from such ascites fluids or from culture supernatants using

standard chromatography methods well known to those of skill in the art.

Human monoclonal Ab to human GP88 can be prepared by immunizing transgenic mice expressing human immunoglobulin genes. Hybridoma produced by using lymphocytes from these transgenic animals will produce human immunoglobulin instead of mouse immunoglobulin.

Since most monoclonal antibodies are derived from murine source and other non-human sources, their clinical efficiency may be limited due to the immunogenicity of rodent mAbs administered to humans, weak recruitment of effector function and rapid clearance from serum. To circumvent these problems, the antigen-binding properties of murine antibodies can be conferred to human antibodies through a process called humanization. A humanized antibody contains the amino-acid sequences for the 6 complementarity-determining regions (CDRs) of the parent murine mAb which are grafted onto a human antibody framework. The low content of non-human sequences in humanized antibodies (around 5%) has proven effective in both reducing the immunogenicity and prolonging the serum half life in humans. Methods such as the ones using monovalent phage display and combinatorial library strategy for humanization of monoclonal antibodies are now widely applied to the humanization of a variety of antibodies and are known to people skilled in the art. These humanized antibodies and human antibodies developed with transgenic animals as described above are of great therapeutic use for several diseases including but not limited to cancer.

Hybridoma supernatants and sera are screened for the presence of antibody specific for GP88 by any number of immunoassays including dot blots and standard immunoassays (EIA or ELISA) which are well known in the art. Once a supernatant has been identified as having an antibody of interest, it may be further screened by Western blotting to identify the size of the antigen to which the antibody binds. One of ordinary skill in the art will know how to prepare and screen such hybridomas without undue experimentation in order to obtain a desired polyclonal or mAb.

Chimeric antibodies have different portions derived from different animal species. For example, a chimeric antibody might have a variable region from a murine mAb and a human immunoglobulin constant region. Chimeric antibodies and methods for their production are also known to those skilled in the art.

An anti-idiotypic (“anti-IdAb”) is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An anti-IdAb can be prepared by immunizing an animal of the same species and genetic type (e.g., mouse strain) as the source of the mAb with the mAb to which an anti-IdAb is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing antibody to these idiotypic determinants (the anti-IdAb). The anti-IdAb may also be used as an immunogen to produce an immune response in yet another animal, producing a so-called anti-anti-IdAb. The anti-anti-IdAb may be epitopically identical to the original m-Ab which induced the anti-IdAb. Thus by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against GP88 may be used to induce human and non-human anti-IdAbs in suitable animals. Spleen cells from such immunized mice are used to produce hybridomas secreting human or non-human anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier

such as Keyhole Limpet Hemocyanin (KLH) or bovine serum albumin (BSA) and used to immunize additional mice. Sera from these mice will contain human or non-human anti-anti-IdAb that have the binding properties of the original mAb specific for a GP88 polypeptide epitope. The anti-Id mAbs thus have their own idiotypic epitopes or idiotypes structurally similar to the epitope being evaluated.

The term antibody is also meant to include both intact molecules as well as fragments thereof such as, for example, Fab and F(ab')₂, which are capable of binding to the antigen. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation and may have less non-specific tissue binding than an intact antibody. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to generate Fab fragments) and pepsin (to generate F(ab')₂ fragments). It will be appreciated that Fab and F(ab')₂ and other fragments of the antibodies useful in the present invention may be used for the detection or quantitation of GP88, and for treatment of pathological states related to GP88 expression, according to the methods disclosed herein for intact antibody molecules.

According to the present invention, antibodies that neutralize GP88 activity *in vitro* can be used to neutralize GP88 activity *in vivo* to treat diseases associated with increased GP88 expression or increased responsiveness to GP88, such as but not limited to cancer and viral infection. A subject, preferably a human subject, suffering from disease associated with increased GP88 expression is treated with an antibody to GP88. Such treatment may be performed in conjunction with other anti-cancer or anti-viral therapy. A typical regimen comprises administration of an effective amount of the antibody specific for GP88 administered over a period of one or several weeks and including between about one and six months. The antibody of the present invention may be administered by any means that achieves its intended purpose. For example, administration may be by various routes including but not limited to subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal and oral. Parenteral administration can be by bolus injection or by gradual perfusion over time. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions and emulsions, which may contain auxiliary agents or excipients known in the art. Pharmaceutical compositions such as tablets and capsules can also be prepared according to routine methods. It is understood that the dosage of will be dependent upon the age, sex and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired. The ranges of effective doses provided below are not intended to limit the invention and merely represent preferred dose ranges. However the most preferred dosage will be tailored to the individual subject as is understood and determinable by one skilled in the art. The total dose required for each treatment may be administered by multiple doses or in a single dose. Effective amounts of antibody are from about 0.01 μg to about 100 mg/kg body weight and preferably from about 10 μg to about 50 mg/kg. Antibody may be administered alone or in conjunction with other therapeutics directed to the same disease.

According to the present invention and concerning the neutralizing antibody, GP88 neutralizing antibodies can be used in all therapeutic cases where it is necessary to inhibit GP88 biological activity, even though there may not necessarily be a change in GP88 expression, including cases where there is an overexpression of GP88 cell surface receptors and this in turn results in an increased biological

activity, or where there is an alteration in GP88 signaling pathways or receptors leading to the fact that the signaling pathways are always "turned on." Neutralizing antibodies to growth factor and to growth factor receptors have been successfully used to inhibit the growth of cells whose proliferation is dependent on this growth factor. This has been the case for IGF-I receptor in human breast carcinoma cells and bombesin for lung cancer. The antibody to GP88 can also be used to deliver compounds such as, but not limited to, cytotoxic reagents such as toxins, oncotoxins, mitotoxins and immunotoxins, or antisense oligonucleotides, in order to specifically target them to cells expressing or responsive to GP88 (30).

One region that allows antigen to develop a neutralizing antibody to GP88 is the 19 amino-acid region defined as K19T in the mouse GP88, and E19V in the human GP88 which is not located within the epithelin/granulin 6 kDa repeats but between these repeats, specifically between granulin A (epithelin 1) and granulin C (5) in what is considered a variant region (see FIG. 10). Without wishing to be bound by theory, it is believed that the region important for the biological activity of GP88 lies outside of the epithelin repeats.

The antibodies or fragments of antibodies useful in the present invention may also be used to quantitatively or qualitatively detect the presence of cells which express the GP88 protein. This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody (see below) with fluorescent microscopic, flow cytometric, or fluorometric detection. The reaction of antibodies and polypeptides of the present invention may be detected by immunoassay methods well known in the art.

The antibodies of the present invention may be employed histologically as in light microscopy, immunofluorescence or immunoelectron microscopy, for *in situ* detection of the GP88 protein in tissues samples or biopsies. *In situ* detection may be accomplished by removing a histological specimen from a patient and applying the appropriately labeled antibody of the present invention. The antibody (or fragment) is preferably provided by applying or overlaying the labeled antibody (or fragment) to the biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the GP88 protein but also its distribution in the examined tissue. Using the present invention, those of ordinary skill in the art will readily perceive that any wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Assays for GP88 typically comprise incubating a biological sample such as a biological fluid, a tissue extract, freshly harvested or cultured cells or their culture medium in the presence of a detectably labeled antibody capable of identifying the GP88 protein and detecting the antibody by any of a number of techniques well known in the art.

The biological sample may be treated with a solid phase support or carrier such as nitrocellulose or other solid support capable of immobilizing cells or cell particles or soluble proteins. The support may then be washed followed by treatment with the detectably labeled anti-GP88 antibody. This is followed by wash of the support to remove unbound antibody. The amount of bound label on said support may then be detected by conventional means. By solid phase support is intended any support capable of binding antigen or antibodies such as but not limited to glass, polystyrene polypropylene, nylon, modified cellulose, or polyacrylamide.

The binding activity of a given lot of antibody to the GP88 protein may be determined according to well known meth-

ods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

Detection of the GP88 protein or functional derivative thereof and of a specific antibody for the protein may be accomplished by a variety of immunoassays well known in the art such as enzyme linked immunoassays (ELISA) or radioimmunoassays (RIA). Such assays are well known in the art and one of skill will readily know how to carry out such assays using the anti-GP88 antibodies and GP88 protein of the present invention.

Such immunoassays are useful to detect and quantitate GP88 protein in serum or other biological fluid as well as in tissues, cells, cell extracts, or biopsies. In a preferred embodiment, the concentration of GP88 is measured in a tissue specimen as a means for diagnosing cancer or other disease associated with increased expression of GP88.

The presence of certain types of cancers and the degree of malignancy are said to be "proportional" to an increase in the level of the GP88 protein. The term "proportional" as used herein is not intended to be limited to a linear or constant relationship between the level of protein and the malignant properties of the cancer. The term "proportional" as used herein, is intended to indicate that an increased level of GP88 protein is related to appearance, recurrence or display of malignant properties of a cancer or other disease associated with increased expression of GP88 at ranges of concentration of the protein that can be readily determined by one skilled in the art.

Another embodiment of the invention relates to evaluating the efficacy of anti-cancer or anti-viral drug or agent by measuring the ability of the drug or agent to inhibit the expression or production of GP88. The antibodies of the present invention are useful in a method for evaluating anti-cancer or anti-viral drugs in that they can be employed to determine the amount of the GP88 protein in one of the above-mentioned immunoassays. Alternatively, the amount of the GP88 protein produced is measured by bioassay (cell proliferation assay) as described herein. The bioassay and immunoassay can be used in combination for a more precise assessment.

An additional embodiment is directed to an assay for diagnosing cancers or other diseases associated with an increase in GP88 expression based on measuring in a tissue or biological fluid the amount of mRNA sequences present that encode GP88 or a functional derivative thereof, preferably using an RNA-DNA hybridization assay. The presence of certain cancers and the degree of malignancy is proportional to the amount of such mRNA present. For such assays the source of mRNA will be biopsies and surrounding tissues. The preferred technique for measuring the amount of mRNA is a hybridization assay using DNA of complementarity base sequence.

Another related embodiment is directed to an assay for diagnosing cancers or other diseases associated with an increase in GP88 responsiveness based on measuring on a tissue biopsy whether treatment with anti-GP88 neutralizing antibody will inhibit its growth or other biological activity.

Another related embodiment is a method for measuring the efficacy of anti-cancer or anti-viral drug or agent which comprises the steps of measuring the agent's effect on inhibiting the expression of mRNA for GP88. Similarly such method can be used to identify or evaluate the efficacy of GP88 antagonizing agents by measuring the ability of said agent to inhibit the production of GP88 mRNA.

Nucleic acid detection assays, especially hybridization assays, can be based on any characteristic of the nucleic acid

molecule such as its size, sequence, or susceptibility to digestion by restriction endonucleases. The sensitivity of such assays can be increased by altering the manner in which detection is reported or signaled to the observer. A wide variety of labels have been extensively developed and used by those of ordinary skill in the art, including enzymatic, radioisotopic, fluorescent, chemical labels and modified bases.

One method for overcoming the sensitivity limitation of a nucleic acid for detection is to selectively amplify the nucleic acid prior to performing the assay. This method has been referred as the "polymerase chain reaction" or PCR (U.S. Pat. Nos. 4,683,202 and 4,582,788). The PCR reaction provides a method for selectively increasing the concentration of a particular nucleic acid sequence even when that sequence has not been previously purified and is present only in a single copy in a particular sample.

GP88 Antisense Components

This invention also provides GP88 antisense components. The constitutive expression of antisense RNA in cells has been shown to inhibit the expression of more than 20 genes and the list continues to grow. Possible mechanisms for antisense effects are the blockage of translation or prevention of splicing, both of which have been observed in vitro. Interference with splicing allows the use of intron sequences which should be less conserved and therefore result in greater specificity, inhibiting expression of a gene product of one species but not its homologue in another species.

The term antisense component corresponds to an RNA sequence as well as a DNA sequence coding therefor, which is sufficiently complementary to a particular mRNA molecule, for which the antisense RNA is specific, to cause molecular hybridization between the antisense RNA and the mRNA such that translation of the mRNA is inhibited. Such hybridization can occur under in vivo conditions. The action of the antisense RNA results in specific inhibition of gene expression in the cells.

According to the present invention, transfection of tumorigenic cells with DNA antisense to the GP88 cDNA inhibits endogenous GP88 expression and inhibits tumorigenicity of the antisense cDNA transfected cells. This antisense DNA must have sufficient complementarity, about 18-30 nucleotides in length, to the GP88 gene so that the antisense RNA can hybridize to the GP88 gene (or mRNA) and inhibit GP88 gene expression regardless of whether the action is at the level of splicing, transcription, or translation. The degree of inhibition is readily discernible to one skilled in the art without undue experimentation given the teachings herein and preferably is sufficient to inhibit the growth of cells whose proliferation is dependent on the expression of GP88. One of ordinary skill in the art will recognize that the antisense RNA approach is but a number of known mechanisms which can be employed to block specific gene expression.

The antisense components of the present invention may be hybridizable to any of several portions of the target GP88 cDNA, including the coding sequence, 3' or 5' untranslated regions, or other intronic sequences, or to GP88 mRNA. As is readily discernible by one of ordinary skill in the art, the minimal amount of homology required by the present invention is that sufficient to result in hybridization to the GP88 DNA or mRNA and in inhibition of transcription of the DNA, or translation or function of the mRNA, preferably without affecting the function of other mRNA molecules and the expression of other unrelated genes.

Antisense RNA is delivered to a cell by transformation or transfection via a vector, including retroviral vectors and plasmids, into which has been placed DNA encoding the antisense RNA with the appropriate regulatory sequences including a promoter to result in expression of the antisense RNA in a host cell. Stable transfection of various antisense expression vectors containing GP88 cDNA fragments in the antisense orientation have been performed. One can also deliver antisense components to cells using a retroviral vector. Delivery can also be achieved by liposomes.

For purpose of antisense technology for in vivo therapy, the currently preferred method is to use antisense oligonucleotides, instead of performing stable transfection of an antisense cDNA fragment constructed into an expression vector. Antisense oligonucleotides having a size of 15–30 bases in length and with sequences hybridizable to any of several portions of the target GP88 cDNA, including the coding sequence, 3' or 5' untranslated regions, or other intronic sequences, or to GP88 mRNA, are preferred. Sequences for the antisense oligonucleotides to GP88 are preferably selected as being the ones that have the most potent antisense effects. Factors that govern a target site for the antisense oligonucleotide sequence are related to the length of the oligonucleotide, binding affinity, and accessibility of the target sequence. Sequences may be screened in vitro for potency of their antisense activity by measuring inhibition of GP88 protein translation and GP88 related phenotype, e.g., inhibition of cell proliferation in cells in culture. In general it is known that most regions of the RNA (5' and 3' untranslated regions, AUG initiation, coding, splice junctions and introns) can be targeted using antisense oligonucleotides.

The preferred GP88 antisense oligonucleotides are those oligonucleotides which are stable, have a high resilience to nucleases (enzymes that could potentially degrade oligonucleotides), possess suitable pharmacokinetics to allow them to traffic to disease tissue at non-toxic doses, and have the ability to cross through membranes.

Phosphorothioate antisense oligonucleotides may be used. Modifications of the phosphodiester linkage as well as of the heterocycle or the sugar may provide an increase in efficiency. With respect to modification of the phosphodiester linkage, phosphorothioate may be used. An N3'-P5' phosphoramidate linkage has been described as stabilizing oligonucleotides to nucleases and increasing the binding to RNA. Peptide nucleic acid (PNA) linkage is a complete replacement of the ribose and phosphodiester backbone and is stable to nucleases, increases the binding affinity to RNA, and does not allow cleavage by RNase H. Its basic structure is also amenable to modifications that may allow its optimization as an antisense component. With respect to modifications of the heterocycle, certain heterocycle modifications have proven to augment antisense effects without interfering with RNase H activity. An example of such modification is C-5 thiazole modification. Finally, modification of the sugar may also be considered. 2'-O-propyl and 2'-methoxyethoxy ribose modifications stabilize oligonucleotides to nucleases in cell culture and in vivo. Cell culture and in vivo tumor experiments using these types of oligonucleotides targeted to c-raf-1 resulted in enhanced potency.

The delivery route will be the one that provides the best antisense effect as measured according to the criteria described above. In vitro cell culture assays and in vivo tumor growth assays using antisense oligonucleotides have shown that delivery mediated by cationic liposomes, by retroviral vectors and direct delivery are efficient. Another possible delivery mode is targeting using antibody to cell

surface markers for the tumor cells. Antibody to GP88 or to its receptor may serve this purpose.

Recombinant GP88

The present invention is also directed to DNA expression systems for expressing a recombinant GP88 polypeptide or a functional derivative thereof substantially free of other mammalian DNA sequences. Such DNA may be double or single stranded. The DNA sequence should preferably have about 20 or more nucleotides to allow hybridization to another polynucleotide. In order to achieve higher specificity of hybridization, characterized by the absence of hybridization to sequences other than those encoding the GP88 protein or a homologue or functional derivative thereof, a length of at least 50 nucleotides is preferred.

The present invention is also directed to the above DNA molecules, expressible vehicles or vectors as well as hosts transfected or transformed with the vehicles and capable of expressing the polypeptide. Such hosts may be prokaryotic, preferably bacteria, or eukaryotic, preferably yeast or mammalian cells. A preferred vector system includes baculovirus expressed in insect cells. The DNA can be incorporated into host organisms by transformation, transduction, transfection, infection or related processes known in the art. In addition to DNA and mRNA sequences encoding the GP88 polypeptide, the invention also provides methods for expression of the nucleic acid sequence. Further, the genetic sequences and oligonucleotides allow identification and cloning of additional polypeptides having sequence homology to the polypeptide GP88 described here.

An expression vector is a vector which (due to the presence of appropriate transcriptional and/or translational control sequences) is capable of expressing a DNA (or cDNA) molecule which has been cloned into the vector and thereby produces a polypeptide or protein. Expression of the cloned sequence occurs when the expression vector is introduced into an appropriate host cell. If a prokaryotic expression vector is employed, then the appropriate host cell would be any prokaryotic cell capable of expressing the cloned sequence. Similarly, if an eukaryotic expression system is employed, then the appropriate host cell would be any eukaryotic cell capable of expressing the cloned sequence. Baculovirus vector, for example, can be used to clone GP88 cDNA and subsequently express the cDNA in insect cells.

A DNA sequence encoding GP88 polypeptide or its functional derivatives may be recombined with vector DNA in accordance with conventional techniques including blunt-ended or staggered ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with proper enzyme ligases. Techniques for such manipulations are discussed in (35).

A nucleic acid molecule is capable of expressing a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are operably linked to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism but shall in general include a promoter region, which in prokaryotes contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which when transcribed into

RNA will signal the initiation of protein synthesis. Such regions will normally include those 5' non-coding sequences involved with the initiation of transcription, translation such as the TATA box, capping sequence, CAAT sequence and the like.

If desired, the 3' non-coding region to the gene sequence encoding the protein may be obtained by described methods (screening appropriate cDNA library or PCR amplification). This region may be retained for the presence of transcriptional termination regulatory sequences such as termination and polyadenylation. Thus, by retaining the 3' region naturally contiguous to the DNA sequence coding for the protein, the transcriptional termination signals may be provided. Where the transcription termination signals are not provided or satisfactorily functional in the expression host cells, then a 3' region from another gene may be substituted.

Two DNA sequences such as a promoter region sequence and GP88 encoding sequence are said to be operably linked if the nature of the linkage between the sequences does not result in the introduction of a frame-shift mutation or interfere with the ability of the promoter sequence to direct transcription of the polypeptide gene sequence.

The promoter sequences may be prokaryotic, eukaryotic or viral. Suitable promoters are inducible, repressible or constitutive. Examples of suitable prokaryotic promoters are reviewed by.

Eukaryotic promoters include but are not limited to the promoter for the mouse methallothionein I gene, the TK promoter of Herpes Virus, the gene gal4 promoter, the SV40 early promoter, the mouse mammary tumor virus (MMTV) promoter, and the cytomegalovirus (CMV) promoter. Strong promoters are preferred. Examples of such promoters are those which recognize the T3, SP6 and T7 polymerases, the PL promoter of bacteriophage lambda, the recA promoter, the promoter of the mouse methallothionein I gene, the SV40 promoter and the CMV promoter.

Diagnostic Methods

The invention also provides GP88 diagnostic methods for determining tumorigenicity and also diagnostic methods for determining whether a patient is resistant to the antineoplastic effects of antiestrogen therapy. As described above, elevated levels of GP88 are indicative of increased tumorigenicity. In addition, the inventors have unexpectedly discovered that increased levels of GP88 are indicative of resistance to the antineoplastic effects of antiestrogens.

Antiestrogen therapy interferes or inhibits the function or synthesis of estrogen. For example, tamoxifen interferes with the binding of estrogen to its receptor. Aromatase inhibitors, such as anastrozole, interfere with enzymes that catalyze the conversion of androgens to estrogens thereby decreasing the levels of estrogen.

While not wishing to be bound by theory, we believe that elevated levels of GP88 contribute to the conversion of a cell from estrogen dependent to estrogen independent growth. Estrogen independent cells are insensitive to the presence or absence of estrogen and therefore are unaffected by antiestrogen therapy. Previously, the presence of the estrogen receptor was used as a much needed prognostic marker for resistance to antiestrogen therapy. However, as GP88 levels increase and the cell achieves estrogen independent growth, presence or absence of the estrogen receptor will no longer be sufficient to indicate whether the patient will be responsive to antiestrogen therapy. The level of GP88, and concomitantly and theoretically, estrogen independence, rather than the presence of the estrogen receptor, serves as an

enhanced and more accurate, earlier and reliable prognostic marker for whether a patient will be resistant or responsive to antiestrogen therapy. As explained above, this new prognostic marker is particularly valuable given the well known propensity for antiestrogens such as tamoxifen to cause ovarian cancer in patients, particularly those that are, until the current invention, unknowingly resistant to the antineoplastic effects of antiestrogen therapy.

Preferred embodiments of the invention are directed to methods of determining whether a patient is resistant to the antineoplastic effects of antiestrogen therapy. GP88 protein or polynucleotide encoding GP88 is detected in biological sample(s) obtained from a patient, and the number of GP88 positive cells in the sample and the ratio of GP88 positive cells compared to the total number of cells are determined. The ratio of GP88 positive cells to the total number of cells is indicative of whether the patient is resistant to the antineoplastic effects of antiestrogen therapy. Alternatively, the amount of GP88 in a biological sample can be determined by detecting GP88 in a biological sample and extrapolating the amount of GP88 from a standard curve generated from detecting known amounts of GP88 protein or nucleic acid ("standard curve technique"). The standard curve technique is a preferred method for measuring the amount of GP88 in a biological sample not readily amenable to detecting GP88 in a discrete number of cells. For example, the amount of GP88 in a biological fluid (e.g., serum or cerebrospinal fluid) can be determined using the standard curve technique. The amount of GP88 in a biological sample is indicative of whether the patient is resistant to the antineoplastic effects of antiestrogen therapy.

Preferred biological samples include, but are not limited to, blood, cerebrospinal fluid, serum, plasma, urine, nipple aspirate, liver, kidney, breast, bone, bone marrow smears, testes, brain, ovary, skin, or lung. The biological sample may be of any size, shape or tissue type and include any number of cells and/or cell types. The biological sample can comprise non-tumorigenic cells and tumorigenic cells. Non-tumorigenic cells can include, without limitation, stromal or epithelial cells from normal tissue, peripheral tissue, benign lobular carcinoma, epithelial hyperplasia and any other benign lesion. The biological sample may comprise biological fluid containing cells or tissue.

As described above, GP88 protein can be detected by any suitable method, including immunostaining (e.g., immunohistochemical staining), western blot, chromatography, microarray, cell sorter using an anti-GP88 antibody. Preferably, the anti GP88 antibody is labeled (e.g., fluorescent dye, biotin, enzymatic, radioisotopic, fluorescent, and chemical labels) for use in a suitable detection system. Detection of GP88 protein or functional derivatives thereof may also be accomplished by a variety of diagnostic imaging techniques. The term "diagnostic imaging" refers to any technique for diagnosing a disease employing a labelled molecule for detection of a marker protein or nucleic acid in an imaging system (e.g., magnetic resonance imaging, ultrasound etc.). Antibody imaging using radiolabelled monoclonal antibodies is available for diagnosis of a variety of cancers (e.g., colon, prostate, lung, and ovarian cancer). Examples of diagnostic imaging systems include monoclonal antibody imaging systems such as the OncoScint® monoclonal antibody imaging system which is currently used to scan or image the body for diagnosis of cancer. Monoclonal antibodies may be employed in conventional scanning technologies such as MRI or ultrasound to specifically detect GP88 and determine the amount of GP88 or the number of GP88 positive cells in a biological sample.

GP88 nucleic acid can be detected with an antisense GP88 nucleic acid by any suitable detection assay (e.g., in situ hybridization, fluorescent in situ hybridization, Reverse Transcriptase-Polymerase Chain Reaction, Northern Blot, Southern blot, Southwestern blot, RNase protection assay, microarray) with anti-GP88 nucleic acid (e.g., GP88 cDNA probe). Preferably, the anti-GP88 nucleic acid is labeled using, for example, an enzymatic, radioisotopic, fluorescent, or chemical label.

GP88 positive cells can be counted by any suitable method known to those of skill in the art (e.g., microscopic examination, MRI, ultrasound, FACS analysis, luminex detection, antibody microarray, digital scanner, and cell sorter systems). For example, the number of GP88 positive cells in a biological sample can be determined by constructing a GP88 index in a manner similar to the Ki-67 index. Ki-67 is a DNA polymerase marker indicative of the proliferation rate of tissues. The number of Ki-67 positive cells is determined, and expressed as the percentage of Ki-67 positively staining cells per 1000 cells counted. Preferably, cells staining positive for GP88 are counted and expressed as the percentage of GP88 positively staining cells per 1000 cells counted. However, any number of cells can be counted. For example, counting more than 1000 cells may be preferred in order to obtain a more representative sample of cells from a patient. Counting fewer than 1000 cells may be preferred if the sample appears to be representative of various cell types and limited amount of sample is available for analysis.

For methods of determining whether a patient is resistant to the antineoplastic effects of antiestrogen therapy, the presence and intensity of GP88 staining in a biological sample is preferably graded as follows: less than about 5% GP88 positive cells is considered negative; about 10–25% GP88 positive cells is considered weakly positive (1+); about 25–50% GP88 positive cells (2+) is considered moderately positive; and more than about 50% GP88 positive cells is considered strongly positive (3+) for GP88. The percentage of GP88 positive cells for each grade may be readjusted depending on the size of the biological sample pool and/or the detection technique used to identify GP88 (e.g., immunostaining, in situ hybridization, fluorescent in situ hybridization (FISH), reverse polymerase chain reaction, northern blot, western blot, southwestern blot, etc.). For example, the percentage of GP88 positive or stained cells for each grade may be adjusted downward when using a more sensitive technique for detecting GP88 or a larger biological sample pool. In accordance with a preferred embodiment of the invention, a percentage of GP88 positive or stained cells of at least about 5%, more preferably 10% and most preferably 25% is indicative of resistance to the antineoplastic effects of antiestrogen therapy. In another preferred embodiment, a percentage of at least about 10% GP88 positive or stained cells in estrogen receptor positive patients is indicative of resistance to the antineoplastic effects of antiestrogen therapy.

The invention also provides methods for diagnosing tumorigenicity comprising obtaining a biological sample from a patient, detecting GP88 protein or polynucleotide encoding GP88 in the biological sample, determining the number of GP88 positive or stained cells in the sample and the ratio of GP88 positive or stained cells compared to the total number of cells in a given sample. The ratio of GP88 positive or stained cells to the total number of cells is indicative of tumorigenicity. Detection of GP88 in biological samples is preferably carried out as described above with reference to methods of determining whether a patient is resistant to the antineoplastic effects of antiestrogen therapy.

In a preferred embodiment of the invention, a grading scale of GP88 staining intensity is constructed based on the percentage of GP88 positive cells according to standard intensity scale techniques known in the art. For methods of diagnosing tumorigenicity, the intensity of GP88 staining in a biological sample is preferably graded as follows: less than about 1% GP88 stained cells is considered negative or 0; about 1–5% GP88 stained cells is considered weakly positive for GP88 (1+); about 5–25% GP88 stained cells (2+) is considered moderately positive; and more than about 25% GP88 stained cells (3+) is considered strongly positive for GP88. The percentage of GP88 stained cells for each grade is readjusted depending on the size of the biological sample pool and the detection technique used to identify GP88 (e.g., immunostaining, in situ hybridization, fluorescent in situ hybridization (FISH), reverse polymerase chain reaction, northern blot, western blot, southwestern blot, etc.). For example, the percentage of stained cells for each grade may be adjusted downward when using a more sensitive technique for detecting GP88 or when the biological sample pool is increased. In accordance with a preferred embodiment of the invention, a percentage of GP88 stained cells of at least about 1% is indicative of tumorigenicity.

The present invention is also directed to kits for diagnosing tumorigenicity or to determine whether a patient is resistant to the antineoplastic effects of antiestrogen therapy. The preferred kits of the invention comprise a container and a molecule for detecting GP88 (e.g., anti-GP88 antibody, GP88 cDNA probe, GP88 oligomer probe). Kits may also include reagents for immunostaining or in situ hybridization of biological samples. In a preferred embodiment, the kits of the invention include reagents for carrying out an enzyme-linked immunoassay (“ELISA”) (e.g., anti-GP88 antibodies, conjugated and unconjugated recombinant GP88 for generated a standard curve, and a substrate for detection). Another preferred embodiment includes reagents for carrying out the reverse polymerase chain reaction method of detecting the presence of GP88 polynucleotides.

The invention also provides methods of treating or preventing cancer, such as breast cancer, comprising determining the amount of GP88 or the percentage of GP88 positive cells in a biological sample obtained from a patient, and administering tamoxifen in an amount sufficient to treat or prevent the breast cancer if the percentage of GP88 positive or stained cells in said breast tissue sample is less than about 5%. In another preferred embodiment of the invention, tamoxifen, or other antiestrogen therapy, is administered to an estrogen receptor positive patient if the percentage of GP88 positive or stained cells in the biological sample is less than about 10%. If the percentage of GP88 positive or stained cells is greater than 5%, anti-GP88 therapy (i.e., use of anti-GP88 antibodies or antisense GP88 polynucleotides) can be administered to restore sensitivity to antiestrogen therapy. The amount of antiestrogen sufficient to treat or prevent cancer is determined on a case-by-case basis. Guidance regarding administering antiestrogen therapy (e.g., Nolvadex®, raloxifene, aromatase inhibitors, estrogen receptor downregulators etc.) to a patient is readily available and known to the treating physician.

It is to be understood that application of the teachings of the present invention to a specific problem or environment will be within the capability of one having ordinary skill in the art in light of the teachings contained herein. The present invention is more fully illustrated by the following non-limiting examples.

EXAMPLE 1

Mediation of Estrogen Mitogenic Effect in Human Breast Cancer MCF-7 Cells by PC-Cell Derived Growth Factor (GP88)

In estrogen-receptor positive cells, 17- β -estradiol (E2), an estrogen replacement compound, transcriptionally stimu-

lated PCDGF expression in a dose and time-dependent fashion. We demonstrate here that PCDGF mediates the mitogenic effect of estradiol (E2) in MCF-7 cells. PCDGF substituted for E2 to stimulate DNA synthesis. The E2 mitogenic effect was inhibited in a dose-dependent fashion by anti-PCDGF neutralizing antibody. Inhibition of PCDGF expression in MCF-7 cells by antisense transfection also inhibited the E2 mitogenic effect. In contrast, overexpression of PCDGF in MCF-7 cells resulted in cells that were able to proliferate in the absence of estrogen and were tamoxifen resistant. Like E2, PCDGF stimulated mitogen activated protein kinase activity. PCDGF could substitute for E2 in stimulating cyclin D1 expression. The cyclin D1 stimulation by E2 was 50% inhibited by anti-PCDGF antibody. In contrast, PCDGF did not stimulate c-myc expression, another molecular target of E2. We conclude that autocrine PCDGF mediates the E2 mitogenic effect via stimulation of cyclin D1.

Screening of human tumor cell lines for PCDGF expression indicated that it was highly expressed in estrogen receptor negative (ER⁻) human breast carcinomas. Inhibition of PCDGF expression in these cells by antisense PCDGF cDNA transfection resulted in a 90% inhibition of tumor incidence and tumor size when injected into nude mice. These data implicated PCDGF in the maintenance of the tumor phenotype. As indicated above, in ER⁺ cells, PCDGF produced in response to estrogen receptor stimulation mediates E2 mitogenic activity. When PCDGF becomes overexpressed, then the cells become estrogen independent and resistant to the antineoplastic effects of antiestrogens.

Results

PCDGF Stimulates DNA Synthesis of MCF-7 Cells in the Absence of Estrogen

We first examined whether addition of PCDGF stimulated DNA synthesis of MCF-7 cells maintained in the absence of E2. Under these conditions, endogenous production of PCDGF was very low. A 2-fold increase in ³H-thymidine incorporation over the control (cells maintained in the absence of E2 and PCDGF) ($P < 0.01$) was observed at 10 ng/ml of PCDGF (FIG. 16A). FIG. 16A illustrates that PCDGF stimulates DNA synthesis of MCF-7 cells in the absence of E2. MCF-7 cells (10^5 cells per well) were plated in a 1:1 mixture of DMEM and Ham's F-12 medium plus 5% FBS. After 48 h, the medium was replaced with PFMEM, followed 24 hr later by serum-free α -MEM medium. Increasing concentrations of human PCDGF were added in triplicate to the medium. EtOH only (CON) or 10^{-9} M E2 (E2) were used as negative and positive controls, respectively. ³H-thymidine (1 μ Ci/ml) was added 24 h later for 5 h. The results are expressed as means \pm SD of triplicate determinations. A 4-fold maximal stimulation was observed with 100 ng/ml PCDGF ($P < 0.001$), similar to the one observed with 10^{-9} M E2. PCDGF stimulated DNA synthesis of another ER⁺ cell line, T47D. A 2.9 ± 0.3 -fold stimulation of DNA synthesis was observed with 100 ng/ml PCDGF.

E2 mitogenic effect is inhibited by treatment of the cells with anti-PCDGF antibody. Based on these results and given that E2 stimulates PCDGF expression in MCF-7 cells, we determined whether endogenous PCDGF could mediate the E2 mitogenic effect via an autocrine loop. For this purpose, we first examined whether treatment with anti-PCDGF antibody that blocks PCDGF produced by the MCF-7 cells would inhibit the E2 mitogenic activity. The addition of affinity-purified anti-human PCDGF antibody inhibited in a

dose-dependent fashion the growth of the cells stimulated by E2 (FIG. 16B). FIG. 16B illustrates that anti-PCDGF antibody specifically inhibits the E2 mitogenic effect. MCF-7 cells were treated with 10^{-9} M E2 (E2) alone or with increasing concentrations of affinity-purified anti-PCDGF antibody: 50 μ g/ml (Ab1), 100 μ g/ml (Ab2), 200 μ g/ml (Ab3) and 300 μ g/ml (Ab4). Cells treated with 10^{-9} M E2 and 300 μ g/ml pre-immune IgG (preIgG) and cells treated with 10 ng/ml of IGF-II (IGF) alone or in the presence of 300 μ g/ml anti-PCDGF antibody (Ab+IGF) were used as controls. The results are expressed in the figure as means \pm SD of triplicate determinations. A 74% inhibition of E2 mitogenic effect was observed with 300 μ g/ml of anti-PCDGF antibody ($P < 0.003$), whereas a similar concentration of non-immune IgG had no effect. This antibody concentration displayed no cellular toxicity. Addition of PCDGF (200 ng/ml) restored the proliferation of the cells treated with anti-PCDGF antibody. The specificity of the PCDGF antibody effect was also demonstrated because it could not inhibit the stimulatory effect of insulin-like growth factor-II, a known growth stimulator of MCF-7 cells. These results show that PCDGF acts as an autocrine growth factor to mediate the mitogenic effect of E2 for MCF-7 cells.

Inhibition of PCDGF expression inhibits the growth of MCF-7 cells in the presence of E2. We next attempted to determine whether inhibiting PCDGF expression in MCF-7 cells would prevent the growth stimulatory effect of E2. For this purpose, we examined the E2 mitogenic effect in MCF-7 cells where PCDGF expression had been inhibited by antisense PCDGF cDNA transfection. The PCDGF levels in two representative antisense clones AS13 and AS22, and empty vector control transfected MCF-7 cells (MCF-7C) were determined by Western blot analysis of samples prepared by using identical cell numbers (FIG. 17A). FIG. 17A illustrates inhibition of PCDGF expression by antisense PCDGF cDNA transfection in MCF-7 cells. PCDGF expression in antisense (AS13 and AS22) and empty vector control transfected cells (MCF-7C) was examined by Western blot analysis. AS13 (lane 1), AS22 (lane 2) and MCF-7C cells (lane 3) were treated with 10^{-9} M E2 in PFMEM. The conditioned media were collected after 24h and normalized to the same cell number (4×10^6 cells) for the measurement of PCDGF expression. AS13 cells displayed an 80% inhibition of PCDGF expression, whereas AS22 showed a 50% inhibition when compared with MCF-7C cells.

The effect of E2 on DNA synthesis then was examined in these antisense and control MCF-7C cells. A [³H] thymidine incorporation assay showed that the E2 stimulatory effect in MCF-7C cells had been reduced in AS13 and AS22 in correlation with the degree of inhibition of PCDGF expression (FIG. 17B). FIG. 17B illustrates that inhibition of PCDGF expression in MCF-7 cells inhibits the E2 mitogenic activity. E2 mitogenic effect was examined in antisense and control MCF-7C cells. AS13 (filled bars), AS22 (hatched bars), and MCF-7C cells (open bars) were treated with 10^{-9} M E2 (E2), 100 ng/ml recombinant PCDGF (PCDGF) or 0.1% EtOH only (Con). ³H-thymidine incorporation was measured as described above. Results are expressed in the figure as means \pm SD. AS 13 showed the highest inhibition of the E2 effect. DNA synthesis mediated by E2 was only 25% ($P < 0.001$) in AS13 and 60% ($P < 0.001$) in AS22, respectively, of that observed in MCF-7C cells. The addition of PCDGF restored the proliferation of the antisense clones to the level found in the control MCF-7 cells cultivated in the presence of E2.

Overexpression of PCDGF Leads to Estrogen-Independence and Tamoxifen Resistance

Based on the above results, we examined the effect of the constitutive overexpression of PCDGF in ER⁺MCF-7 cells

on their estrogen and tamoxifen responsiveness. MCF-7 cells were transfected with a pcDNA3 expression vector containing PCDGF cDNA under the control of the cytomegalovirus promoter. Several PCDGF overexpressing clones were obtained including a representative clone termed O4. FIG. 18A shows that O4 cells produced elevated levels of PCDGF when compared with control MCF-7C cells in E2-depleted medium. FIG. 18A illustrates PCDGF expression in O4 cells and control MCF-7C cells. O4 and empty vector control MCF-7 cells were cultivated in PFMEM. The conditioned media were collected after 24 h to determine PCDGF expression.

Thymidine incorporation in O4 cells was 2.2-fold higher than in MCF-7C cells ($P < 0.01$) demonstrating the ability of O4 cells to proliferate in the absence of E2 (FIG. 18B). FIG. 18B shows proliferation of O4 and control MCF-7 cells in estrogen-depleted PFMEM medium. Cells were plated in triplicate in estrogen-depleted PFMEM medium. Cells were either maintained in the absence of E2 (controls: Con) or were treated for 24 hours with either 10^{-9} M E2 or with 200 ng/ml PCDGF. ^3H -thymidine was then added to measure DNA synthesis. The results are expressed as means \pm SD. Moreover, there was no significant difference between the thymidine incorporation level for O4 cells in E2-depleted medium and for MCF-7C cells treated with either PCDGF or E2 ($P > 0.05$). The addition of E2 or PCDGF to O4 cells did not have any significant additional effect in contrast to MCF-7C cells. In long-term growth assays, whereas MCF-7C cells could not grow in the absence of E2, O4 cells proliferated with a doubling time of 42 h, close to the 36 h doubling time of MCF-7C cells in the presence of E2. These data indicate that PCDGF overexpression provided a growth advantage in the absence of E2.

Based on these results, the tamoxifen-responsiveness of O4 and MCF-7C cells was examined. FIG. 18C shows a comparison of the response of MCF-7 and O4 cells to tamoxifen. MCF-7C cells (filled bars) and O4 cells (open bars) received 10^{-9} M E2 only or in the presence of increasing concentrations of tamoxifen. MCF-7C cells treated with 200 ng/ml of human PCDGF and 1 μM tamoxifen (T+P) were also examined. DNA synthesis was measured as described in FIG. 16. The results were expressed as the percentage of the DNA synthesis values in cells treated with E2 only. Tamoxifen inhibited the proliferation of MCF-7C cells in a dose-dependent fashion with a 60% inhibition of E2 effect at 100 nM, an 80% inhibition at 500 nM and a maximal 90% inhibition at 1 μM tamoxifen (FIG. 18C). In contrast, O4 cells displayed a markedly decreased responsiveness to tamoxifen, inasmuch as their proliferation was not affected by 100 nM tamoxifen, whereas only a 20% and a 55% inhibition of E2 effect was observed at 500 nM and 1 μM tamoxifen, respectively ($P < 0.001$). Interestingly, the addition of PCDGF to MCF-7 cells reduced the tamoxifen inhibition from 90% to 38% ($P < 0.0001$). These data show that PCDGF can overcome the tamoxifen inhibition and that increased PCDGF expression in MCF-7 cells leads to tamoxifen resistance.

The status of other parameters associated with estrogen responsiveness, such as estrogen receptor expression, activation of an estrogen response element by E2, and stimulation of progesterone receptor mRNA expression by E2, was assessed in all transfected cells. No difference was observed in these assays between antisense, control, and PCDGF overexpressing cells (data not shown). These data indicate that the changes in E2 proliferative response observed in the antisense or overexpressing cells were not due to an overall alteration in ER number or function but rather correlated with changes of PCDGF expression.

PCDGF Stimulates DNA Synthesis in MCF-7 Cells Via Activation of Mitogen Activating Protein Kinase ("MAP Kinase") Pathway

We next examined the signal transduction pathways stimulated by PCDGF in MCF-7 cells, in comparison with E2. It has been shown that E2 stimulates progression through the G1 phase of the cell cycle. Some of the underlying molecular targets have now been elucidated. It is known that E2 stimulates MAP kinase activity in MCF-7 cells. Downstream, E2 activates the c-myc pathway and D-type cyclin/cdk complexes. Recent studies have postulated that the c-myc and cyclin D1 pathways were independently activated by E2 in breast cancer cells. Thus, we attempted to determine whether PCDGF could replace E2 to stimulate MAP kinase, cyclin D1 and/or c-myc pathways in MCF-7 cells.

Two approaches showed the involvement of MAP kinase in mediating PCDGF action in MCF-7 cells. First, the stimulatory effect of PCDGF on DNA synthesis was inhibited in a dose-dependent fashion by the MEK inhibitor PD098059 (FIG. 19A). FIG. 19A shows the effect of MAP kinase inhibitor on the mitogenic effect of PCDGF. MCF-7 cells were cultivated as described in FIG. 16. Cells were pretreated for 60 min with 10 μM (PD10) or 30 μM (PD30) of PD098059 prior to addition of vehicle only (Con) or 100 ng/ml of recombinant human PCDGF alone (P). Thymidine incorporation data are presented as means \pm SD. The PCDGF effect was almost completely abolished by 10 μM PD098059. Complete inhibition was observed with 30 μM PD098059, a concentration known to completely inhibit MAP kinase activity in other systems. Second, by using an in vitro MAP kinase assay, we showed that PCDGF (200 ng/ml) caused a three-fold increase in MAP kinase activity in MCF-7 cells (FIG. 19B). This effect was abolished by PD098059 (30 μM). FIG. 19B illustrates activation of MAP kinase by PCDGF. Cells were cultivated in PFMEM medium for 24 h followed by serum-free α -MEM medium. The samples examined were: MCF-7 cells treated for 10 minutes with 0, 50 ng/ml and 200 ng/ml of human PCDGF, (lanes 1-3, respectively); and MCF-7 cells treated for 10 minutes with 200 ng/ml of PCDGF and 30 μM PD098059 (lane 4). The kinase assay was conducted as described herein. The intensity of the phosphorylated MBP bands was analyzed by densitometric scanning. An equal amount of original supernatant was used to check the expression of MAP kinase (Erk1 and Erk2) as an internal control to normalize MAP kinase activity.

PCDGF Stimulates Cyclin D1 but not c-myc Expression in MCF-7 Cells

Experiments were performed to determine the effect of PCDGF on cyclin D1 expression. PCDGF in a time-dependent fashion stimulated cyclin D1 expression in MCF-7 cells reaching a maximum of 4-fold over untreated controls after 5 h (FIG. 20A). The cyclin D1 stimulatory effect was abolished by PD98095 in an identical manner to its inhibition of the PCDGF mitogenic effect. FIG. 20A shows stimulation of cyclin D1 expression by PCDGF. MCF-7 cells were cultivated in triplicates in PFMEM medium and synchronized by treatment with 1 μM tamoxifen. Medium was replaced by fresh medium supplemented with 200 ng/ml PCDGF alone or with 30 μM PD98095 (upper) or with 10^{-9} M E2 (lower). Cells were lysed at the indicated times to determine cyclin D1 expression by Western blot analysis with 60 μg of whole cell lysates, using anti-cyclin D1 antibody. Samples were MCF-7 cells treated

with 200 ng/ml PCDGF at 0 h (lane 1), 3 h (lane 2), 5 h (lane 3) and 12 h (lane 4); untreated MCF-7 cells at 5 hr (lane 5); MCF-7 cells treated with 200 ng/ml PCDGF and PD08950 at 5 hr (lane 6); and MCF-7 cells treated with 10^{-9} M E2 at time 0, 3 hr and 5 hr (lanes 7–9, respectively).

The stimulation of cyclin D1 expression by PCDGF was accompanied by the increase in the phosphorylation and expression of pRb, strictly required for G1 phase progression. After 6 hours of PCDGF treatment, pRb became hyperphosphorylated. After 24 hr, all of the pRb was in the hyper-phosphorylated form with a 5-fold increase in protein expression when compared to untreated cells. This effect was also blocked by PD98095 (data not shown).

We next compared the abilities of E2 and PCDGF to stimulate c-myc expression, another target for estrogen action in MCF-7 cells. As shown in FIG. 20B, E2 (10^{-9} M) stimulated a rapid increase in c-myc expression in steroid-deprived MCF-7 cells with a maximal 4-fold induction within 3 hours. The level of c-myc induction by E2 was sustained for more than 10 hours. In contrast, PCDGF had no effect on c-myc protein expression during the same period of time (FIG. 20B). FIG. 20B illustrates the effect of E2 and PCDGF on c-myc expression. MCF-7 cells were synchronized by treatment with 1 μ M tamoxifen. Cells were then treated with 10^{-9} M E2 (upper) or 200 ng/ml PCDGF (lower) for the indicated periods of time. Western blot detection of c-myc expression was performed by using 60 μ g of whole cell lysates and anti-myc polyclonal antibody.

Because PCDGF induced cyclin D1 but not c-myc expression, we determined whether the increase of endogenous PCDGF was involved in the ability of E2 to stimulate cyclin D1 expression. The treatment of MCF-7 cells with anti-PCDGF neutralizing antibody (300 μ g/ml) resulted in a $52 \pm 8\%$ inhibition of the stimulation of cyclin D1 by E2, 5 hrs after addition of the antibody. This degree of inhibition was sustained 12 hours after antibody addition. These data show that the ability of E2 to stimulate cyclin D1 in MCF-7 cells is mediated, at least partially, by the endogenously produced PCDGF.

Discussion

We have reported previously that ER human mammary carcinoma cells express PCDGF and that E2 stimulates PCDGF expression in a dose and time-dependent fashion. We demonstrate herein that PCDGF mediates the growth stimulatory effect of E2. We show that PCDGF stimulates the proliferation of the human breast cancer cells MCF-7 and T47D maintained in the absence of E2. We next show that blocking the PCDGF autocrine pathway with anti-PCDGF antibody inhibits the mitogenic effect of E2. This effect was specific to PCDGF because the antibody was unable to inhibit the IGF-II mitogenic effect. The involvement of PCDGF in the mitogenic effect of E2 was further demonstrated by showing that the inhibition of PCDGF expression by antisense cDNA transfection reduced the E2 mitogenic effect on MCF-7 cells in correlation with the degree of inhibition of PCDGF expression. The addition of exogenous PCDGF restored the proliferation of these antisense cells. In addition, increased expression of PCDGF in MCF-7 cells led to cells able to proliferate in the absence of E2 and that were tamoxifen resistant. The fact that there was no change in other parameters of E2 responsiveness such as ER expression, activation of estrogen-response element-luciferase reporter gene, or stimulation of progesterone receptor expression indicated that alteration of E2 mitogenic effect in transfected cells was not due to a change in E2 receptors but specifically to a change in PCDGF expression.

Our results show that PCDGF upregulates cyclin D1 to mediate the mitogenic effect of E2 in human breast cancer cells. In support of this hypothesis, E2 inhibited the stimulation of cyclin D1 expression was inhibited by 52% within 5–12 hours following treatment of MCF-7 cells with anti-PCDGF neutralizing antibody. In conclusion, the studies described herein identify PCDGF as a novel mediator, at least in part, of the mitogenic effect of E2 on the human breast cancer cell line MCF-7.

EXAMPLE 2

Determination of Tamoxifen Resistance

We have shown that inhibiting the expression of GP88 by antisense cDNA transfection in ER negative breast cancer cells resulted in inhibition of tumorigenicity indicating the importance of GP88 overexpression in the tumorigenic properties of breast cancer cells. Because breast cancer is heterogeneous in nature and can affect various cellular functional compartments of the gland, it is preferable to examine biopsies representative of the various stages of the progression model for expression of GP88. It has been shown that detection of tumor markers is the preferred approach to evaluate and predict the clinical course of breast cancer at the time of diagnosis or primary treatment and is important in determining the choice of therapy. These studies are directly relevant to women's health because they provide an analysis of the novel growth factor PCDGF as a potential prognosis marker of breast cancer.

Expression of GP88 was followed by immunohistochemical analysis of sections from paraffin embedded biopsies using an affinity purified anti-human GP88 antibody. (FIG. 21). The progressive stages of the disease examined include benign hyperplasia \rightarrow atypical ductal hyperplasia (ADH) \rightarrow (Ductal carcinoma in situ) DCIS \rightarrow Invasive Ductal Carcinoma (IDC) \rightarrow Metastatic Disease. Selected markers representative of genetic and biological alterations occurring during breast cancer progression were also examined in parallel.

For each stage of the disease examined (i.e.: benign stages including epithelial hyperplasia and fibrocystic changes, atypical ductal hyperplasia, ductal carcinoma in situ, invasive ductal carcinoma; and metastatic mammary carcinoma), GP88 expression was detected in parallel with detection of other markers such as Ki-67 (proliferation marker), p53 (tumor suppressor), ER/PR (responsiveness), cerbB2 expression (growth factor receptor), and correlated with grade, stage of disease and with lymph node status.

The biopsies were classified by histopathological determination of the stage and type of lesion and by examining several other prognostic markers such as Ki-67, and ER/PR status. Results indicated GP88 expression in the epithelial cells in ER-/PR ductal carcinoma grade 3 corresponding to tumors with poor prognosis. GP88 expression in the epithelial cells of normal tissue or benign lobular carcinoma were negative.

Methods

Preparation of tissues. 4 μ m thick tissue sections were cut from paraffin blocks and mounted on electrically charged glass slides. Deparaffinization, fixation, antigen retrieval and rehydration of sections was done by standard procedures.

Preparation of anti-human GP88 antibody: A preferred method of detecting GP88 expression is by immunostaining using affinity purified anti-human PCDGF antibody. Polyclonal or monoclonal anti-human GP88 antibody was devel-

oped by immunizing rabbits or mice with human recombinant PCDGF expressed and purified in the inventor's laboratory. For polyclonal antibodies, anti-GP88 IgG fraction was prepared from rabbit antiserum by sodium sulfate precipitation and dialysis. This fraction was affinity purified by chromatography on GP88 conjugated Sepharose column prepared by coupling pure human GP88 to CNBr activated Sepharose. This highly purified antibody fraction was analyzed by SDS-PAGE chromatography and shown to contain a single band corresponding to the IgG and shown by immunostaining to provide a highly specific staining with no background on fixed cells and on tissue sections. Alternatively, anti-GP88 monoclonal antibodies may be employed.

Immunostaining was done by incubation of tissue sections prepared as described above for 1 hour at room temperature in moisture chambers with the specific antibody followed by incubation with anti-GP88 antibody. Antibody binding is preferably detected by incubation with biotinylated secondary antibodies and streptavidin peroxidase complex (Ventana kit). Chromogenic development is preferably obtained by the immersion of sections in 3,3'-diaminobenzidine solution (0.25 mg/mL with 3% hydrogen peroxide). The slides are then counterstained, preferably with hematoxylin, and coverslipped.

Analysis of the immunostaining data was done by microscopic examination. Scoring of selected markers was done using published methods as indicated below. Prior art staining intensity scoring methods include the following: ER and PR status and the presence of p53 mutated form are determined by grading the nuclear staining by universally used method (<5% of nuclei staining-negative; positive staining is assigned an intensity score from weak (1+) to moderate (2+) to strong (3+). c-erbB-2 staining is assessed by the presence and intensity of the membrane staining and is graded as follows: <5% staining-negative; positive staining graded from weak (1+) to moderate (2+) to strong (3+). Stromelysin-3 expression is graded separately in the tumor cells and the surrounding stroma and the scoring is preferably performed using the method used for ER/PR, p53 and c-erbB-2. The staining intensity grading methodologies are directly applicable to detection of GP88.

Our results show that in normal tissue and in benign lesion, the epithelial cells do not stain for GP88. In contrast, in ER-/PR: invasive ductal carcinoma, epithelial cells display a very strong positive staining for GP88. The presence of epithelial cells positive for GP88 can be scored and a grade can be assigned following the methods routinely used for scoring and indexing other prognosis markers of breast cancer. The following scoring and indexing scale was used to assess GP88 staining: less than about 5% staining is negative; about 10-25% positive staining is graded from weak (1+) to (moderate 25-50% positive cells (2+)) to strong (3+) (more than 50% positive cells). The percentage of positive cells for each grade is readjusted downward whenever an analysis of a large number of samples is obtained.

In addition to the method and type of biological samples described above, the detection of GP88 expression for predicting antiestrogen therapy response can be extended to measuring the increase of GP88 expression in biological fluids such as serum or plasma, urine or nipple aspirate. The detection of GP88 expression for predicting antiestrogen therapy response can also be readily extended to measuring the increase of GP88 expression in cell extracts.

The detection methods include, without limitation, enzyme linked immunoassay, such as an antibody directly

conjugated to an enzyme (such as, without limitation, peroxidase, alkaline phosphatase, biotin) or to a fluorescent tag, or the use of a conjugated secondary antibody (such as, without limitation, peroxidase, alkaline phosphatase, streptavidin or a fluorescent tag).

Preferred methods for scoring of selected markers are well known in the art and described in the following references, the contents of which are hereby incorporated by reference into this application: (1) Ferno, M. (1998) Prognostic factors in breast cancer, *Anticancer Research*, 18, 2167-2172; (2) Harbeck N., Dettmar, P., Thomssen, C., Henzelmann, B., Kuhn, W., Ulm, K., Janicke, F., Hofler, H., Graeff, H., Schmitt, M. (1998), Prognostic impact of tumor biological factors on survival in node-negative breast cancer, *Anticancer Res.* 18, 2187-2198; (3) Allred, D. C., Harvey, J. M., Berardo, M., Clark, G. M. (1998), Prognostic and predictive factors in breast cancer by immunohistochemical analysis, *Mod. Pathol.* 11,155-168; and (4) Thor, A. D., Moore, D. H., Edgerton, S. M., Kawasaki, E. S., Reihnsaus, E., Lynch, H. T., Marcus, J. N., Scwartz, L., Chen, L. C., Mayall, B. H. (1992), Increased accumulation of the p53 suppressor gene product is an independent prognostic variable for breast cancer, *I. Natl. Cancer. Inst.*, 84, 845-855.

GP88 Staining with anti-GP88 Antibody

Paraffin embedded breast tissue biopsies from breast cancer patients were stained with anti-GP88 antibody using immunohistochemical techniques. As shown in FIG. 21, benign lesions exhibited no GP88 staining while invasive ductal carcinoma ("IDC") samples exhibited strong staining of GP88 positive cells. GP88 expression is elevated in tumorigenic ductal invasive carcinoma compared to benign lesions.

GP88 Staining Intensity In Estrogen Receptor Positive (ER+) and Estrogen Receptor Negative (ER-) Invasive Ductal Carcinoma (IDC) Tumors

Biological samples of IDC tumors were immunostained with anti-GP88 antibody and classified according their estrogen receptor status (ER+=estrogen receptor positive, ER-=estrogen receptor negative) and GP88 staining intensity grade. As shown in FIG. 22, an increase in GP88 staining intensity directly correlates with loss of estrogen receptor in IDC tumors. At a staining intensity of 0, all the IDC tumor samples analyzed were ER+. At a staining intensity of 1, 80% of the IDC tumors were ER+and 20% were ER-. At a staining intensity of 2, 50% of the IDC tumors were ER+and 50% were ER-. 20% of the IDC tumors were ER+at a staining intensity of 3 while 80% were ER-. Thus, as GP88 staining intensity increases, the percentage of ER+IDC tumors decreases.

Tamoxifen Responsiveness of ER+IDC Tumors Classified According To GP88 Staining Intensity

An increase in GP88 staining intensity indicates a cell has lost dependance on estrogen for growth and has become estrogen independent. The growth of the cell is no longer controlled by external signals from hormones such as estrogen, but rather is controlled by autocrine growth signals from GP88. Previously, it was thought that the presence of the estrogen receptor was indicative of whether the cell was capable of responding to antiestrogen therapy. However, when a cell has converted from estrogen dependant to estrogen independent growth, the presence of the estrogen receptor is irrelevant to whether antiestrogen therapy will be effective since the cellular growth signal is constitutive and

not controlled by the presence or absence of estrogens or antiestrogens. Thus, estrogen receptor positive tumors with high levels of GP88 (+2 or greater) are antiestrogen therapy resistant despite the fact the cells display the estrogen receptor.

In order to examine the effect of increased GP88 levels on antiestrogen resistance, IDC tumor samples were obtained from patients resistant to tamoxifen treatment and from patients responsive to tamoxifen treatment. The tumor samples were immunostained with anti-human GP88 antibody as described above. As shown in FIG. 23, IDC tumors were classified according to their GP88 staining intensity on a grading scale of 0 to 3 (x-axis). The percentage of total IDC tumors that were estrogen receptor positive (ER+) was determined and classified as either tamoxifen responsive (white bars) or tamoxifen resistant (black bars) along the y-axis. At a staining intensity grade of 0, all of the ER+IDC tumors (40% of the total number of tumors) were from tamoxifen responsive patients. At staining intensity grade of 1, all of the ER+IDC tumors (60% of the total number of tumors) were from tamoxifen responsive patients. However, as the staining intensity moved from grade 1 to grade 2, all of the ER+IDC tumors (80% of the total number of tumors) were from tamoxifen resistant patients. At a staining intensity grade of 3, all of the ER+ tumors (30% of the total number of tumors) were from tamoxifen resistant patients. Thus, even ER+IDC tumors become increasingly resistant to

the effects of tamoxifen increases as the GP88 intensity increases from grade 0 through grade 3. Furthermore, when the staining intensity increases from a grade 1 to 2, the ER+IDC tumors completely shifted from tamoxifen responsive to tamoxifen resistant. While not wishing to be bound by theory, it appears that the subsequent decrease in the percentage of tamoxifen resistant tumors from stage 3 to stage 4 is attributable to the loss of estrogen receptor (i.e., the percentage of ER+IDC tumors) as the level of GP88 increases. Therefore, FIG. 23 establishes that increasing levels of GP88 shift cells from an antiestrogen therapy responsive (grades 0 and 1) to antiestrogen therapy resistant (grades 2 and 3) stage even prior to the loss of the estrogen receptor. Thus, compared to the presence of the estrogen receptor, GP88 serves as a more accurate and reliable prognostic indicator of whether a patient will respond to, or be unduly harmed by, antiestrogen therapy.

The above description and accompanying drawings are only illustrative of exemplary embodiments, which can achieve the features and advantages of the present invention. It is not intended that the invention be limited to the embodiments shown and described in detail herein. The invention can be modified to incorporate any number of variations, alterations, substitutions or equivalent arrangements not heretofore described, but which are commensurate with the spirit and scope of the invention. The invention is only limited by the scope of the following claims.

SEQUENCE LISTING

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 <221> NAME/KEY: CDS
 <222> LOCATION: (23)..(1789)
 <223> OTHER INFORMATION: The sequence is identical to that of the published mouse granulin except for one nucleotide (T instead of G) at position 1071 of GP88 cDNA (position 1056 of mouse granulin).

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Ala Ala Gly Leu Val Ala Gly Thr Gln Cys Pro Asp Gly Gln Phe Cys
                    15                20                25

cct gtt gcc tgc tgc ctt gac cag gga gga gcc aac tac agc tgc tgt      148
Pro Val Ala Cys Cys Leu Asp Gln Gly Gly Ala Asn Tyr Ser Cys Cys
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aac cct ctt ctg gac aca tgg cct aga ata acg agc cat cat cta gat      196
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ctc act gtg tct ggg act tcc agc tgc tgc ccg ttc tct aag ggt gtg      292
Leu Thr Val Ser Gly Thr Ser Ser Cys Cys Pro Phe Ser Lys Gly Val
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Ala	Asp	Gly	Lys	Ser	Cys	Phe	Gln	Met	Ser	Asp	Asn	Pro	Leu	Gly	Ala	
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Asp	Met	Glu	Val	Ser	Cys	Pro	Glu	Gly	Tyr	Thr	Cys	Cys	Arg	Leu	Asn	
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Pro	Cys	Asp	Asp	Phe	Thr	Arg	Cys	Pro	Thr	Asn	Asn	Thr	Cys	Cys	Lys	
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<223> OTHER INFORMATION: Antisense oligonucleotide to human GP88

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<400> SEQUENCE: 15
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<222> LOCATION: (13)..(1791)
<223> OTHER INFORMATION: Nucleotide sequence of human granulin/
epithelin precursor (human GP88). Human Granulin Genebank
M75161.

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ctg gtg gct gga acg cgg tgc cca gat ggt cag ttc tgc cct gtg gcc 99

-continued

acc tgt gaa cag ggg ccc cac cag gtg ccc tgg atg gag aag gcc cca Thr Cys Glu Gln Gly Pro His Gln Val Pro Trp Met Glu Lys Ala Pro 335 340 345	1059
gct cac ctc agc ctg cca gac cca caa gcc ttg aag aga gat gtc ccc Ala His Leu Ser Leu Pro Asp Pro Gln Ala Leu Lys Arg Asp Val Pro 350 355 360 365	1107
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cgc cag cac tgc tgc ccg gct ggc tac acc tgc aac gtg aag gct cga Arg Gln His Cys Cys Pro Ala Gly Tyr Thr Cys Asn Val Lys Ala Arg 480 485 490	1491
tcc tgc gag aag gaa gtg gtc tct gcc cag cct gcc acc ttc ctg gcc Ser Cys Glu Lys Glu Val Val Ser Ala Gln Pro Ala Thr Phe Leu Ala 495 500 505	1539
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 Arg Gly Ser Leu Ser His Pro Arg Asp Ile Gly Cys Asp Gln His Thr
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 Ser Cys Pro Val Gly Gly Thr Cys Cys Pro Ser Gln Gly Gly Ser Trp
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 Ala Cys Cys Gln Leu Pro His Ala Val Cys Cys Glu Asp Arg Gln His
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 Lys Glu Val Val Ser Ala Gln Pro Ala Thr Phe Leu Ala Arg Ser Pro
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 Ala Gly Phe Arg Cys Ala Arg Arg Gly Thr Lys Cys Leu Arg Arg Glu
 565 570 575
 Ala Pro Arg Trp Asp Ala Pro Leu Arg Asp Pro Ala Leu Arg Gln Leu
 580 585 590

Leu

What is claimed is:

1. A method for diagnosing tumorigenicity in a human patient, comprising:
 - obtaining a breast tissue sample containing cells from said patient;
 - detecting the protein encoded by SEQ ID NO. 16 in said cells of said breast tissue sample;
 - determining the number of cells containing said protein in said sample; and
 - determining the ratio of cells containing said protein to the total number of cells in said sample, wherein a ratio greater than about 5% is indicative of numorigenicity.
2. The method of claim 1, wherein said breast tissue sample comprises a material selected from the group consisting of blood, serum, and plasma.
3. The method of claim 1, wherein said patient has been diagnosed with cancer.
4. The method of claim 3, wherein said cancer is breast cancer.
5. The method of claim 1, wherein said protein is detected by immunostaining with an anti-human GP88 antibody.
6. The method of claim 5, wherein said antibody is labeled.
7. The method of claim 6, wherein said label is selected from the group consisting of biotin, enzymatic, radioisotopic, fluorescent, and chemical labels.
8. The method of claim 1, wherein said number of cells is determined by microscopic examination.
9. The method of claim 1, wherein said number of cells is determined by a technique selected from group consisting of

FACS analysis, luminex detection, antibody microarray digital scanner, and cell sorter.

10. The method of claim 1 wherein said ratio is at least about 10%.
11. The method of claim 1 wherein said ratio is at least about 25%.
12. The method of claim 1 wherein said ratio is at least about 50%.
13. A method of determining whether a human patient is resistant to the antineoplastic effects of antiestrogen therapy, comprising:
 - obtaining a breast tissue sample containing cells from said patient; and
 - detecting the presence of the protein encoded by SEQ ID NO. 16 in said sample wherein the presence of said protein in a ratio of greater than about 5% of the cells of the sample is indicative of resistance to the antineoplastic effects of antiestrogen therapy.
14. A method of determining whether a human patient is resistant to the antineoplastic effects of antiestrogen therapy, comprising:
 - obtaining a breast tissue sample containing cells from said patient;
 - detecting the protein encoded by SEQ ID NO. 16 in said cells of said breast tissue sample;
 - determining the number of cells containing said protein in said sample; and
 - determining the ratio of cells containing said protein to the total number of cells in said breast tissue sample

wherein a ratio greater than about 5% is indicative of resistance to the antineoplastic effects of antiestrogen therapy.

15. The method of claim 13, wherein said sample comprises a material selected from the group consisting of blood, serum, and plasma.

16. The method of claim 13, wherein said patient has been diagnosed with cancer.

17. The method of claim 16, wherein said cancer is breast cancer.

18. The method of claim 13, wherein said protein is detected by immunostaining with an anti-human GP88 antibody.

19. The method of claim 18, wherein said antibody is labeled.

20. The method of claim 19, wherein said label is selected from the group consisting of biotin, enzymatic, radioisotopic, fluorescent, and chemical labels.

21. The method of claim 14, wherein said number of cells is determined by a technique selected from group consisting of FACS analysis, luminex detection, antibody microarray, digital scanner, and cell sorter.

22. The method of claim 14 wherein said ratio is at least about 10%.

23. The method of claim 22 wherein said patient is estrogen receptor positive.

24. The method of claim 22 wherein said ratio is at least about 25%.

25. The method of claim 24 wherein said patient is estrogen receptor positive.

26. The method of claim 24 wherein said ratio is at least about 50%.

27. The method of claim 26 wherein said patient is estrogen receptor positive.

28. The method of claim 13 wherein the ratio is at least about 10%.

29. The method of claim 28 wherein said patient is estrogen receptor positive.

30. The method of claim 13 wherein the ratio is at least about 25.

31. The method of claim 30 wherein said patient is estrogen receptor positive.

32. The method of claim 13 wherein the ratio is at least about 50%.

33. The method of claim 32 wherein said patient is estrogen receptor positive.

34. A method for diagnosing tumorigenicity, comprising: obtaining a breast tissue sample containing cells from a patient;

detecting the protein encoded by SEQ ID NO. 16 in said cells of said breast tissue sample by immunostaining with anti-human GP88 antibody;

determining the number of cells containing said protein in said sample by microscopic examination; and

determining the ratio of cells containing said protein to the total number of cells in said breast tissue sample wherein a ratio of at least about 5% indicates tumorigenicity.

35. A method of determining whether an estrogen receptor positive patient is resistant to the antineoplastic effects of tamoxifen, comprising:

obtaining a breast tissue sample containing cells from said patient;

detecting GP88 in said cells of said breast tissue sample by immunohistochemical staining with anti-human GP88 antibody;

determining the number of GP88 positive cells in said sample by microscopic examination; and

determining the ratio of GP88 positive cells to the total number of cells in said biological sample wherein a ratio of at least about 10% indicates said patient is resistant to the antineoplastic effects of tamoxifen.

* * * * *

专利名称(译)	用于诊断致瘤性和确定抗雌激素疗法的抗肿瘤作用的抗性的方法和试剂盒		
公开(公告)号	US6881548	公开(公告)日	2005-04-19
申请号	US09/880842	申请日	2001-06-15
[标]申请(专利权)人(译)	SERRERO GINETTE		
申请(专利权)人(译)	SERRERO GINETTE		
当前申请(专利权)人(译)	A & G制药, INC.		
[标]发明人	SERRERO GINETTE		
发明人	SERRERO, GINETTE		
IPC分类号	C07K14/435 C07K16/22 C07K14/475 C07K16/18 A61K38/00 A61K45/00 A61K31/138 A61K39/395 A61K48/00 A61P35/00 A61P43/00 C07K16/30 C12N15/09 C12Q1/02 C12Q1/68 G01N33/574 G01N37/00 G01N33/543 G01N33/577 G01N33/53		
CPC分类号	C07K14/475 C07K16/22 A61K38/00 A61K2039/505 G01N2333/475 C07K2316/96 C07K2317/73 C12N2799/026 A61P15/00 C07K2317/76		
代理机构(译)	迪克斯坦夏皮罗MORIN & OSHINSKY LLP		
审查员(译)	HELMS, 拉里R.		
优先权	61/863079 1997-05-23 US		
其他公开文献	US20020025543A1		
外部链接	Espacenet USPTO		

摘要(译)

用于诊断致瘤性和确定癌症患者是否对抗雌激素疗法的药理学作用具有抗性的方法和试剂盒。PCDGF (GP88) 生长因子水平的增加表明致瘤性和抗雌激素疗法的药理学作用的抗性。本发明的方法和试剂盒可用于评估来自患者的生物样品的致瘤性, 并确定患者是否是抗雌激素的候选物, 包括他莫昔芬, 治疗。

