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(54) **MATERIALS AND METHODS FOR TREATMENT OF CANCER**

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MATIERES ET METHODES DE TRAITEMENT DU CANCER

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WO-A-03/006611

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- **YU W. ET AL.: "GPC5 is a possible target for the 13q31-q32 amplification detected in lymphoma cell lines" JOURNAL OF HUMAN GENETICS, vol. 48, 29 April 2003 (2003-04-29), pages 331-335, XP002346988 cited in the application**
- **FITZGIBBON J. ET AL.: "Refinement and evaluation of candidate genes within the 13q amplicon in germinal centre lymphoma" BLOOD, vol. 100, no. 11, 16 November 2002 (2002-11-16), page ABS. NO. 4258, XP008052951**

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Description**Field of the Invention**

5 **[0001]** The present invention relates to the treatment of cancer, and in particular to the use of glypican 5 (GPC5) antagonists and binding agents for the treatment of cancers.

Background to the Invention

10 **[0002]** Amplification of genomic regions is frequently observed in human tumors and is one mechanism leading to the upregulation of genes that may critically affect cellular behaviour and drive tumour progression. Therefore, identifying the genes involved in amplification events represents a useful approach to increasing understanding of tumorigenic processes and may provide clinically useful markers.

15 **[0003]** Rhabdomyosarcomas (RMS) are the most common soft tissue sarcomas of childhood and account for around 5% of all childhood cancers. There are two main histological subtypes of RMS namely, alveolar (ARMS) and embryonal (ERMS). Both subtypes consist of cells that resemble and have markers for developing skeletal muscle. The alveolar subtype is generally associated with a poorer prognosis than ERMS and often has a t(2;13) (q35;q14) or t(1;13) (p36;q14) translocation which fuses the PAX3 or PAX7 genes, respectively, to FOXO1A (1-3). In addition to these translocations a number of other aberrations have been defined including regions of genomic amplification (4-10). There are a small but significant group of ERMS which demonstrate a poor response to treatment; the genetics of this group is not well defined.

20 **[0004]** Our previous work on RMS showed amplification of the 13q31-q32 chromosomal region in around 20% of ARMS studied (4). In addition, samples from a number of other tumour types have been reported with amplification of the 13q31-32 region including other sarcomas (leiomyosarcomas (11), malignant fibrous histiocytomas (12), lymphomas (13), breast cancers (14), small cell lung carcinomas and various neurological tumours (15-17)). Also, the widely available leukaemic cell line K562 has been shown to have amplification of the 13q31-32 region in addition to the translocation associated with the BCR-ABL fusion gene (18). Investigation of this region in tumour samples showed expression of the GPC5 and GPC6 genes (Fitzgibbon J. et al, BLOOD, vol. 100, no. 11 2002 Abs. No 4258). More recently, work on a few lymphoma cell lines derived from different types of lymphoma defined a minimum region of amplification at 13q31-32 to an approximately 4 megabase region (13). This region contained the glypican 5 gene (GPC5) which was shown to be expressed and was suggested as a possible target for the amplification event in lymphomas. Numerous GPC genes have been suggested as modulators of the IRRTK and p21 pathways, although a causal link between GPC-pathway modulation and disorders associated with defects in this pathway has not been shown (WO 03/006611 A). In addition, recent work has suggested that GPC5 is not likely to be the target gene for 13q31-q32 amplification (33). Therefore, whether this gene plays a functional role in lymphomas and whether the same gene is involved in other tumour types with genomic aberrations in this region remains to be determined.

35 **[0005]** In a previous analysis of RMS samples we used a new approach to profile global changes in differential expression which targets chromosomes called comparative expressed sequence hybridization (CESH) (19). The data from 45 cases was used in a study to examine the classification potential of these profiles (20). Here we compare chromosomal level genetic and expression data for the 13q31-32 region and suggest that amplification is not the only mechanism leading to increased expression of gene(s) from this region. In view of the frequent differential expression from the 13q31-32 region in addition to its amplification we have sought to implicate gene(s) from this region in the development of RMS. This could provide a target for therapeutic approaches to treat these, and potentially other tumours.

Summary of the Invention

45 **[0006]** As described above, amplification of 13q31 has been observed in alveolar RMS and a number of other cancer types. Yu et al. (13) have further shown that the GPC5 gene is overexpressed in

50 **[0007]** lymphoma cell lines having an amplicon at 13q31-32, as compared to cell lines lacking that amplicon. While those authors speculated that GPC5 might play a role in the pathogenesis of lymphomas with amplification of 13q31-32, they did not provide any evidence of this. Tumour cells are notoriously genetically unstable, being prone to acquiring genetic abnormalities, such as chromosomal amplifications, after transformation. It is therefore possible that the observed amplification was acquired after transformation, or alternatively is simply not involved in the transformation process. Accordingly, there is no proof in the literature to date that GPC5 has any role in normal or abnormal cell proliferation.

55 **[0008]** The present inventors have demonstrated that downregulation of GPC5 expression in cells which overexpress it reduces the ability of these cells to form colonies *in vitro*. The inventors have therefore shown for the first time that GPC5 expression is directly linked to cellular proliferation, thus providing a novel therapeutic target.

[0009] Furthermore, the inventors have found that *GPC5* is overexpressed in tumours which do not show chromosomal

amplification at 13q31.

[0010] The inventors have also found evidence to suggest that GPC5 may be regulated by the Wilms' Tumour (WT1) gene product. WT1 is a zinc finger transcription factor which has been shown to be inappropriately and/or over expressed in leukaemias and a wide range of solid tumours including prostate, breast and lung, as well as thyroid, testicular and ovarian carcinomas, melanoma and mesothelioma (reviewed in Reddy, J. C., and Licht, J. D. (1996) *Biochim Biophys Acta* 1287, 1-28; Scharnhorst, V., van der Eb, A. J., and Jochemsen, A. G. (2001) *Gene* 273, 141-161). The fact that GPC5 is associated with *MYCN* and WT1 which are genes of known significance in tumours is consistent with GPC5 itself being of importance in tumorigenesis.

[0011] In tumour cells grown either *in vitro* or *in vivo*, downregulation of WT1 results in the concomitant downregulation of GPC5. Conversely, upregulation of WT1 results in the upregulation of GPC5. Direct transcriptional regulation of GPC5 by WT1 is likely since GPC5 has two WT1 consensus binding sites in its promoter region. Overexpression of WT1 may therefore drive the overexpression of GPC5 in tumours which do not carry chromosomal 13q31-32 amplicons and GPC5 expression may be modulated indirectly by targeting the expression of WT1. Downregulation of WT1 using antisense oligonucleotides results in inhibition of cell proliferation and induction of apoptosis (Algar, E. M., Khromykh, T., Smith, S. I., Blackburn, D. M., Bryson, G. J., and Smith, P. J. (1996) *Oncogene* 12, 1005-1014). Inhibition of proliferation may be wholly or partially mediated by downregulated GPC5 in some cell types.

[0012] Expression of high levels of *WT1* is associated with poor prognosis in leukaemias and breast cancer. Evidence in the literature suggests that WT1 may contribute to drug resistance mechanisms through interference with cell checkpoint control and apoptotic pathways. However definitive evidence is lacking. The inventors have demonstrated upregulation of *WT1* in chemoresistant tumour cell lines treated with cytotoxic drugs, a phenomenon absent in sensitive cell lines. Associated upregulation of GPC5 has also been demonstrated. Upregulation of GPC5 may mediate some of the effects of upregulated WT1 and contribute to chemoresistance mechanisms. Similarly, overexpression of *GPC3* has recently been implicated in resistance to mitoxantrone and etoposide in a cell line model (Wichert et al. *Oncogene* 23: 945-955 2004). Inhibition of GPC5 activity either by direct or indirect downregulation of expression, or by blocking its activity, may increase the potency of some classes of cytotoxic drugs, particularly in cancers which inappropriately express or overexpress WT1.

[0013] The inventors' work therefore suggests a number of ways in which GPC5 may be targeted therapeutically. GPC5 is a cell surface molecule; agents capable of binding to GPC5 may therefore be used to direct therapeutic agents to target cells. Additionally or alternatively, antagonists which inhibit the expression or function of GPC5 at the cell surface can be used to inhibit cell proliferation directly. Furthermore, such GPC5 antagonists may also be used to increase the sensitivity of target cells to other chemotherapeutic agents, and so may be of significance in treating tumours that have become resistant to therapy.

[0014] Thus the therapeutic application of the inventors' findings extends far beyond those few cancers carrying chromosomal 13q31 amplicons.

[0015] Thus, in a first aspect, the present invention provides an *in vitro* method of inhibiting proliferation of a target cell, comprising contacting the cell with a GPC5 antagonist comprising nucleic acid sequence complementary to the sequence of GPC5 mRNA or pre-mRNA or an antibody which is capable of binding to and inhibiting the activity of GPC5. The present invention also provides an *in vitro* method of inhibiting proliferation said therapeutic comprising contacting the target cell with an antibody capable of binding GPC5 and a therapeutic agent, said therapeutic agent being associated with said antibody or capable of binding to said antibody. In this aspect of the invention, a GPC5 binding agent is an agent capable of binding to GPC5 protein, that is to say to the GPC5 core protein and/or its associated heparan sulphate chains.

[0016] The GPC5 binding agent is used to direct a therapeutic agent capable of inhibiting proliferation of the target cell to the appropriate cell type.

[0017] The therapeutic agent may be part of, or associated with (covalently or non-covalently bonded or otherwise linked to), the binding agent. Alternatively the binding agent may be used to label a target cell in order that a suitable therapeutic agent can then be directed to the cell in preference to unlabelled cells, or activated in the vicinity of the cell. In such embodiments the therapeutic agent may be capable of binding to the GPC5 binding agent.

[0018] The skilled person will be aware of numerous possible mechanisms by which suitable therapeutic agents can be directed to a target cell via a GPC5 binding agent.

[0019] The therapeutic agent may comprise a cell or molecule of the immune system. For example, an anti-GPC5 antibody bound to a target cell may be capable of recruiting various effector mechanisms of the immune system to attack that cell. These include cellular mechanisms, such as antibody-directed cell-mediated cytotoxicity, which is mediated by polymorphonuclear cells, mononuclear cells and K cells, as well as molecular mechanisms such as the complement cascade.

[0020] Alternatively, the therapeutic agent may comprise a molecule capable of directly killing or inhibiting proliferation of the cell, such as a toxin or drug. This approach includes the use of precursor molecules capable of being converted to toxin or drug molecules by action of an enzyme expressed by the cell or associated with the GPC5 binding agent. An

example of such a method is often referred to as ADEPT therapy (see below).

[0021] In yet further alternative embodiments the therapeutic agent may comprise a vector, such as a viral vector, comprising nucleic acid encoding a toxic or inhibitory agent to be synthesised within the cell. In such cases the agent encoded by the vector may itself be a GPC5 antagonist as described elsewhere in this specification.

[0022] The present inventions also provides use of a GPC5 antagonist for the manufacture of a medicament for inhibiting proliferation of a target cell, wherein the GPC5 antagonist comprises a nucleic acid sequence complementary to the sequence of GPC5 mRNA or pre-mRNA or wherein the GPC5 antagonist is an antibody which is capable of binding to and inhibiting the activity of GPC5.

[0023] The present invention further provides a GPC5 antagonist for use in a method of treatment comprising inhibiting proliferation of a target cell, wherein said GPC5 antagonist comprises a nucleic acid sequence complementary to the sequence of GPC5 mRNA or pre-mRNA or wherein the GPC5 antagonist is an antibody which is capable of binding to and inhibiting the activity of GPC5.

[0024] The present invention further provides use of an antibody capable of binding to GPC5 for the manufacture of a medicament for inhibiting proliferation of a target cell, wherein said medicament is for inhibiting proliferation of a target cell in combination with a therapeutic agent, said therapeutic agent being associated with said antibody or capable of binding to said antibody.

[0025] The present invention additionally provides an antibody capable of binding to GPC5 for use in a method of treatment comprising inhibiting proliferation of a target cell, said method further comprising contacting the target cell with a therapeutic agent, said therapeutic agent being associated with said antibody or capable of binding to said antibody.

[0026] These approaches may be used individually or in combination. Other suitable embodiments will be apparent to the skilled person.

[0027] A GPC5 binding agent may (but need not) have GPC5 antagonist activity in its own right.

[0028] A GPC5 antagonist is an agent which inhibits either the activity of GPC5 or the expression of functional GPC5 on the cell surface. Thus a GPC5 antagonist may inhibit cellular proliferation by directly blocking the proliferative effects of GPC5.

[0029] GPC5 antagonists which affect GPC5 activity (rather than expression) are typically GPC5 binding agents which prevent or inhibit the GPC5 protein from exerting its physiological activity, e.g. by blocking GPC5 from binding to a ligand, receptor, and/or co-receptor. For example, an antibody, peptide, small molecule or the like which performs any one of these functions may be regarded as a GPC5 antagonist as well as a GPC5 binding agent.

[0030] GPC5 antagonists which inhibit GPC5 expression may act at any one of a number of points in the generation of mature GPC5 protein. For example, the antagonist may inhibit transcription of the GPC5 gene, processing of GPC5 pre-mRNA, translation of GPC5 mRNA into protein, glycosylation of GPC5 (i.e. addition of carbohydrate residues to the GPC5 core protein) or processing of the carbohydrate chains into mature heparan sulphate (HS) chains.

[0031] Preferred antagonists of GPC5 expression comprise nucleic acid sequences complementary to the sequence of GPC5 mRNA or pre-mRNA. These include antisense RNA, dsRNA molecules (including RNAi and siRNA), and ribozymes.

[0032] As set out above, GPC5 antagonists may sensitise target cells to cytotoxic agents. Thus the therapy may comprise the further step of contacting the cell with a cytotoxic agent, wherein the GPC5 antagonist increases the sensitivity of the cell to the cytotoxic agent. This may be particularly useful in treatment of cancer patients whose cells have become resistant to a chemotherapeutic agent; the GPC5 antagonist may be used in combination with the chemotherapy to increase the efficacy of the chemotherapeutic agent.

[0033] Preferred aspects involve GPC5 antagonists or binding agents for use in methods comprising administration to subjects suffering from cancer, in order to inhibit proliferation of cancer cells.

[0034] Thus, also provided is use of a GPC5 antagonist or a GPC5 binding agent as described in the claims in the preparation of a medicament for the treatment of cancer.

[0035] In all of the therapeutic methods and compositions described herein, the GPC5 antagonists and binding agents may be used alone or in combination with other therapeutic agents, including cytotoxic agents (see above).

[0036] In a further aspect, the invention provides an in vitro method of determining the susceptibility of a cancer to treatment with a GPC5 antagonist or binding agent, comprising determining the presence, absence or level of expression of GPC5 in a cell from said cancer. Additionally or alternatively, the method may comprise the step of determining the presence, absence or degree of chromosomal amplification at 13q31, e.g. determining the genomic copy number of the GPC5 gene. Additionally or alternatively the method may comprise the step of determining the presence, absence or degree of WT1 expression in the cell. The cell may previously have been found to express WT1 inappropriately or to overexpress WT1. Determinations may be qualitative, quantitative or semi-quantitative.

[0037] The method will typically be performed in vitro using a sample isolated from a subject suffering from the cancer in question. The sample may comprise whole cells or cell extracts and may be derived from a biopsy, a body fluid such as blood, or any other suitable sample suspected or known to contain one or more cancer cells. The method may comprise the step of isolating the sample from the subject.

[0038] Typically the method will comprise the step of contacting the sample with a GPC5-binding agent, which in this aspect of the invention may be capable of binding to GPC5 protein, DNA or RNA. The method may further comprise the step of determining the amount of binding agent bound to GPC5 and correlating the results obtained with a likelihood that the cancer will be susceptible to treatment with a GPC5 antagonist or binding agent.

5 **[0039]** The presence or level of free circulating GPC5 (i.e. GPC5 protein not bound to a cell surface via a GPI anchor) may also serve as a marker for a cancer in which GPC5 is overexpressed. Thus in a further aspect the invention provides a method of screening for the presence of a cancer in a patient, the method comprising contacting a sample derived from the patient with a GPC5 binding agent. Preferably the sample is a sample of a body fluid, such as whole blood, serum, plasma urine, etc.

10 **[0040]** The method may further comprise the step of determining the amount of binding agent bound to GPC5 and correlating the results obtained with a likelihood that the patient has cancer.

[0041] The inventors have also found that overexpression of GPC5 in breast cancer samples correlates well with the stage of that cancer. In particular, tumours which overexpress GPC5 are significantly more likely to be stage 3 tumours than stage 1 or stage 2 tumours. This implies that GPC5 can be used as a prognostic marker for breast cancer.

15 **[0042]** Therefore the invention provides a method for determining a prognosis for a patient with breast cancer comprising assigning a prognosis to the patient based on the expression levels of GPC5 in a breast tumour from that patient.

[0043] The method comprises determining the presence, absence or degree of expression of GPC5 in a sample containing breast cancer cells. The method is performed in vitro using a sample isolated from the patient. The sample may be contacted with a GPC5 binding agent capable of binding to GPC5 mRNA or protein.

20 **[0044]** "Prognosis" is intended in its most general sense, and may be quantitative or qualitative. It may be expressed in general terms, such as a "good" or "bad" prognosis, and/or in terms of likely clinical outcomes, such as duration of disease free survival (DFS), likelihood of survival for a defined period of time, and/or probability of distant metastasis within a defined period of time. Quantitative measures of prognosis will generally be probabilistic. Additionally or alternatively, and especially for communicating the prognosis to or between medical practitioners, the prognosis may be expressed in terms of another indicator of prognosis, such as the NPI scale.

25 **[0045]** In general, a patient with a 'good prognosis' tumour would probably be treated with a conventional treatment regimen. A patient with a 'poor prognosis' tumour might be treated with an alternative or more aggressive regimen. The 'poor prognosis' patient would usually not have to wait for the conventional treatment regimen to fail before moving onto the more aggressive one. Furthermore, having an understanding of the likely clinical course of the disease allows a patient to prepare a realistic plan for future, which is an important social aspect of cancer treatment.

30 **[0046]** For the avoidance of doubt, the term "determining" need not imply absolute certainty in prognosis. Rather, the expression levels of GPC5 in a tumour will generally be indicative of the likely prognosis of the patient.

35 **[0047]** Those patients whose tumours are found to overexpress GPC5 are likely to have stage 3 tumours, which may result in them being assigned a poor prognosis. It will be understood, though that GPC5 will not necessarily be the sole marker used in determination of prognosis. Rather, it may be used in combination with other prognostic makers to assist in reaching a detailed prognosis.

40 **[0048]** The inventors envisage that GPC5 expression may also be used to monitor the progress (e.g. success or failure) of a treatment for a cancer previously found to express GPC5. Such methods typically involve monitoring GPC5 expression in cells of the cancer. This may involve determining the level of expression of GPC5 within cells of the cancer. A reduction in the level of GPC5 expression over time may be taken as an indication that the treatment is effective. Additionally or alternatively, the method may involve determining the number or density of cells in a given sample expressing or overexpressing GPC5. This may assist in determining whether the size of the tumour is being reduced, so giving an indication of whether the treatment is having the desired effect of reducing tumour size.

45 **[0049]** The method will typically involve comparing the results obtained with results of an equivalent assay performed for the same patient before treatment, and/or at an earlier stage of treatment.

[0050] The method typically comprises determining the presence, absence or degree of expression of GPC5, or the number or density of cells expressing or overexpressing GPC5, in a sample containing breast cancer cells from the patient. It is performed in vitro using a sample isolated from the patient. The sample is typically contacted with a GPC5 binding agent capable of binding to GPC5 mRNA or protein.

50 **[0051]** In a further aspect the invention further provides in vitro methods of screening which may be used to identify therapeutic agents. In particular the invention provides a method of screening for an agent capable of killing or inhibiting proliferation of a target cell, comprising the steps of:

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- (i) contacting GPC5 protein with one or more candidate substances;
 - (ii) selecting one or more candidate substances based on their ability to bind GPC5 protein;
 - (iii) contacting said one or more selected substances with a target cell; and

(iv) determining the effect of said selected substance(s) on proliferation of said cell.

[0052] The cell typically expresses GPC5, and preferably inappropriately expresses or overexpresses GPC5. The cell may naturally express GPC5 (e.g. it may be derived from a cancer in which GPC5 is expressed or overexpressed) or it may have been engineered to express GPC5, e.g. by transformation with a vector comprising nucleic acid encoding GPC5. The cell may previously have been found to inappropriately express or overexpress WT1.

[0053] The method may comprise the step of further selecting one or more substances found to inhibit proliferation of the target cell. The selected substances may be subjected to one or more rounds of modification, to increase activity and/or suitability for in vivo administration and re-tested for the ability to inhibit cellular proliferation. Suitable substances may be formulated for therapeutic administration, e.g. as a pharmaceutical composition.

[0054] In the various methods described above, the target cell or cancer cell typically overexpresses GPC5 mRNA and/or GPC5 protein. The cell may also carry a chromosomal amplicon comprising part or all of the 13q31 region; i.e. the cell may carry more than the normal two genomic copies of the GPC5 gene. In preferred embodiments, though, the cell overexpresses GPC5 but does not carry any amplification of that chromosomal region. The target cell is typically a cancer cell, and preferably one which overexpresses GPC5.

[0055] Cancers previously found to carry 13q31-32 amplicons include examples of rhabdomyosarcomas, including both embryonal and alveolar RMS, lymphomas including follicular lymphoma, mantle cell lymphoma and primary cutaneous B-cell lymphoma, non-small cell lung cancer, bladder cancer, a small proportion of breast cancers, neuroglial tumours including malignant peripheral nerve sheath tumours, squamous cell carcinoma of the head and neck, chronic myeloid leukemia, leiomyosarcoma, liposarcoma, malignant fibrous histiocytoma of bone and soft tissues, (See Gordon et al., 2000, Yu et al., 2003, and references cited therein).

[0056] In view of the finding that GPC5 overexpression occurs in the absence of chromosomal amplification in RMS, prostate cancer and breast cancer, it is likely that examples of the above described cancer types will also overexpress GPC5 without 13q31 amplification and so any of the above are envisaged as suitable for treatment by the methods and compositions described in this specification.

[0057] GPC5 expression appears to be regulated (at least in part) by WT1. Accordingly, cancers which show inappropriate expression (e.g. overexpression) of WT1 may also be suitable for treatment by the methods described. These include leukaemias and a wide range of solid tumours including prostate, breast and lung, as well as thyroid, testicular and ovarian carcinomas, melanoma and mesothelioma (Scharnhorst, V., van der Eb, A. J., and Jochemsen, A. G. (2001) Gene 273, 141-161).

[0058] WT1 expression has also been implicated in the resistance of cancer cells to chemotherapeutic agents. Thus, cancers which are resistant to treatment with one or more cytotoxic agents may also be suitable for treatment according to the present invention. The cells may have been resistant ab initio or may have developed a resistance over the course of treatment. Typically such cells will be characterised by overexpression of WT1 and GPC5.

[0059] In addition, improved efficacy of GPC5 antagonists in increasing chemosensitivity may be achieved by concomitant down regulation of WT1 using WT1 antagonists. Preferred WT1 antagonists comprise nucleic acid sequences complementary to the sequence of WT1 mRNA or pre-mRNA. These include antisense RNA, dsRNA molecules (including RNAi and siRNA), and ribozymes.

Description of the Drawings

[0060]

Figure 1: FISH results using BACs spanning the amplified 13q31q32 region in two primary RMS samples and the cell line K562. The minimal region of amplification is defined by RP11-51a2 at the centromeric end and by GPC6 Taqman at the telomeric end which shows no amplification. Therefore, the region of minimal amplification flanks the GPC5 gene but does not extend into the next telomeric gene GPC6. The gene C13ORF25A, located proximally centromeric to GPC5, has been suggested to be implicated in lymphoma (33).

Figure 2A: Log distribution of GPC5 expression relative to normal muscle; left (Embryonal), right (Alveolar).

Figure 2B: Expression of C13ORF25A in rhabdomyosarcoma relative to normal muscle. Bars marked "A" indicate samples which show genomic amplification of C13ORF25A.

Figure 3 shows the effect of GPC5 antisense oligonucleotides on GPC5 mRNA levels and cell survival in K562 cells. Panel A shows data for 13 representative GPC5-targeted oligonucleotides relative to the control oligonucleotide 15770. Panels B and C show dose-response curves for one active oligonucleotide (276107) relative to a different control oligonucleotide, designated 276124.

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Figure 4A shows the level of GPC5 mRNA expression in 18 prostate cancer samples and 6 benign prostate hyperplasia samples, as compared to normal prostate. Figure 4B shows the overall difference in GPC5 expression between prostate cancer and benign prostatic hyperplasia.

Figure 5A shows GPC5 mRNA expression in breast cancer samples relative to normal breast biopsy tissue. Figure 5B shows the number of tumours of stage 1, 2 or 3 which overexpress GPC5 relative to normal breast tissue in a sample of 44 breast tumours.

Figure 6 shows the effect of WT1 antisense oligonucleotides on WT1 and GPC5 mRNA levels in K562 cells, both 24 and 48 hours after treatment, suggesting that WT1 may regulate GPC5 expression.

Figure 7 shows upregulation of both WT1 and GPC5 mRNA in PC3M tumors treated with docetaxol.

Figure 8 shows the effect of GPC5 overexpression in the RMS cell line T91-95. A: Difference in proliferation between 5 GPC5 overexpressing colonies and 5 mock-transfected control colonies. B: Difference in expression of GPC5 as measured by TaqMan assay between GPC5 overexpressing colonies and control colonies. C: Western Blot of an In Vitro Translation; Lane 1 water (negative control), Lane 2 pCMV-TNT-GPC5 (63Kda), Lane 3 Luciferase (61Kda positive control). Protein was translated so as to incorporate biotin labelled lysine residues. Colour was developed using streptavidin-alkaline phosphatase and appropriate colorimetric reagents.

Detailed Description of the Invention

Glypicans

[0061] The glypicans are cell surface heparan sulphate proteoglycan (HSPG) molecules which consist of a protein core carrying heparan sulphate (HS) glycosaminoglycan chains and linked to the plasma membrane of the cell through a GPI anchor. For general reviews see Perrimon and Bernfield (2000) Nature 404: 725-728 and Selleck (1999) Am J Hum Genet 64: 372-377.

[0062] The HS chains themselves are attached to the serine residues of consensus SGXG "glycanation" sites. They each consist of a tetrasaccharide linker (-GlcA-Gal-Gal-Xyl) to which is added a linear polymer of a repeating disaccharide unit made up of GlcNAc and GlcA. After the chains have been synthesised, they are subjected to processing in the Golgi by the enzymes N-deacetylase/N-sulphotransferase (NDST), uronosyl C5-epimerase, 2-O-sulphotransferase (2-OST), 6-O-sulphotransferase (6-OST) and 3-O-sulphotransferase (3-OST).

[0063] The HSPGs are increasingly thought to play specific roles in cell signalling. Some HSPGs may act as co-receptors for growth factor signalling (see the reviews by Perrimon and Bernfield, and Selleck, above). Glypican-3 has been shown to co-immunoprecipitate with FGF2 and BMP-7 (Int J Cancer 2003 103:455-65), while glypican-1 has been shown to co-immunoprecipitate with FGF2 and HB-EGF and to increase the growth stimulatory action of these growth factors (J Clin Invest 1998 102(9) 1662-73). Glypican-4 modulates the function of FGF2 by suppressing its growth stimulatory properties (Dev Dyn 219(3):353-67).

[0064] The mRNA and protein sequence of human GPC5 is found in Veugelers et al "Characterization of Glypican-5 and chromosomal localization of human Glypican-5; new member of glypican gene family" Genomics 1997 40(1):24-30. The genomic structure of the gene is described in Veugelers et al "A 4MB BAC/PAC contig + complete genomic structure of the GPC5/GPC6 gene cluster on chromosome 13q32" Matrix Biol 2001 20(5-6):375-85. The GenBank reference sequence accession number is AF001462 (gi:3015541) for mRNA and NP004457 for protein. Homologous proteins have also been identified in mouse (NP 780709) and rat (XP 224489) having protein sequence identity of 82% and 87% respectively to the human sequence. This is greater than the next most homologous gene within the human genome GPC3 (NP 004475) which has a protein sequence identity of 46% with human GPC5.

[0065] "GPC5 protein" is used herein as a general term to include both the core protein and/or its associated heparan sulphate chains. References to the protein should be construed accordingly.

Cells

[0066] The term "cancer cell" is used throughout this specification to refer to any transformed cell including cells from cancers and tumours occurring in vivo, as well as laboratory cell lines adapted to continuous culture. Such cell lines typically display characteristics such as unlimited capacity for in vitro replication, loss of contact inhibition, ability to form tumours in animals, etc.. They may historically be derived from a cancer, or may have been transformed in the laboratory.

[0067] The cell may be of any suitable species, although mammalian cells are preferred. Particularly preferred are human and rodent (e.g. mouse or rat) cells.

[0068] Target cells of the various methods described typically overexpress GPC5. The cells may have been engineered to overexpress GPC5, e.g. by transformation with a vector comprising nucleic acid encoding GPC5, or may naturally overexpress GPC5, in that they overexpresses GPC5 without having been deliberately manipulated to do so.

[0069] A cell is considered to overexpress GPC5 if it shows a higher level of RNA or protein than normally found in that cell type. A tumour cell may be considered to overexpress GPC5 if it shows a level of expression greater than that found in a corresponding cell type from which the tumour is thought to be derived. For example, RMS cells can be compared to normal muscle cells or muscle precursor cells, breast cancer cells to healthy breast tissue or normal breast epithelial cells and prostate cancer cells to healthy prostate tissue or normal prostate epithelium.

[0070] Thus a cell type which does not normally express GPC5 may be considered to overexpress, or inappropriately express, GPC5 if it shows a detectable level of GPC5 expression. A cell which would normally show detectable GPC5 expression may be considered to overexpress GPC5 if it shows double the normal level of RNA or protein for that cell type, more preferably 5 times, 10 times, 50 times or 100 times the normal level for that cell type. The brain is the only normal human adult tissue which has been reported to display detectable expression of GPC5.

[0071] The same considerations, mutatis mutandis, apply to determining whether whole tissues, body fluids etc. display elevated levels of GPC5.

[0072] WT1 is normally only expressed in specific cell types in kidney, gonads, haematopoietic and nervous system, and mesothelium (Reddy, J. C., and Licht, J. D. (1996) *Biochim Biophys Acta* 1287, 1-28). Overexpression of WT1 in these tissues or cancers derived therefrom may be determined as described above for GPC5. Expression in other cell types may be considered inappropriate.

Antagonists

[0073] The term "GPC5 antagonist" encompasses two different classes of agent.

[0074] An "antagonist of GPC5 activity" is an agent which prevents the mature GPC5 protein from exerting its normal function when expressed at the cell surface. Typically these will be binding agents for GPC5 protein which are capable of binding to GPC5 protein (core protein and/or HSPG chains) and inhibiting its pro-proliferative function.

[0075] An "antagonist of GPC5 expression" is an agent capable of inhibiting or blocking expression of the mature protein at the cell surface, although it will be appreciated that the ultimate effect of such agents is also to inhibit GPC5 function or activity.

[0076] GPC5 antagonists may therefore be identified by screening candidate compounds or substances for the ability to bind GPC5. Suitable assay methods are described below.

[0077] Those candidates which show suitable binding may be screened (subsequently or in parallel) for their ability to inhibit proliferation of a cell overexpressing GPC5. Suitable cells include those which naturally overexpress GPC5 (such as cell lines derived from GPC5-overexpressing cancers), as well as those engineered to overexpress GPC5 (e.g. by transformation with a vector encoding GPC5).

[0078] By contrast, antagonists of GPC5 expression typically comprise nucleic acid molecules capable of hybridising to GPC5 genomic DNA, precursor mRNA, mRNA, or cDNA, which may be single stranded or double stranded. Such modulators include antisense RNA or DNA, triple helix-forming molecules, RNAi, siRNA and ribozymes. (Such antagonists may also be considered to be GPC5 binding agents as described below.) Nucleic acids comprising chemically modified nucleotides (such as locked nucleic acids, or propynyl, methyl or G-clamped pyrimidine nucleotides) as well as nucleic acid analogues having modified sugar residues (e.g. 2'-O-methyl and 2'-methoxyethyl modifications) or backbone structure (e.g. by incorporation of phosphoramidite or morpholino linkages, or peptide nucleic acids (PNAs)) are also included within this definition. For more details of these modifications and antisense techniques in general, see Dean and Bennett (2003) *Oncogene* 22: 9087-9096 and references cited therein.

[0079] Antisense oligonucleotides hybridise with complementary sequences of RNA generally by Watson-Crick base pairing. The resultant double stranded complex prevents translation of the message into protein product either by steric blocking at the ribosome or activation of RNase H that cleaves the RNA strand of the duplex. With respect to antisense DNA, oligodeoxy-ribonucleotides derived from the translation initiation site, e.g. between the -10 and +10 regions of the target gene nucleotide sequence of interest, are preferred.

[0080] In using antisense genes or partial gene sequences to down-regulate gene expression, a nucleotide sequence is placed under the control of a promoter in a "reverse orientation" such that transcription yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene.

[0081] The complete sequence corresponding to the coding sequence need not be used. For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of the coding sequence to optimise the level of antisense inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A further possibility is to target a conserved sequence of a gene, e.g. a sequence that is characteristic of one or more genes, such as a regulatory sequence.

[0082] The sequence employed may be 500 nucleotides or less, possibly about 400 nucleotides, about 300 nucleotides, about 200 nucleotides, or about 100 nucleotides. It may be possible to use oligonucleotides of much shorter lengths, 14-23 nucleotides, although longer fragments, and generally even longer than 500 nucleotides are preferable where possible.

5 **[0083]** It may be preferable that there is complete sequence identity in the sequence used for down-regulation of expression of a target sequence, and the target sequence, though total complementarity or similarity of sequence is not essential. One or more nucleotides may differ in the sequence used from the target gene. Thus, a sequence employed in a down-regulation of gene expression in accordance with the present invention may be a wild-type sequence (e.g. gene) selected from those available, or a mutant, derivative, variant or allele, by way of insertion, addition, deletion or substitution of one or more nucleotides, of such a sequence. The sequence need not include an open reading frame or specify an RNA that would be translatable. It may be preferred for there to be sufficient homology for the respective anti-sense and sense RNA molecules to hybridise. There may be down regulation of gene expression even where there is about 5%, 10%, 15% or 20% or more mismatch between the sequence used and the target gene.

10 **[0084]** Double stranded RNA (dsRNA) has been found to be even more effective in gene silencing than antisense strands alone (Fire A. et al Nature, Vol 391, (1998)). dsRNA mediated silencing is gene specific and is often termed RNA interference (RNAi). RNA interference is a two step process. First, dsRNA is cleaved within the cell to yield short interfering RNAs (siRNAs) of about 21-23nt length with 5' terminal phosphate and 3' short overhangs (~2nt) The siRNAs target the corresponding mRNA sequence specifically for destruction (Zamore P.D. Nature Structural Biology, 8, 9, 746-750, (2001) RNAi may be also be efficiently induced using chemically synthesized siRNA duplexes of the same structure with 3'-overhang ends (Zamore PD et al Cell, 101, 25-33, (2000)). Synthetic siRNA duplexes have been shown to specifically suppress expression of endogenous and heterologous genes in a wide range of mammalian cell lines (Elbashir SM. et al. Nature, 411, 494-498, (2001)). See also Fire (1999) Trends Genet. 15: 358-363, Sharp (2001) Genes Dev. 15: 485-490, Hammond et al. (2001) Nature Rev. Genes 2: 1110-1119 and Tuschl (2001) Chem. Biochem. 2: 239-245.

15 **[0085]** Ribozymes are enzymatic RNA molecules capable of catalysing the specific cleavage of RNA. (For a review, see Rossi, J., 1994, Current Biology 4: 469-471). The mechanism of ribozyme action involves sequence specific hybridisation of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. The composition of ribozyme molecules must include one or more sequences complementary to the target protein mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see US Pat. No. 5,093,246.

20 As such, within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyse endonucleolytic cleavage of RNA sequences encoding target proteins.

25 **[0086]** Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the molecule of interest for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short sequences of between 15 and 20 ribonucleotides corresponding to the region of the target protein gene containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate sequences may also be evaluated by testing their accessibility to hybridise with complementary oligonucleotides, using ribonuclease protection assays.

30 **[0087]** Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC⁺ triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementary to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

35 **[0088]** Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so-called "switchback" nucleic acid molecule. Switchback molecules are synthesised in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

40 **[0089]** WT1 antagonists are agents which prevent WT1 exerting its function as a transcription factor. As it is an intracellular protein, preferred WT1 antagonists are those which interfere with WT1 expression. Particularly preferred WT1 antagonists comprise nucleic acid sequences complementary to the sequence of WT1 mRNA or pre-mRNA. These include antisense RNA, dsRNA molecules (including RNAi and siRNA), and ribozymes. For exemplary WT1 sequences, see GenBank accession numbers NM_000378.2, NM_024426.2, NM_02442S.1, NM_024424.1. All accession numbers cited in this application are taken from GenBank release 140.0, updated 20 February 2004.

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Binding agents

[0090] In most aspects of the invention, GPC5 binding agents are referred to in the context of agents capable of binding to GPC5 core protein and/or its associated heparan sulphate chains. However in some aspects of the invention, such as the diagnostic methods described below, agents capable of binding to nucleic acid encoding GPC5, and in particular to GPC5 pre-mRNA, mRNA, or cDNA derived therefrom, may also be considered to be GPC5 binding agents.

[0091] In preferred embodiments the binding agents which are used may be regarded to constitute a specific binding pair with GPC5. The term "specific binding pair" may be used to describe a pair of molecules comprising a specific binding member (sbm) and a binding partner (bp) therefor which have particular specificity for each other and which in normal conditions bind to each other in preference to binding to other molecules. Examples of specific binding pairs are antigens and antibodies, ligands (such as hormones, etc.) and receptors, avidin/streptavidin and biotin, lectins and carbohydrates, and complementary nucleotide sequences.

[0092] By "specific" is meant that the particular binding sites of the agent which interact with GPC5 will not show any significant binding to molecules other than GPC5 which are likely to be encountered by the binding agent (e.g. other molecules in an assay or on a given cell surface). For example the interaction between the binding agent and GPC5 may have a K_D of the order of 10^{-6} to $10^{-9}M^{-1}$ or smaller.

[0093] The binding agent may bind to the protein core or the HS chains of the GPC5 molecule, but in preferred embodiments bind to the protein core, preferably to hydrophilic regions of the core, e.g. to part or all of the sequence CKSYTQRWNGNIKAQ.

[0094] The binding agent may be a protein or polypeptide of 50 amino acids in size or greater, or a peptide of up to 50 amino acids in length. Typically a peptide will be from 5 to 50 amino acids in length, more typically 10 to 20 amino acids in length. Alternatively the binding agent may be a small molecule e.g. of 1000 Da or less, preferably 750 Da or less, preferably 500 Da or less.

[0095] Antibodies are preferred examples of binding agents. Thus preferred assay formats for diagnosis are immunological assays including ELISA assays, and immunohistochemistry, which may be carried out on whole cells or tissue sections, other forms of immunostaining for FACS analysis, confocal microscopy or the like, which may be carried out on single cells or populations of dispersed cells, and immunoblotting, which is suitable for analysis of cell extracts.

[0096] It has been shown that fragments of a whole antibody can perform the function of binding antigens. The term "antibody" is therefore used herein to encompass any molecule comprising the binding fragment of an antibody. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')₂ fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding member (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988).

Diagnostic methods and other assays

[0097] As well as being useful as GPC5 antagonists and targeting agents, binding agents for GPC5 may also be used to detect the presence of GPC5 in biological samples. This has a number of applications within the scope of the invention.

[0098] The binding agents described herein may be used to assess the susceptibility of a particular cancer to treatment with GPC5 antagonists or binding agents by assessing the level of expression of GPC5 in that cancer. A cancer found to overexpress or have an elevated level of GPC5 may be treatable by means of the methods and compositions described herein.

[0099] The method typically comprises contacting a sample with a GPC5 binding agent, the sample having been isolated from a subject suffering from the cancer in question. The sample may comprise one or more whole cells or extracts of cells, and may be derived from any suitable biological sample suspected or known to contain one or more cancer cells. Examples include tissue samples (e.g. biopsies) and samples of body fluid, e.g. blood, serum or plasma.

[0100] The binding agent may detect expression of either GPC5 protein or mRNA.

[0101] In preferred embodiments the method further comprises comparing the level of GPC5 expression with that found in one or more reference samples, which may be pre-determined. Suitable reference samples include samples of the same tissue type, or a comparable tissue type, as that from which the cancer or suspected cancer is derived. The reference samples may be obtained from the same individual as the test sample, or from different individuals. The reference samples may also include samples of cancer cells of known type, optionally of known GPC5 expression level, which may serve as positive controls.

[0102] The sample may be derived from a cancer previously identified to inappropriately express or overexpress WT1. Alternatively the method may further comprise the step of determining the level of expression of WT1 to see whether combined therapy with one or more WT1 antagonists would be beneficial.

[0103] The invention also provides methods of determining whether a subject is suffering from a cancer characterised by overexpression of GPC5, the method comprising contacting a sample from the subject with a GPC5 binding agent. Preferably the method comprises determining the level of circulating free (i.e. not cell-associated) GPC5. In such cases, the sample is preferably blood, serum or plasma, and the binding agent detects GPC5 protein.

[0104] Again, the results obtained may be compared with suitable positive and/or negative control samples to arrive at an indication of the subject's clinical status.

[0105] Methods for determining the concentration of analytes in samples from individuals are well known in the art and readily adapted by the skilled person in the context of the present invention to determine expression of GPC5 protein or mRNA as appropriate. Such assays may allow a physician to optimise the treatment of a disorder and, thus, the methods described allow for planning of appropriate therapy, permitting streamlining of treatment by targeting those most likely to benefit.

[0106] Assay methods for determining the concentration of protein markers typically employ binding agents having binding sites capable of specifically binding to protein markers, or fragments thereof, or antibodies in preference to other molecules. Examples of binding agents include antibodies, receptors and other molecules capable of specifically binding the analyte of interest. Conveniently, the binding agents are immobilised on a solid support, e.g. at defined, spatially separated locations, to make them easy to manipulate during the assay.

[0107] The sample is generally contacted with the binding agent(s) under appropriate conditions which allow the analyte in the sample to bind to the binding agent(s). The fractional occupancy of the binding sites of the binding agent (s) can then be determined either by directly or indirectly labelling the analyte or by using a developing agent or agents to arrive at an indication of the presence or amount of the analyte in the sample. Typically, the developing agents are directly or indirectly labelled (e.g. with radioactive, fluorescent or enzyme labels, such as horseradish peroxidase) so that they can be detected using techniques well known in the art. Directly labelled developing agents have a label associated with or coupled to the agent. Indirectly labelled developing agents may be capable of binding to a labelled species (e.g. a labelled antibody capable of binding to the developing agent) or may act on a further species to produce a detectable result. Thus, radioactive labels can be detected using a scintillation counter or other radiation counting device, fluorescent labels using a laser and confocal microscope, and enzyme labels by the action of an enzyme label on a substrate, typically to produce a colour change. In further embodiments, the developing agent or analyte is tagged to allow its detection, e.g. linked to a nucleotide sequence which can be amplified in a PCR reaction to detect the analyte. Other labels are known to those skilled in the art are discussed below. The developing agent(s) can be used in a competitive method in which the developing agent competes with the analyte for occupied binding sites of the binding agent, or non-competitive method, in which the labelled developing agent binds analyte bound by the binding agent or to occupied binding sites. Both methods provide an indication of the number of the binding sites occupied by the analyte, and hence the concentration of the analyte in the sample, e.g. by comparison with standards obtained using samples containing known concentrations of the analyte.

[0108] In alternative embodiments, the analyte can be tagged before applying it to the support comprising the binding agent.

[0109] Preferred formats are ELISA assays and immunostaining (e.g. immunohistochemistry).

[0110] There is also an increasing tendency in the diagnostic field towards miniaturisation of such assays, e.g. making use of binding agents (such as antibodies or nucleic acid sequences) immobilised in small, discrete locations (microspots) and/or as arrays on solid supports or on diagnostic chips. These approaches can be particularly valuable as they can provide great sensitivity (particularly through the use of fluorescent labelled reagents), require only very small amounts of biological sample from individuals being tested and allow a variety of separate assays can be carried out simultaneously. This latter advantage can be useful as it provides an assay employing a plurality of analytes to be carried out using a single sample. Examples of techniques enabling this miniaturised technology are provided in WO84/01031, WO88/1058, WO89/01157, WO93/8472, WO95/18376/ WO95/18377, WO95/24649 and EP 0 373 203 A. Thus, in a further aspect, the present invention provides a kit comprising a support or diagnostic chip having immobilised thereon a plurality of binding agents capable of specifically binding different protein markers or antibodies, optionally in combination with other reagents (such as labelled developing reagents) needed to carrying out an assay. In this connection, the support may include binding agents specific for analytes such as vimentin, e.g. as disclosed in US Patent No: 5,716,787.

[0111] Such assay methods may also be used to screen for binding agents capable of binding to GPC5 protein. Candidate agents identified by such screens may be subjected to one or more rounds of modification and re-testing in order to identify further agents having improved properties. The skilled person will be aware of numerous suitable screening methods and will be able to design appropriate protocols for identification of candidate binding agents.

[0112] Alternatively the binding agent may be a nucleic acid molecule capable of binding to mRNA or precursor mRNA. Thus mRNA or precursor mRNA encoding GPC5 may be detected by hybridisation with a probe having a suitable complementary sequence, e.g. by Northern blotting or in situ hybridisation. Such protocols may use probes of at least about 20-80 bases in length. The probes may be of 100, 200, 300, 400 or 500 bases in length or more. Binding assays may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning A Laboratory

Manual (New York: Cold Spring Harbor Laboratory Press, 1989 or later editions).

[0113] Alternatively, conventional RT PCR procedures (including quantitative PCR procedures) may be used to analyse the presence or amount of mRNA or precursor mRNA in a given sample. A suitable primer having at least 15 to 20 bases complementary to the GPC5 mRNA or precursor mRNA sequence will typically be used to prime cDNA synthesis. Subsequently, a segment of the cDNA is amplified in a PCR reaction using a pair of nucleic acid primers. The skilled person will be able to design suitable probes or primers based on the publicly available sequence data for GPC5 (see above).

[0114] Whether it is a protein, peptide, small molecule or nucleic acid, the binding agent may also act as an antagonist of GPC5.

Pharmaceutical compositions

[0115] GPC5 antagonists and binding agents can be formulated in pharmaceutical compositions. These compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes or topical application.

[0116] Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

[0117] For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

[0118] Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Suitable carriers, adjuvants, excipients, etc. can be found in standard pharmaceutical texts, for example Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

[0119] Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons; for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

[0120] Instead of administering these agents directly, they could be produced in the target cells by expression from an encoding gene introduced into the cells, eg in a viral vector (a variant of the VDEPT technique - see below). The vector could be targeted to the specific cells to be treated, or it could contain regulatory elements which are switched on more or less selectively by the target cells. The targeting method may itself make use of the expression of GPC5 on the surface of the target cells.

[0121] Alternatively, the agent could be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT or VDEPT; the former involving targeting the activating agent to the cells by conjugation to a cell-specific antibody, while the latter involves producing the activating agent, e.g. an enzyme, in a vector by expression from encoding DNA in a viral vector (see for example, EP-A-415731 and WO 90/07936).

[0122] A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

[0123] Expression of GPC5 in healthy adult human tissue is largely restricted to the brain. Therefore when designing pharmaceutical and other compositions for in vivo administration according to the invention, it may be desirable to make use of components (and particularly active ingredients) which tend not to cross the blood-brain barrier.

[0124] The blood-brain barrier is most permeable to small (up to approx. 700 Da) and/or lipophilic molecules such as water, carbon dioxide, oxygen and anaesthetic molecules, while being almost impermeable to plasma proteins and non-

lipid-soluble large organic molecules. It may therefore be preferable that the compositions of the invention comprise GPC5 antagonists and/or binding agents which are relatively hydrophilic, and/or above at least 1kDa. Peptides and proteins may be preferred. Strategies for screening and design of GPC5 binding agents and antagonists may be designed accordingly.

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Gene therapy

[0125] Nucleic acids encoding modulators (antagonists) of GPC5 expression (e.g. antisense, RNAi, siRNA or ribozyme molecules) may be used in methods of gene therapy. A construct capable of expressing such nucleic acid may be introduced into cells of a recipient by any suitable means, such that the relevant sequence is expressed in the cells.

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[0126] The construct may be introduced in the form of naked DNA, which is taken up by some cells of animal subjects, including muscle cells of mammals. In this aspect of the invention the construct will generally be carried by a pharmaceutically acceptable carrier alone. The construct may also be formulated in a liposome particle, as described above.

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[0127] Such methods of gene therapy further include the use of recombinant viral vectors such as adenoviral or retroviral vectors which comprise a construct capable of expressing a polypeptide of the invention. Such viral vectors may be delivered to the body in the form of packaged viral particles. The viral vectors may themselves be targeted to the appropriate cells via GPC5 binding agents.

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[0128] Constructs of the invention, however formulated and delivered, will be for use in treating tumours in conjunction with therapy. The construct will comprise the relevant nucleic acid linked to a promoter capable of expressing it in the target cells. The constructs may be introduced into cells of a human or non-human mammalian recipient either in situ or ex-vivo and reimplanted into the body. Where delivered in situ, this may be by for example injection into target tissue (s) or in the case of liposomes, inhalation.

[0129] Gene therapy methods are widely documented in the art and may be adapted for use in the expression of the required sequence.

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MATERIALS AND METHODS

Patient samples and cell lines

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[0130] Samples were collected from patients with a diagnosis of RMS from the Royal Marsden NHS Trust or participating UKCCSG (United Kingdom Children's Cancer Study Group) centres around the time of first diagnosis. In addition, 22 samples were collected at the University hospital in Leuven, Belgium and two samples were collected from University hospital Dusseldorf. Samples were snap frozen and material taken adjacent to samples were taken to confirm high tumour content. A pathological diagnosis of RMS was made in the majority of cases by the pathological review committee of the MMT studies. In cases where there was no central review of pathology, morphology and immunohistochemistry reports were examined to ensure RMS pathology. The diagnosis of ARMS was consistent with the current histopathological criteria whereby any alveolar foci are sufficient to result in ARMS classification. Clinical data for the majority of tumours were obtained from the UKCCSG data centre (Leicester, UK) otherwise data was collected directly from participating hospitals. Only patients under the age of 21 were used in survival analysis. The majority of patients were treated using the SIOP (Societe Internationale de Oncologie Paediatric) MMT89 (Malignant Mesenchymal Tumour) protocol or the closely related MMT95 and MMT98 protocols. Some patients were treated using local treatment protocols that were comparable to the MMT protocols. Tumour samples from breast and prostate cancer patients were snap frozen after removal. DNA and RNA was extracted from samples as previously described (20). K562 cl.6 cells, a subclone of the parent erythroleukaemia were kindly provided by Professor Adrian Newland and Dr Xu-Rong Jiang, (The London Hospital Medical College, UK) and the RMS cell line T91-95 used in the transfection studies was a kind gift from Jaclyn Biegel (Children's Hospital of Philadelphia).

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Chromosomal level data on genetic imbalances and differential expression

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[0131] Data from previous studies on RMS included comparative genomic hybridization (CGH) analysis for genomic imbalances ($n = 127$) (4-10)(unpublished data) and comparative expressed sequence hybridization (CESH) data for chromosomal level differential expression ($n=45$)(19, 20). These data were used here to compare the genomic changes with differential expression relative to muscle at 13q31-q32.

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Fluorescence In Situ Hybridization - FISH

[0132] Interphase fluorescence in situ hybridization (FISH) was performed as described previously (21). We used touch preps from two ARMS primary samples and the K562 cell line which possess a 13q31-32 amplicon. BAC (Bacterial

Artificial Chromosome) clones spanning the 13q31-32 region were obtained from the Sanger Centre and included RP11-51a2, RP11-16n13, RP11-121J7, RP11-215m7, RP11-57f10 RP11-169i15, RP11-210-3.

Real Time Quantification of GPC5/6 DNA and RNA levels

[0133] Five sets of primers and probes were designed in order to measure the amount of genomic and mRNA copies of GPC5 and GPC6 (see Table 1). All primers and probes were designed in accordance with Applied Biosystems' TaqMan® standard requirements. Primers and a probe were designed within intron 2 of the GPC5 gene to detect genomic copies of GPC5. To detect copies of GPC6 primers and a probe were designed within exon 3. So as to correct for aneuploidy the gene GJB2 was chosen as an endogenous control. GJB2 is a gene that is not believed to be involved in tumorigenesis and located in a region of chromosome 13 (13q11) not frequently altered in RMS. Primers and a probe were designed within exon 1.

[0134] To measure the amount of mature mRNA copies of GPC5 and GPC6 the probe was designed across the exon 1-exon 2 boundary and the exon 7-exon 8 boundary respectively. Applied Biosystems' Predeveloped GAPDH (Glycer-aldehyde 3-phosphate dehydrogenase) was used as an endogenous control. 25µL multiplex PCR reactions were run using 2X Universal TaqMan® Master Mix (Applied Biosystems Pt No. 4304437), the concentration of primers and probes shown in Table 1 and 10ng of DNA or cDNA. These samples were run in triplicate under standard operating conditions on an ABI7700 SDS TaqMan® Machine (Applied Biosystems, CA). Limiting primer conditions were determined and template titrations showed that the comparative method was appropriate for both genomic and expression reactions (data not shown). Thus, the amount of GPC5 and GPC6 was measured relative to either normal genomic DNA in the case of genomic measurements and to normal muscle in the case of expression measurements. Normal genomic DNA was extracted from the blood of a healthy donor, normal muscle cDNA was produced from RNA extracted from a pool of 11 normal muscle biopsies.

Statistics

[0135] All statistics tests were performed using the SPSS 10.0 package and tested to the 5% significance level. Failure free survival was defined as the time from diagnosis to relapse, progression, death or if event free to the date of last contact. Time to death was defined as the time from diagnosis to death or if event free to the date of last contact.

Real Time Quantification of WT1 mRNA levels.

[0136] The primer pair and probe for the quantification of *WT1* were designed using the Primer Express program (Applied Biosystems) according to the recommended guidelines and as described above.: WTI- Forward primer, 5'-TACCCAGGCTGCAATAAGAGATATTTAAAG-3', reverse primer, 5'-CCTTTGGTGTCTTTTGAGCTGGTC-3', and probe, 5'-CACTGGTGAGAAACCATAACCAGTGTGACTTCAAGGACT-3'. Each assay sample was analysed in triplicate as above, and multiplexed to facilitate the measurement of gene expression levels relative to GAPDH (*in vivo* tumour samples) or 18s ribosomal RNA expression (ribosomal RNA control reagents, Applied Biosystems)(*in vitro* cell line samples) using the standard curve method.

Polyclonal Antibody Production, Purification and Western Blotting

[0137] As no commercial antibody existed for GPC5 a custom polyclonal antibody was raised to the epitope peptide H2N-CKSYTQRWNGIKAQ -COOH (16aa) by immunising two rabbits (performed by Eurogentec, Belgium). 5 mg of epitope peptide was immobilised to 8ml of SulfoLink coupling Gel (Pierce Biotechnology, IL) and used to affinity purify final bleed serum from the rabbit with the highest antibody titre. Antibody specificity was confirmed by western blotting, performed as previously described (22), using 2µL of a GPC5 *in vitro* translation reaction and 2µL of a luciferase *in vitro* translation reaction as a control. A single clean band of the correct size was obtained for GPC5. Membrane protein was extracted from cell lines using MEM-PER extraction reagent (Pierce Biotechnology, IL) ultra-filtered using YM-30 Filter (Millipore) to remove detergent and the protein concentration determined using the BCA assay kit (Pierce Biotechnology, IL).

Table 1 - Primers and Probes

Primers and Probes for Genomic Quantification of GPC5		
GPC5 Forward	5'- CCCACCCAAATCTCATCTAGAATT -3'	300 nM
GPC5 Probe - FAM Labelled	5'- CCGGGTTCCTCCCTTGCACATG -3'	100nM

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(continued)

Primers and Probes for Genomic Quantification of GPC5		
GPC5 Reverse	5'- ACGCATTGCCAGTTGTTAGA -3'	300 nM
GJB2 Forward	5'- TGGTTGCATTTAAGGTCAGAATCTT -3'	50 nM
GJB2 Probe - Vic Labelled	5'- CTAGCGACTGAGCCTTGACAGCTGAGC -3'	100 nM
GJB2 Reverse	5'-GCAGAGGCACGTTTCAGGAA-3'	300 nM
Primers and Probes for Expression Quantification of GPC5		
GPC5 Forward	5'- GGGCTGCCGGATTTCG -3'	300 nM
GPC5 Probe - FAM Labelled	5'- CGCGGGCAGGACCTGATCTTCA -3'	100 nM
GPC5 Reverse	5'- CTGGTGCAACATGTAGGCTTTT -3'	300 nM
GAPDH PDAR	Applied Biosystems Part No. 4310884E	1X
Primers and Probes for Genomic Quantification of GPC6		
GPC6 Forward	5'-TGACCAGCTCAAGCCATTG -3'	50 nM
GPC6 Probe - FAM Labelled	5'- AGACGTGCCCCGGAAACTGAAGATTC -3'	100 nM
GPC6 Reverse	5'- TGAAGGCGCGGGTAACC -3'	300nM
Primers and Probes for Expression Quantification of GPC6		
GPC6 Forward	5'- AACGAGGAGGAATGCTGGAA -3'	300 nM
GPC6 Probe - FAM Labelled	5'- CACAGCAAAGCCAGATACTTGCCTGAGATC -3'	100 nM
GPC6 Reverse	5'- CTGGTTGGTGAGCCCATCAT -3'	50 nM
Primers for amplification of GPC5 sequence including restriction sites and kozak sequence		
GPC5 Forward	5'-TATAAGCTTCCACCATGGACGCACAGACCTGGCCCCG-3'	300 nM
GPC5 Reverse	5'- CGCGTGCAGCTTACCAAATCCCGGGAAGTA -3'	300 nM

Antisense Oligonucleotides (ASOs) Targeted to GPC5 and WT1.

[0138] 20mer, 2'-O-methoxyethyl (2'-MOE) chimeric oligonucleotides consisting of a central window of eight 2'-deoxy unmodified sugar residues with flanking 2'-MOE regions and a fully thioated backbone were synthesized by Isis Pharmaceuticals Inc., as described previously (Baker, B. F., Lot, S. S., Condon, T. P., Cheng-Flournoy, S., Lesnik, E. A., Sasmor, H. M., and Bennett, C. F. (1997) *J Biol Chem* 272, 11994-12000). Twenty antisense oligonucleotides targeting predicted accessible GPC5 mRNA sequences over the full length mRNA product were provided and screened for activity in K562 cells. ISIS 15770, sequence 5'-ATGCATTCTGCCCCCAAGGA-3', a 5-10-5 gapmer targeting murine c-raf kinase was used as a control in this screen. The two active compounds identified were ISIS 276107 sequence 5'-CAGCCCCCT-GACAGCTCCCA-3', and ISIS 276119 sequence 5'-CCATCTGCAGCAGCTAATTC-3'. Also used as a control was ISIS 276124, sequence 5'-TGGATTTGCTTTACATCACT-3'.

[0139] The previously identified WT1 ASOs were ISIS 16609, sequence 5'-GCCCTTCTGTCCATTTCACT-3', targeting WT1 exon 5 (ASWT1exon 5) and ISIS 16601, sequence 5'-CACATACACATGCCCTGGCC-3', targeting the 3'-UTR region of WT1 (ASWT13'UTR). The control ASO was ISIS 105730, sequence 5'-CCATCGACCTGCACCGATCA-3', a scrambled sequence of ASWT13'UTR, (ASWT1scram).

Assessment of GPC5 and WT1 Antisense Activity.

[0140] Antisense or control oligonucleotides were dissolved in PBS and introduced into K562 cells by low voltage electroporation: 40µL of appropriately diluted ASOs were combined with 360µL of cell suspension at 2x10⁷ cells/ml and cells were electroporated (Bio-Rad Gene Pulser® II Electroporation system with Pulse Controller Plus capacitance extender accessory module, Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK) using 300V and a capacitor value of 1000µF, and diluted to 10ml with complete medium. From each sample appropriate duplicate aliquots of cells were serially diluted in complete medium for assessment of cytotoxicity while the remaining cells were incubated at 37°C for 24 hours and RNA extracted for quantification of GPC5 and/or WT1 expression levels.

Clonogenic Cell Survival Assay.

[0141] In this assay, cells are grown at low density in suspension in soft agar following treatment. Each colony formed derives from a single surviving cell. 2ml aliquots of diluted cells were added to polystyrene tubes (Elkay Products (UK) Ltd., Basingstoke, Hampshire, UK) containing 3ml of medium supplemented with 20% FCS and 0.2% Agar Noble (DIFCO Laboratories, Detroit, Michigan), incubated at 37°C, and colonies containing at least 32 cells counted after 14 days. The number of colonies formed following drug treatment were compared to the number obtained following sham electroporation or a control scrambled oligonucleotide and expressed as % control treatment. In a number of separate experiments, control plating efficiencies ranged from 22-38%, with 800-1600 cells typically plated. In addition, cells from the dilution series (approx $10^3 - 10^4$ cells/ml) were allowed to grow up in suspension culture in parallel for 3 - 5 days in order to confirm cytotoxicity.

Production of GPC5 Construct and Transfection

[0142] Image clone 5744533 containing the full coding region of human GPC5 was obtained from the ATCC (American Type Culture Collection at LGC Promochem, UK). The coding region was amplified using the Xpand High fidelity PCR kit (Roche, Switzerland) and primers to produce a product containing an in frame kozak sequence at the 5' end (see Table 1). This product was TA cloned into the vector pCR4-TOPO (Invitrogen, CA) and sequence confirmed using Applied Biosystems Big Dye Sequencing kit Version 1 and a 377 ABI Prism sequencing machine. The insert was restriction digested with EcoR1 and re-ligated into an EcoR1 digested vector pCMV-TnT (Promega, UK) using T4 DNA ligase (Invitrogen, CA). Purified pCMV-TnT-GPC5 and a control T7-luciferase plasmid were in vitro translated using Promega's Quick coupled TnT T7 in vitro translation system including biotin labelled lysine (Promega, UK) as per manufacturers instructions. Products were separated by SDS-PAGE, blotted onto Immobilon-P PVDF membrane (Millipore, UK) and colour developed using a streptavidin-Alkaline phosphatase and western blue colorimetric substrate (Promega, UK) as per manufacturers instructions.

[0143] Following cleanup of plasmids using Wizard Purefection kit (Promega, UK) plasmids were transfected into the RMS cell line T91-95 at ~75% confluence using FuGene6 transfection reagent (Roche, Switzerland) in a ratio of 3 μ l FuGene: 1 μ g DNA. pCMV-TnT-GPC5 was co-transfected in a molar ratio of 10:1 with pTK-Hyg (Clontech, CA). A control transfection using empty pCMV-TnT vector in a 10:1 molar ratio with pTK-Hyg was also performed. Cells were grown in Dulbecco's Modified Eagle Media (DMEM) and 10% foetal calf serum. 200 μ g per ml of hygromycin (Clontech, CA) was added 48hr post transfection in order to produce stable colonies. After two weeks healthy stable colonies were selected and expanded. Overexpression of GPC5 was confirmed by TaqMan RT-PCR using DNase treated cDNA (DNA Free, Ambion, TX) and increased protein was confirmed by western blot analysis.

Cell Proliferation Assay

[0144] In order to assess the growth characteristics of stably transfected cells a method based on the ability of the metabolic enzyme hexosaminidase to produce a coloured product by breaking down p-Nitrophenyl-N-acetyl- β -D-glucosaminide was used as previously described to measure cell numbers (23).

RESULTS**Comparison of CGH and CESH data**

[0145] Meta-analysis of all the CGH data available in the literature showed that the region 13q31-32 was gained in 21/87 (25%) of ARMS and 9/40 (22%) of ERMS. 5/87 (6%) of the ARMS were defined as amplifying this region (4-10) (unpublished data). CESH analysis which detects gross differential expression on a region by region basis showed that 7/27 (26%) of ARMS and 4/19 (21%) of ERMS showed overexpression from 13q31-32 relative to normal muscle. Where CGH and CESH data was available from the same sample the majority of samples with gain at 13q31-32 also showed overexpression from the region 13q31-32 7/8 (88%). In addition, 3 samples showed overexpression from the region without any apparent gain by CGH.

Interphase FISH analysis of 13q31-32 amplicon

[0146] Using BACs mapped to 13q31-32 it was possible to place the region of amplification in two primary RMS samples and the cell line K562 to a region of 13q31-32 approximately 3.8Mb in length from ~88,200K to ~91,900K on the physical map (Build 32) of chromosome 13 (see Figure 1). 2Mb of this amplified region had the highest copy number in both RMS samples and this 2Mb interval contains the gene GPC5 (Glypican 5). At ~ 2Mb in length GPC5 is the second

largest gene in the human genome to date (24). Amplification levels in K562 appeared lower and stopped short of GPC6 the next annotated gene telomeric of GPC5. In addition to GPC5, the only other curated genes within this amplified region are pseudogenes (www.ncbi.nlm.nih.gov/locuslink) and the recently-identified C13ORF25 (33). The FISH mapping of the 13q31-q32 amplification in 5 lymphoma samples by Yu et al defined a minimal amplification which spanned the same region but extended an extra 1 Mb centromeric of GPC5 (13).

Quantification of GPC5/GPC6 genomic copy number and expression

[0147] Genomic copy number of GPC5 was measured in primary tumour samples taken from 102 individuals with a diagnosis of RMS, of which 45 were ARMS, 51 were ERMS and 5 were RMS - Not Otherwise Specified (RMS-NOS). 13 out of 102 RMS (13%) show gain of GPC5 copies > 1.5 times relative to normal DNA; by subtype 7/45 ARMS (16%), 6/51 ERMS (12%). The largest amplification showed ~90 times more genomic copies than normal genomic DNA. This data was concurrent with CGH and FISH data for samples where data was available (4). There was no significant difference between genomic copy number in ARMS and ERMS. All samples with gain of GPC5 copies were measured for their GPC6 copy number and showed no gain of genomic copy number.

[0148] Expression of GPC5 was measured in 85 individuals of which 42 were diagnosed ARMS, 39 were diagnosed ERMS and 4 were diagnosed RMS-NOS. Expression of GPC5 is consistently greater than normal muscle and spans several orders of magnitude; median = 83.5 times greater than normal muscle (see Figure 2A). Although expression in samples with GPC5 amplification is always in the top quartile, overexpression is also apparent in some samples without a GPC5 amplification. Consequently there is no significant correlation between copy number and expression. Furthermore, there is no significant difference in GPC5 expression between ERMS and ARMS: ARMS median = 80.5 times greater than relative to normal muscle, ERMS median = 126 times greater than normal muscle. GPC6 expression was virtually undetectable in all but one RMS sample tested.

[0149] Expression of C13ORF25 was also determined in the same set of rhabdomyosarcoma cDNA samples. Results are shown in Figure 2B. Whilst a majority of samples with genomic amplification also show relatively high expression of C13ORF25, crucially there are 2 samples which show genomic gain but no expression. This is not the case with GPC5. Furthermore, roughly half of the samples do not show detectable expression of C13ORF25, in contrast to GPC5 where many of the samples show expression levels much higher than that seen in muscle. Whilst these data suggest that expression of C13ORF25A is affected by genomic gain, its impact on rhabdomyosarcomagenesis (if any) is likely to be less significant than that of GPC5.

Clinico-Pathological Correlations

[0150] Genomic gain of GPC5 copies appears to occur primarily in younger children. Of the 83 samples with associated age data aged <21, all 13 amplified samples were aged between 0-10 years old whereas 42 of the non-amplified samples were aged 0-10 years and the remaining 28 were aged 10-21. There is significant heterogeneity in the age at diagnosis of patients with gain of genomic copies of GPC5 and those without gain of genomic copies of tumours: Likelihood Ratio = 8.3, 32 n = 83 p = 0.0038. There is no significant difference observed between GPC5 expression or GPC5 amplification and grade, stage, time to death or event free survival.

GPC5 Downregulation and Cell Proliferation.

[0151] Twenty GPC5 targeted antisense oligonucleotides were screened for the ability to significantly reduce both GPC5 levels and cell survival. Two active compounds, ISIS 276107 and ISIS 276119, were identified in this primary screen, reducing GPC5 levels to less than 30% control levels and reducing cell survival by greater than 80% (Figure 3A). These results are compared to two other typical ASOs from this screen where both GPC5 levels and cell survival were unaffected. Although preliminary, these data suggest a correlation between downregulation of GPC5 expression and loss of cell viability.

[0152] Figures 3B and C show the effects of ISIS 276107 and a control compound (ISIS 276124) at varying concentrations in the same assay. Panel B shows the percentage of colonies in a soft agar clonogenic assay compared to sham-treated levels following treatment with the active compound ISIS 107 and a control compound ISIS 276124, which has been found not to affect cell viability or GPC5 expression (data not shown). HL-60, a control leukaemic cell line which does not express GPC5, shows no decrease in colony number following treatment with either the active ISIS 107 or the control ISIS 124 compound. Panel C shows the percentage expression of GPC5 relative to sham-treated levels 24 hours after treatment.

GPC5 expression in prostate and breast cancer

[0153] For prostate samples, the assay was carried out as for the rhabdomyosarcoma samples described above, except that expression was measured relative to normal prostate cDNA synthesised from a commercially available normal prostate RNA pool as opposed to normal muscle cDNA.

[0154] Figure 4A shows the level of expression of GPC5 in a number of individual samples. Several samples show greater expression than normal prostate. Furthermore, there is a significantly greater expression in prostate cancer compared with Benign Prostatic Hyperplasia (BPH) Mann-Whitney U = 16 p = 0.011 N= 24. However the significance of results should be interpreted with caution as there are only six BPH samples. Taken together this data does suggest a potential role for GPC5 overexpression in the development of prostate cancer.

[0155] Figure 4B shows overall GPC5 expression in a larger sample of prostate adenocarcinoma (n=45) and benign prostate hyperplasia (n=25) relative to normal prostate tissue. The samples used here include those for which results are shown in Figure 4A.

[0156] For the breast cancer samples shown in Figure 5A, measurement was performed relative to normal breast cDNA biopsy tissue as opposed to normal muscle cDNA, and was performed in duplicate instead of triplicate.

[0157] The data shows relative overexpression of GPC5 in some samples. The highest value was in biopsies taken from lymph nodes with metastasis. This suggests potential involvement of GPC5 in tumourigenesis in these samples.

[0158] Expression of GPC5 was then measured by TaqMan analysis in seven normal breast samples and 44 breast cancer samples. The mean normal breast GPC5 expression and 95% confidence intervals were calculated. Any tumour sample in which GPC5 expression exceeded the upper confidence interval for normal samples was said to overexpress GPC5. The samples were grouped according to the stage of the disease (stage 1, 2 or 3). 5 out of the 6 samples which show overexpression are stage 3. There is a significant difference in the stage of those samples which overexpress GPC5; Fisher's Exact Test p = 0.017 n = 44 (Figure 5B).

Correlation with MYCN Expression

[0159] For some of the samples we already had information about expression of MYCN from a previous TaqMan study (data not shown). It was found that in ERMS and ARMS with a confirmed PAX/FOXO1A translocation that expression of GPC5 correlated significantly with expression of MYCN : Spearman's Rho = 0.497 n = 38 p = 0.002 and Spearman's Rho = 0.399 n = 26 p = 0.043 respectively.

Regulation of GPC5 expression by the Wilms' Tumour gene (WT1) product.

[0160] Previous gene profiling studies following treatment of K562 cells with WT1 directed ASOs, identified GPC5 as a putative WT1 target gene (manuscript in preparation). Downregulation of GPC5 expression following WT1 antisense treatment and correlating with reduced WT1 expression was confirmed in independent experiments (see Figure 6). Similarly, preliminary *in vivo* studies have demonstrated downregulation of GPC5 in PC3M prostate cancer cells grown as subcutaneous implants in athymic nude mice treated with WT1 antisense: A 50% decrease in WT1 expression levels in ASWT1exon5 mice was reflected by a 30% reduction in GPC5 expression (data not shown). Direct transcriptional regulation of GPC5 by WT1 is likely since GPC5 has two WT1 consensus binding sites in its promoter region.

[0161] Using the same *in vivo* model system we have obtained preliminary evidence of upregulation of both WT1 and GPC5 expression in PC3M tumours following treatment of the mice with docetaxol (15mg/kg): A nine fold increase in WT1 expression was reflected by a 5 fold increase in GPC5 expression. These studies raise the possibility that GPC5 overexpression may be induced by cancer chemotherapy. The PC3M tumour model is relatively resistant to cytotoxic drug treatment giving rise to the speculation that upregulation of GPC5 expression may contribute to poor response to therapy.

GPC5 Overexpression and Cell Proliferation

[0162] In order to test if overexpression of GPC5 could confer oncogenic properties, a GPC5 construct was made under the control of a CMV (Cyto-Megalo Virus) promoter in order to constitutively express GPC5. This construct was tested by *in vitro* translation and was shown to produce the appropriate size protein (63 K Daltons) (see Figure 8C). RMS cell line T91-95 was stably transfected and healthy colonies were picked. T91-95 is an RMS cell line which expresses both GPC5 and MYCN at levels similar to that of normal muscle. 5 GPC5 transfected and 5 empty vector control transfected colonies were randomly selected and subjected to a cell proliferation assay.

[0163] Cells were plated at 7500 cells per 24 well plate in triplicate repeats. In order to allow cells to recover and to normalise for potential variations in plating efficiency and cell counting errors the first measurement was taken at 16hrs. The proliferation assay uses a colorimetric PNNAG assay in which absorbance is proportional to number of cells. Log

Normalised Growth is calculated as the mean natural logarithm of absorbance of triplicates at 64 hours minus the mean natural logarithm of absorbance of triplicates at 16 hours. There is a significant difference between the Log Normalised Growth in GPC5 overexpressing colonies compared to control colonies $p = 0.027$ $t = 2.70$ $n = 10$ (Figure 8A).

[0164] Figure 8B shows the expression level of GPC5 as measured by TaqMan assay in GPC5 overexpressing colonies and control colonies, as compared to normal muscle tissue.

DISCUSSION

[0165] Our data support and characterize a potential role for the GPC5 gene, a cell surface heparan sulfate proteoglycan, in the development of RMS. Analysis of previous data indicated differential overexpression corresponding to the 13q31-32 region, which harbors the GPC5 gene, was found in cases which amplify the region and also in some cases without amplification of both the alveolar and embryonal subtypes. This data was mirrored by the quantitative analysis of GPC5 copy number and expression and together suggests that overexpression of GPC5 is important in the pathogenesis of RMS of both subtypes. The data is also consistent with gene expression being up-regulated by mechanisms other than genomic amplification.

[0166] The Glypicans are a family of conserved cell surface heparan sulfate proteoglycans which are believed to modulate the activity of heparan binding growth factors such as FGFs (fibroblast growth factors) and Wnts (25). Deregulation of other members of the glypican family has been implicated in development of a number of tumors. GPC1 (Glypican-1) has been shown to be overexpressed in human pancreatic cancer and to positively regulate the action of HB-EGF (Heparan Binding Epidermal Growth Factor) and FGF2 (Fibroblast Growth Factor 2) (26). Furthermore, stable transfection of a GPC1 antisense construct in the GPC1 overexpressing pancreatic cancer cell line PANC-1 decreased tumourigenicity (27). GPC1 is similarly overexpressed in breast cancer and modulates the activity of a number of heparan binding growth factors (28). GPC3 has been shown to aberrantly overexpress in 7/10 neuroblastoma cell lines, 4/4 primary neuroblastoma samples and 7/7 primary Wilms tumour samples (29). Expression in these tumours is shown to correlate with IGF2 expression (Insulin-like Growth Factor II) a gene which is frequently overexpressed in RMS (30).

[0167] Examination of the only two annotated genes in the 13q31 region has demonstrated that the 13q31 amplification in RMS includes the gene GPC5 but not GPC6. Although no other sequences from the region have been shown to be expressed, it remains possible that as yet uncharacterised genes exert an effect. In contrast to Yu et al our amplification appears to peak centrally at the gene GPC5. As we have demonstrated a functional consequence of elevated protein levels of GPC5 in RMS cells it is likely that the overexpression of this gene associated with amplification in lymphoma cell lines is exerting a similar effect. In addition, we note amplification and/or overexpression from 13q31-32 in another type of soft tissue sarcoma, namely leiomyosarcomas which resemble smooth muscle tissue (11, 20)(Unpublished Data). Other sarcoma types have also been documented with amplification of this region and therefore it is possible that up regulation of this gene is a more general feature of soft tissue sarcomas. This may extend to other tumour types described with amplification of this region such as breast cancer, small-cell lung cancer, medulloblastoma and glioblastoma (11-17). Preliminary data for the expression levels of GPC5 in prostate and breast cancers relative to corresponding normal tissues suggests that some of these tumours aberrantly overexpress this gene.

[0168] Whilst amplification and/or overexpression of GPC5 are a frequent occurrence in RMS, we provide no evidence that these are likely to be of use as clinical markers of prognosis. In the series studied there does not appear to be a significant difference in the survival characteristics of patients with tumors amplifying or overexpressing GPC5 compared to those that do not. Furthermore, amplification seems to predominate in patients between 0-10 years; a clinical group with generally improved prognosis (31). Further analysis of a larger cohort of samples, perhaps using a tissue array, may identify sub-groups of rhabdomyosarcoma in which deregulation of GPC5 is clinically significant. Another possibility is that detection of GPC5 protein in blood serum of patients may serve as a marker of RMS or other cancers in a similar manner to which GPC1 protein in the serum identifies patients with hepatocellular carcinoma (32).

[0169] The fact that expression of GPC5 correlates with expression of the oncogene MYCN is further evidence to support the role of GPC5 in the tumourigenesis of RMS. It is unclear from this study whether the relationship is causal. Certainly GPC5 overexpression in our in vitro model does not cause overexpression of MYCN. It is noteworthy, however, that the sequence proximally upstream of the start of GPC5 transcription contains an E-box (MYC trans-activation site) consensus sequence suggesting potential direct trans-activation of GPC5 by MYCN.

[0170] Potentially as significant, is the apparent regulation of GPC5 by the WT1 gene product. Although not proven to be oncogenic, WT1 may contribute to the maintenance of a malignant phenotype in leukaemias and the large range of solid tumours where its expression is deregulated. In addition, WT1 has been implicated in drug resistance mechanisms and is being investigated as a potential target for cancer therapy. Our studies so far indicate that GPC5 may well mediate at least some of the downstream biological effects of WT1 expression making GPC5, potentially, the more effective target for therapeutic intervention.

[0171] In conclusion, we have established that GPC5 is amplified and/or overexpressed in RMS and that GPC5 is a novel oncogene. GPC5 is particularly attractive target for novel therapies for a number of reasons. First, because it is

a cell surface protein it is physically accessible to a number of potential anti-GPC5 therapies. Second, because it potentially acts as a modulator of multiple growth factors therapies which reduce the function of GPC5 could therefore affect multiple tumorigenic pathways. Third, because it is likely to be important in the tumorigenesis of a number of other cancer types.

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Claims

1. An in vitro method of inhibiting proliferation of a target cell, comprising contacting the cell with a GPC5 antagonist comprising a nucleic acid sequence complementary to the sequence of GPC5 mRNA or pre-mRNA or an antibody which is capable of binding to and inhibiting the activity of GPC5.
2. Use of a GPC5 antagonist for the manufacture of a medicament for inhibiting proliferation of a target cell, wherein the GPC5 antagonist comprises a nucleic acid sequence complementary to the sequence of GPC5 mRNA or pre-mRNA or wherein the GPC5 antagonist is an antibody which is capable of binding to and inhibiting the activity of GPC5.
3. A GPC5 antagonist for use in a method of treatment comprising inhibiting proliferation of a target cell, wherein said GPC5 antagonist comprises a nucleic acid sequence complementary to the sequence of GPC5 mRNA or pre-mRNA or wherein the GPC5 antagonist is an antibody which is capable of binding to and inhibiting the activity of GPC5.
4. A method according to claim 1, use according to claim 2 or GPC5 antagonist for use in a method of treatment according to claim 3, wherein the target cell inappropriately expresses or overexpresses GPC5.

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5. A method, use or GPC5 antagonist for use in a method of treatment according to any one of the preceding claims wherein the cell inappropriately expresses or overexpresses WT1.
- 5 6. A method, use or GPC5 antagonist for use in a method of treatment according to any one of the preceding claims wherein the cell is a cancer cell.
7. A method, use or GPC5 antagonist for use in a method of treatment according to claim 6 wherein the cancer is rhabdomyosarcoma, lymphoma, non-small cell lung cancer, bladder cancer, breast cancer, prostate cancer, a neuroglial tumour, squamous cell carcinoma of the head and neck, leukemia, leiomyosarcoma, liposarcoma, malignant fibrous histiocytoma of bone or soft tissues, melanoma, mesothelioma, thyroid cancer, lung cancer, testicular cancer or ovarian cancer.
10
8. A method, use or GPC5 antagonist for use in a method of treatment according to any one of the preceding claims wherein the cell does not carry a chromosomal amplicon at 13q31.
15
9. A method, use or GPC5 antagonist for use in a method of treatment according to any one of the preceding claims wherein the antibody binds to the GPC5 core protein and/or its associated heparan sulphate chains.
10. A method, use or GPC5 antagonist for use in a method of treatment according to any one of claims 1 to 8 wherein the GPC5 antagonist comprises antisense RNA, dsRNA such as RNAi or siRNA, or a ribozyme.
20
11. A method, use or GPC5 antagonist for use in a method of treatment according to any one of the preceding claim further comprising contacting the cell with a therapeutic agent.
- 25 12. A method, use or GPC5 antagonist for use in a method of treatment according to claim 11 wherein the GPC5 antagonist increases the sensitivity of the cell to the therapeutic agent.
13. An in vitro method of inhibiting proliferation of a target cell, comprising contacting the cell with an antibody capable of binding to GPC5 and a therapeutic agent, said therapeutic agent being associated with said antibody or capable of binding to said antibody.
30
14. Use of an antibody capable of binding to GPC5 for the manufacture of a medicament for inhibiting proliferation of a target cell, wherein said medicament is for inhibiting proliferation of a target cell in combination with a therapeutic agent, said therapeutic agent being associated with said antibody or capable of binding to said antibody.
35
15. An antibody capable of binding to GPC5 for use in a method of treatment comprising inhibiting proliferation of a target cell, said method further comprising contacting the target cell with a therapeutic agent, said therapeutic agent being associated with said antibody or capable of binding to said antibody.
- 40 16. The method, use, GPC5 antagonist or antibody for use in a method of treatment, according to any one of claims 12 to 15, wherein the therapeutic agent comprises a cytotoxic molecule or a precursor molecule capable of being converted into a cytotoxic molecule by enzyme action, or a cell or molecule of the immune system.
17. An in vitro method of determining the susceptibility of a cancer to treatment with a GPC5 antagonist or binding agent, comprising determining the presence, absence or level of expression of GPC5 and/or WT1 in a cell from said cancer.
45
18. An in vitro method according to claim 17 further comprising the step of determining the presence, absence or degree of chromosomal amplification at 13q31 in a cell from said cancer.
- 50 19. An in vitro method of determining the susceptibility of a cancer to treatment with a GPC5 antagonist or binding agent, comprising determining the presence, absence or degree of chromosomal amplification at 13q31.
20. A method according to claim 19 further comprising determining the presence, absence or level of expression of GPC5 and/or WT1 in a cell from said cancer.
55
21. A method according to any one of claims 17 to 20 wherein the cancer is rhabdomyosarcoma, lymphoma, non-small cell lung cancer, bladder cancer, breast cancer, prostate cancer, a neuroglial tumour, squamous cell carcinoma of the head and neck, leukemia, leiomyosarcoma, liposarcoma, malignant fibrous histiocytoma of bone or soft tissues,

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melanoma, mesothelioma, thyroid cancer, lung cancer, testicular cancer or ovarian cancer.

22. An in vitro method of screening for the presence of a cancer in a patient, the method comprising determining the presence, absence or level of circulating GPC5 in a sample derived from the patient.

23. A method according to claim 22 wherein the sample is blood, serum or plasma.

24. A method according to claim 22 or claim 23 wherein the method comprises contacting the sample with an antibody capable of binding to GPC5.

25. A method according to any one of claims 22 to 24 wherein the cancer is rhabdomyosarcoma, lymphoma, non-small cell lung cancer, bladder cancer, breast cancer, prostate cancer, a neuroglial tumour, squamous cell carcinoma of the head and neck, leukemia, leiomyosarcoma, liposarcoma, malignant fibrous histocytoma of bone or soft tissues, melanoma, mesothelioma, thyroid cancer, lung cancer, testicular cancer or ovarian cancer.

26. An in vitro method of screening for an agent capable of inhibiting proliferation of a target cell, comprising the steps of:

- (i) contacting GPC5 protein with one or more candidate substances;
- (ii) selecting one or more candidate substances based on their ability to bind GPC5 protein;
- (iii) contacting said one or more selected substances with a target cell; and
- (iv) determining the effect of said selected substance(s) on proliferation of said cell.

27. A method according to claim 26 wherein the cell inappropriately expresses or overexpresses GPC5.

28. A method according to claim 26 or claim 27 wherein the cell is a cancer cell.

29. A method according to claim 28 wherein the cancer is rhabdomyosarcoma, lymphoma, non-small cell lung cancer, bladder cancer, breast cancer, prostate cancer, a neuroglial tumour, squamous cell carcinoma of the head and neck, leukemia, leiomyosarcoma, liposarcoma, malignant fibrous histocytoma of bone or soft tissues, melanoma, mesothelioma, thyroid cancer, lung cancer, testicular cancer or ovarian cancer.

30. An in vitro method for determining a prognosis for a patient with breast cancer comprising assigning a prognosis to the patient based on the expression levels of GPC5 in a breast tumour from that patient.

31. A method according to claim 30 which comprises determining the presence; absence or degree of expression of GPC5 in vitro using a sample containing breast cancer cells from the patient.

32. A method according to claim 31 which comprises contacting the sample with an antibody capable of binding to GPC5.

33. A method for monitoring the success of a treatment for a cancer previously found to express GPC5, comprising determining GPC5 expression in cells of the cancer, wherein said method is performed in vitro using a sample containing cancer cells from the patient.

34. A method according to claim 33 wherein said step of determining GPC5 expression comprises contacting cells of the cancer with an antibody capable of binding to GPC5.

35. A method according to claim 33 or 34 comprising comparing the results obtained with results of an equivalent assay performed for the same patient before treatment and/or at an earlier stage of treatment.

36. A method according to claim 33 or 34 comprising determining the level of expression of GPC5 within cells of the cancer, or determining the number or density of cells expressing or overexpressing GPC5.

Patentansprüche

1. In-vitro-Verfahren zum Hemmen der Proliferation einer Zielzelle, umfassend das Kontaktieren der Zelle mit einem GPC5-Antagonisten, der eine Nucleinsäuresequenz umfasst, welche zu der Sequenz von GPC5-mRNA oder -Prä-mRNA komplementär ist, oder mit einem Antikörper, der in der Lage ist, an GPC5 zu binden und dessen Aktivität

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zu hemmen.

2. Verwendung eines GPC5-Antagonisten zur Herstellung eines Medikaments zum Hemmen der Proliferation einer Zielzelle, worin der GPC5-Antagonist eine Nucleinsäuresequenz umfasst, welche zu der Sequenz von GPC5-mRNA oder -Prä-mRNA komplementär ist, oder worin der GPC5-Antagonist ein Antikörper ist, der in der Lage ist, an GPC5 zu binden und dessen Aktivität zu hemmen.
3. GPC5-Antagonist zur Verwendung in einem Behandlungsverfahren, das das Hemmen der Proliferation einer Zielzelle umfasst, worin der GPC5-Antagonist eine Nucleinsäuresequenz umfasst, welche zu der Sequenz von GPC5-mRNA oder -Prä-mRNA komplementär ist, oder worin der GPC5-Antagonist ein Antikörper ist, der in der Lage ist, an GPC5 zu binden und dessen Aktivität zu hemmen.
4. Verfahren nach Anspruch 1, Verwendung nach Anspruch 2 oder GPC5-Antagonist zur Verwendung in einem Behandlungsverfahren nach Anspruch 3, worin die Zielzelle GPC5 unangemessen exprimiert oder überexprimiert.
5. Verfahren, Verwendung oder GPC5-Antagonist zur Verwendung in einem Behandlungsverfahren nach einem der vorangegangenen Ansprüche, worin die Zelle WT1 unangemessen exprimiert oder überexprimiert.
6. Verfahren, Verwendung oder GPC5-Antagonist zur Verwendung in einem Behandlungsverfahren nach einem der vorangegangenen Ansprüche, worin die Zelle eine Krebszelle ist.
7. Verfahren, Verwendung oder GPC5-Antagonist zur Verwendung in einem Behandlungsverfahren nach einem der vorangegangenen Ansprüche, worin der Krebs Rhabdomyosarkom, Lymphom, nichtkleinzelliger Lungenkrebs, Blasenkrebs, Brustkrebs, Prostatakrebs, ein neuroglialer Tumor, Plattenepithelkarzinom im Kopf-Hals-Bereich, Leukämie, Leiomyosarkom, Liposarkom, malignes fibröses Histiozytom des Knochen- oder Weichteilgewebes, Melanom, Mesotheliom, Schilddrüsenkrebs, Lungenkrebs, Hodenkrebs oder Eierstockkrebs ist.
8. Verfahren, Verwendung oder GPC5-Antagonist zur Verwendung in einem Behandlungsverfahren nach einem der vorangegangenen Ansprüche, worin die Zelle an 13q31 kein chromosomales Amplicon trägt.
9. Verfahren, Verwendung oder GPC5-Antagonist zur Verwendung in einem Behandlungsverfahren nach einem der vorangegangenen Ansprüche, worin der Antikörper an das GPC5-Kernprotein bindet und/oder mit dessen Heparansulfatketten assoziiert ist.
10. Verfahren, Verwendung oder GPC5-Antagonist zur Verwendung in einem Behandlungsverfahren nach einem der Ansprüche 1 bis 8, worin der GPC5-Antagonist Antisense-RNA, dsRNA, wie z.B. RNAi oder siRNA, oder ein Ribozym umfasst.
11. Verfahren, Verwendung oder GPC5-Antagonist zur Verwendung in einem Behandlungsverfahren nach einem der vorangegangenen Ansprüche, weiters umfassend das Kontaktieren der Zelle mit einem therapeutischen Mittel.
12. Verfahren, Verwendung oder GPC5-Antagonist zur Verwendung in einem Behandlungsverfahren nach Anspruch 11, worin der GPC5-Antagonist die Empfindlichkeit der Zelle für das therapeutische Mittel erhöht.
13. In-vitro-Verfahren zum Hemmen der Proliferation einer Zielzelle, umfassend das Kontaktieren der Zelle mit einem Antikörper, der in der Lage ist, an GPC5 zu binden, und einem therapeutischen Mittel, wobei das therapeutische Mittel mit dem Antikörper assoziiert ist oder in der Lage ist, an den Antikörper zu binden.
14. Verwendung eines Antikörpers, der in der Lage ist, an GPC5 zu binden, zur Herstellung eines Medikaments zum Hemmen der Proliferation einer Zielzelle, worin das Medikament dem Hemmen der Proliferation einer Zielzelle in Kombination mit einem therapeutischen Mittel dient, wobei das therapeutische Mittel mit dem Antikörper assoziiert ist oder in der Lage ist, an den Antikörper zu binden.
15. Antikörper, der in der Lage ist, an GPC5 zu binden, zur Verwendung zur Herstellung eines Medikaments zum Hemmen der Proliferation einer Zielzelle, wobei das Verfahren weiters das Kontaktieren der Zielzelle mit einem therapeutischen Mittel umfasst, wobei das therapeutische Mittel mit dem Antikörper assoziiert ist oder in der Lage ist, an den Antikörper zu binden.

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- 5
16. Verfahren, Verwendung, GPC5-Antagonist oder Antikörper zur Verwendung in einem Behandlungsverfahren nach einem der Ansprüche 12 bis 15, worin das therapeutische Mittel ein zytotoxisches Molekül oder ein Vorläufermolekül, das in der Lage ist, durch Enzymwirkung in ein zytotoxisches Molekül überführt zu werden, oder eine Zelle oder ein Molekül des Immunsystems umfasst.
- 10
17. In-vitro-Verfahren zur Bestimmung der Empfänglichkeit eines Krebses für die Behandlung mit einem GPC5-Antagonisten oder -Bindemittel, umfassend das Bestimmen der Gegenwart, der Abwesenheit oder des Expressionsausmaßes von GPC5 und/oder WT1 in einer Zelle aus dem Krebs.
- 15
18. In-vitro-Verfahren nach Anspruch 17, weiters umfassend den Schritt des Bestimmens der Gegenwart, der Abwesenheit oder des Grades der chromosomalen Amplifikation an 13q31 in einer Zelle aus dem Krebs.
19. In-vitro-Verfahren zur Bestimmung der Empfänglichkeit eines Krebses für die Behandlung mit einem GPC5-Antagonisten oder -Bindemittel, umfassend das Bestimmen der Gegenwart, der Abwesenheit oder des Grades der chromosomalen Amplifikation an 13q31.
20. Verfahren nach Anspruch 19, weiters umfassend das Bestimmen der Gegenwart, der Abwesenheit oder des Expressionsausmaßes von GPC5 und/oder WT1 in einer Zelle aus dem Krebs.
- 20
21. Verfahren nach einem der Ansprüche 17 bis 20, worin der Krebs Rhabdomyosarkom, Lymphom, nichtkleinzelliger Lungenkrebs, Blasenkrebs, Brustkrebs, Prostatakrebs, ein neuroglialer Tumor, Plattenepithelkarzinom im Kopf-Hals-Bereich, Leukämie, Leiomyosarkom, Liposarkom, malignes fibröses Histiozytom des Knochen- oder Weichteilgewebes, Melanom, Mesotheliom, Schilddrüsenkrebs, Lungenkrebs, Hodenkrebs oder Eierstockkrebs ist.
- 25
22. In-vitro-Verfahren zum Screenen nach dem Vorliegen eines Krebses bei einem Patienten, wobei das Verfahren das Bestimmen der Gegenwart, der Abwesenheit oder des Ausmaßes von zirkulierendem GPC5 in einer dem Patienten entnommenen Probe umfasst.
- 30
23. Verfahren nach Anspruch 22, worin die Probe Blut, Serum oder Plasma ist.
24. Verfahren nach Anspruch 22 oder Anspruch 23, worin das Verfahren das Kontaktieren der Probe mit einem Antikörper umfasst, der in der Lage ist, an GPC5 zu binden.
- 35
25. Verfahren nach einem der Ansprüche 22 bis 24, worin der Krebs Rhabdomyosarkom, Lymphom, nichtkleinzelliger Lungenkrebs, Blasenkrebs, Brustkrebs, Prostatakrebs, ein neuroglialer Tumor, Plattenepithelkarzinom im Kopf-Hals-Bereich, Leukämie, Leiomyosarkom, Liposarkom, malignes fibröses Histiozytom des Knochen- oder Weichteilgewebes, Melanom, Mesotheliom, Schilddrüsenkrebs, Lungenkrebs, Hodenkrebs oder Eierstockkrebs ist.
- 40
26. In-vitro-Verfahren zum Screenen nach einem Mittel, das in der Lage ist, die Proliferation einer Zielzelle zu hemmen, umfassend die folgenden Schritte:
- 45
- (i) das Kontaktieren von GPC5-Protein mit einer oder mehreren Kandidatensubstanzen;
 - (ii) das Selektieren einer oder mehrerer Kandidatensubstanzen auf Grund ihrer Fähigkeit, an GPC5-Protein zu binden;
 - (iii) das Kontaktieren der einen oder mehreren selektierten Substanzen mit einer Zielzelle; und
 - (iv) das Bestimmen der Wirkung der selektierten Substanz(en) auf die Proliferation der Zelle.
27. Verfahren nach Anspruch 26, worin die Zelle GPC5 unangemessen exprimiert oder überexprimiert.
- 50
28. Verfahren nach Anspruch 26 oder Anspruch 27, worin die Zelle eine Krebszelle ist.
29. Verfahren nach Anspruch 28, worin der Krebs Rhabdomyosarkom, Lymphom, nichtkleinzelliger Lungenkrebs, Blasenkrebs, Brustkrebs, Prostatakrebs, ein neuroglialer Tumor, Plattenepithelkarzinom im Kopf-Hals-Bereich, Leukämie, Leiomyosarkom, Liposarkom, malignes fibröses Histiozytom des Knochen- oder Weichteilgewebes, Melanom, Mesotheliom, Schilddrüsenkrebs, Lungenkrebs, Hodenkrebs oder Eierstockkrebs ist.
- 55
30. In-vitro-Verfahren zur Bestimmung einer Prognose für einen Patienten mit Brustkrebs, umfassend das Erstellen einer Prognose für den Patienten auf Basis der Expressionsausmaße von GPC5 in einem Brusttumor dieses Pati-

enten.

- 5
31. Verfahren nach Anspruch 30, umfassend das Bestimmen der Gegenwart, der Abwesenheit oder des Grades der Expression von GPC5 *in vitro* unter Verwendung einer Probe, die Brustkrebszellen des Patienten enthält.
- 10
32. Verfahren nach Anspruch 31, umfassend das Kontaktieren der Probe mit einem Antikörper, der in der Lage ist, an GPC5 zu binden.
- 15
33. Verfahren zur Überwachung des Erfolgs einer Behandlung für einen Krebs, von dem zuvor herausgefunden wurde, dass er GPC5 exprimiert, umfassend das Bestimmen der GPC5-Expression in Zellen des Krebses, worin das Verfahren *in vitro* unter Verwendung einer Probe durchgeführt wird, die Krebszellen des Patienten enthält.
- 20
34. Verfahren nach Anspruch 33, worin der Schritt des Bestimmens der GPC5-Expression das Kontaktieren der Zellen des Krebses mit einem Antikörper umfasst, der in der Lage ist, an GPC5 zu binden.
- 25
35. Verfahren nach Anspruch 33 oder 34, umfassend das Vergleichen der erhaltenen Ergebnisse mit Ergebnissen eines gleichwertigen Tests, der an demselben Patienten vor der Behandlung und/oder in einem früheren Stadium der Behandlung vorgenommen wurde.
- 30
36. Verfahren nach Anspruch 33 oder 34, umfassend das Bestimmen des Expressionsausmaßes von GPC5 in Zellen des Krebses oder das Bestimmen der Anzahl oder der Dichte der Zellen, die GPC5 exprimieren oder überexprimieren.

Revendications

- 25
1. Procédé *in vitro* pour inhiber la prolifération d'une cellule cible, consistant à mettre la cellule en contact avec un antagoniste de GPC5 comprenant une séquence d'acide nucléique complémentaire à la séquence d'ARNm ou de pré-ARNm de GPC5, ou un anticorps qui est capable de se lier à et d'inhiber l'activité de GPC5.
- 30
2. Utilisation d'un antagoniste de GPC5 pour la fabrication d'un médicament destiné à inhiber la prolifération d'une cellule cible, dans laquelle l'antagoniste de GPC5 comprend une séquence d'acide nucléique complémentaire à la séquence d'ARNm ou de pré-ARNm de GPC5, ou dans laquelle l'antagoniste de GPC5 est un anticorps qui est capable de se lier à et d'inhiber l'activité de GPC5.
- 35
3. Antagoniste de GPC5 destiné à être utilisé dans un procédé de traitement qui consiste à inhiber la prolifération d'une cellule cible, dans lequel ledit antagoniste de GPC5 comprend une séquence d'acide nucléique complémentaire à la séquence d'ARNm ou de pré-ARNm de GPC5, ou dans lequel l'antagoniste de GPC5 est un anticorps qui est capable de se lier à et d'inhiber l'activité de GPC5.
- 40
4. Procédé selon la revendication 1, utilisation selon la revendication 2, ou antagoniste de GPC5 destiné à être utilisé dans un procédé de traitement selon la revendication 3, où la cellule cible exprime de manière inappropriée ou surexprime GPC5.
- 45
5. Procédé, utilisation ou antagoniste de GPC5 destiné à être utilisé dans un procédé de traitement selon l'une quelconque des revendications précédentes, où la cellule exprime de manière inappropriée ou surexprime WT1.
- 50
6. Procédé, utilisation ou antagoniste de GPC5 destiné à être utilisé dans un procédé de traitement selon l'une quelconque des revendications précédentes, où la cellule est une cellule cancéreuse.
- 55
7. Procédé, utilisation ou antagoniste de GPC5 destiné à être utilisé dans un procédé de traitement selon la revendication 6, où le cancer est un rhabdomyosarcome, un lymphome, un cancer du poumon non à petites cellules, un cancer de la vessie, un cancer du sein, un cancer de la prostate, une tumeur neurogliale, un carcinome à cellules squameuses de la tête et du cou, une leucémie, un léiomyosarcome, un liposarcome, un histiocytome fibreux malin des os ou des tissus mous, un mélanome, un mésothéliome, un cancer de la thyroïde, un cancer du poumon, un cancer des testicules ou un cancer des ovaires.
8. Procédé, utilisation ou antagoniste de GPC5 destiné à être utilisé dans un procédé de traitement selon l'une quelconque des revendications précédentes, où la cellule ne porte par d'amplicon chromosomique à 13q31.

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9. Procédé, utilisation ou antagoniste de GPC5 destiné à être utilisé dans un procédé de traitement selon l'une quelconque des revendications précédentes, où l'anticorps se lie à la protéine centrale de GPC5 et/ou à ses chaînes de sulfate d'héparane associées.
- 5 10. Procédé, utilisation ou antagoniste de GPC5 destiné à être utilisé dans un procédé de traitement selon l'une quelconque des revendications 1 à 8, où l'antagoniste de GPC5 comprend un ARN antisens, un ARNdb comme un ARNi ou un ARNsi, ou un ribozyme.
- 10 11. Procédé, utilisation ou antagoniste de GPC5 destiné à être utilisé dans un procédé de traitement selon l'une quelconque des revendications précédentes, consistant en outre à mettre la cellule en contact avec un agent thérapeutique.
- 15 12. Procédé, utilisation ou antagoniste de GPC5 destiné à être utilisé dans un procédé de traitement selon la revendication 11, où l'antagoniste de GPC5 augmente la sensibilité de la cellule à l'agent thérapeutique.
- 20 13. Procédé *in vitro* pour inhiber la prolifération d'une cellule cible, consistant à mettre la cellule en contact avec un anticorps capable de se lier à GPC5 et un agent thérapeutique, ledit agent thérapeutique étant associé audit anticorps ou étant capable de se lier audit anticorps.
- 25 14. Utilisation d'un anticorps capable de se lier à GPC5 pour la fabrication d'un médicament destiné à inhiber la prolifération d'une cellule cible, où ledit médicament est destiné à inhiber la prolifération d'une cellule cible en combinaison avec un agent thérapeutique, ledit agent thérapeutique étant associé audit anticorps ou étant capable de se lier audit anticorps.
- 30 15. Anticorps capable de se lier à GPC5 qui est destiné à être utilisé dans un procédé de traitement consistant à inhiber la prolifération d'une cellule cible, ledit procédé consistant en outre à mettre la cellule cible en contact avec un agent thérapeutique, ledit agent thérapeutique étant associé audit anticorps ou étant capable de se lier audit anticorps.
- 35 16. Procédé, utilisation, antagoniste de GPC5 ou anticorps destiné à être utilisé dans un procédé de traitement selon l'une quelconque des revendications 12 à 15, où l'agent thérapeutique comprend une molécule cytotoxique ou une molécule précurseur capable d'être convertie en une molécule cytotoxique par une action enzymatique, ou une cellule ou une molécule du système immunitaire.
- 40 17. Procédé *in vitro* pour déterminer la sensibilité d'un cancer à un traitement avec un antagoniste de GPC5 ou un agent de liaison, consistant à déterminer la présence, l'absence ou le taux d'expression de GPC5 et/ou de WT1 dans une cellule dudit cancer.
- 45 18. Procédé *in vitro* selon la revendication 17, comprenant en outre l'étape consistant à déterminer la présence, l'absence ou le degré d'amplification chromosomique à 13q31 dans une cellule dudit cancer.
- 50 19. Procédé *in vitro* pour déterminer la sensibilité d'un cancer à un traitement avec un antagoniste de GPC5 ou un agent de liaison, consistant à déterminer la présence, l'absence ou le degré d'amplification chromosomique à 13q31.
- 55 20. Procédé selon la revendication 19, consistant en outre à déterminer la présence, l'absence ou le taux d'expression de GPC5 et/ou de WT1 dans une cellule dudit cancer.
21. Procédé selon l'une quelconque des revendications 17 à 20, dans lequel le cancer est un rhabdomyosarcome, un lymphome, un cancer du poumon non à petites cellules, un cancer de la vessie, un cancer du sein, un cancer de la prostate, une tumeur neurogliale, un carcinome à cellules squameuses de la tête et du cou, une leucémie, un léiomyosarcome, un liposarcome, un histiocytome fibreux malin des os ou des tissus mous, un mélanome, un mésothéliome, un cancer de la thyroïde, un cancer du poumon, un cancer des testicules ou un cancer des ovaires.
22. Procédé *in vitro* pour dépister la présence d'un cancer chez un patient, le procédé consistant à déterminer la présence, l'absence ou le taux de GPC5 circulante dans un échantillon dérivé du patient.
23. Procédé selon la revendication 22, dans lequel l'échantillon est du sang, du sérum ou du plasma.
24. Procédé selon la revendication 22 ou la revendication 23, où le procédé consiste à mettre l'échantillon en contact

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avec un anticorps capable de se lier à GPC5.

- 5
25. Procédé selon l'une quelconque des revendications 22 à 24, dans lequel le cancer est un rhabdomyosarcome, un lymphome, un cancer du poumon non à petites cellules, un cancer de la vessie, un cancer du sein, un cancer de la prostate, une tumeur neurogliale, un carcinome à cellules squameuses de la tête et du cou, une leucémie, un léiomyosarcome, un liposarcome, un histiocytome fibreux malin des os ou des tissus mous, un mélanome, un mésothéliome, un cancer de la thyroïde, un cancer du poumon, un cancer des testicules ou un cancer des ovaires.
- 10
26. Procédé *in vitro* pour cribler un agent capable d'inhiber la prolifération d'une cellule cible, comprenant les étapes consistant à:
- 15
- (i) mettre une protéine GPC5 en contact avec une ou plusieurs substances candidates;
 - (ii) sélectionner une ou plusieurs substances candidates en se basant sur son/leur aptitude à se lier à la protéine GPC5;
 - (iii) mettre ladite/lesdites une ou plusieurs substances sélectionnées en contact avec une cellule cible; et
 - (iv) déterminer l'effet de ladite/lesdites substance(s) sur la prolifération de ladite cellule.
- 20
27. Procédé selon la revendication 26, dans lequel la cellule exprime de manière inappropriée ou surexprime GPC5.
- 25
28. Procédé selon la revendication 26 ou la revendication 27, dans lequel la cellule est une cellule cancéreuse.
- 30
29. Procédé selon la revendication 28, dans lequel le cancer est un rhabdomyosarcome, un lymphome, un cancer du poumon non à petites cellules, un cancer de la vessie, un cancer du sein, un cancer de la prostate, une tumeur neurogliale, un carcinome à cellules squameuses de la tête et du cou, une leucémie, un léiomyosarcome, un liposarcome, un histiocytome fibreux malin des os ou des tissus mous, un mélanome, un mésothéliome, un cancer de la thyroïde, un cancer du poumon, un cancer des testicules ou un cancer des ovaires.
- 35
30. Procédé *in vitro* pour déterminer un pronostic pour un patient souffrant d'un cancer du sein, consistant à attribuer un pronostic au patient en se basant sur les taux d'expression de GPC5 dans une tumeur mammaire de ce patient.
- 40
31. Procédé selon la revendication 30, qui consiste à déterminer la présence, l'absence ou le degré d'expression de GPC5 *in vitro* en utilisant un échantillon contenant des cellules du cancer du sein du patient.
- 45
32. Procédé selon la revendication 31, qui consiste à mettre l'échantillon en contact avec un anticorps capable de se lier à GPC5.
- 50
33. Procédé pour surveiller la réussite du traitement d'un cancer ayant antérieurement montré qu'il exprimait GPC5, consistant à déterminer l'expression de GPC5 dans des cellules du cancer, où ledit procédé est réalisé *in vitro* en utilisant un échantillon contenant des cellules cancéreuses du patient.
- 55
34. Procédé selon la revendication 33, dans lequel ladite étape de détermination de l'expression de GPC5 consiste à mettre les cellules du cancer en contact avec un anticorps capable de se lier à GPC5.
35. Procédé selon la revendication 33 ou 34, consistant à comparer les résultats obtenus avec les résultats d'un essai équivalent qui est réalisé pour le même patient avant le traitement et/ou à un stade plus précoce du traitement.
36. Procédé selon la revendication 33 ou 34, consistant à déterminer le taux d'expression de GPC5 dans des cellules du cancer, ou à déterminer le nombre ou la densité de cellules exprimant ou surexprimant GPC5.

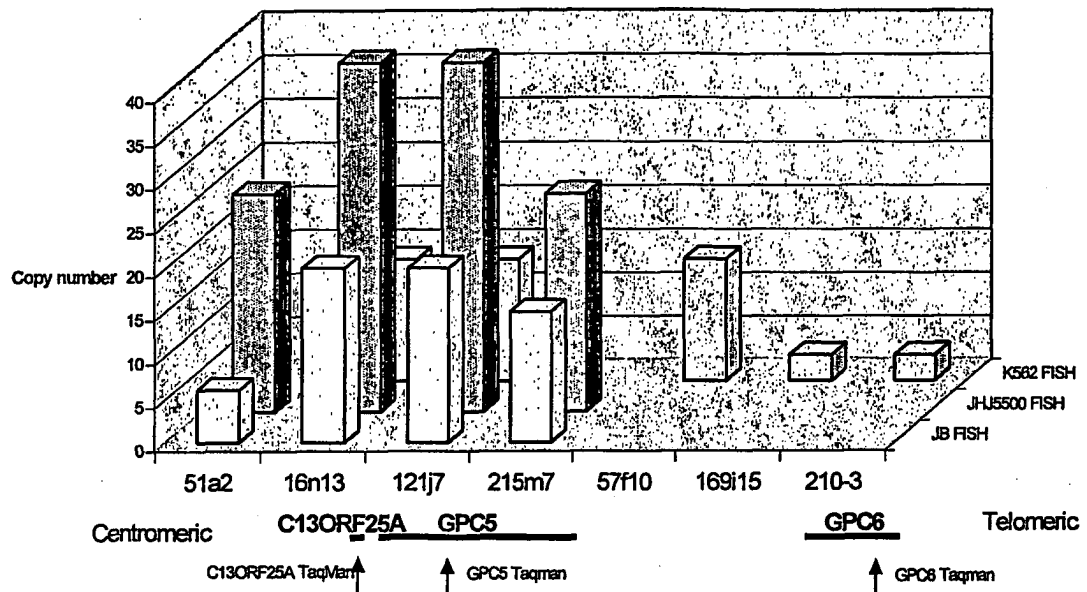


Figure 1

(A)

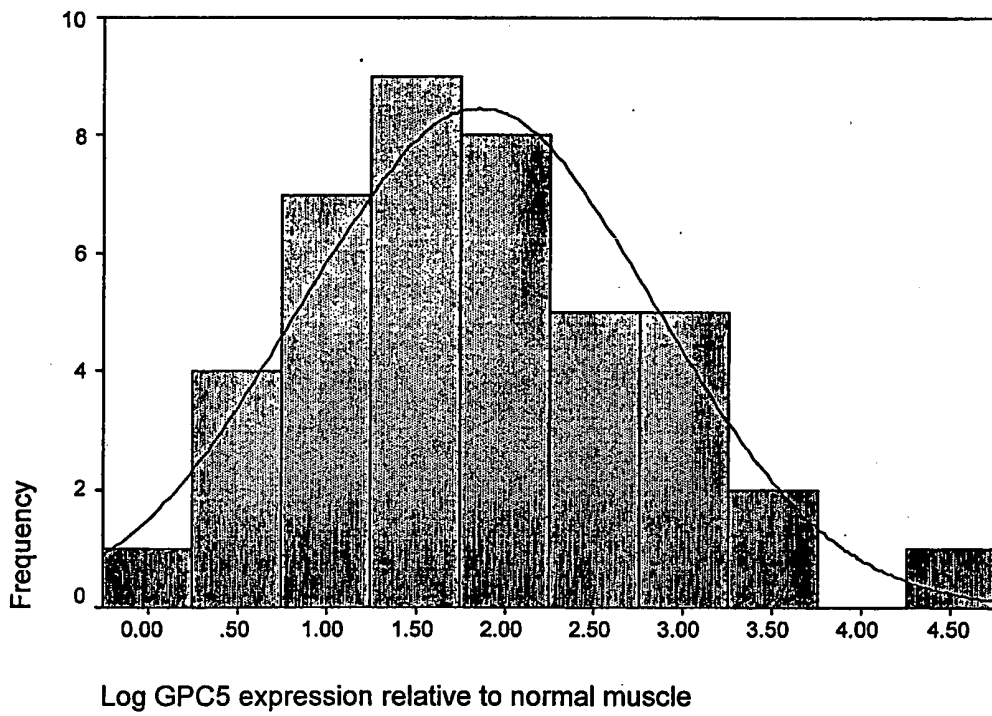
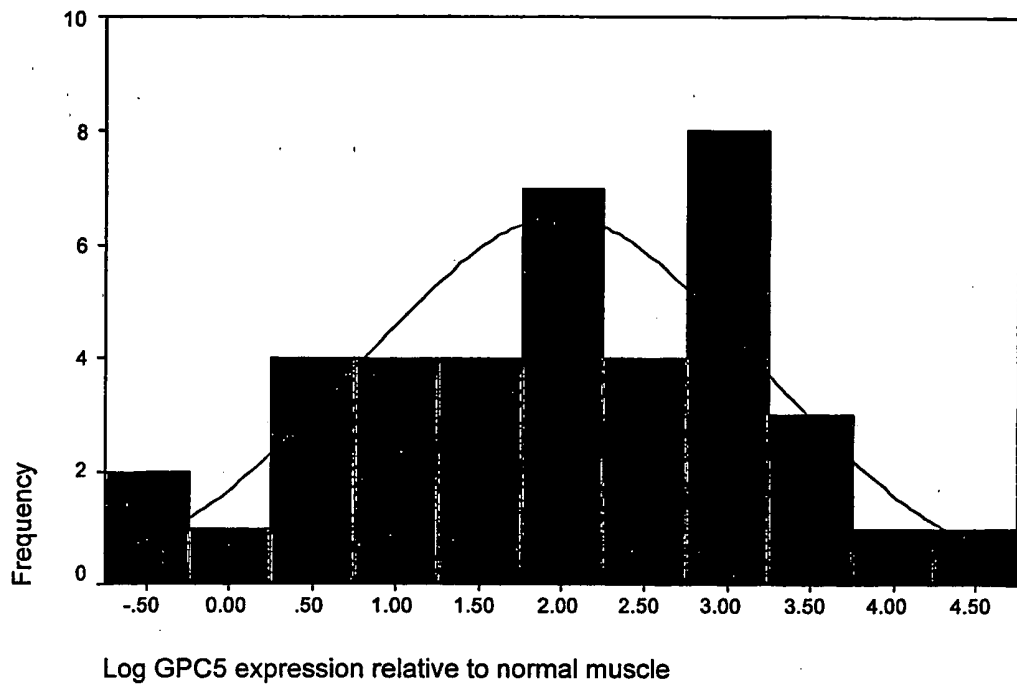


Figure 2

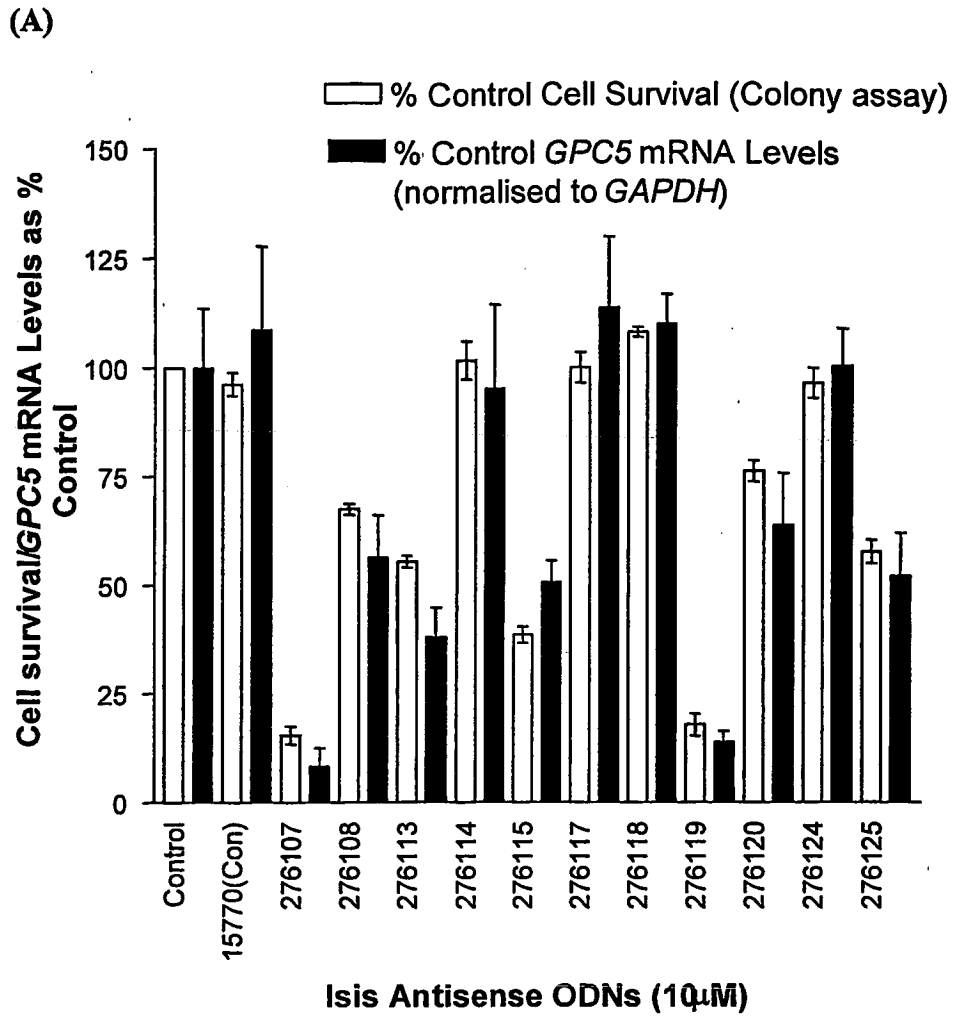
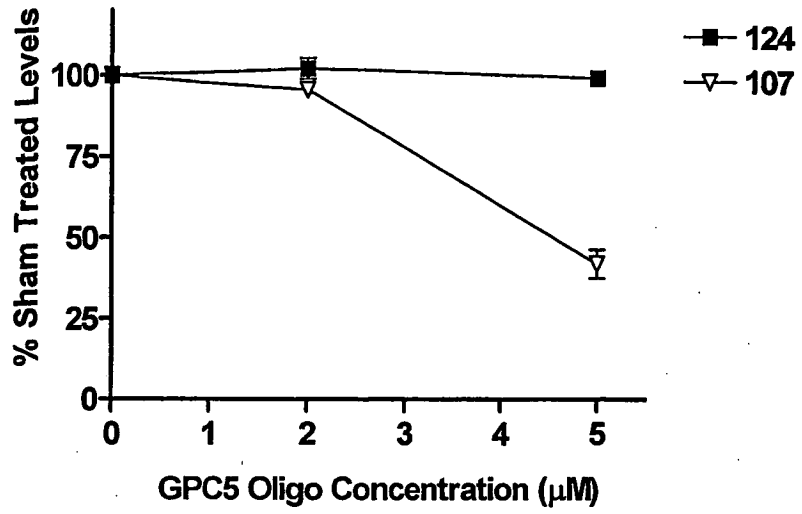


Figure 3

(B)



(C)

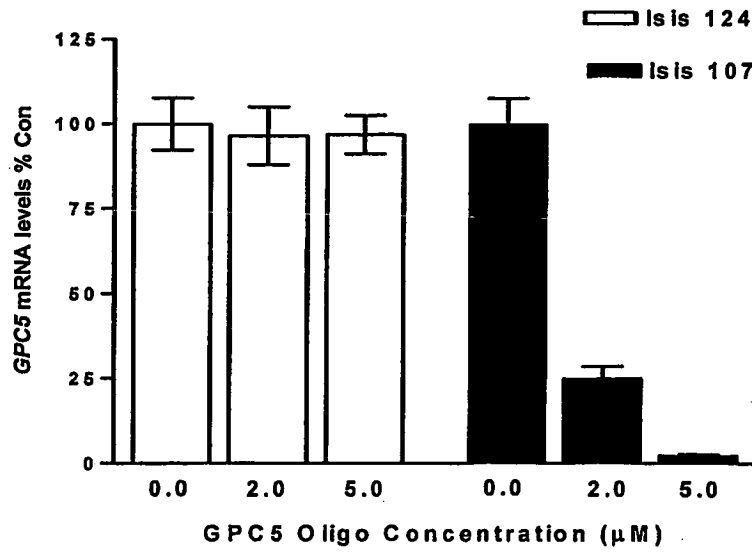
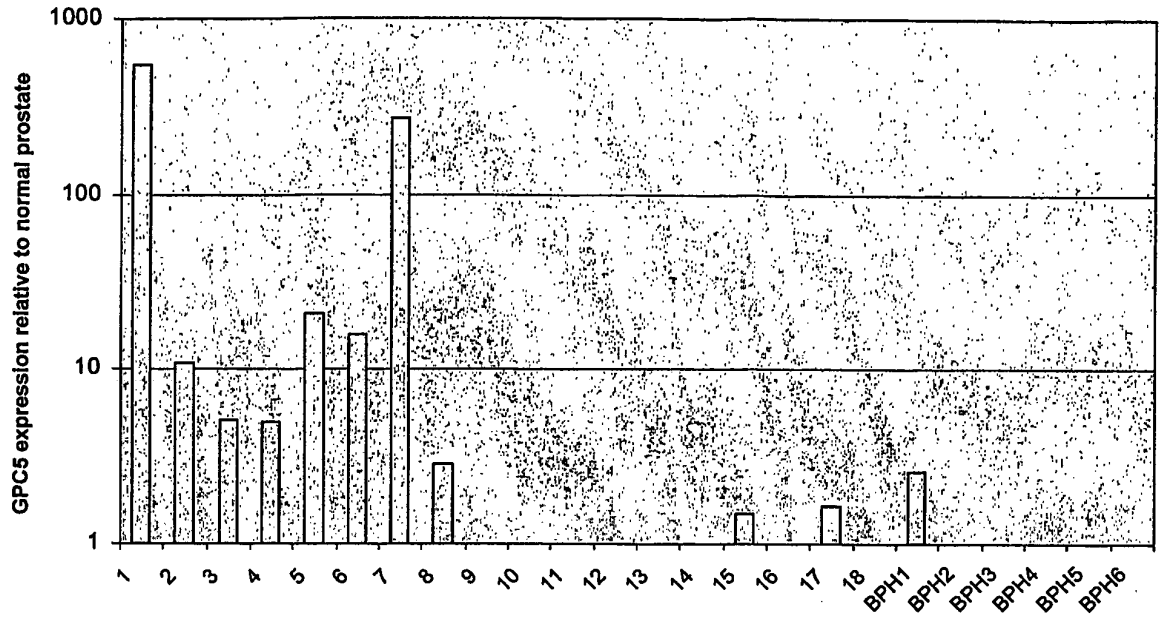


Figure 3 (contd.)

(A)



(B)

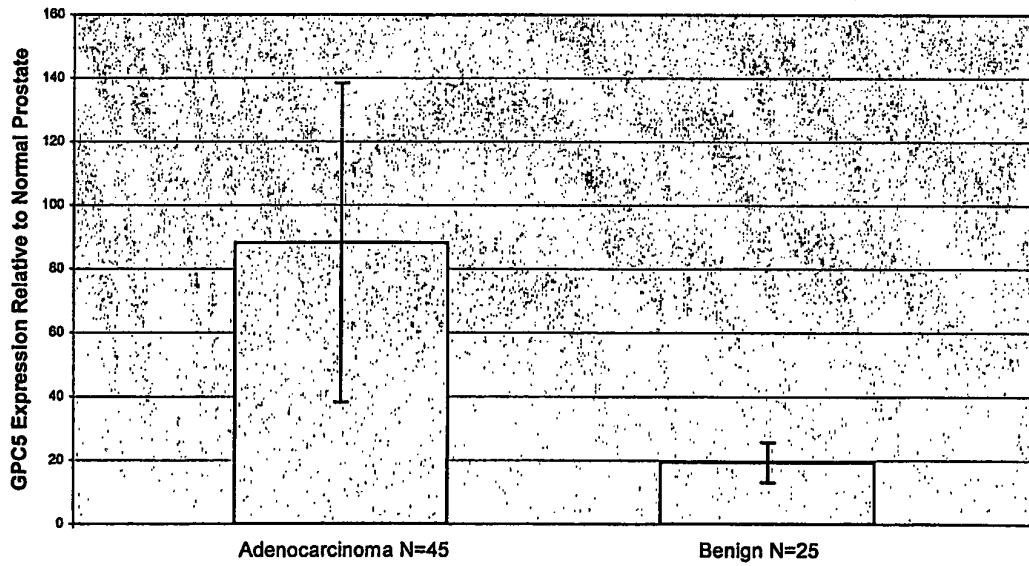
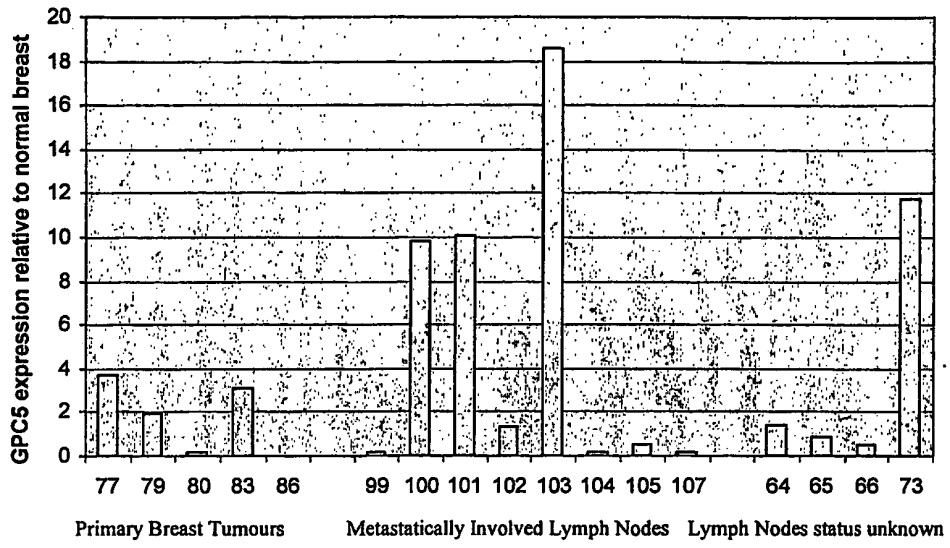


Figure 4

(A)



(B)

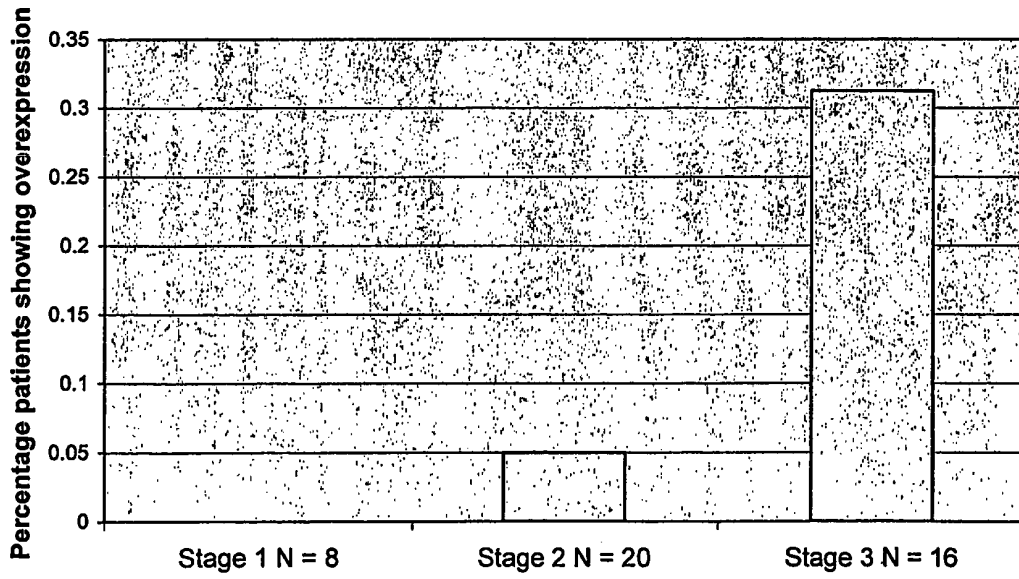


Figure 5

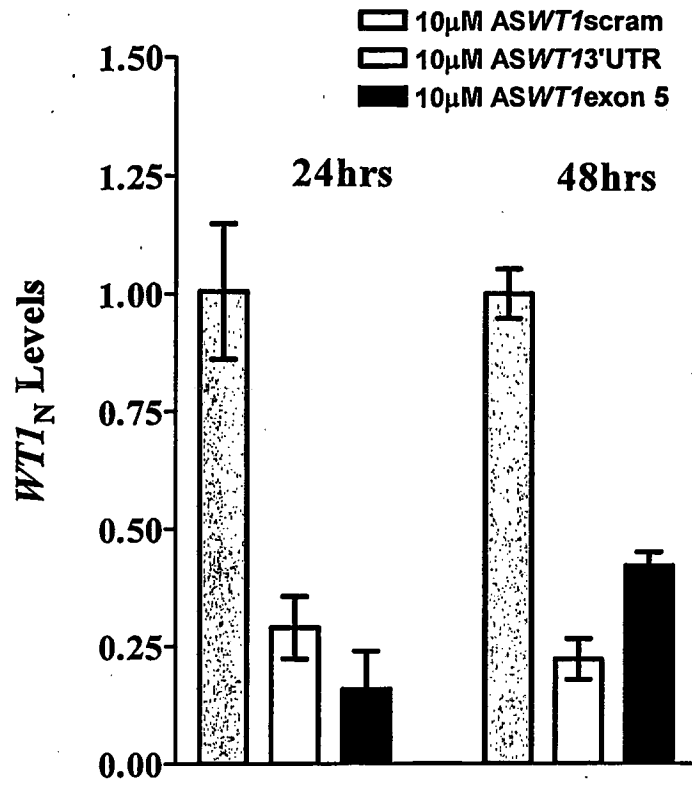


Figure 6(a)

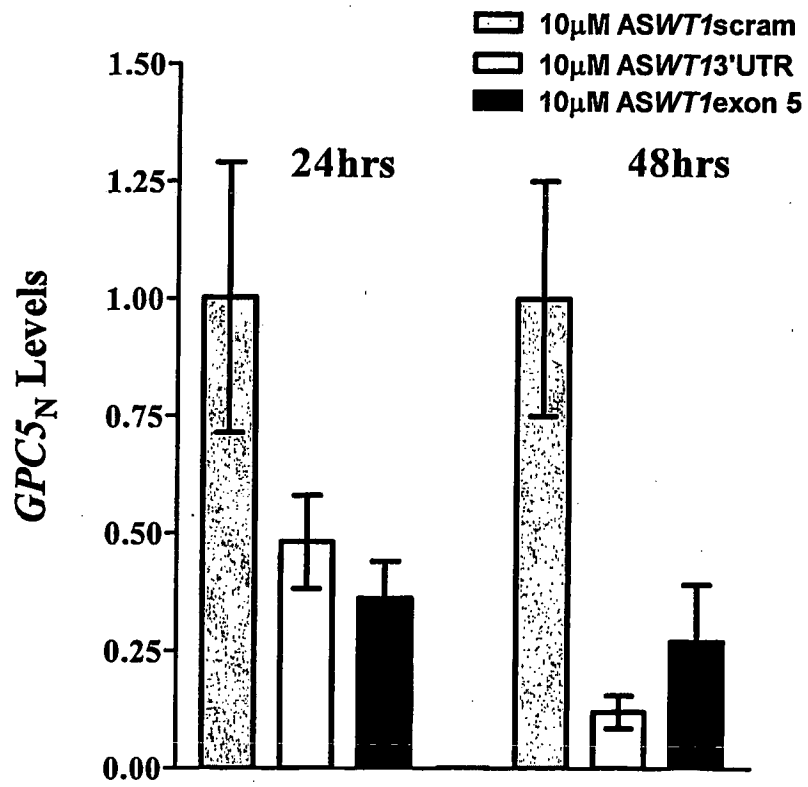


Figure 6(b)

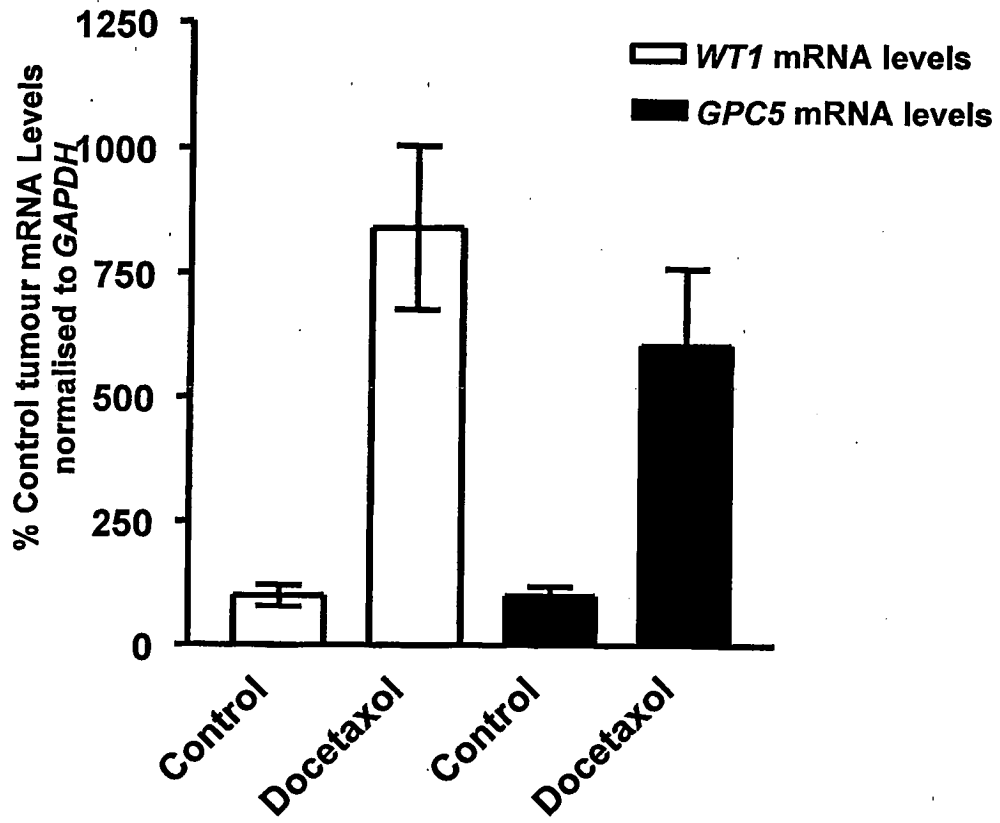


Figure 7

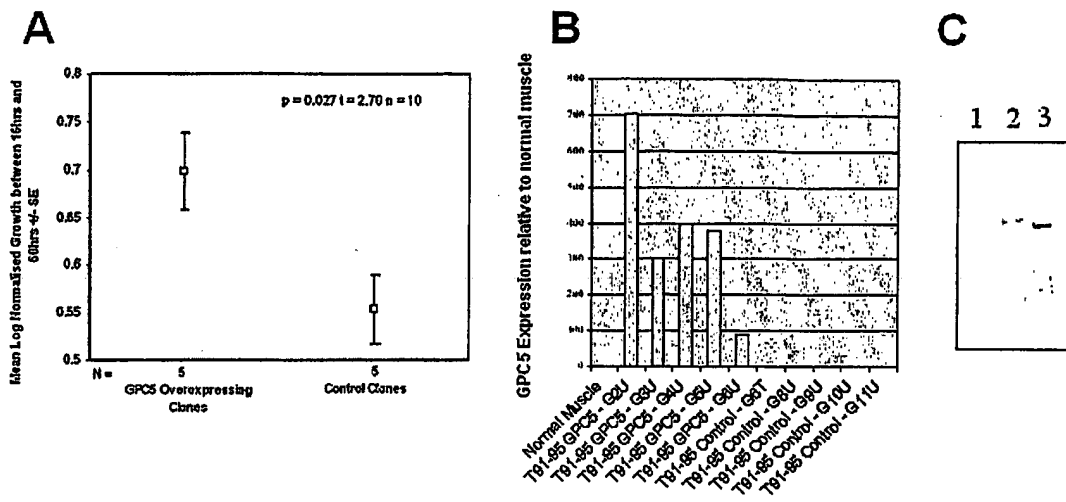


Figure 8

REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	用于治疗癌症的材料和方法		
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当前申请(专利权)人(译)	癌症研究学院 : ROYAL肿瘤医院		
发明人	SHIPLEY, JANET, INSTITUTE OF CANCER RESEARCH WILLIAMSON, DANIEL, INSTITUTE OF CANCER RESEARCH RENSHAW, JANE, INSTITUTE OF CANCER RESEARCH ORR, ROSEANNE, INSTITUTE OF CANCER RESEARCH		
IPC分类号	C12N15/12 C07K14/705 C12N15/11 G01N33/50 G01N33/53 G01N33/68 C07K16/28 A61K31/7088 A61K38/00 C12Q1/68 C12N15/113		
CPC分类号	A61P35/00 A61P35/02 A61P43/00 C12N15/1138 C12N2310/11 C12N2310/315 C12N2310/321 C12N2310/341 C12N2310/3521		
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摘要(译)

磷脂酰肌醇蛋白聚糖5首次显示出在癌细胞增殖中起作用，包括在13q31处未显示染色体扩增的肿瘤。公开了使用磷脂酰肌醇蛋白聚糖5 (GPC5) 拮抗剂和结合剂治疗癌症，特别是横纹肌肉瘤和乳腺癌。

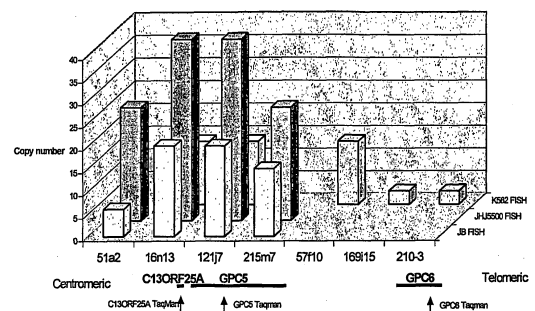


Figure 1