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DESCRIPTION

FIELD OF THE INVENTION

[0001] The present invention relates generally to the field of prostate cancer diagnostics. In particular, the present invention relates to the identification of biomarkers in biological samples, which can be used in the detection of prostate cancer. As described herein, the identified markers may also be used in determining prognosis and monitoring response to treatment for prostate cancer patients.

BACKGROUND OF THE INVENTION

[0002] Prostate cancer is the most frequently diagnosed visceral cancer and the second leading cause of cancer death for men in the United States. The American Cancer Society estimates that in 2013 about 238,590 new cases of prostate cancer will be diagnosed and 29,720 men will die of the disease. Overall, one in six men will be diagnosed with prostate cancer in their lifetime.

[0003] Currently, prostate cancer can be detected by either digital rectal exam (DRE) or by the measurement of prostate-specific antigen (PSA) in the blood of patients. However, neither test is entirely conclusive, and both can lead to false negatives (leaving real cancers undetected), and false positives (signaling cancer where there is none). For example, standard PSA tests conducted at the recommended 4.0 ng/ml cutoff, are 86% sensitive to cancer patients but only 33% specific, producing false positives in roughly 67% of non-cancer patients (Hoffman et al. 2002). False positives are usually followed by invasive and painful biopsies. Suhovskih et al. (2013) describe glypican-1 expression in normal human prostate tissue and prostate cancer.

[0004] A need exists for prostate cancer diagnostic tests with improved accuracy and/or sensitivity.

SUMMARY OF THE INVENTION

[0005] The present invention is based, in part, on the discovery that soluble glypican-1 heparan sulfate proteoglycan (GPC-1) levels are elevated in the body fluids or tissues of prostate cancer patients. The present inventors have discovered that soluble glypican-1 is a new marker for prostate cancer. Accordingly, the present invention provides for methods of detecting soluble glypican-1 to determine the existence of prostate cancer in patients.

[0006] The invention provides an in vitro method of detecting prostate cancer in a patient, the method comprising measuring the level of soluble glypican-1 in a body fluid sample selected from the group consisting of blood, serum, plasma, and urine, obtained from a patient and determining that said patient has prostate cancer or an increased likelihood of developing

prostate cancer based upon the level of soluble glypican-1 in the body fluid sample, wherein the measured levels of soluble glypican-1 in the body fluid sample of the patient are compared against: a control sample of body fluid obtained from a cancer-free patient; or pre-determined reference values or ranges of reference values. In some embodiments the levels of one or more additional prostate markers are measured in the body fluid sample, and the determination that the patient has cancer is based upon the levels of soluble glypican-1 and the levels of the one or more additional markers in the patient body fluid sample. In some embodiments, the cancer is prostate cancer, and the additional marker is PSA. In some embodiments, the anti-glypican-1 antibody is MIL-38. In other embodiments, the anti-glypican-1 antibody is not MIL-38. In some embodiments, the anti-glypican-1 antibody is an antibody fragment or recombinant antibody capable of binding glypican-1. The anti-glypican-1 antibody can be labeled for easy detection. The antibody label can be a fluorescent label, a biotin-avidin amplification system, a chemiluminescence system, microspheres or colloidal gold among others.

[0007] The body fluid sample obtained from the patient is a blood, serum, plasma, or urine sample.

[0008] In one embodiment, the anti-glypican-1 antibody binding to the patient's body fluid sample is compared to the level of anti-glypican-1 antibody binding of a control sample; wherein increased anti-glypican-1 antibody binding of the body fluid sample over the control sample is associated with the presence of prostate cancer. In some embodiments, said control sample comprises the body fluid from an age-matched prostate cancer-free patient.

[0009] In other embodiments, the level of anti-glypican-1 antibody binding to the patient's body fluid is compared to the level of anti-glypican-1 antibody binding to a reference standard, wherein increased anti-glypican-1 antibody binding of the body fluid sample over the reference standard sample is associated with the presence of prostate cancer. In some embodiments, said reference standard comprises a sample with known soluble glypican-1 content. In some embodiments, the comparison of the anti-glypican-1 binding to the body fluid or tissue is compared to the anti-glypican-1 binding to the glypican-1 standards to quantify the amount of soluble glypican-1 in said body fluid.

[0010] Soluble glypican-1 content higher than about: 0.1 ng/ml, 0.5ng/ml, 1ng/ml, 5ng/ml, 10ng/ml, 15ng/ml or 20ng/ml in the body fluid sample can be indicative of prostate cancer.

[0011] Diagnostic methods described herein may further comprise administering one or more prostate cancer treatments to a patient, and following changes in the level of glypican-1 in body fluids or tissues as a mechanism to monitor patient recovery or responses to the prostate cancer treatments. Antiglypican-1 antibody binding can be detected via techniques such as immunofluorescence, radiolabeling, immunoblotting, enzyme-linked immunoassay, flow cytometry, optical density, and chemiluminescence.

[0012] The present disclosure also includes kits for detecting glypican-1 in the body fluids or tissues of patients. As described herein, the kit for detecting prostate cancer comprises a first anti-glypican-1 antibody, a pharmaceutically acceptable carrier, and glypican-1 standards;

wherein said kit is capable of detecting glypican-1 in the body fluid or tissue of a patient. As described herein, the kit further comprises a secondary ligand. As described herein, the secondary ligand is a second anti-glypican-1 antibody. As described herein, the second anti-glypican-1 antibody is the same as the first anti-glypican-1 antibody.

[0013] As described herein, the secondary ligand is conjugated to a label for rapid detection of said ligand.

[0014] The present invention thus relates an in vitro method of detecting prostate cancer in a patient, the method comprising measuring the level of soluble glypican-1 in a body fluid sample selected from the group consisting of blood, serum, plasma, and urine, obtained from a patient and determining that said patient has prostate cancer or an increased likelihood of developing prostate cancer based upon the level of soluble glypican-1 in the body fluid sample, wherein the measured levels of soluble glypican-1 in the body fluid sample of the patient are compared against: a control sample of body fluid obtained from a cancer-free patient; or pre-determined reference values or ranges of reference values.

[0015] Other embodiments are defined by the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016]

Figure 1. Characterization of the cell bound MIL-38 glypican-1 antigen.

DU-145 prostate cancer cells were processed with a Membrane Protein Extraction Kit (MPEK, Merck) and incubated with the MIL-38 antibody attached to magnetic beads. The antigen was then immunoprecipitated and washed on the magnetic beads before eluting the antigen and subjecting said antigen to mass spectrometry analysis. Results of mass spec analysis are shown identifying glypican-1 as the MIL-38 antigen. The 18 unique peptide sequences spanning the glypican-1 protein are underlined.

Figure 2. MIL-38 immunoprecipitation and size-exclusion chromatography.

DU-145 prostate cancer cell membrane extracts were immunoprecipitated using MIL-38 and run through a size-exclusion chromatography column. The figure shows western blot analyses of even numbered chromatography fractions using either MIL-38 antibody or secondary antibody control. The MIL-38 antigen is shown at 60Kd in fractions A28 and A30.

Figure 3. Mass spectrometry of size-exclusion chromatography purified MIL-38 antigen.

Fraction number A29 from size-exclusion chromatography separation of Figure 2 was analyzed via mass spectrometry. Results of mass spec analysis are shown identifying glypican-1 as the MIL-38 antigen with 8 unique peptide sequences spanning the glypican-1 protein underlined.

Figure 4. MIL-38 and anti-GPC-1 antibodies show overlapping reactivity on 2D gel western blot.

Membrane protein extracts of DU-145 prostate cancer cells were separated on 2D gel (pI

gradient-horizontal, and molecular mass vertical). Western blots using MIL-38 antibody and commercial rGPC-1 rabbit polyclonal antibodies show overlapping reactivity marking a 60Kd protein (circled in figure). **Lane D** is a one dimension separation for DU-145 extracts as a control. **Lane M** is a one dimension separation lane for molecular size markers as controls.

Figure 5. MIL-38 can detect immunoprecipitates of GPC-1 antibodies and vice versa.

MIL-38 and rabbit anti-GPC-1 antibodies were each used to immunoprecipitate their antigens from DU-145 prostate cancer or C3 (MIL-38 negative) cell membrane protein extracts. Shown are the western blots of the immunoprecipitations detected with either MIL-38 or anti-GPC-1 antibody. **Figure 5A** depicts GPC-1 detection of MIL-38 immunoprecipitates (left) and MIL-38 detection of GPC-1 immunoprecipitates (right). **Figure 5B** depicts MIL-38 detection of MIL-38 immunoprecipitates as a control. Lanes are: **Magic Mark**- commercial protein marker as control; **DU145 MPEK**- prostate cancer membrane protein extract (not immunoprecipitated); **DU145 FT**- prostate cancer flow through from immune precipitation; **DU145 IP**- immunoprecipitate using antibody; **C3 MPEK**- (MIL-38 negative) control membrane protein extract (not immunoprecipitated); **C3 FT**- (MIL-38 negative) cell flow through from immune precipitation; **C3 IP elution**- (MIL-38 negative) cell immunoprecipitate using antibody. MIL-38 can detect the immunoprecipitate from rGPC-1 antibody and vice versa. MIL-38 can also bind to all controls including DU145 MPEK and to IP conducted by MIL-38.

Figure 6. MIL-38 detects recombinant glypican-1

A recombinant glypican-1 produced in NS0 cells was tested for reactivity with MIL-38 and anti-GPC-1 antibodies. Western blots show reactivity of both MIL-38 and rabbit polyclonal anti-GPC-1 antibody with the recombinant glypican-1. Lanes are: **Magic Mark**-commercial protein marker as control; **GPC-1**- recombinant Glypican-1 protein; **GPC-1 red**-recombinant Glypican-1 protein with reducing agent.

Figure 7. MIL-38 can detect antigen secreted into cell culture supernatant.

DU-145 prostate cancer cells were incubated with serum free media for 36 hours to produce cell free conditioned media. The conditioned media without any cells was immunoprecipitated using the MIL-38 antibody and compared to a standard IP using DU-145 MPEK membrane protein extracts. Shown is a western blot of the immunoprecipitates using the MIL-38 antibody. The MIL-38 antibody can detect antigens of 40 and 55kD in the conditioned liquid media.

Figure 8. MIL-38 antigen detected in cell culture supernatant is glypican-1

DU-145 prostate cancer cells were incubated with serum free media for 36 hours to produce cell free conditioned media. The conditioned media was immunoprecipitated using the MIL-38 antibody and the immunoprecipitates were sent for mass spectrometry analysis. **Figure 8A**. A sample containing both the 40 and 55kD MIL-38 reactive antigens was subjected to mass spec analysis. Results of mass spec analysis are shown identifying glypican-1 as the MIL-38 antigen from the conditioned liquid media with 9 unique peptide sequences spanning the N-terminus of the glypican-1 protein underlined. **Figure 8B**. A sample containing the 40 kDa MIL-38 reactive antigen was subjected to mass spec analysis. Results of mass spec analysis are shown identifying glypican-1 as the MIL-38 antigen from the conditioned liquid media with 10 unique peptide sequences spanning the N-terminus of the glypican-1 protein underlined. **Figure 8C**. A sample containing the 55 kDa MIL-38 reactive antigen was subjected to mass spec analysis.

Results of mass spec analysis are shown identifying glypican-1 as the MIL-38 antigen from the conditioned liquid media with 9 unique peptide sequences spanning the N-terminus of the glypican-1 protein underlined.

Figure 9. MIL-38 antibody can detect glypican-1 in the plasma of prostate cancer patients and in membrane extracts of prostate cancers.

Figure 9A. Plasma samples from one normal control patient and one prostate cancer patient were immunoprecipitated with the MIL-38 antibody. Shown are western blots with both the MIL-38 and rGPC1 antibodies. MIL-38 immunoprecipitated higher levels of glypican-1 protein in the plasma of the prostate cancer patient than the plasma of the control patient. Lanes are: **046 IP NT-** IP from prostate cancer plasma; **046 IP HepI-** IP from prostate cancer plasma treated with heparinase; **042 IP NT-** IP from normal control plasma; **042 IP HepI-** IP from normal control plasma treated with heparinase; **Magic Mark**-commercial protein marker as control. **Figure 9B.** Membrane protein extracts from one normal prostate and one prostate cancer were obtained from Novus Bio. Equivalent amounts of protein were western blotted using MIL-38 antibody. The prostate cancer extract demonstrated higher expression of the MIL-38 antigen.

Figure 10. MIL-38 can detect cancer in the urine of prostate cancer patients.

Urine samples from 125 age-matched patients were collected and tested for the presence of prostate cancer using MIL-38 antibody in an indirect immunofluorescence assay. Patients were classified as healthy controls, benign prostatic hypertrophy (BPH) or prostate cancer (CaP) based on either biopsy confirmation (BPH, CaP) or analysis of risk factors (healthy controls). Figure shows exemplary pictures of CaP experimental samples; **DU145** positive control samples; and C3 negative control samples.

Figure 11. MIL-38 can detect recombinant glypican-1 in a variety of ELISA formats

Sandwich ELISAs were performed using 0, 0.1, 1 or 10ng recombinant human NS0-produced glypican-1 protein (rhGPC1) as analyte. **Figure 11A.** Capture with MIL-38 antibody, detection with rabbit polyclonal anti-GPC1 (α-GPC1). **Figure 11B.** Capture with anti-glypican-1 antibody, detection with MIL-38. **Figure 11C.** Capture with MIL-38, detection with biotinylated MIL-38.

Figure 12. Comparative sandwich ELISAs performed using different MIL-38 antibody preparations as capture antibodies.

Figure 12A shows comparative sandwich ELISAs using AM3 and AM4 as capture antibodies. **Figure 12B** shows comparative sandwich ELISAs using either a mixed preparation (34A) or a clonal population (AM4 1F5) as capture antibodies.

Figure 13. Box plot graphs of raw data of antibody screening.

The binding of AM4 MIL-38 antibody to each of the synthesized peptides was tested in a PEPSCAN-based ELISA. The bottom and top of the boxes are the 25th and 75th percentile of the data. The band near the middle of the box is the 50th percentile (the median). The whiskers are at 1.5 the inter-quantile range, an indication of statistical outliers within the dataset (Mcgill et al., The American Statistician, 32: 12-16, 1978);

Figure 14. Letterplot representation of MIL38-AM4 probed on the substitution analysis of set 3 (Example 13).

DETAILED DESCRIPTION

[0017] The present invention is based, in part, on the discovery that glypican-1 heparan sulfate proteoglycan (GPC-1) levels are elevated in the body fluids of prostate cancer patients. The present inventors have discovered that glypican-1 is a new marker for prostate cancer. Accordingly, the present invention provides for methods of detecting the existence of prostate cancer in patients.

[0018] Normal human cells are only capable of forty to sixty cellular divisions before telomeric shortenings make them unviable. Prostate cancer cells however, are not subject to the Hayflick limit of divisions and continue to divide indefinitely causing abnormal growths.

[0019] The most common manifestation of cancer is the formation of tumors in the bodies of patients. The prostate cancer tumors can be painless and asymptomatic. The tumors can cause physical discomfort or other localized symptoms such as fluid blockages or bleeding. Prostate cancer may cause systemic symptoms such as those caused by disrupting normal body functions. The symptoms of prostate cancers can include change in bowel habits or bladder function.

[0020] One of the distinguishing factors between benign prostate tumors (non cancerous) and malignant prostate tumors (cancerous) is the ability to metastasize. Metastasis is the ability of cancers to spread (metastasize) to other parts of the body. Prostate cancer in patients is further categorized into stages according to the progression of the disease. The most common staging system is the TNM system, which categorizes cancer based on the size and extent of the primary tumor (T), the spread of the cancer to nearby lymph nodes (N), and the presence of secondary tumor formed by the metastasis (M) of the primary tumor to other parts of the body (American Cancer Society). Table 1 shows example definitions for each cancer stage. **Table 1.** Definitions for cancer stages of the TNM system, adapted from the American Cancer Society.

Stage	Definition
Stage 0	Carcinoma in situ
Stage I, Stage II, and Stage III	Higher numbers indicate more extensive disease: Larger tumor size and/or spread of the cancer beyond the organ in which it first developed to nearby lymph nodes and/or tissues or organs adjacent to the location of the primary tumor
Stage IV	The cancer has spread to distant tissues or organs

[0021] Methods described herein can detect cancers at any one or more stages.

[0022] Glypican-1 is encoded by **SEQ ID NO: 1**. Glypican-1 protein is the full amino acid sequence of **SEQ ID NO: 2**. In some examples, the glypican-1 protein does not include the signal peptide of **SEQ ID NO: 3**. In some examples, the glypican-1 protein does not include the

propeptide of **SEQ ID NO: 4**. In some examples, the glypican-1 protein is **SEQ ID NO: 5**. In some examples, glypican-1 includes glypican-1 variants such as isoforms, splice variants, and allotypes. The present disclosure also provides a method of determining a prognosis for a patient with prostate cancer. The method comprises obtaining a body fluid or tissue test sample from a patient, measuring the levels of glypican-1 in said body fluid or tissue, and comparing said levels with a fixed range of values wherein higher glypican-1 levels are associated with poorer prognosis or less favorable patient outcome.

[0023] Non-limiting examples of prostate cancers that may be detected with the present invention include prostatic intraepithelial neoplasia, adenocarcinoma, leiomyosarcoma, and rhabdomyosarcoma

[0024] One of the most powerful tools against cancer is early detection. Earlier stages of cancer tend to be easier to treat, and the prognosis for most cancers is generally better if the disease is still localized. There are many tests that can help diagnose cancer. In some examples, the present disclosure uses glypican-1 alone to detect prostate cancer. In other examples GPC-1 is used together with another antigen wherein the presence of prostate cancer is determined by the detection of both antigens. In one example the other antigen is PSA.

[0025] The present invention provides methods for detecting prostate cancer. Prostate cancer is the most frequently diagnosed visceral cancer and the second leading cause of cancer death for men in the United States. The American Cancer Society estimates that in 2013 about 238,590 new cases of prostate cancer will be diagnosed and 29,720 men will die of the disease. Overall, one in six men will be diagnosed with prostate cancer in their lifetime. Prostate cancers have been associated with many symptoms including difficulty urinating, erectile dysfunction and pain. Although most prostate cancers are slow growing, there are cases of aggressive prostate cancers which can metastasize and can ultimately lead to death.

[0026] There are two major prostate cancer detection tests currently used by medical professionals: a digital rectal exam (DRE), and the measurement of prostate-specific antigen (PSA) in the blood of patients. Unfortunately, neither of these tests is entirely conclusive and both can result in false negatives (leaving real cancers undetected), and false positives (signaling cancer where there is none). For example, standard PSA tests conducted at the recommended 4.0 ng/ml cutoff are 86% sensitive to cancer patients but only 33% specific, producing false positives in roughly 67% of non-cancer patients (Hoffman et al. 2002). The present invention describes methods for combining soluble glypican-1 measurements with another prostate cancer antigen, PSA, wherein the presence of prostate cancer is determined based on the levels of glypican-1 in the body fluid of the patient and the results of the PSA test,

[0027] BLCA-38 (also known as MIL-38) is an IgG₁ murine raised antibody against human bladder cancer cell line UCRU-BL-17CL (Walker et al., 1989). The resulting antibody was shown to bind to most human bladder cancer lines (Russell et al., 2004). The antibody was described as binding a cell surface protein of 30Kd and to be useful in detecting certain kinds of bladder carcinoma (US PAT 5,622,836).

[0028] The present invention describes for the first time the identity of the MIL-38 antigen. The present inventors discovered the antigen through a series of immunoprecipitations, westerns blot analyses, mass spectrometry analyses, and 2D gels described below in Examples 1-8. In accordance with the present invention, any suitable agent and/or any suitable technique as known to those of skill in the art can be used to measure the levels of soluble glypican-1 in a given sample (i.e. a body fluid sample), and use the measurement to diagnose prostate cancer in a patient from which the sample is derived. In some embodiments, the agent is an anti-glypican-1 antibody. MIL-38 antibody is used to bind to and detect a 60kD glypican-1 proteoglycan. The MIL-38 antibody can be used to detect the glypican-1 antigen on the surface of prostate cancer cells. According to the invention, the MIL-38 antibody is used to detect soluble glypican-1 in the body fluids of prostate cancer patients. The MIL-38 antibody has binding specificity for a glypican-1 epitope comprising a first segment KVNPPQGGPE (**SEQ ID NO: 6**) or KVNPPQGGP (**SEQ ID NO: 7**). The epitope may further comprise a second segment TQNARA (**SEQ ID NO: 8**) or TQNARAFRD (**SEQ ID NO: 9**). It is described herein that MIL-38's ability to bind to prostate cancer tissue is based on the presence of the glypican-1 antigen and further demonstrates the use of other anti-glypican-1 antibodies to detect cancerous prostate cells. Therefore in some embodiments the anti-glypican-1 antibody is not MIL-38. Further the present invention demonstrates the ability to detect prostate cancer by detecting soluble glypican-1 levels in the body fluids of patients. The present inventors have thus discovered that soluble glypican-1 in body fluids being selected from blood, serum, plasma and urine obtained from a patient is a marker for prostate cancer.

[0029] According to the invention soluble glypican-1 levels in body fluids can be detected using any suitable technique (e.g. any proteomic technique). In some embodiments, the soluble glypican-1 levels can be detected using an anti-glypican-1 antibody. For example, the soluble glypican-1 levels can be detected using an anti-glypican-1 antibody that comprises: a heavy chain variable region comprising a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 50-54 of **SEQ ID NO: 10**; a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 69-85 of **SEQ ID NO: 10**; a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 118-126 of **SEQ ID NO: 10**; and comprise a light chain variable region comprising a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 44-54 of **SEQ ID NO: 11**; a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 70-76 of **SEQ ID NO: 11**; a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 109-117 of **SEQ ID NO: 11**. The anti-glypican-1 antibody used to detect the soluble glypican-1 levels may not comprise: a light chain variable region comprising a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 48-58 of **SEQ ID NO: 12**; a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 74-80 of **SEQ ID NO: 12**; and a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 113-121 of **SEQ ID NO: 12**. The anti-glypican-1 antibody may be produced by or otherwise identical to an antibody generated by hybridoma cells as deposited on 22 August 2014 at CellBank Australia (CBA) under accession number

Company	Cat#	Reactivity	Use	Host	M/P	Biding specificity	Immunogen
antibodies-online	ABIN1174125	Human	IHC, WB, ELISA	Rabbit	poly		immunogen. Biotin conjugated
antibodies-online	ABIN740109	Human, mouse, rat, dog, cow, horse	WB, ELISA, IHC-P&F	Rabbit	poly	C-term	Synthetic peptide derived from human glypican 1 C-terminus. Enquire about sequence info. HRP conjugated
antibodies-online	ABIN653109	Human	WB, IHC, FACS, ELISA	Rabbit	poly	N-term	KLH conjugated sythetic peptide between 12-41aa from the N-terminal region of human Glypican-1
antibodies-online	ABIN952553	human	ELISA, IHC-p, WB, FACS	Rabbit	poly	N-term	KLH conjugated synthetic peptide between 12-41aa from the N-terminal region of human Glypican-1
antibodies-online	ABIN797896	human	IHC, WB	Rabbit	poly	N-term	synthetic peptide derived from N-terminal domain of human GPC1
antibodies-online	ABIN347483	mouse, rat, human	IHC, WB	Rabbit	poly	N-term	synthetic peptide derived from N-terminal domain of human GPC1
							synthetic peptide

Company	Cat#	Reactivity	Use	Host	M/P	Biding specificity	Immunogen
antibodies-online	ABIN330371	human	WB, ELISA	goat	poly	aa24-530	rhGlypican 1 aa24-530
antibodies-online	ABIN1479675	human	FACS, IHC, WB, ELISA	Rabbit	poly	aa12-41	KLH conjugated synthetic peptide from N-terminal region of human GPC1

[0031] In some aspects, a new anti-glypican-1 antibody can be generated from glypican-1 protein or a fragment or derivative thereof. By way of non-limiting example only, the anti-glypican-1 antibody can be raised against a glypican-1 epitope comprising a first segment KVNPGQGPGE (**SEQ ID NO: 6**) or KVNPGQGGP (**SEQ ID NO: 7**). The epitope may further comprise a second segment TQNARA (**SEQ ID NO: 8**) or TQNARAFRD (**SEQ ID NO: 9**). One skilled in the art will recognize that many procedures are available for the production of antibodies, for example, the methods described in (Harlow et al., 1988). In some examples, the glypican-1 immunogen used to create the anti-glypican-1 antibodies will include the post translational modifications of the native protein (e.g. folding). In some examples, the glypican-1 immunogen will not include the signal peptide (**SEQ ID No: 3**), or the C-terminal propeptide (**SEQ ID No: 4**) sequences. In some examples, the glypican-1 immunogens are obtained from human or other mammalian cells such as transformed murine NS0, wild type DU-145, or other glypican-1 expressing cell line. In some embodiments the glypican-1 antigen can be cells expressing glypican-1. In some examples, the glypican-1 immunogen can be whole cells or cell parts with glypican-1 protein on their surface. One skilled in the art will also appreciate that binding fragments or Fab fragments can be prepared from genetic information by various well-known procedures such as those described in (Borrebaeck et al., 1995; and US patent 7,960,517).

[0032] Polyclonal antibodies targeting glypican-1 may be created for the detection of prostate cancer. Again by way of non-limiting example only, the polyclonal antibody targeting glypican-1 can be raised against a series of glypican-1 epitopes including a glypican-1 epitope comprising a first segment KVNPGQGPGE (**SEQ ID NO: 6**) or KVNPGQGGP (**SEQ ID NO: 7**). The epitope may further comprise a second segment TQNARA (**SEQ ID NO: 8**) or TQNARAFRD (**SEQ ID NO: 9**). Various procedures known in the art may be used for the production of polyclonal antibodies to glypican-1 or a fragment of glypican-1. The glypican-1 protein or fragment thereof may be injected into a host animal. In some examples the host animals can include but are not limited to rabbits, mice, rats, etc. In some examples the resulting sera is purified and tested for its ability to react with glypican-1 via techniques well known in the art such as westerns, ELISAs, immunofluorescence screens, flow cytometry, Fluorescence Activated Cell Sorting (FACS) or others.

[0033] In another example, monoclonal antibodies (mAbs) directed against glypican-1 may be

created for the detection of prostate cancer. Again by way of non-limiting example only, the monoclonal antibody targeting glypican can be raised against a glypican-1 epitope comprising a first segment KVMPQGGPE (**SEQ ID NO: 6**) or KVNPGGPG (**SEQ ID NO: 7**). The epitope may further comprise a second segment TQNARA (**SEQ ID NO: 8**) or TQNARAFRD (**SEQ ID NO: 9**). In one example, anti-glypican-1 antibodies are created via the hybridoma technique (Kohler and Milstein 1975), or other techniques (Cole et al, 1985; or US PAT 6,116,013). For more details and examples on antibody production see US PAT 7,985,560.

[0034] In some embodiments of the present invention, soluble glypican-1 is detected in the body fluids of patients by an anti-glypican-1 antibody. According to the invention, the body fluid sample obtained from the patient is a blood, serum, plasma, or urine sample. As described herein, glypican-1 is detected in tissue samples of patients. In some examples, the tissue samples include tumor biopsies or other patient tissue. In some aspects, the antibody detects glypican-1 via Western blot analysis, Enzyme-linked immunosorbent assays (ELISAs), fluorescent cell sorting or FACS, immunofluorescence, radiolabeling, immunoprecipitation, immunohistochemistry, immunoblotting, chemiluminescence, and/or other known techniques to detect protein with an antibody or other ligand such as a protein capable of binding glypican-1. In some examples, glypican-1 is detected via a biofilm test, or affinity ring test as described in US application 2013/016,736. In some examples glypican-1 is detected via glypican-1 binding agents coated to transparent surfaces (e.g. polycarbonate slides). Binding of glypican-1 or glypican-1 bearing cells can be detected by changes in optical density. In some examples, anti-glypican-1 antibody binding to said body fluid or tissue sample is compared to the anti-glypican-1 antibody binding to one or more glypican-1 calibration standards; wherein the anti-glypican-1 antibody binding of the calibration standards is used to quantify the amount of glypican-1 in said body fluid sample. In one example, the calibration standards comprise one or more samples with known glypican-1 concentrations.

[0035] In some examples the measurement of glypican-1 is accomplished by contacting said body fluid sample with a glypican-1 ligand. In some examples the ligand can be an anti-glypican-1 antibody capable of binding the glypican-1 proteoglycan.

[0036] In some examples, the body fluid of a patient may require a pre-treatment prior to detection by the anti-glypican-1 ligand. In some examples, said pre-treatment may include treatment with agents such as heparinase PNGaseF, N-Glycosidase, alkaline phosphatase, or heparitinase among others. In other examples, said pre-treatment may include tissue lysis, membrane purification, blood plasma or serum fractionation, cell purification, or protein purification among others.

[0037] In some embodiments the measured levels of soluble glypican-1 in the body fluid of a patient are compared against a control sample of body fluid from a cancer-free patient. In other embodiments the measured levels of soluble glypican-1 in the body fluid of a patient are compared against pre-determined reference values or ranges of reference values. In other examples, the levels of glypican-1 in body fluids of a patient are indicative of prostate tumor size, or progression.

[0038] In some examples, the detection of glypican-1 from body fluid samples is conducted via Enzyme-linked immunosorbent assays (ELISAs). ELISAs comprise those based on colorimetry, chemiluminescence, and fluorometry. ELISAs have been successfully applied in the determination of low amounts of drugs and other antigenic components in body tissues or fluids such as blood, serum, and plasma samples, and are well known in the art. An ELISA that is useful in the present disclosure may employ any suitable capture reagent and detectable reagent including antibodies and derivatives thereof, protein ligands and the like. In certain examples, the ELISA is cell-based. In other examples, the ELISA detects cell-free antigens. In some examples, the biological sample suspected of containing glypican-1 is contacted and incubated with the capture (or coat) antibodies so that the capture antibodies capture or bind to the glypican-1. The detection step involves use of the detectable antibody or detectable protein ligands, which can bind to said glypican-1 and be used to detect the presence or amount of glypican-1 based on detection of its label.

[0039] In some examples, the biological sample is contacted and incubated with the immobilized capture (or coat) reagent, which can be a glypican-1 antibody. This antibody may be from any species, but in some examples, the antibody is a murine or rat antibody. In other examples the antibody is a murine antibody. In other embodiments the antibody is derived from a hybridoma. In some examples, the glypican-1 antibody is a recombinant antibody or antibody fragment. Immobilization conventionally is accomplished by insolubilizing the capture reagent either before the assay procedure, as by adsorption to a water-insoluble matrix or surface (U.S. Pat. No. 3,720,760) or non-covalent or covalent coupling (for example, using glutaraldehyde or carbodiimide cross-linking, with or without prior activation of the support with, e.g., nitric acid and a reducing agent as described in U.S. Pat. No. 3,645,852 or in Rotmans et al., 1983), or afterward, e.g., by immunoprecipitation.

[0040] In some examples, the solid phase used for immobilization may be any inert support or carrier that is essentially water insoluble and useful in immunometric assays, including supports in the form of, e.g., surfaces, particles, porous matrices, etc. Examples of commonly used supports include small sheets, Sephadex, polyvinyl chloride, plastic beads, and assay plates or test tubes manufactured from polyethylene, polypropylene, polystyrene, and the like including 96-well microtiter plates, as well as particulate materials such as filter paper, agarose, cross-linked dextran, and other polysaccharides. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are suitably employed for capture reagent immobilization. In one example the immobilized capture reagent is coated on a microtiter plate, and in particular the solid phase used is a multi-well microtiter plate that can be used to analyze several samples at one time, e.g., a microtest 96-well ELISA plate such as that sold as Nunc Maxisorb or Immulon. In certain embodiments, the plate is a MICROTEST™ or MAXISGRP™ 96-well ELISA plate such as that sold as NUNC MAXISORB™ or IMMULON™.

[0041] In some examples the solid phase is coated with the capture reagent as defined above, which may be linked by a non-covalent or covalent interaction or physical linkage as desired. Techniques for attachment include those described in U.S. Pat. No. 4,376,110 and the

references cited therein. If covalent, the plate or other solid phase is incubated with a cross-linking agent together with the capture reagent under conditions well known in the art, e.g., such as for 1 hour at room temperature.

[0042] In other examples, commonly used cross-linking agents for attaching the capture reagent to the solid phase substrate include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxy-succinimide esters, for example, esters with 4-azido-salicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propionimide yield photoactivatable intermediates capable of forming cross-links in the presence of light.

[0043] In some examples, 96-well plates are utilized. In some examples the 96-well plates are coated with the capture reagent (typically diluted in a buffer such as 0.05 M sodium carbonate by incubation for at least about 10 hours, more preferably at least overnight, at temperatures of about 4-20°C, or about 4-8°C, and at a pH of about 8-12, or about pH 9-10, or about pH 9.6). If shorter coating times are desired, in some examples the plate can be coated for less time, e.g. at room temperature for two hours or less. In some examples, the plates may be stacked and coated long in advance of the assay itself, and then the assay can be carried out simultaneously on several samples in a manual, semi-automatic, or automatic fashion, such as by using robotics.

[0044] In some examples, the coated plates can be treated with a blocking agent that binds non-specifically to and saturates the binding sites to prevent unwanted binding of the free ligand to the excess sites on the wells of the plate. Non-limiting examples of appropriate blocking agents for this purpose include, e.g., gelatin, bovine serum albumin, egg albumin, casein, and non-fat milk. In some examples the blocking treatment can take place under conditions of ambient temperatures for about 1-4 hours. In other examples the blocking can take place over the course of 1 to 3 hours or less. In other examples the blocking can take place overnight at 0-4°C.

[0045] In some examples the glypican-1 standard (e.g. purified glypican-1 protein) or the biological sample to be analyzed, appropriately diluted, is added to the immobilized phase. In some examples, the dilution rate is about 1-15% by volume. In some examples, the glypican-1 protein standard will include the post-translational modifications of the native protein. In some examples, the glypican-1 protein standards are obtained from human or other mammalian cells such as transformed NS0, wild type DU-145, or other glypican-1 expressing cell line. In other examples, the glypican-1 protein may be purified from body fluids or tissues. In some examples, the glypican-1 standard will be a partial glypican-1 peptide or other epitope detected by the glypican-1 detection antibody, or ligand. In some examples the standard can be a cell expressing glypican-1. In some examples the dilution is about 10% by volume. A non-limiting group of buffers that may be used for dilution for this purpose include (a) PBS containing 0.5% BSA, 0.05% TWEEN 20™ detergent (P20), 0.05% PROCLIN™ 300 antibiotic, 5 mM EDTA, 0.25% CHAPS surfactant, 0.2% bovine γ -globulins, and 0.35M NaCl, pH 7.4; (b) PBS containing 0.5% bovine serum albumin, 0.05% polysorbate 20, 5 mM EDTA, 0.25% CHAPS, 0.2% bovine γ -globulins, and 0.35 M NaCl; pH 7.4 (c) PBS containing 0.5% BSA, 0.05% polysorbate 20 (P20),

and 0.05% PROCLIN™ 300, pH 7; (d) PBS containing 0.5% BSA, 0.05% P20, 0.05% PROCLIN™ 300, 5 mM EDTA, and 0.35 M NaCl, pH 6.35; (e) PBS containing 0.5% BSA, 0.05% P20, 0.05% PROCLIN™ 300, 5 mM EDTA, 0.2% bovine γ -globulins, and 0.35 M NaCl, pH 7.4; and (f) PBS containing 0.5% BSA, 0.05% P20, 0.05% PROCLIN™ 300, 5 mM EDTA, 0.25% CHAPS, and 0.35 M NaCl, pH 7.4. PROCLIN™ 300 acts as a preservative, and TWEEN 20™ acts as a detergent to eliminate non-specific binding.

[0046] While the concentration of the capture reagents will generally be determined by the concentration range of interest of the glypican-1 taking any necessary dilution of the biological sample into account, the final concentration of the capture reagent will normally be determined empirically to maximize the sensitivity of the assay over the range of interest.

[0047] The conditions for incubation of sample and immobilized capture reagent are selected to maximize sensitivity of the assay and to minimize dissociation. In some examples, the incubation is accomplished at fairly constant temperatures, ranging from about 0°C to about 40°C. In other examples the incubation is conducted from about 20 to 25 °C. The time for incubation depends primarily on the temperature, being generally no greater than about 10 hours to avoid an insensitive assay. In some examples the incubation time is from about 0.5 to 3 hours. In other examples the incubation is about 1.5-3 hours or less at room temperature to maximize binding of free glypican-1 to capture reagents. The duration of incubation may be longer if a protease inhibitor is added to prevent proteases in the biological fluid from degrading the glypican-1.

[0048] In some examples, the detection method is a competitive ELISA. In some examples the incubation step includes the addition of unbound and unlabeled antibody. In some embodiments the known concentration of unlabeled antibody binds the free glypican-1 antigen and prevents it from becoming immobilized on the plate. In some examples the incubation step includes the addition of labeled glypican-1 protein of known concentrations. In some examples the amount of glypican-1 in the body fluid sample is detected as a diminishing binding of the mixed labeled glypican-1 protein. In other examples the ELISA is a sandwich ELISA.

[0049] At this stage, the pH of the incubation mixture will ordinarily be in the range of about 4-9.5. In other embodiments the pH range will be about 6-9. In yet another examples the pH range will be about 7-8. In another embodiment the pH of the assay (ELISA) diluent is pH 7.4, The pH of the incubation buffer is chosen to maintain a significant level of specific binding of the capture reagent to the glypican-1 being captured. Various buffers may be employed to achieve and maintain the desired pH during this step, including borate, phosphate, carbonate, Tris-HCl or Tris-phosphate, acetate, barbital, and the like. The particular buffer employed is not critical to the invention, but in individual assays one buffer may be preferred over another.

[0050] In some examples, the biological sample is separated (preferably by washing) from the immobilized capture reagent to remove uncaptured molecules. The solution used for washing is generally a buffer ("washing buffer") with a pH determined using the considerations and buffers described above for the incubation step. In one example, the pH range of the washing buffer is about 6-9. The washing may be done one or more times. The temperature of washing is generally from refrigerator to moderate temperatures, with a constant temperature maintained

during the assay period, typically from about 0-40°C. In other examples the washing temperature is about 4-30°C. For example, the wash buffer can be placed in ice at 4°C in a reservoir before the washing, and a plate washer can be utilized for this step, A cross-linking agent or other suitable agent may also be added at this stage to allow the now-bound glypican-1 to be covalently attached to the capture reagent if there is any concern that the captured glypican-1 may dissociate to some extent in the subsequent steps.

[0051] In some examples, the immobilized capture reagent is contacted with detectable antibodies. In some embodiments the detectable antibodies are anti-glypican-1 antibodies. In some embodiments the anti-glypican-1 antibody is MIL-38. In other examples the antibodies are those described in table 2. In other examples the detectable antibodies are any antibody capable of detecting glypican-1. The detectable antibody is contacted with the immobilized glypican-1 at a temperature of about 20-40°C. In other examples the detectable antibody is contacted at about 20-25°C with the exact temperature and time for contacting the two being dependent primarily on the detection means employed. For example, when streptavidin-peroxidase and 3,3',5,5'-tetramethyl benzidine are used as the means for detection, e.g., in one example, the contacting is carried out (e.g., about 1 hour or more) to amplify the signal to the maximum. In some examples, a molar excess of an antibody or ligand with respect to the maximum concentration of expected free glypican-1 is added to the plate after it is washed. This antibody is directly or indirectly detectable. The detectable antibody may be a polyclonal or monoclonal antibody, e.g., in certain examples, it is a monoclonal antibody, in one example murine, and in one embodiment MIL-38. Also, the detectable antibody can be directly detectable, and in one example has a colorimetric label, and in another example has a fluorimetric label. In other examples the detectable antibody is biotinylated and the detection means is avidin or streptavidin-peroxidase and 3,3',5,5'-tetramethyl benzidine. In some examples the detectable antibody may be labeled with a biotin-avidin amplification system, a chemiluminescence system, microspheres, or colloidal gold. The readout of the detection means can be fluorimetric or colorimetric among others. The affinity of the antibody must be sufficiently high that small amounts of the free glypican-1 can be detected.

[0052] In some examples, the glypican-1 that is bound to the capture reagent is measured using a detection means for the detectable antibody. If the body fluid sample is from patient, the measuring step comprises comparing the reaction that occurs as a result of the above steps with a standard curve to determine the level of glypican-1 in said body fluid or tissue sample. In other examples the reaction that occurs as a result of the above steps is compared to a similar reaction using a control body fluid sample such as the body fluid of a age-matched cancer-free individual.

[0053] In other glypican-1 detection examples, the glypican-1 in the body fluid of patients is detected via a Western Blot Analysis. In some examples this assay separates the proteins in a complex sample using electrophoresis. In other examples the electrophoresis separation is performed in a size exclusion gel such as a sodium dodecyl sulfate polyacrylamide gel (commonly known as SDS-PAGE). In one example the separated proteins are then transferred to a membrane. One skilled in the art will recognize that there are a variety of materials that can be used for westerns. In some embodiments the membrane is made from nitrocellulose or

polyvinylidene fluoride, PVDF. In some examples the transfer occurs in a protein transfer box such that the proteins retain the same separation pattern on the membrane as they had in the gel. In some examples the membrane is then incubated in diluted protein solutions, e.g. non-fat dry milk or bovine serum albumin (BSA), to block the non-specific binding sites. The blocked membrane can then be incubated with a primary antibody that is specific for the glypican-1 target protein. In some examples the membrane is then washed and incubated, with a secondary antibody that targets the first antibody. In some examples the first or the second antibody is conjugated to a detectable label such that it can be easily detected. In some examples the label includes a fluorescent label, a chemiluminescent label, a radiolabel, or another label well known in the art. In some examples, said labels conjugated to the secondary ligand are chosen from a group consisting of a radiolabel, a fluorescent label, a biotin-avidin amplification system, a chemiluminescence system, microspheres, and colloidal gold.

[0054] Optionally, some aspects of the disclosure teach that once a user has determined whether a target protein is present in the sample, the primary and (optional) secondary antibodies can be stripped from the membrane, and the membrane can be incubated with an alternative primary antibody that is specific for the same or another protein. In some examples the second protein may be used as a loading control. In other examples the second protein is another marker for patient health.

[0055] In one example, glypican-1 is detected via flow cytometry. In some examples glypican-1 is detected on the cell surface of cells in patient body fluids or tissues. In certain aspects of the disclosure the detection of glypican-1 for flow cytometry may be conducted as outlined below. In some examples, cells from body fluids are purified. The purification of the cells can include a neutralization step. In some examples the neutralization step comprises storing the cells in neutralization buffer. The neutralization buffer can be made by combining 39ml of 0.2 M NaH_2PO_4 to 61ml of 0.2M Na_2HPO_4 and adding water to 200ml. In some examples, the cells are centrifuged and resuspended in different solutions. In other examples, the cells are sorted without purification. In some examples the cells are resuspended or washed in CytoLyt solution. In other examples the cells are resuspended or washed in phosphate buffered saline (PBS). In some examples the cell suspension is treated with ammonium chloride to lyse the red blood cells. In some examples the cells are fixed onto slides. In other examples the cells remain free. In some examples, cells are contacted with a primarily anti-glypican-1 antibody or other ligand. In some embodiments the primary antibody is MIL-38. In some embodiments the primary antibody is not MIL-38. In other examples the primary antibody is any other anti-glypican-1 antibody (Table 2). In certain examples, the cells are further contacted with a second detection antibody conjugated to a detection label. In alternative examples, the antigen may be detected directly by the primary antibody if the primary antibody is conjugated to a detection label. In particular cases, in addition to being labeled with the glypican-1 antibody, the cells may be distinguishably labeled with other probes, including, but not limited to, antibodies to cell surface markers that distinguish one cell type from another. In some aspects the other probes may be used to normalize signals or total cell counts. In some examples the other probes label intracellular antigens. In other examples the other probes label extracellular antigens. Once labeled, the labeled cells may be isolated by FACS flow cytometry. In some examples, the FACS machine may isolate labeled cells singly (i.e., as single cells). In other examples, the labeled cells may be

isolated as a mixed population, and then diluted into single cells after FACS. In some examples the second label can be a dye. In some examples the dye label is DAPI. In some examples DAPI labeling is used to quantify the number of cells in the sample.

[0056] In examples in which the cell is labeled with a plurality of different labels, the cells may be selected using a plurality of different properties. For example, cells may first be sorted by one probe and then another. In some examples cells can first be sorted by cell type and later be sorted by glypican-1 concentration. Similarly cells may be sorted in any sequence as designed together with the probes and the detection of the FACS machine. The general principles of fluorescence activated cell sorting, including methods by which single cell suspensions can be made, methods by which cells can be labeled using, e.g., fluorescently labeled probes, methods by which cells can be separated from one another, as well as hardware that can be employed in flow cytometry, including flow cells, reagents, and computer control systems are known and are reviewed in a variety of publications, including, but not limited to: (Orfao et al., 1996; Johnson et al., 2007; Tung et al., 2007; and Dainiak et al., 2007).

[0057] The present disclosure also includes kits for detecting glypican-1 in the body fluids or tissues of patients. The kit for detecting cancer comprises the materials necessary to conduct any of the detection assays described in this The kit for detecting cancer comprises a first anti-glypican-1 antibody or other ligand, a pharmaceutically acceptable carrier, and glypican-1 standards; wherein said kit is capable of detecting glypican-1 in the body fluid or tissue of a patient. The first anti-glypican-1 antibody may or may not be MIL-38. The first anti-glypican-1 antibody may comprise: a heavy chain variable region comprising a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 50-54 of **SEQ ID NO: 10**; a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 69-85 of **SEQ ID NO: 10**; a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 118-126 of **SEQ ID NO: 10**; and comprise a light chain variable region comprising a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 44-54 of **SEQ ID NO: 11**; a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 70-76 of **SEQ ID NO: 11**; a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 109-117 of **SEQ ID NO: 11**. The anti-glypican-1 antibody used to detect the glypican-1 levels may not comprise: a light chain variable region comprising a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 48-58 of **SEQ ID NO: 12**; a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 74-80 of **SEQ ID NO: 12**; and a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 113-121 of **SEQ ID NO: 12**. The first anti-glypican-1 antibody may be produced by or otherwise identical to an antibody generated by hybridoma cells as deposited on 22 August 2014 at CellBank Australia (CBA) under accession number CBA20140026.

[0058] The kit will require the additional use of standard laboratory tools or machinery. Necessary tools include pipettes, cell sorting machines, plate readers, centrifuges etc as are

known to those being skilled in the art. Use of the kit may require additional standard laboratory reagents such as pipette tips, membranes, buffers, or chemicals as are well known by those being skilled in the art. The kit further comprises a secondary ligand. The secondary ligand is a second anti-glypican-1 antibody. The second anti-glypican antibody is the same as the first anti-glypican-1 antibody. The secondary ligand is conjugated to a label for rapid detection of said ligand. The antibodies of the kit can be antibody fragments or antibody combinations as described in this application.

[0059] The detection of glypican-1 in the body fluids or tissues of a patient is indicative of the presence of prostate cancer. The presence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 600, 700, 800, 900, 1000 pg/ml of glypican-1 in the body fluid of a patient is indicative of prostate cancer.

[0060] The detection of glypican-1 in the body fluids or tissues of a patient is indicative of the presence of prostate cancer. The presence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 600, 700, 800, 900, 1000 ng/ml of glypican-1 in the body fluid of a patient is indicative of prostate cancer.

[0061] The detection of glypican-1 in the body fluids or tissues of a patient is indicative of the presence of prostate cancer. The presence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 μ g/ml of glypican-1 in the body fluid or tissue of a patient is indicative of prostate cancer.

[0062] Elevated levels of glypican-1, or glypican-1 detection signal in the body fluid of a patient is indicative of prostate cancer. In some cases, the glypican-1 levels of cancer patients are 1%, 2%, 3%, 4, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 210%, 220%, 230%, 240%, 250%, 260%, 270%, 280%, 290%, 300%, 310%, 320%, 330%, 340%, 350%, 360%, 370%, 380%, 390%, 400%, 410%, 420%, 430%, 440%, 450%, 460%, 470%, 480%, 490%, 500, 600%, 700%, 800%, 900%, 1000%, 5,000%,

10,000%, >15,000% higher than the glypican-1 levels or glypican-1 detection signal of a control non-cancerous body fluid. The control non-cancerous body fluid will be age matched to the patient.

EXAMPLES**Example 1. Characterization of the cell bound MIL-38 antigen.**

[0063] Despite initial reports of the MIL-38 antigen as a 30kD protein (Russell *et al* 2004), work by the present inventors has indicated that the MIL-38 antibody predominantly detects a 60kDa antigen in a range of cell extracts. Western blot reactivity of MIL-38 with the antigen is lost if the sample has been incubated with reducing agents prior to gel electrophoresis.

[0064] Using MIL-38 antibody for immunoprecipitation experiments we were able to specifically isolate the 60kDa protein from a variety of cell extracts. The presence of the 60kDa antigen on the cell surface was investigated using live cell immunoprecipitations. In these experiments, live cells were incubated on ice with serum-free media containing the MIL-38 antibody. Cells were then washed, lysates prepared and incubated with protein G beads to isolate any antibody associated with the cells. The 60kDa band was present in these immunoprecipitates indicating that the antigen was recognized on the cell surface by the MIL-38 in the media.

Example 2. MIL-38 antigen immunoprecipitation and mass spectrometry.

[0065] DU-145 prostate cancer cells were processed through a membrane protein extraction kit (MPEK). Membrane extracts were immunoprecipitated with MIL-38 cross-linked to magnetic beads. The immunoprecipitates were either run into a gel and excised for mass spectrometry analysis or were eluted directly from the beads and then subjected to mass spectrometry. Antigens bound to the MIL-38 antibody were then analyzed via mass spectrometry analysis, which can identify peptides based on mass/charge data.

[0066] The mass spectrometer identified glypican-1 with a peptide score of 4278 and sequence coverage of 46% including 18 distinct sequences (Figure 1).

Example 3. MIL-38 antigen immunoprecipitation, size exclusion chromatography and mass spectrometry.

[0067] DU-145 prostate cancer cells were processed through a membrane protein extraction kit (MPEK). Membrane extracts were immunoprecipitated with MIL-38 cross-linked to magnetic beads. Following extensive washing, the immunoprecipitate was eluted in TBS (tris buffered saline) containing 2% SDS. The eluate was subjected to size exclusion chromatography (SEC)

and every second fraction was acetone precipitated, resuspended in sample loading buffer and used for MIL-38 western blots. Fractions 28 and 30 contained high amounts of MIL-38 antigen (Figure 2), indicating that fraction 29 would also contain high concentrations of the MIL-38 antigen. Fraction 29 was subjected to mass spec analysis and glypican-1 was identified with sequence coverage of 14% (Figure 3). This further confirmed that the antigen for the MIL-38 antibody was glypican-1.

Example 4. MIL-38 and anti-glypican-1 (anti-GPC-I) antibodies show overlapping reactivity on 2D western blots.

[0068] A rabbit anti-GPC-1 antibody was purchased and showed reactivity with the glypican-1 core protein at a molecular weight of approximately 60 kDa - the same molecular weight as detected by MIL-38. To confirm that MIL-38 recognized glypican-1, prostate cancer DU-145 MPEK extracts were subjected to 2D electrophoresis and western blotting.

[0069] As shown in Figure 4, MIL-38 antibody and the anti-GPC-1 antibodies showed overlapping reactivity detecting a protein with 60 kDa molecular weight and isoelectric points ranging from 5 to 7.

Example 5. MIL-38 is detected in anti-GPC-1 immunoprecipitates and vice versa.

[0070] MIL-38 or rabbit anti-GPC-1 antibodies were used to immunoprecipitate their respective antigens from DU-145 or C3 (MIL-38 negative) MPEK extracts. The immunoprecipitates (IPs) were western blotted with either MIL-38 or anti-GPC-1 antibody (Figure 5).

[0071] A 60kDa GPC-1 reactive band was detected in MIL-38 IPs blotted with anti-GPC-1, while a 60 kDa MIL-38 reactive band was detected in anti-GPC-1 IPs blotted with MIL-38. No reactivity was detected with the secondary only controls. Furthermore, immunoprecipitating with MIL-38 antibody resulted in almost complete depletion of both MIL-38 and anti-GPC-1 antigens, strongly suggesting that the MIL-38 antigen is glypican-1.

Example 6. MIL-38 detects recombinant GPC-1

[0072] Two sources of purified recombinant glypican-1 were tested for reactivity with MIL-38 and anti-GPC-1 antibodies. The first source was a truncated form produced from wheat germ extracts (note this would not contain appropriate mammalian post-translational modifications). The second source was full-length glypican-1 produced in murine NSO cells. No MIL-38 reactivity was observed with the wheat germ expressed glypican-1, however it could be detected with the rabbit anti-GPC-1 antibody (data not shown). In contrast, very strong reactivity with MIL-38 and anti-GPC-1 antibodies was observed for the glypican-1 produced in NSO cells (Figure 6).

Example 7. MIL-38 can detect antigen secreted into cell culture supernatant.

[0073] To date, there has been no experimental evidence for secretion of MIL-38 antigen. To test this, DU-145 cells were washed in serum-free media then incubated with serum-free media for 36 hrs. The resulting conditioned media was immunoprecipitated with MIL-38 and the resulting samples compared with a standard IP from DU-145 MPEK extracts. Bands of approximately 40 and 55 kDa were observed in the conditioned medium IP, compared to the 60 kDa band isolated from DU-145 extracts (Figure 7).

[0074] The conditioned medium IP sample containing the 40 and 55kD bands was subjected to mass spec analysis. Glypican-1 (SEQ ID No: 2) was identified with 16% peptide coverage (Figure 8a). Separate analyses of just the 40kD band (Figure 8b) or the 55kD band (Figure 8c) both identified as the glypican-1 peptide (SEQ ID No: 2).

[0075] These results suggest that a MIL-38 reactive form of glypican-1 can be released into cell culture supernatant from the DU-145 prostate cancer cell line.

Example 8. GPC-1 can be detected by MIL-38 in prostate cancer plasma samples and in membrane extracts from prostate cancer patients (comparative example).

[0076] To date, there has been no experimental evidence for secretion of MIL-38 antigen in plasma samples from normal or prostate cancer patients. To test this, plasma samples from one normal patient (042) and one prostate cancer patient (046) were immunoprecipitated with MIL-38 antibody and the IP sample western blotted with MIL-38 and anti-GPC-1 antibodies (Figure 9a).

[0077] Both antibodies detected specific bands of approx. 70kDa in both plasma samples. The signals were markedly higher (darker bands) for both MIL-38 and anti-GPC-1 antibodies in the prostate cancer patient plasma compared to the normal patient plasma, suggesting that this soluble form of glypican-1 may be elevated in prostate cancer patients.

[0078] To determine if MIL-38 antigen could be detected in membrane protein extracts from normal prostate and prostate cancer, one sample of each was obtained from Novus Bio. Equivalent amounts of protein were western blotted using MIL-38 antibody (Figure 9b). The prostate cancer extract demonstrated much higher expression of the MIL-38 antigen than the normal prostate sample.

Example 9. Detection of MIL-38 antigen in patient urine.

[0079] MIL-38 can detect cells in the urine of prostate cancer patients. To test the sensitivity and specificity of this detection method, 125 age-matched urine samples were obtained. Cells were

spun down from the urine and analyzed by the MIL-38 indirect immunofluorescence assay. A total of 47 healthy controls, 37 benign prostatic hypertrophy (BPH) and 41 biopsy-confirmed prostate cancers were analyzed. Examples of positive prostate cancer cells, DU-145 positive controls and C3 negative cells are shown (Figure 10).

[0080] The MIL-38 immunofluorescence assay (IFA) test demonstrated a sensitivity of 71% and a specificity of 73% in identifying prostate cancers within the cohort. The test showed 71% sensitivity and 76% specificity in identifying prostate cancers compared to BPH patients. (Table 3).

Table 3. Sensitivity and specificity calculations of prostate cancer detection in patient urine.

Sensitivity and Specificity Calculations	
True Positive	False Positive
29	12
False Negative	True Negative
23	61
Sensitivity and Specificity Calculations for BPH only	
True Positive	False Positive
29	12
False Negative	True Negative
9	28

Example 10. Combination of MIL-38 antigen detection with PSA level increases ability to detect prostate cancer.

[0081] When the MIL-38 immunofluorescence assay (IFA) test is combined with the PSA test there are increases in sensitivity and specificity. These increases vary according to the cut-off value applied to the PSA test. When the cutoff for a positive diagnosis is greater than 2 ng/mL then specificity increases from 73% for just the IFA test to 83% for the two tests combined. When the cutoff for a positive diagnosis is greater than 4 ng/mL then specificity increases from 73% for just the IFA test to 89% for the two tests combined.

[0082] This is further illustrated by the logistic regression analysis which shows increases in OR and 95% CI when the two tests are combined.

Table 4. Increases in sensitivity and specificity when the MIL-38 IFA test is combined with PSA scores

Stratified by 2 ng/ml

PSA < 2 ng/ml					
	Cancer		Cancer		
	1	0	1	0	
	Sensitivity =		GPC-I		Sensitivity = 67%
			IFA		Specificity = 83%
			1	24	4
			0	12	20

GPC-1	1	5	17	100%	<u>Stratified by 4 ng/ml</u>
IFA	0	0	36	Specificity = 68%	

PSA \geq 2 ng/ml PSA < 4 ng/ml

		Cancer		
		1	0	
GPC-1	1	10	20	Sensitivity = 77%
IFA	0	3	48	Specificity = 71%

PSA \geq 4 ng/ml

		Cancer		
		1	0	
GPC-1	1	19	1	Sensitivity = 68%
IFA	0	9	8	Specificity = 89%

Logistic regression

	OR	95% CI
GPC-1 IFA	6.4	2.8-14.9
GPC-1 IFA (adjusted for PSA <4)	10.2	3.2-32.8
GPC-1 IFA (adjusted for PSA <2)	13.4	4.0-44.7

Example 11. MIL-38 can detect recombinant glypican-1 in a variety of ELISA formats.

[0083] Three ELISA assay formats were conducted as shown in Figure 11. Recombinant glypican-1 was tested at 0, 0.1, 1 and 10 ng/ml concentrations using MIL-38 as a capture antibody and rabbit anti-GPC-1 as a detection antibody. Similar experiments were conducted using the rabbit anti-GPC-1 as the capture antibody and the MIL-38 as the detection antibody. A single antibody ELISA was also tested using the MIL-38 antibody as the capture antibody, and a biotinylated MIL-38 antibody as the detection antibody. The results indicate that anti-GPC antibodies can be used in a variety of ELISA formats and that the NS0 GPC-1 might represent a suitable positive control.

Example 12. Detection of glypican-1 antigen using AM4 MIL-38 antibodies

[0084] Experiments performed by the present inventors determined that an original deposit of the hybridoma for MIL-38 antibody (ATCC accession no. HB11783: murine hybridoma BLCA-38), then referred to as the "BLCA-38 hybridoma" is a mixed population of hybridoma cells that produces two distinct antibody populations, referred to here as "AM3" and "AM4". Hybridoma cells responsible for producing each different antibody population were separated, and the "AM4" hybridoma cells were deposited on 22 August 2014 at CellBank Australia (CBA) under accession number C8A20140026.

[0085] Ninety-six well plates were coated with MIL-38 preps AM3 or AM4 (1 µg/well) in carbonate buffer pH 9.5 overnight. Plates were blocked with PBS-Tween (0.1%) containing 5% skim milk at 37°C and washed. Antigen (GPC-1 NS0) was diluted in Buffer II (20mM HEPES pH 7.5, 0.5mM EDTA, 0.5% Triton X-100) with the addition of 150mM NaCl and incubated overnight at 37°C. Detection was performed with biotinylated AM4 antibody followed by detection with avidin HRP (1 µg/mL). TMB (Sigma cat no T0440) was added and stopped with TMB stop solution (Sigma S5814). Absorbance was read at 450nm. Results are shown in **Figure 12A**.

[0086] In a second experiment, ninety-six well plates were coated with MIL-38 preps 34A (a mixture of AM3 and AM4 antibodies) or AM4 (2.5 µg/well) in PBS pH 7.2 for in at room temperature. Plates were blocked with Blocker Casein (Thermo) in PBS-Tween (0.05%) for 1h at 37°C. Following washing, antigen (GPC-1 NS0) was diluted in TBS pH 7.2 containing 50mM Tricine and 150 mM NaCl and incubated at 37°C for 1h. Detection was performed with biotinylated AM4 clone 1F5 followed by detection with avidin HRP (1µg/ml). TMB (Sigma cat no T0440) was added and stopped with TMB stop solution (Sigma S5814). Absorbance was read 450nm. Results are shown in **Figure 12B**.

[0087] The first ELISA described above was developed using MIL-38 to capture NS0 produced GPC-1 (i.e. MIL-38 antigen). This experiment compared monoclonal AM3 MIL-38 and monoclonal AM4 MIL-38 for capture. AM3 did not function as a capture agent in a sandwich ELISA assay, whereas AM4 was shown to do so (**Figure 12A**).

[0088] The second ELISA described above compared the ELISA signal obtained when a mixed population of MIL-38 (AM3 and AM4) was compared to that obtained from a monoclonal AM4 1F5 clone. Using AM4 1F5 as a capture agent provided a higher ELISA signal than using the mixed 34A antibody population (**Figure 12B**).

[0089] The sandwich ELISA results demonstrate that only the AM4-like forms of the monoclonal MIL-38 antibody have utility in detecting glypican-1 antigen as a capture reagent and that a capture agent containing a monoclonal population provides a superior ELISA signal to that consisting of a mixed population.

Example 13 sequence analysis of AM4 and AM3 MIL-38 antibody populations

Materials and Methods

- Heavy and light chain sequencing (DNA)

[0090] Three separate sequencing runs were performed. The first run (coded 224945) utilised bi-clonal hybridoma cells from a mixed (AM4 and AM3) preparation named 1-O. The second run

(coded 449295-1) utilised cells from Alfio I a hybridoma stock that was used to generate AM4. The third run (coded 449295-5) utilised cells from Alfio II, a hybridoma stock that was used to generate AM3.

[0091] For sequencing runs 224945 (1-O) and 449295-1 (Alfio I), total RNA was extracted from frozen hybridoma cells and cDNA was synthesized from the RNA. RT-PCR was then performed to amplify the variable regions (heavy and light chains) and constant regions of the antibody, which were then cloned into a standard cloning vector separately and sequenced.

[0092] Total RNA was isolated from the hybridoma cells following the technical manual of TRIzol® Plus RNA Purification System. The total RNA was analysed by agarose gel electrophoresis. Total RNA was reverse transcribed into cDNA using isotype-specific antisense primers or universal primers following the technical manual of SuperScript™ III First-Strand Synthesis System. The antibody fragments of V_H, V_L, C_H and C_L were amplified according to the standard operating procedure of RACE of GenScript.

[0093] Amplified antibody fragments were separately cloned into a standard cloning vector using standard molecular cloning procedures.

[0094] Colony PCR screening was performed to identify clones with inserts of correct sizes. No less than five single colonies with inserts of correct sizes were sequenced for each antibody fragment.

[0095] V_H and V_L plasmids encoded the full-length variable regions of the antibody and a part of C_{H1} and C_L. C_H plasmid encoded a part of C_{H1} and full-length C_{H2} and C_{H3}. C_L plasmid encoded a part of C_L. In order to get full-length constant regions or heavy/light chain, the part of constant regions encoded by V_H and V_L plasmids and the part of constant regions encoded by C_H and C_L plasmids were amplified by PCR separately, and then overlap extension PCR was employed to obtain full-length DNAs. Five single colonies with correct V_H, V_L, C_H and C_L insert sizes were sent for sequencing.

[0096] Sequencing run 449295-5 (Alfio II) encountered difficulty obtaining sequence corresponding to the expected IgG1 heavy chain sequence. Two RNA preparations were performed. For the 1st batch of cells, oligo-dT primer and CDS III primers were used for reverse transcription (RT). V_H/C_H and V_K/C_K were amplified by PCR using IgG1 and IgK specific primers, partial mouse β-actin gene was amplified as positive control. Normal light chain bands were obtained easily while only weak V_H could be observed on the gel. Five individual colonies with correct V_K and C_K insert sizes were sent for sequencing. The V_K and C_K genes of five different clones were found to be nearly identical. The consensus light chain sequences from the Alfio II hybridoma is listed below. One unproductive heavy chain sequence was obtained from eight randomly sequenced V_H positive clones, shown as below. Three kinds of heavy chain constant region sequences were obtained from ten randomly sequenced C_H positive clones (one IgG₁C_B, one IgG_{2a}C_H and eight IgG_{2b}C_H). In order to avoid the influence of potential class switching, amplification of the C_H using IgM specific primer was performed, but no target PCR

product was obtained. There was also no target PCR product when full-length heavy chain (V_H-C_H) was amplified using heavy chain FR1 degenerate primers.

[0097] As no productive heavy chain could be obtained after several attempts, isolation of heavy chain sequence from the 2nd vial of Alfio II cells was attempted. For the 2nd vial of cells, oligo-dT primer was used for reverse transcription initially. V_H was amplified using IgG1, IgG2b, IgM, IgA specific primers and IgG degenerate primer, respectively, and V_K was amplified using IgK specific primers. Productive light chain and unproductive heavy chain, which were identical with previous results, were obtained. Reverse transcription using Random 6 mers primer was also attempted without success.

[0098] In summary, multiple attempts to isolate light chain and heavy chain sequence were made. One rearranged light chain sequence was consistently obtained after different attempts on two batches of cells. However, only weak V_H target PCR products were observed and sequencing did not result in any consistent heavy chain sequence.

Results

- Sequence Summary Table

[0099] Table 5 below provides an overview of heavy and light chain nucleic acid and protein sequences of the antibodies studied, indicating the positions of various internal regions.

Internal regions	HFR2	HCDR2	HFR3	HCDR3	HFR4	CHI-CH3	Hinge
	353-394	205-255	256-352	353-375	379-411	412-1361	703-743
	LFR2	LCDR2	LFR3	LCDR3	LFR4	CL	
	463-507	208-228	275-329	325-351	352-361	362-702	
	575-619	120-140	241-335	337-353	354-395	394-714	
	HFR2	HCDR2	HFR3	HCDR3	HFR4	CH	Hinge
	55-68	69-85	86-117	118-126	127-157	138-461	235-247
	LFR2	LCDR2	LFR3	LCDR3	LFR4	CL	
	55-69	70-76	77-108	109-117	118-127	128-234	
	59-73	74-80	81-112	113-121	122-131	132-238	

*in complementarity determining region; CH = heavy chain constant region
 complementarity determining region; CL = light chain constant region
 in column 1 by SEQ ID NO.*

CAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAACTGTCACCATCACATGT
 TGGTATCAGCAGAAACAGGGAAAATCTCCTCAACTCCTGGGTAT
 GGTGTGCCATCAAGGTTCAAGTGGCAGTGGATCAGGAACACAATATCTCTCAAGATCAATAGCCTGCAGCCT
 GAAGATTTTGGGACTTATTACTGT
 TTDGGETGGAGGCACCAAGCTGGAA
 ATCAAA
 TGA

Individual regions of mouse heavy chain encoded sequence are highlighted with alternating shaded/unshaded text. Positions: 1-57 = leader sequence; 58-147 = framework region (HFR1); 148-162 = complementarity determining region (HCDR1); 163-204 = HFR2; 205-255 = HCDR2; 256-351 = HFR3; 352-378 = HCDR3; 379-411 = HFR4; 412-1383 = constant region (CH1-CH3); 703-741 = hinge region (underlined); 1384-1386 = stop codon.

AM4 MIL-38 Mouse Kappa Light Chain DNA Consensus Sequence (SEQ ID NO: 14)

CACATCCAGATGACT
 CAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAACTGTCACCATCACATGT
 TGGTATCAGCAGAAACAGGGAAAATCTCCTCAACTCCTGGGTAT
 GGTGTGCCATCAAGGTTCAAGTGGCAGTGGATCAGGAACACAATATCTCTCAAGATCAATAGCCTGCAGCCT
 GAAGATTTTGGGACTTATTACTGT
 TTDGGETGGAGGCACCAAGCTGGAA
 ATCAAA
 TAG

Individual regions of mouse light chain encoded sequence are highlighted with alternating shaded/unshaded text. Positions: 1-60 = leader sequence; 61-129 = framework region (LFR1); 130-162 = complementarity determining region (LCDR1); 163-207 = LFR2; 208-228 = LCDR2; 229-324 = LFR3; 325-351 = LCDR3; 352-381 = LFR4; 382-702 = constant regions (CK); 703-705 = stop codon.

[0103] The heavy and light chain AM4 MIL-38 consensus DNA sequences above translate to the following heavy chain and light chain amino acid sequences:

AM4 MIL-38 Mouse IgG1 Heavy Chain Amino Acid Consensus Sequence (SEQ ID NO:

10)

MAKVWTLLELMAAACSIOAQIQLVQSGPELKKPGETVKISCKASGYAFTDYSMWVVKQAPGKGLRWMGWINTETG
 EPTYLDEKGRFAPSLETSASTAFLQINNLRNEDTATYFCARHYDYCGEPIYWGQGLVTVSAAKTTPFESVYPLAE
 GSAACINSMVTLGCLVKGYPPEVTVTWNSGSLSSGVHTYPAVLQSDLYTLSSSVTVPSSTWPESETVYCKVAHPA
 SSTRVERRKIVPRDCGKPCICTVPEVNSVYIIPPKPKRVLVITLTERVICWVVDISKDDPEVQESWPFVDEWEVHT
 ACTOPREKQFNSTFESVSELPIHQDNLNGKEFNCRVNSAAFEKPIEKTISKTKGRFFAPQVYITPPEKEQMAKE
 KVELTCNITCFPPEDITVLEKQWNGQPAENYKNTQEIIMDTDGSYFVYSKLNVDKSWAAGNFTTGSVLHEGLNHR
 TEKSLSHSEGK*

Individual regions of mouse IgG1 heavy chain sequence are indicated in the amino acid sequence above. Positions 1-19 = leader sequence; 20-49 = framework region (HFR1); 50-54 = complementarity determining region 1 (HCD1); 55-68 = HFR2; 69-85 = HCDR2; 86-117 = HFR3; 118-126 = HCDR3; 127-137 = HFR4 (also called the joining region or J-region); 138-461 = IgG1 chain constant regions (CH1 - CH3) & stop codon (*). Hinge region = is underlined in the sequence above.

AM4 consensus MIL-38 Light Chain Amino Acid Consensus Sequence (SEQ ID NO: 11)

MSYLTQVLAALLLINTGARGDIQMTQSPASLSASVGETVTITCRASGYNZHNYLEWYQQKQKNSPQLLVYAKTLA

GCVPSRFSGSGSGTQYSLKINSLOPEDFGTYTC
HFWSNQWTFGGGTRLEIKRADAAPTIVSIFPPPSSEQLTSGG
SVVCFLNNEFYKDIIVKWKIDGSEKQNGVLNSWTDQISKDSSTYSMSSTLTLTKBEYERHNSYTC
EATHKTTSTPE
TKSFRNRFC*

Individual regions of light chain amino acid sequence are indicated as labelled: Positions 1-20 - Leader sequence; framework region (LFR1); 21-43 - complementarity determining region 1 (LCDR1); 44-54 - LFR2; 55-69 - LCDR2; 70-76 - LFR3; 77-108 = LCDR3; 109-117 - LFR4; 118-234 - kappa constant region (CK) & stop codon(*)

AM3 consensus sequences

[0104] No consistent heavy chain sequence could be obtained from the AM3-iike Alfio II cells. The light chain sequence obtained from sequencing run 449295-5 (Alfio II) was consistently obtained and showed clear differences in both the framework regions and the complementarity-determining regions compared to the sequence for the other two sequencing runs.

AM3 MIL-38 Kappa Light Chain DNA Consensus Sequence (SEQ ID NO: 15)

GAC
ATTGTGATGACCCAGTCTCAAAAAGTTCATGTCCACATCAATAGGAGACAGGGTCAGCGTCACCTGC
TGGPTTCAGCAGAAACCAGGSCAATCTCCTAAAGCACTGATTAC
GGAGTCACTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAAC
AATGTCCACTCTGAACACTTCGCCAGACTATTTCTGT
TTCGGTTC
CCCG
ACAAAGTTGGAAATAAAA
TAG

* Individual regions of light chain encoded sequence are highlighted with alternating shaded-unshaded text. Positions: 1-72 = leader sequence; 73-141 = framework region (LFR1); 142-174 = complementarity determining region (LCDR1); 175-219 = LFR2; 220-240 = LCDR2; 241-336 = LFR3; 337-363 = LCDR3; 364-393 = LFR4; 394-714 = constant region (CK); 715-717 = stop codon

AM3 MIL-38 Light Chain Amino Acid Consensus Sequence (SEQ ID NO: 12)

NCILIMESQTQVFVYMLLNLSQVEGDIVMTQSQKFMSTSIQDRVSVTC
KASQNVGSHVAKFQQKPCQSPKALTY
SIRYISGVTDTRFTGSGSGTDFTLTINNVQSEDLAEYFCQYNSFPFIFGSGTKLEIKRADAAPTIVSIFPPPSSEQLT
SGGASVVCFLNNEFYKDIIVKWKIDGSEKQNGVLNSWTDQISKDSSTYSMSSTLTLTKBEYERHNSYTC
EATHKTTSTPE
TKSFRNRFC*

Individual regions of light chain amino acid sequence are indicated as labelled; Positions 1-24 = Leader sequence; 25-47 = framework region (LFR1); 48-58 = complementarity determining region 1 (LCDR1); 59-73 = LFR2; 74-80 = LCDR2; 81-112 = LFR3; 113-121 = LCDR3; 122-131 = LFR4; 132-238 = kappa constant region (CK) & stop codon(*)

Example 14 identification and characterisation of glypican-1 epitope bound by AM4 anti-glypican-1 antibodies

Materials and Methods

[0105] Table 6 below provides information on the AM4 anti-glypican-1 antibodies used in this study.

Table 6: description of AM4 antibodies used

Name	Origin	Concentration	Location	Status
MIL38-AM4*	mouse	4.6 mg/ml	-20 °C/ 73	OK
* Produced by hybridoma cells as deposited at Cellbank Australia under accession number CBA20140026				

- Peptides

[0106] The human glypican-1 (GPC-1) sequence on which this study was based is defined in SEQ ID NO: 16. The following sequences of residues were used:

GPC1_residues #343-366	GNPKVNPQGPGEKRRRGKLAP	(SEQ ID NO: 17)
GPC1_residues #140-149	GELYTQNARAFRDLYSELR	(SEQ ID NO: 18)

- Peptide synthesis

[0107] Peptide synthesis was performed using the methods referred in Example 1. Chemically synthesized linear and CLIPS peptides were synthesized according to the designs shown below:

- Chemically Linked Peptides on Scaffolds (CLIPS) technology

[0108] The following provides description of general principles of the CLIPS technology utilised.

[0109] CLIPS technology structurally fixes peptides into defined three-dimensional structures. This results in functional mimics of even the most complex binding sites. CLIPS technology is now routinely used to shape peptide libraries into single, double or triple looped structures as well as sheet- and helix-like folds.

[0110] The CLIPS reaction takes place between bromo groups of the CLIPS scaffold and thiol sidechains of cysteines. The reaction is fast and specific under mild conditions. Using this chemistry, native protein sequences are transformed into CLIPS constructs with a range of structures including single T2 loops, T3 double loops, conjugated T2+T3 loops, stabilized beta sheets, and stabilized alpha helices (Timmerman et al., J. Mol. Recognit. 2007; 20: 283- 29).

[0111] CLIPS library screening starts with the conversion of the target protein into a library of up to 10,000 overlapping peptide constructs, using a combinatorial matrix design. On a solid carrier, a matrix of linear peptides is synthesized, which are subsequently shaped into spatially defined CLIPS constructs. Constructs representing both parts of the discontinuous epitope in the correct conformation bind the antibody with high affinity, which is detected and quantified. Constructs presenting the incomplete epitope bind the antibody with lower affinity, whereas constructs not containing the epitope do not bind at all. Affinity information is used in iterative screens to define the sequence and conformation of epitopes in detail.

[0112] The target protein containing a discontinuous conformational epitope is converted into a matrix library. Combinatorial peptides are synthesized on a proprietary minicard and chemically converted into spatially defined CLIPS constructs. Binding of antibodies is quantified.

- Peptide synthesis

[0113] To reconstruct discontinuous epitopes of the target molecule a library of structured peptides was synthesized. This was done using Chemically Linked Peptides on Scaffolds (CLIPS) technology. CLIPS technology allowed the generation of structured peptides in single loops, double-loops, triple loops, sheet-like folds, helix-like folds and combinations thereof. CLIPS templates were coupled to cysteine residues. The side-chains of multiple cysteines in the peptides were coupled to one or two CLIPS templates. For example, a 0.5 mM solution of the T2 CLIPS template 1,3-bis (bromomethyl) benzene was dissolved in ammonium bicarbonate (20 mM, pH 7.9)/acetonitrile (1:1(v/v)). This solution was added onto the peptide arrays. The CLIPS template bound to side-chains of two cysteines as present in the solid-phase bound peptides of the peptide-arrays (455 wells plate with 3 µl wells). The peptide arrays were gently shaken in the solution for 30 to 60 minutes while completely covered in solution. Finally, the peptide arrays were washed extensively with excess of H₂O and sonicated in disrupt-buffer containing 1 percent SDS/0.1 percent beta-mercaptoethanol in PBS (pH 7.2) at 70°C for 30 minutes, followed by sonication in H₂O for another 45 minutes. The T3 CLIPS carrying peptides were made in a similar way but with three cysteines.

[0114] Linear and CLIPS peptides were chemically synthesized according to the following designs:

Set 1

Mimic	Discontinuous epitope mimics
Type	
Label	MAT.A, MAT.B
Description	Constrained peptide mimics of varying length. From the two starting sequences (SEQ ID NO: 17 and SEQ ID NO: 18) all 10 to 22, and 10 to 18 mer peptides with stepsize 4 have been made, and these have been paired. At the termini and in between the two peptides cysteines are placed. These are linked by a T3 CLIPS.

Set 2

Mimic	Linear peptides
Type	
Label	RN.PKVNPQGGPGPEEKRR (SEQ ID NO: 19)
Description	Substitution analysis, starting from the base sequence PKVNPQGGPGPEEKRR (SEQ ID NO: 20), all individual amino acids are replaced by all naturally occurring amino acids, except cysteine.

Set 3

Mimic	Constrained peptides
Type	
Label	RN.PKVNPQGGPGPEEKRR_LOOP (SEQ ID NO: 19),
	RN.ELCTQNCRAFRDLYS_heli3 (SEQ ID NO: 21)
	RN.ELCTQNCRAFRDLYS_LOOP (SEQ ID NO: 21)
Description	Substitution analyses, starting from the base sequences as indicated in the label names, all individual amino acids are replaced by all naturally occurring amino acids, except cysteine.

- ELISA screening

[0115] The binding of antibody to each of the synthesized peptides was tested in a PEPSCAN-based ELISA. The peptide arrays were incubated with primary antibody solution (overnight at 4°C). After washing, the peptide arrays were incubated with a 1/1000 dilution of an antibody peroxidase conjugate (SBA, cat.nr.2010-05) for one hour at 25°C. After washing, the peroxidase substrate 2,2'-azio-di-3-ethylbenzthiazoline sulfonate (ABTS) and 2 µl/ml of 3 percent H₂O₂ were added. After one hour, the color development was measured. The color development was quantified with a charge coupled device (CCD) - camera and an image processing system.

- Data processing

[0116] The values obtained from the CCD camera range from 0 to 3000 mAU, similar to a standard 96-well plate ELISA-reader. The results are quantified and stored into the Peplab database. Occasionally a well contains an air-bubble resulting in a false-positive value, the cards are manually inspected and any values caused by an air-bubble are scored as 0.

- Synthesis quality control

[0117] To verify the quality of the synthesized peptides, a separate set of positive and negative control peptides was synthesized in parallel. These were screened with antibody 57.9 (*ref.* Posthumus et al., J. Virology, 1990, 64:3304-3309).

- Screening details

[0118] Table 7 summarises antibody binding conditions. For the Pepscan Buffer and Preconditioning (SQ), the numbers indicate the relative amount of competing protein (a combination of horse serum and ovalbumin).

Table 7: screening conditions

serum	dilution	samplebuffer	preconditioning
MIL-38 AM4	10 µg/ml	10%SQ	50%SQ

Results

- Primary experimental results and signal to noise ratio determination

[0119] A graphical overview of the complete dataset of raw ELISA results generated by the screening is shown in **Figure 13**. Here a box plot depicts each dataset and indicates the average ELISA signal, the distribution and the outliers within each dataset. Depending on experiment conditions (amount of antibody, blocking strength etc) different distributions of ELISA data are obtained.

- Antibody MIL 38-AM4

[0120] In earlier analyses carried out by the present inventors it was established that MIL 38-AM4 binds glypican on stretch $_{348}\text{VNPQGPGPEEK}_{358}$ (**SEQ ID NO: 22**), and also binds to the stretch $_{135}\text{TQNARA}_{140}$ (**SEQ ID NO: 8**)/ $_{135}\text{TQNARAFRD}_{143}$ (**SEQ ID NO: 9**), which was taken as an indication for a discontinuous epitope.

[0121] The looped constructs containing the main stretch pinpoint the residues that are most critical to binding. The results of this study demonstrated that residues V348, Q351, G352, and P353 do not tolerate replacement, with significant requirement for K347, N349 and P350, and to a lesser extent from G354, P355, and E356 (**Figure 14**).

- Conclusions

[0122] The conformational epitope of antibody MIL38- AM4 was profiled.

Leads obtained in earlier analyses that point to a discontinuous epitope for MIL38 - AM4 were used to generate a matrix array in which the loops have different lengths. In addition, full substitution analyses of the individual lead sequences were made. All arrays were probed with MIL38-AM4 antibody.

[0123] For recognition of glypican-1, the MIL38 - AM4 antibody investigated in this study binds to an epitope that exclusively or mainly consists of the flexible loop between residues 347 and 358.

[0124] Monoclonal antibody MIL38-AM4 mainly binds glypican-1 on the loop between residues 347-355, but this antibody clearly benefits from the addition of residues from the range 135-140 or 135-143 to the peptide.

Table 8: Epitope of the MIL38-AM4 antibody

Antibody	Epitope	Most important residues	Conservation
MIL38-AM4	₃₄₇ KVNPQGGP ₃₅₅ (SEQ ID NO: 7)	V348, Q351, G352, P353	Y

[0125] Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials are described herein.

[0126] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

[0127] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

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Ala	Asp	Ala	Ala	Pro	Thr	Val	Ser	Ile	Phe	Pro	Pro	Ser	Ser	Glu	Gln
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aacagttgga	ctgatcagga	cagcaagac	agcaoctaca	gcattgagcag	cacctcaccg	600
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<211> 558

<212> PRT

<213> Homo sapiens

<400> 16

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          35           40           45
Val Pro Gln Ala Glu Ile Ser Gly Glu His Leu Arg Ile Cys Pro Gln
          50           55           60
Gly Tyr Thr Cys Cys Thr Ser Glu Met Glu Glu Asn Leu Ala Asn Arg
65           70           75           80
Ser His Ala Glu Leu Glu Thr Ala Leu Arg Asp Ser Ser Arg Val Leu
          85           90           95
Gln Ala Met Leu Ala Thr Gln Leu Arg Ser Phe Asp Asp His Phe Gln
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His Leu Leu Asn Asp Ser Glu Arg Thr Leu Gln Ala Thr Phe Pro Gly
          115          120          125
Ala Phe Gly Glu Leu Tyr Thr Gln Asn Ala Arg Ala Phe Arg Asp Leu
          130          135          140
Tyr Ser Glu Leu Arg Leu Tyr Tyr Arg Gly Ala Asn Leu His Leu Glu
          145          150          155          160
Glu Thr Leu Ala Glu Phe Trp Ala Arg Leu Leu Glu Arg Leu Phe Lys
          165          170          175
Gln Leu His Pro Gln Leu Leu Leu Pro Asp Asp Tyr Leu Asp Cys Leu
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Gly Lys Gln Ala Glu Ala Leu Arg Pro Phe Gly Glu Ala Pro Arg Glu
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Ala His Cys Leu Gly Val Pro Gly Ala Arg Pro Cys Pro Asp Tyr Cys
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Glu Trp Arg Asn Leu Leu Asp Ser Met Val Leu Ile Thr Asp Lys Phe
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Trp Gly Thr Ser Gly Val Glu Ser Val Ile Gly Ser Val His Thr Trp
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Cys Ser Glu Lys Met Ala Leu Ser Thr Ala Ser Asp Asp Arg Cys Trp
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Pro Asp Met Thr Ile Arg Gln Gln Ile Met Gln Leu Lys Ile Met Thr
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<211> 17

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<223> Description of Artificial Sequence: Labeled homo sapiens Glypican-1 sequence

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<210> 23
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 115 120 125
 Ala Phe Gly Glu Leu Tyr Thr Gln Asn Ala Arg Ala Phe Arg Asp Leu
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 Tyr Ser Glu Leu Arg Leu Tyr Tyr Arg Gly Ala Asn Leu His Leu Glu

 145 150 155 160
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 Gln Leu His Pro Gln Leu Leu Leu Pro Asp Asp Tyr Leu Asp Cys Leu
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 Gly Lys Gln Ala Glu Ala Leu Arg Pro Phe Gly Glu Ala Pro Arg Glu
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 225 230 235 240
 Pro Leu Gly Pro Glu Cys Ser Arg Ala Val Met Lys Leu Val Tyr Cys
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 260 265 270

Arg Asn Val Leu Lys Gly Cys Leu Ala Asn Gln Ala Asp Leu Asp Ala
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<211> 17

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<213> Homo sapiens

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REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. En *in vitro*-metode til detektion af prostatakraft hos en patient, metoden omfatter måling af niveauet af opløseligt glypican-1 i en kropsvæskeprøve, der er
5 indsamlet fra den gruppe, der består af blod, serum, plasma og urin, som er indhentet fra en patient, og fastsættelse af, at nævnte patient har prostatakraft eller en øget sandsynlighed for at udvikle prostatakraft baseret på niveauet af opløseligt glypican-1 i kropsvæskeprøven, hvori de målte niveauer af opløseligt glypican-1 i kropsvæskeprøven fra patienten sammenlignes med:
10 en kontrolprøve af kropsvæske, der er opnået fra en kræftfri patient; eller forudbestemte referenceværdier eller områder af referenceværdier.

2. *In vitro*-metoden til detektion af prostatakraft hos en patient i krav 1 omfatter trinnene at:
15 (a) bringe nævnte kropsvæskeprøve, der er opnået fra patienten, i kontakt med et anti-glypican-1-antistof; og
(b) fastsætte, at nævnte patient har prostatakraft eller en øget sandsynlighed for at udvikle prostatakraft baseret på binding af nævnte anti-glypican-1-antistof til nævnte kropsvæskeprøve;
20 hvori anti-glypican-1-antistofbinding til nævnte kropsvæskeprøve sammenlignes med anti-glypican-1-antistofbindingen i en kontrolprøve;
hvori øget anti-glypican-1-antistofbinding af kropsvæskeprøven over kontrolprøven associeres med forekomsten af prostatakraft.

- 25 3. *In vitro*-metoden i krav 2, hvori nævnte anti-glypican-1-antistof er MIL-38.

4. *In vitro*-metoden i krav 2, hvori nævnte kropsvæskeprøve bringes i kontakt med en population af antistoffer, hvori: antistoffer af populationen omfatter:
30 (a) et variabelt område med svær kæde, som omfatter:
et komplementaritetsbestemmende område 1 (CDR1), der omfatter eller består af en aminosyresekvens, der er defineret af positionerne 50-54 i **SEQ ID NO: 10**;

et komplementaritetsbestemmende område 2 (CDR2), der omfatter eller består af en aminosyresekvens, der er defineret af positionerne 69-85 i **SEQ ID NO:10;**

5 et komplementaritetsbestemmende område 3 (CDR3), der omfatter eller består af en aminosyresekvens, der er defineret af positionerne 118-126 i **SEQ ID NO: 10;** og

(b) et variabelt område med let kæde, som omfatter:

10 et komplementaritetsbestemmende område 1 (CDR1), der omfatter eller består af en aminosyresekvens, der er defineret af positionerne 44-54 i **SEQ ID NO: 11;**

et komplementaritetsbestemmende område 2 (CDR2), der omfatter eller består af en aminosyresekvens, der er defineret af positionerne 70-76 i **SEQ ID NO: 11;**

15 et komplementaritetsbestemmende område 3 (CDR3), der omfatter eller består af en aminosyresekvens, der er defineret af positionerne 109-117 i **SEQ ID NO: 11;** og

antistoffer af populationen udgør ikke et variabelt område med let kæde, som omfatter:

20 et komplementaritetsbestemmende område 1 (CDR1), der omfatter eller består af en aminosyresekvens, der er defineret af positionerne 48-58 i **SEQ ID NO: 12;**

et komplementaritetsbestemmende område 2 (CDR2), der omfatter eller består af en aminosyresekvens, der er defineret af positionerne 74-80 i **SEQ ID NO: 12;**

25 et komplementaritetsbestemmende område 3 (CDR3), der omfatter eller består af en aminosyresekvens, der er defineret af positionerne 113-121 i **SEQ ID NO: 12.**

5. *In vitro*-metoden i krav 4, hvori antistofpopulationen produceres af eller på anden
30 måde er identisk med en antistofpopulation, som genereret af hybridomaceller, der blev deponeret den 22. august 2014 hos CellBank Australia (CBA) under adgangsnummer CBA20140026.

6. *In vitro*-metoden i krav 2, hvori nævnte anti-glypican-1-antistof ikke er MIL-38.
7. *In vitro*-metoden i ethvert af kravene 2 til 4, hvori nævnte anti-glypican-1-antistof
5 er et antistoffragment eller rekombinant antistof, som kan binde glypican-1.
8. *In vitro*-metoden i krav 1, hvori nævnte niveau af opløseligt glypican-1 i den
kropsvæskeprøve, der er opnået fra en patient, sammenlignes med niveauet af
opløseligt glypican-1 i en kontrolprøve; hvori øget anti-glypican-1-antistofbinding
10 af kropsvæskeprøven over kontrolprøven, fortrinsvis en stigning på 50 % eller
mere i niveauet af opløseligt glypican-1 i nævnte kropsvæskeprøve over niveauet
af opløseligt glypican-1 i kontrolprøven, er associeret med tilstedeværelsen af
prostatakræft.
- 15 9. *In vitro*-metoden i ethvert af kravene 2 til 8, hvori en stigning på 50 % eller mere i
anti-glypican-1-antistofbinding til nævnte kropsvæskeprøve over niveauet af anti-
glypican-1-antistofbinding af kontrolprøven er indikativ for prostatakræft.
- 10 10. *In vitro*-metoden i ethvert af kravene 2 til 7 eller 9, hvori anti-glypican-1-
antistofbindingen til nævnte kropsvæskeprøve sammenlignes med anti-glypican-
1-antistofbinding til én eller flere glypican-1-standarder; hvori anti-glypican-1-
antistofbindingen af standarderne bruges til at kvantificere mængden af opløseligt
glypican-1 i nævnte kropsvæskeprøve.
- 25 11. *In vitro*-metoden i ethvert af kravene 1 til 10, hvori et opløseligt glypican-1-
indhold over 10 ng/ml i kropsvæskeprøven er indikativ for prostatakræft.
12. *In vitro*-metoden i ethvert af kravene 1 til 11 omfatter desuden:
måling af niveauet af prostataspecifikt antigen (PSA) i en kropsvæskeprøve,
30 der er opnået fra patienten, og fastsættelse af, at nævnte patient har prostatakræft
eller en øget sandsynlighed for at udvikle prostatakræft baseret på (i) niveauet af

PSA, der er målt i kropsvæskeprøven, og (ii) binding af nævnte anti-glypican-1-antistof til nævnte kropsvæskeprøve.

- 5 **13.** *In vitro*-metoden i krav 12, hvori niveauet af prostataspecifikt antigen (PSA) i den målte kropsvæskeprøve sammenlignes med niveauet af PSA, der er målt i en kontrolprøve; hvori øgede PSA-niveauer i kropsvæskeprøven over kontrolprøven er associeret med tilstedeværelsen af prostatakraft.
- 10 **14.** Brug af et sæt omfatter:
et første anti-glypican-1-antistof og glypican-1-standarder;
i *in vitro*-metoden i henhold til krav 1, hvori det første anti-glypican-1-antistof bruges til at detektere opløseligt glypican-1 i kropsvæskeprøven.
- 15 **15.** Brugen i krav 14, hvori nævnte anti-glypican-1-antistof:
(i) ikke er MIL-38; eller
(ii) er MIL-38; eller
(iii) er det antistof, der henvises til i ethvert af kravene 4, 5 eller 7.

DRAWINGS

SEQ_ID: 212223 Mass: 61641 Score: 4276 Matches: 166(134) Sequences: 21(18) emPAI: 5.50
 ClpY1cat-1 O6-Homo sapiens E0=CFC1 E2=1 S1=2

Sequence Coverage: 46%

Matched peptides are underlined

1 MEIDRRRGWVLC LCAAALVAC ARGDFAKSR SCGEVRQYIG AKGFSLSQVP
 51 QAEISGEHLR ICPQGYTCCT SEMEENLANK SHAELETALR DSSRVLQAML
 101 ATQLSEFDH FQHLNDSEK TLQATFFGAF GELYIONRR FRDLYSELR
 151 YVRCANLHL ETLAEFWARL LERLFKQLR QLLFPDDYLD CIGKQAELR
 201 FFGEARREL LRATRAFVAA RSFYQGLGYA SDVVRKVAV RIGPECSRAY
 251 MKLVYCAHL GVPGARPCD YCRNVLKGL ANQADIDAEW RMLLDSMYLI
 301 TDKFWGTSV ESVIGSVHW LAEAINALQD NRDYLTAVI QCCGNPKWV
 351 QSPGFEKRR RGKIAPEER PSGTLEKLV EAKAQLRIVQ DFWISLESTL
 401 CSEKMALST SDDRCWNGMA RGRYLPEVMG DGLANQINP EVEVDITKFD
 451 MTIRQIMQL KIMNRLRSA YNGNDVDFQD ASDDGSGSG GDGCLDUCS
 501 RVSRKSSSS RPLTHALFG LSEQEGOKT AASCPQPTF LLPILLFLAL
 551 TVARFRNR (SEQ ID NO: 23)

Figure 1

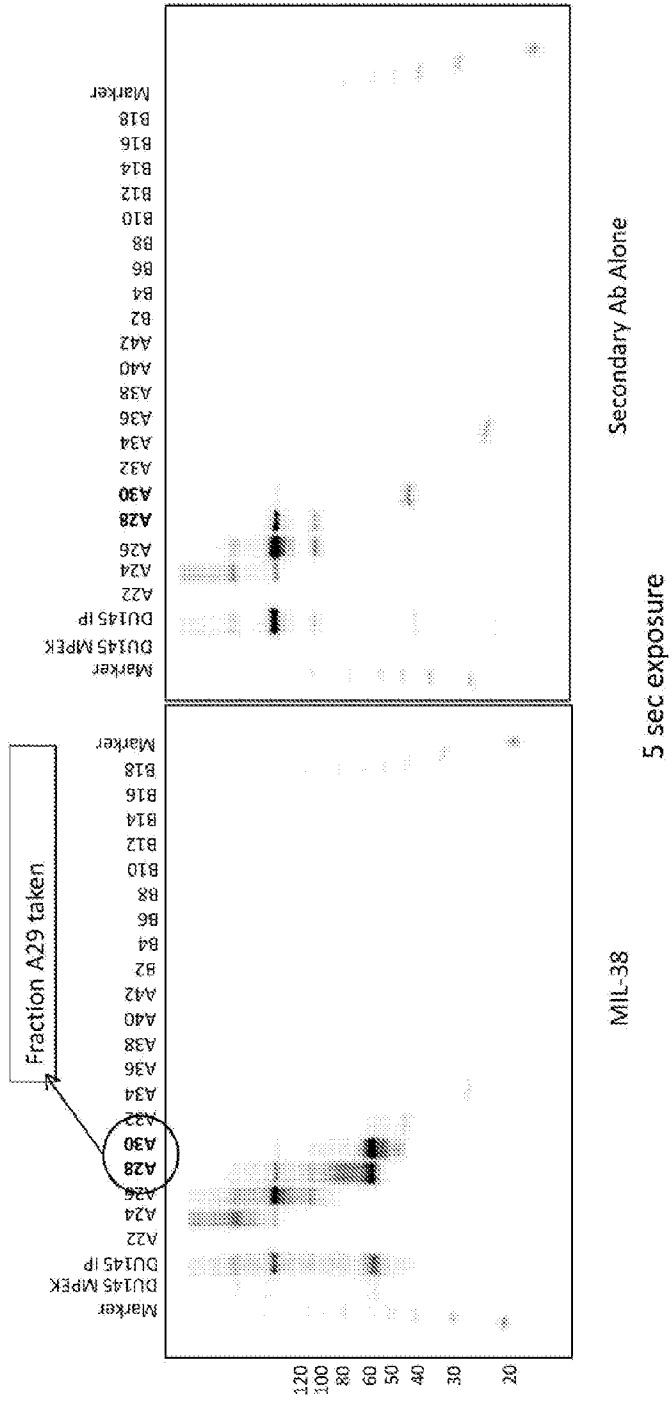


Figure 2

Mass Spectrometry of Size Exclusion HPLC Fraction #29

SEC1_HUMAN Mass: 61641 Score: 290 Matches: 6 (8) Sequences: 6 (8) eMRFI: 0.52
 Glypican-1 OS=Homo sapiens GN=GPC1 PE=M1 SV=2

Sequence Coverage: 14%

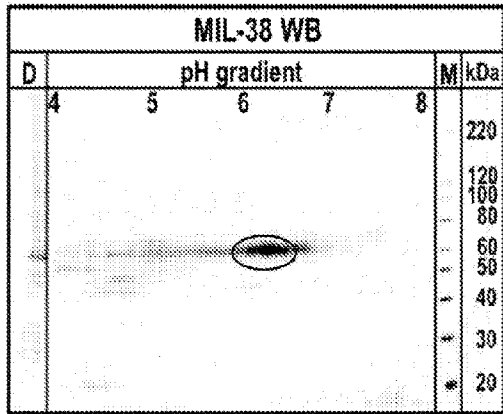
Matched peptides are underlined

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1  MELRAGQWML LCAAAALVAC AKSDPAKSR SCQEVKQIYG AKDFSLSDVF
51  QAEISEHLR ICFQGYTCT SEMENLAKR SHAEETALR DSHVYQAME
101  ATQLRSTDDH FQHLLNDSR TLAATPPGAF GELVYQWRA FRLYSELRL
151  YRGARILE ERLAEFWRL LERLFRQLR QLLPDDYLD CLGQAEALR
201  FFGELRRLR LRATRAVAA RSTYQGLGVA SDVVRVYQY ELSPCEKRV
251  NELVYQWHL GYFGRPCFD YCFNVLAKSL ANQADLDAEW RNLDSMVLV
301  FDFWQTSQV ESVIGSVHW LAELINALQ MDITLALVI QCCQPKYVF
351  QCFQFEENR RGLAPRERF PSEFTEKLV EAKAGLRYQ DFWLSLFTL
401  CSEKALSTA SDRCWNGMA KGRYLEKYS DELANQINP EYVYDIKFD
451  MTRQQIMQL KIMTRLESA YNGNDVDFQ ASDKSGSGS GSGCIDILCS
501  KYSRKSSS RPLTHALFG LSEQESQKTS AASCPQPTF LFLILLFLAL
551  TYAREWR (SEQ ID NO: 23)
    
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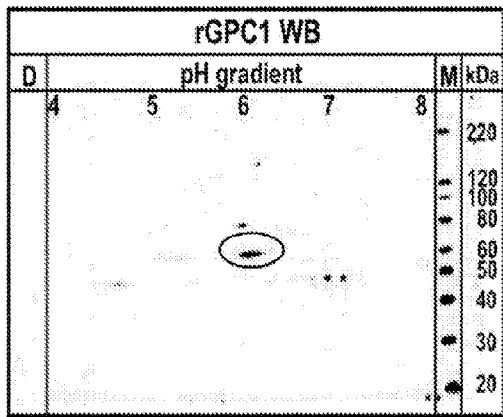
Figure 3

A



30 sec exposure

B



1 min exposure

Figure 4

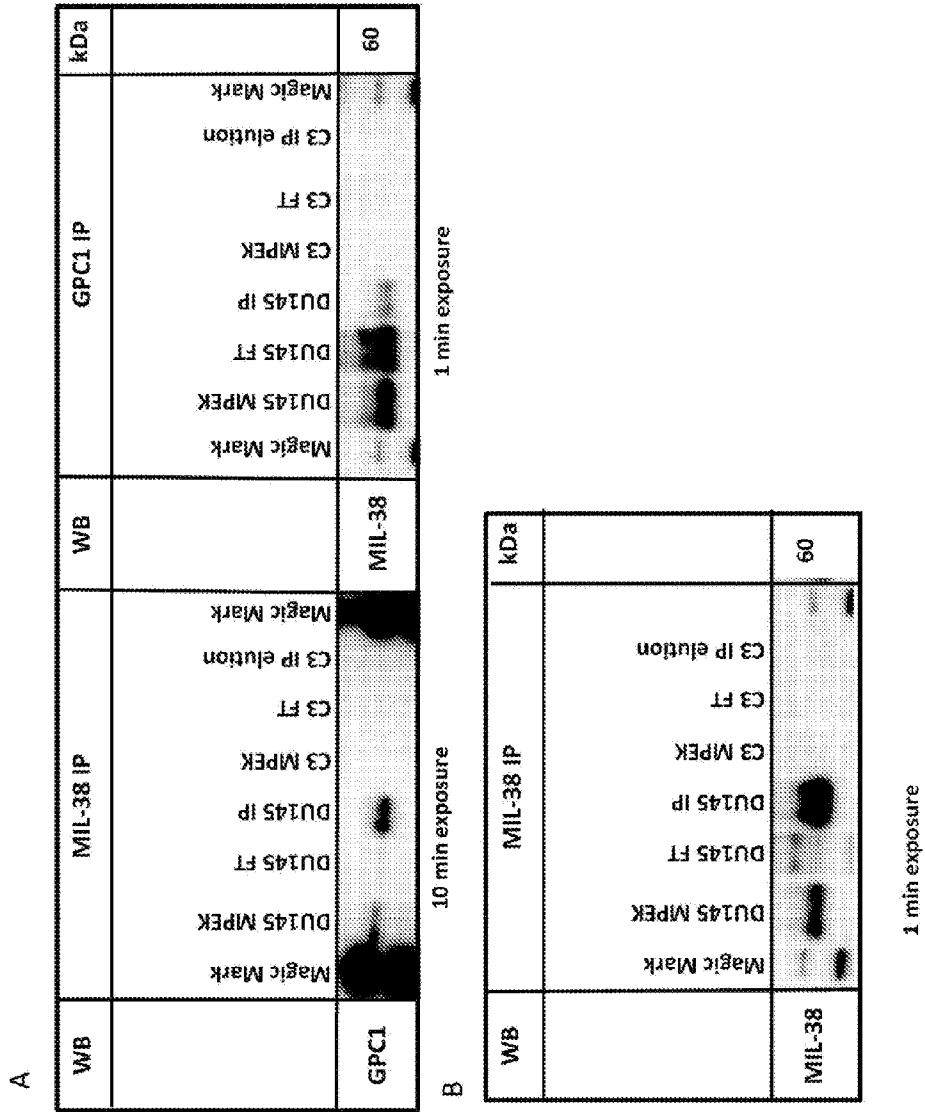


Figure 5

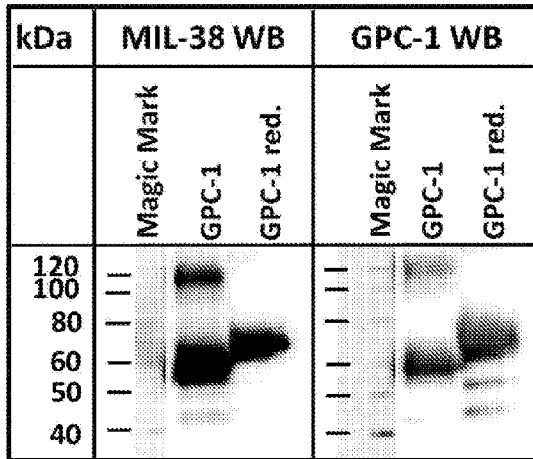
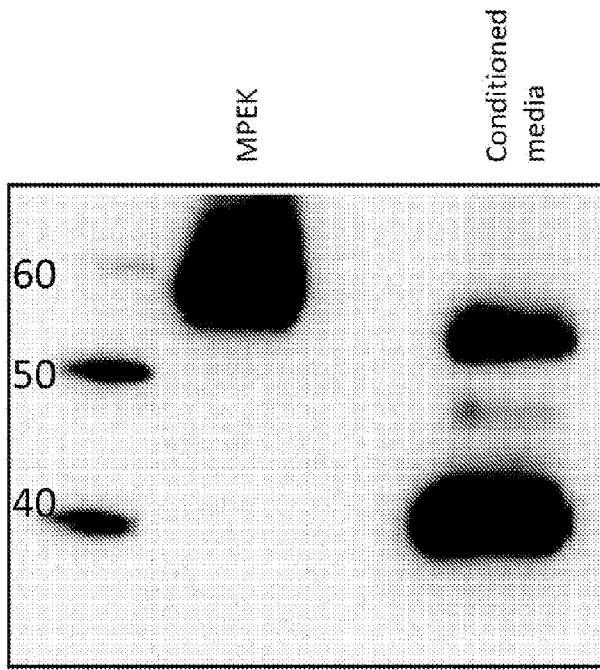


Figure 6



5 min exposure

Figure 7

A
 SPC1_HUMAN Mass: 61641 Score: 852 Matches: 30 (22) Sequences: 11 (9) eanFAI: 0.68
 Glypican-1 Os=Homo sapiens GH=SPC1 FE=1 SV=2

Sequence Coverage: 16%

Matched peptides are underlined

1 MELRARIQWIL ICAAAALVAC ARGDEASKSR SCGEVRIQYG AKGFSLSDVP
 51 CAETISEHLR ICPQYTOCT SEMENIANKR SHAELETALR DSSRVLOAML
 101 AIQLRSFDH FOHLNDSEK TIQATFFGAF GELIYONARA FEDLYSELR
 151 YIRGANLHE ETLAEFWRL LERLTKLHF QLLPPDDYLD CIGKQAEAIR
 201 FECEAPREL LRAIRAFVA RSEVQGLGVA SDVVRKVAQV PLGPECRRAY
 251 MKLVYCAHCL GYFGRKCED YCENVIKGL ANQADLDAEW RNLIDSWLL
 301 TDKFWETSGV ESVGSVHTW LAEAINALQD NRDTITAKVI QGCGNPFVNP
 351 QGRGFEERR RGLAFRRR PSSTLEKLV EAKQLRUVQ DEWISLPSLL
 401 CSEKVALSTA SDDRCKGGE RGRYLFEVME DGLANQINP EVEVDITKED
 451 MTIRCOIMQL KIMTWLRSA YNGNDVDFQD ASDDGSGSGS GDCGLDILCS
 501 RKYSRKSSSS RFLTHALPS LSEQEGKTS AASCPQPTT LLFLLLFLAL
 551 TVAREEER (SEQ ID NO: 23)

Figure 8

B

SEQ_ID: HUMAN Mass: 61642 Score: 1551 Matches: 48 (43) Sequences: 11 (10) eMRFAT: 1.42
 Glypican-1 OS=Homo sapiens GN=GPC1 PE=1 SV=2

Sequence Coverage: 18%

Matched peptides are underlined

1 MEIARRGHW ICAAALIVAC ARGDPASKR SGEVRQIYG AKGFELSDVE
 51 QALISSEHLR ICFQYTCCT SEMEMLANR SHAELETALR DSSRVLOAML
 101 ATQLRSEDDH FCHLNDSEK TLQATFFCAL GELTYQNRK FEDLYSEELR
 151 YVRGNLHLR ETLAEFWARL LERLTKQLRP QLLAFDDYLD CIGKQAEALR
 201 PEGEARHLR LRATRAFVAA RSEVQGLAVY SDVVRKVRQY ELGPECERAV
 251 MKLVICARCL GVFGARCFD YCRNVLKGL ANQADLDREN RMLDSEMTLI
 301 TDKFNSTSGV ESVIGSVHTW LAELINALQD NRDILIRKVI QSCGRKKNR
 351 QGGPEERR RGRIAFRRP PSGILEKLYS EAKAQIRIVQ DFWISLPSIL
 401 CSEKMLSTA EDDRCWNGMA RGRYLPEVMG DGLANQINMP EVEVDITKPD
 451 MTTRDQIMDL KIMTRILRSA YNGMDVDFD ASDDGSSSES GTGCGIDKICS
 501 KVYERKSSS RPLTHALPS LSEQEGQKTS AAACPEQPTF LIPILLFLAL
 551 TVARERRR (SEQ ID NO: 23)

Figure 8

C

GPC1_HUMAN Mass: 61641 Score: 1364 Matches: 39(66) Sequences: 12(3) eucPAI: 1.12
 Glypican-1 OS=Homo sapiens GN=GPC1 PE=1 SV=2

Sequence Coverage: 24%

Matched peptides are underlined

1 MELRRRGWNL LCAAAALVAC ARGDFASKR SCGEYRQYIG AKRFELSDVF
 51 QAEISGEHLR ICPOGIYTCI SEMEENIANR SHAELETLAR DSSNVLQAML
 101 ATQLRSFDDH FCHLLNDSER TLQATFFGAF GELTYQWRA FRDLYSELRL
 151 YRGNLHLE ETLAEFWAHL LERLTKQLHE QLLLFDDVLD CIGKDAFLR
 201 EGEARRELRL LEATRAEVAA RSEVQSLGYA SDVVRKVAQV PLGPECSRAY
 251 MKLVYCAHCL GYFGARPCFD YCRNVLNGCL ANQADLDAAW RNLIDSMYLI
 301 EDKFWGISGV ESYIGSVHIM LAELINALGD NRDTLEAVI QGCEKRVNF
 351 QGFQPEKRR RGKLAFRERF PSETLEKLYS EANKQLRIVQ DFWISLFCFL
 401 CSEKMAIATA SDRRCWNGMA RGRYLFPEYMG DELANQIRNF EYEVHITKED
 451 MTIRQIMQL KIMTNELRSA YNENVDVFDQ ASDDGGSGGS GEGCLDGLCS
 501 RKYSRKSSSS RTPFLTHALFG LSEKQEQKTS AASCPQPTT LFLFLFLAL
 551 TVARFRNR (SEQ ID NO: 23)

Figure 8

A

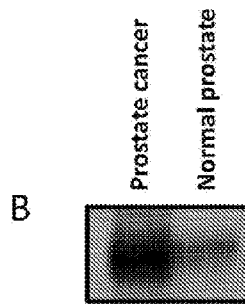
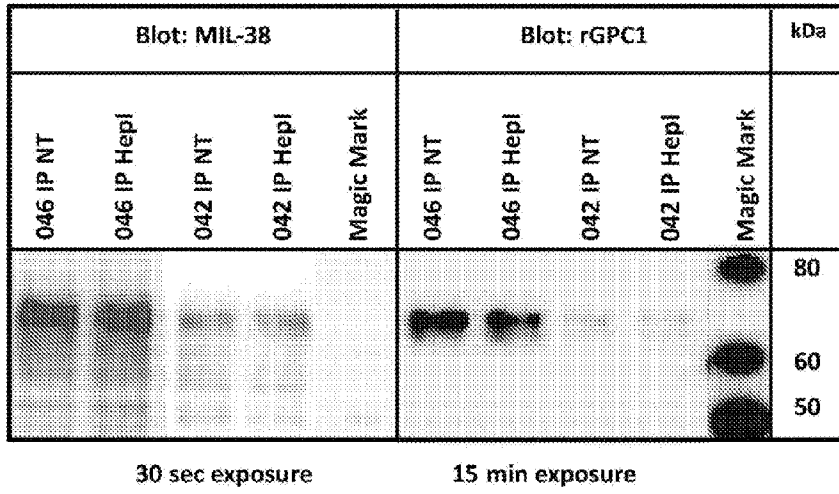


Figure 9

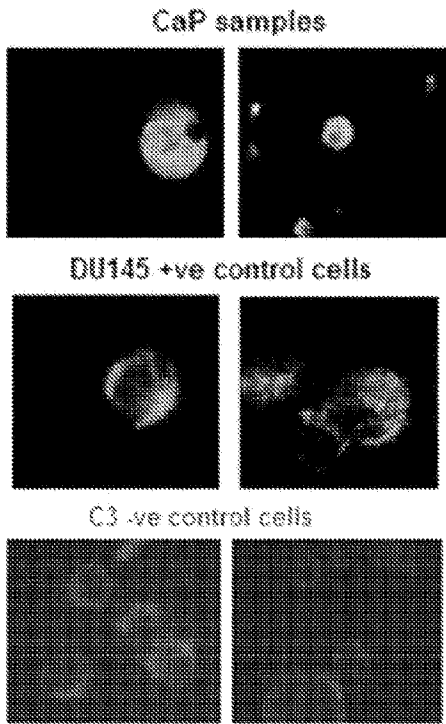


Figure 10

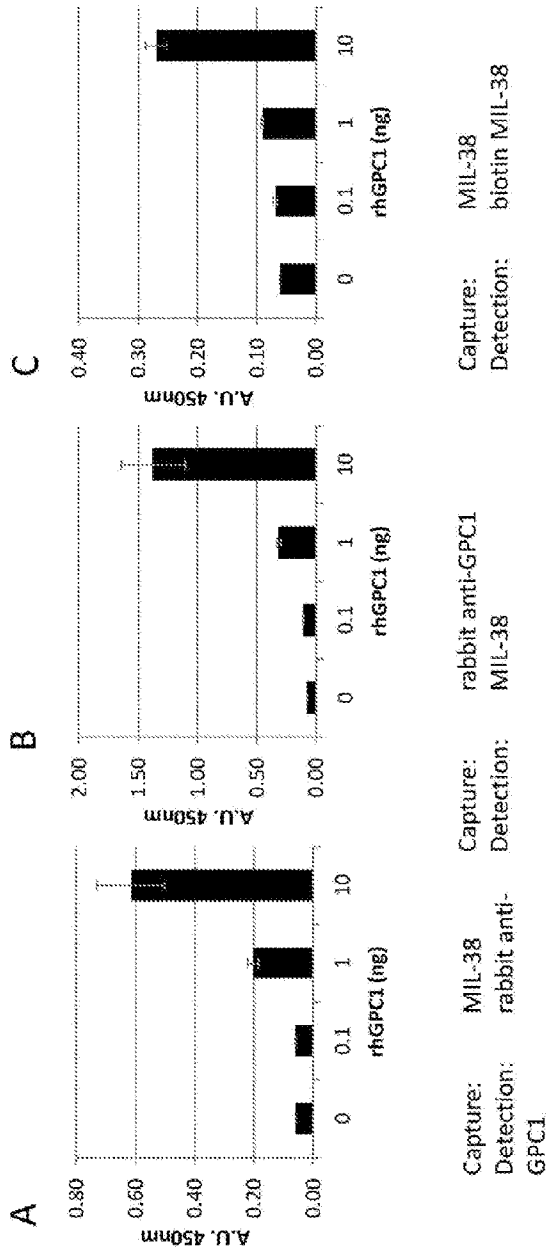


Figure 11

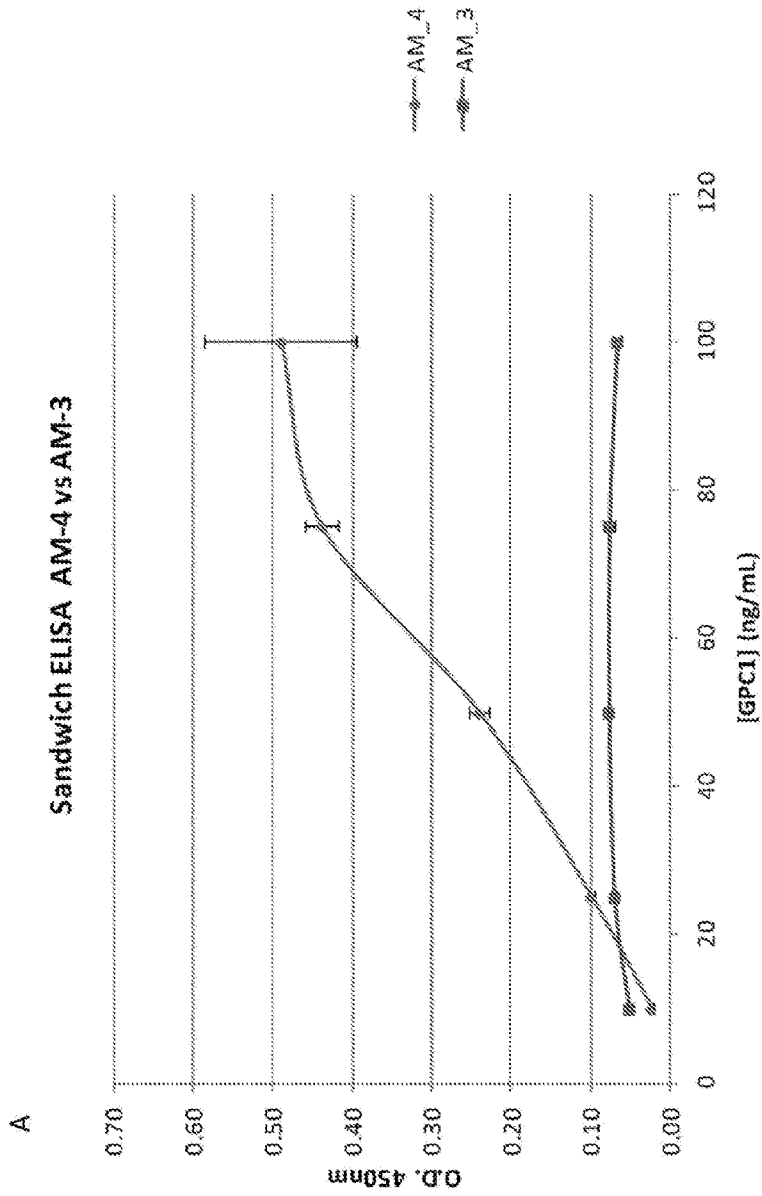


Figure 12

B

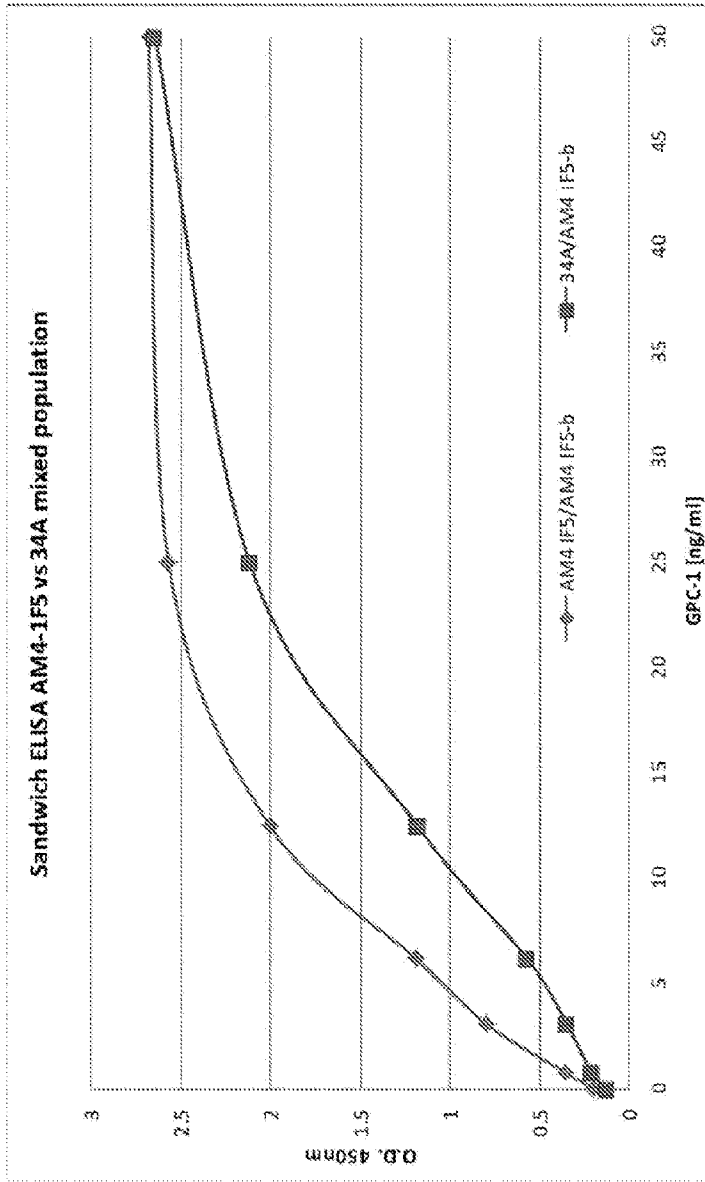


Figure 12

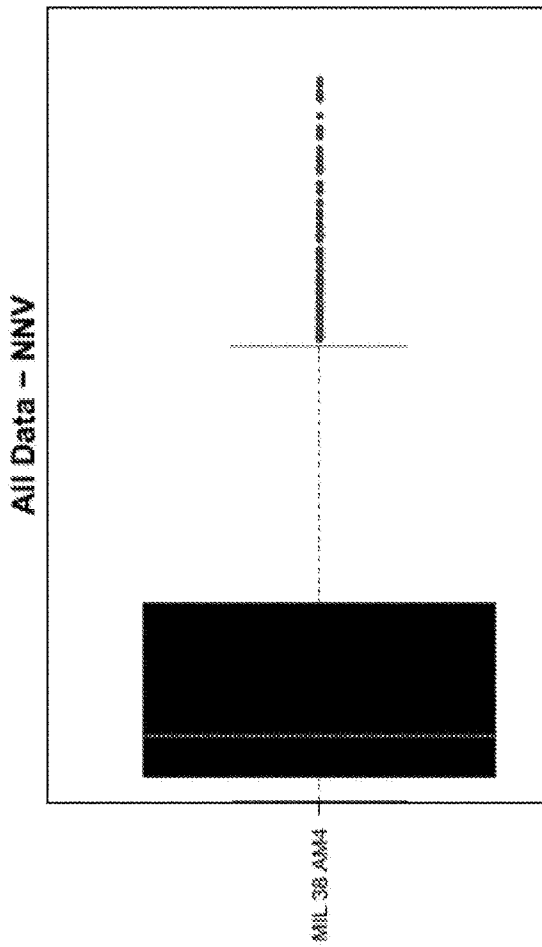


Figure 13

