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(57) Abrégé/Abstract:

The present invention provides compositions and methods of detecting prostate cancer in the body fluids or tissues of patients. Prostate cancer is detected by measuring the level of glypican-1 in a body fluid sample. In one embodiment prostate cancer is detected by contacting a body fluid sample with an anti-glypican-1 antibody, such as MIL-38. The invention includes kits for detection of prostate cancer in a body fluid sample, comprising an anti-glypican-1 antibody and glypican-1 standards.



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(54) Title: CELL SURFACE PROSTATE CANCER ANTIGEN FOR DIAGNOSIS

(57) Abstract: The present invention provides compositions and methods of detecting prostate cancer in the body fluids or tissues of patients. Prostate cancer is detected by measuring the level of glypican-1 in a body fluid sample. In one embodiment prostate cancer is detected by contacting a body fluid sample with an anti-glypican-1 antibody, such as MIL-38. The invention includes kits for detection of prostate cancer in a body fluid sample, comprising an anti-glypican-1 antibody and glypican-1 standards.



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CELL SURFACE PROSTATE CANCER ANTIGEN FOR DIAGNOSIS

The present application claims priority from United States of America provisional patent application number 61/928,776 filed on 17 January 2014.

FIELD OF THE INVENTION

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. The identified markers may also be used in determining prognosis and monitoring response to treatment for prostate cancer patients.

BACKGROUND OF THE INVENTION

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.

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.

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

SUMMARY OF THE INVENTION

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 or tissues 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 glypican-1 to determine the existence of prostate cancer in patients.

In one embodiment, the invention provides for methods for detecting prostate cancer in a patient comprising obtaining a body fluid or tissue sample from a patient, contacting said sample with an anti-glypican-1 antibody, determining that said patient has prostate cancer or an increased likelihood of developing prostate cancer based upon binding of said anti-glypican-1 antibody to said body fluid or tissue sample. In some embodiments the levels of one or more additional prostate markers are measured in the body fluid or tissue sample, and the determination that the patient has cancer is based upon the levels of glypican-1 and the levels of the one or more additional markers in the patient body fluid or tissue 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. In some embodiments, the anti-glypican-1 antibody is labeled for easy detection. In some embodiments the antibody label can be a fluorescent label, a biotin-avidin amplification system, a chemiluminescence system, microspheres or colloidal gold among others.

In some embodiments the body fluid sample obtained from the patient is a blood, serum, plasma, or urine sample.

In one embodiment, the anti-glypican-1 antibody binding to the patient's body fluid or tissue 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 or tissue 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.

In other embodiments, the level of anti-glypican-1 antibody binding to the patient's body fluid or tissue 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 or tissue 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 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 glypican-1 in said body fluid.

5 In some embodiments, glypican-1 content higher than about: 0.1ng/ml, 0.5ng/ml, 1ng/ml, 5ng/ml, 10ng/ml, 15ng/ml or 20ng/ml in the body fluid sample is indicative of prostate cancer.

The diagnostic methods of the invention may further comprise administering one or more prostate cancer treatments to a patient, and following changes in the level of glypican-1
10 in body fluids or tissues as a mechanism to monitor patient recovery or responses to the prostate cancer treatments. In some embodiments, the anti-glypican-1 antibody binding is detected via techniques such as immunofluorescence, radiolabeling, immunoblotting, enzyme-linked immunoassay, flow cytometry, optical density, and chemiluminescence.

The present invention also includes kits for detecting glypican-1 in the body fluids or
15 tissues of patients. In one embodiment 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. In some embodiments the kit further comprises a secondary ligand. In some embodiments the secondary ligand is a second anti-glypican-1 antibody. In one embodiment, the second anti-
20 glypican-1 antibody is the same as the first anti-glypican-1 antibody.

In some embodiments, the secondary ligand is conjugated to a label for rapid detection of said ligand.

The present invention thus relates at least to the following series of numbered embodiments below:

25 Embodiment 1: A method of detecting prostate cancer in a patient, the method comprising measuring the level of glypican-1 in a body fluid sample from a patient and determining that said patient has prostate cancer or an increased likelihood of developing prostate cancer based upon the level of glypican-1 in the body fluid sample.

Embodiment 2: The method of detecting prostate cancer in a patient of embodiment 1,
30 comprising the steps of:

- (a) obtaining a body fluid sample from a patient;
- (b) contacting said body fluid sample with an anti-glypican-1 antibody; and

(c) determining that said patient has prostate cancer or an increased likelihood of developing prostate cancer based upon binding of said anti-glypican-1 antibody to said body fluid sample.

Embodiment 3: The method of embodiment 2, wherein said anti-glypican-1 antibody
5 is MIL-38.

Embodiment 4: The method of embodiment 2, wherein said body fluid sample is contacted with a population of antibodies, wherein:

antibodies of the population comprise:

- (a) a heavy chain variable region comprising:
- 10 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
15 an amino acid sequence defined by positions 118-126 of **SEQ ID NO: 10**; and
- (b) 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
20 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**; and
- antibodies of the population do not comprise a light chain variable region comprising:
- 25 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**;
- a complementarity determining region 3 (CDR3) comprising or consisting of
30 an amino acid sequence defined by positions 113-121 of **SEQ ID NO: 12**.

Embodiment 5: The method of embodiment 4, wherein the antibody population is produced by or otherwise identical to an antibody population as generated by hybridoma cells

deposited on 22 August 2014 at CellBank Australia (CBA) under accession number CBA20140026.

Embodiment 6: The method of embodiment 2, wherein said anti-glypican-1 antibody is not MIL-38.

5 Embodiment 7: The method of any one of embodiments 2 to 4, wherein said anti-glypican-1 antibody is an antibody fragment or recombinant antibody capable of binding glypican-1.

Embodiment 8: The method of any one of embodiments 2 to 7, wherein said anti-glypican-1 antibody is labeled.

10 Embodiment 9: The method of embodiment 8, wherein said label is chosen from a group consisting of a radiolabel, a fluorescent label, a biotin-avidin amplification system, a chemiluminescence system, microspheres, and colloidal gold.

Embodiment 10: The method of any one of embodiments 2 to 9, wherein anti-glypican-1 antibody binding is detected via a technique selected from the group consisting of
15 immunofluorescence, radiolabeling, immunoblotting, Western blotting, enzyme-linked immunosorbent assay (ELISA), flow cytometry, immunoprecipitation, immunohistochemistry, biofilm test, affinity ring test, antibody array optical density test, and chemiluminescence.

Embodiment 11: The method of embodiment 1, wherein said level of glypican-1 in
20 the body fluid sample from a patient is compared to the level of glypican-1 in 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.

Embodiment 12: The method of embodiment 11, wherein a 50% or more increase in
25 the level of glypican-1 of said body fluid sample over the level of glypican-1 in the control sample is indicative of prostate cancer.

Embodiment 13: The method of any one of embodiments 2 to 10, wherein anti-glypican-1 antibody binding to said body fluid sample is compared to anti-glypican-1 antibody binding of a control sample; wherein increased anti-glypican-1 antibody binding of
30 the body fluid sample over the control sample is associated with the presence of prostate cancer.

Embodiment 14: The method of embodiment 13, wherein a 50% or more increase in the anti-glypican-1 antibody binding to said body fluid sample over the level of anti-glypican-1 antibody binding of the control sample is indicative of prostate cancer.

Embodiment 15: The method of any one of embodiments 2 to 10, 13 or 14 wherein anti-glypican-1 antibody binding to said body fluid sample is compared to anti-glypican-1 antibody binding to one or more glypican-1 standards; wherein the anti-glypican-1 antibody binding of the standards is used to quantify the amount of glypican-1 in said body fluid sample.

Embodiment 16: The method of any one of embodiments 1 to 15, wherein a glypican-1 content higher than about 10ng/ml in the body fluid sample is indicative of prostate cancer.

Embodiment 17: The method of any one of embodiments 1 to 16, further comprising: measuring the level of prostate-specific antigen (PSA) in a body fluid sample from the patient, and

determining that said patient has prostate cancer or an increased likelihood of developing prostate cancer based upon (i) the level of PSA measured in the body fluid sample, and (ii) binding of said anti-glypican-1 antibody to said body fluid sample.

Embodiment 18: The method of embodiment 17, wherein the level of prostate-specific antigen (PSA) is measured in a blood sample from the patient.

Embodiment 19: The method of embodiment 17 or embodiment 18, wherein the level of prostate-specific antigen (PSA) in the body fluid sample measured is compared to the level of PSA measured in a control sample; wherein increased PSA levels in the body fluid sample over the control sample is associated with the presence of prostate cancer.

Embodiment 20: The method of any one of embodiments 1 to 19, wherein said body fluid is selected from the group consisting of blood, serum, plasma, and urine.

Embodiment 21: A kit for detecting prostate cancer comprising 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 of a patient.

Embodiment 22: The kit of embodiment 21, wherein the anti-glypican-1 antibody is not MIL-38.

Embodiment 23: The kit of embodiment 21, wherein the anti-glypican-1 antibody is MIL-38.

Embodiment 24: The kit of embodiment 21, wherein the anti-glypican-1 antibody is the antibody referred to in any one of embodiments 4, 5 or 7.

Embodiment 25: The kit of any one of embodiments 21 to 24, further comprising a secondary ligand.

Embodiment 26: The kit of embodiment 25, wherein said secondary ligand is a second anti-glypican-1 antibody or an aptamer capable of binding to glypican-1; wherein said second anti-glypican-1 antibody can be the same as the first anti-glypican-1 antibody.

Embodiment 27: The kit of embodiment 25 or embodiment 26, wherein said
5 secondary ligand is conjugated to a label for rapid detection of said ligand.

Embodiment 28: The kit of embodiment 27, wherein said label is for use in a detection method selected from the group consisting of immunofluorescence, radiolabeling, immunoblotting, Western blotting, enzyme-linked immunosorbent assay (ELISA), flow cytometry, immunoprecipitation, immunohistochemistry, biofilm test, affinity ring test,
10 antibody array optical density test, and chemiluminescence.

Embodiment 29: The kit of any one of embodiments 22 to 28, wherein the kit comprises components for conducting an ELISA.

BRIEF DESCRIPTION OF THE DRAWINGS

15 **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
20 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
25 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
30 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

5 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 (a-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**

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 or cells 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.

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.

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

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

In some embodiments, the present invention can detect cancers at any one or more stages.

In some embodiments, the glypican-1 of the present invention is encoded by **SEQ ID NO: 1**. In some embodiments the glypican-1 protein is the full amino acid sequence of **SEQ ID NO: 2**. In some embodiments, the glypican-1 protein does not include the signal peptide of **SEQ ID NO: 3**. In some embodiments, the glypican-1 protein does not include the propeptide of **SEQ ID NO: 4**. In some embodiments, the glypican-1 protein of the present invention is **SEQ ID NO: 5**. In some embodiments glypican-1 of the present invention includes glypican-1 variants such as isoforms, splice variants, and allotypes. The present invention also provides a method of determining a prognosis for a patient with prostate cancer. In one embodiment, 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.

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

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 embodiments, the present invention uses glypican-1 alone to detect prostate cancer. In other embodiments GPC-1 is used together with another antigen wherein the presence of prostate cancer is determined by the detection of both antigens. In one embodiment, the other antigen is PSA.

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.

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 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 or tissue of the patient and the results of the PSA test.

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).

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 glypican-1 in a given sample (e.g. a body fluid sample), and use the measurement to diagnose and/or prognose prostate cancer in a patient from which the sample is derived. In some embodiments, the agent is an anti-glypican-1 antibody. In some embodiments of the invention MIL-38 antibody is used to bind to and detect a 60kD glypican-1 proteoglycan. In some embodiments the MIL-38 antibody is used to detect the glypican-1 antigen on the surface of prostate cancer cells. In other embodiments, the MIL-38 antibody is used to detect soluble glypican-1 in the body fluids or tissues of prostate cancer patients. In some embodiments, the MIL-38 antibody has binding specificity for 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**). The present invention shows 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 glypican-1

levels in the body fluids or tissues of patients. The present inventors have thus discovered that glypican-1 is a marker for prostate cancer.

According to the invention glypican-1 levels in body fluids or tissues can be detected using any suitable technique (e.g. any proteomic technique). In some embodiments, the glypican-1 levels can be detected using an anti-glypican-1 antibody. For example, the 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 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 CBA20140026.

In some embodiments of the invention, one or more other anti-glypican-1 antibodies may be used to detect glypican-1 in the body fluids or tissues of patients. In some embodiments, said other anti-glypican-1 antibodies can be any of the antibodies listed in (Table 2) of this application. In yet another embodiment, the antibody used to detect glypican-1 in the body fluids or tissues of patients could be any antibody capable of binding glypican-1. In some embodiments the antibodies of the present invention include antibody fragments or recombinant antibodies. In some embodiments the antibodies of the present

invention include human antibodies, humanized antibodies and chimeric antibodies. In some embodiments the antibodies of the present invention are conjugated antibodies. A non-limiting list of the antibody fragments of the present invention include fragment antigen binding Fab, F(ab')₂, ScFv, Di-scFv sdAb, chemically linked F(ab')₂, bispecific antibodies, 5 trispecific antibodies Fab3, Bis-scFv, Minibody bivalent antibodies, triabody trivalent antibodies, diabody bispecific antibodies, tetrabody tetravalent antibodies. A review of antibody fragments and domain combinations can be found in (Holliger and Hudson 2005, and US 2003/0077282).

Table 2. Anti-Glypican-1 Antibodies

Company	Cat#	Reactivity	Use	Host	M/P	Binding specificity	Immunogen
R&D	BAF4519	Human	WB, FC	Goat	poly	aa24-530	mouse myeloma cell line NS0-derived recombinant human Glypican-1, Asp24-Ser530 (Accession# P35052)
Bioss	bs-2426R-Biotin	Human, mouse, rat, dog, cow, horse	WB, ELISA, IHC-P&F	Rabbit	poly		Unknown immunogen. Biotin conjugated
Bioss	bs-2426-HRP	Human, mouse, rat, dog, cow, horse	WB, ELISA, IHC-P&F	Rabbit	poly		Unknown immunogen. HRP conjugated
antibodies-online	ABIN740102	Human, mouse, rat, dog, cow, horse	WB, ELISA, IHC-P&F	Rabbit	poly	C-term	Synthetic peptide derived from human glypican 1 C-terminus. Biotin conjugated.
antibodies-online	ABIN1174125	Human	IHC, WB, ELISA	Rabbit	poly		Unknown 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
antibodies-online	ABIN347484	human	IHC, WB, ICC, ELISA	Rabbit	poly	N-term	synthetic peptide derived from N-terminal domain of human GPC1
antibodies-online	ABIN740100	Human, mouse, rat, dog, cow, horse	WB, ELISA, IHC-P&F, IF	Rabbit	poly	C-term	Synthetic peptide derived from human glypican 1 C-terminus. Enquire about sequence info.
antibodies-online	ABIN207433	human	WB, ELISA	Rabbit	poly	C-term	synthetic peptide corresponding to C-terminal residues of human GPC1 precursor
antibodies-online	ABIN964659	human, mouse, rat	WB, ELISA	Rabbit	poly	internal region	synthetic peptide corresponding to an internal region of human GPC-1
antibodies-online	ABIN349638	human	WB, ELISA	Rabbit	poly	internal region	synthetic peptide corresponding to human GPC1
antibodies-online	ABIN1101824	human	WB, ELISA	Rabbit	poly	internal region	synthetic peptide corresponding to an internal region of human GPC-1
antibodies-online	ABIN595376	human	WB, ELISA	Rabbit	poly	internal region	synthetic peptide corresponding to an internal region of human GPC-1
antibodies-online	ABIN330371	human	WB, ELISA	goat	poly	aa24-530	NS0-derived 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

In some aspects of the invention, 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 KVNPGPGPE (SEQ ID NO: 6) or KVNPGPGP (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 embodiments, 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 embodiments 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 embodiments, 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 embodiments 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).

In another embodiment of the present invention, 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 KVNPGPGPE (SEQ ID NO: 6) or KVNPGPGP (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. In one embodiment of the invention, the glypican-1 protein or fragment thereof may be injected into a host animal. In some embodiments the host animals can include but are not limited to rabbits, mice, rats, etc. In some embodiments 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.

In another embodiment, 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 KVNPPQGPGPE (**SEQ ID NO: 6**) or KVNPPQGPGP (**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 embodiment, 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.

In some embodiments of the present invention, glypican-1 is detected in the body fluids or tissues of patients by an anti-glypican-1 antibody. In some embodiments the body fluid sample obtained from the patient is a blood, serum, plasma, or urine sample. In other embodiments, glypican-1 is detected in tissue samples of patients. In some embodiments, the tissue samples include tumor biopsies or other patient tissue. In some aspects of this invention, 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 embodiments, glypican-1 is detected via a biofilm test, or affinity ring test as described in US application 2013/016,736. In some embodiments 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 embodiments, 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 embodiment, the calibration standards comprise one or more samples with known glypican-1 concentrations.

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

In some embodiments, the tissue or body fluid of a patient may require a pre-treatment prior to detection by the anti-glypican-1 ligand. In some embodiments, said pre-treatment may include treatment with agents such as heparinase PNGaseF, N-Glycosidase, alkaline phosphatase, or heparitinase among others. In other embodiments, said pre-treatment

may include tissue lysis, membrane purification, blood plasma or serum fractionation, cell purification, or protein purification among others.

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

In some embodiments, the detection of glypican-1 from body fluid or tissue 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 invention may employ any suitable capture reagent and detectable reagent including antibodies and derivatives thereof, protein ligands and the like. In certain embodiments, the ELISA is cell-based. In other embodiments, the ELISA detects cell-free antigens. In some embodiments 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.

In some embodiments, 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 embodiments the antibody is a murine or rat antibody. In other embodiments the antibody is a murine antibody. In other embodiments the antibody is derived from a hybridoma. In some embodiments, 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.

In some embodiments, 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 embodiment 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 MICROTTEST™ or MAXISORP™ 96-well ELISA plate such as that sold as NUNC MAXISORB™ or IMMULON™.

In some embodiments 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.

In other embodiments, 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]propiolate yield photoactivatable intermediates capable of forming cross-links in the presence of light.

In some embodiments, 96-well plates are utilized. In some embodiments 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 embodiments the plate can be coated for less time, e.g. at room temperature for two hours or less. In some embodiments, 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.

In some embodiments, 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 embodiments the blocking treatment can take place under conditions of ambient temperatures for about 1-4 hours. In other embodiments the blocking can take place over the course of 1 to 3 hours or less. In other embodiments the blocking can take place overnight at 0-4°C.

In some embodiments 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 embodiments, the dilution rate is about 1-15% by volume. In some embodiments, the glypican-1 protein standard will include the post-translational modifications of the native protein. In some embodiments, 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 embodiments, the glypican-1 protein may be purified from body fluids or tissues. In some embodiments, 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 embodiments the standard can be a cell expressing glypican-1. In some embodiments 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.

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.

The conditions for incubation of sample and immobilized capture reagent are selected to maximize sensitivity of the assay and to minimize dissociation. In some embodiments, the incubation is accomplished at fairly constant temperatures, ranging from about 0°C to about 40°C. In other embodiments 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 embodiments the incubation time is from about 0.5 to 3 hours. In other embodiments 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.

In some embodiments, the detection method is a competitive ELISA. In some embodiments 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 embodiments the incubation step includes the addition of labeled glypican-1 protein of known concentrations. In some embodiments the amount of glypican-1 in the body fluid or tissue sample is detected as a diminishing binding of the mixed labeled glypican-1 protein. In other embodiments the ELISA is a sandwich ELISA.

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 embodiment 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.

In some embodiments, 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 embodiment, 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 embodiments 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.

In some embodiments, 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 embodiments, the antibodies are those described in table 2. In other embodiments 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 embodiments 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 embodiment, the contacting is carried out (e.g., about 1 hour or more) to amplify the signal to the maximum. In some embodiments, 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 embodiments, it is a monoclonal antibody, in one embodiment murine, and in one embodiment MIL-38. Also, the detectable antibody can be directly detectable, and in one embodiment has a colorimetric label, and in another embodiment has a flurometric label. In other embodiments the detectable antibody is biotinylated and the detection means is avidin or streptavidin-peroxidase and 3,3',5,5'-tetramethyl benzidine. In some embodiments 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.

In some embodiments, the glypican-1 that is bound to the capture reagent is measured using a detection means for the detectable antibody. If the body fluid or tissue 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 embodiments the reaction that occurs as a result of the above steps is compared to a similar reaction using a control body fluid or tissue sample such as the body fluid or tissue of a age-matched cancer-free individual.

In other glypican-1 detection embodiments, the glypican-1 in the body fluid of patients is detected via a Western Blot Analysis. In some embodiments this assay separates the proteins in a complex sample using electrophoresis. In other embodiments 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 embodiment 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 embodiments 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 embodiments 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 embodiments the membrane is then washed and incubated with a secondary antibody that targets the first antibody. In some embodiments the first or the second antibody is conjugated to a detectable label such that it can be easily detected. In some embodiments the label includes a fluorescent label, a chemiluminescent label, a radiolabel, or another label well known in the art. In some embodiments, 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.

Optionally, some aspects of the invention 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 embodiments the second protein may be used as a loading control. In other embodiments the second protein is another marker for patient health.

In one embodiment, glypican-1 is detected via flow cytometry. In some embodiments glypican-1 is detected on the cell surface of cells in patient body fluids or tissues. In certain aspects of the invention the detection of glypican-1 for flow cytometry may be conducted as

outlined below. In some embodiments, cells from body fluids or tissues are purified. The purification of the cells can include a neutralization step. In some embodiments 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 embodiments, the cells are centrifuged and resuspended in different solutions. In other embodiments, the cells are sorted without purification. In some embodiments the cells are resuspended or washed in CytoLyt solution. In other embodiments the cells are resuspended or washed in phosphate buffered saline (PBS). In some embodiments the cell suspension is treated with ammonium chloride to lyse the red blood cells. In some embodiments the cells are fixed onto slides. In other embodiments the cells remain free. In some embodiments, 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 embodiments the primary antibody is any other anti-glypican-1 antibody (Table 2). In certain embodiments, the cells are further contacted with a second detection antibody conjugated to a detection label. In alternative embodiments, 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 embodiments the other probes label intracellular antigens. In other embodiments the other probes label extracellular antigens. Once labeled, the labeled cells may be isolated by FACS flow cytometry. In some embodiments, the FACS machine may isolate labeled cells singly (i.e., as single cells). In other embodiments, the labeled cells may be isolated as a mixed population, and then diluted into single cells after FACS. In some embodiments the second label can be a dye. In some embodiments the dye label is DAPI. In some embodiments DAPI labeling is used to quantify the number of cells in the sample.

In embodiments 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 embodiments 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).

The present invention also includes kits for detecting glypican-1 in the body fluids or tissues of patients. In one embodiment the kit for detecting cancer comprises the materials necessary to conduct any of the detection assays described in this application. In some embodiments 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.

In some embodiments, the kit will require the additional use of standard laboratory tools or machinery. In some embodiments, necessary tools include pipettes, cell sorting machines, plate readers, centrifuges etc as are known to those being skilled in the art. In some embodiments, 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. In some embodiments the kit further comprises a secondary ligand. In some embodiments the secondary ligand is a second anti-glypican-1 antibody. In one embodiment, the second anti-glypican antibody is the same as the first anti-glypican-1 antibody. In some embodiments, the secondary ligand is conjugated to a label for rapid detection of said ligand. In some embodiments the antibodies of the kit can be antibody fragments or antibody combinations as described in this application.

In some embodiments the detection of glypican-1 in the body fluids or tissues of a patient is indicative of the presence of prostate cancer. In some embodiments, 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.

In some embodiments the detection of glypican-1 in the body fluids or tissues of a patient is indicative of the presence of prostate cancer. In some embodiments, 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.

In some embodiments the detection of glypican-1 in the body fluids or tissues of a patient is indicative of the presence of prostate cancer. In some embodiments, 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 $\mu\text{g/ml}$ of glypican-1 in the body fluid or tissue of a patient is indicative of prostate cancer.

In some embodiments elevated levels of glypican-1, or glypican-1 detection signal in the body fluid or tissue 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 or tissue. In some embodiments the control non-cancerous body fluid or tissue will be age matched to the patient.

EXAMPLES

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

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.

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.

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.

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.

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-1) antibodies show overlapping reactivity on 2D western blots.

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.

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.

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).

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

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 NS0 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 NS0 cells (Figure 6).

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

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).

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).

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.

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).

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.

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.

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).

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.

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.

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		
		1	0	
GPC-I	1	5	17	Sensitivity = 100% Specificity = 68%
IFA	0	0	36	

PSA ≥ 2 ng/ml

		Cancer		
		1	0	
GPC-I	1	24	4	Sensitivity = 67% Specificity = 83%
IFA	0	12	20	

Stratified by 4 ng/ml

PSA < 4 ng/ml

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

PSA $\geq$ 4 ng/ml

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

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.

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

Experiments performed by the present inventors determined that an original deposit of the hybridoma for MIL-38 antibody (ATCC accession no. HB11785: 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 CBA20140026.

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 (1µg/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**.

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 1h 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**.

The first ELISA described above was developed using MIL-38 to capture NSO 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**).

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**).

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)

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.

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.

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 anti-sense primers or universal primers following the technical manual of SuperScript™ III

First-Strand Synthesis System. The antibody fragments of VH, VL, CH and CL were amplified according to the standard operating procedure of RACE of GenScript.

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

5 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.

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 10 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.

15 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). VH/CH and VK/CK were amplified by PCR using IgG1 and IgK specific primers, partial mouse β -actin gene was amplified as positive control. Normal light 20 chain bands were obtained easily while only weak VH could be observed on the gel. Five individual colonies with correct VK and CK insert sizes were sent for sequencing. The VK and CK 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 VH positive clones, shown as below.

25 Three kinds of heavy chain constant region sequences were obtained from ten randomly sequenced CH positive clones (one IgG₁CH, one IgG_{2a}CH and eight IgG_{2b}CH). 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 30 degenerate primers.

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 VK 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.

5 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 VH target PCR products were observed and sequencing did not result in any consistent heavy chain sequence.

10 ***Results***

- Sequence Summary Table

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.

Table 5: Overview of AM4 and AM3 antibody sequences and internal regions

DNA Seq ID#	Leader	HFR1	HCDR1	HFR2	HCDR2	HFR3	HCDR3	HFR4	CH1-CH3	Hinge
13	AM4 Heavy	58-147	148-162	163-204	205-255	256-351	352-378	379-411	412-1383	703-741
14	AM4 Light	61-129	130-162	163-207	208-226	229-324	325-351	352-381	382-702	
15	AM3 light	73-141	142-174	175-219	220-240	241-335	337-363	364-393	394-714	
AA Seq ID#	Leader	HFR1	HCDR1	HFR2	HCDR2	HFR3	HCDR3	HFR4	CH	Hinge
10	AM4 heavy	1-19	20-49	50-54	55-68	69-85	86-117	118-136	137-137	138-461
11	AM4 light	1-20	21-43	44-54	55-69	70-76	77-108	109-117	118-127	128-234
12	AM3 light	1-24	25-47	48-58	59-73	74-80	81-112	113-121	122-131	132-238

Notes: HFR = heavy chain framework region; HCDR = heavy chain complementarity determining region; CH = heavy chain constant region

LFR = light chain framework region; LCDR = light chain complementarity determining region; CL = light chain constant region

Grey Boxes are indicative of positions within sequence defined in column 1 by SEQ ID NO.

The V_H , V_L , C_H and C_L genes of five different clones were nearly identical. The consensus sequence, listed below, was determined to be the sequence of the antibody produced by the monoclonal hybridoma population (AM-4).

[illegible]

40

GACATCCAGATGACT
 CAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAACTGTCACCATCACATGT
 TGGTATCAGCAGAAACAGGGAAAATCTCCTCAACTCCTGCTCTAT
 GGTGTGCCATCAAGGTTCACTGGCAGTGGATCAGGAACACAATATTCTCTCAAGATCAATAGCCTGCAGCCT
 GAAGATTTTGGGACTTATTACTGT
 ATCAAA
 TAG

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

MAKVVWILLFLM¹AAQSIQAQIQLVQSGFELKKPGETVKISCKASGYAFTDYSMNWVKQAPGKGLRWMCWINTETG
EFTYTLDEKGRFAFSLETSASTAFLQINNLRNEDTATYFCARHYDTGCGFPIWGQGTLLVTVSAAKTTFP²SVYPLAF
GSAAQINSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTTFAVLQSDLYTLSSSVTVFPPSTWPESTVTCNVAHPA
SSSTRVDDKKIVPRDCGCKPCICTVPEVSSVFIFPPKPKQVLTITLTIPAVTECVVVDISKDDPEVQFSNFWDDVEVHT
AQTQPREEQFNSTFPRSVSELPIMHQDWLNGKEFKCRVNSAAFEAP³IEKTI⁴SKTKGRPKAPQVYTIPEPKQMAKD
KVS⁵LT⁶CMIT⁷DEFFPEDITVEWQWNGQPAENYKNTQPI⁸MDT⁹DG¹⁰SYFVYSKLN¹¹V¹²Q¹³EN¹⁴WEAG¹⁵NT¹⁶FTCS¹⁷VL¹⁸HEGL¹⁹HN²⁰H
TEKSLSHSPGK*

41

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

MGIMESQTQVFVYMLLNLGGVGGDIVMTQSQKFMSTSIGDRVSVTCKASQNVGSHVAVFQQKPGQSPKALTYSA
 SYRISGVTDRTFTGSGSGTDFTLTINNVSQSEDLAEYFCQYNSFPFTFGSGTKLEIKRADAAPTVSIFPPSSEQLT
 SGGASVVCFLNNFYPKDIINVKKIDGSEKQNGVLNSWTQDSKDSITYSMSSLTTLTKDEYERHNSYTCEATHKTS
 TSPIVKSEFNEEC*

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

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

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 GNPKNVPQGGPGPEBKRRRGKLAP (**SEQ ID NO: 17**)

GPC1_residues #140-149 GELYTQNARAFRDLYSELK (**SEQ ID NO: 18**)

Peptide synthesis

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*

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

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.

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 helixes (Timmerman *et al.*, J. Mol. Recognit. 2007; 20: 283-29).

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.

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*

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 μ l 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.

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.PKVNPQGPGPEEKRR (SEQ ID NO: 19)
Description Substitution analysis, starting from the base sequence PKVNPQGPGPEEKRR (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.PKVNPQGPPEEKRR_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*

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'-azino-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.

10

- *Data processing*

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.

15

- *Synthesis quality control*

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*).

20

- *Screening details*

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

25

Table 7: screening conditions

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

Results*- Primary experimental results and signal to noise ratio determination*

5 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.

10

- Antibody MIL 38-AM4

In earlier analyses carried out by the present inventors it was established that MIL 38-AM4 binds glypican on stretch ₃₄₈VNPQGPGEK₃₅₈ (**SEQ ID NO: 22**), and also binds to the stretch ₁₃₅TQNARA₁₄₀ (**SEQ ID NO: 8**)/₁₃₅TQNARAFRD₁₄₃ (**SEQ ID NO: 9**), which
15 was taken as an indication for a discontinuous epitope.

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**).

20

- Conclusions

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,
25 full substitution analyses of the individual lead sequences were made. All arrays were probed with MIL38-AM4 antibody.

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.

30 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	Conformation sensitive
MIL38-AM4	³⁴⁷ KVNPQGPGP ₃₅₅	V348, Q351, G352, P353	Y
(SEQ ID NO: 7)			

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.

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.

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.

REFERENCES

- Aikawa T, Whipple C, Lopez M, Gunn J, Young A, Lander A, Korc M. 2008, "Glypican-1 modulates the angiogenic and metastatic potential of human and mouse cancer cells" *The Journal of Clinical Investigation* Vol 118:1
- 5 Borrebaeck et al., 1995 *Antibody Engineering: A Practical Approach* (Borrebaeck, C, ed.), 1995, Oxford University Press, Oxford; *J. Immunol.* 149, 3914-3920 (1992)
- 10 Cole et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96
- Dainak M, Kumar A, Galaev IY, Mattiasson B. 2007 "Methods in Cell Separations" Vol 106:1-18
- 15 David G, Lories V, Decock B, Marynen P, Cassiman JJ, Van den Berghe H. 1990. "Molecular Cloning of a Phosphatidylinositol-anchored Membrane Heparan Sulfate Proteoglycan from Human Lung Fibroblasts" *The Journal of Cell Biology* Vol 111:6-2 3165-3176
- 20 Filmus J, Capurro M, Rast J. "Glypicans" *Genome Biology* 2008, 9:224
- Filmus J, Selleck SB: Glypicans: proteoglycans with a surprise. *J Clin Invest* 2001, 108:497-501.
- 25 Fujise M, Takeo S, Kamimura K, Matsuo T, Aigaki T, Izumi S, Nakato H. Dally regulates DPP morphogen gradient formation in the *Drosophila* wing. *Development* 2003, 130:1515-1522.
- 30 Han C, Belenkaya TY, Wang B, Lin X. "Drosophila glypicans control the cell-to-cell movement of hedgehog by a dynamin-independent process". *Development* 2004, 131:601-611.
- Harlow et al 1988. *Antibodies, A Laboratory Manual*, Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988), Cold Spring Harbor, N.Y.

- Harmon J Eyre, Robert A Smith, and Curtis J Mettlin, "Chapter 28 Cancer Screening and Early Detection" Holland-Frei Cancer Medicine 5th Edition. Bast RC Jr, Kufe DW, Pollock RE, editors Hamilton (ON): BC Decker; 2000.
- 5 Hoffman R., Gililand F., Cameron M., Hunt W., and Key C. "Prostate-specific antigen testing accuracy in community practice" BMC Fam Pract. 2002, 3:19.
- Holliger P and Hudson P. 2005. "Engineered antibody fragments and the rise of single
10 domains" Nature Biotech 23 1126-1136.
- Johnson KW, Dooner M, Queensberry PJ. 2007 "Fluorescence Activated Cell Sorting: A Window on the Stem Cell" Vol 8:133-9
- 15 Kleef J, Ishiwata T, Kumbasar A, Friess H, Buchler M, Lander A. "The Cell-surface heparan sulfate proteoglycan glypican-1 regulated growth factor action in pancreatic carcinoma cells and is overexpressed in human pancreatic cancer" J Clin Invest. Vol 102 No 9 Nov 1998
- Kohler and Milstein 1975 Continuous cultures of fused cells secreting antibody of predefined
20 specificity Nature 256:495-497
- Matsuda K, Maruyama H, Guo F, Kleef J, Matsumoto Y, Lander AD, Korc M. "Glypican-1 is overexpressed in human breast cancer and modulates the mitogenic effects of multiple heparin-binding growth factors in breast cancer cells." Cancer Res 2001 Jul 15, 61(14)5562-
25 9.
- Orfao A, Ruiz-arguelles, Alejandro. 1996 "General concepts about cell sorting techniques" Clin. Biochem. Vol 29:1 5-9
- 30 -Qiao D, Meyer K, Mundhenke C, Friedl S, Friedl D. 2003 "Heparan Sulfate Proteoglycans as Regulators of Fibroblast Growth Factor-2 Signaling in Brain Endothelial Cells: SPECIFIC ROLE FOR GLYPICAN-1 IN GLIOMA ANGIOGENESIS" J. Biol. Chem. 16045-16053.

- Rotmans et al.1983 Cross-linking of *Schistosoma mansoni* antigens and their covalent binding on the surface of polystyrene microtitration trays for use in the ELISAJ. Immunol. Methods 57:87-98 (1983)
- 5 Russell PJ., Ow KT., Tam PN., Juarez EA., Qu CF., Li Y., Cozzi PJ., and Martiniello-Wilks. "Immunohistochemical characterization of the monoclonal antibody BLCA-38 for the detection of prostate cancer" Cancer Immunol. Immunother (2004) 53: 995-1004
- Spaltro, Andrea Doctoral Dissertation University of Bologna 2012
- 10 Su G, Meyer, K, Nandini C, Qiao D, Salamat S, Friedl A. "Glypican-1 is frequently overexpressed in human gliomas and enhances FGF-2 signaling in glioma cells" Am J Pathol 2006 June 168(6) 2014-2026.
- 15 Suhovskih A, MostovichL, Kunin I, Mekhroziddin B, Nepomnyashchikh G, Aidaulova S, Griorieva E. "Proteoglycan expression in normal human prostate tissue and prostate cancer" Oncology Volume 2013 ID 680136
- Tung Jw, Heydari K, Tirouvanziam R, Sahaf B, Parks DR, Herzenberg LA. 2007 "Modern
- 20 Flow Cytometry: A Practical Approach" Vol 27:453-68
- Veugelers M, De Cat B, Ceulemans H, Bruystens AM, Coomans C, Durr J, Vermeesch J, Marynen P, David G. "Glypican-6, a new member of the glypican family of cell surface proteoglycans." J Biol Chem 1999 274:26968-26977
- 25 Walker KZ, Russell PJ, Kingsley EA, Philips J, Raghavan D. "Detection of malignant cells in voided urine from patients with bladder cancer, a novel monoclonal assay. J Urol 142:1578 1989.
- 30 Whipple C, Yong A, RLATG, Korc M. 2012. "A Kras^{G12D}-driven Genetic Mouse Model of Pancreatic Cancer Requires Glypican-1 for Efficient Proliferation and Angiogenesis" Oecogene May 17 31(20) 2535-2544.

CLAIMS

1. A method of detecting prostate cancer in a patient, the method comprising measuring a level of soluble glypican-1 in a body fluid sample from a patient and determining that said patient has prostate cancer based upon the level of soluble glypican-1 in the body fluid sample, wherein:

the level of the soluble glypican-1 measured in the body fluid sample of the patient is increased compared to:

- (i) a control body fluid sample obtained from a cancer-free patient; or
 - (ii) pre-determined reference values or ranges of reference values;
- and the body fluid sample comprises whole blood, serum, plasma, or urine.

2. The method of claim 1, wherein said level of soluble glypican-1 in the body fluid sample is measured using an anti-glypican-1 antibody, and wherein said anti-glypican-1 antibody is MIL-38.

3. The method of claim 1, wherein said body fluid sample is contacted with a population of antibodies, wherein:

antibodies of the population comprise:

- (a) 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
 - (b) 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; and
- antibodies of the population do not comprise a light chain variable region

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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;

a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 113-121 of SEQ ID NO: 12.

4. The method of claim 3, wherein the antibody population is produced by or otherwise identical to an antibody population as generated by hybridoma cells deposited on 22 August 2014 at CellBank Australia (CBA) under accession number CBA20140026.

5. The method of claim 1, wherein said level of soluble glypican-1 in the body fluid sample is measured using an anti-glypican-1 antibody, and said anti-glypican-1 antibody is not MIL-38.

6. The method of claim 1, wherein said level of soluble glypican-1 in the body fluid sample is measured using an anti-glypican-1 antibody, and wherein said anti-glypican-1 antibody is an antibody fragment or recombinant antibody capable of binding glypican-1.

7. The method of any one of claims 2, 5, or 6, wherein said anti-glypican-1 antibody is labeled.

8. The method of claim 7, wherein said label is selected from the group consisting of a radiolabel, a fluorescent label, a component of a biotin-avidin amplification system, a component of a chemiluminescence system, and colloidal gold.

9. The method of any one of claims 2, 5, or 8, wherein binding of the anti-glypican-1 antibody to the soluble glypican-1 is measured via a technique selected from the group consisting of immunofluorescence, radiolabeling, immunoblotting, Western blotting, enzyme-linked immunosorbent assay (ELISA), flow cytometry, immunoprecipitation, immunohistochemistry, biofilm test, affinity ring test, antibody array optical density test, and chemiluminescence.

REPLACEMENT SHEET

10. The method of claim 1, wherein said level of soluble glypican-1 in the body fluid sample from the patient is compared to the level of soluble glypican-1 in the control sample using anti-glypican-1 antibodies, whereby detecting increased anti-glypican-1 antibody binding to the soluble glypican-1 of the body fluid sample over the control sample is associated with the presence of prostate cancer.

11. The method of claim 10, wherein a 50% or more increase in the level of soluble glypican-1 of said body fluid sample over the level of soluble glypican-1 in the control sample is indicative of prostate cancer.

12. The method of any one of claims 2, 5, 8, or 9, wherein binding of the anti-glypican-1 antibody to the soluble glypican-1 in said body fluid sample is compared to binding of the anti-glypican-1 antibody to soluble glypican-1 within the control sample; wherein increased binding of the anti-glypican-1 antibody in the body fluid sample over the control sample is associated with the presence of prostate cancer.

13. The method of claim 12, wherein a 50% or more increase in the anti-glypican-1 antibody binding to said body fluid sample over the level of anti-glypican-1 antibody binding of the control sample is indicative of prostate cancer.

14. The method of any one of claims 2, 5, 8, 9, 12, or 13, wherein binding of the anti-glypican-1 antibody to the soluble glypican-1 in said body fluid sample is compared to binding of the anti-glypican-1 antibody to soluble glypican-1 in one or more glypican-1 standards; wherein the anti-glypican-1 antibody binding of the standards is used to quantify the amount of soluble glypican-1 in said body fluid sample.

15. The method of any one of claims 1 to 14, wherein a soluble glypican-1 content higher than 10ng/ml in the body fluid sample is indicative of prostate cancer.

16. The method of any one of claims 1 to 15, further comprising:
measuring the level of prostate-specific antigen (PSA) in the body fluid sample or a different body fluid sample from the patient, and

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determining that said patient has prostate cancer based upon (i) the level of PSA measured in the body fluid sample, and (ii) said level of the soluble glypican-1 measured in the body fluid sample of the patient.

17. The method of claim 16, wherein the body fluid sample from the patient is a blood sample.

18. The method of claim 16, wherein the level of prostate-specific antigen (PSA) in the body fluid sample measured is compared to the level of PSA measured in a control sample; wherein increased PSA levels in the body fluid sample over the control sample is associated with the presence of prostate cancer.

19. The method of claim 16, wherein said body fluid sample from the patient is selected from the group consisting of serum, plasma, and urine.

20. Use of a kit comprising:
a first anti-glypican-1 antibody, and
glypican-1 standards;
for detecting prostate cancer in a body fluid sample from a patient, wherein the first anti-glypican-1 antibody is used to detect soluble glypican-1 in the body fluid, and wherein the body fluid sample comprises whole blood, serum, plasma or urine.

21. The use of claim 20, wherein the first anti-glypican-1 antibody is not MIL-38.

22. The use of claim 20, wherein the first anti-glypican-1 antibody is MIL-38.

23. The use of claim 20, wherein the first anti-glypican-1 antibody is the antibody referred to in any one of claims 3, 4 or 6.

24. The use of any one of claims 20 to 23, further comprising a further ligand of glypican-1.

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25. The use of claim 24, wherein said further ligand is a second anti-glypican-1 antibody or an aptamer capable of binding to soluble glypican-1; wherein said second anti-glypican antibody is the same as the first anti-glypican-1 antibody.
26. The use of claim 24 or claim 25, wherein said further ligand is conjugated to a label for rapid detection of said further ligand.
27. The use of claim 26, wherein said label is for use in a detection method selected from the group consisting of immunofluorescence, radiolabeling, immunoblotting, Western blotting, enzyme-linked immunosorbent assay (ELISA), flow cytometry, immunoprecipitation, immunohistochemistry, biofilm test, affinity ring test, antibody array optical density test, and chemiluminescence.
28. The use of any one of claims 20 to 27, wherein the kit comprises components for conducting an enzyme-linked immunosorbent assay (ELISA).

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QPC1_H12224 Mass: 61641 Score: 4278 Matches: 166 (134) Sequences: 21 (18) empAI: 5.50
 Glypican-1 CS-Homo sapiens GN=GPC1 PE=1 SV=2

Sequence Coverage: 46%

Matched peptides are underlined

1	<u>MEIRARGWNL</u>	<u>LCAAAALVAC</u>	<u>ARGDEASKSR</u>	<u>SCGEVRQIYG</u>	<u>AKGFSLSDVP</u>
51	<u>QAEISGEHLR</u>	<u>ICPQGYTCCT</u>	<u>SEMEENLANR</u>	<u>SHAELETALR</u>	<u>DSSRVLOAML</u>
101	<u>ATQLRSFDDH</u>	<u>FQHLNDSEK</u>	<u>TLQATFPGAF</u>	<u>GELYTONARA</u>	<u>FRDLYSELRL</u>
151	<u>YYRGANLHLE</u>	<u>ETLAEFWARL</u>	<u>LERLFKQLHP</u>	<u>QLLEPDDYLD</u>	<u>CLGKQAEALR</u>
201	<u>EFGEAPRELR</u>	<u>LRATRAFVAA</u>	<u>RSEVQGLGVA</u>	<u>SDVVRKVAQV</u>	<u>PLGPECRAV</u>
251	<u>MKLVYCAHCL</u>	<u>GVPGARPCPD</u>	<u>YCRNVLGCL</u>	<u>ANQADLDAEW</u>	<u>RNLDSMVL</u>
301	<u>TDKFWGTSGV</u>	<u>ESVIGSVHTW</u>	<u>LAFAINALQD</u>	<u>NRDTLTAKVI</u>	<u>QGCGRKKNP</u>
351	<u>QGECEEEKRR</u>	<u>RGKLAERERP</u>	<u>PSGTLEKLYS</u>	<u>EAKAQLRIVQ</u>	<u>DFWISLESTL</u>
401	<u>CSEKMAISTA</u>	<u>SDDRCWNGMA</u>	<u>RGRYLPEVMG</u>	<u>DGLANQINNP</u>	<u>EVEVDITKPD</u>
451	<u>MTIRQQIMQL</u>	<u>KIMTNRLRSA</u>	<u>YNGNDVDFQD</u>	<u>ASDDGSGSGS</u>	<u>GDGCLDDLCS</u>
501	<u>RKVSRRKSSSS</u>	<u>RTEPLTHALEG</u>	<u>LSEQEGQKTS</u>	<u>AASCPQPTTF</u>	<u>LLPLLELAL</u>
551	<u>TVARERWR</u>				

(SEQ ID NO: 23)

Figure 1

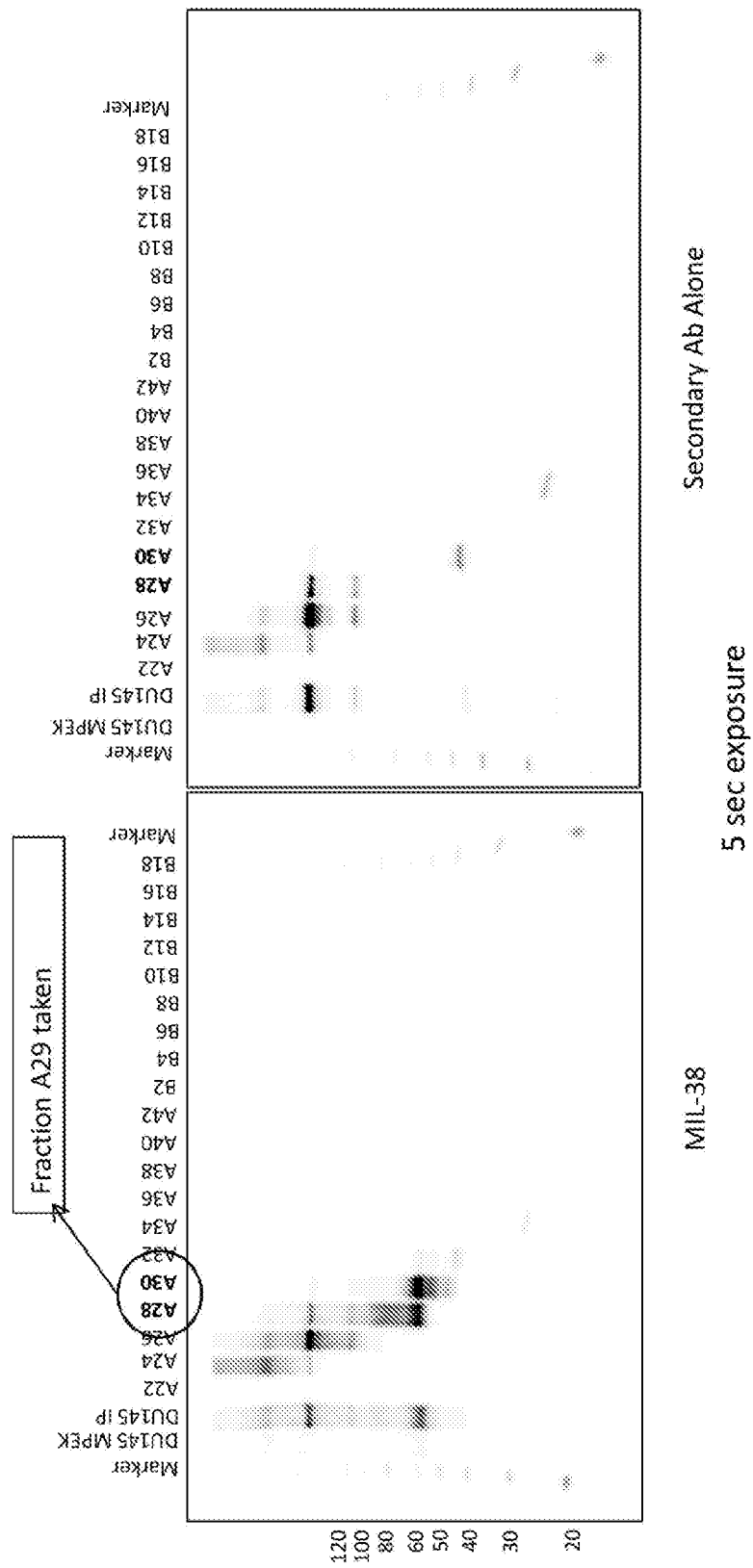


Figure 2

Mass Spectrometry of Size Exclusion HPLC Fraction #29

SECI_HUMAN Mass: 61641 Score: 290 Matches: 8(8) Sequences: 8(8) eMFAI: 0.52
Glypican-1 OS=Homo sapiens GN=GPC1 PE=1 SV=2

Sequence Coverage: 14%

Matched peptides are underlined

1 MEIIRARGSWL LCAAAALVAC ARGDPAKSR SCGEVQIYG ARGFSLDVP
51 QAEISGEHLR ICPQGYTCT SEMEENIANR SHALETALR DSSRYLCAML
101 ATGLRSFTDH FOHLNDSER TLOATFGAF GELYTONARA FEDLYSELRL
151 YTEGANLME ETLAEFWARL LERLFKQLHP QLLLPDDYLD CIGKQAEAIR
201 PFGEARRELK LRA TRAFVAA RSFVQGLGVA SDVVRKVQV ELGFECSRIV
251 MLIVYCAHCL GYFGARPCD YCRNVLKGL ANQADIDAEW RNLDSMWLI
301 TDHFWGTSGV ESVIGSVHW LAEAINALQD NRDILTAKVI QCCGMPKVP
351 QGEGFEERK RGLAFRRRP PSFTLEKLVS EAKAQLADVQ DFWISLPGTL
401 CSEKNALSTA SDDRCWNGMA RGRILPEVMG DGLANQINP EVEVDITKED
451 MTIRQQIMQL KIMTNRLRSA YNENVDVFDQ ASDDGSGSGS GDGCLDILCS
501 RKVSRKSSSS KTEPLTHALPG LSEQEGQKTS AASCPQPTTF LIFLILLFAL
551 TVAREWR (SEQ ID NO: 23)

Figure 3

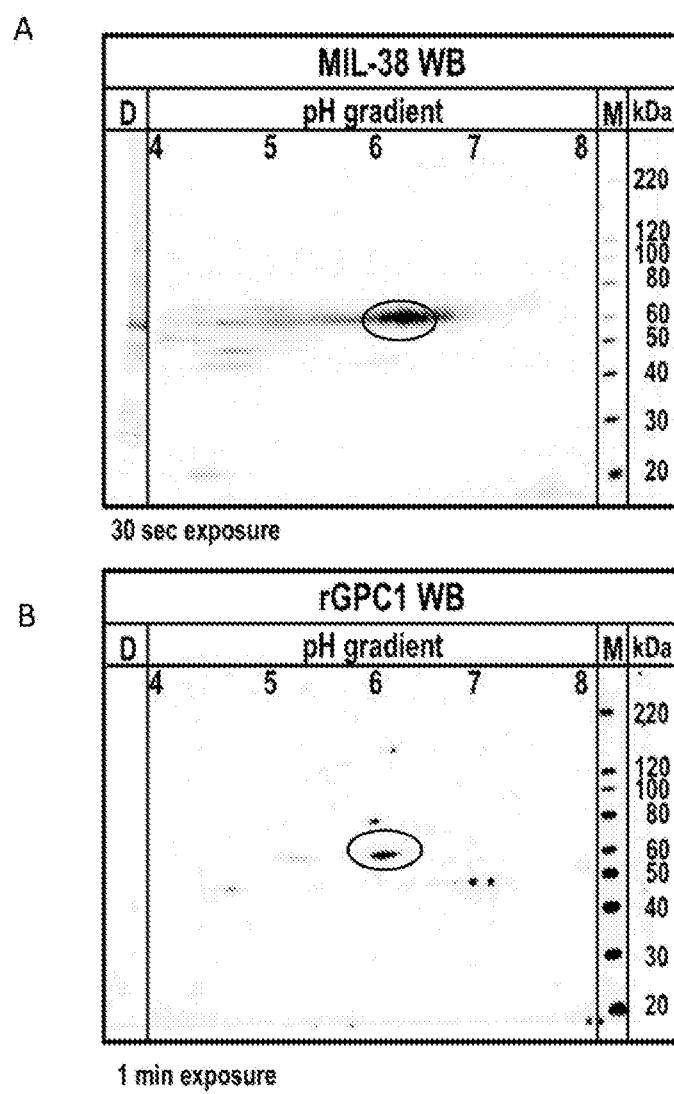
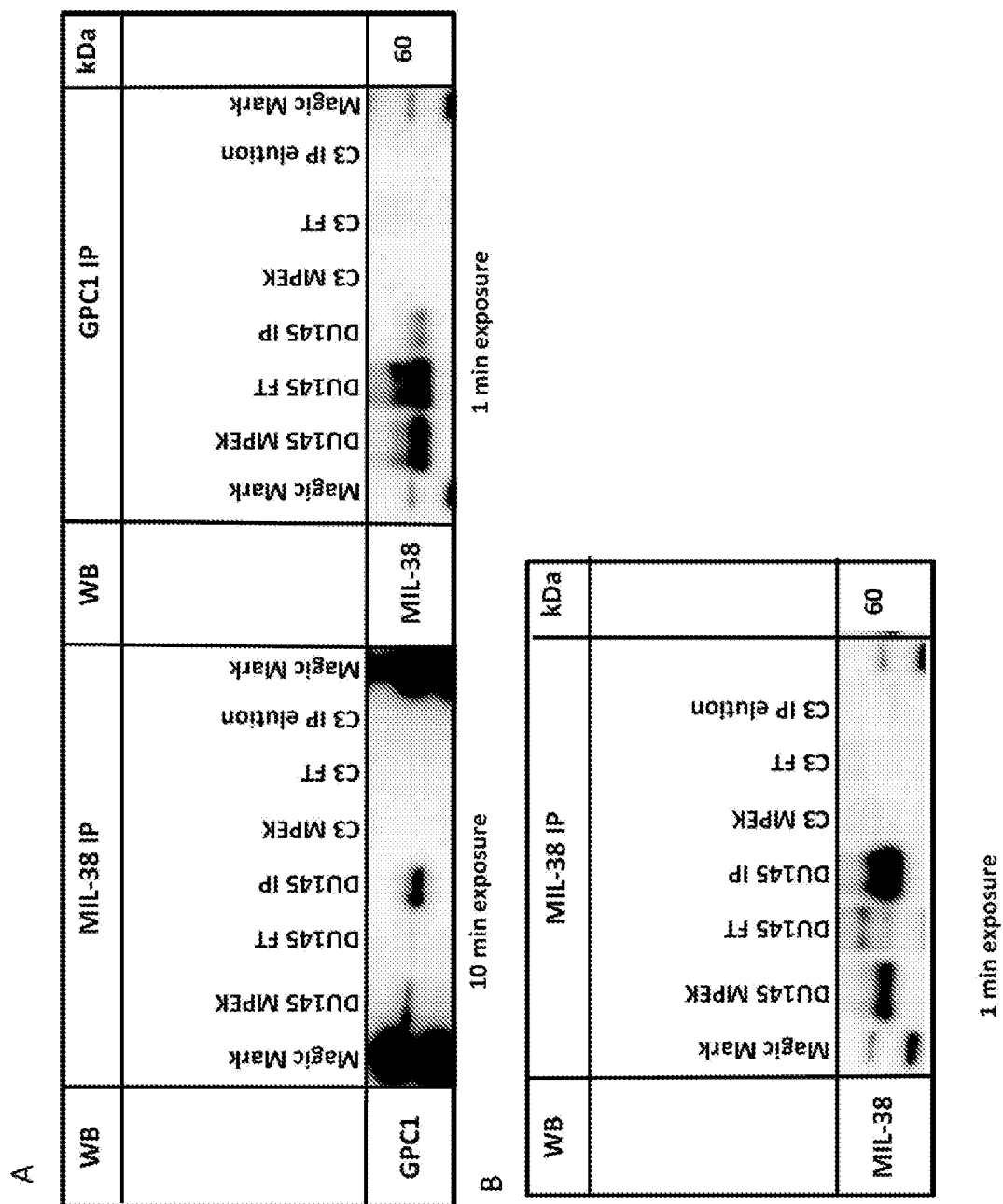


Figure 4



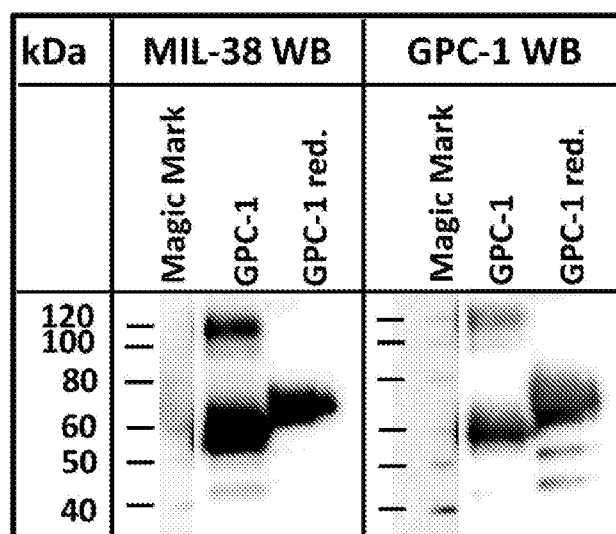


Figure 6

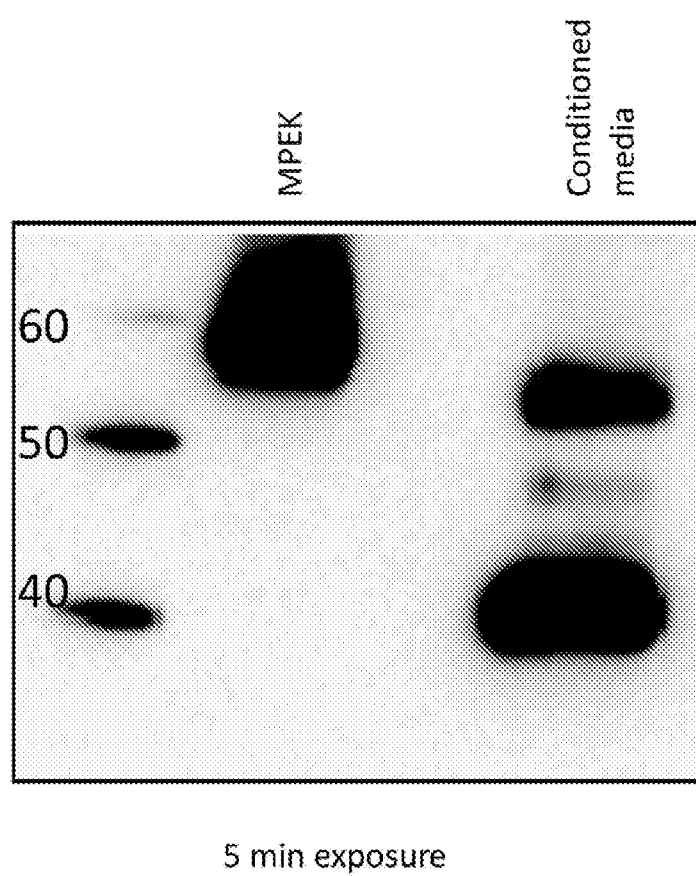


Figure 7

A

SPC1_HUMAN Mass: 61641 Score: 852 Matches: 30 (22) Sequences: 11 (9) emPAI: 0.89
 Glypican-1 OS=Homo sapiens GN=GPC1 PE=1 SV=2

Sequence Coverage: 16%

Matched peptides are underlined

1 MELRARGWNL LCAAAALVAC ARGDEASKSR SCGEVRQIYG AKGFSLSQVP
 51 QAEISGEHLR ICPQGYTOCT SEMEENIANR SHAELETALR DSSRVLOAML
 101 ATQLRSFDDH FOHLNDSEK TDAQTFPGAF GELYTONARA FEDLYSELRL
 151 YYRGANLHLE ETLAEFWARL LERLEKQLHP QULLPDDYLD CLGKQAEALR
 201 FFGEAFRELR LRATRAEFVA RSEVQGLGVA SDVVRKYAQV FLGPECRAV
 251 MRLVYCAHCL GVPGARPCPD YCENVLKGCL ANQADLDAEW RNLLDSWLL
 301 TDKFWGTSGV ESYIGSVHTW LAZAINALQD NRDITLAKVI QGCENFTVNP
 351 QGSGFEKRR RGLAPRRP PSGTLEKLYS EAKAQLRDVQ DEWISLPGTL
 401 CSEKMAISTA SDDRQWNGMA RGRYLPEVMG DGLANQINNP EVEVDITKPD
 451 MTIROQIMQL KIMTNRLRSA YNGNDVDFOD ASDDGSQSGS GDGCLDMLCS
 501 RKVSRKSSSS RPLTHALPS LSEQEGQKTS AASCQPPTF LLELLFLAL
 551 TVAREWR (SEQ ID NO: 23)

Figure 8

B

SEQ1_HUMAN Mass: 61642 Score: 1531 Matches: 48 (43) Sequences: 11 (10) eMPI: 1.42
 Glypican-1 OS=Homo sapiens GN=GPC1 PE=1 SV=2

Sequence Coverage: 18%

Matched peptides are underlined

1 MELRARGWNL LCAAAALVAC ARGDEASKR SCGEVRQIYG AKGEELSDVP
 51 QAEISGEHLR ICPQGYTCCT SEMEENLANR SHALETALR DSSRYLQAML
 101 ATQLRSFDH FQHLNDSEK TLQATFFGAF GELYQNARA FRDLYSELRL
 151 YVRGANHLR ETLAEFWARL LERLFKQLRP QLLPDDYLD CLGKQAEALR
 201 PFGEAPRELK LRATRAFWAA RSFVQGLGVA SDVVRKVAVQV PLGPECRAV
 251 MKLVIYCAHCL GVPGARPCFD YCRNVLGCL ANQADLDAEW RNLDSMWLI
 301 TDKFWGTSGV ESVIGSVHTW LAELINALQD NRDILTAKVI QGCENPKVNP
 351 QGPGEPEKRR RGKLAERERF PSGTLEKLYS EAKAQLRUQ DEWISLESTL
 401 CSEKMAISTA SDDRCWNGMA RGRYLPEVMG DGLANQINNP EVEVDITKPD
 451 MTTRQOIMOL KIMTNRLRSA YNGMDVDFOD ASDNGSGSGS GNGCIDNDCS
 501 RRVSRKSSSS RTELTHALPS LSEQEGQKTS AASCEQPETF LLPILLFLAL
 551 TVARERWR (SEQ ID NO: 23)

Figure 8

C

GPC1_HUMAN Mass: 61641 Score: 1364 Matches: 39 (33) Sequences: 13 (9) eMPI: 1.18
 Glypican-1 OS=Homo sapiens GN=GPC1 PE=1 SV=2

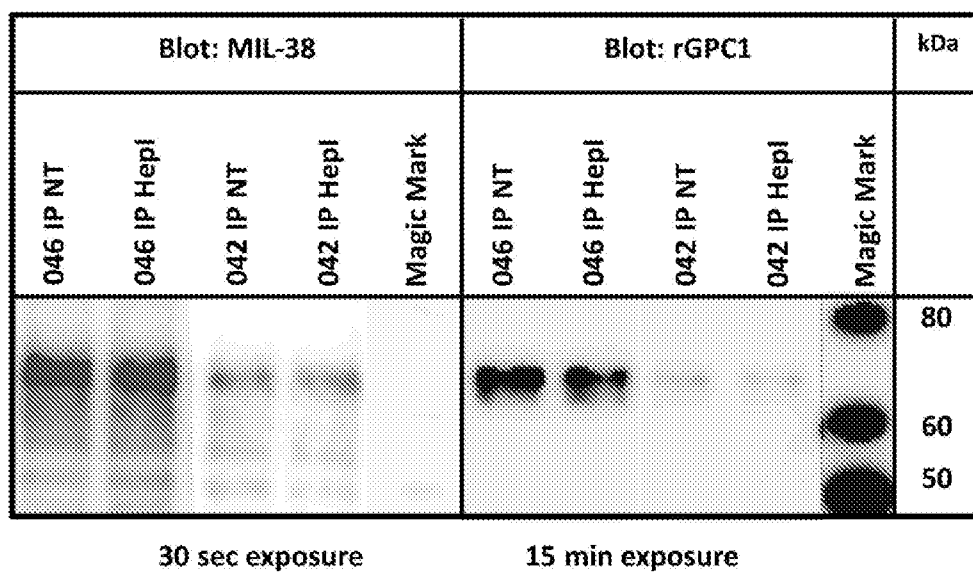
Sequence Coverage: 24%

Matched peptides are underlined

1 MELRARGWNL ICAAAALVAC ARGDPASKSR SCGEVRQLYG AKETSLSDVP
 51 QAEISGEHLR ICEQGYTOCT SEMEENIANR SHALEETALR DSENVLQAML
 101 ATQLRSFDDH FQHLLNDSEK TLQATFFGAF GELYTONARA PRDLYSELRL
 151 YIRGANLHLE ETLAEFWARL LERLFKQLHP QLLFFDDYLD CLGKQAEALR
 201 EFGAEAPRELK LRATRAFYAA RSEVQGLGYA SDVVRKVAQV PLGPECRAV
 251 WKLVCARHCL GVEGARPCFD YCRNVLNGCL ANQADLDAEW RNLLDSMYLI
 301 TDKFWGTSGV ESVGSVHTW LAFAINALQD NRDELTAKVI QCGCNFEVNP
 351 QGPGPEEKRR RGNLAFRERP PSGTLEKLYS EAKAQIRDVQ DFWISLPGTL
 401 CSEKMAISTA SDDRCWNGMA RGRYLFEVNG DGLANQINNF EVEVDITKFD
 451 MTIRQIMQL KIMTNRLRSA YNGNDVDFQD ASDDSGSGS GDGCLDDLCS
 501 RKVSRKSSSS RTFLTHALPG LSEQEGQKTS AASCPQPTF LFLFLFLAL
 551 TVARERNR (SEQ ID NO: 23)

Figure 8

A



B

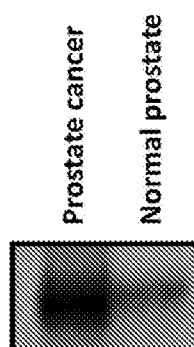


Figure 9

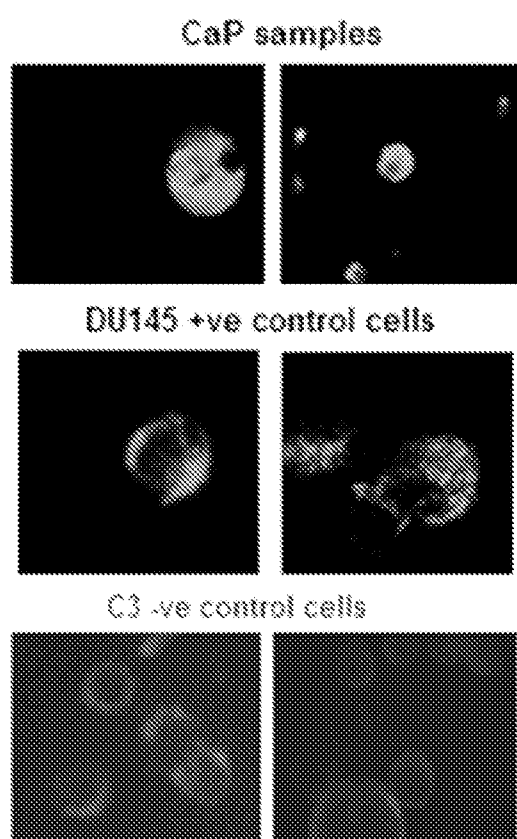


Figure 10

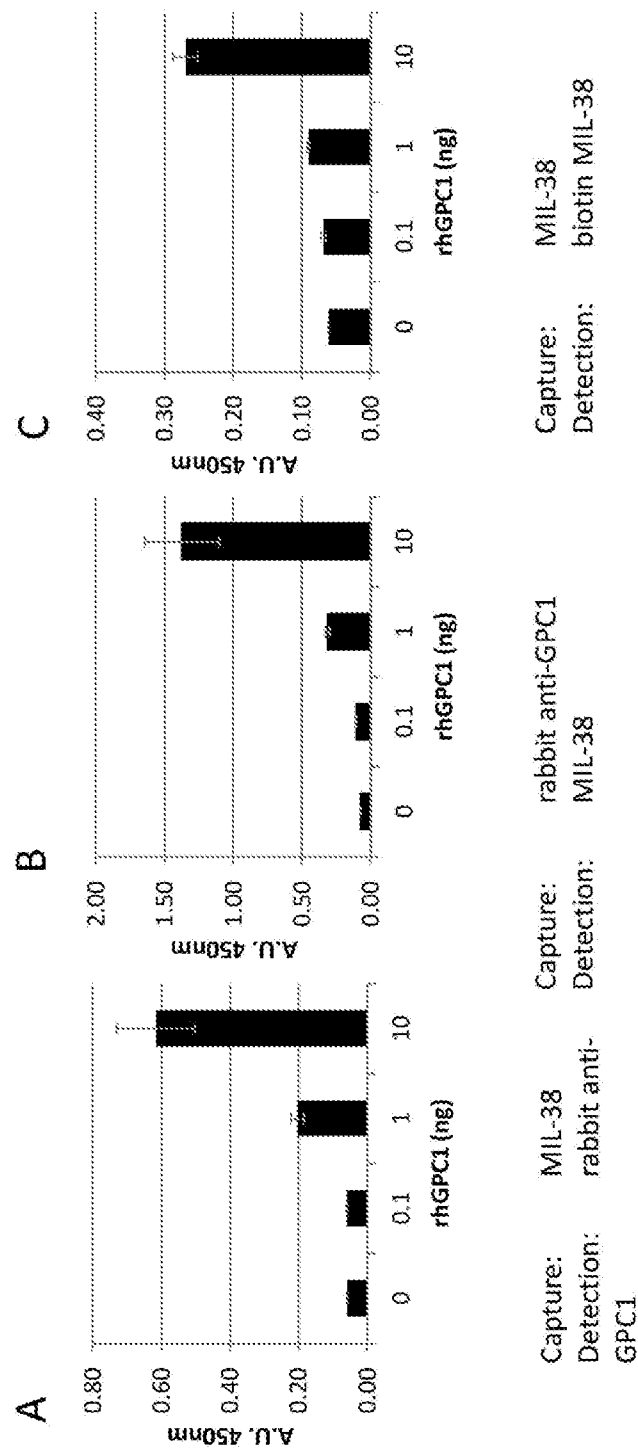


Figure 11

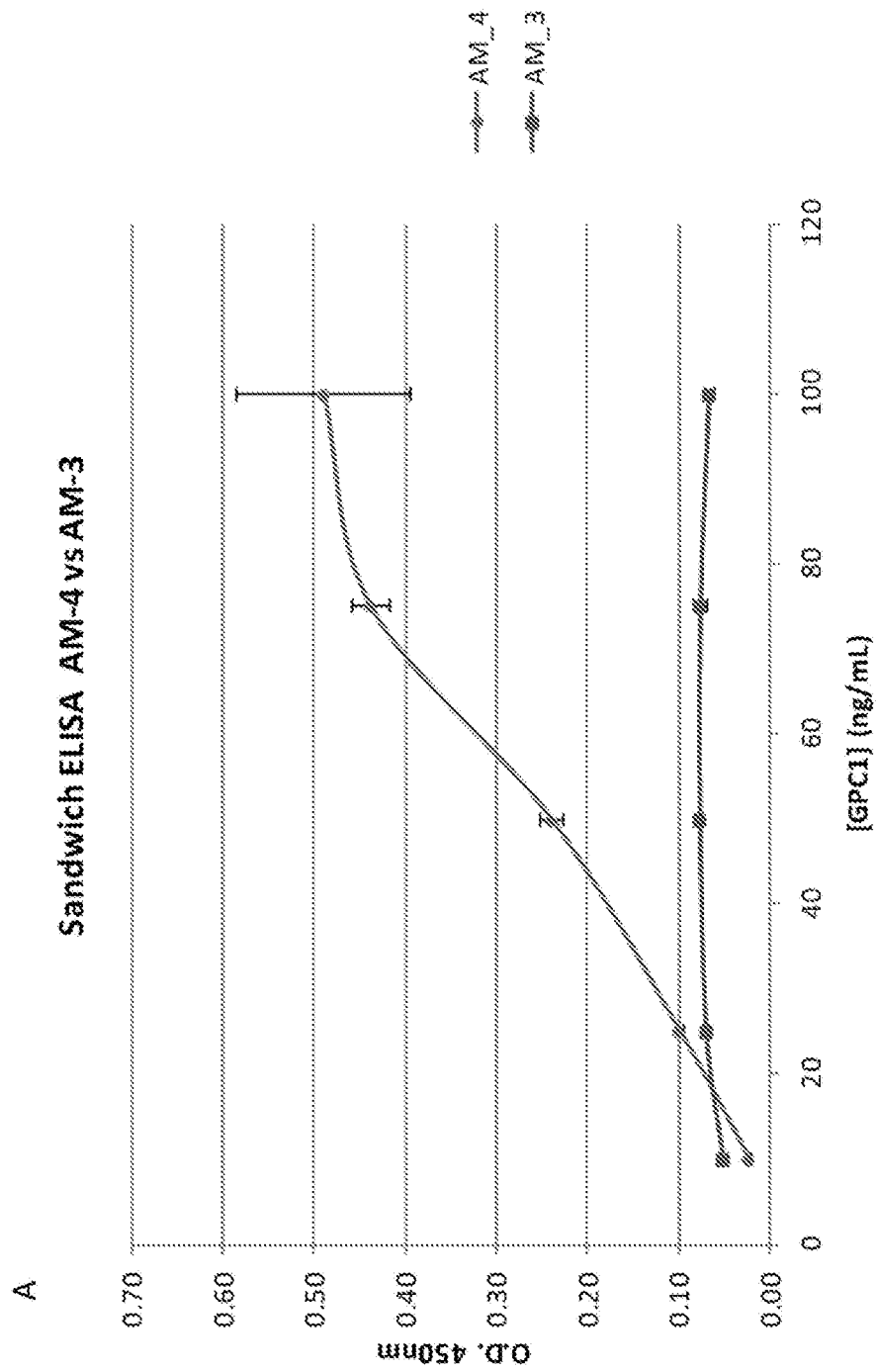


Figure 12

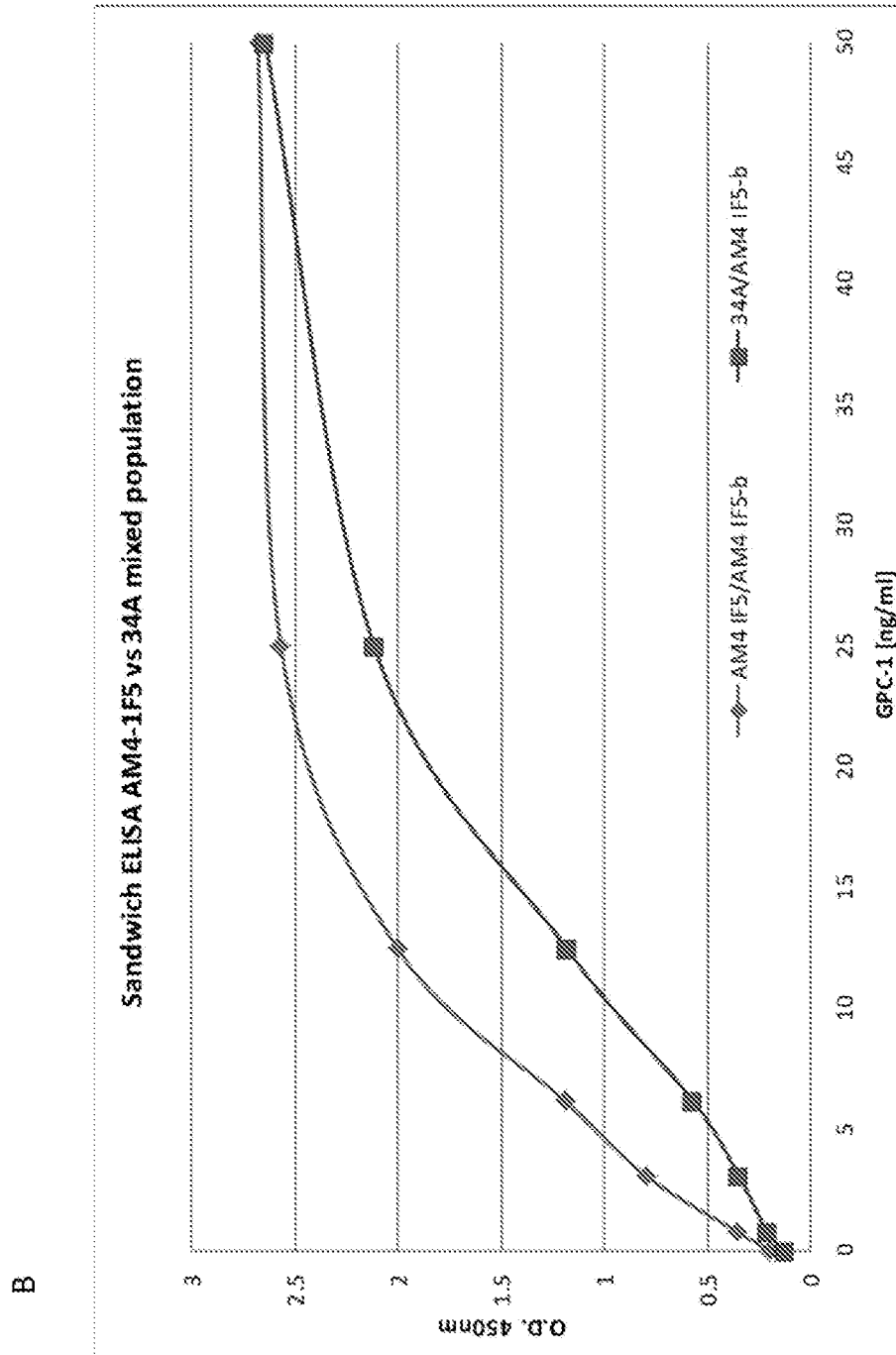


Figure 12

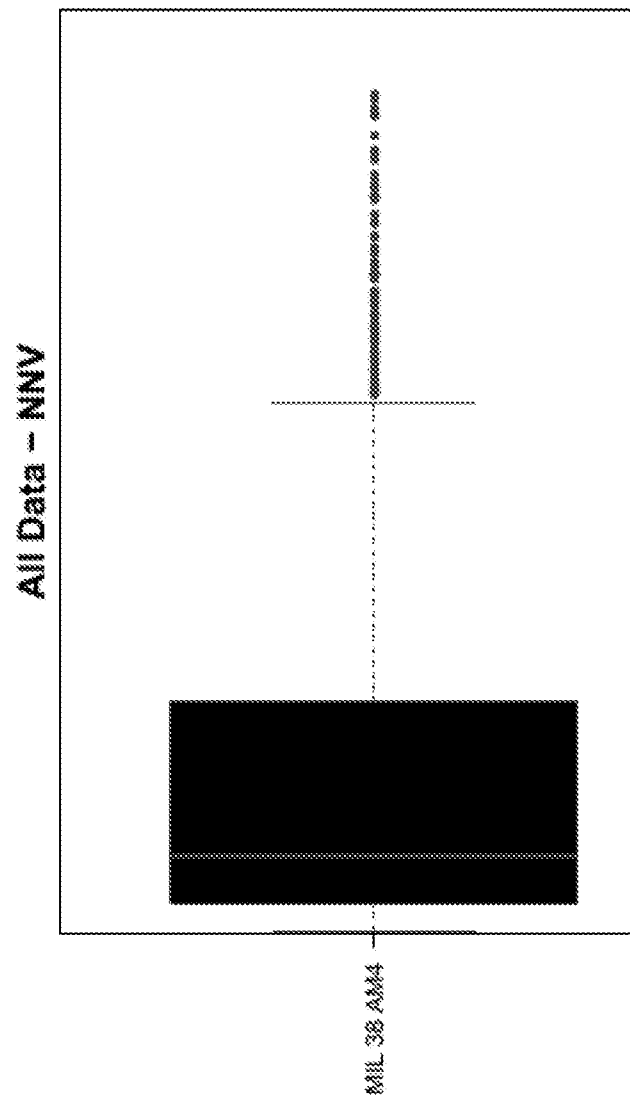


Figure 13

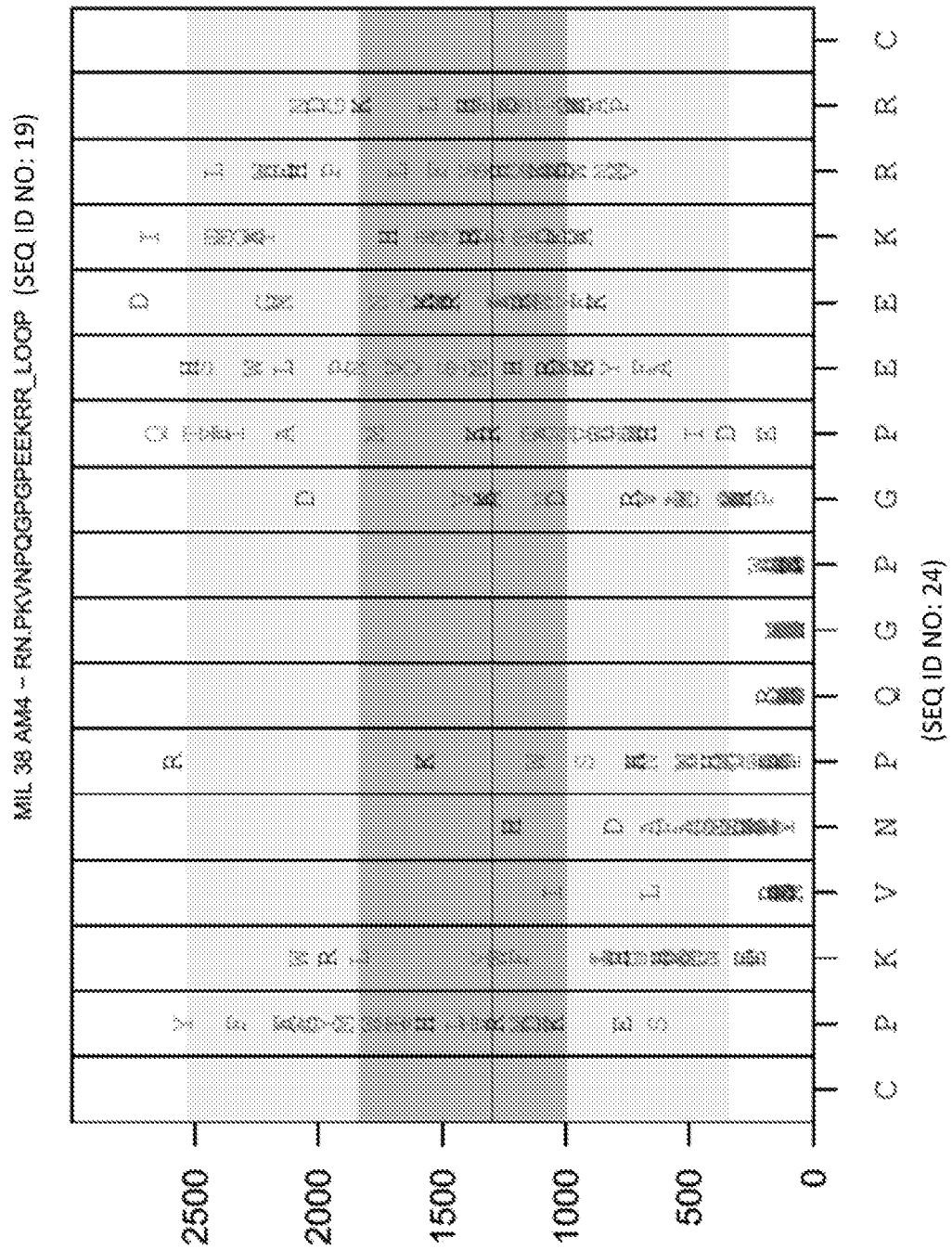


Figure 14