

cancer along with liver cirrhosis, non-alcoholic fatty liver disease, alcoholic liver disease, Wilson's disease, hereditary hemochromatosis, autoimmune hepatitis, documented aflatoxin exposure, schistosomiasis and diabetes mellitus. The increasing prevalence of these conditions throughout the world makes it imperative that a quick and non-invasive test for liver cancer is devised.

In recent years it has become apparent that antibodies, and in particular autoantibodies, can serve as biological markers of disease or disease susceptibility. Autoantibodies are naturally occurring antibodies directed to an antigen which an individual's immune system recognises as foreign even though that antigen actually originated in the individual. They may be present in the circulation as circulating free autoantibodies or in the form of circulating immune complexes consisting of autoantibodies bound to their target protein. Differences between a wild type protein expressed by "normal" cells and an altered form of the protein produced by a diseased cell or during a disease process may, in some instances, lead to the altered protein being recognised by an individual's immune system as "non-self" and thus eliciting an immune response in that individual. This may be a humoral (i.e. B cell-mediated) immune response leading to the production of autoantibodies immunologically specific for the altered protein.

Assays which measure the immune response of an individual to the presence of tumour marker proteins in terms of autoantibody production provide an alternative to the direct measurement or detection of tumour marker proteins in bodily fluids. Such assays essentially constitute indirect detection of the presence of a tumour marker protein. The nature of the immune response means it is likely that autoantibodies can be elicited by a very small amount of circulating tumour marker protein and indirect methods which rely on detecting the immune response to tumour marker proteins will consequently be more sensitive than methods for the direct measurement of tumour marker protein levels in bodily fluids. Assay methods based on the detection of autoantibodies may therefore be of particular value early in the disease process.

The inventors have surprisingly determined four tumour marker antigens previously not known to be associated with liver cancer. Through the detection of autoantibodies directed to any one of these tumour marker antigens, optionally in combination with one or more additional tumour marker antigens, the inventors have devised an effective and non-invasive screening method for liver cancer, and a corresponding kit.

Summary

The inventors have surprisingly established that autoantibodies immunologically specific for any one of the tumour marker proteins matrix metalloproteinase 9 (MMP9), allograft

inflammatory factor 1 (AIF1), epithelial cell adhesion molecule (EpCAM) and cyclin-dependent kinase inhibitor 1B (CDKN1B) are indicative of the presence of liver cancer. Therefore, the detection of autoantibodies immunologically specific for any one of these tumour marker proteins can be used for the diagnosis of liver cancer.

In one aspect, the present disclosure refers to a method of detecting liver cancer in a mammalian subject by detecting an antibody in a test sample comprising a bodily fluid from the mammalian subject, wherein the antibody is an autoantibody immunologically specific for a tumour marker protein selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B, which method comprises the steps of:

- (a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and

- (b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample;

whereby the presence of said complexes is indicative of the presence of liver cancer.

According to an example of the disclosure there is provided a method of detecting an antibody in a test sample comprising a bodily fluid from a mammalian subject, wherein the antibody is an autoantibody immunologically specific for a tumour marker protein selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B, which method comprises the steps of:

- (a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and

- (b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample.

Within this example the subject is preferably suspected of having liver cancer.

According to an example of the disclosure there is provided a method of detecting liver cancer in a mammalian subject by detecting an antibody in a test sample comprising a bodily fluid from the mammalian subject, wherein the antibody is an autoantibody immunologically specific for a tumour marker protein selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B, which method comprises the steps of:

- (a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and

- (b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample;

whereby the presence of said complexes is indicative of the presence of liver cancer.

According to an example of the disclosure there is provided a method of diagnosing and treating liver cancer in a mammalian subject by detecting an antibody in a test sample comprising a bodily fluid from the mammalian subject, wherein the antibody is an autoantibody immunologically specific for a tumour marker protein selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B, which method comprises the steps of:

- (a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B;
- (b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample;
- (c) diagnosing the subject with liver cancer when complexes of the tumour marker antigen bound to autoantibodies present in the test sample are detected; and

(d) administering a liver cancer treatment to the diagnosed subject.

In another aspect, the present disclosure refers to a method of predicting response to an anti-liver cancer treatment, the method comprising detecting an antibody in a test sample comprising a bodily fluid from a mammalian subject, wherein the antibody is an autoantibody immunologically specific for a tumour marker protein selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B, which method comprises the steps of:

- (a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B;
 - (b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample;
 - (c) detecting the amount of specific binding between the tumour marker antigen and autoantibodies present in the test sample; and
 - (d) comparing the amount of specific binding between the tumour marker antigen and the autoantibody with a previously established relationship between the amount of binding and the likely outcome of treatment;
- whereby a change in the amount of specific binding, when compared to controls, predicts that the patient will or will not respond to the anti-liver cancer treatment.

Within this example of the disclosure the anti-liver cancer treatment may be selected from the group consisting of chemotherapy, radiofrequency ablation, liver resection, liver transplant, vaccination, anti-growth factor or signal transduction therapies, endocrine therapy, human antibody therapy, transcatheter arterial chemoembolization, percutaneous ethanol injection, microwave ablation, sorafenib administration and radioembolisation.

In another aspect, the present disclosure refers to the use of a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B in a method of detecting liver cancer in a mammalian subject by detecting an autoantibody immunologically specific for MMP9, AIF1, EpCAM or CDKN1B in a test sample comprising a bodily fluid from the mammalian subject, which method comprises the steps of:

- (a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and
 - (b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample;
- whereby the presence of said complexes is indicative of the presence of liver cancer.

In another aspect, the present disclosure refers to a kit for the detection of liver cancer, wherein the kit comprises:

- (a) a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and
- (b) a reagent capable of detecting complexes of the tumour marker antigen bound to autoantibodies present in the test sample;
optionally further comprising:
- (c) means for contacting the tumour marker antigen with a test sample comprising a bodily fluid from a mammalian subject.

According to an example of the disclosure there is provided a kit for the detection of autoantibodies in a test sample comprising a bodily fluid from a mammalian subject comprising:

- (a) a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and
- (b) a reagent capable of detecting complexes of the tumour marker antigen bound to autoantibodies present in the test sample.

In another aspect, the present disclosure refers to an *in vitro* method of determining an antibody profile of an individual suffering from liver cancer in a test sample comprising a bodily fluid from the mammalian subject wherein the antibody is an autoantibody immunologically specific for a tumour marker protein selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B, which method comprises the steps of:

- a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and
- b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample, wherein the method is repeated to build up a profile of antibody production.

In all examples of the disclosure the mammalian subject is preferably a human. Herein the terms “mammalian subject” and “subject” will be used interchangeably to refer to a subject who is mammalian, preferably human.

In all examples of the disclosure the method is preferably carried out *in vitro* on a test sample comprising a bodily fluid obtained or prepared from the mammalian subject.

The surprising discovery that autoantibodies immunologically specific for a tumour marker protein selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B can be used as markers for liver cancer has permitted the inventors to devise methods for the detection of such autoantibodies, which can be used to detect and diagnose liver cancer. Such detection can be performed using a kit, and these methods and kits form the core of the present disclosure.

Brief description of Figures

Figure 1. Diagrammatic representation to demonstrate the derivation of secondary curve parameters: Figure 1A = Slope, Intercept, Area under the Curve (AUC) and SlopeMax; Figure 1B = dissociation constant (Kd).

Figure 2. Autoantibody microtitre plate layouts: Figure 2A = high-throughput assay (HTPA) layout; Figure 2B = titration layout.

Claims

1. A method of detecting liver cancer in a mammalian subject by detecting an antibody in a test sample comprising a bodily fluid from the mammalian subject, wherein the antibody is an autoantibody immunologically specific for a tumour marker protein selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B, which method comprises the steps of:

(a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and

(b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample;

whereby the presence of said complexes is indicative of the presence of liver cancer.

2. The method of claim 1,

wherein the mammalian subject has tested positive for alpha-fetoprotein (AFP), des-gamma carboxyprothrombin (DCP) or lectin-reactive alpha-fetoprotein (AFP-L3); or

wherein the mammalian subject has tested positive for liver cancer using ultrasound surveillance; or

wherein the mammalian subject has liver cirrhosis, non-alcoholic fatty liver disease, alcoholic liver disease, Wilson's disease, hereditary hemochromatosis, autoimmune hepatitis, hepatitis B, hepatitis C, documented aflatoxin exposure, schistosomiasis or diabetes mellitus; or

wherein the liver cancer is hepatocellular carcinoma (HCC).

3. A method of predicting response to an anti-liver cancer treatment, the method comprising detecting an antibody in a test sample comprising a bodily fluid from a mammalian subject, wherein the antibody is an autoantibody immunologically specific for a tumour marker protein selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B, which method comprises the steps of:

(a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B;

(b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample;

(c) detecting the amount of specific binding between the tumour marker antigen and autoantibodies present in the test sample; and

(d) comparing the amount of specific binding between the tumour marker antigen and the autoantibody with a previously established relationship between the amount of binding and the likely outcome of treatment;

whereby a change in the amount of specific binding, when compared to controls, predicts that the patient will or will not respond to the anti-liver cancer treatment.

4. The method of claim 3, wherein the liver cancer treatment is selected from the group consisting of chemotherapy, radiofrequency ablation, liver resection, liver transplant, vaccination, anti-growth factor or signal transduction therapies, endocrine therapy, human antibody therapy, transcatheter arterial chemoembolization, percutaneous ethanol injection, microwave ablation, sorafenib administration and radioembolisation.

5. The method of any one of claims 1-4, wherein two or more autoantibodies are detected, and wherein the method comprises the step of:

(a) contacting the test sample with a panel of two or more tumour marker antigens comprising a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B and one or more further tumour marker antigens immunologically specific for at least one of said autoantibodies; optionally

wherein the panel comprises two or more tumour marker antigens which are distinct antigens; optionally

wherein the panel comprises two or more antigen variants of one or more of the distinct antigens.

6. The method of claim 5, wherein the panel of two or more tumour marker antigens comprises MMP9, AIF1, EpCAM and CDKN1B.

7. The method of claim 5, wherein the panel of two or more tumour marker antigens comprises one or more tumour marker antigens selected from the group consisting of NY-ESO-1, vimentin, HSPA4, transferrin, HNRNP-L, HSPD1, HNRNP-A2, SALL4, Cyclin B1, AFP, NPM1, YWHAZ, DDX3X, p62, CAGE, MAGE A4, RalA, GBU4-5, SOX2, AKR1B10, ApoA1, BCL2, CD44, CK18, CPS1, FUCA1, GLUL, HSPA2, IL-8, MDM2, PEBP1, prolactin, RGN, SPP1, SSX2 and TGFB1.

8. The method of claim 7, wherein the panel of two or more tumour marker antigens comprises or consists of:

(i) CAGE, NY-ESO-1, MMP9, transferrin, MAGE A4, RalA, HSPA4, SALL4, Cyclin B1, EpCAM, DDX3X, and AIF1;

(ii) CAGE, NY-ESO-1, MMP9, transferrin, MAGE A4, RalA, HSPA4, SALL4, Cyclin B1, EpCAM, DDX3X, AIF1, SOX2 and AFP;

(iii) MMP9, AIF1, EpCAM, NY-ESO-1, HSPA4, vimentin, HNRNP-L and transferrin;

(iv) AIF1, EpCAM, HSPA4 and CPS1, optionally wherein the subject is female;

- (v) AIF1, CAGE, HSPD1, SOX2, SALL4, HSPA4 and transferrin, optionally wherein the subject is female;
- (vi) EpCAM, NY-ESO-1, vimentin, HSPA2, HSPA4 and HNRNP-L, optionally wherein the subject is male;
- (vi) EpCAM, CAGE, SOX2, RalA, MAGE A4, DDX3X and NY-ESO-1, optionally wherein the subject is male;
- (viii) MMP9, AIF1, EpCAM, DDX3X, SALL4, MAGE A4, NY-ESO-1, CAGE, RalA and SOX2;
- (ix) NY-ESO-1, vimentin, HSPA4, transferrin, HNRNP-L, HSPD1, HNRNP-A2, SALL4, Cyclin B1, AFP, NPM1, YWHAZ, DDX3X, p62, CAGE, MAGE A4, RalA, GBU4-5, SOX2, AKR1B10, ApoA1, BCL2, CD44, CK18, CPS1, FUCA1, GLUL, HSPA2, IL-8, MDM2, PEBP1, prolactin, RGN, SPP1, SSX2 and TGFB1; or
- (x) MMP9, AIF1, EpCAM, CDKN1B, NY-ESO-1, vimentin, HSPA4, transferrin, HNRNP-L, HSPD1, HNRNP-A2, SALL4, Cyclin B1, AFP, NPM1, YWHAZ, DDX3X, p62, CAGE, MAGE A4, RalA, GBU4-5, SOX2, AKR1B10, ApoA1, BCL2, CD44, CK18, CPS1, FUCA1, GLUL, HSPA2, IL-8, MDM2, PEBP1, prolactin, RGN, SPP1, SSX2 and TGFB1.

9. An *in vitro* method of determining an antibody profile of an individual suffering from liver cancer in a test sample comprising a bodily fluid from the mammalian subject wherein the antibody is an autoantibody immunologically specific for a tumour marker protein selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B, which method comprises the steps of:

- a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and
- b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample, wherein the method is repeated to build up a profile of antibody production.

10. The method of any one of the preceding claims, wherein the tumour marker antigen is a naturally occurring protein or polypeptide, a recombinant protein or polypeptide, a synthetic protein or polypeptide, a synthetic peptide, a peptide mimetic, a polysaccharide or a nucleic acid; or

wherein the bodily fluid is selected from the group consisting of plasma, serum, whole blood, urine, sweat, lymph, faeces, cerebrospinal fluid, ascites fluid, pleural effusion, seminal fluid, sputum, nipple aspirate, post-operative seroma, saliva, amniotic fluid, tears and wound drainage fluid.

11. The method of any one of the preceding claims, wherein the method further comprises detecting alpha-fetoprotein (AFP), des-gamma carboxyprothrombin (DCP) or lectin-reactive

alpha-fetoprotein (AFP-L3) in a test sample comprising a bodily fluid from the mammalian subject.

12. The method of claim 11, wherein the method comprises detecting alpha-fetoprotein (AFP) in a test sample comprising a bodily fluid from the mammalian subject, optionally wherein autoantibodies immunologically specific for the tumour marker proteins CAGE, NY-ESO-1, MMP9, transferrin, MAGE A4, RalA, HSPA4, SALL4, Cyclin B1, EpCAM, DDX3X, and AIF1 are detected in a test sample comprising a bodily fluid from the mammalian subject; or optionally

wherein autoantibodies immunologically specific for the tumour marker proteins CAGE, NY-ESO-1, MMP9, transferrin, MAGE A4, RalA, HSPA4, SALL4, Cyclin B1, EpCAM, DDX3X, AIF1, SOX2 and AFP are detected in a test sample comprising a bodily fluid from the mammalian subject.

13. Use of a tumour marker antigen selected from the group consisting of MMP9 AIF1, EpCAM and CDKN1B in a method of detecting liver cancer in a mammalian subject by detecting an autoantibody immunologically specific for MMP9, AIF1, EpCAM or CDKN1B in a test sample comprising a bodily fluid from the mammalian subject, which method comprises the steps of:

(a) contacting the test sample with a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and

(b) determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies present in the test sample;

whereby the presence of said complexes is indicative of the presence of liver cancer.

14. A kit for the detection of liver cancer, wherein the kit comprises:

(a) a tumour marker antigen selected from the group consisting of MMP9, AIF1, EpCAM and CDKN1B; and

(b) a reagent capable of detecting complexes of the tumour marker antigen bound to autoantibodies present in the test sample;

optionally further comprising:

(c) means for contacting the tumour marker antigen with a test sample comprising a bodily fluid from a mammalian subject.

15. The kit of claim 14, wherein the means for contacting the tumour marker antigen with a test sample comprising a bodily fluid from a mammalian subject comprises the tumour marker antigen immobilised on a chip, slide, plate, wells of a microtitre plate, bead, membrane or nanoparticle.

16. The kit of claim 14 or claim 15, wherein the tumour marker antigen is present within a panel of two or more tumour marker antigens; optionally

wherein the panel comprises two or more tumour marker antigens which are distinct antigens.

17. The kit of claim 16, wherein the panel of two or more tumour marker antigens comprises MMP9, AIF1, EpCAM and CDKN1B.

18. The kit of claim 16, wherein the panel of two or more tumour marker antigens comprises one or more tumour marker antigens selected from the group consisting of NY-ESO-1, vimentin, HSPA4, transferrin, HNRNP-L, HSPD1, HNRNP-A2, SALL4, Cyclin B1, AFP, NPM1, YWHAZ, DDX3X, p62, CAGE, MAGE A4, RalA, GBU4-5, SOX2, AKR1B10, ApoA1, BCL2, CD44, CK18, CPS1, FUCA1, GLUL, HSPA2, IL-8, MDM2, PEBP1, prolactin, RGN, SPP1, SSX2 and TGFB1.

19. The kit of claim 18, wherein the panel of two or more tumour marker antigens comprises or consists of:

(i) CAGE, NY-ESO-1, MMP9, transferrin, MAGE A4, RalA, HSPA4, SALL4, Cyclin B1, EpCAM, DDX3X, and AIF1;

(ii) CAGE, NY-ESO-1, MMP9, transferrin, MAGE A4, RalA, HSPA4, SALL4, Cyclin B1, EpCAM, DDX3X, AIF1, SOX2 and AFP;

(iii) MMP9, AIF1, EpCAM, NY-ESO-1, HSPA4, vimentin, HNRNP-L and transferrin;

(iv) AIF1, EpCAM, HSPA4 and CPS1, optionally wherein the subject is female;

(v) AIF1, CAGE, HSPD1, SOX2, SALL4, HSPA4 and transferrin, optionally wherein the subject is female;

(vi) EpCAM, NY-ESO-1, vimentin, HSPA2, HSPA4 and HNRNP-L, optionally wherein the subject is male;

(vii) EpCAM, CAGE, SOX2, RalA, MAGE A4, DDX3X and NY-ESO-1, optionally wherein the subject is male;

(viii) MMP9, AIF1, EpCAM, DDX3X, SALL4, MAGE A4, NY-ESO-1, CAGE, RalA and SOX2;

(ix) NY-ESO-1, vimentin, HSPA4, transferrin, HNRNP-L, HSPD1, HNRNP-A2, SALL4, Cyclin B1, AFP, NPM1, YWHAZ, DDX3X, p62, CAGE, MAGE A4, RalA, GBU4-5, SOX2, AKR1B10, ApoA1, BCL2, CD44, CK18, CPS1, FUCA1, GLUL, HSPA2, IL-8, MDM2, PEBP1, prolactin, RGN, SPP1, SSX2 and TGFB1; or

(x) MMP9, AIF1, EpCAM, CDKN1B, NY-ESO-1, vimentin, HSPA4, transferrin, HNRNP-L, HSPD1, HNRNP-A2, SALL4, Cyclin B1, AFP, NPM1, YWHAZ, DDX3X, p62, CAGE, MAGE A4, RalA, GBU4-5, SOX2, AKR1B10, ApoA1, BCL2, CD44, CK18, CPS1, FUCA1, GLUL, HSPA2, IL-8, MDM2, PEBP1, prolactin, RGN, SPP1, SSX2 and TGFB1.

20. The kit of any one of claims 14-19, wherein the bodily fluids is selected from the group consisting of plasma, serum, whole blood, urine, sweat, lymph, faeces, cerebrospinal fluid, ascites fluid, pleural effusion, seminal fluid, sputum, nipple aspirate, post-operative seroma, saliva, amniotic fluid, tears and wound drainage fluid.