(12) STANDARD PATENT (11) Application No. **AU 2011251854 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Method for the diagnosis/prognosis of colorectal cancer

(51) International Patent Classification(s)
G01N 33/574 (2006.01)

(21) Application No: **2011251854** (22) Date of Filing: **2011.05.13**

(87) WIPO No: **WO11/141612**

(30) Priority Data

(31) Number	(32) Date	(33) Country
P201030708	**2010.05.13**	**ES**

(43) Publication Date: **2011.11.17**
(44) Accepted Journal Date: **2015.12.17**

(71) Applicant(s)
Consejo Superior de Investigaciones Cientificas

(72) Inventor(s)
Casal Alvarez, Jose Ignacio;Barderas Manchado, Rodrigo;Babel, Ingrid Henriette Suzanne

(74) Agent / Attorney
Collison & Co, Gpo Box 2556, Adelaide, SA, 5001

(56) Related Art
KLEIN-SCORY et al., BMC Cancer, 2010, vol. 10, page 70

(12) SOLICITUD INTERNACIONAL PUBLICADA EN VIRTUD DEL TRATADO DE COOPERACIÓN EN MATERIA DE PATENTES (PCT)

(19) Organización Mundial de la Propiedad Intelectual
Oficina internacional

(43) Fecha de publicación internacional
17 de noviembre de 2011 (17.11.2011)

PCT

(10) Número de Publicación Internacional
WO 2011/141612 A3

(51) Clasificación Internacional de Patentes:
G01N 33/574 (2006.01)

(21) Número de la solicitud internacional:
PCT/ES2011/070346

(22) Fecha de presentación internacional:
13 de mayo de 2011 (13.05.2011)

(25) Idioma de presentación: español

(26) Idioma de publicación: español

(30) Datos relativos a la prioridad:
P201030708 13 de mayo de 2010 (13.05.2010) ES

(71) Solicitante *(para todos los Estados designados salvo US)*: **CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS** [ES/ES]; C/ Serrano, 117, E-28006 Madrid (ES).

(72) Inventores; e

(75) Inventores/Solicitantes *(para US solamente)*: **CASAL ÁLVAREZ, José Ignacio** [ES/ES]; Laboratorio de Proteómica Funcional, Centro de Investigaciones Biológicas (CSIC), C/ Ramiro de Maeztu, 9, E-28040 Madrid (ES). **BARDERAS MANCHADO, Rodrigo** [ES/ES]; Laboratorio de Proteómica Funcional, Centro de Investigaciones Biológicas (CSIC), C/ Ramiro de Maeztu, 9, E-28040 Madrid (ES). **BABEL HENZIETT, Ingrid** [FR/ES]; Laboratorio de Proteómica Funcional, Centro de Investigaciones Biológicas (CSIC), C/ Ramiro de Maeztu, 9, E-28040 Madrid (ES).

(74) Mandatario: **ARIAS SANZ, Juan**; Avenida de Burgos, 16D, Edificio EUROMOR, E-28036 Madrid (ES).

(81) Estados designados *(a menos que se indique otra cosa, para toda clase de protección nacional admisible)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Estados designados *(a menos que se indique otra cosa, para toda clase de protección regional admisible)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), euroasiática (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europea (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Publicada:

— *con informe de búsqueda internacional (Art. 21(3))*

— *antes de la expiración del plazo para modificar las reivindicaciones y para ser republicada si se reciben modificaciones (Regla 48.2(h))*

— *con la parte de lista de secuencias de la descripción (Regla 5.2(a))*

(88) Fecha de publicación del informe de búsqueda internacional:
5 de enero de 2012

(54) Title: METHOD FOR THE DIAGNOSIS/PROGNOSIS OF COLORECTAL CANCER

(54) Título : MÉTODO DE DIAGNÓSTICO/PRONÓSTICO DEL CÁNCER COLORRECTAL

(57) Abstract: The invention relates to a method for obtaining useful data for the diagnosis, prognosis or monitoring of the evolution of colorectal cancer (CRC), to a CRC diagnosis method, to a CRC prognosis method and to a kit for carrying out said methods.

(57) Resumen: La presente invención se refiere a un método de obtención de datos útiles para el diagnóstico, el pronóstico o la monitorización de la evolución de cáncer colorrectal (CCR), a un método de diagnóstico de CCR, a un método de pronóstico de CCR y a un kit para llevar a cabo dichos métodos.

WO 2011/141612 A3

METHOD FOR THE DIAGNOSIS/PROGNOSIS OF COLORECTAL CANCER

Field of the Invention

The present invention is comprised within the field of biomedicine. It specifically relates to a method for obtaining useful data for the diagnosis, prognosis or monitoring of colorectal cancer (CRC) progression, to a method for the diagnosis of CRC, to a method for the prognosis of CRC and to a kit for carrying out said methods.

Background of the Invention

Colorectal cancer (CRC) is the second most prevalent cancer in the Western world. The disease develops over decades and involves multiple genetic events. Despite the fact that CRC is one of the best characterized solid tumors from a genetic viewpoint, it continues to be one of the main causes of death in developed countries because of the late diagnosis of patients due to the waiting time that passes to perform certain diagnostic tests, such as colonoscopy.

Today there are few proteins that have been described as effective biomarkers of CRC (carcinoembryonic antigen (CEA), CA19.9 and CA125) (Crawford *et al.* 2003. Journal of surgical oncology 84 (4), 239-248; Duffy *et al.* 2007 Eur J Cancer 43 (9), 1348-1360) and they are not specific enough to perform clinical screenings with a view to detect CRC (Locker *et al.* 25 2006. J Clin Oncol 24 (33), 5313-5327).

Proteomic analyses are being actively used for identifying new biomarkers. In different earlier proteomic studies, differentially expressed proteins in CRC tissue have been identified by means of using antibody microarrays and 2D-DIGE, including isoforms and post-transductional modifications responsible for modifications in signaling pathways (Alfonso *et al.* 2005. Proteomics 5(10), 2602-2611; Kopf *et al.* 2005. Proteomics 5(9), 2412-2416; Madoz-Gurpide *et al.* 2007. Mol Cell Proteomics 6 (12), 2150-2164; Alfonso *et al.* 2008. Journal of Proteome Research 7 (10), 4247-4255). These two

approaches have allowed identifying a broad collection of potential tumor markers of CRC tissue which are currently under research.

However, the implementation of non-invasive and simpler diagnostic methods which allow early detection of CRC must be based on identifying proteins or antibodies detectable in serum or plasma (Hanash *et al.* 2008. Nature 452 (7187), 571-579; Hudson *et al.* 2007. Proceedings of the National Academy of Sciences of the United States of America 104 (44), 17494-17499). The existence of an immune response to cancer in humans has been shown by the presence of autoantibodies in serum from cancer patients. Different human proteins (autoantigens) can thereby be affected before or during the formation of the tumor, being able to produce an immune response once released (Hudson *et al.* 2007. Proceedings of the National Academy of Sciences of the United States of America 104 (44), 17494-17499; Wang *et al.* 2005. The New England Journal of Medicine 353 (12), 1224-1235; Sreekumar *et al.* 2004. J Natl Cancer Inst 96 (11), 834-843). Said autoantibodies can be detected in early stages of the disease and even before the cancer can be detected by means of other techniques, indicating their enormous potential as biomarkers of the disease. These tumor proteins can be affected by point mutations, have anomalous folding, be overexpressed, aberrantly glycosylate, be truncated or undergo aberrant degradation as is the case of p53, HER2, NY-ESO1 or MUC1, respectively (Chen *et al.* 1997. Proceedings of the National Academy of Sciences of the United States of America 94 (5), 1914-1918; Schubert *et al.* 2000. Nature 404 (6779), 770-774; Ulanet *et al.* 2003. Proceedings of the National Academy of Sciences of the United States of America 100 (21), 12361-12366). In fact, tumor-associated autoantigens (TAAs) have previously been characterized in CRC using different approaches (Scanlan *et al.* 1998. International Journal of Cancer 76 (5), 652-658). Several authors have described some

TAA panels as biomarkers of CRC, among which STK4/MST1 protein is found (Tan *et al.* 2009. Journal 276: 6880-6904; Babel *et al.* 2009. Molecular and Cellular Proteomics 8: 2382-2395; WO 2010/136629).

Nevertheless, the diagnostic validity of the autoantibodies associated with CRC identified until now still requires an independent validation for their generalized use in the diagnosis/prognosis of CRC.

Therefore, there is a need for biomarkers which allow the diagnosis of CRC, its classification in the different stages of tumor progression, the prognosis of disease progression, the evaluation of its response to a specific treatment and the detection of the recurrence or the spread of CRC, by means of a simple, effective and non-invasive method.

Brief Description of the Invention

The present invention relates to a method for obtaining useful data for the diagnosis, prognosis or monitoring of colorectal cancer (CRC) progression, to a method for the diagnosis of CRC, to a method for the prognosis of CRC and to a kit for carrying out said methods.

The present invention therefore provides a response to the need for biomarkers which allow the diagnosis of CRC, its classification in the different stages of tumor progression, the prognosis of disease progression, the evaluation of its response to a specific treatment and the detection of the recurrence or the spread of CRC, by means of a simple, effective and non-invasive method.

Blood is usually the optimal biological fluid based on non-invasive methods for massive screening of large populations of patients for diagnostic purposes. On one hand, serum and plasma are easy to obtain, and on the other hand, blood circulation facilitates the contact of the blood with all the tissues of the human body, including contact with tumor tissue and its representative antigens in the case of cancer patients. The release of these tumor associated

antigens probably occurs at a very low concentration in plasma and probably experience proteolysis in a short time period. In contrast, antibodies are very stable molecules which have been used for years in different clinical immunoassays, which facilitates standardizing assays. The use of autoantibodies is also beneficial because the immune system amplifies the response, facilitating identification and quantification.

Phage microarrays (occasionally identified in this description as "phages-peptides") have been used in the present invention to identify autoantibodies present in serum from CRC patients at different stages.

Six phages containing sequences homologous to NHSL1, GRN, MST1, SULF1, SREBF2 and GTF2i proteins were selected. The combination of MST1 and SULF1 recombinant proteins with the 4 other phage sequences allowed predicting the disease with 72% sensitivity and 87% specificity, with an Area Under the Curve (AUC) of 0.83. If the age of the patient is further taken into account, the AUC is 0.91. These markers further allow grouping the results by discriminating not only the sick individuals but also the different stages of the disease. The detection of this panel of autoantibodies in serum is therefore a simple and non-invasive method for the diagnosis/prognosis of CRC.

The present invention provides new biomarkers for the diagnosis, the prognosis, the tracking of CRC and its diagnosis from a blood sample from a subject in which the autoantibodies against an antibody capturing entity (ACE) are detected and/or quantified, wherein said ACE is selected from the group consisting of:

(i) an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody;

(ii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an ACE comprising the amino acid sequence shown in

SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said ACE is not MST1 protein;

(v) an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

(vi) an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and

(vii) any combination of said ACEs (i)-(vi).

Generally, the greater the number of biomarkers used, the more precise the diagnosis/prognosis of CRC.

The present invention therefore provides a specific autoantibody signature of CRC consisting of the presence of new disease specific biomarkers, with potential for diagnosing CRC using sera from patients with greater specificity and sensitivity than the biomarkers described until now.

The present invention further provides a method for the diagnosis of CRC based on determining SULF1 protein levels.

The inventive aspects of the present invention are described in the attached claims.

Brief Description of the Drawings

Figure 1 shows the response of the autoantibodies to the six specific phages. Intensity of the signal of each phage with the CRC sera and the control sera. The results show the data after normalization and on a scale of arbitrary units (a.u.).

Figure 2 shows the competitive analysis between the phage peptides and their respective homologous proteins. A. An ELISA competitive assay was conducted between the phages displaying peptides with homology to SULF1 and MST1 and the respective recombinant proteins. GST was used as a negative

inhibition control. Increasing amounts of the recombinant proteins were pre-incubated with the serum from patients and their respective phage binding was tested by means of ELISA (Vertical bars: light gray, recombinant protein; dark gray, GST). EBNA1 protein was used as a control to demonstrate that inhibition was specific of the protein used as inhibitor and that no error was introduced in the assay (data not shown). B. Schematic location of the peptides with homology to SULF1 and MST1 within the recombinant proteins. The position of the peptide in the protein is highlighted in the figure. The vertical lines correspond to potential phosphorylation sites. The different amino acids between the phage and protein sequence are in lower case letters.

Figure 3 shows the analysis of SULF1, MST1, GTF2i, NHSL1, GRN and SREBF2 expression in cell lines and tissue from CRC patients. A. The gene expression levels of the proteins the peptide of which is displayed in T7 phages was determined by means of meta-analysis using the Oncomine database. The p values are indicated in the figure. Relative gene expression levels were found for NHSL1, SREBF2, GTF2i, SULF1, MST1 and GRN. B. Immunodetection in SULF1 and MST1 membrane in colorectal cancer cell lines compared with control cell lines and paired tissues of CRC belonging to stages I, II and III. An anti-tubulin antibody was used as a loading control. C, Data of the tissue microarray for GTF2i and GRN obtained from the Human Protein Atlas WebPage.

Figure 4 shows the predictive values of MST1 and SULF1 proteins. A. Mean absorbance values obtained with CRC sera and the controls by means of indirect ELISA. The dots represent the individual value for each serum. The error bars represent the standard deviation value. The images of the polyacrylamide gels correspond to the recombinant proteins used in the ELISA assays. B. Both proteins were capable of discriminating control sera from sera from CRC patients with p values < 0.0001 and 0.0006 for MST1 and SULF1, respectively. The AUC

for MST1 was 0.75 (95% CI= 0.647-0.829) with sensitivity and specificity of 60.0% and 82.6%, respectively, using 0.63 as a cutoff point. The AUC was 0.72 (95% CI= 0.617-0.805) for SULF1, with sensitivity and specificity of 68% and 67.4%, respectively, using 0.36 as a cutoff point. C and D, ROC curves for CEA and the combination of 4 phages, 2 proteins and the age variable, resulting in AUC values of 0.81 and 0.89, respectively.

Figure 5 shows the survival analysis using autoantibodies against MST1 and NHSL1 proteins. The Kaplan-Meier survival curves were calculated using an independent set of 95 sera from CRC patients to analyze the effect of the presence of autoantibodies on absolute survival of CRC patients.

Figure 6 shows the validation of the combination of four phages with MST1 and SULF1 proteins in the diagnosis of colorectal cancer. Behavior of the combination of MST1 and NHSL1phages GTF2i, NHSL1, GRN and SREBF2 and MST1 and NHSL1 proteins in the validation test. A. Behavior of CRC samples versus healthy controls. B. Behavior of CRC samples versus reference sera. C. Behavior of healthy sera versus tumor sera. D. Dotplot showing the individual probability of being classified as a CRC patient for each of the subjects with different pathologies. Most of the samples were classified below the probability value of 0.5 (dotted vertical line).

Detailed Description of the Invention

Definitions

The meaning of some terms and expressions as they are used in the present description are indicated below to aid in understanding.

As it is used herein, the term "antibody" refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules containing an antigen fixing site binding specifically (immunoreacting) with an antigen, such as a protein for example. There are 5

isotypes or main classes of immunoglobulins: immunoglobulin M (IgM), immunoglobulin D (IgD), immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin E (IgE).

As it is used herein, the term "autoantibody" applies to an antibody reacting against an antigen present in the subject's organism, even if the reaction occurs only *in vitro*, and whether or not it causes pathological effects *in vivo*.

As it is used herein, the term "colorectal cancer" or "CRC", also called colon cancer, includes any type of neoplasias of the colon, rectum and appendix, as well as any histological subtype typically occurring in colon cancer, e.g., transitional carcinoma cells, squamous carcinoma and adenocarcinoma cells, any clinical subtype, e.g., surface, invasive muscle or metastatic disease cancer, or any TNM stage including T0-T4, N0-N2 and M0-M1 tumors. Patients can be classified in different groups with respect to the stage of the tumor. The classification of colon cancer is an estimate of the penetration of a particular cancer. It is carried out for investigational purposes, diagnostic purposes and for determining the best method of treatment. The system for the classification of colorectal cancer depends on the extent of local invasion, on the degree of lymphatic nodes involved and on if distant metastasis exists. The most common classification system is the TNM (for tumors/nodes/metastasis) system, of the American Joint Committee on Cancer (AJCC). The TNM system assigns a number based on three categories. "T" indicates the degree of invasion of the intestinal wall, "N" the degree of involvement of lymphatic nodes and "M" the degree of metastasis. The broadest stage of cancer is usually mentioned as a number I, II, III, IV derived from the TNM value grouped by the prognosis, a higher number indicates a more advanced cancer and a worse prognosis. Details of the classification are indicated in Table 1.

Table 1

TNM system for the classification of CRC

AJCC Stage	**TNM Stage**	**Criteria of TNM stages for CRC**
Stage 0	Tis N0 M0	Tis: The tumor confined to the mucosa; cancer-*in-situ*
Stage I	T1 N0 M0	T1: The tumor invades the mucosa
Stage I	T2 N0 M0	T2: The tumor invades the actual muscles
Stage II-A	T3 N0 M0	T3: The tumor invades the subserosal layer or beyond (other organs not involved)
Stage II-B	T4 N0 M0	T4: The tumor invades adjacent organs or perforates the visceral peritoneum
Stage III-A	T1-2 N1 M0	N1: Metastasis of 1 to 3 regional lymphatic nodes. T1 or T2.
Stage III-B	T3-4 N1 M0	N1: Metastasis of 1 to 3 regional lymphatic nodes. T3 or T4.
Stage III-C	any T, N2 M0	N2: Metastasis of 4 or more regional lymphatic nodes. Any T.
Stage IV	any T, any N, M1	M1: Presence of distant metastasis. Any T, any N.

As it is used herein, the term "antibody capturing entity" (ACE) refers to a macromolecular entity binding specifically to an antibody (or autoantibody). In a particular embodiment, said ACE comprises a peptide or a protein binding specifically to an antibody (or autoantibody). Said peptide can either be immobilized on a support or exposed on the phage surface. In a preferred particular embodiment, said ACE is a peptide, a protein or a phage on the surface of which said peptide or said protein is exposed. If desired, said ACE can be immobilized on a solid support.

As it is used herein, the term "sample" refers but is

not limited to a tissues and/or biological fluids from a subject, obtained by means of any method known by a person skilled in the art which serves for carrying out any of the methods provided by the present invention; i.e., said biological sample must be a sample susceptible to containing antibodies, e.g., autoantibodies against SULF1, MST1 proteins, etc., or against ACEs comprising the amino acid sequences shown in SEQ ID NO: 1-6, or variants thereof containing epitopes recognizable by autoantibodies, etc. By way of non-limiting illustration, said biological sample can be a blood, urine, saliva, serum, or plasma sample, a buccal or buccal-pharyngeal swab, a surgical specimen, a specimen obtained from a biopsy or autopsy, etc. In a particular embodiment, said sample is a biological fluid. In a preferred embodiment for the detection of autoantibodies, the sample from the subject is blood, plasma or blood serum. In another particular embodiment, said sample is a tissue sample. In a preferred embodiment for the quantification of SULF1 protein level, said sample is preferably a colorectal tissue sample or tumor tissue sample, etc., obtained by conventional methods, for example, by means of a biopsy, resection, etc.

As it is used herein, the term "SULF1 protein" includes SULF1 protein and variants thereof; in a particular embodiment, said protein is the protein with NCBI database accession number (May 1, 2011 version) EAW86954.1 and its amino acid sequence is SEQ ID NO: 10.

As it is used herein, the term "NHSL1 protein" includes NHSL1 protein and variants thereof; in a particular embodiment, said protein is the protein with NCBI database accession number (May 1, 2011 version) NP_001137532.1 and its amino acid sequence is SEQ ID NO: 7.

As it is used herein, the term "GRN protein" includes GRN protein and variants thereof; in a particular embodiment, said protein is the protein with NCBI database accession number (May 1, 2011 version) 2JYT and its amino acid sequence

is SEQ ID NO: 8.

As it is used herein, the term "MST1 protein" includes MST1 protein and variants thereof; in a particular embodiment, said protein is the protein with NCBI database accession number (May 1, 2011 version) AAA83254.1 and its amino acid sequence is SEQ ID NO: 9.

As it is used herein, the term "SREBF2 protein" includes SREBF2 protein and variants thereof; in a particular embodiment, said protein is the protein with NCBI database accession number (May 1, 2011 version) NP_004590.2 and its amino acid sequence is SEQ ID NO: 11.

As it is used herein, the term "GTF2i protein" includes GTF2i protein and variants thereof; in a particular embodiment, said protein is the protein with NCBI database accession number (May 1, 2011 version) NP_001157108.1 and its amino acid sequence is SEQ ID NO: 12.

As it is used herein, the term "variant" refers to a protein or peptide substantially homologous to another protein or peptide, for example, to the peptides the amino acid sequences of which are shown in SEQ ID NO:1 to 6, to SULF1, MST1, NHSL1, GRN, SREBF2 or GTF2i proteins, etc. A variant generally includes additions, deletions or substitutions of one or more amino acids. The person skilled in the art will understand that the amino acid sequences referred to in this description can be chemically modified, for example, by means of physiologically relevant chemical modifications, such as phosphorylations, acetylations, glycosylations or methylations. According to the present invention, said variants are recognized by autoantibodies against the protein or peptide in question. Variants of said peptides or proteins include peptides or proteins showing at least 25%, at least 40%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity with respect to certain amino acid sequences of peptides or proteins. The degree of identity

between two amino acid sequences can be determined by conventional methods, for example, by means of standard sequence alignment algorithms known in the state of the art, such as BLAST for example (Altschul S.F. *et al.* Basic local alignment search tool. J Mol Biol. 1990; 215(3):403-10).

Method for detecting autoantibodies in subjects with CRC

In a first aspect, the invention relates to a method for detecting an autoantibody in a subject suspected of having colorectal cancer (CRC), hereinafter first method of the invention, comprising:

a) contacting a sample from said subject with an antibody capturing entity (ACE), wherein said ACE is selected from the group consisting of:

(i) an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody;

(ii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said ACE is not MST1 protein;

(v) an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

(vi) an ACE comprising the amino acid sequence

shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and

(vii) any combination of said ACEs (i)-(vi); and

b) detecting the formation of an autoantibody-ACE complex,

wherein the detection of said autoantibody-ACE complex is indicative of the presence of said autoantibody in said subject.

The sample will generally be a biological sample susceptible to containing antibodies from a subject, and it can be obtained by conventional methods known by those of average skill in the art, depending on the nature of the sample. In a particular embodiment, said biological sample is a blood, serum or plasma sample which can be obtained by any conventional method, for example, by means of a blood extraction, etc. Blood is usually the optimal biological fluid to be used in non-invasive methods for massive screening of large populations of subjects for diagnostic purposes. On one hand, serum and plasma are easy to obtain, and on the other hand, blood circulation facilitates the contact of the blood with all the tissues of the human body, including contact with tumor tissue and its representative antigens in the case of cancer patients.

The first method of the invention comprises contacting a sample from a subject suspected of having CRC with an ACE selected from ACEs (i) to (vi), indicated in Table 2, and their combinations [step a)], under conditions allowing the formation of an autoantibody-ACE complex.

Table 2

ACEs

(i) ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an

autoantibody.

(ii) ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody.

(iii) ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody.

(iv) ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody; wherein said ACE is not MST1 protein.

(v) ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody.

(vi) ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody.

The suitable conditions for the formation of the autoantibody-ACE complex to take place are known by the persons skilled in the art. If the sample contains autoantibodies against said ACEs, then the corresponding autoantibody-ACE complex will be formed; otherwise, said complex will not be formed.

Although said ACEs could be together in the same medium, in practice it is advantageous for said ACEs to be separated from one another. The ACEs can be in solution or suspension in a suitable medium, or can alternatively be deposited or supported on a support [e.g., a microtiter plate, beads (magnetic or non-magnetic), columns, matrices, membranes, etc.] These materials can be used in the suitable forms, such

as films, sheets, plates, etc., or they can be used to coat inert carriers (e.g., paper, glass, plastic films, etc.). In a particular embodiment, the sample to be analyzed is contacted with said ACEs, separated from one another, and deposited on a suitable support.

The detection of said autoantibodies against the ACEs mentioned can be carried out by conventional methods known by those of average skill in the art. In a particular embodiment, the detection of said autoantibodies is carried out by means of immunoassay; illustrative, non-limiting examples of immunoassays known in the state of the art include immunoblot, Enzyme-linked Immunosorbent Assay (ELISA), linear immunoassay (LIA), radioimmunoassay (RIA), immunofluorescence (IF), immunohistochemistry (IHC), protein microarrays, phage microarrays, etc. The person skilled in the art will also understand that other methods based for example on electrophoretic or chromatographic techniques can be used for detecting said autoantibodies.

In a particular embodiment, the detection of autoantibodies against one or more ACEs is done by means of an ELISA. The ELISA technique is based on the premise that an immunoreagent (e.g., an antigen or an antibody) is immobilized on a solid support, and then that system is contacted with a fluid phase containing the complementary reagent which can be bound to a marker compound. There are different types of ELISA, for example, direct ELISA, indirect ELISA or sandwich ELISA.

The detection of autoantibodies against one or more ACE/ACEs by means of ELISA, for example by means of indirect ELISA, generally comprises the following steps: (a) covering a solid support with one or more ACEs, preferably separated from one another; (b) incubating the covered support of step (a) with a sample, such as a biological sample from the subject to be studied, under conditions allowing the formation of an autoantibody-ACE complex; and (c) adding a secondary antibody,

which recognizes the autoantibody against the ACE/ACEs, conjugated or bound to a marker compound.

In another particular embodiment, the detection of autoantibodies against one or more ACEs is done by means of a protein microarray. A protein microarray consists of a collection of proteins immobilized on a solid support in a regular and pre-established arrangement. There are several important factors to be taken into account in the design of protein microarrays, among which, for example, the nature of the support on which the proteins (or suitable fragments thereof) are immobilized, the protein immobilization technique, the format of the microarray, the capturing agent used or the method of detection to be used are found. Different formats, supports and techniques which can be used for carrying out this inventive aspect are known in the state of the art.

The detection of autoantibodies against one or more ACEs by means of a protein microarray generally comprises the following steps: (a) covering a solid support with said ACE/ACEs, preferably separated from one another; (b) incubating the covered support of step (a) with a sample, such as a biological sample from the subject to be studied, under conditions allowing the formation of an immunocomplex of the autoantibody against the ACE/ACEs present in said sample with the corresponding antigenic determinants present in said ACEs; and (c) adding a secondary antibody, which recognizes the autoantibody against the ACE/ACEs, conjugated or bound to a marker compound.

In another particular embodiment, the detection of autoantibodies against one or more ACEs is done by means of a phage microarray. A phage microarray consists of a collection of peptides exposed on the surface of phages. In a particular embodiment, said peptides are fused to T7 phage capsid protein 10B. Said phages are immobilized on a solid support in a regular and pre-established arrangement. There are several

important factors to be taken into account in the design of phage microarrays such as, for example, the nature of the support on which the phages are immobilized, the immobilization technique, the format of the microarray or the method of detection to be used. Different formats, supports and techniques that can be used for carrying out this preferred aspect of the method of the invention are known in the state of the art.

The detection of autoantibodies against one or more ACE/ACEs by means of a phage microarray generally comprises the following steps: (a) covering a solid support with a phage lysate, for example, a T7 phage lysate, having a peptide or an amino acid sequence susceptible to being recognized by an autoantibody exposed on the phage surface; (b) incubating the covered support of step (a) with a sample, such as a biological sample from the subject to be studied, under conditions allowing the formation of an autoantibody-ACE complex; and (c) adding a secondary antibody, which recognizes the autoantibody against the ACE/ACEs, conjugated or bound to a marker compound. In a specific embodiment, said phage microarray comprises a phage selected from the group consisting of:

(i) a phage comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface;

(ii) a phage comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface;

(iii) a phage comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody, wherein

said amino acid sequence is exposed on the phage surface;

(iv) a phage comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface;

(v) a phage comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface;

(vi) a phage comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface; and

(vii) any of the combinations of (i) to (vi).

The marker bound to the secondary antibody referred to in these techniques is a compound capable of giving rise to a chromogenic, fluorogenic, radioactive and/or chemiluminescent signal which allows the detection, identification and, optionally, quantification of the amount of the autoantibody versus the ACE/ACEs present in the analyzed sample. In a particular embodiment, said marker compound is selected from the group consisting of radioisotopes, enzymes, fluorophores or any molecule susceptible to being conjugated with another molecule or detected and/or quantified directly. This marker compound can bind to the autoantibody directly, or through another compound. Illustrative non-limiting examples of said marker compounds binding directly to the autoantibody include enzymes, such as alkaline phosphatase, peroxidase, etc., radioactive isotopes, such as ^{32}P, ^{35}S, etc., fluorochromes, such as fluorescein, etc., or metal particles, for their direct detection by means of colorimetry, auto-radiography,

fluorometry, or metallography, respectively.

The detection of the autoantibodies can be carried out by applying a single technique or it can be carried out by applying a combination of two or more techniques; by way of illustration, some autoantibodies can be detected by means of an ELISA and others by means of a protein microarray, or some by means of an ELISA and others by means of a phage microarray, or some by means of a protein microarray and others by means of a phage microarray, etc.

In a particular embodiment, the sample to be analyzed is contacted with a single ACE selected from the group of ACEs (i)-(vi) shown in Table 2, and their combinations, under conditions allowing the formation of an autoantibody-ACE complex for the purpose of identifying autoantibodies against said ACE. In another particular embodiment, said biological sample is contacted with two or more of said ACEs susceptible to being recognized by said autoantibodies, separated from one another, optionally deposited on a suitable support, for the purpose of identifying autoantibodies against said ACEs.

In a particular embodiment, the first method of the invention comprises the detection of an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody, and furthermore the detection of an autoantibody selected from the group consisting of: (i) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody; (ii) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody; (iii) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody; (iv) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a

variant thereof containing an epitope recognizable by an autoantibody; (v) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and (vi) any combination of autoantibodies (i) to (v). In a more specific form of said particular embodiment, said first method of the invention comprises the detection of an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody, and furthermore the detection of any 1, 2, 3, 4, or 5 of said autoantibodies (i) to (v) previously indicated.

In a specific embodiment, said ACE is SULF1 protein or a variant or fragment thereof containing an epitope recognizable by an autoantibody, whereas in another specific embodiment, said ACE is a phage comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface.

In another particular embodiment, the first method of the invention comprises the detection of an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody, and furthermore the detection of an autoantibody selected from the group consisting of: (i') an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody; (ii') an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody; (iii') an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody; (iv') an autoantibody against an ACE comprising the amino acid sequence shown in SEQ

ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody; (v') an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody; and (vi') any combination of autoantibodies (i') to (v'). In a more specific form, said first method of the invention comprises the detection of an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody, and furthermore the detection of any 1, 2, 3, 4, or 5 of said autoantibodies (i') to (v') previously indicated.

Step b) of the method for detecting autoantibodies of the invention comprises detecting the formation of an autoantibody-ACE complex. This step can be carried out by conventional methods known by those of average skill in the art, for the detection of the formation of antibody-antigen complexes (in this case, autoantibody-ACE).

In a particular embodiment, by way of non-limiting illustration, for the detection of said complex, a conjugate comprising an antibody recognizing the autoantibody and a marker (labeled secondary antibody) can be added under conditions allowing the formation of an (autoantibody-ACE)-antibody/marker complex and detecting the formation of said complex. If the biological sample contains autoantibodies against one or more of said ACEs, then the autoantibody-ACE complex will have been previously formed, whereby when said complex is contacted with said conjugate comprising the antibody and the marker in suitable conditions, (autoantibody-ACE)-antibody/marker complex is formed, which will be viewed by means of the suitable technique depending on the marker used, as mentioned below; whereas, otherwise, i.e., when the biological sample does not contain autoantibodies against said ACE/ACEs then said (autoantibody-ACE)-antibody/marker complex will not be formed. The suitable conditions for the formation

of this latter complex to take place are known by the persons skilled in the art.

Virtually any indicator reagent which allows detecting said (autoantibody-ACE)-antibody/marker complex can be used in putting the present invention into practice. By way of non-limiting illustration, said marker can be an enzyme catalyzing a detectable reaction (e.g., peroxidase, glycosidase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, β-galactosidase, β-glucosidase, β-glucuronidase, etc.), a compound generating a signal when it forms part of said complex (e.g., a fluorescent compound or fluorophore, such as fluorescein, rhodamine, etc.; a (chemi)luminescent compound, such as a dioxetane, an acridinium, a phenanthridinium, ruthenium, luminol, etc.), a radioactive element (e.g., sulfur, iodine, etc.), etc. In a particular embodiment, said marker is a peroxidase. The selection of a particular marker is not critical, provided that it is capable of producing a signal by itself or together with one or more additional substances. The (autoantibody-ACE)-antibody/marker complex formed can thus be detected or displayed by any suitable technique, depending on the chosen marker, known by those of average skill in the art, using the suitable devices, for example, by means of techniques based on colorimetric, fluorometric, (chemi)luminescent, radioactive methods, etc., all of them known by those of average skill in the art.

The conjugate comprising said antibody which recognizes said autoantibody and said marker can be obtained by conventional methods known by those of average skill in the art.

By way of illustration, when the marker is an enzyme, the detection of the complex in question can be carried out by contacting said complex with a suitable substrate and, optionally, with suitable enzymatic amplification agents and/or activators. Illustrative non-limiting examples of said

substrates include:

- For alkaline phosphatase:

  Chromogenic: substrates based on p-nitrophenyl phosphate (p-NPP), 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NPT), etc.

  Fluorogenic: 4-methylumbeliphenyl phosphate (4-MUP), 2-(5´-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (CPPCQ), 3,6-fluorescein-diphosphate (3,6-FDP), etc.

- For peroxidases:

  Chromogenic: substrates based on 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic) (ABTS) acid, o-phenylenediamine (OPT), 3,3',5,5'-tetramethylbenzidine (TMB), o-dianisidine, 5-aminosalicylic acid, 3-dimethylaminobenzoic (DMAB) acid and 3-methyl-2-benzothiazolinehydrazone (MBTH), 3-amino-9-ethylcarbazole (AEC) and 3,3'-diaminobenzidine (DAB) tetrachloride, etc.

  Fluorogenic: 4-hydroxy-3-methoxyphenylacetic acid, reduced phenoxazines and reduced benzothiazines, including the reagent Amplex® Red, Amplex UltraRed, reduced dihydroxanthenes, etc.

- For glycosidases:

  Chromogenic: substrates based on o-nitrophenyl-β-D-galactoside (o-NPG), p-nitrophenyl-β-D-galactoside and 4-methylumbeliphenyl-β-D-galactoside (MUG) for β-D-galactosidase, etc.

  Fluorogenic: resorufin β-D-galactopyranoside, fluorescein digalactoside (FDG), fluorescein diglucuronide, 4-methylumbelliferyl beta-D-galactopyranoside, carboxyumbelliferyl beta-D-galactopyranoside, fluorinated coumarin beta-D-galactopyranosides, etc.

Therefore, by means of putting the first method of the invention into practice, it is possible to detect and obtain

autoantibodies against the ACEs indicated in Table 2. Additionally, the level or amount of said autoantibodies against said ACEs present in the sample under study could be determined (quantified) if desired because the signal generated by some markers (e.g., enzymes, etc.) is proportional to the amount of autoantibody present in said sample.

Optionally, if desired, the autoantibody-ACE complex can be isolated by means of conventional techniques, for example, by means of using immunoprecipitation techniques, etc., and the sequence of the autoantibody binding to the ACE can be subsequently sequenced by means of conventional proteomic methods described in the art, such as the determination of the peptide fingerprint or MS/MS analysis (Vikas Dhingraa, *et al.* 2005. International Journal of Pharmaceutics 299 (1-2):1-18; Hanash SM *et al.* Nature. 2008 Apr 3;452(7187):571-9).

According to the first method of the invention, the detection of the autoantibody-ACE complex is indicative of the presence of the corresponding specific autoantibody (or autoantibodies) against said ACE/ACEs in the analyzed sample and, therefore, in the analyzed subject.

In a particular embodiment, the formation of said autoantibody-ACE complex in said sample can be correlated with a diagnosis of CRC in the subject the analyzed sample is from, or with the prognosis of said disease, or with tracking said disease progression. In the sense used in this description, the term "correlate" refers to comparing the presence or amount of the indicator in a subject (e.g., a subject suspected of having CRC) with its presence or amount in subjects having said disease (CRC), or predisposed to develop it, or in subjects free of said disease.

As it is used herein, the term "diagnosis" generally refers to the process whereby a disease, nosological entity, syndrome, or any disease-health condition is identified. Particularly, the term "diagnosis of colorectal cancer (or

CRC)" refers to the capacity to identify or detect the presence of CRC; this detection, as it is understood by a person skilled in the art, does not claim to be correct in 100% of the analyzed samples. However, it requires that a statistically significant amount of the analyzed samples are classified correctly. The amount that is statistically significant can be established by a person skilled in the art by using different statistical tools; illustrative, non-limiting examples of said statistical tools include determining confidence intervals, determining the p-value, the Student's t-test or Fisher's discriminant functions, etc. (see, for example, Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York 1983). The confidence intervals are preferably at least 90%, at least 95%, at least 97%, at least 98% or at least 99%. The p-value is preferably less than 0.1, less than 0.05, less than 0.01, less than 0.005 or less than 0.0001. The teachings of the present invention advantageously allow correctly detecting CRC in at least 50%, preferably in at least 60%, more preferably in at least 70%, even more preferably in at least 80%, or still even more preferably in at least 90% of the subjects of a specific group or population analyzed.

In another particular embodiment, the first method of the invention comprises comparing the immunoreactivity of the analyzed sample with the immunoreactivity of a second sample from the same subject in a later time period. It is therefore possible to either evaluate disease progression or to evaluate the efficacy of the treatment if said second sample has been obtained after the subject has been treated for CRC. In the sense used in this description, the term "immunoreactivity" refers to the presence or level of binding of an antibody or antibodies in a sample to one or more target antigens, for example, the ACEs of Table 2. An "immunoreactivity pattern" refers to a binding profile of antibodies in a sample (autoantibodies) to a plurality of target antigens (e.g., the

ACEs of Table 2).

In another embodiment, the first method of the invention further comprises analyzing the presence of one or more additional markers of CRC, for example, CEA or autoantibodies against Pim1, SRC, MAPKAPK3, FGFR4, STK4 and/or ACVR2B proteins.

Method for detecting autoantibodies

In another aspect, the invention relates to a method for detecting an autoantibody in a sample, hereinafter second method of the invention, comprising:

(a) contacting a sample with an antibody capturing entity (ACE), wherein said ACE is selected from the group consisting of:

(i) an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody;

(ii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said ACE is not MST1 protein;

(v) an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

(vi) an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and

(vii) any combination of said ACEs (i)-(vi); and

(b) detecting the formation of an autoantibody-ACE complex, wherein the detection of said autoantibody-ACE complex is

indicative of the presence of said autoantibody in said sample.

Generally, the characteristics of said ACEs referred to in the second method of the invention are the same as the characteristics of the ACEs referred to in the first method of the invention. The techniques for detecting the autoantibodies according to the second method of the invention are the same as those mentioned in relation to the first method of the invention, so they are herein incorporated by reference.

The particular embodiments of the first method of the invention, as well as the definitions of the terms used also apply to the second method of the invention, so they are incorporated in this second method of the invention by reference.

This second method of the invention allows correlating the results obtained with those pathologies in which immune responses with the subsequent production of autoantibodies are generated. Illustrative, non-limiting examples of said pathologies include some types of cancer, e.g., CRC, hepatocellular carcinoma (Imai, H *et al*. Intervirology 35:73-85), breast cancer, prostate cancer (Wang X et al. N Engl J Med. 2005; 353(12):1224-35), lung cancer, etc. and autoimmune diseases.

In a particular embodiment, the second method of the invention comprises the detection of an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody, and furthermore the detection of an autoantibody selected from the group consisting of:

(i) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody;

(ii) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or

a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

(v) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and

(vi) any combination of autoantibodies (i) to (v).

In another particular embodiment, the second method of the invention comprises the detection of an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody, and furthermore the detection of an autoantibody selected from the group consisting of:

(i) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody;

(ii) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant

thereof containing an epitope recognizable by an autoantibody;

(v) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody; and

(vi) any combination of autoantibodies (i) to (v).

Antibody capturing entity (ACE)

In another aspect, the invention relates to an antibody capturing entity (ACE), hereinafter ACE of the invention, selected from the group consisting of:

(i) an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said ACE is not SULF1 protein;

(ii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said ACE is not GRN protein;

(iii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said ACE is not GTF2i protein; and

(iv) an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said ACE is not MST1 protein;

(v) an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said ACE is not SREBF2 protein;

(vi) an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said ACE is not NHSL1 protein; and

(vii) any combination of ACEs (i) to (vi).

As previously defined, an ACE is a macromolecular entity, for example, a peptide, a protein or a phage, binding specifically to an antibody (or autoantibody). In a particular embodiment, said ACE comprises a peptide or a protein binding specifically to an antibody (or autoantibody). Said peptide can either be immobilized on a support or exposed on the phage surface. In a preferred particular embodiment, said ACE is a peptide, a protein or a phage on the surface of which said peptide or said protein is exposed. Said ACE can be immobilized on a solid support if desired.

In a particular embodiment, the ACE of the invention is selected from the group consisting of:

(i) a phage comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface;

(ii) a phage comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface;

(iii) a phage comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface;

(iv) a phage comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface;

(v) a phage comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing

an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface;

(vi) a phage comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody, wherein said amino acid sequence is exposed on the phage surface; and

(vii) any of the combinations of (i) to (vi).

Said phages can be obtained by conventional methods known by those of average skill in the art, and more specifically by means of the process described in Example 1.

The ACEs provided by this invention can be used in the detection of antibodies or autoantibodies against said ACEs in a sample, particularly against the amino acid sequences identified as SEQ ID NOs: 1-6 present in said ACEs, and the presence of said autoantibodies in said sample can be correlated with the diagnosis, prognosis, monitoring the progression, or efficacy of the treatment, of a disease, so that said autoantibodies are markers, for example, of CRC.

Composition of the invention

In another aspect, the invention relates to a composition, hereinafter composition 1 of the invention, comprising an ACE of the invention. As previously indicated, in a particular embodiment, said ACE can be a peptide, a protein or a phage.

In a particular embodiment, composition 1 of the invention comprises at least one ACE of the invention. In another particular embodiment, composition 1 of the invention comprises at least 2 ACEs of the invention, for example, 2, 3, 4, 5 or even the 6 ACEs of the invention.

In another particular embodiment, composition 1 of the invention comprises an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody, and furthermore at

least one ACE selected from the group consisting of:

(i) an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody;

(ii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

(v) an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and

(vi) any combination of ACEs (i) to (v).

In another particular embodiment, composition 1 of the invention comprises an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody, and furthermore at least one ACE selected from the group consisting of:

(i) an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody;

(ii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

(v) an ACE comprising the amino acid sequence shown in

SEQ ID NO: 6 a variant thereof containing an epitope recognizable by an autoantibody; and

(vi) any combination of ACEs (i) to (v).

In another particular embodiment, composition 1 of the invention comprises at least one ACE of the invention and at least one protein selected from the group consisting of SULF1 protein or a variant thereof, MST1 protein or a variant thereof, and their combinations. The person skilled in the art will note that it is possible to use SULF1 or MST1 proteins of different species; nevertheless, in a preferred embodiment, composition 1 of the invention includes SULF1 or MST1 proteins of a human origin, such as human SULF1 protein, with NCBI database accession number (May 1, 2011 version) EAW86954.1 and its amino acid sequence is SEQ ID NO: 10, and human MST1 protein with NCBI database accession number (May 1, 2011 version) AAA83254.1 and its amino acid sequence is SEQ ID NO: 9. The term "variant" has already been defined above in the "Definitions" section.

In another particular embodiment, composition 1 of the invention comprises:

a) an ACE selected from the group consisting of:

(i) an ACE comprising the amino acid sequence shown in SEQ ID NO: 4 or a variant thereof containing an epitope recognizable by an autoantibody,

(ii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof

containing an epitope recognizable by an autoantibody;

(v) an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

(vi) an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and

(vii) any combination of ACEs (i) to (vi); and

b) a protein selected from the group consisting of SULF1 protein or a variant thereof, MST1 protein or a variant thereof, and their combinations.

In a preferred embodiment, said composition 1 of the invention comprises:

a) an ACE selected from the group consisting of:

(i) an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody,

(ii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and

(v) any combination of ACEs (i) to (iv); and

b) a protein selected from the group consisting of SULF1

protein or a variant thereof, MST1 protein or a variant thereof, and their combinations.

This composition 1 of the invention has provided good results in the diagnosis of CRC [Example 4].

In another aspect, the invention relates to a composition, hereinafter composition 2 of the invention, comprising SULF1 protein or a variant thereof and MST1 protein or a variant thereof. The characteristics of said SULF1 and MST1 proteins, and of their variants, have been mentioned previously.

In a particular embodiment, composition 2 of the invention further comprises at least one ACE of the invention. In another particular embodiment, composition 2 of the invention comprises at least 2 ACEs of the invention.

In another particular embodiment, said composition 2 of the invention comprises:

a) SULF1 protein or a variant thereof;

b) MST1 protein or a variant thereof; and

c) an ACE selected from the group consisting of:

(i) an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody,

(ii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and

(v) any combination of ACEs (i) to (iv).

In a particular embodiment, both composition 1 of the invention and composition 2 of the invention are supported on a solid support.

Kit of the invention and applications

In another aspect, the invention relates to a kit, hereinafter kit 1 of the invention, comprising a composition of the invention. In a particular embodiment, said composition of the invention is composition 1 of the invention. In another particular embodiment, said composition of the invention is composition 2 of the invention.

In another aspect, the invention relates to the use of kit 1 of the invention for:

- detecting an antibody in a sample,
- detecting an autoantibody in a subject suspected of having colorectal cancer (CRC),
- diagnosing whether a subject has CRC,
- determining the risk of a subject developing CRC,
- monitoring CRC progression in a subject,
- evaluating the efficacy of a treatment against CRC, or
- predicting survival of a subject who has CRC.

For said applications, kit 1 of the invention will include the reagents necessary for detecting autoantibodies against at least one ACE of Table 2.

In a particular embodiment, kit 1 of the invention comprises an ACE of the invention. In another particular embodiment, the kit of the invention comprises composition 1 of the invention. In another particular embodiment, the kit of the invention comprises composition 2 of the invention. Therefore, in a particular embodiment, kit 1 of the invention comprises a protein selected from the group consisting of SULF1 protein or a variant thereof, MST1 protein or a variant thereof, and their combinations.

In another aspect, the invention relates to a kit,

hereinafter kit 2 of the invention, comprising a reagent for detecting SULF1 protein or a variant thereof. In a particular embodiment, said kit 2 of the invention further comprises a reagent for detecting MST1 protein or a variant thereof. In another particular embodiment, kit 2 of the invention comprises an ACE selected from the group of ACEs mentioned in Table 2.

Kit 2 of the invention can be used in the same applications as kit 1 of the invention.

Both kit 1 of the invention and kit 2 of the invention can further contain all those reagents necessary for detecting the amount of autoantibodies against the ACEs defined previously, or against the SULF1, MST1 proteins or their variants, such as but not being limited to the following for example

- secondary antibodies labeled with a marker specifically recognizing the autoantibody-ACE complexes;
- substrates for the markers present in said labeled secondary antibodies; and
- positive and/or negative controls.

Likewise, said kits 1 and 2 of the invention can further include, without any type of limitation, buffers, agents for preventing contamination, protein degradation inhibitors, etc. In addition, the kit of the invention can include all the supports and containers necessary for being put into practice and for optimization. Preferably, the kit further comprises instructions for use.

Method for the diagnosis of CRC

The authors of the present invention have additionally found that the overexpression of SULF1 protein is correlated with CRC, as shown in Figure 3.

Therefore, in another aspect, the invention relates to a method for diagnosing whether a subject suffers colorectal cancer (CRC), hereinafter third method of the invention, comprising determining SULF1 protein level in a sample from

said subject, wherein if said SULF1 protein level is greater than the SULF1 protein level of a reference sample, it is indicative of the subject having CRC.

For putting the third method of the invention into practice, the sample can preferably be a tissue sample, such as a colon or tumor tissue sample.

The term "diagnosis" has already been defined above.

The methods for determining protein level (concentration) are well-known by a person skilled in the art and include a number of alternatives. Virtually any method which allows determining (quantifying) SULF1 protein level can be used in putting the third method of the invention into practice.

In a particular embodiment, SULF1 protein level is quantified by means of a conventional method allowing detecting and quantifying said protein in a sample to be studied, such as a sample from a subject. By way of non-limiting illustration, said SULF1 protein level can be determined by means of an immunoassay, for example, ELISA, etc., by means of nuclear magnetic resonance (NMR) or by means of any other suitable technique known in the state of the art. In a preferred embodiment, protein level is determined by means of an immunoassay. In a preferred particular embodiment, said immunoassay is an immunoblot (Western blot or membrane immunodetection). To that end, briefly, a protein extract is obtained from a biological sample isolated from a subject and the protein is separated by electrophoresis in a support medium capable of retaining it. Once the proteins are separated, they are transferred to a different support or membrane where they can be detected by using specific antibodies recognizing the protein in question (SULF1). Said membrane is hybridized with a first specific antibody (or primary antibody) recognizing SULF1 protein. Then the membrane is hybridized with a second antibody (or secondary antibody) specifically recognizing the primary antibody and conjugated

or bound with a marker compound. In an alternative embodiment, the antibody recognizing a SULF1 protein is conjugated or bound to a marker compound, and the use of a secondary antibody is not necessary. Different formats, supports and techniques that can be used for performing this preferred aspect of the third method of the invention are known.

In another preferred particular embodiment, the immunoassay comprises an immunohistochemical assay. Immunohistochemistry techniques allow the identification of characteristic antigenic determinants in tissue and cytology samples. Analysis by means of immunohistochemistry (IHC) is performed on tissue sections, either frozen or included in paraffin, from a biological sample isolated from a subject. These sections are hybridized with a specific antibody or primary antibody recognizing specific antibodies recognizing a SULF1 protein. The sections are then hybridized with a secondary antibody capable of specifically recognizing the primary antibody and is conjugated or bound to a marker compound. In an alternative embodiment, the antibody recognizing SULF1 protein is conjugated or bound to a marker compound, and the use of a secondary antibody is not necessary.

By way of non-limiting illustration, "SULF1 protein level" refers but is not limited to a quantifiable, semiquantifiable, or relative amount of said SULF1 protein, as well as to any other value or parameter related to said protein or which can be derived therefrom. Said values or parameters comprise signal intensity values obtained from any of the physical or chemical properties of said protein obtained either by means of direct measurement, e.g., intensity values of mass spectroscopy, nuclear magnetic resonance, etc., or by means of indirect measurement, e.g., by means of any of the systems of measurement described herein, for example, by means of the measurement obtained from a secondary component or a biological measurement system (e.g.,

the measurement of cell responses, ligands, "tags" or enzymatic reaction products). The SULF1 protein level determined in a sample, such as a biological sample from the subject subjected to study, is said to be "greater" than the reference level of said SULF1 protein when, according to the invention, the level of said protein in the biological sample to be analyzed is at least 1.5 times, 5 times, 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times, 100 times or even more, with respect to the reference level of said protein.

As it is used herein, the term "reference level" generally refers to the level of a product, for example, SULF1 protein, present in control subjects. In a particular embodiment, said control subjects are subjects who do not suffer a specific disease (e.g., CRC), whereas in another particular embodiment, said control subject is the actual subject under study, which is particularly useful for evaluating the tracking of a disease (e.g., CRC) or for evaluating the efficacy of a treatment for said disease (e.g., CRC), etc., for which the reference level of a given product can be the level of said product determined in a sample from the same subject under study but taken days, weeks, months or even years before for the purpose of evaluating the tracking of the disease, or taken before, for example, the application in the subject of a treatment for said disease for the purpose of evaluating its efficacy.

Due to the variability that can occur between the different subjects in terms of the production of SULF1 protein, the reference level could be obtained from a set of samples from a population of healthy subjects (e.g., subjects who do not suffer CRC) and by calculating the mean level of the product in question (SULF1 protein) in said population of healthy subjects.

The reference level of a certain product, for example, SULF1 protein, can be determined from a reference sample

which can be analyzed, for example, simultaneously or consecutively, together with the sample to be analyzed (test sample). The reference level can generally be derived from the normal distribution limits of a physiological amount found in a population of control subjects. Said physiological amount can be determined by several well-known techniques, as described in this description.

According to the present invention, said reference level allows discriminating the presence of CRC and can therefore be used in the diagnosis, prognosis or tracking of CRC progression.

The markers and methods provided by the present invention are suitable for diagnosing CRC, as well as for predicting the development of a CRC, monitoring the progression of a CRC and/or evaluating the efficacy of treatment administered to a subject who has CRC.

Assays performed by the inventors have clearly shown that the phages identified as MST1, SULF1, NHSL1, SREBF2, GRN and GTF2i allow discriminating between tumor sera (CRC) and control sera by ELISA. Although sensitivity was relatively low for the individual phages, ranging between 46% and 60%, specificity was greater, between 50% and 73.9%, and furthermore the combination of the 6 phages as a predictor of CRC provided an AUC of 0.82 with sensitivity and specificity of 70% and 73.9% [Example 4].

In turn, the combination of SULF1 and MST1 recombinant proteins together with NHSL1, GRN, SREBF2 and GTF2i phages allowed predicting the disease (CRC) with 72% sensitivity and 87% specificity, with an AUC of 0.83. If the age of the patient is further taken into account, the AUC is of 0.91. These markers further allow grouping results by discriminating not only sick individuals, but also different stages of the disease. Likewise, autoantibodies against MST1 and NHSL1 are associated with the clinical prognosis of CRC patients. Therefore, the detection of this panel of autoantibodies in

serum is therefore a simple and non-invasive method for the diagnosis/prognosis of CRC.

It has additionally been observed that SULF1 protein overexpression is correlated with the diagnosis of CRC [Figure 3].

EXAMPLES

The invention will be illustrated below by means of assays conducted by the inventors, which clearly show the specificity and effectiveness of the method for the diagnosis/prognosis of CRC based on the detection in serum of antibodies against the tumor autoantigens described.

EXAMPLE 1

Analysis of sera from CRC patients with microarrays printed with T7 phages

I. MATERIALS AND METHODS

CRC and reference sera

The sera used in microarray and survival analyses were obtained from patients at Hospital Universitario de Bellvitge, Instituto Catalán de Oncología de Barcelona, Hospital Puerta de Hierro de Madrid and Hospital de Cabueñes de Gijón, after obtaining the written consent of all the patients included in the study.

3 sera from CRC patients with Duke's stage B, 3 with stage C and 6 with stage D (3 with liver metastasis and 3 with lung metastasis) were used to select the libraries of CRC-specific T7 phages. 15 sera from CRC patients with different stages, with a mean age of 66.3 years (age range 54-82) and 15 sera from control individuals with a similar mean age and the same sex ratio as the CRC patients were selected for microarray screening.

For the survival analysis, another panel of 95 CRC sera with over 10 years of tracking was tested. The median age was 66.2 years (range between 23-90 years). The clinical data of all the patients are included in Table 3. All the samples were managed anonymously according to the ethical and legal

standards of the Consejo Superior de Investigaciones Científicas (CSIC) (Superior Council for Scientific Research).

Table 3. Clinical-pathological information of patients whose serum was used for the identification and validation of the autoantibodies.

CRC patients (n)	160	Healthy donors (n)	61
Mean age (years)	67.7	Mean age (years)	61.7
Age range (years)	23-91	Age range (years)	34-89
Sex:		Sex:	
Male	65.6%	Male	60.7%
Female	34.4%	Female	39.3%
Duke's stage:			
I	40.6%		
II	19.4%		
III	15.6%		
IV	24.4%		
Prognosis:			
Dead	33.1%		
Alive	60%		
Unknown	6.9%		
Mean survival time (months)	57.5		

An independent group of sera was used for validation; 50 CRC sera representative of all Duke's stages (A-D), 46 control sera, 10 asymptomatic patients with family history, 2 with hyperplastic polyps, 2 with ulcerative colitis and 43 sera of other types of cancer (bladder, breast, lung, pancreas and stomach).

The sera used were processed in the same manner in the different hospitals; the blood samples were left at room temperature for at least 30 minutes (and a maximum of 60

minutes) to allow clot formation. The samples were subsequently centrifuged at 3000 g at 4°C for 10 minutes. The sera were frozen and stored at -80°C until use.

Synthesis of T7 phage cDNA library and selection rounds

The total RNA of 3 tumor tissues from CRC patients in Duke's stages A (samples A) and 3 with stage C (samples C), was isolated by means of the reagent Trizol (Invitrogen). 4 µg of each RNA were used, mixing samples A and samples C separately, for the synthesis of cDNA. The T7 phage cDNA libraries of CRC in were constructed using the OrientExpress cDNA synthesis and cloning system (Novagen) according to the manufacturer's instructions. The cDNA was synthesized by means of RT-PCR using the oligonucleotide oligo(dT). The cloning was performed indistinctly in vectors T7Select 415-1 and T7Select 10-3b, which differ in the size of the insert they allow. Phage growth was obtained in *E. coli* strains BL21 and BLT 5403, respectively. The four T7 libraries were titrated making serial dilutions of the T7 phages in Petri dishes. The sizes of the libraries were greater than 10^6 pfu/mL in all cases. The phage selection rounds were performed using on one hand the combination of the 2 libraries constructed with the T7Select 415-1 vector and on the other the combination of the 2 libraries constructed with the T7Select 10-3b vector.

Negative selection was performed first. To that end, protein A/G coupled magnetic particles (Invitrogen) were incubated with a mixture of 8 control sera (120 µL of mixture of control sera, diluted 1:50, at 4°C overnight) to bind the IgGs of the control subjects. The phages were subsequently incubated with said magnetic particles to remove those phages bound to the IgGs of the control sera. Secondly, 4 mixtures of sera (stage B mixture: from 3 CRC patients with Duke's stage B, stage C mixture: from 3 patients with Duke's stage C, stage D-H mixture: from 3 patients with Duke's stage D and liver metastasis and stage D-P mixture: from 3 patients with Duke's stage D and lung metastasis) were incubated with protein A/G

coupled magnetic particles to enrich the phage libraries with CRC specific phages. The phages not retained in the negative selection were incubated with the magnetic particles previously incubated with the different mixtures of sera from CRC patients. The phages bound to said magnetic particles were eluted with 100 µL of 1% SDS and amplified in *E. coli* BLT5406 or BL21. A total of 4 selection rounds were conducted to enrich T7 phage libraries with CRC specific phages. Individual clones of the 8 selections which were printed in nitrocellulose microarrays were finally amplified.

Phage microarrays

After the amplification of monoclonal phages, the bacteria lysates were centrifuged and the supernatants containing the phages were diluted 1:2 in PBS with 0.1% Tween 20 (PBST) for printing in duplicate in nitrocellulose microarrays (Whatman/Schleicher & Schuell's) with the OmniGrid Spotter robot (GeneMachines, San Carlos, CA). The negative controls used in the printing were BSA (Sigma Aldrich), printing buffer or empty gaps. T7 and human IgG protein (Sigma-Aldrich) were printed as positive controls to verify the quality of the array.

Thirty sera (15 from CRC patients and 15 from normal individuals) were incubated with the phage microarrays as described previously (Chaterjee, M *et al.* 2006. Cancer Res. 66:1181-1190). Briefly, the slides were equilibrated with PBS at room temperature for 5 minutes and blocked with 3% skim milk in PBS (3% MPBS) for 1 hour at room temperature under stirring. The arrays were subsequently incubated with 6.6 µL of human serum (dilution 1:300), 120 µg of *E. coli* lysate and 0.3 µg of anti T7-tag monoclonal antibody (Novagen) diluted in 2 mL of 3% MPBS for 90 minutes at room temperature. The slides were washed 3 times with PBST for 10 minutes to eliminate non-specific binding and were incubated with an AlexaFluor 647-conjugated anti-human IgG antibody (Invitrogen) diluted 1:2,000 and an AlexaFluor 555-conjugated anti-mouse IgG

antibody (Invitrogen) diluted 1:40,000 in 3% MPBS for detecting the human antibodies bound to T7 phages and the T7 phages, respectively. Subsequently, the microarrays were washed 3 times with PBST, once with PBS and were dried by means of centrifugation at 1200 rpm for 3 minutes. Finally, the slides were scanned in a ScanArrayTM5000 (Packard BioChip Technologies). The Genepix Pro 7 image analysis program (Axon Laboratories) was used to quantify the intensity of the points.

Statistical analysis

The microarray data were normalized and processed using the Asterias applications (http://asterias.bioinfo.cnio.es/), an interface for using software packages, Limma and marrayNorm from Bioconductor. After applying a background noise correction and global Loess normalization (http://dnmad.bioinfo.cnio.es/), the data were processed to filter the missing values or points with too high of a variance, to combine duplicates and then obtain a single log transformed base 2 value for each phage (http://prep.bioinfo.cnio.es/). The groups of CRC patients and healthy individuals were compared by performing a t-test with the Pomelo II program (http://pomelo2.bioinfo.cnio.es/), where p values were obtained by means of 200000 permutations. The Pomelo II program generated a graph showing the phages with a positive result false discovery rate (FDR) value below 0.15 and an unadjusted p value below 0.05.

The bootstrapping analyses were adjusted by means of a logistic regression model where the probability of being a tumor against the probability of being normal was modeled as a function of the variables (phages and proteins). The age and sex of the patients were also included in the model to correct the possible effects of these variables. The area under the ROC curve (AUC) was calculated to evaluate the predictive ability of the models. The AUC calculated directly with the original model and the complete data set is biased towards

high values. Therefore, bootstrapping with 1,000 replicate samples was used to obtain a corrected AUC not biased towards high values, giving an estimate of the AUC that could be obtained with an independent future validation (Efron B. J. Am. Stat Assoc. 1983;78:316-331). The models were adjusted using the Harrell design library (Harrel F. Springer. 2001) with the statistical computing system R (Team RDC, 2009).

II. RESULTS

The tissue RNA of CRC from 6 patients (three with Duke's stage A and three with Duke's stage C) was used to construct T7 phage libraries containing cDNA fragments in 2 vectors (T7Select 415-1 or T7select 10-3b). After selecting CRC specific phages 8 different libraries enriched in tumor specific phages, depending on the vector and the mixture of sera (B, C, H and P) used during selection, were obtained. A total of 1,536 individual phages were amplified (192 individual phages of each selection) and were printed in duplicate in nitrocellulose arrays. An anti-T7 antibody which allowed observing the presence of a homogenous signal in the array was used as a control of the amount of phage printed in the array. The intensity of the 2 points corresponding to the same phage within the same array and between two different arrays was represented for the purpose of determining intra- and inter- array reproducibility. It was determined that intra- and inter-array reproducibility was good with R2 values of 0.9703 and 0.9091, respectively.

The arrays with 30 sera (15 from patients with different stages of CRC and 15 from healthy controls) were incubated to evaluate the immune response in CRC patients. After quantifying the images and normalizing the data, the signal of the tumor sera was compared with the healthy sera using a t-test with 200,000 permutations. Between the 2 groups 128 phages showed different reactivities, with an FDR < 0.22. Out of those phages, 78 showed increased reactivity in CRC whereas 50 had reduced reactivity in sera with CRC. The representation

of the supervised analysis of the 45 phages with the lowest FDR (<0.15) showed a clear separation between CRC patients and healthy individuals.

EXAMPLE 2

Identification of the phage-displayed sequences

I. MATERIALS AND METHODS

Sequencing and analysis of the internal sequences by means of BLASTp

The DNA inserted in the phage genome was amplified by PCR using forward primer T7_up2: 5'-TGCTAAGGACAACGTTATCGG-3´ (SEQ ID NO:13) and reverse primer T7_down2: 5'-TTGATACCGGACGTTCAC-3´ (SEQ ID NO:14). The PCR products were precipitated with ethanol and sequenced directly with forward primer T7_up2.

A search was conducted in the NCBI database with BLASTp software to find sequence homology for each peptide displayed on the selected phage surface.

Proteins, antibodies and cell lines

MST1/STK4 and SULF1 human recombinant proteins were expressed in *E. coli*. MST1 cDNA was sub-cloned into pET28a vector (Novagen). SULF1 cDNA was cloned into pDONR221 vector and subsequently into pDEST17 expression vector. The 2 6xHis-MST1 and 6xHis-SULF1 fusion proteins were expressed in *E. coli* strain BL21 (DE3) and purified to homogeneity by means of HisTrap column affinity chromatography (GE Healthcare). Finally, the proteins were dialyzed against PBS and concentrated. EBNA1 protein used as a positive control in ELISA assays was purchased from the company Tebu-Bio.

Antibodies against MST1/STK4, SULF1 and tubulin used in the membrane immunodetection were purchased from the companies Atlas antibodies, Santa Cruz Biotechnology and Sigma, respectively. The TrueBlot peroxidase-conjugated anti-rabbit IgG antibody was purchased from the company eBioscence and the peroxidase-conjugated anti-mouse IgG and anti-human IgG antibodies were purchased from DakoCytomation.

Colorectal cancer cell lines RKO, Caco2, Hct15, Hct116, Colo320, SW480, SW48, KM12C, KM12SM, HT29, Colo205 and reference cell lines (HEK293 and MOLT4) were grown according to protocols established. Peripheral blood lymphocytes (PBL) and monocytes were isolated from a healthy donor.

Western-blot analysis

The preparation of the cell lines and paired tissue extracts was performed according to the following protocol. The cells and the tissues were washed twice with PBS before lysis with 500 µL of 0.5% SDS with protease inhibitors (Roche Applied Science). The concentration of the extracts was determined by means of the 2D-Quant kit (GE Healthcare) after clarifying the sample by means of centrifugation at 12,000 g for 15 minutes at 4°C.

25 µg of protein extract were separated in 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Hybond-C Extra) according to established protocols (Babel *et al*. Mol. Cell Proteomics 2009; 8:2382-95). The membrane was blocked with 3% MPBS and incubated overnight at 4°C with the antibodies against MST1 (1:1,000 dilution), SULF1 (1:3,000 dilution) or tubulin (1:5,000 dilution). Immunodetection was performed using an HRP-conjugated anti-mouse IgG antibody (1:5,000 dilution) or an HRP-conjugated anti-rabbit IgG antibody (1:5,000 dilution). Antibody binding was finally detected using ECL (GE Healthcare) or SuperSignal Femto (Pierce).

ELISA

T7 phage capture ELISA plates (Novagen) were blocked for 2 hours at 37°C with 3% MPBS and incubated overnight with 100 µL of the bacterial lysate of the phages diluted in 3% MPBS. After washing 3 times with PBST, the plates were blocked with 3% MPBS for 1 hour at 37°C and incubated with 100 µL of human serum (1:50 dilution in 3% MPBS) for 1 hour at 37°C. After 3 additional washes, peroxidase-labeled anti-human IgG antibody (1:3,000 in 3% MPBS) was added for 2 hours at room

temperature. The signal was detected with 3,3',5,5'-tetramethylbenzidine substrate (Sigma) for 10 minutes, stopping the reaction with 1 M HCl and measuring the signal at 450 nm.

The competitive assay between the peptides displayed on phage surfaces and the recombinant proteins was performed using the T7 phage capture plates (Novagen) following the preceding protocol, except the human sera were pre-incubated overnight at 4°C with serial dilutions of MST1, SULF1 or GST proteins. The sera thus pre-incubated were tested against EBNA1 in ELISA plates (Maxisorp, Nunc) as a positive control to verify that the competition for IgGs between the phage and its respective recombinant protein was specific.

The ELISAs with MST1, SULF1 and EBNA1 proteins were performed as described previously (Babel *et al.* Mol. Cell Proteomics 2009; 8:2382-95). The concentration of CEA in the sera from CRC patients and the control sera was determined by means of a specific immunological test following manufacturer's recommendations (MP Biomedicals).

Statistical analysis

The ELISA data for each individual marker (full length phage or protein) were evaluated calculating an ROC curve (receiver operating characteristic curve). The corresponding area under the curve (AUC) was calculated using the JMP7 program (SAS). The mean and standard deviation of the immunohistochemistry results were calculated using the Microsoft Office Excel 2007 program. The one-tailed Student's t-test was carried out using the immunohistochemistry results, assuming that the unequal variances for determining the means of the normal and tumor groups were significantly different from one another.

II. RESULTS

Forty-three unique amino acid sequences were obtained fused to T7 phage capsid protein 10B among the 78 phages showing increased reactivity in sera from CRC patients.

Out of the 43 unique phages those phages which contained between 8 and 20 residues with high homology to known protein sequences, which appeared a greater number of times with the same amino acid sequence and which had a low p value, were selected to verify the results. Sequences homologous to MST1/STK4, SULF1, NHSL1, SREBF2, GRN and GTF2i proteins were identified. All of them had a significantly greater signal in the microarray with the serum from CRC patients than with the control sera (Figure 1). The phages were identified by the name of the protein with which homologous sequences were identified. MST1/STK4 protein was identified previously as a tumor-associated antigen in CRC using commercial protein microarrays (Babel *et al.* Mol. Cell Proteomics 2009;8:2382-95) and the SULF1 gene was described as being overexpressed in a transcriptome analysis of CRC (Madoz-Gurpide *et al.* Mol Cell Proteomics, 2006;5:1471-83).

A competitive IgG assay was performed between the phages and SULF1 and MST1 human recombinant proteins for the purpose of confirming that the phage-displayed peptides the homologous sequence of which belonged to SULF1 and MST1 proteins. The binding of the immunoglobulins present in the human sera to the 2 phages was inhibited in a dose-dependent manner with MST1 and SULF1 recombinant proteins (Figure 2A). GST did not affect the binding of IgGs to phages (negative control). As a specific inhibition control, it was observed that the binding of the antibodies of the patients to EBNA protein was not affected by incubation with MST1 or SULF1 proteins.

In addition, it was determined that the sequences of the phage-displayed peptides were located in the C-terminal region of MST1 and in the N-terminal region of SULF1 (Figure 2B).

All these results confirm that the displayed peptides correspond to immunodominant epitopes of MST1 and SULF1 proteins.

EXAMPLE 3

<u>The identified proteins are overexpressed in colorectal cancer</u>

Tumor-associated antigens recognized by autoantibodies are generally overexpressed in cell lines and in tumor tissues. Meta analysis of mRNA expression levels of the homologous proteins corresponding to the 6 phages selected [MST1/STK4, SULF1, NHSL1, SREBF2, GRN and GTF2i] was performed with the Oncomine microarray database (Rhodes *et al.* Neoplasia 2004;6:1-6) (Figure 3A). It was found that SULF1 was the most overexpressed gene in colon cancer, followed by GTF2i, MST1, GRN, NHSL1 and SREBF2. In addition, membrane immunodetection was performed with the antibodies against MST1 and SULF1 using 11 CRC cell lines and tumor tissues from CRC patients representing the different stages of disease progression (Figure 3B). It was found that MST1 and SULF1 proteins were expressed in most colon cancer cell lines. The greater expression of SULF1 was observed in metastatic cell lines (SW48, HT29 and COLO205) and in CRC tumor tissue in late stages.

The cellular expression patterns of the selected proteins were characterized by means of immunohistochemistry (TMA) using independent CRC tumors arranged in microarrays or by means of meta analysis of tissue microarray data obtained from the Human Protein Atlas (Berglund *et al.* Mol Cell Proteomics. 2008;7:2019-27) (Figure 3C). In all cases, more abundant expression of the protein studied was detected in tumor tissues.

Therefore, there is good correlation between the presence of autoantibodies, the abundance of proteins and gene expression.

EXAMPLE 4

<u>Validation of the predictor formed by phage-displayed peptides and their homologous proteins</u>

An independent set of 96 serum samples (50 with colorectal cancer with 19 samples in early stages (A+B) and 46 healthy controls) were used for the validation of the results. MST1, SULF1, NHSL1, SREBF2, GRN and GTF2i phages were tested

for their ability to discriminate between tumor sera and control sera by ELISA. ROC curves were constructed for each of the markers with the ELISA results. While sensitivity was relatively low for individual phages, ranging between 46% and 60%, specificity was higher, between 50% and 73.9%. The data was fitted to a logistic curve performing linear regressions and producing different models with different combinations of phages to investigate if different combinations of phages showed greater precision in discriminating healthy individuals from cancer patients. Therefore, the result of the combination of the 6 phages as a predictor of CRC gives an AUC of 0.82 with sensitivity and specificity of 70% and 73.9%, respectively (Table 4).

Table 4. Data from the ROC curves obtained from the ELISA values of the validation of both individual phages and of combined phages.

Phage-displayed peptide	Specificity (%)	Sensitivity (%)	AUC
SULF1	73.9	50.0	0.63
NHSL1	50.0	56.0	0.59
MST1	71.7	46.0	0.58
GTF2i	52.2	60.0	0.57
SREBF2	69.6	54.0	0.61
GRN	50.0	58.0	0.53
Combination of 6 phages	73.9	70.0	0.82

The following step consisted of seeing if the replacement of the phages with their MST1 and SULF1 recombinant proteins would improve the discriminatory power of the model (Figure 4). The results confirmed a significant improvement of the prediction using the recombinant proteins, with AUCs of 0.71 and 0.74 for SULF1 and MST1 proteins against

0.63 and 0.58 of the respective phages (Table 2). By combining the two proteins (SULF1 and MST1) and the four phages (NHSL1, SREBF2, GRN and GTF2i), the AUC increased to 0.86 with sensitivity of 82.6% and specificity of 70% (Figure 6A). The CEA values were lower (AUC 0.81) and when combined with the remaining predictions they barely improved the model (AUC 0.89). Different AUC estimations were further performed in the validation step to compare not only CRC versus healthy, but also CRC versus reference sera and healthy versus other tumors (Figure 6). The most relevant result was the capacity of the model to discriminate not only CRC from healthy sera (AUC 0.86) (Figure 6A), but also CRC from all the reference sera, which included other colon-related pathologies (AUC 0.85) (Figure 6B). Notably, the panel seemed to not suitably discriminate healthy controls from other tumors (AUC 0.63) (Figure 6C). The panel further seemed to significantly discriminate healthy controls from asymptomatic patients with a family history of CRC (AUC 0.78).

Bootstrapping analysis

Bootstrapping was also performed to obtain the corrected AUC. The initial model included linear terms for all the phages and proteins, together with two other variables: sex and age of the patients. The corrected AUC value was 0.83 with this model.

This model was probably more complex than necessary. For that reason, a variable selection was performed with the Akaike information criterion as the endpoint. The final model only retained 3 proteins (GRN, MST1 and SULF1), in addition to the age of the patients (Table 5). However, to prevent an overestimate of the predictive capacity of the model, estimated corrected AUC values were obtained by means of bootstrapping the entire variable selection process (i.e., the complete model with 8 variables was performed and the Akaike information criterion was used for each bootstrap sample). The corrected AUC was 0.84. Bootstrapping also provided

information on selection process stability; most bootstrapping models contained four, five, six or seven variables. Some of the variables appeared in most of the models; the GRN phage in 976, protein SULF1 in 954, age in 952 and MST1 protein in 833.

This model was further used for predicting the probability of being CRC from a group of 57 sera that comprised various pathologies. A dot-plot (Figure 6D) was generated, showing the individual probability for each subject. Great variability in probability was observed within each group, but the median was way below 0.5, indicating a low probability of having CRC.

Table 5. Final model with bootstrapping after the selection model

	Estimate	Std Error.	z value	Pr(>|z|)
(Intercept*)	-5.92318	2.15625	-2.747	0.00601**
GRN-phage	-6.28345	2.91418	-2.156	0.03107*
NHSL1- phage	6.40976	2.8367	2.26	0.02385*
GTF2B- phage	-9.00788	2.63827	-3.414	0.00064***
SREBF2-phage	10.31184	3.30888	3.116	0.00183**
MST1- phage	3.53576	1.32002	2.679	0.00739**
SULF1- phage	7.26445	2.97056	2.445	0.01447*
Sex	-0.98593	0.65076	-1.515	0.12976
Age	0.05674	0.0232	2.446	0.01444*

**The intercept is the log value (p/(1-p)), where p is the probability of being a tumor, when the value of the other variables in the model is 0. Estimate, estimated coefficient (slope); Std. Error, standard error of the specified variable; z value or Wald statistic, which is exactly equal to the estimated coefficient divided by its standard error; Pr(>|z|), p value of the Wald test for that specific coefficient comparing the z value to the normal standard; *, degree of significance.*

The predictor was subsequently tested according to the stage of the patient, using the model with 6 markers (4 phages + 2 proteins) plus the age of the patients. The AUC corrected using bootstrapping was 0.786 for stages A+B; 0.857 for stage C; and 0.849 for stage D. If the same test with CEA values is applied, the corrected AUC values were 0.742 for stages A+B, 0.770 for stage C and 0.973 for stage D. These results indicate clear superiority of the predictive model for the diagnosis of CRC in stages A, B and C, CEA being better for stage D, as was expected.

EXAMPLE 5

Autoantibodies against MST1 and NHSL1 are associated with the clinical prognosis of CRC patients

After having demonstrated that the predictive panel was capable of identifying patients with tumors in both early and late stages, the potential prognosis of autoantibodies was investigated by analyzing their association with absolute patient survival.

Survival was estimated by means of standard Kaplan-Meier method. The prognostic value of candidate antibodies was evaluated by means of stage-stratified Cox's proportional hazards models. The p values were obtained from the probability ratio test. Only the antibodies found as being significant for diagnosis were evaluated for prognosis in a step-by-step multivariate process.

Patients with low levels of antibodies against MST1 showed a lower cumulative survival than those with high levels of autoantibodies (p=0.08). In contrast, patients with higher titers of antibodies against NHSL1 were associated with lower survival (p=0.06). The combination of both effects improves the statistical value of the prediction (0.032) (Figure 5) and confirms the survival prediction. Collectively, these data indicate a correlation between the presence of antibodies against MST1 and NHSL1 with the clinical prognosis of

colorectal cancer patients.

This prognostic value was most important for early stages of the disease with a risk index of 5.1; though it was not significant ($p=0.12$) because only 8 events were observed in the 47 patients in stages I-II. A similar effect was further observed in the analysis of disease-free survival when the patients were restricted to tumor-free surgical resection margins.

2011251854 13 Nov 2015

CLAIMS

1. A method for detecting an autoantibody in a subject suspected of having colorectal cancer (CRC), comprising:
   a) contacting a sample from said subject with an antibody capturing entity (ACE), wherein said ACE is an ACE comprising the amino acid sequence shown in SEQ ID NO: 4; and
   b) detecting the formation of an autoantibody-ACE complex,

   wherein the detection of said autoantibody-ACE complex is indicative of the presence of said autoantibody in said subject.

2. The method according to claim 1, comprising the detection of an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 4, and furthermore the detection of an autoantibody selected from the group consisting of:
   (i) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody;
   (ii) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody;
   (iii) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody;
   (iv) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

2011251854 13 Nov 2015

(v) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and

(vi) any combination of autoantibodies (i) to (v).

3. The method according to claim 1, further comprising the detection of an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody.

4. The method according to claim 1, wherein said sample comprises a biological fluid.

5. The method according to claim 4, wherein said biological fluid comprises blood, plasma or blood serum.

6. The method according to claim 1, wherein said ACE is a peptide, a protein or a phage.

7. The method according to claim 1, wherein said ACE is SULF1 or a variant or fragment thereof containing an epitope recognizable by an autoantibody.

8. The method according to claim 1, wherein said ACE is a phage comprising the amino acid sequence shown in SEQ ID NO: 4, wherein said amino acid sequence is exposed on the phage surface.

9. The method according to any of claims 1 to 8, wherein the detection of said autoantibodies is carried out by means of an immunoassay.

10. The method according to claim 9, wherein said

2011251854 13 Nov 2015

immunoassay comprises an immunoblot, an Enzyme-linked Immunosorbent Assay (ELISA), a protein microarray and/or a phage microarray.

11. The method according to claim 1, wherein said ACE is immobilized on a solid support.

12. The method according to claim 1, further comprising correlating the formation of the autoantibody-ACE complex in the sample from the subject with a diagnosis of CRC.

13. The method according to claim 1, further comprising comparing the immunoreactivity of the sample with the immunoreactivity of a second sample from the same subject in a later time period.

14. The method according to claim 13, wherein said second sample from the subject has been obtained after said subject has been treated for CRC.

15. A method for detecting an autoantibody in a sample, comprising:

a) contacting a sample from said subject with an antibody capturing entity (ACE), wherein said ACE is an ACE comprising the amino acid sequence shown in SEQ ID NO: 4;and

b) detecting the formation of an autoantibody-ACE complex,

wherein the detection of said autoantibody-ACE complex is indicative of the presence of said autoantibody in said sample.

16. The method according to claim 15, comprising the detection of an autoantibody against an ACE comprising

2011251854 13 Nov 2015

the amino acid sequence shown in SEQ ID NO: 4, and furthermore the detection of an autoantibody selected from the group consisting of:

(i) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 2 or a variant thereof containing an epitope recognizable by an autoantibody;

(ii) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 6 or a variant thereof containing an epitope recognizable by an autoantibody;

(iii) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 3 or a variant thereof containing an epitope recognizable by an autoantibody;

(iv) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 5 or a variant thereof containing an epitope recognizable by an autoantibody;

(v) an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody; and

(vi) any combination of autoantibodies (i) to (v).

17. The method according to claim 15, further comprising the detection of an autoantibody against an ACE comprising the amino acid sequence shown in SEQ ID NO: 1 or a variant thereof containing an epitope recognizable by an autoantibody.

18. An antibody capturing entity (ACE) comprising the amino acid sequence shown in SEQ ID NO: 4, wherein said ACE is

2011251854 13 Nov 2015

not SULF1 protein.

19. The antibody capturing entity (ACE) according to claim 18, wherein said ACE is a peptide, a protein or a phage.

20. The antibody capturing entity (ACE) according to claim 18, wherein said ACE is a phage comprising the amino acid sequence shown in SEQ ID NO: 4, wherein said amino acid sequence is exposed on the phage surface.

21. A composition comprising an antibody capturing entity (ACE) according to claim 18.

22. The composition according to claim 21, comprising at least one ACE according to claim 18, and at least one protein selected from the group consisting of SULF1 protein or a variant thereof, MST1 protein or a variant thereof, and their combinations.

23. A composition comprising SULF1 protein or a variant thereof and MST1 protein or a variant thereof and an antibody capturing entity (ACE) according to claim 18.

24. The composition according to any of claims 21 to 23, supported on a solid support.

25. A kit comprising a composition according to any of claims 21 to 23.

26. The kit according to claim 25, comprising an ACE according to claim 18, and a protein selected from the group consisting of SULF1 protein or a variant thereof, MST1 protein or a variant thereof, and their combinations.

2011251854 13 Nov 2015

27. Use of a kit according to any of claims 25 or 26 for detecting an antibody in a sample, or for detecting an autoantibody in a subject suspected of having colorectal cancer (CRC), or for diagnosing whether a subject has CRC, or for determining the risk of a subject developing CRC, or for monitoring CRC progression in a subject, or for evaluating the efficacy of a treatment against CRC, or for predicting survival of a subject who has CRC.

Figure 1

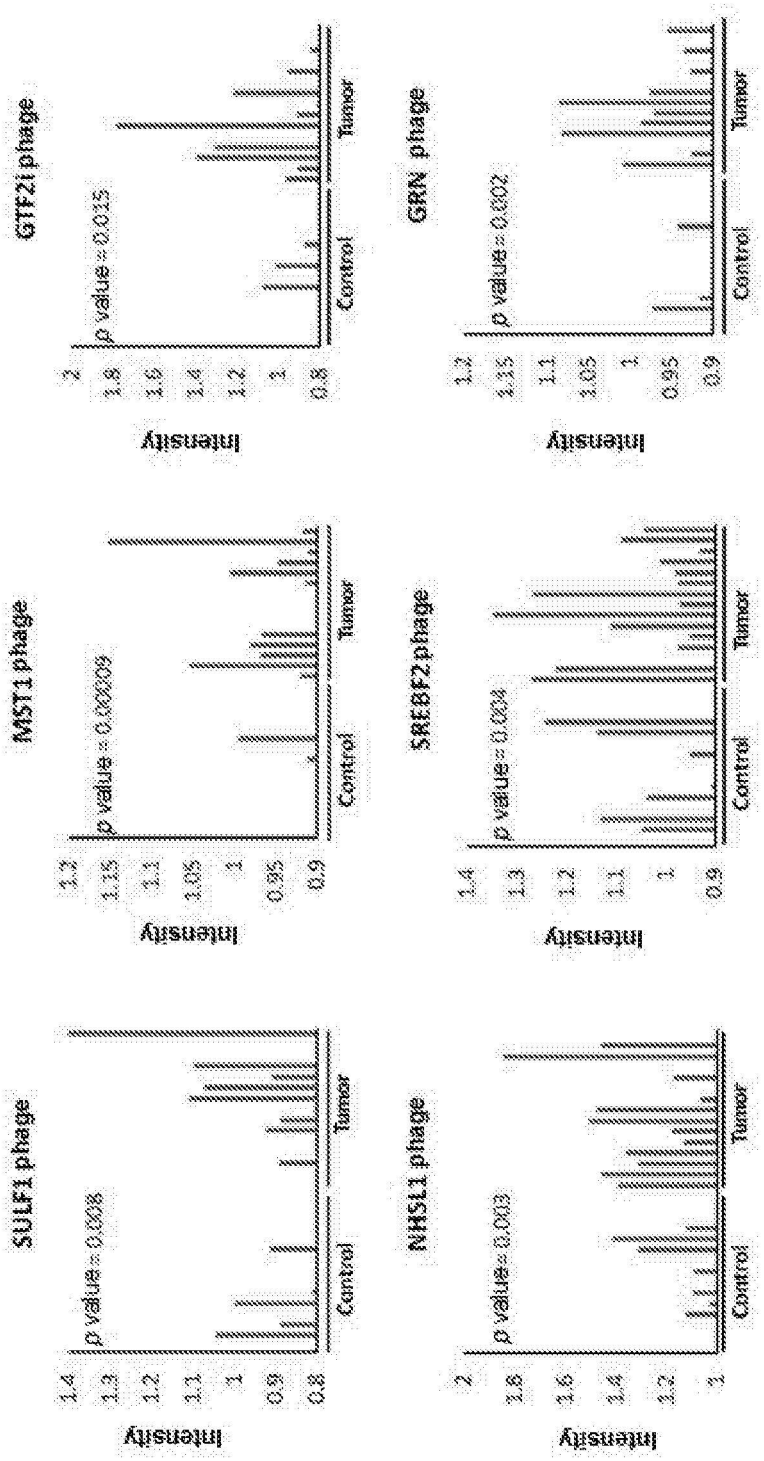

GTF2i phage
p value = 0.015
Intensity
Control
Tumor
GRN phage
p value = 0.002
MST1 phage
p value = 0.00009
SRSF2 phage
p value = 0.004
SULF1 phage
p value = 0.008
NHSL1 phage
p value = 0.003

Figure 2

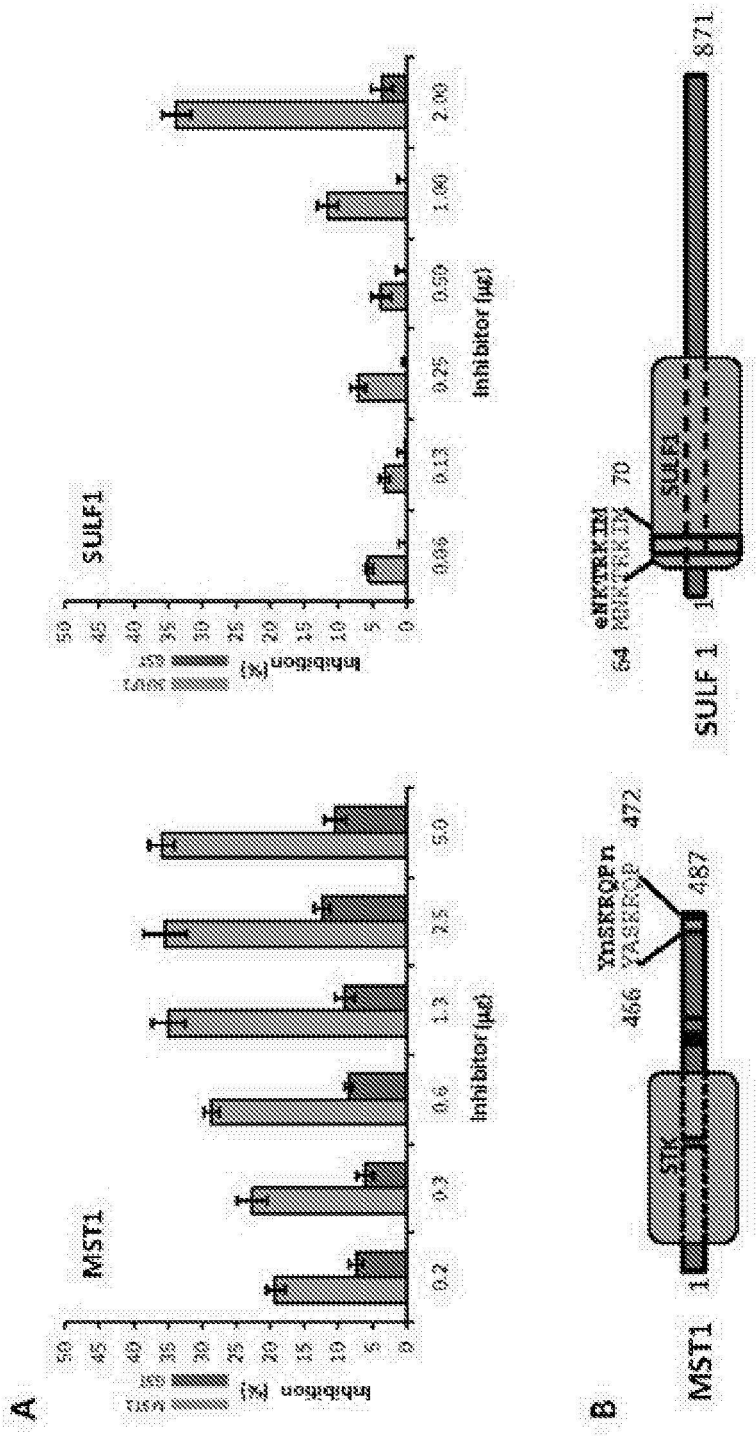

A
MST1
SULF1
Inhibition (%)
Inhibitor (µg)
B
MST1
SULF 1
SPK
SULF1
1
487
472
466
70
64
871

Figure 3A

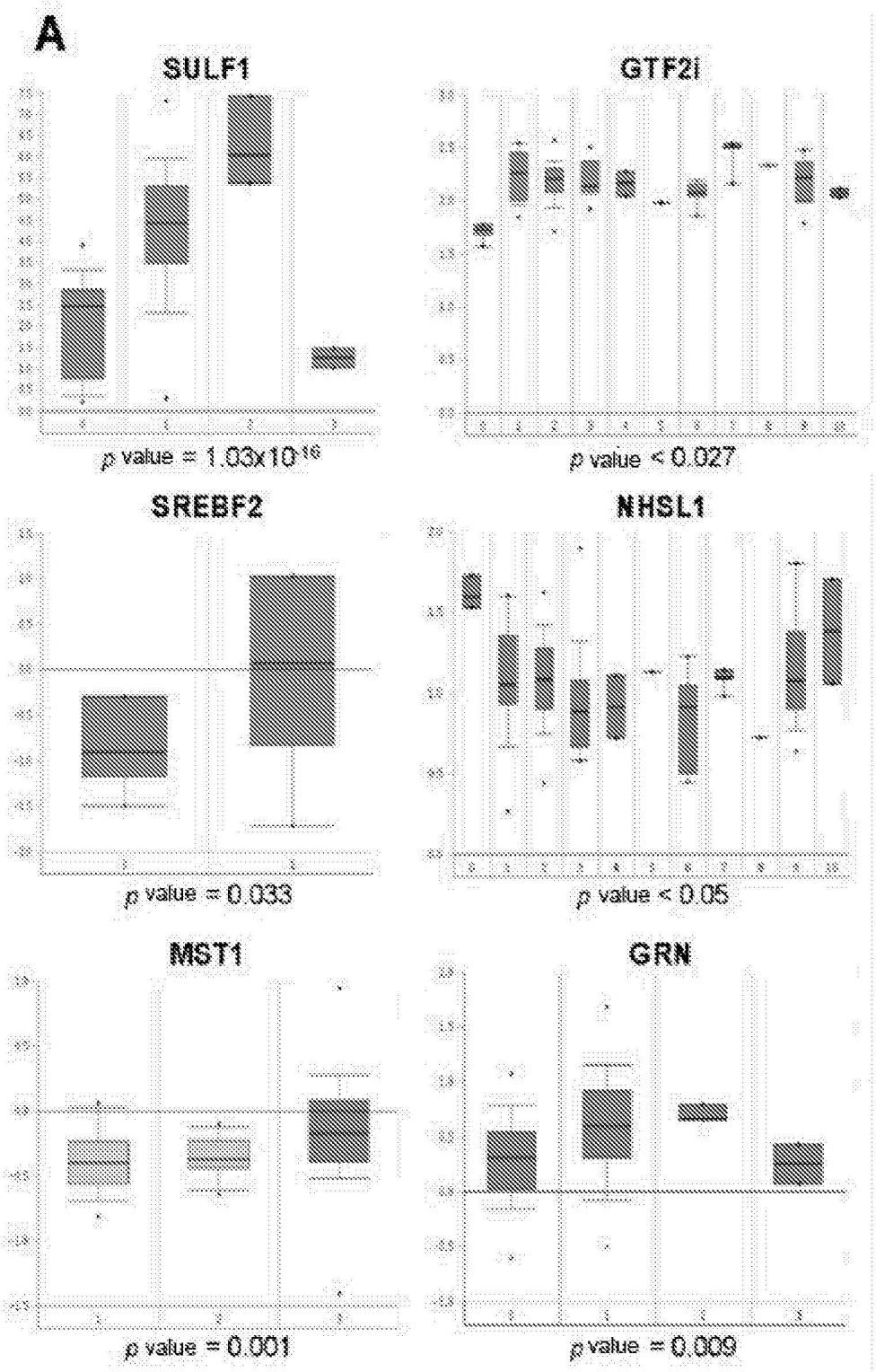

A
SULF1
p value = 1.03x10-16
GTF2i
p value < 0.027
SREBF2
p value = 0.033
NHSL1
p value < 0.05
MST1
p value = 0.001
GRN
p value = 0.009

Figure 3B

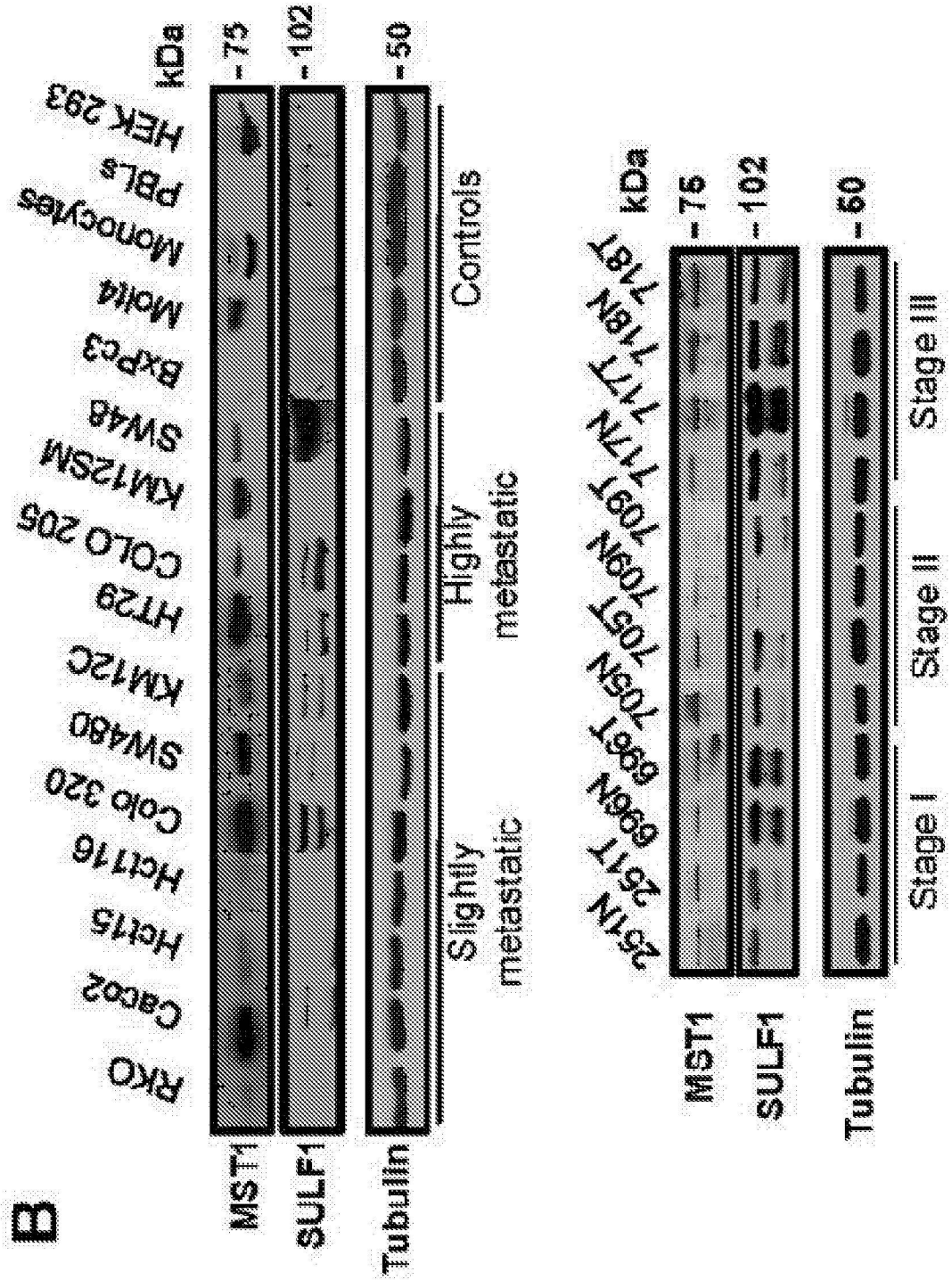

B
kDa
HEK 293
PBLs
Monocytes
Molt4
BxPc3
SW48
KM12SM
COLO 205
HT29
KM12C
SW480
Colo 320
Hct116
Hct15
Caco2
RKO
- 75
- 102
- 50
MST1
SULF1
Tubulin
Controls
Highly metastatic
Slightly metastatic
kDa
- 75
- 102
- 50
MST1
SULF1
Tubulin
Stage I
Stage II
Stage III

Figure 3C

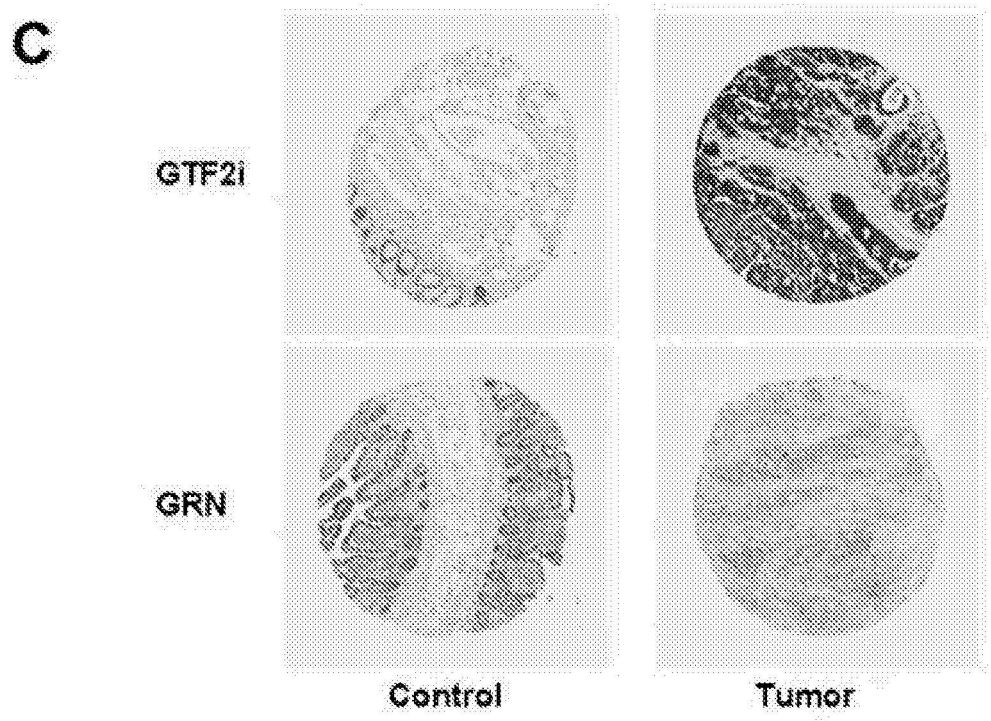


Figure 4

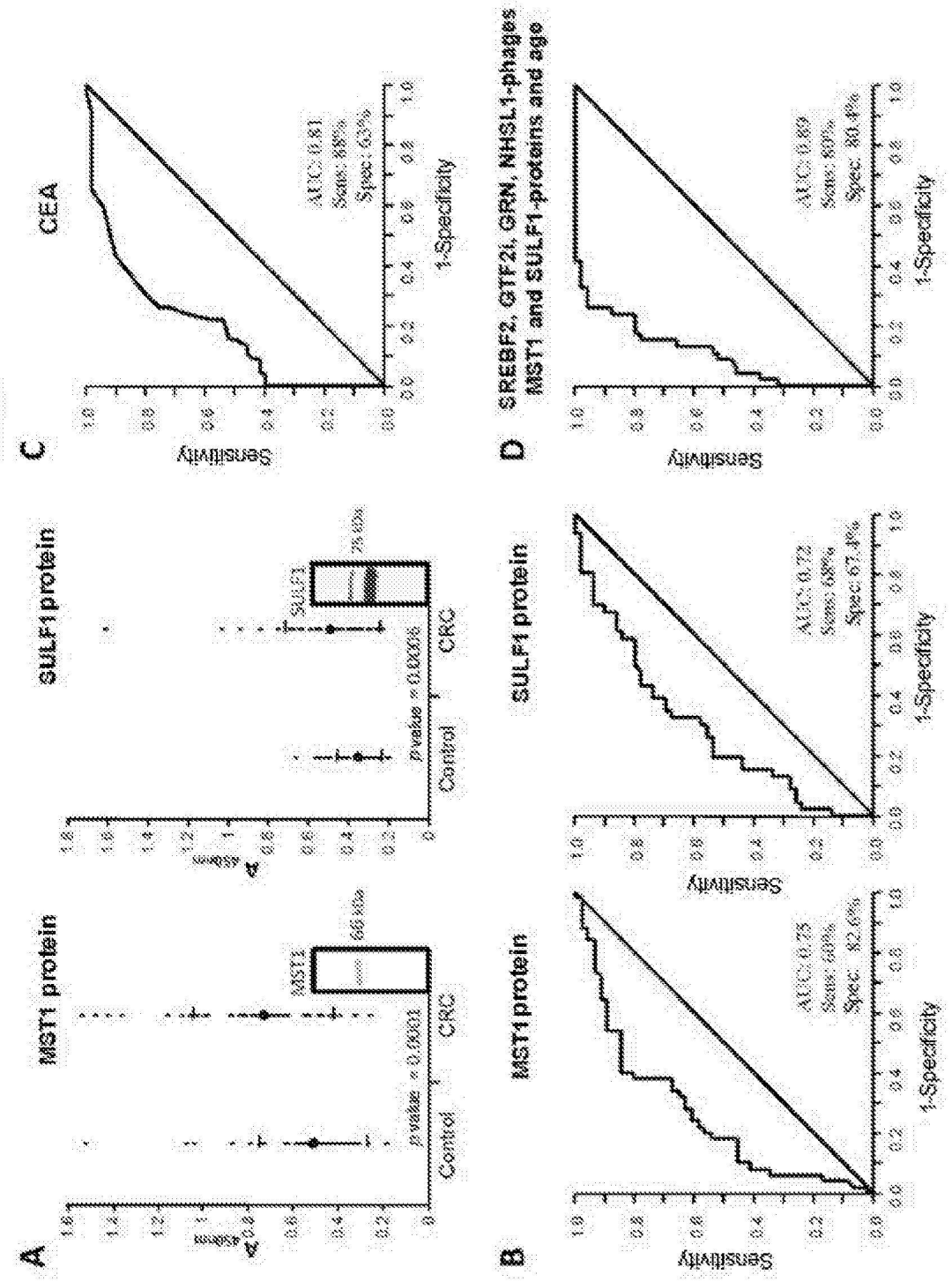

A
MST1 protein
p value = 0.0001
Control
CRC
MST1
66 kDa
SULF1 protein
p value = 0.0001
Control
CRC
SULF1
26 kDa
C
CEA
AUC: 0.61
Sens: 85%
Spec: 63%
Sensitivity
1-Specificity
D
SREBF2, GTF2I, GRN, NHSL1-phages
MST1 and SULF1-proteins and age
AUC: 0.89
Sens: 80%
Spec: 80.4%
Sensitivity
1-Specificity
B
MST1 protein
AUC: 0.73
Sens: 68%
Spec: 82.6%
Sensitivity
1-Specificity
SULF1 protein
AUC: 0.72
Sens: 68%
Spec: 67.4%
Sensitivity
1-Specificity

Figure 5

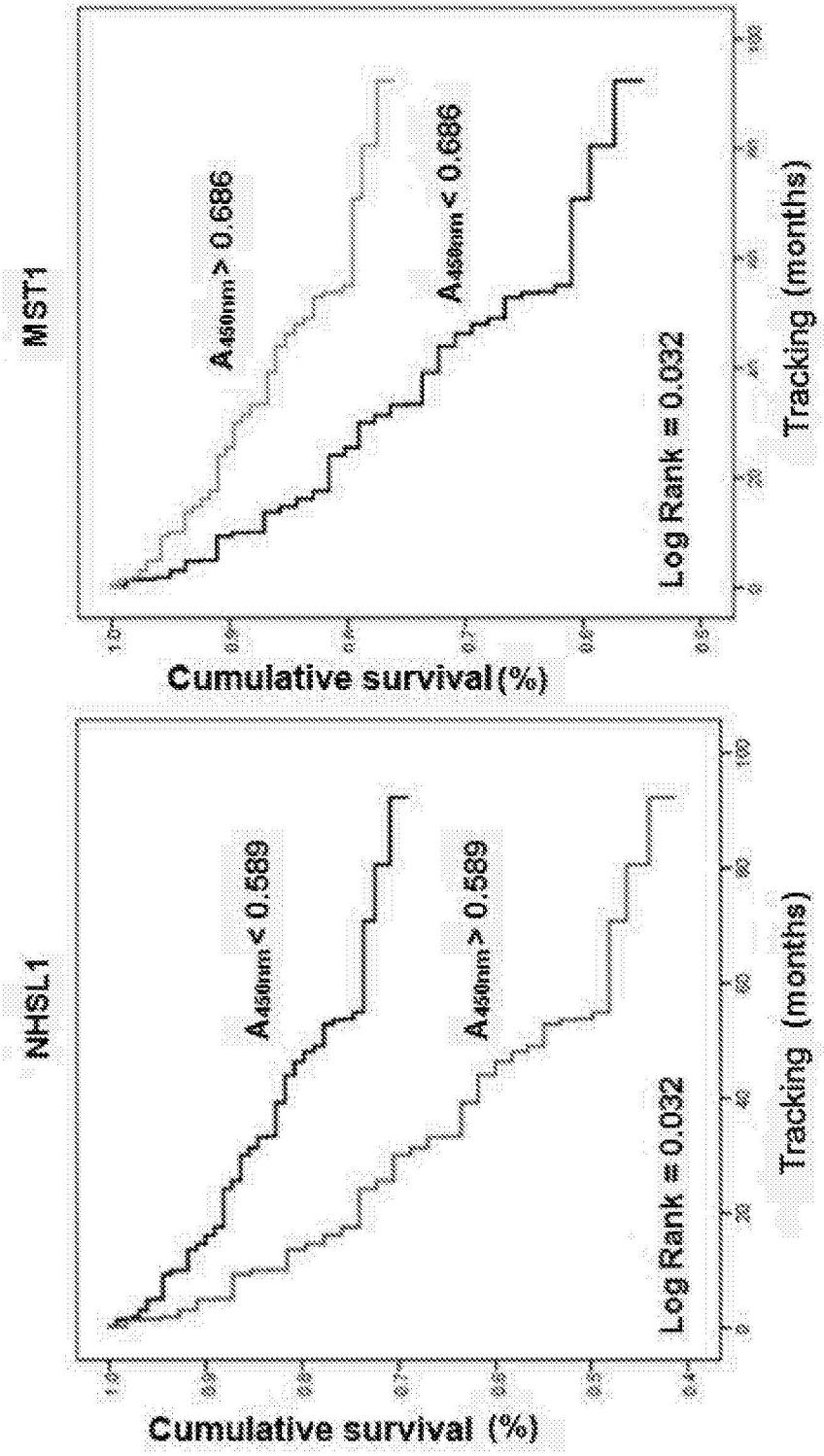

MST1
A_{450nm} > 0.686
A_{450nm} < 0.686
Log Rank = 0.032
Cumulative survival (%)
Tracking (months)
NHSL1
A_{450nm} < 0.589
A_{450nm} > 0.589
Log Rank = 0.032
Cumulative survival (%)
Tracking (months)

Figure 6

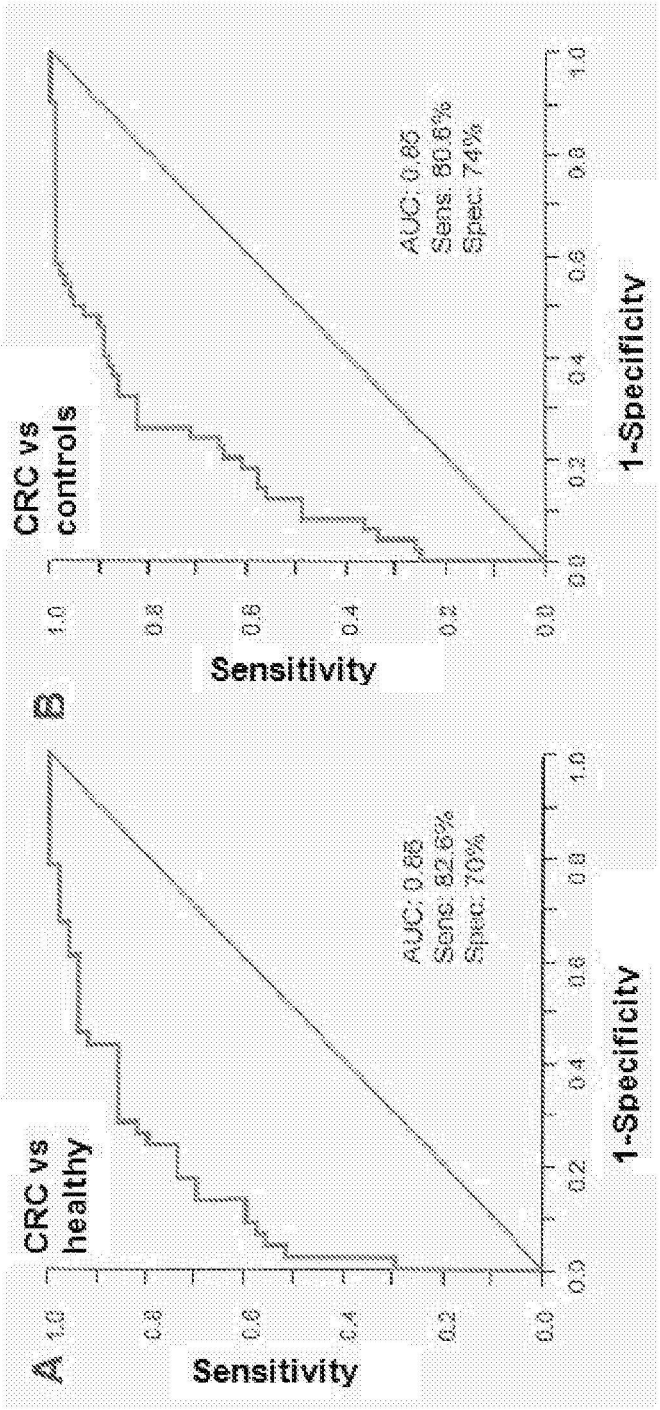
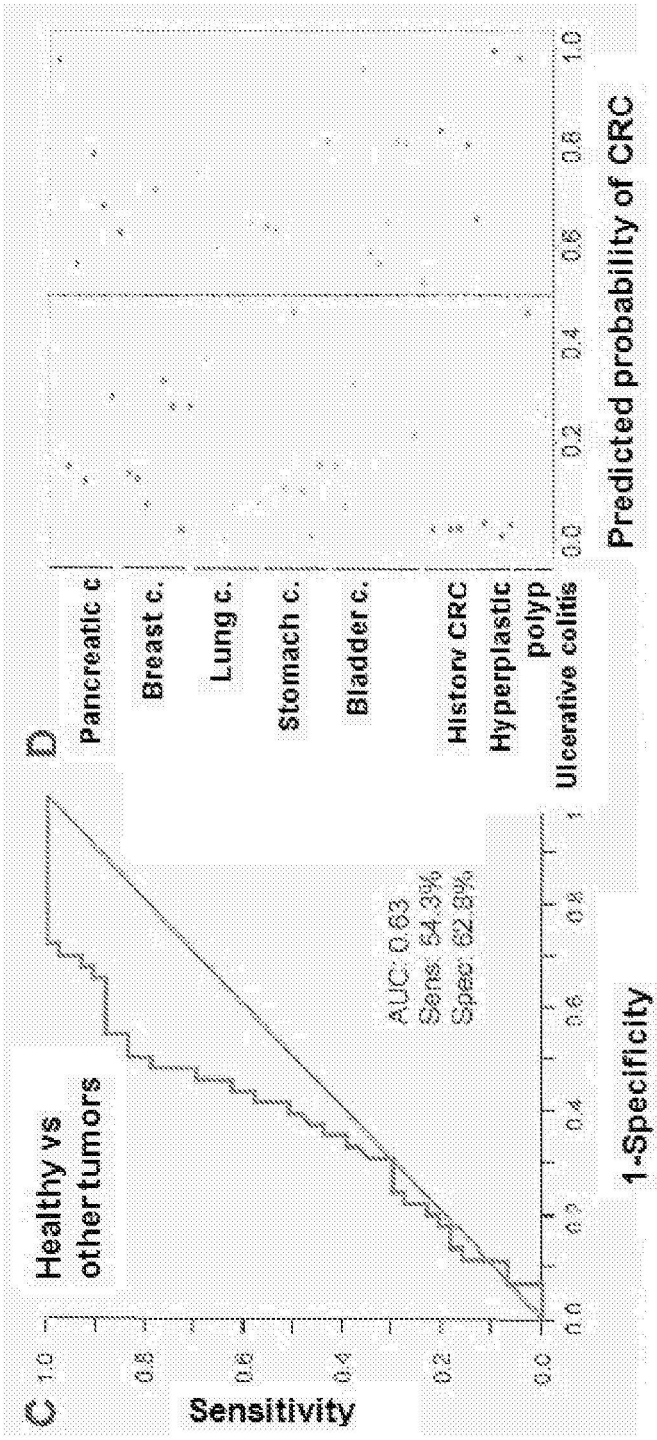

A
CRC vs healthy
Sensitivity
1-Specificity
AUC: 0.88
Sens: 82.6%
Spec: 70%
B
CRC vs controls
Sensitivity
1-Specificity
AUC: 0.85
Sens: 80.8%
Spec: 74%

Figure 6 (cont.)



SEQUENCE LISTING

<110> CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

<120> METHOD FOR THE DIAGNOSIS/PROGNOSIS OF COLORECTAL CANCER

<130> P6917PC00

<150> ES201030708
<151> 2010-05-13

<160> 14

<170> PatentIn version 3.5

<210> 1
<211> 7
<212> PRT
<213> Homo sapiens

<400> 1

Ser Arg Ala Thr Met Pro Gly
1 5

<210> 2
<211> 14
<212> PRT
<213> Homo sapiens

<400> 2

Ser Leu Gly Gln Arg Ser Gln Lys Leu Ala Ala Ala Leu Glu
1 5 10

<210> 3
<211> 8
<212> PRT
<213> Homo sapiens

<400> 3

Tyr Asn Ser Lys Arg Gln Pro Asn
1 5

<210> 4
<211> 8
<212> PRT
<213> Homo sapiens

<400> 4

Glu Asn Lys Thr Arg Lys Ile Met
1 5

<210> 5
<211> 10
<212> PRT
<213> Homo sapiens

<400> 5

Arg Asp Arg Ala Thr Ala Ile Gln Pro Gly
1 5 10

<210> 6
<211> 5
<212> PRT
<213> Homo sapiens

<400> 6

Ser Ser Lys Ser Pro
1 5

<210> 7
<211> 1606
<212> PRT
<213> Homo sapiens

<400> 7

Met Val Val Phe Ile Asn Ala Lys Ile Lys Ser Leu Ile Lys Leu Phe
1 5 10 15

Lys Lys Lys Thr Val Ser Asn Leu Asp Glu Glu Ser Arg Trp Thr Val
20 25 30

His Tyr Thr Ala Pro Trp His Gln Gln Glu Asn Val Phe Leu Pro Thr
35 40 45

Thr Arg Pro Pro Cys Val Glu Asp Leu His Arg Gln Ala Lys Leu Asn
50 55 60

Leu Lys Ser Val Leu Arg Glu Cys Asp Lys Leu Arg His Asp Gly Tyr
65 70 75 80

Arg Ser Ser Gln Tyr Tyr Ser Gln Gly Pro Thr Phe Ala Ala Asn Ala
85 90 95

Ser Pro Phe Cys Asp Asp Tyr Gln Asp Glu Asp Glu Glu Thr Asp Gln
100 105 110

Lys Cys Ser Leu Ser Ser Ser Glu Glu Glu Arg Phe Ile Ser Ile Arg
115 120 125

Arg Pro Lys Thr Pro Ala Ser Ser Asp Phe Ser Asp Leu Asn Thr Gln
130 135 140

Thr Asn Trp Thr Lys Ser Leu Pro Leu Pro Thr Pro Glu Glu Lys Met
145 150 155 160

Arg Gln Gln Ala Gln Thr Val Gln Ala Asp Val Val Pro Ile Asn Ile
165 170 175

Thr Gly Glu Asn Phe Asp Arg Gln Ala Ser Leu Arg Arg Ser Leu Ile
180 185 190

```
Tyr Thr Asp Thr Leu Val Arg Arg Pro Lys Lys Val Lys Arg Arg Lys
        195                 200                 205

Thr Ile Thr Gly Val Pro Asp Asn Ile Gln Lys Glu Leu Ala Ser Gly
    210                 215                 220

Thr Gly Gln Asp Asp Ala Asp Gly His Ser Val Tyr Thr Pro Asp His
225                 230                 235                 240

Tyr Ser Thr Leu Gly Arg Phe Asn Ser Cys Arg Ser Ala Gly Gln Arg
                245                 250                 255

Ser Glu Thr Arg Asp Ser Ser Cys Gln Thr Glu Asp Val Lys Val Val
            260                 265                 270

Pro Pro Ser Met Arg Arg Ile Arg Ala Gln Lys Gly Gln Gly Ile Ala
        275                 280                 285

Ala Gln Met Gly His Phe Ser Gly Ser Ser Gly Asn Met Ser Val Leu
    290                 295                 300

Ser Asp Ser Ala Gly Ile Val Phe Pro Ser Arg Leu Asp Ser Asp Ala
305                 310                 315                 320

Gly Phe His Ser Leu Pro Arg Ser Gly Ala Arg Ala Asn Ile Gln Ser
                325                 330                 335

Leu Glu Pro Arg Leu Gly Ala Leu Gly Pro Ala Gly Asp Met Asn Gly
            340                 345                 350

Thr Phe Leu Tyr Gln Arg Gly His Pro Gln Ala Asp Glu Asn Leu Gly
        355                 360                 365

His Leu Gly Gly Ala Ser Gly Thr Gly Thr Leu Leu Arg Pro Lys Ser
    370                 375                 380

Gln Glu Leu Arg His Phe Glu Ser Glu Asn Ile Met Ser Pro Ala Cys
385                 390                 395                 400

Val Val Ser Pro His Ala Thr Tyr Ser Thr Ser Ile Ile Pro Asn Ala
                405                 410                 415

Thr Leu Ser Ser Ser Ser Glu Val Ile Ala Ile Pro Thr Ala Gln Ser
            420                 425                 430

Ala Gly Gln Arg Glu Ser Lys Ser Ser Gly Ser Ser His Ala Arg Ile
        435                 440                 445

Lys Ser Arg Asp His Leu Ile Ser Arg His Ala Val Lys Gly Asp Pro
    450                 455                 460
```

```
Gln Ser Pro Gly Arg His Trp Asn Glu Gly His Ala Thr Ile Leu Ser
465                 470                 475                 480

Gln Asp Leu Asp Pro His Ser Pro Gly Glu Pro Ala Leu Leu Ser Leu
                485                 490                 495

Cys Asp Ser Ala Val Pro Leu Asn Ala Pro Ala Asn Arg Glu Asn Gly
            500                 505                 510

Ser Gln Ala Met Pro Tyr Asn Cys Arg Asn Asn Leu Ala Phe Pro Ala
        515                 520                 525

His Pro Gln Asp Val Asp Gly Lys Ser Glu Ser Ser Tyr Ser Gly Gly
    530                 535                 540

Gly Gly His Ser Ser Ser Glu Pro Trp Glu Tyr Lys Ser Ser Gly Asn
545                 550                 555                 560

Gly Arg Ala Ser Pro Leu Lys Pro His Leu Ala Thr Pro Gly Tyr Ser
                565                 570                 575

Thr Pro Thr Ser Asn Met Ser Ser Cys Ser Leu Asp Gln Thr Ser Asn
            580                 585                 590

Lys Glu Asp Ala Gly Ser Leu Tyr Ser Glu Asp His Asp Gly Tyr Cys
        595                 600                 605

Ala Ser Val His Thr Asp Ser Gly His Gly Ser Gly Asn Leu Cys Asn
    610                 615                 620

Ser Ser Asp Gly Phe Gly Asn Pro Arg His Ser Val Ile Asn Val Phe
625                 630                 635                 640

Val Gly Arg Ala Gln Lys Asn Gln Gly Asp Arg Ser Asn Tyr Gln Asp
                645                 650                 655

Lys Ser Leu Ser Arg Asn Ile Ser Leu Lys Lys Ala Lys Lys Pro Pro
            660                 665                 670

Leu Pro Pro Ser Arg Thr Asp Ser Leu Arg Arg Ile Pro Lys Lys Ser
        675                 680                 685

Ser Gln Cys Asn Gly Gln Val Leu Asn Glu Ser Leu Ile Ala Thr Leu
    690                 695                 700

Gln His Ser Leu Gln Leu Ser Leu Pro Gly Lys Ser Gly Ser Ser Pro
705                 710                 715                 720

Ser Gln Ser Pro Cys Ser Asp Leu Glu Glu Pro Trp Leu Pro Arg Ser
                725                 730                 735
```

```
Arg Ser Gln Ser Thr Val Ser Ala Gly Ser Ser Met Thr Ser Ala Thr
            740                 745                 750

Thr Pro Asn Val Tyr Ser Leu Cys Gly Ala Thr Pro Ser Gln Ser Asp
        755                 760                 765

Thr Ser Ser Val Lys Ser Glu Tyr Thr Asp Pro Trp Gly Tyr Tyr Ile
    770                 775                 780

Asp Tyr Thr Gly Met Gln Glu Asp Pro Gly Asn Pro Ala Gly Gly Cys
785                 790                 795                 800

Ser Thr Ser Ser Gly Val Pro Thr Gly Asn Gly Pro Val Arg His Val
                805                 810                 815

Gln Glu Gly Ser Arg Ala Thr Met Pro Gln Val Pro Gly Gly Ser Val
            820                 825                 830

Lys Pro Lys Ile Met Ser Pro Glu Lys Ser His Arg Val Ile Ser Pro
        835                 840                 845

Ser Ser Gly Tyr Ser Ser Gln Ser Asn Thr Pro Thr Ala Leu Thr Pro
    850                 855                 860

Val Pro Val Phe Leu Lys Ser Val Ser Pro Ala Asn Gly Lys Gly Lys
865                 870                 875                 880

Pro Lys Pro Lys Val Pro Glu Arg Lys Ser Ser Leu Ile Ser Ser Val
                885                 890                 895

Ser Ile Ser Ser Ser Ser Thr Ser Leu Ser Ser Ser Thr Ser Thr Glu
            900                 905                 910

Gly Ser Gly Thr Met Lys Lys Leu Asp Pro Ala Val Gly Ser Pro Pro
        915                 920                 925

Ala Pro Pro Pro Pro Pro Val Pro Ser Pro Pro Phe Pro Cys Pro Ala
    930                 935                 940

Asp Arg Ser Pro Phe Leu Pro Pro Pro Pro Pro Val Thr Asp Cys Ser
945                 950                 955                 960

Gln Gly Ser Pro Leu Pro His Ser Pro Val Phe Pro Pro Pro Pro Pro
                965                 970                 975

Glu Ala Leu Ile Pro Phe Cys Ser Pro Pro Asp Trp Cys Leu Ser Pro
            980                 985                 990

Pro Arg Pro Ala Leu Ser Pro Ile  Leu Pro Asp Ser Pro  Val Ser Leu
        995                 1000                1005
```

```
Pro Leu  Pro Pro Pro Leu Leu  Pro Ser Ser Glu Pro  Pro Pro Ala
    1010                 1015                 1020

Pro Pro  Leu Asp Pro Lys Phe  Met Lys Asp Thr Arg  Pro Pro Phe
    1025                 1030                 1035

Thr Asn  Ser Gly Gln Pro Glu  Ser Ser Arg Gly Ser  Leu Arg Pro
    1040                 1045                 1050

Pro Ser  Thr Lys Glu Glu Thr  Ser Arg Pro Pro Met  Pro Leu Ile
    1055                 1060                 1065

Thr Thr  Glu Ala Leu Gln Met  Val Gln Leu Arg Pro  Val Arg Lys
    1070                 1075                 1080

Asn Ser  Gly Ala Glu Ala Ala  Gln Leu Ser Glu Arg  Thr Ala Gln
    1085                 1090                 1095

Glu Gln  Arg Thr Pro Val Ala  Pro Gln Tyr His Leu  Lys Pro Ser
    1100                 1105                 1110

Ala Phe  Leu Lys Ser Arg Asn  Ser Thr Asn Glu Met  Glu Ser Glu
    1115                 1120                 1125

Ser Gln  Pro Ala Ser Val Thr  Ser Ser Leu Pro Thr  Pro Ala Lys
    1130                 1135                 1140

Ser Ser  Ser Gln Gly Asp His  Gly Ser Ala Ala Glu  Arg Gly Gly
    1145                 1150                 1155

Pro Val  Ser Arg Ser Pro Gly  Ala Pro Ser Ala Gly  Glu Ala Glu
    1160                 1165                 1170

Ala Arg  Pro Ser Pro Ser Thr  Thr Pro Leu Pro Asp  Ser Ser Pro
    1175                 1180                 1185

Ser Arg  Lys Pro Pro Pro Ile  Ser Lys Lys Pro Lys  Leu Phe Leu
    1190                 1195                 1200

Val Val  Pro Pro Pro Gln Lys  Asp Phe Ala Val Glu  Pro Ala Glu
    1205                 1210                 1215

Asn Val  Ser Glu Ala Leu Arg  Ala Val Pro Ser Pro  Thr Thr Gly
    1220                 1225                 1230

Glu Glu  Gly Ser Val His Ser  Arg Glu Ala Lys Glu  Ser Ser Ala
    1235                 1240                 1245

Ala Gln  Ala Gly Ser His Ala  Thr His Pro Gly Thr  Ser Val Leu
    1250                 1255                 1260
```

```
Glu Gly  Gly Ala Ala Gly Ser  Met Ser Pro Ser Arg  Val Glu Ala
    1265                 1270                 1275

Asn Val  Pro Met Val Gln Pro  Asp Val Ser Pro Ala  Pro Lys Gln
    1280                 1285                 1290

Glu Glu  Pro Ala Glu Asn Ser  Ala Asp Thr Gly Gly  Asp Gly Glu
    1295                 1300                 1305

Ser Cys  Leu Ser Gln Gln Asp  Gly Ala Ala Gly Val  Pro Glu Thr
    1310                 1315                 1320

Asn Ala  Ala Gly Ser Ser Ser  Glu Ala Cys Asp Phe  Leu Lys Glu
    1325                 1330                 1335

Asp Gly  Asn Asp Glu Val Met  Thr Pro Ser Arg Pro  Arg Thr Thr
    1340                 1345                 1350

Glu Asp  Leu Phe Ala Ala Ile  His Arg Ser Lys Arg  Lys Val Leu
    1355                 1360                 1365

Gly Arg  Arg Asp Ser Asp Asp  Asp His Ser Arg Asn  His Ser Pro
    1370                 1375                 1380

Ser Pro  Pro Val Thr Pro Thr  Gly Ala Ala Pro Ser  Leu Ala Ser
    1385                 1390                 1395

Pro Lys  Gln Val Gly Ser Ile  Gln Arg Ser Ile Arg  Lys Ser Ser
    1400                 1405                 1410

Thr Ser  Ser Asp Asn Phe Lys  Ala Leu Leu Leu Lys  Lys Gly Ser
    1415                 1420                 1425

Arg Ser  Asp Thr Ser Ala Arg  Met Ser Ala Ala Glu  Met Leu Lys
    1430                 1435                 1440

Asn Thr  Asp Pro Arg Phe Gln  Arg Ser Arg Ser Glu  Pro Ser Pro
    1445                 1450                 1455

Asp Ala  Pro Glu Ser Pro Ser  Ser Cys Ser Pro Ser  Lys Asn Arg
    1460                 1465                 1470

Arg Ala  Gln Glu Glu Trp Ala  Lys Asn Glu Gly Leu  Met Pro Arg
    1475                 1480                 1485

Ser Leu  Ser Phe Ser Gly Pro  Arg Tyr Gly Arg Ser  Arg Thr Pro
    1490                 1495                 1500

Pro Ser  Ala Ala Ser Ser Arg  Tyr Ser Met Arg Asn  Arg Ile Gln
    1505                 1510                 1515
```

```
Ser Ser  Pro Met Thr Val Ile  Ser Glu Gly Glu Gly  Glu Ala Val
    1520                1525                1530

Glu Pro  Val Asp Ser Ile Ala  Arg Gly Ala Leu Gly  Ala Ala Glu
    1535                1540                1545

Gly Cys  Ser Leu Asp Gly Leu  Ala Arg Glu Glu Met  Asp Glu Gly
    1550                1555                1560

Gly Leu  Leu Cys Gly Glu Gly  Pro Ala Ala Ser Leu  Gln Pro Gln
    1565                1570                1575

Ala Pro  Gly Pro Val Asp Gly  Thr Ala Ser Ala Glu  Gly Arg Glu
    1580                1585                1590

Pro Ser  Pro Gln Cys Gly Gly  Ser Leu Ser Glu Glu  Ser
    1595                1600                1605
```

<210> 8
<211> 69
<212> PRT
<213> Homo sapiens

<400> 8

```
Ala Met Val Pro Cys Asp Asn Val Ser Ser Cys Pro Ser Ser Asp Thr
1               5                   10                  15

Cys Cys Gln Leu Thr Ser Gly Glu Trp Gly Cys Cys Pro Ile Pro Glu
            20                  25                  30

Ala Val Cys Cys Ser Asp His Gln His Cys Cys Pro Gln Gly Tyr Thr
        35                  40                  45

Cys Val Ala Glu Gly Gln Cys Gln Lys Leu Ala Ala Ala Leu Glu His
    50                  55                  60

His His His His His
65
```

<210> 9
<211> 487
<212> PRT
<213> Homo sapiens

<400> 9

```
Met Glu Thr Val Gln Leu Arg Asn Pro Pro Arg Arg Gln Leu Lys Lys
1               5                   10                  15

Leu Asp Glu Asp Ser Leu Thr Lys Gln Pro Glu Glu Val Phe Asp Val
            20                  25                  30

Leu Glu Lys Leu Gly Glu Gly Ser Tyr Gly Ser Val Tyr Lys Ala Ile
        35                  40                  45
```

```
His Lys Glu Thr Gly Gln Ile Val Ala Ile Lys Gln Val Pro Val Glu
    50                  55                  60

Ser Asp Leu Gln Glu Ile Ile Lys Glu Ile Ser Ile Met Gln Gln Cys
65                  70                  75                  80

Asp Ser Pro His Val Val Lys Tyr Tyr Gly Ser Tyr Phe Lys Asn Thr
                85                  90                  95

Asp Leu Trp Ile Val Met Glu Tyr Cys Gly Ala Gly Ser Val Ser Asp
            100                 105                 110

Ile Ile Arg Leu Arg Asn Lys Thr Leu Thr Glu Asp Glu Ile Ala Thr
        115                 120                 125

Ile Leu Gln Ser Thr Leu Lys Gly Leu Glu Tyr Leu His Phe Met Arg
    130                 135                 140

Lys Ile His Arg Asp Ile Lys Ala Gly Asn Ile Leu Leu Asn Thr Glu
145                 150                 155                 160

Gly His Ala Lys Leu Ala Asp Phe Gly Val Ala Gly Gln Leu Thr Asp
                165                 170                 175

Thr Met Ala Lys Arg Asn Thr Val Ile Gly Thr Pro Phe Trp Met Ala
            180                 185                 190

Pro Glu Val Ile Gln Glu Ile Gly Tyr Asn Cys Val Ala Asp Ile Trp
        195                 200                 205

Ser Leu Gly Ile Thr Ala Ile Glu Met Ala Glu Gly Lys Arg Pro Tyr
    210                 215                 220

Ala Asp Ile His Pro Met Arg Ala Ile Phe Met Ile Pro Thr Asn Pro
225                 230                 235                 240

Pro Pro Thr Phe Arg Lys Pro Glu Leu Trp Ser Asp Asn Phe Thr Asp
                245                 250                 255

Phe Val Lys Gln Cys Leu Val Lys Ser Pro Glu Gln Arg Ala Thr Ala
            260                 265                 270

Thr Gln Leu Leu Gln His Pro Phe Val Arg Ser Ala Lys Gly Val Ser
        275                 280                 285

Ile Leu Arg Asp Leu Ile Asn Glu Ala Met Asp Val Lys Leu Lys Arg
    290                 295                 300

Gln Glu Ser Gln Gln Arg Glu Met Asp Gln Asp Asp Glu Glu Asn Ser
305                 310                 315                 320
```

```
Glu Glu Asp Glu Met Asp Ser Gly Thr Met Val Arg Ala Val Gly Asp
                325                 330                 335

Glu Met Gly Thr Val Arg Val Ala Ser Thr Met Thr Asp Gly Ala Asn
            340                 345                 350

Thr Met Ile Glu His Asp Asp Thr Leu Pro Ser Gln Leu Gly Thr Met
        355                 360                 365

Val Ile Asn Ala Glu Asp Glu Glu Glu Glu Gly Thr Met Lys Arg Arg
    370                 375                 380

Asp Glu Thr Met Gln Pro Ala Lys Pro Ser Phe Leu Glu Tyr Phe Glu
385                 390                 395                 400

Gln Lys Glu Lys Glu Asn Gln Ile Asn Ser Phe Gly Lys Ser Val Pro
                405                 410                 415

Gly Pro Leu Lys Asn Ser Ser Asp Trp Lys Ile Pro Gln Asp Gly Asp
            420                 425                 430

Tyr Glu Phe Leu Lys Ser Trp Thr Val Glu Asp Leu Gln Lys Arg Leu
        435                 440                 445

Leu Ala Leu Asp Pro Met Met Glu Gln Glu Ile Glu Glu Ile Arg Gln
    450                 455                 460

Lys Tyr Gln Ser Lys Arg Gln Pro Ile Leu Asp Ala Ile Glu Ala Lys
465                 470                 475                 480

Lys Arg Arg Gln Gln Asn Phe
                485
```

<210> 10
<211> 882
<212> PRT
<213> Homo sapiens

<400> 10

```
Met Lys Tyr Ser Cys Cys Ala Leu Val Leu Ala Val Leu Gly Thr Glu
1               5                   10                  15

Leu Leu Gly Ser Leu Cys Ser Thr Val Arg Ser Pro Arg Phe Arg Gly
            20                  25                  30

Arg Ile Gln Gln Glu Arg Lys Asn Ile Arg Pro Asn Ile Ile Leu Val
        35                  40                  45

Leu Thr Asp Asp Gln Asp Val Glu Leu Gly Ser Leu Gln Val Met Asn
    50                  55                  60
```

```
Lys Thr Arg Lys Ile Met Glu His Gly Gly Ala Thr Phe Ile Asn Ala
65                  70                  75                  80

Phe Val Thr Thr Pro Met Cys Cys Pro Ser Arg Ser Ser Met Leu Thr
                85                  90                  95

Gly Lys Tyr Val His Asn His Asn Val Tyr Thr Asn Asn Glu Asn Cys
            100                 105                 110

Ser Ser Pro Ser Trp Gln Ala Met His Glu Pro Arg Thr Phe Ala Val
        115                 120                 125

Tyr Leu Asn Asn Thr Gly Tyr Arg Thr Ala Phe Phe Gly Lys Tyr Leu
    130                 135                 140

Asn Glu Tyr Asn Gly Ser Tyr Ile Pro Pro Gly Trp Arg Glu Trp Leu
145                 150                 155                 160

Gly Leu Ile Lys Asn Ser Arg Phe Tyr Asn Tyr Thr Val Cys Arg Asn
                165                 170                 175

Gly Ile Lys Glu Lys His Gly Phe Asp Tyr Ala Lys Asp Tyr Phe Thr
            180                 185                 190

Asp Leu Ile Thr Asn Glu Ser Ile Asn Tyr Phe Lys Met Ser Lys Arg
        195                 200                 205

Met Tyr Pro His Arg Pro Val Met Met Val Ile Ser His Ala Ala Pro
    210                 215                 220

His Gly Pro Glu Asp Ser Ala Pro Gln Phe Ser Lys Leu Tyr Pro Asn
225                 230                 235                 240

Ala Ser Gln His Ile Thr Pro Ser Tyr Asn Tyr Ala Pro Asn Met Asp
                245                 250                 255

Lys His Trp Ile Met Gln Tyr Thr Gly Pro Met Leu Pro Ile His Met
            260                 265                 270

Glu Phe Thr Asn Ile Leu Gln Arg Lys Arg Leu Gln Thr Leu Met Ser
        275                 280                 285

Val Asp Asp Ser Val Glu Arg Leu Tyr Asn Met Leu Val Glu Thr Gly
    290                 295                 300

Glu Leu Glu Asn Thr Tyr Ile Ile Tyr Thr Ala Asp His Gly Tyr His
305                 310                 315                 320

Ile Gly Gln Phe Gly Leu Val Lys Gly Lys Ser Met Pro Tyr Asp Phe
                325                 330                 335
```

```
Asp Ile Arg Val Pro Phe Phe Ile Arg Gly Pro Ser Val Glu Pro Gly
            340                 345                 350

Ser Ile Val Pro Gln Ile Val Leu Asn Ile Asp Leu Ala Pro Thr Ile
        355                 360                 365

Leu Asp Ile Ala Gly Leu Asp Thr Pro Pro Asp Val Asp Gly Lys Ser
    370                 375                 380

Val Leu Lys Leu Leu Asp Pro Glu Lys Pro Gly Asn Arg Phe Arg Thr
385                 390                 395                 400

Asn Lys Lys Ala Lys Ile Trp Arg Asp Thr Phe Leu Val Glu Arg Gly
                405                 410                 415

Lys Phe Leu Arg Lys Lys Glu Glu Ser Ser Lys Asn Ile Gln Gln Ser
            420                 425                 430

Asn His Leu Pro Lys Tyr Glu Arg Val Lys Glu Leu Cys Gln Gln Ala
        435                 440                 445

Arg Tyr Gln Thr Ala Cys Glu Gln Pro Gly Gln Lys Trp Gln Cys Ile
    450                 455                 460

Glu Asp Thr Ser Gly Lys Leu Arg Ile His Lys Cys Lys Gly Pro Ser
465                 470                 475                 480

Asp Leu Leu Thr Val Arg Gln Ser Thr Arg Asn Leu Tyr Ala Arg Gly
                485                 490                 495

Phe His Asp Lys Asp Lys Glu Cys Ser Cys Arg Glu Ser Gly Tyr Arg
            500                 505                 510

Ala Ser Arg Ser Gln Arg Lys Ser Gln Arg Gln Phe Leu Arg Asn Gln
        515                 520                 525

Gly Thr Pro Lys Tyr Lys Pro Arg Phe Val His Thr Arg Gln Thr Arg
    530                 535                 540

Ser Leu Ser Val Glu Phe Glu Gly Glu Ile Tyr Asp Ile Asn Leu Glu
545                 550                 555                 560

Glu Glu Glu Glu Leu Gln Val Leu Gln Pro Arg Asn Ile Ala Lys Arg
                565                 570                 575

His Asp Glu Gly His Lys Gly Pro Arg Asp Leu Gln Ala Ser Ser Gly
            580                 585                 590

Gly Asn Arg Gly Arg Met Leu Ala Asp Ser Ser Asn Ala Val Gly Pro
        595                 600                 605
```

```
Pro Thr Thr Val Arg Val Thr His Lys Cys Phe Ile Leu Pro Asn Asp
    610                 615                 620

Ser Ile His Cys Glu Arg Glu Leu Tyr Gln Ser Ala Arg Ala Trp Lys
625                 630                 635                 640

Asp His Lys Ala Tyr Ile Asp Lys Glu Ile Glu Ala Leu Gln Asp Lys
                645                 650                 655

Ile Lys Asn Leu Arg Glu Val Arg Gly His Leu Lys Arg Arg Lys Pro
            660                 665                 670

Glu Glu Cys Ser Cys Ser Lys Gln Ser Tyr Tyr Asn Lys Glu Lys Gly
        675                 680                 685

Val Lys Lys Gln Glu Lys Leu Lys Ser His Leu His Pro Phe Lys Glu
    690                 695                 700

Ala Ala Gln Glu Val Asp Ser Lys Leu Gln Leu Phe Lys Glu Asn Asn
705                 710                 715                 720

Arg Arg Arg Lys Lys Glu Arg Lys Glu Lys Arg Arg Gln Arg Lys Gly
                725                 730                 735

Glu Glu Cys Ser Leu Pro Gly Leu Thr Cys Phe Thr His Asp Asn Asn
            740                 745                 750

His Trp Gln Thr Ala Pro Phe Trp Asn Leu Gly Ser Phe Cys Ala Cys
        755                 760                 765

Thr Ser Ser Asn Asn Asn Thr Tyr Trp Cys Leu Arg Thr Val Asn Glu
    770                 775                 780

Thr His Asn Phe Leu Phe Cys Glu Phe Ala Thr Gly Phe Leu Glu Tyr
785                 790                 795                 800

Phe Asp Met Asn Thr Asp Pro Tyr Gln Leu Thr Asn Thr Val His Thr
                805                 810                 815

Val Glu Arg Gly Ile Leu Asn Gln Leu His Val Gln Leu Met Glu Leu
            820                 825                 830

Arg Ser Cys Gln Gly Tyr Lys Gln Cys Asn Pro Arg Pro Lys Asn Leu
        835                 840                 845

Asp Val Gly Asn Lys Asp Gly Gly Ser Tyr Asp Arg Thr Val Met Gly
    850                 855                 860

Trp Met Gly Arg Leu Ile Ser Pro Val Ser Leu Gln Thr Ser Thr Gly
865                 870                 875                 880
```

Lys Ala

<210> 11
<211> 1141
<212> PRT
<213> Homo sapiens

<400> 11

```
Met Asp Asp Ser Gly Glu Leu Gly Gly Leu Glu Thr Met Glu Thr Leu
1               5                  10                  15

Thr Glu Leu Gly Asp Glu Leu Thr Leu Gly Asp Ile Asp Glu Met Leu
            20                  25                  30

Gln Phe Val Ser Asn Gln Val Gly Glu Phe Pro Asp Leu Phe Ser Glu
        35                  40                  45

Gln Leu Cys Ser Ser Phe Pro Gly Ser Gly Gly Ser Gly Ser Ser Ser
    50                  55                  60

Gly Ser Ser Gly Ser Ser Ser Ser Ser Ser Asn Gly Arg Gly Ser Ser
65                  70                  75                  80

Ser Gly Ala Val Asp Pro Ser Val Gln Arg Ser Phe Thr Gln Val Thr
                85                  90                  95

Leu Pro Ser Phe Ser Pro Ser Ala Ala Ser Pro Gln Ala Pro Thr Leu
            100                 105                 110

Gln Val Lys Val Ser Pro Thr Ser Val Pro Thr Thr Pro Arg Ala Thr
        115                 120                 125

Pro Ile Leu Gln Pro Arg Pro Gln Pro Gln Pro Gln Pro Gln Thr Gln
    130                 135                 140

Leu Gln Gln Gln Thr Val Met Ile Thr Pro Thr Phe Ser Thr Thr Pro
145                 150                 155                 160

Gln Thr Arg Ile Ile Gln Gln Pro Leu Ile Tyr Gln Asn Ala Ala Thr
                165                 170                 175

Ser Phe Gln Val Leu Gln Pro Gln Val Gln Ser Leu Val Thr Ser Ser
            180                 185                 190

Gln Val Gln Pro Val Thr Ile Gln Gln Gln Val Gln Thr Val Gln Ala
        195                 200                 205

Gln Arg Val Leu Thr Gln Thr Ala Asn Gly Thr Leu Gln Thr Leu Ala
    210                 215                 220
```

```
Pro Ala Thr Val Gln Thr Val Ala Ala Pro Gln Val Gln Gln Val Pro
225                 230                 235                 240

Val Leu Val Gln Pro Gln Ile Ile Lys Thr Asp Ser Leu Val Leu Thr
                245                 250                 255

Thr Leu Lys Thr Asp Gly Ser Pro Val Met Ala Ala Val Gln Asn Pro
            260                 265                 270

Ala Leu Thr Ala Leu Thr Thr Pro Ile Gln Thr Ala Ala Leu Gln Val
        275                 280                 285

Pro Thr Leu Val Gly Ser Ser Gly Thr Ile Leu Thr Thr Met Pro Val
    290                 295                 300

Met Met Gly Gln Glu Lys Val Pro Ile Lys Gln Val Pro Gly Gly Val
305                 310                 315                 320

Lys Gln Leu Glu Pro Pro Lys Glu Gly Glu Arg Arg Thr Thr His Asn
                325                 330                 335

Ile Ile Glu Lys Arg Tyr Arg Ser Ser Ile Asn Asp Lys Ile Ile Glu
            340                 345                 350

Leu Lys Asp Leu Val Met Gly Thr Asp Ala Lys Met His Lys Ser Gly
        355                 360                 365

Val Leu Arg Lys Ala Ile Asp Tyr Ile Lys Tyr Leu Gln Gln Val Asn
    370                 375                 380

His Lys Leu Arg Gln Glu Asn Met Val Leu Lys Leu Ala Asn Gln Lys
385                 390                 395                 400

Asn Lys Leu Leu Lys Gly Ile Asp Leu Gly Ser Leu Val Asp Asn Glu
                405                 410                 415

Val Asp Leu Lys Ile Glu Asp Phe Asn Gln Asn Val Leu Leu Met Ser
            420                 425                 430

Pro Pro Ala Ser Asp Ser Gly Ser Gln Ala Gly Phe Ser Pro Tyr Ser
        435                 440                 445

Ile Asp Ser Glu Pro Gly Ser Pro Leu Leu Asp Asp Ala Lys Val Lys
    450                 455                 460

Asp Glu Pro Asp Ser Pro Pro Val Ala Leu Gly Met Val Asp Arg Ser
465                 470                 475                 480

Arg Ile Leu Leu Cys Val Leu Thr Phe Leu Cys Leu Ser Phe Asn Pro
                485                 490                 495
```

```
Leu Thr Ser Leu Leu Gln Trp Gly Gly Ala His Asp Ser Asp Gln His
            500                 505                 510

Pro His Ser Gly Ser Gly Arg Ser Val Leu Ser Phe Glu Ser Gly Ser
        515                 520                 525

Gly Gly Trp Phe Asp Trp Met Met Pro Thr Leu Leu Leu Trp Leu Val
    530                 535                 540

Asn Gly Val Ile Val Leu Ser Val Phe Val Lys Leu Leu Val His Gly
545                 550                 555                 560

Glu Pro Val Ile Arg Pro His Ser Arg Ser Ser Val Thr Phe Trp Arg
                565                 570                 575

His Arg Lys Gln Ala Asp Leu Asp Leu Ala Arg Gly Asp Phe Ala Ala
            580                 585                 590

Ala Ala Gly Asn Leu Gln Thr Cys Leu Ala Val Leu Gly Arg Ala Leu
        595                 600                 605

Pro Thr Ser Arg Leu Asp Leu Ala Cys Ser Leu Ser Trp Asn Val Ile
    610                 615                 620

Arg Tyr Ser Leu Gln Lys Leu Arg Leu Val Arg Trp Leu Leu Lys Lys
625                 630                 635                 640

Val Phe Gln Cys Arg Arg Ala Thr Pro Ala Thr Glu Ala Gly Phe Glu
                645                 650                 655

Asp Glu Ala Lys Thr Ser Ala Arg Asp Ala Ala Leu Ala Tyr His Arg
            660                 665                 670

Leu His Gln Leu His Ile Thr Gly Lys Leu Pro Ala Gly Ser Ala Cys
        675                 680                 685

Ser Asp Val His Met Ala Leu Cys Ala Val Asn Leu Ala Glu Cys Ala
    690                 695                 700

Glu Glu Lys Ile Pro Pro Ser Thr Leu Val Glu Ile His Leu Thr Ala
705                 710                 715                 720

Ala Met Gly Leu Lys Thr Arg Cys Gly Gly Lys Leu Gly Phe Leu Ala
                725                 730                 735

Ser Tyr Phe Leu Ser Arg Ala Gln Ser Leu Cys Gly Pro Glu His Ser
            740                 745                 750

Ala Val Pro Asp Ser Leu Arg Trp Leu Cys His Pro Leu Gly Gln Lys
        755                 760                 765
```

```
Phe Phe Met Glu Arg Ser Trp Ser Val Lys Ser Ala Ala Lys Glu Ser
    770                 775                 780

Leu Tyr Cys Ala Gln Arg Asn Pro Ala Asp Pro Ile Ala Gln Val His
785                 790                 795                 800

Gln Ala Phe Cys Lys Asn Leu Leu Glu Arg Ala Ile Glu Ser Leu Val
                805                 810                 815

Lys Pro Gln Ala Lys Lys Lys Ala Gly Asp Gln Glu Glu Glu Ser Cys
            820                 825                 830


Glu Phe Ser Ser Ala Leu Glu Tyr Leu Lys Leu Leu His Ser Phe Val
        835                 840                 845

Asp Ser Val Gly Val Met Ser Pro Pro Leu Ser Arg Ser Ser Val Leu
    850                 855                 860

Lys Ser Ala Leu Gly Pro Asp Ile Ile Cys Arg Trp Trp Thr Ser Ala
865                 870                 875                 880

Ile Thr Val Ala Ile Ser Trp Leu Gln Gly Asp Asp Ala Ala Val Arg
                885                 890                 895

Ser His Phe Thr Lys Val Glu Arg Ile Pro Lys Ala Leu Glu Val Thr
            900                 905                 910

Glu Ser Pro Leu Val Lys Ala Ile Phe His Ala Cys Arg Ala Met His
        915                 920                 925

Ala Ser Leu Pro Gly Lys Ala Asp Gly Gln Gln Ser Ser Phe Cys His
    930                 935                 940

Cys Glu Arg Ala Ser Gly His Leu Trp Ser Ser Leu Asn Val Ser Gly
945                 950                 955                 960

Ala Thr Ser Asp Pro Ala Leu Asn His Val Val Gln Leu Leu Thr Cys
                965                 970                 975

Asp Leu Leu Leu Ser Leu Arg Thr Ala Leu Trp Gln Lys Gln Ala Ser
            980                 985                 990

Ala Ser Gln Ala Val Gly Glu Thr  Tyr His Ala Ser Gly  Ala Glu Leu
        995                 1000                1005

Ala Gly  Phe Gln Arg Asp Leu  Gly Ser Leu Arg Arg  Leu Ala His
    1010                1015                1020

Ser Phe  Arg Pro Ala Tyr Arg  Lys Val Phe Leu His  Glu Ala Thr
    1025                1030                1035
```

```
Val Arg  Leu Met Ala Gly Ala  Ser Pro Thr Arg Thr  His Gln Leu
    1040                1045                1050

Leu Glu  His Ser Leu Arg Arg  Arg Thr Thr Gln Ser  Thr Lys His
    1055                1060                1065

Gly Glu  Val Asp Ala Trp Pro  Gly Gln Arg Glu Arg  Ala Thr Ala
    1070                1075                1080


Ile Leu  Leu Ala Cys Arg His  Leu Pro Leu Ser Phe  Leu Ser Ser
    1085                1090                1095

Pro Gly  Gln Arg Ala Val Leu  Leu Ala Glu Ala Ala  Arg Thr Leu
    1100                1105                1110

Glu Lys  Val Gly Asp Arg Arg  Ser Cys Asn Asp Cys  Gln Gln Met
    1115                1120                1125

Ile Val  Lys Leu Gly Gly Gly  Thr Ala Ile Ala Ala  Ser
    1130                1135                1140
```

<210> 12
<211> 976
<212> PRT
<213> Homo sapiens

<400> 12

```
Met Ala Gln Val Ala Met Ser Thr Leu Pro Val Glu Asp Glu Glu Ser
1               5                  10                  15

Ser Glu Ser Arg Met Val Val Thr Phe Leu Met Ser Ala Leu Glu Ser
            20                  25                  30

Met Cys Lys Glu Leu Ala Lys Ser Lys Ala Glu Val Ala Cys Ile Ala
        35                  40                  45

Val Tyr Glu Thr Asp Val Phe Val Val Gly Thr Glu Arg Gly Arg Ala
    50                  55                  60

Phe Val Asn Thr Arg Lys Asp Phe Gln Lys Asp Phe Val Lys Tyr Cys
65                  70                  75                  80

Val Glu Glu Glu Glu Lys Ala Ala Glu Met His Lys Met Lys Ser Thr
                85                  90                  95

Thr Gln Ala Asn Arg Met Ser Val Asp Ala Val Glu Ile Glu Thr Leu
            100                 105                 110

Arg Lys Thr Val Glu Asp Tyr Phe Cys Phe Cys Tyr Gly Lys Ala Leu
        115                 120                 125
```

```
Gly Lys Ser Thr Val Val Pro Val Pro Tyr Glu Lys Met Leu Arg Asp
    130                 135                 140

Gln Ser Ala Val Val Val Gln Gly Leu Pro Glu Gly Val Ala Phe Lys
145                 150                 155                 160

His Pro Glu Asn Tyr Asp Leu Ala Thr Leu Lys Trp Ile Leu Glu Asn
                165                 170                 175

Lys Ala Gly Ile Ser Phe Ile Ile Lys Arg Pro Phe Leu Glu Pro Lys
            180                 185                 190

Lys His Val Gly Gly Arg Val Met Val Thr Asp Ala Asp Arg Ser Ile
        195                 200                 205

Leu Ser Pro Gly Gly Ser Cys Gly Pro Ile Lys Val Lys Thr Glu Pro
    210                 215                 220

Thr Glu Asp Ser Gly Ile Ser Leu Glu Met Ala Ala Val Thr Val Lys
225                 230                 235                 240

Glu Glu Ser Glu Asp Pro Asp Tyr Tyr Gln Tyr Asn Ile Gln Gly Pro
                245                 250                 255

Ser Glu Thr Asp Asp Val Asp Glu Lys Gln Pro Leu Ser Lys Pro Leu
            260                 265                 270

Gln Gly Ser His His Ser Ser Glu Gly Asn Glu Gly Thr Glu Met Glu
        275                 280                 285

Val Pro Ala Glu Asp Asp Asp Tyr Ser Pro Pro Ser Lys Arg Pro Lys
    290                 295                 300

Ala Asn Glu Leu Pro Gln Pro Pro Val Pro Glu Pro Ala Asn Ala Gly
305                 310                 315                 320

Lys Arg Lys Val Arg Glu Phe Asn Phe Glu Lys Trp Asn Ala Arg Ile
                325                 330                 335

Thr Asp Leu Arg Lys Gln Val Glu Glu Leu Phe Glu Arg Lys Tyr Ala
            340                 345                 350

Gln Ala Ile Lys Ala Lys Gly Pro Val Thr Ile Pro Tyr Pro Leu Phe
        355                 360                 365

Gln Ser His Val Glu Asp Leu Tyr Val Glu Gly Leu Pro Glu Gly Ile
    370                 375                 380

Pro Phe Arg Arg Pro Ser Thr Tyr Gly Ile Pro Arg Leu Glu Arg Ile
385                 390                 395                 400
```

```
Leu Leu Ala Lys Glu Arg Ile Arg Phe Val Ile Lys Lys His Glu Leu
                405                 410                 415

Leu Asn Ser Thr Arg Glu Asp Leu Gln Leu Asp Lys Pro Ala Ser Gly
            420                 425                 430

Val Lys Glu Glu Trp Tyr Ala Arg Ile Thr Lys Leu Arg Lys Met Val
        435                 440                 445

Asp Gln Leu Phe Cys Lys Lys Phe Ala Glu Ala Leu Gly Ser Thr Glu
    450                 455                 460

Ala Lys Ala Val Pro Tyr Gln Lys Phe Glu Ala His Pro Asn Asp Leu
465                 470                 475                 480

Tyr Val Glu Gly Leu Pro Glu Asn Ile Pro Phe Arg Ser Pro Ser Trp
                485                 490                 495

Tyr Gly Ile Pro Arg Leu Glu Lys Ile Ile Gln Val Gly Asn Arg Ile
            500                 505                 510

Lys Phe Val Ile Lys Arg Pro Glu Leu Leu Thr His Ser Thr Thr Glu
        515                 520                 525

Val Thr Gln Pro Arg Thr Asn Thr Pro Val Lys Glu Asp Trp Asn Val
    530                 535                 540

Arg Ile Thr Lys Leu Arg Lys Gln Val Glu Glu Ile Phe Asn Leu Lys
545                 550                 555                 560

Phe Ala Gln Ala Leu Gly Leu Thr Glu Ala Val Lys Val Pro Tyr Pro
                565                 570                 575

Val Phe Glu Ser Asn Pro Glu Phe Leu Tyr Val Glu Gly Leu Pro Glu
            580                 585                 590

Gly Ile Pro Phe Arg Ser Pro Thr Trp Phe Gly Ile Pro Arg Leu Glu
        595                 600                 605

Arg Ile Val Arg Gly Ser Asn Lys Ile Lys Phe Val Val Lys Lys Pro
    610                 615                 620

Glu Leu Val Ile Ser Tyr Leu Pro Pro Gly Met Ala Ser Lys Ile Asn
625                 630                 635                 640

Thr Lys Ala Leu Gln Ser Pro Lys Arg Pro Arg Ser Pro Gly Ser Asn
                645                 650                 655

Ser Lys Val Pro Glu Ile Glu Val Thr Val Glu Gly Pro Asn Asn Asn
            660                 665                 670
```

```
Asn Pro Gln Thr Ser Ala Val Arg Thr Pro Thr Gln Thr Asn Gly Ser
        675                 680                 685

Asn Val Pro Phe Lys Pro Arg Gly Arg Glu Phe Ser Phe Glu Ala Trp
    690                 695                 700

Asn Ala Lys Ile Thr Asp Leu Lys Gln Lys Val Glu Asn Leu Phe Asn
705                 710                 715                 720

Glu Lys Cys Gly Glu Ala Leu Gly Leu Lys Gln Ala Val Lys Val Pro
                725                 730                 735

Phe Ala Leu Phe Glu Ser Phe Pro Glu Asp Phe Tyr Val Glu Gly Leu
            740                 745                 750

Pro Glu Gly Val Pro Phe Arg Arg Pro Ser Thr Phe Gly Ile Pro Arg
        755                 760                 765

Leu Glu Lys Ile Leu Arg Asn Lys Ala Lys Ile Lys Phe Ile Ile Lys
    770                 775                 780

Lys Pro Glu Met Phe Glu Thr Ala Ile Lys Glu Ser Thr Ser Ser Lys
785                 790                 795                 800

Ser Pro Pro Arg Lys Ile Asn Ser Ser Pro Asn Val Asn Thr Thr Ala
                805                 810                 815

Ser Gly Val Glu Asp Leu Asn Ile Ile Gln Val Thr Ile Pro Asp Asp
            820                 825                 830

Asp Asn Glu Arg Leu Ser Lys Val Glu Lys Ala Arg Gln Leu Arg Glu
        835                 840                 845

Gln Val Asn Asp Leu Phe Ser Arg Lys Phe Gly Glu Ala Ile Gly Met
    850                 855                 860

Gly Phe Pro Val Lys Val Pro Tyr Arg Lys Ile Thr Ile Asn Pro Gly
865                 870                 875                 880

Cys Val Val Val Asp Gly Met Pro Pro Gly Val Ser Phe Lys Ala Pro
                885                 890                 895

Ser Tyr Leu Glu Ile Ser Ser Met Arg Arg Ile Leu Asp Ser Ala Glu
            900                 905                 910

Phe Ile Lys Phe Thr Val Ile Arg Pro Phe Pro Gly Leu Val Ile Asn
        915                 920                 925

Asn Gln Leu Val Asp Gln Ser Glu Ser Glu Gly Pro Val Ile Gln Glu
    930                 935                 940
```

Ser Ala Glu Pro Ser Gln Leu Glu Val Pro Ala Thr Glu Glu Ile Lys
945 950 955 960

Glu Thr Asp Gly Ser Ser Gln Ile Lys Gln Glu Pro Asp Pro Thr Trp
965 970 975

<210> 13
<211> 21
<212> DNA
<213> Artificial

<220>
<223> Forward primer T7_up2

<400> 13
tgctaaggac aacgttatcg g 21

<210> 14
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Reverse primer T7_down2

<400> 14
ttgataccgg acgttcac 18