



US011105809B2

(12) **United States Patent**
Fishelson et al.

(10) **Patent No.: US 11,105,809 B2**
(45) **Date of Patent: Aug. 31, 2021**

(54) **METHODS AND KITS FOR PREDICTING PROGNOSIS OF CANCER USING SOLUBLE MORTALIN IN BLOOD**

(71) Applicant: **Ramot at Tel-Aviv University Ltd.,**
Tel-Aviv (IL)

(72) Inventors: **Zvi Fishelson, Tel-Aviv (IL); Zoltan Prohaszka, Budapest (HU)**

(73) Assignee: **Ramot at Tel-Aviv University Ltd.,**
Tel-Aviv (IL)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 459 days.

(21) Appl. No.: **14/434,164**

(22) PCT Filed: **Oct. 9, 2013**

(86) PCT No.: **PCT/IL2013/050817**

§ 371 (c)(1),

(2) Date: **Apr. 8, 2015**

(87) PCT Pub. No.: **WO2014/057490**

PCT Pub. Date: **Apr. 17, 2014**

(65) **Prior Publication Data**

US 2015/0268242 A1 Sep. 24, 2015

Related U.S. Application Data

(60) Provisional application No. 61/711,327, filed on Oct. 9, 2012.

(51) **Int. Cl.**

G01N 31/00 (2006.01)

G01N 33/53 (2006.01)

G01N 33/574 (2006.01)

(52) **U.S. Cl.**

CPC . **G01N 33/57488** (2013.01); **G01N 33/57407** (2013.01); **G01N 33/57419** (2013.01); **G01N 2333/4703** (2013.01)

(58) **Field of Classification Search**

CPC C12Q 1/6811; C12Q 1/6886; G01N 33/57488; C12N 15/115; C12N 2310/16

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,627,039 A * 5/1997 Pereira-Smith G01N 33/57496 435/7.23

7,700,307 B2 * 4/2010 Murray G01N 33/57419 435/7.23

7,883,702 B2 2/2011 Wadhwa et al.

8,293,716 B2 * 10/2012 Fishelson C07K 16/18 514/44 A

8,470,793 B2 * 6/2013 Fishelson A61K 31/713 424/138.1

2006/0270622 A1 11/2006 Fishelson et al.

2009/0023657 A1 1/2009 Gabriele et al.

FOREIGN PATENT DOCUMENTS

EP	1243923 A1 *	9/2002	G01N 33/5011
WO	WO 2004/079368	9/2004		
WO	WO 2007/071045	6/2007		
WO	WO 2008/032324	3/2008		
WO	WO 2009/040819	4/2009		
WO	WO 2011/071099	6/2011		
WO	WO 2013/190075	12/2013		
WO	WO 2014/057490	4/2014		

OTHER PUBLICATIONS

Iosefson et al. (Cell Stress and Chaperones, Jan. 2012, vol. 17, pp. 57-66).*

Iosefson et al. (Cell Stress and Chaperones, vol. 17, pp. 57-66, 2012).*

StressMarq Biosciences Inc., Victoria Canada Product Sheet 2018.*

Santa Cruz Biotechnology, Santa Cruz, CA, Product Sheet, 2018.*

Santa Cruz Biotechnology, Santa Cruz, CA, Product List, 2018.*

Iosefson et al. (Cell Stress and Chaperones, 2012, vol. 17, pp. 57-66).*

Molvarec et al. (Journal of Human Hypertension, vol. 20, pp. 780-786, 2006) (Year: 2006).*

Wadhwa et al. (Biochem J. vol. 391, pp. 185-190, 2005) (Year: 2005).*

Kocsis et al. (Cell Stress & Chaperones, Mar. 2010, vol. 15, No. 2, pp. 143-151) (Year: 2010).*

Srokowski et al. "Expression and Localization of GRP75 in Human Epithelial Tumors and Normal Tissues", Applied Immunohistochemistry & Molecular Morphology, XP008179660, 12(2): 132-138, Jun. 2004. Title, Abstract, Materials and Method Section, Figs.3, 4.

International Preliminary Report on Patentability dated Apr. 23, 2015 From the International Bureau of WIPO Re. Application No. PCT/IL2013/050817.

International Search Report and the Written Opinion dated Dec. 29, 2013 From the International Searching Authority Re. Application No. PCT/IL2013/050817.

Czarniecka et al. "Mitochondrial Chaperones in Cancer. From Molecular Biology to Clinical Diagnostics", Cancer Biology & Therapy, 5(7): 714-720, Jul. 31, 2006.

De Maio "Extracellular Heat Shock Proteins, Cellular Export Vesicles, and the Stress Observation System: A Form of Communication During Injury, Infection, and Cell Damage", Cell Stress and Chaperones, 16: 235-249, Published Online Oct. 21, 2010.

Dundas et al. "Mortalin Is Over-Expressed by Colorectal Adenocarcinomas and Correlates With Poor Survival", Journal of Pathology, 205(1): 74-81, Mar. 31, 2005. Abstract.

(Continued)

Primary Examiner — Lisa V Cook

(57) **ABSTRACT**

Provided are methods of determining a presence or level of soluble mortalin in a body fluid of a subject, and predicting prognosis of a subject diagnosed with cancer (e.g., solid tumor) by determining the presence or level of soluble mortalin in a body fluid of the subject. Also provided are kits for determining presence and/or level of soluble mortalin in a body fluid of the subject and/or predicting prognosis of a subject diagnosed with cancer, comprising at least two distinct antibodies to mortalin directed against different epitopes of said mortalin.

11 Claims, 3 Drawing Sheets

Specification includes a Sequence Listing.

(56)

References Cited

OTHER PUBLICATIONS

Kocsis et al. "High Levels of Acute Phase Proteins and Soluble 70 kDa Heat Shock Proteins Are Independent and Additive Risk Factors for Mortality in Colorectal Cancer", *Cell stress and Chaperones*, 16: 49-55, Published Online Aug. 22, 2010.

Kocsis et al. "Serum Level of Soluble 70-kD Heat Shock Protein Is Associated With High Mortality in Patients With Colorectal Cancer Without Distant Metastasis", *Cell Stress and Chaperones*, 15: 143-151, Published Online Jul. 4, 2009.

Kocsis et al. "Serum Level of Soluble 70-kD Heat Shock Protein Is Associated With High Mortality in Patients With Colorectal Cancer Without Distant Metastasis", *Cell Stress and Chaperones*, 15: 143-151, Published Online Jul. 4, 2009.

Koji et al. "Significance of Mortalin Expression in Gastric Cancer With Normal P53", General Poster Session A: Cancers of the Esophagus and Stomach, 2012 Gastrointestinal Cancers Symposium, Jul. 31, 2012, *Journal of Clinical Oncology*, 30(Suppl.4): # 25, Jul. 31, 2012.

Pilzer et al. "Emission of Membrane Vesicles: Roles in Complement Resistance, Immunity and Cancer", *Springer Seminars in Immunopathology*, 27(3): 375-387, Nov. 2005.

Pilzer et al. "Mortalin Inhibitors Sensitize K562 Leukemia Cells to Complement-Dependent Cytotoxicity", *International Journal of Cancer*, 126: 1428-1435, 2010.

Pilzer et al. "Mortalin/GRP75 Promotes Release of Membrane Vesicles From Immune Attacked Cells and Protection From Complement-Mediated Lysis", *International Immunology*, 17(9): 1239-1248, Aug. 9, 2005.

Rozenberg et al. "Elevated Levels of Mitochondrial Mortalin and Cytosolic HSP70 in Blood as Risk Factors in Patients With Colorectal Cancer", *International Journal of Cancer*, 133(2): 514-518, Jul. 15, 2013. Abstract. Abstract.

Takano et al. "Elevated Levels of Mortalin Expression in Human Brain Tumors", *Experimental Cell Research*, 237: 38-45, 1997.

Yi et al. "Association of Mortalin (HSPA9) With Liver Cancer Metastasis and Prediction for Early Tumor Recurrence", *Molecular & Cellular Proteomics*, 7(2): 315-325, 2008.

* cited by examiner

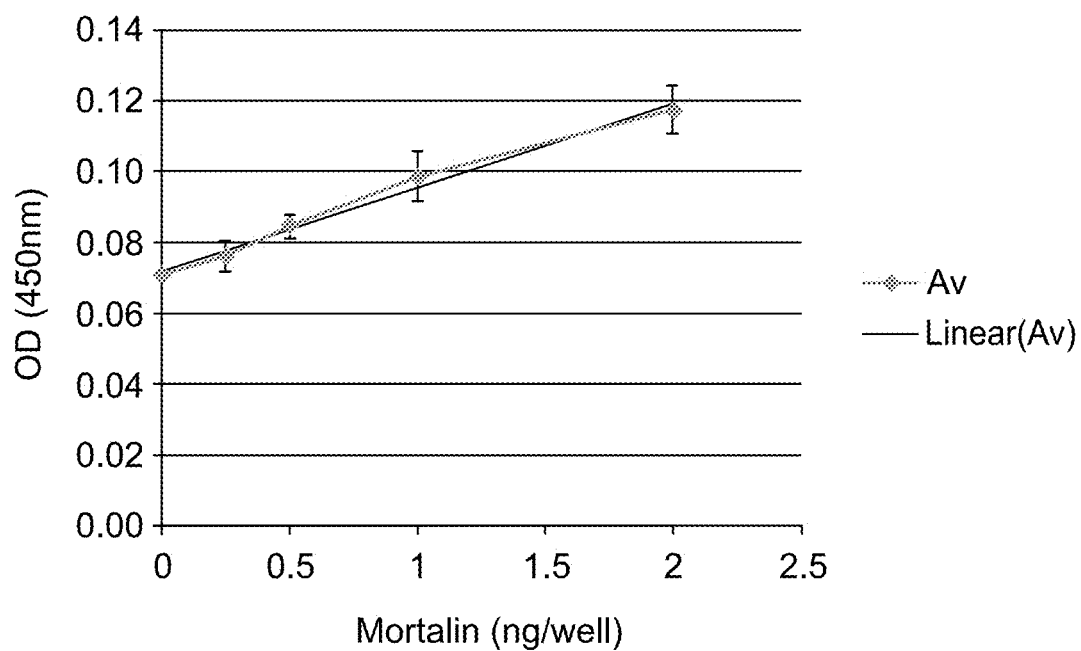


Figure 1

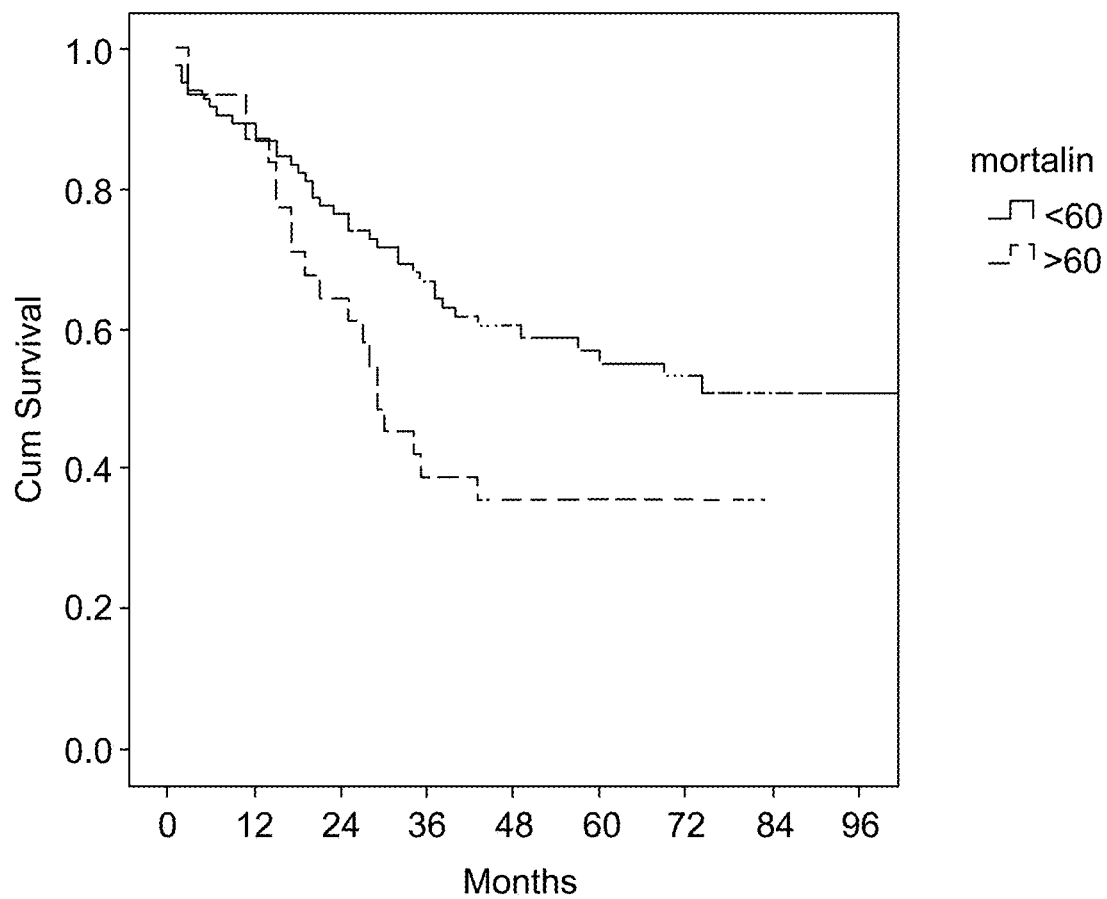


Figure 2

	Antibody 1:20 (18% lysis)		Antibody 1:16 (25% lysis)	
PBS	HIS	NHS	HIS	NHS

Figure 3

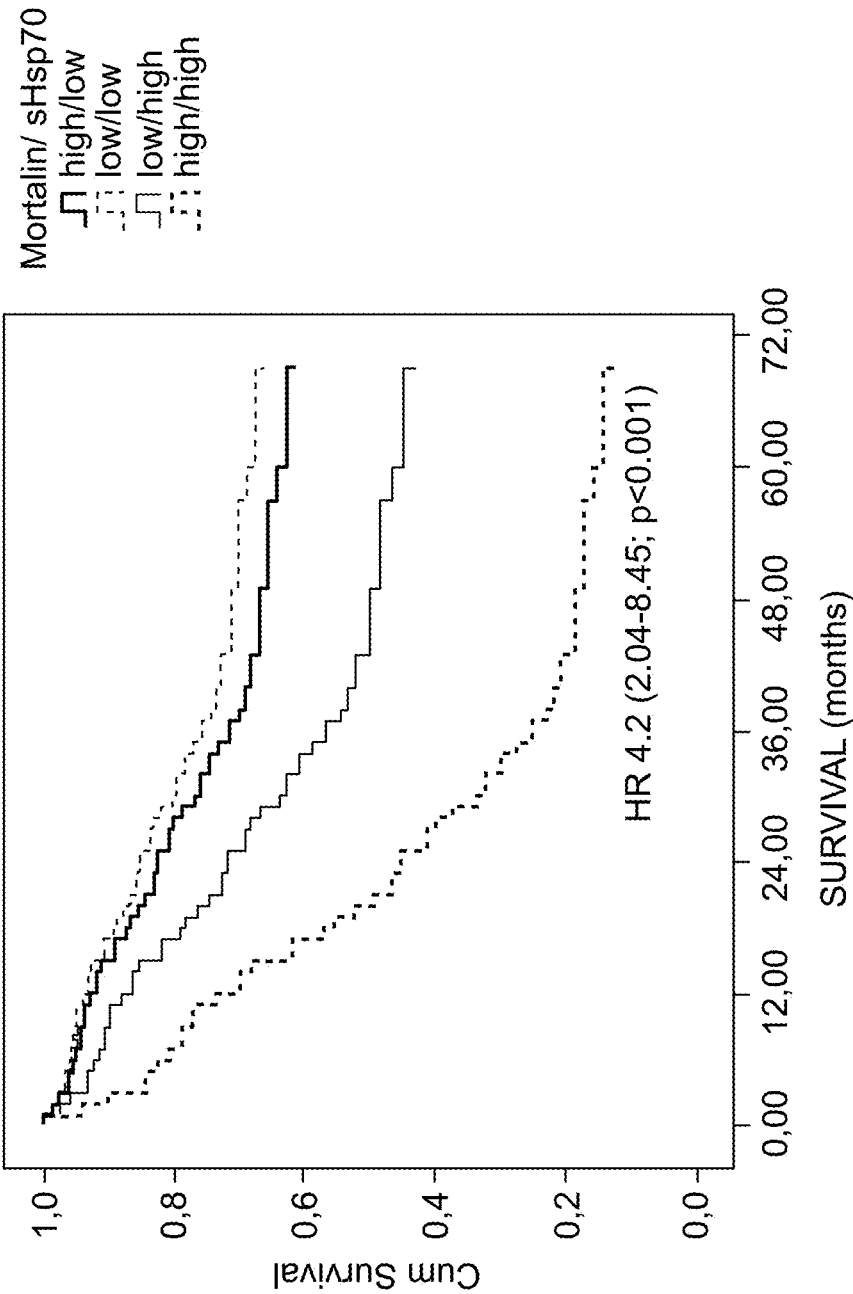


Figure 4

1

METHODS AND KITS FOR PREDICTING PROGNOSIS OF CANCER USING SOLUBLE MORTALIN IN BLOOD

RELATED APPLICATIONS

This application is a National Phase of PCT Patent Application No. PCT/IL2013/050817 having International filing date of Oct. 9, 2013, which claims the benefit of priority under 35 USC § 119(e) of U.S. Provisional Patent Application No. 61/711,327 filed on Oct. 9, 2012. The contents of the above applications are all incorporated by reference as if fully set forth herein in their entirety.

SEQUENCE LISTING STATEMENT

The ASCII file, entitled 62212SequenceListing.txt, created on Mar. 24, 2015, comprising 48,340 bytes, submitted concurrently with the filing of this application is incorporated herein by reference.

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods and kits for determining the presence and/or level of mortalin in a biological sample such body fluids of a subject, e.g., blood, and methods and kits for determining prognosis of a subject having a solid cancerous tumor, e.g., colorectal cancer, based on the level of mortalin in the biological sample (e.g., blood).

Mortalin (mthsp70/grp75), the mitochondrial heat shock protein 70, plays a major role in import and refolding of mitochondrial proteins. Mortalin is ubiquitously and constitutively expressed in all eukaryotic cells and its expression is not heat-induced, yet may be affected following ionizing radiation, glucose deprivation and calorie restriction. Mortalin is essential for cell growth and mitochondrial biogenesis. Over expression of mortalin protected cells from glucose deprivation and reactive oxygen species (ROS) accumulation and from serum deprivation, reduced oxidative stress, antagonized ischemic damage and promoted tumorigenesis. In contrast, knock down of mortalin by RNA interference caused senescence-like growth arrest in immortalized cells. Synthesized with a mitochondrial-targeting sequence, mortalin is mainly located in mitochondria, yet few reports identified it in other cellular compartments such as the cytosol and plasma membrane.

Several human transformed and tumor cells have been shown to express elevated levels of mortalin (14). In human colorectal adenocarcinoma, higher mortalin expression in situ (in tumors) correlated with poor patient survival (15). Mortalin plays a role in protection of cancer cells from complement-dependent cytotoxicity (16, 17) and it facilitates elimination of the complement membrane attack complex (MAC) from the cell surface by exo-vesiculation (16). Mortalin inhibitors sensitized the cells to complement-dependent cytotoxicity and inhibited the shedding of mortalin with the MAC (17).

Contrary to mortalin, the cytosolic hsp70 (also referred to as "sHSP70" hereinafter) was known to be released from cells (19), and was identified in blood plasma of patients with colorectal cancer (20). sHSP70 was found to be a useful, stage-independent prognostic marker in colorectal cancer, especially in patients without distant metastasis. Plasma levels of sHSP70 and acute phase proteins could independently predict survival in patients with colorectal

2

cancer but their combined measurements gave even a higher prediction value in specific subgroups of patients (21).

Additional related art include Pilzer, D., and Z. Fishelson. 2005 (Int Immunol 17:1239-1248); Pilzer, D., et al., 2005 (Springer Semin Immunopathol 27:375-387); WO2008032324 and WO2009/040819.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of predicting prognosis of a subject diagnosed with cancer (e.g., a solid tumor), comprising: determining presence and/or level of soluble mortalin in a body fluid of the subject, wherein

(i) when a level of the soluble mortalin is above a predetermined threshold, then the subject is predicted to have poor prognosis as compared to an expected prognosis based on the subject's tumor stage, age and/or gender; and
(ii) when a level of the soluble mortalin is below a predetermined threshold, then the subject has good prognosis as compared to an expected prognosis based on the subject's tumor stage, age and/or gender,

thereby predicting the prognosis of the subject diagnosed with the cancer (e.g., the solid tumor).

According to an aspect of some embodiments of the present invention there is provided a method of analyzing a presence and/or level of soluble mortalin in a biological sample (e.g., body fluid) of a subject, comprising: determining a presence or level of soluble mortalin in the biological sample, thereby analyzing the soluble mortalin in the biological sample (e.g., the body fluid) of the subject.

According to an aspect of some embodiments of the present invention there is provided a kit for determining presence and/or level of soluble mortalin in a biological sample (e.g., body fluid) of a subject and/or predicting prognosis of a subject diagnosed with a solid tumor, comprising: at least two distinct antibodies to mortalin directed against different epitopes of the mortalin.

According to some embodiments of the invention, the method of some embodiments of the invention, further comprising:

classifying the subject as having good or poor prognosis based on the level of the soluble mortalin, thereby predicting the prognosis of the subject diagnosed with the cancer (e.g., the solid tumor).

According to some embodiments of the invention, determining the presence or the level of the soluble mortalin is effected using an immunological method or a mass-spectroscopy.

According to some embodiments of the invention, the immunological method comprising:

(a) contacting the biological sample (e.g., body fluid) with an anti-mortalin antibody under conditions which allow immunocomplex formation; and

(b) determining a presence or level of the immunocomplex,

thereby determining the presence or the level of the soluble mortalin in the biological sample (e.g., body fluid).

According to some embodiments of the invention, the anti-mortalin antibody is bound to a solid support.

According to some embodiments of the invention, a presence or level of the immunocomplex is determined by surface plasmon resonance.

According to some embodiments of the invention, a presence or level of the immunocomplex is determined by Enzyme-linked immunosorbent assay (ELISA).

3

According to some embodiments of the invention, the soluble mortalin is not attached to a cell, to a cell membrane, or to a cell organelle.

According to some embodiments of the invention, wherein when the solid tumor is colorectal cancer, then the poor prognosis is characterized about 43 months mean survival time.

According to some embodiments of the invention, wherein when the solid tumor is colorectal cancer, then the good prognosis is characterized about 72 months mean survival time.

According to some embodiments of the invention, the method of some embodiments of the invention, further comprising:

(a) determining the level of soluble heat shock protein 70 (sHSP70) in the biological sample (e.g., the body fluid), wherein when a level of the soluble mortalin and the sHSP70, each is above a predetermined threshold in the biological sample (e.g., the body fluid), then the subject is predicted to have a poor prognosis as compared to an expected prognosis based on the subject's tumor's stage, age and/or gender; and

(b) classifying the subject as having good or poor prognosis based on the mortalin and the sHSP70 level,

thereby predicting the prognosis of the subject diagnosed with the solid tumor.

According to some embodiments of the invention, the kit of some embodiments of the invention, further comprising a secondary antibody for binding one of the at least two antibodies.

According to some embodiments of the invention, the kit of some embodiments of the invention, further comprising an Enzyme-linked immunosorbent assay (ELISA) plate.

According to some embodiments of the invention, the kit of some embodiments of the invention, further comprising an anti-soluble heat shock protein 70 (sHSP70) antibody.

According to some embodiments of the invention, the biological sample (e.g., the body fluid) is selected from the group consisting of blood, urine, tear, saliva, stool, cerebrospinal fluid, synovial fluid, lymph fluids, external secretions of a respiratory tract, external secretions of an intestinal tract, external secretions of a genitourinary tract, milk, amniotic fluid and chorionic villi.

According to some embodiments of the invention, the blood sample comprises serum.

According to some embodiments of the invention, the biological sample (e.g., the body fluid) is a serum sample of the subject.

According to some embodiments of the invention, the solid tumor comprises colorectal cancer.

According to some embodiments of the invention, the immunocomplex is employed in an immunoprecipitation assay.

According to some embodiments of the invention, the immunoprecipitation assay is followed by mass spectroscopy or nephelometry.

According to some embodiments of the invention, one antibody of the at least two antibodies is a mouse monoclonal against human mortalin.

According to some embodiments of the invention, one antibody of the at least two antibodies is a goat polyclonal against human mortalin.

According to some embodiments of the invention, the mouse monoclonal antibody against human mortalin is attached to a solid support.

According to some embodiments of the invention, the kit further comprising a calibration standard for mortalin levels.

4

According to some embodiments of the invention, the level of the mortalin immunocomplex is between about 10 nanogram per milliliter (ng/ml) to about 214 ng/ml.

According to some embodiments of the invention, the predetermined threshold is about 60 ng/ml of mortalin in the biological sample (e.g., body fluid).

According to some embodiments of the invention, the predetermined threshold is about 60 ng/ml of mortalin in the serum.

According to some embodiments of the invention, the tumor stage is determined by Classification of Malignant Tumours (TNM) criteria.

According to some embodiments of the invention, the TNM is TNM-T based on tumor size and state of invasion to nearby tissue.

According to some embodiments of the invention, the TNM is TNM-N based on extent of lymph node involvement.

According to some embodiments of the invention, the TNM is TNM-M based on presence or absence of distant metastases.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how to embodiments of the invention may be practiced.

In the drawings:

FIG. 1 is a calibration curve of mortalin ELISA. Recombinant human mortalin (0.25-2.0 ng per well) was analyzed by the capture ELISA described under Methods. The calibration curve was used to quantify the amount of mortalin in patients' sera.

FIG. 2 is a graph depicting cumulative survival (Kaplan-Meier) of colorectal cancer patients. Patients divided according to mortalin levels in their serum; high (≥ 60 ng/ml) or low (< 60 ng/ml) mortalin in their serum. Log Rank overall comparison showed significant equality of survival distribution ($P=0.042$). Table 1 (Examples section below) provides the survival data of the patients presented in this Figure.

FIG. 3 is a Western blot analysis depicting release of mortalin from HCT116 colon carcinoma cells. HCT116 cells (2.5×10^6) were treated for 30 minutes on ice with diluted (1:16 or 1:20) rabbit anti-human cancer cells antiserum (prepared by the present inventors). Then, normal human serum (NHS) or heat-inactivated serum (HIS) (50% final concentration) was added as a source of complement and control, respectively. After 10 minutes of incubation at 37°C ., the cells were washed and suspended in Hank's Balanced

5

Salt Solution (HBSS) buffer. After 10 minutes incubation at 37° C., the cells were removed by centrifugation and the supernatant was further subjected to centrifugation for 15 minutes at 5,000 g at 4° C. The final supernatant was subjected to SDS-PAGE and Western Blotting with mouse anti-mortalin mAb and a secondary peroxidase-conjugated goat anti-mouse IgG antibody. The extracellular mortalin bands are shown. Cells were also treated with the antibody and complement for 60 minutes at 37° C. to ensure that the conditions are indeed sublytic (lysis percentages shown in parentheses). Basal mortalin release is shown with cells incubated with PBS alone. As shown, NHS induces release of mortalin more than HIS.

FIG. 4 is a graph depicting cumulative survival of the colorectal cancer patients divided according to their mortalin (low: <60 ng/ml, high: >60 ng/ml) and sHSP70 (low: <1.65 ng/ml., high: >1.65 ng/ml) levels. Analysis by Cox regression HR (with 95% CI) of patients with both high mortalin and high HSP70 vs. both low mortalin and low sHSP70 is indicated. Table 4 (Examples section below) provides the survival data of the patients presented in this Figure.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods and kits for determining the presence and/or level of mortalin in a biological sample (e.g., a body fluid of a subject) such as blood (e.g., serum), and methods and kits for determining prognosis of a subject having a solid cancerous tumor, e.g., colorectal cancer, based on the level of mortalin in the biological sample (e.g., the body fluid such as blood or serum).

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

The present inventors have uncovered, following laborious experimentations, a novel and sensitive method for determining the presence and level of mortalin in a biological sample (e.g., body fluids of the subject) such as blood, e.g., serum. By using this ELISA the present inventors have identified mortalin in serum of colorectal cancer patients, and found that higher levels of mortalin in the serum of the patients correlates with faster disease progression and shorter survival time of the patients having colorectal cancer.

Thus, as shown in the Examples section which follows, the novel ELISA method is based on the combination of two anti-mortalin antibodies which are added sequentially, such that a highly sensitive detection of mortalin levels is obtained in a biological sample (Examples 1 of the Examples section which follows and FIG. 1). Using this method, the level of mortalin in serum samples of colorectal cancer patients was determined and was found to be between 14-215 ng/ml (Example 2 of the Examples section which follows). The samples of colorectal cancer patients were then divided based on Receiver operating characteristic (ROC) calculation to yield optimal stratification of patients regarding mortalin levels and survival. Thus, colorectal patients having serum mortalin levels which are below 60 ng/ml (also referred to as "low mortalin" levels hereinafter) were compared to those having a mortalin level which is equal or higher than 60 ng/ml (also referred to as "high mortalin" levels hereinafter). As shown in Example 2 of the Examples section which follows, a significant correlation

6

($R=0.335$, $p<0.001$) was found between serum mortalin and C1-Inhibitor concentrations. In addition, univariate survival analysis revealed mortalin as a predictor for patient's survival (Example 3 of the Examples section which follows) with 113 months estimated median survival in the "low mortalin" group and 29 months median survival in the "high mortalin" group (FIG. 2 and Table 1). Moreover, multivariate analysis in which the survival of the patients stratified according to sHSP70 and mortalin levels was analyzed (FIG. 4 and Table 4) revealed that patients with concomitant high mortalin and high sHSP70 concentrations (>1.67 ng/ml) had a 4.2 times higher hazard of mortality than patients with both low sHSP70 and low mortalin levels, indicating the additive nature of these two biomarkers (FIG. 4). Median survival time of the patients with high mortalin/high sHSP70 and low mortalin/low sHSP70 levels were 27.5 (13.3-34.3) months and 57.0 (31.0-81.0) months, respectively. In addition, the mean survival time (FIG. 4) of patients with high mortalin/high sHsp70 was 24.9 ± 14.0 months and that of patients with low mortalin/low sHsp70 was 54.6 ± 30.8 months. In addition, as described in Example 3 of the Examples section which follows and in Table 5, the addition of the biomarkers sHsp70 and mortalin to the baseline model which includes the basic demographic variables and tumor stage significantly increased the survival-predicting value of the model (the likelihood-ratio test, $\chi^2=26.846$; $p<0.001$). In the subgroup of patients with concomitantly high sHsp70 and mortalin levels, a hazard rate of 8.176 (95% CI 3.267-20.463, $P<0.001$) was observed, indicating that the baseline model (clinical stage, age and sex) and the biomarkers are independent and additive predictors of mortality in colorectal cancer. The hazard index was found to be very high when levels of both mortalin and sHSP70 in serum were elevated and even higher when combined with the TNM cancer stage. Altogether, these findings demonstrate the development of novel and sensitive methods of determining presence and/or level of mortalin in a sample and using these methods for predicting prognosis of a subject having cancer.

Thus, according to an aspect of some embodiments of the invention, there is provided a method comprising determining a presence and/or level of soluble mortalin in a body fluid of a subject.

The term "mortalin" refers to the heat shock 70 KDa protein 9 (HSPA9). Preferably, the mortalin is human mortalin, which is also known as CSA; MOT; MOT2; PBP74; GRP75; HSPA9B; MTHSP75, and encodes a member of the heat shock protein 70 gene family. The encoded protein is primarily localized to the mitochondria but is also found in the endoplasmic reticulum, plasma membrane and cytoplasmic vesicles. This protein is a heat-shock cognate protein, which plays a role in cell proliferation, stress response and maintenance of the mitochondria. The human mortalin polypeptide is set forth in SEQ ID NO:1 (GenBank Accession NO. NP_004125.3). The human mortalin mRNA is provided in SEQ ID NO:2 (GenBank Accession No. NM_004134, gi|296080701, nuclear gene encoding mitochondrial protein, mRNA).

According to some embodiments of the invention, the soluble mortalin is set forth in SEQ ID NO:1.

As used herein the term "soluble" mortalin refers to a mortalin polypeptide which is at least partially separated from any cellular moiety such as a cell membrane, a nuclear membrane, cytoplasmic vesicles, cell organelle, e.g., mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, nucleus and the like.

According to some embodiments of the invention, the soluble mortalin is not attached to a cell, to a cell membrane or to a cell organelle.

According to some embodiments of the invention, the mortalin is a circulating soluble mortalin which is not attached to a cell or to any cellular moiety such as those described above.

According to some embodiments of the invention, the soluble mortalin is not present on, in or attached to a solid cancerous tumor.

According to some embodiments of the invention, the method is performed in vitro.

According to some embodiments of the invention, the method is performed ex vivo.

According to some embodiments of the invention, the biological sample is a bodily fluid (body fluid of the subject) such as whole blood, serum, plasma, cerebrospinal fluid, synovial fluid, urine, lymph fluids, and various external secretions of the respiratory, intestinal and genitourinary tracts, tears, saliva, milk as well as white blood cells, amniotic fluid sample, chorionic villi sample and the like from the subject.

Methods of obtaining body fluids of a subject are well known in the art and include for example, collection of urine, saliva and tears, aspiration of cerebrospinal fluid, synovial fluid, lymph fluids, and various external secretions of the respiratory, intestinal and genitourinary tracts, the use of a needle and/or a catheter for drawing a blood sample, amniotic fluid sample, chorionic villi sample and the like from the subject.

According to some embodiments of the invention, the body fluid is at least partially removed from the subject.

According to some embodiments of the invention, the body fluid is removed from the subject (e.g., completely separated from the subject).

According to some embodiments of the invention, the body fluid is taken from the subject using a non-invasive method.

According to some embodiments of the invention, the body fluid is an archival sample of the subject.

According to some embodiments of the invention, the body fluid is a clinical sample available from the subject.

According to some embodiments of the invention, the biological sample is a cell suspension of body fluids.

According to some embodiments of the invention, the blood sample can be whole blood, serum, and/or plasma.

According to some embodiments of the invention, the biological sample is a blood sample, e.g., a serum sample.

According to some embodiments of the invention, the blood sample is a serum sample.

According to some embodiments of the invention, the blood sample is a non-cultured blood sample obtained from a subject.

According to some embodiments of the invention, the blood sample comprises supernatant collected from in vitro cultured blood cells.

According to some embodiments of the invention, the biological sample (e.g., body fluid) is not a sample of a solid tumor (e.g., tissue biopsy of a solid tumor, fine needle aspirate of a solid tumor).

According to some embodiments of the invention, the biological sample (e.g., body fluid) is obtained from a subject having a solid cancerous tumor.

As used herein the term "subject" refers to a mammal, e.g., a human being. The subject can be a male or a female at any age.

According to some embodiments of the invention, the subject is diagnosed with a solid cancerous tumor.

According to some embodiments of the invention, the cancer is any solid tumor and/or cancer metastasis thereof, including, but not limited to, tumors of the gastrointestinal tract (colon carcinoma, rectal carcinoma, colorectal carcinoma, colorectal cancer, colorectal adenoma, hereditary nonpolyposis type 1, hereditary nonpolyposis type 2, hereditary nonpolyposis type 3, hereditary nonpolyposis type 6; colorectal cancer, hereditary nonpolyposis type 7, small and/or large bowel carcinoma, esophageal carcinoma, tylosis with esophageal cancer, stomach carcinoma, pancreatic carcinoma, pancreatic endocrine tumors), endometrial carcinoma, dermatofibrosarcoma protuberans, gallbladder carcinoma, Biliary tract tumors, prostate cancer, prostate adenocarcinoma, renal cancer (e.g., Wilms' tumor type 2 or type 1), liver cancer (e.g., hepatoblastoma, hepatocellular carcinoma, hepatocellular cancer), bladder cancer, embryonal rhabdomyosarcoma, germ cell tumor, trophoblastic tumor, testicular germ cells tumor, immature teratoma of ovary, uterine, epithelial ovarian, sacrococcygeal tumor, choriocarcinoma, placental site trophoblastic tumor, epithelial adult tumor, ovarian carcinoma, serous ovarian cancer, ovarian sex cord tumors, cervical carcinoma, uterine cervix carcinoma, small-cell and non-small cell lung carcinoma, nasopharyngeal, breast carcinoma (e.g., ductal breast cancer, invasive intraductal breast cancer, sporadic; breast cancer, susceptibility to breast cancer, type 4 breast cancer, breast cancer-1, breast cancer-3; breast-ovarian cancer), squamous cell carcinoma (e.g., in head and neck), neurogenic tumor, astrocytoma, ganglioblastoma, neuroblastoma, lymphomas (e.g., Hodgkin's disease, non-Hodgkin's lymphoma, B cell, Burkitt, cutaneous T cell, histiocytic, lymphoblastic, T cell, thymic), gliomas, adenocarcinoma, adrenal tumor, hereditary adrenocortical carcinoma, brain malignancy (tumor), various other carcinomas (e.g., bronchogenic large cell, ductal, Ehrlich-Lettré ascites, epidermoid, large cell, Lewis lung, medullary, mucoepidermoid, oat cell, small cell, spindle cell, spinocellular, transitional cell, undifferentiated, carcinosarcoma, choriocarcinoma, cystadenocarcinoma), ependimoblastoma, epithelioma, erythroleukemia (e.g., Friend, lymphoblast), fibrosarcoma, giant cell tumor, glial tumor, glioblastoma (e.g., multiforme, astrocytoma), glioma, hepatoma, heterohybridoma, heteromyeloma, histiocytoma, hybridoma (e.g., B cell), hypernephroma, insulinoma, islet tumor, keratoma, leiomyoblastoma, leiomyosarcoma, leukemia (e.g., acute lymphatic, acute lymphoblastic, acute lymphoblastic pre-B cell, acute lymphoblastic T cell leukemia, acute-megakaryoblastic, monocytic, acute myelogenous, acute myeloid, acute myeloid with eosinophilia, B cell, basophilic, chronic myeloid, chronic, B cell, eosinophilic, Friend, granulocytic or myelocytic, hairy cell, lymphocytic, megakaryoblastic, monocytic, monocytic-macrophage, myeloblastic, myeloid, myelomonocytic, plasma cell, pre-B cell, promyelocytic, subacute, T cell, lymphoid neoplasm, predisposition to myeloid malignancy, acute nonlymphocytic leukemia), lymphosarcoma, melanoma, mammary tumor, mastocytoma, medulloblastoma, mesothelioma, metastatic tumor, monocyte tumor, multiple myeloma, myelodysplastic syndrome, myeloma, nephroblastoma, nervous tissue glial tumor, nervous tissue neuronal tumor, neurinoma, neuroblastoma, oligodendroglioma, osteochondroma, osteomyeloma, osteosarcoma (e.g., Ewing's), papilloma, transitional cell, pheochromocytoma, pituitary tumor (invasive), plasmacytoma, retinoblastoma, rhabdomyosarcoma, sarcoma (e.g., Ewing's, histiocytic cell, Jensen, osteogenic, reticulum cell), schwannoma, subcutaneous tumor, teratocarcinoma (e.g., pluripotent), teratoma, testicular tumor, thymoma and trichoepithelioma, gastric cancer,

fibrosarcoma, glioblastoma multiforme; multiple glomus tumors, Li-Fraumeni syndrome, liposarcoma, lynch cancer family syndrome II, male germ cell tumor, medullary thyroid, multiple meningioma, endocrine neoplasia myxosarcoma, paraganglioma, familial nonchromaffin, pilomatricoma, papillary, familial and sporadic, rhabdoid predisposition syndrome, familial, rhabdoid tumors, soft tissue sarcoma, and Turcot syndrome with glioblastoma.

According to some embodiments of the invention, the cancer is colorectal cancer.

According to specific embodiments of the invention, the subject does not have a non-solid tumor (e.g., leukemia).

As used herein the phrase “determining a presence . . .” refers to the determination of presence or absence of mortalin in the sample as assayed by a method such as ELISA, Western blot analysis, radio immunoassay (RIA), Fluorescence activated cell sorting (FACS), surface plasmon resonance, immunohistochemistry, immunofluorescence, immunoprecipitation, nephelometry and mass spectroscopy when compared to a negative control sample which is devoid of mortalin.

According to some embodiments of the invention, determining the presence and/or the level of the soluble mortalin is effected by a method selected from the group consisting of an immunological assay, mass-spectroscopy, nephelometry and surface Plasmon resonance.

According to some embodiments of the invention, determining the presence and/or the level of the soluble mortalin is effected using an immunological method or a mass-spectroscopy method.

The mass spectroscopy detects peptide sequences and is able to identify the presence of a protein in a sample.

For example, for mass spectroscopy, the samples are trypsinized and the tryptic peptides are analyzed by Liquid Chromatography-Mass Spectrometry (LC-MSMS) on the Orbitrap mass spectrometer (Thermo). The data can be analyzed using the Sequest 3.31 software versus the *e coli* section of the NCBI-NR (non-redundant National Center for Biotechnology Information) database and versus the whole Uniprot database and vs the human section of it. Thus, mass spectroscopy analysis can detect presence of mortalin in a sample and further determine the level of mortalin in the sample.

According to some embodiments of the invention, determining the presence and/or the level of the soluble mortalin is effected by an immunological detection assay or method.

According to an aspect of some embodiments of the invention, the method of determining presence or level of soluble mortalin in a biological sample (e.g., body fluid) of a subject is effected by: (a) contacting the biological sample with an anti-mortalin antibody under conditions which allow immunocomplex formation; and (b) determining a presence or level of the immunocomplex, thereby determining the presence and/or level of the soluble mortalin in the biological sample.

According to some embodiments of the invention, determining the presence or level of mortalin is performed using a mortalin specific antibody or antibodies which are added (or applied) to a sample of body fluid of the subject, e.g., anti-mortalin antibodies which are added to a serum sample of a subject and form an immunocomplex.

As used herein the term “immunological” refers to using an antigen-antibody interaction, by formation of an immunocomplex, in order to determine presence and/or level of mortalin.

As used herein the phrase “immunocomplex” refers to a complex formed by binding of an antibody to its antigen.

According to some embodiments of the invention, binding of the antibody to the antigen depends on affinity between the antibody to an epitope on or in the antigen.

According to some embodiments of the invention, the complex is a non-covalent complex.

As used herein, the term “epitope” refers to any antigenic determinant on an antigen to which the paratope of an antibody binds.

Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The term “antibody” as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')₂, and Fv that are capable of binding to macrophages. These functional antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')₂, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')₂ is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) Single chain antibody (“SCA”), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

Antibody fragments according to some embodiments of the invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. [Biochem. J. 73: 119-126 (1959)]. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar et al. [Proc. Nat'l Acad. Sci. USA 69:2659-62 (1972)]. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by [Whitlow and Filpula, Methods 2: 97-105 (1991); Bird et al., Science 242:423-426 (1988); Pack et al., Bio/Technology 11:1271-77 (1993); and U.S. Pat. No. 4,946,778], which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry [Methods, 2: 106-10 (1991)].

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature 332:323-327 (1988); Verhoeven et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies

are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies [Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology 10: 779-783 (1992); Lonberg et al., Nature 368: 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

According to some embodiments of the invention, the anti-mortalin antibody is a monoclonal antibody.

Commercially available monoclonal anti-mortalin antibodies include, but are not limited to mouse monoclonal antibody directed to human mortalin (StressMarq Biosciences Inc., Victoria, Canada), mouse monoclonal anti-human mortalin (Enzo® Life Sciences, Cat. No. ADI-SPS-825), and Rabbit Anti-Mortalin antibody (Monoclonal, Cell Signaling Technology [D13H4] Cat. No. 3593P).

According to some embodiments of the invention, the anti-mortalin antibody is a polyclonal antibody.

Commercially available polyclonal anti-mortalin antibodies include, but are not limited to and treated with a goat anti-human mortalin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.); rabbit polyclonal anti-mortalin (Enzo® Life Sciences, Catalogue Number ADI-SPS-827-D); Rabbit Anti-Mortalin antibody, Polyclonal Cell Signaling Technology Cat. No. 2816S.

According to some embodiments of the invention, the anti-mortalin antibody comprises a combination of at least two anti-mortalin antibodies, each binding to a different epitope of mortalin. For example, a mouse monoclonal antibody and a goat polyclonal antibody. For example, the mouse monoclonal antibody available from StressMarq can be used, along with the Goat polyclonal antibody, available from Santa Cruz.

The antibody or antibody fragment may be attached to any of various functional moieties. An antibody or antibody fragment, such as that of some embodiments of the invention, attached to a functional moiety may be referred to in the art as an "immunoconjugate".

According to some embodiments of the invention, the functional moiety is a detectable moiety or a toxin.

The detectable moiety attached to the antibody or antibody fragment can be a reporter moiety enabling specific detection of the immunocomplex.

While various types of reporter moieties may be utilized to detect the immunocomplex, depending on the application and purpose, the reporter moiety can be a fluorophore or an enzyme. Alternately, the reporter moiety may be a radioisotope, such as [125]iodine. Further examples of reporter moieties, including those detectable by Positron Emission Tomography (PET) and Magnetic Resonance Imaging (MRI), are well known to those of skill in the art.

A fluorophore may be advantageously employed as a detection moiety enabling detection of the immunocomplex via any of numerous fluorescence detection methods. Depending on the application and purpose, such fluorescence detection methods include, but are not limited to, fluorescence activated flow cytometry (FACS), immunofluorescence confocal microscopy, fluorescence in-situ hybridization (FISH), fluorescence resonance energy transfer (FRET), and the like.

Various types of fluorophores, depending on the application and purpose, may be employed to detect the immunocomplex.

Examples of suitable fluorophores include, but are not limited to, phycoerythrin (PE), fluorescein isothiocyanate (FITC), Cy-chrome, rhodamine, green fluorescent protein (GFP), blue fluorescent protein (BFP), TEXAS RED (MOLECULAR PROBES, INC. sulforhodamine 101 acid chloride), PE-Cy5, and the like.

Preferably, the fluorophore is phycoerythrin.

Ample guidance regarding fluorophore selection, methods of linking fluorophores to various types of molecules, such as an antibody or antibody fragment of the invention, and methods of using such conjugates to detect molecules which are capable of being specifically bound by antibodies or antibody fragments comprised in such immunconjugates is available in the literature of the art [for example, refer to: Richard P. Haugland, "Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals 1992-1994", 5th ed., Molecular Probes, Inc. (1994); U.S. Pat. No. 6,037,137 to Oncoimmunin Inc.; Hermanson, "Bioconjugate Techniques", Academic Press New York, N.Y. (1995); Kay M. et al., 1995. *Biochemistry* 34:293; Stubbs et al., 1996. *Biochemistry* 35:937; Gakamsky D. et al., "Evaluating Receptor Stoichiometry by Fluorescence Resonance Energy Transfer," in "Receptors: A Practical Approach," 2nd ed., Stanford C. and Horton R. (eds.), Oxford University Press, UK. (2001); U.S. Pat. No. 6,350,466 to Targesome, Inc.].

Alternately, an enzyme may be advantageously utilized as the detectable moiety to enable detection of the immunocomplex via any of various enzyme-based detection methods. Examples of such methods include, but are not limited to, enzyme linked immunosorbent assay (ELISA; for example, to detect the immunocomplex in a solution), enzyme-linked chemiluminescence assay (for example, to detect the immunocomplex in an electrophoretically separated protein mixture), and enzyme-linked immunohistochemical assay (for example, to detect the immunocomplex in a fixed tissue).

Numerous types of enzymes may be employed to detect the immunocomplex, depending on the application and purpose. For example, an antibody or antibody fragment attached to an enzyme such as horseradish peroxidase can be used to effectively detect the immunocomplex, such as via ELISA, or enzyme-linked immunohistochemical assay.

Examples of suitable enzymes include, but are not limited to, horseradish peroxidase (HPR), beta-galactosidase, and alkaline phosphatase (AP).

Ample guidance for practicing such enzyme-based detection methods is provided in the literature of the art (for

example, refer to: Khatkhatay M I. and Desai M., 1999. *J Immunoassay* 20:151-83; Wisdom G B., 1994. *Methods Mol Biol.* 32:433-40; Ishikawa E. et al., 1983. *J Immunoassay* 4:209-327; Oellerich M., 1980. *J Clin Chem Clin Biochem.* 18:197-208; Schuurs A H. and van Weemen B K., 1980. *J Immunoassay* 1:229-49).

The functional moiety may be attached to the antibody or antibody fragment in various ways, depending on the context, application and purpose.

A functional moiety may also be attached to the antibody or antibody fragment using standard chemical synthesis techniques widely practiced in the art [for example, refer to the extensive guidelines provided by The American Chemical Society (for example at: [hypertexttransferprotocol://worldwideweb\(dot\)chemistry\(dot\)org/portal/Chemistry](http://hypertexttransferprotocol://worldwideweb(dot)chemistry(dot)org/portal/Chemistry))]. One of ordinary skill in the art, such as a chemist, will possess the required expertise for suitably practicing such chemical synthesis techniques.

Alternatively, a functional moiety may be attached to the antibody or antibody fragment by attaching an affinity tag-coupled antibody or antibody fragment of the invention to the functional moiety conjugated to a specific ligand of the affinity tag.

Various types of affinity tags may be employed to attach the antibody or antibody fragment to the functional moiety.

Examples of detectable moieties that can be used in the invention include but are not limited to radioactive isotopes, phosphorescent chemicals, chemiluminescent chemicals, fluorescent chemicals, enzymes, fluorescent polypeptides and epitope tags. The detectable moiety can be a member of a binding pair, which is identifiable via its interaction with an additional member of the binding pair, and a label which is directly visualized. In one example, the member of the binding pair is an antigen which is identified by a corresponding labeled antibody. In one example, the label is a fluorescent protein or an enzyme producing a colorimetric reaction.

When the detectable moiety is a polypeptide, the immunolabel (i.e. the antibody conjugated to the detectable moiety) may be produced by recombinant means or may be chemically synthesized by, for example, the stepwise addition of one or more amino acid residues in defined order using solid phase peptide synthetic techniques. Examples of polypeptide detectable moieties that can be linked to the antibodies of the invention using recombinant DNA technology include fluorescent polypeptides, phosphorescent polypeptides, enzymes and epitope tags.

Expression vectors can be designed to fuse proteins encoded by the heterologous nucleic acid insert to fluorescent polypeptides. For example, antibodies can be expressed from an expression vector fused with a green fluorescent protein (GFP)-like polypeptide. A wide variety of vectors are commercially available that fuse proteins encoded by heterologous nucleic acids to the green fluorescent protein from *Aequorea victoria* ("GFP"), the yellow fluorescent protein and the red fluorescent protein and their variants (e.g., Evrogen). In these systems, the fluorescent polypeptide is entirely encoded by its amino acid sequence and can fluoresce without requirement for cofactor or substrate. Expression vectors that can be employed to fuse proteins encoded by the heterologous nucleic acid insert to epitope tags are commercially available (e.g., BD Biosciences, Clontech).

Alternatively, chemical attachment of a detectable moiety to the antibodies of the invention can be effected using any suitable chemical linkage, direct or indirect, as via a peptide bond (when the detectable moiety is a polypeptide), or via

covalent bonding to an intervening linker element, such as a linker peptide or other chemical moiety, such as an organic polymer. Such chimeric peptides may be linked via bonding at the carboxy (C) or amino (N) termini of the peptides, or via bonding to internal chemical groups such as straight, branched or cyclic side chains, internal carbon or nitrogen atoms, and the like. Such modified peptides can be easily identified and prepared by one of ordinary skill in the art, using well known methods of peptide synthesis and/or covalent linkage of peptides. Description of fluorescent labeling of antibodies is provided in details in U.S. Pat. Nos. 3,940,475, 4,289,747, and 4,376,110.

Exemplary methods for conjugating two peptide moieties are described herein below:

SPDP Conjugation:

Any SPDP conjugation method known to those skilled in the art can be used. For example, in one illustrative embodiment, a modification of the method of Cumber et al. (1985, *Methods of Enzymology* 112: 207-224) as described below, is used.

A peptide, such as an identifiable, (1.7 mg/ml) is mixed with a 10-fold excess of SPDP (50 mM in ethanol) and the antibody is mixed with a 25-fold excess of SPDP in 20 mM sodium phosphate, 0.10 M NaCl pH 7.2 and each of the reactions incubated, e.g., for 3 hours at room temperature. The reactions are then dialyzed against PBS.

The peptide is reduced, e.g., with 50 mM DTT for 1 hour at room temperature. The reduced peptide is desalted by equilibration on G-25 column (up to 5% sample/column volume) with 50 mM KH₂PO₄ pH 6.5. The reduced peptide is combined with the SPDP-antibody in a molar ratio of 1:10 antibody:peptide and incubated at 4° C. overnight to form a peptide-antibody conjugate.

Glutaraldehyde Conjugation:

Conjugation of a peptide (e.g., an identifiable) with an antibody can be accomplished by methods known to those skilled in the art using glutaraldehyde. For example, in one illustrative embodiment, the method of conjugation by G. T. Hermanson (1996, "Antibody Modification and Conjugation, in *Bioconjugate Techniques*, Academic Press, San Diego) described below, is used.

The antibody and the peptide (1.1 mg/ml) are mixed at a 10-fold excess with 0.05% glutaraldehyde in 0.1 M phosphate, 0.15 M NaCl pH 6.8, and allowed to react for 2 hours at room temperature. 0.01 M lysine can be added to block excess sites. After the reaction, the excess glutaraldehyde is removed using a G-25 column equilibrated with PBS (10% v/v sample/column volumes).

Carbodiimide Conjugation:

Conjugation of a peptide with an antibody can be accomplished by methods known to those skilled in the art using a dehydrating agent such as a carbodiimide. Most preferably the carbodiimide is used in the presence of 4-dimethyl aminopyridine. As is well known to those skilled in the art, carbodiimide conjugation can be used to form a covalent bond between a carboxyl group of peptide and an hydroxyl group of an antibody (resulting in the formation of an ester bond), or an amino group of an antibody (resulting in the formation of an amide bond) or a sulfhydryl group of an antibody (resulting in the formation of a thioester bond).

Likewise, carbodiimide coupling can be used to form analogous covalent bonds between a carbon group of an antibody and a hydroxyl, amino or sulfhydryl group of the peptide. See, generally, J. March, *Advanced Organic Chemistry: Reaction's, Mechanism, and Structure*, pp. 349-50 & 372-74 (3d ed.), 1985. By means of illustration, and not limitation, the peptide is conjugated to an antibody via a

covalent bond using a carbodiimide, such as dicyclohexyl-carbodiimide. See generally, the methods of conjugation by B. Neises et al. (1978, *Angew Chem., Int. Ed. Engl.* 17:522; A. Hassner et al. (1978, *Tetrahedron Lett.* 4475); E. P. Boden et al. (1986, *J. Org. Chem.* 50:2394) and L. J. Mathias (1979, *Synthesis* 561)).

Preferably, the affinity tag is a biotin molecule, more preferably a streptavidin molecule.

A biotin or streptavidin affinity tag can be used to optimally enable attachment of a streptavidin-conjugated or a biotin-conjugated functional moiety, respectively, to the antibody or antibody fragment due to the capability of streptavidin and biotin to bind to each other with the highest non covalent binding affinity (i.e., with a K_d of about 10⁻¹⁴ to 10⁻¹⁵). A biotin affinity tag may be highly advantageous for applications benefiting from. Thus, the antibody of invention can be a multimeric form of the antibody or antibody fragment, which may be optimally formed by conjugating multiple biotin-attached antibodies or antibody fragments of the invention to a streptavidin molecule, as described in further detail below.

Various methods, widely practiced in the art, may be employed to attach a streptavidin or biotin molecule to a molecule such as the antibody or antibody fragment to a functional moiety.

For example, a biotin molecule may be advantageously attached to an antibody or antibody fragment of the invention attached to a recognition sequence of a biotin protein ligase. Such a recognition sequence is a specific polypeptide sequence serving as a specific biotinylation substrate for the biotin protein ligase enzyme. Ample guidance for biotinylating a target polypeptide such as an antibody fragment using a recognition sequence of a biotin protein ligase, such as the recognition sequence of the biotin protein ligase BirA, is provided in the literature of the art (for example, refer to: Denkberg, G. et al., 2000. *Eur. J. Immunol.* 30:3522-3532). Preferably, such biotinylation of the antibody or antibody fragment is effected as described and illustrated in the Examples section below.

Alternately, various widely practiced methods may be employed to attach a streptavidin molecule to an antibody fragment, such as a single chain Fv (for example refer to Cloutier S M. et al., 2000. *Molecular Immunology* 37:1067-1077; Dubel S. et al., 1995. *J Immunol Methods* 178:201; Huston J S. et al., 1991. *Methods in Enzymology* 203:46; Kipriyanov S M. et al., 1995. *Hum Antibodies Hybridomas* 6:93; Kipriyanov S M. et al., 1996. *Protein Engineering* 9:203; Pearce L A. et al., 1997. *Biochem Molec Biol Intl* 42:1179-1188).

Functional moieties, such as fluorophores, conjugated to streptavidin are commercially available from essentially all major suppliers of immunofluorescence flow cytometry reagents (for example, Pharmingen or Becton-Dickinson). Standard recombinant DNA chemical techniques are preferably employed to produce a fusion protein comprising streptavidin fused to a polypeptidic functional moiety. Standard chemical synthesis techniques may also be employed to form the streptavidin-functional moiety conjugate. Extensive literature is available providing guidance for the expression, purification and uses of streptavidin or streptavidin-derived molecules (Wu S C. et al., 2002. *Protein Expression and Purification* 24:348-356; Gallizia A. et al., 1998. *Protein Expression and Purification* 14:192-196), fusion proteins comprising streptavidin or streptavidin-derived molecules (Sano T. and Cantor C R., 2000. *Methods Enzymol.* 326: 305-11), and modified streptavidin or streptavidin-derived molecules (see, for example: Sano T. et al., 1993. *Journal of*

Biological Chemistry 270:28204-28209), including for streptavidin or streptavidin-derived molecules whose gene sequence has been optimized for expression in *E. coli* (Thompson L. D. and Weber P. C., 1993. Gene 136:243-6).

According to some embodiments of the invention, contacting the biological sample (e.g., the blood sample) with an anti-mortalin antibody is effected under conditions which allow immunocomplex formation. Such conditions may include buffers, temperature, salts, which increase the binding between the antibody to the antigen, yet avoids binding of the antibody to non-specific proteins or determinants, such as the vial, plate or vessel to which the antibody is added.

According to some embodiments of the invention, the anti-mortalin antibody is bound to a solid support.

According to some embodiments of the invention, the method further comprises determining a presence or level of the immunocomplex.

Methods of determining presence (or absence) of an immunocomplex and determining the presence and/or level of the antigen in a sample based on detection of the immunocomplex are known in the art, and include, for example, ELISA and Western blot analysis, radio immunoassay (RIA), Fluorescence activated cell sorting (FACS), surface plasmon resonance, immunohistochemistry, immunofluorescence, immunoprecipitation, nephelometry and the like.

Following is a non-limiting description of the immunological detection methods.

Enzyme linked immunosorbent assay (ELISA): This method involves fixation of a sample (e.g., fixed cells or a proteinaceous solution) containing a protein substrate to a surface such as a well of a microtiter plate. A substrate specific antibody coupled to an enzyme is applied and allowed to bind to the substrate. Presence of the antibody is then detected and quantitated by a colorimetric reaction employing the enzyme coupled to the antibody. Enzymes commonly employed in this method include horseradish peroxidase and alkaline phosphatase. If well calibrated and within the linear range of response, the amount of substrate present in the sample is proportional to the amount of color produced. A substrate standard is generally employed to improve quantitative accuracy.

Western blot: This method involves separation of a substrate from other protein by means of an acrylamide gel followed by transfer of the substrate to a membrane (e.g., nylon or PVDF). Presence of the substrate is then detected by antibodies specific to the substrate, which are in turn detected by antibody binding reagents. Antibody binding reagents may be, for example, protein A, or other antibodies. Antibody binding reagents may be radiolabeled or enzyme linked as described hereinabove. Detection may be by autoradiography, colorimetric reaction or chemiluminescence. This method allows both quantitation of an amount of substrate and determination of its identity by a relative position on the membrane which is indicative of a migration distance in the acrylamide gel during electrophoresis.

Radio-immunoassay (RIA): In one version, this method involves precipitation of the desired protein (i.e., the substrate) with a specific antibody and radiolabeled antibody binding protein (e.g., protein A labeled with I^{125}) immobilized on a precipitable carrier such as agarose beads. The number of counts in the precipitated pellet is proportional to the amount of substrate.

In an alternate version of the RIA, a labeled substrate and an unlabeled antibody binding protein are employed. A sample containing an unknown amount of substrate is added

in varying amounts. The decrease in precipitated counts from the labeled substrate is proportional to the amount of substrate in the added sample.

Fluorescence activated cell sorting (FACS): This method involves detection of a substrate in situ in cells by substrate specific antibodies. The substrate specific antibodies are linked to fluorophores. Detection is by means of a cell sorting machine which reads the wavelength of light emitted from each cell as it passes through a light beam. This method may employ two or more antibodies simultaneously.

Immunohistochemical analysis: This method involves detection of a substrate in situ in fixed cells by substrate specific antibodies. The substrate specific antibodies may be enzyme linked or linked to fluorophores. Detection is by microscopy and subjective or automatic evaluation. If enzyme linked antibodies are employed, a colorimetric reaction may be required. It will be appreciated that immunohistochemistry is often followed by counterstaining of the cell nuclei using for example Hematoxyline or Giemsa stain.

Nephelometry: This technique is used in medicine to quantify proteins based on antibody binding and measuring intensity of light scattering by the sample.

According to some embodiments of the invention, the immunocomplex is employed in an immunoprecipitation assay.

According to some embodiments of the invention, the immunoprecipitation assay is followed by mass spectroscopy or nephelometry.

According to some embodiments of the invention, the immunological method uses an anti-mortalin antibody (or antibodies) which are conjugated to a surface plasmon resonance. The anti-mortalin antibody, coated to the surface of the chip, captures mortalin, and the response elicited by the binding is detected.

According to some embodiments of the invention, the level of the immunocomplex is determined by an Enzyme-linked immunosorbent assay (ELISA).

According to some embodiments, the immunological method is followed by mass spectroscopy analysis of the immuno-precipitant. For example, the immuno-precipitant can be dissociated with an enzyme (e.g., trypsin) and be subject to mass spectroscopy using known parameters.

According to some embodiments, the level of immunocomplex is compared to a control or reference sample (e.g., from a healthy subject, devoid of the cancer).

According to some embodiments of the invention, the reference sample is obtained from a subject of the same species e.g. human.

According to some embodiments of the invention, the control or reference sample is obtained from a healthy subject (e.g., devoid of the cancer) matched with the same ethnic group, age, sex, and/or body mass index (BMI).

According to some embodiments of the invention, the control or reference sample is of the same type as the biological sample (e.g., body fluid) from the subject in need of diagnosis. For example, if the biological sample of the subject is a serum sample, then, the control or reference sample is also a serum sample.

According to some embodiments, the level of immunocomplex is compared to a calibration curve or calibration database obtained using known amounts of mortalin such as that produced by the present inventors and shown in FIG. 1 and described in Example 1 of the Examples section which follows.

Thus, the methods described herein can be used to determine the presence and/or level of soluble mortalin in a sample of the subject and can then be compared to the level in a reference sample.

As described above and in the Examples section which follows, the present inventors have uncovered, for the first time, that the level of mortalin in a blood sample of a subject having a solid tumor such as colorectal cancer can be used as a powerful predictor for the prognosis of the subject having the cancer.

Thus, according to an aspect of some embodiments of the invention, there is provided a method of predicting prognosis of a subject diagnosed with a solid cancerous tumor, the method is effected by determining a level of soluble mortalin according to the method of some embodiments of the invention, wherein

(i) when a level of the soluble mortalin is above a pre-determined threshold, then the subject is predicted to have poor prognosis as compared to an expected prognosis based on the subject's tumor's stage, age and/or gender; and

(ii) when a level of the soluble mortalin is below a pre-determined threshold, then the subject has good prognosis as compared to an expected prognosis based on the subject's tumor's stage, age and/or gender.

As used herein the phrase "predicting prognosis" refers to determining the likelihood of a subject diagnosed with the cancer to have good or poor prognosis as compared to the expected prognosis based the subject's tumor's stage, age and/or gender.

It should be noted that the prognosis of a subject diagnosed with a disease such as cancer is currently determined based on known measurements, known as the gold standard. For example, tumor stage can be determined using criteria which are based on histological evaluation of the tumor (e.g., by a Pathologist), presence or absence of metastases, invasion into healthy nearby tumor, metastases in lymph nodes or distance metastases.

According to some embodiments of the invention, the tumor stage is determined by Classification of Malignant Tumours (TNM) criteria.

According to some embodiments of the invention, TNM is TNM-T based on tumor size and state of invasion to nearby tissue.

According to some embodiments of the invention, TNM is TNM-N based on extent of lymph node involvement.

According to some embodiments of the invention, TNM is TNM-M based on presence or absence of distant metastases.

According to some embodiments of the invention, the prognosis refers to the survival period of the subject having the solid tumor.

According to some embodiments of the invention, the survival period of the subject having the solid tumor is from the time of diagnosing the cancer until death occurs.

According to some embodiments of the invention, the method of determining the prognosis of the subject is performed shortly after diagnosing the cancer in the subject.

According to some embodiments of the invention, the method of determining the prognosis of the subject is performed within a few days (e.g., 1-30 days), a few weeks (e.g., 2-10 weeks) or a few months (e.g., within 1-12 months) from the date of diagnosing the cancer in the subject.

For example, as described in the Examples section which follows, testing the level of mortalin in the blood sample of

the colorectal cancer patients was performed within 4-6 weeks after surgery when the patients came for a routine follow up at the clinic.

According to some embodiments of the invention, a good prognosis refers to better health condition and/or longer survival time (in months or years) as compared to the expected prognosis of the subject as determined using the gold standard parameters.

According to some embodiments of the invention, when the solid tumor is colorectal cancer, then a good prognosis is characterized by about 72 months mean survival time.

According to some embodiments of the invention, a poor prognosis refers to worse health condition and/or shorter survival time (in months or years) as compared to the expected prognosis of the subject as determined using the gold standard parameters.

According to some embodiments of the invention, when the solid tumor is colorectal cancer, then a poor prognosis is characterized by about 43 months mean survival time.

According to some embodiments of the invention, the level of mortalin in a blood sample of a subject having colorectal cancer is between about 5-300 nanogram per milliliter (ng/ml), e.g., between about 8-250 ng/ml, e.g., between about 10-214 ng/ml.

According to some embodiments of the invention, the level of the mortalin immunocomplex in a biological sample (e.g., body fluid, e.g., serum) is between about 10 nanogram per milliliter (ng/ml) to about 214 ng/ml.

According to some embodiments of the invention, the level of the mortalin in a blood sample of a control, healthy subject (e.g., devoid of the cancer), is apparently 0 ng/ml.

As used herein the phrase "pre-determined threshold" refers to level of mortalin [e.g., concentration of mortalin in a biological sample (e.g., body fluid) which can be measured in nanogram per milliliter; or in molar units (e.g., milimolar or molar)] which discriminates between subjects diagnosed with the cancer and having a good prognosis and subjects diagnosed with the cancer and having a poor prognosis.

As described in the Examples section which follows, the predetermined threshold can be determined retrospectively using archive samples with a known clinical outcome, e.g., a known survival period. Tools for determining such a threshold which discriminates between the two groups of patients, e.g., those having good prognosis from those having poor prognosis, are known in the art, and include, for example, the receiver operating characteristic (ROC) calculation which yields optimal stratification of patients regarding mortalin levels and survival (Example 2 of the Examples section which follows).

Once determined, the threshold of mortalin concentration in the body fluid of the subject can be used to predict the prognosis of a subject having the solid tumor.

For example, as shown in Example 2 below, the predetermined threshold of 60 ng/ml of mortalin in the serum was found to accurately discriminate between patients with colorectal cancer and having a poor prognosis (e.g., having a concentration of mortalin which is equal to or higher than 60 ng/ml) or a good prognosis (e.g., having a concentration of mortalin which is lower than 60 ng/ml).

According to some embodiments of the invention, the predetermined threshold is about 60 ng/ml of mortalin in the biological sample (e.g., body fluid).

According to some embodiments of the invention, the level of mortalin in the biological sample (e.g., body fluid, e.g., blood) enables the classification of a subject as having good or poor prognosis (e.g., long or short survival periods).

According to some embodiments of the invention, the method further comprising: classifying the subject as having good or poor prognosis based on the level of the soluble mortalin, thereby predicting the prognosis of the subject diagnosed with the cancer.

As used herein the term “diagnosing” refers to determining presence or absence of a pathology (e.g., a disease, disorder, condition or syndrome), classifying a pathology or a symptom, determining a severity of the pathology, monitoring pathology progression, forecasting an outcome of a pathology and/or prospects of recovery and screening of a subject for a specific disease.

According to some embodiments of the invention, screening of the subject for a specific disease is followed by substantiation of the screen results using gold standard methods.

As shown in Tables 4 and 5 and Example 3 of the Examples section which follows, determining the level of mortalin along with the level of sHSP70 results in an even higher predicting power for prognosis of colorectal cancer patients.

Thus, according to some embodiments of the invention, the method further comprising:

(a) determining the level of soluble heat shock protein 70 (sHSP70) in the sample, wherein when a level of the soluble mortalin and the sHSP70, each is above a predetermined threshold in the biological sample (e.g., blood sample), then the subject is predicted to have a poor prognosis as compared to an expected prognosis based the subject's tumor's stage, age and/or gender; and

(b) classifying the subject as having good or poor prognosis based on the mortalin and the sHSP70 levels in the biological sample (e.g., body fluid),

thereby predicting the prognosis of the subject diagnosed with the solid tumor.

As used herein the soluble heat shock protein 70 (sHSP70) refers to a 70 kDa protein present in the cytosol of the cell.

Non-limiting examples of cytosolic HSP70 protein which can be identified in the biological sample (e.g., blood sample) according to the method or kit of some embodiments of the invention include HSPA1A (GenBank Accession No. NP_005336.3; SEQ ID NO:3), HSPA1B (GenBank Accession No. NP_005337.2; SEQ ID NO:4), HSPA2 (GenBank Accession No. NP_068814.2; SEQ ID NO:5), HSPA1L (GenBank Accession No. NP_005518.3; SEQ ID NO:6), HSPA6 (GenBank Accession No. NP_002146.2; SEQ ID NO:7) and HSPA8 (GenBank Accession Nos. NP_006588.1 and NP_694881; SEQ ID NOs:8 and 9).

According to some embodiments of the invention, the cytosolic (soluble) HSP70 which is identified by the method or kit of the invention is HSPA1A.

Antibodies directed against soluble HSP70 are available from various commercial suppliers such as R&D systems [e.g., an ELISA kit, DYC1663E, Human/Mouse/Rat Total HSP70/HSPA1A DuoSet IC Econ Pk, 15 Plt].

According to some embodiments of the invention, the predetermined level of sHSP70 is 1.67 ng/ml in a body fluid of a subject such as a serum sample.

According to some embodiments of the invention, the method further comprising informing the subject of the predicted prognosis (i.e., good or poor prognosis) as compared to the expected prognosis based on the gold standard criteria for determining prognosis of the subject having the solid tumor, e.g., as predicted based on tumor's stage, subject's age and/or subject's gender (i.e., male or female).

As used herein the phrase “informing the subject” refers to advising the subject on the predicted prognosis based on the level of mortalin or the level of mortalin and sHSP70 in the biological sample (e.g., body fluid) of the subject (e.g., in the blood sample).

Once the predicted prognosis is determined, the results can be recorded in the subject's medical file, which may assist in selecting a treatment regimen for the subject.

The agents of some embodiments of the invention which are described hereinabove for determining the presence and/or level of mortalin and/or sHSP70 may be included in a diagnostic kit/article of manufacture preferably along with appropriate instructions for use and labels indicating FDA approval for use in determining the presence and/or level of mortalin in a blood sample and/or predicting prognosis of a subject diagnosed with cancer.

Such a kit can include, for example, at least one container including at least one of the above described diagnostic agents (e.g., anti-mortalin antibody, and/or anti soluble HSP70 antibody) and an imaging reagent packed in another container (e.g., enzymes, secondary antibodies, buffers, chromogenic substrates, fluorogenic material). The kit may also include appropriate buffers and preservatives for improving the shelf-life of the kit.

According to an aspect some embodiments of the invention, there is provided a kit which comprises at least two distinct antibodies to mortalin directed against different epitopes of the mortalin.

According to an aspect some embodiments of the invention, the kit is for determining presence and/or level of soluble mortalin in a biological sample (e.g., body fluid) and/or predicting prognosis of a subject diagnosed with solid tumor.

According to an aspect some embodiments of the invention, the kit includes instructions for use in determining presence and/or level of soluble mortalin in a biological sample (e.g., body fluid) and/or predicting prognosis of a subject diagnosed with solid tumor.

According to some embodiments of the invention, one antibody of the at least two antibodies is a mouse monoclonal against human mortalin.

According to some embodiments of the invention, one antibody of the at least two antibodies is a goat polyclonal against human mortalin.

According to some embodiments of the invention, the mouse monoclonal antibody against human mortalin is attached to a solid support.

According to some embodiments of the invention, the kit further comprising a secondary antibody for binding one of the at least two antibodies.

According to some embodiments of the invention, the kit further comprising an Enzyme-linked immunosorbent assay (ELISA) plate.

According to some embodiments of the invention, the kit further comprising an anti-soluble heat shock protein 70 (sHSP70) antibody.

According to some embodiments of the invention, the kit further comprising a reference sample. For example, the kit may comprise a biological sample such as blood which is obtained from a healthy subject, or from a subject diagnosed with cancer and having a known clinical outcome, e.g., prognosis, e.g., known survival period.

According to some embodiments of the invention, the kit further comprises a calibration standard for mortalin levels, e.g., with serial dilutions of known concentrations of mortalin. The calibration standard can be in a solid or liquid form (e.g., solution), and can be also attached to a solid

surface. According to some embodiments of the invention, the calibration standard has mortalin diluted in normal serum, or in a biological sample (e.g., body fluid) of a normal (healthy, control) subject.

According to some embodiments of the invention, the kit further comprises a calibration standard for sHSP70 levels, e.g., with serial dilutions of known concentrations of sHSP70. The calibration standard can be in a solid or liquid form (e.g., solution), and can be also attached to a solid surface. According to some embodiments of the invention, the calibration standard has sHSP70 diluted in normal serum, or in a biological sample (e.g., body fluid) of a normal (healthy, control) subject.

As used herein the term "about" refers to $\pm 10\%$.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

When reference is made to particular sequence listings, such reference is to be understood to also encompass sequences that substantially correspond to its complementary sequence as including minor sequence variations, resulting from, e.g., sequencing errors, cloning errors, or other alterations resulting in base substitution, base deletion or base addition, provided that the frequency of such variations is less than 1 in 50 nucleotides, alternatively, less than 1 in 100 nucleotides, alternatively, less than 1 in 200 nucleotides, alternatively, less than 1 in 500 nucleotides, alternatively, less than 1 in 1000 nucleotides, alternatively, less than 1 in 5,000 nucleotides, alternatively, less than 1 in 10,000 nucleotides.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Md. (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, Conn. (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed.

(1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, Calif. (1990); Marshak et al., "Strategies for Protein Purification and Characterization—A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

General Materials and Experimental Methods

Serum samples—Serum samples of colorectal cancer patients were collected at the outpatient oncology clinic of the 3rd Department of Internal Medicine, Semmelweis University, Budapest. A number of 175 consecutive patients, diagnosed with colorectal cancer, who were willing to give informed consent for the study, were enrolled regardless of tumor stage. Serum samples of 121 of these patients were available for this study and the median follow-up time was increased from 33 to 42 months. This patient cohort was already tested for the association between sHsp70 levels and survival (20) and between acute phase proteins plus sHsp70 and survival (21), except for four patients with no available serum samples for the present study. In the majority of cases, the primary tumor was removed surgically, according to relevant international guidelines, and patients were enrolled 4-6 weeks after surgery when blood was collected for the current study. In 16 cases, the primary tumor could not be removed before inclusion; these patients had advanced, metastatic tumors and were referred for primary chemotherapy. The patients were followed during and after chemotherapy in a protocol-based manner and health status and disease outcome were registered. More information on the patients is available in earlier publications (20, 21). The study received approvals by the ethical committees of Semmelweis University, Budapest and Tel Aviv University, Tel Aviv. 163 of the 175 patients were followed up and their survival recorded.

Mortalin EUSA—To be able to measure serum levels of Mortalin the present inventors developed the following capture enzyme-linked immunosorbent assay (ELISA). Wells of 96 well MaxiSorp Nunc-Immuno plate (Thermo Fisher Scientific, Rochester, N.Y.) were coated overnight at 4° C. with 1 mg/ml mouse monoclonal antibody directed to human mortalin (StressMarq Biosciences Inc., Victoria, Canada) in TB buffer (50 mM Tris Base pH 7.0, 100 mM NaCl). The wells were blocked with bovine serum albumin 15 mg/ml (Sigma-Aldrich, Rehovot, Israel) in TBS (Iris-buffered saline) for 1 hour at 37° C. and then serum samples diluted 1:3 in TB were added to the wells for 2 hours at 37° C., Next, the wells were washed with TBT (TB with 0.05% TWEEN®-20 (Croda International PLC. Polyoxyethylene-sorbitan monolaurate) and treated with a goat anti-human mortalin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) diluted in TB for 1 hour at 37° C. Finally, after a wash with TBT, peroxidase-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa.) diluted in TB was added for 1. hour at 25° C. Antibody binding was quantified by using TMB One Component Microwell Substrate (Southern Biotech, Birmingham, Ala.) and absorbance was read at 450 nm in a Microplate Reader (Spectrafluor plus, Tecan, Austria). Calibration

of the quantity of mortalin was included in each ELISA plate with 0-2.5 ng purified recombinant mortalin mixed with normal human serum (same dilution as the tested samples). Specificity of the anti-mortalin antibodies used was confirmed in Western Blots showing a single protein band in whole cell lysates [similar to the bands shown in (17)]. Purity of the recombinant mortalin was also confirmed (17). Absorbance of normal serum without recombinant mortalin was subtracted from all samples in the plate. A typical calibration curve is shown in FIG. 1.

Statistical analysis—Statistical analysis was performed using the SPSS 15.0 software (SPSS Inc., Chicago, Ill.). Patients' survival was tested by the Kaplan-Meier survival analysis, using the Log Rank test. The power of the study to identify the observed difference in mortality rate (0.18) between low- and high-mortalin groups was 0.78. The impact of soluble mortalin and other factors on patients' survival was tested by the Cox Proportional hazards analysis (univariate and multivariate analyses). The results of the Cox regression models are presented as hazard ratios, the corresponding 95% confidence intervals (CI) and the Wald chi-square and p values of likelihood ratio tests.

Example 1

Development of a Sensitive ELISA for Determining Mortalin Levels in a Blood Sample

Experimental Results

Mortalin is shed in vitro from complement attacked viable human erythroleukemia cells (16). Release of mortalin in vitro was also demonstrated from human colon carcinoma HCT116 cells (FIG. 3), mouse colorectal CT26 cells, mouse lymphoma EL4 cells, mouse bladder carcinoma MBT2 cells and human B lymphoma Raji cells (data not shown).

Calibration of the mortalin ELISA assay for quantitation of mortalin concentrations in a biological sample—In order to measure mortalin level in patients' blood, the present inventors have developed a sensitive mortalin ELISA with a low background reading. Several alternative protocols were tested and finally the captured ELISA described in details under the "General Materials and Experimental Methods" section above was found to be satisfactory. This mortalin ELISA gave a reproducible and sensitive detection of 0.25 ng of mortalin in human serum diluted 3 folds. Each ELISA plate had wells with normal human serum alone or human serum with increasing amounts of recombinant mortalin. Normal human serum gave a low background readout to that was subtracted from all samples. The recombinant mortalin samples gave a linear dose-dependent calibration curve (FIG. 1) that was used to convert the optical density readout of the clinical samples into nanogram (ng) of mortalin per sample.

Example 2

Determination of Mortalin Levels in Serum of Patients with Colorectal Cancer

Experimental Results

Determination of the concentration of mortalin in sera of patients with colorectal cancer—By using this ELISA, significant amounts of soluble mortalin were detected in the serum samples of all colorectal cancer patients (14-215 ng/ml). The patients were divided into two groups, according to their serum mortalin level. The low mortalin group (n=88) had serum mortalin concentration below 60 ng/ml,

whereas the high mortalin group (n=33) had levels equal to or higher than 60 ng/ml (60 ng/ml). This concentration of mortalin (60 ng/ml) was chosen based on Receiver operating characteristic (ROC) calculation to yield optimal stratification of patients regarding mortalin levels and survival.

Correlation between mortalin levels and other tumor determinants—Mortalin concentrations were analyzed in relation to the tumor stage of the patients determined in earlier studies (20, 21). There was no difference in the mortalin levels among the patients with different size of tumors (TNM-T) (p=0.151, Kruskal-Wallis test), among patients with different extent of lymph node involvement (TNM-N) (p=0.346, Kruskal-Wallis test) or between patients with or without distant metastases (TNM-M) (p=0.205, Mann-Whitney test). Likewise, there was no significant association between mortalin levels and different Duke's grade categories and grade of primary tumor (both p>0.050, data not shown). Similarly, data was available on the serum levels of sHSP70, C-reactive protein (CRP) and C1-Inhibitor in these patients (20, 21). Mortalin concentrations did not show correlation to the sHSP70 concentrations (Spearman correlation coefficient R=0.051, p=0.580) or CRP levels (R=0.018, p=0.878) whereas a significant correlation (R=0.335, p<0.001) was found between serum mortalin and C1-Inhibitor concentrations (28).

Example 3

Mortalin as a Predictor of Colorectal Prognosis and Patient's Survival

Experimental Results

Univariate survival analysis revealed mortalin as a predictor for patient's survival—Kaplan-Meier survival analysis of patients with low (<60 ng/ml) versus (vs.) high (≥60 ng/ml) mortalin level in serum during a 10 year follow-up gave a Log Rank overall comparison of P=0.042 between the two groups, with 113 months estimated median survival in the low mortalin group and 29 months median survival in the high mortalin group (FIG. 2 and Table 1 below). Univariate analysis by Cox regression of the variables: age and sex of the patients, TNM-N and TNM-M stage of the disease as well as serum mortalin and sHsp70 concentrations are presented in Table 2 below. Only patients without missing data were included in this analysis (n=99). Age was found to be a highly significant factor with a hazard of 1.04 per year (P=0.002). Females had a Hazard of 1.62 (P=0.029) relative to males. Both TNM-N (metastases in the regional lymph node) and TNM-M (distant metastases) were highly significant predictors of mortality while TNM-T was not (data not shown). High sHSP70 serum concentration (>1.65 ng/ml) was associated with mortality with a hazard ratio (HR) of 1.86 (p=0.006). High mortalin (≥60 ng/ml) level is a significant factor (P=0.048) with a hazard ratio of 1.73. In this analysis, patients with high and low serum mortalin levels had a mean survival time of 43.19 (32.39-53.99) months and 72.52 months (62.78-82.76), respectively (p=0.046).

TABLE 1

Provided are the survival data of patients at risk with low or high mortalin. Data is also presented in FIG. 2.						
Mortalin	Patients at risk (0-60 months)					
Low	89	80	70	56	50	47
High	33	29	22	13	12	12

TABLE 2

Univariate Cox-regression analysis of the association between age, sex of the colorectal cancer patients, TNM staging, as well as sHsp70 or mortalin serum levels and their survival.				
Variable*	Significance (P)	Hazard Ratio	95.0% confidence interval	
Age at diagnosis	0.002	1.041	1.015	1.068
Sex	0.029	1.620	1.049	2.500
TNM-N	0.001	3.211	1.654	6.231
TNM-M	<0.001	3.346	1.925	5.815
sHsp70	0.006	1.858	1.190	2.901
Mortalin	0.048	1.729	1.004	2.946

*Age (years); Sex (female/male); TMN-N (1 or 2 metastasis in the regional lymph nodes vs. 0, yes/no); TNM-M (distant metastasis, yes/no); sHSP70 (high: >median 1.67 ng/ml, low: <1.67 ng/ml); Mortalin (high: >median, 60 ng/ml, low: <60 ng/ml).

Multivariate analysis: Independent and additive effects of risk factors—The association of the same variables with mortality of the cancer patients was evaluated by multivariate Cox regression analysis (Table 3, below). The effects of age and sex were found to be insignificant, while the hazard of TNM-N1+TNM-N2 stages vs. TNM-N0 stage (lymph nodes involved, yes vs. no) remained highly significant (HR 3.34 (p=0.001). Similarly, patients with distant metastasis had significantly higher risk (HR: 4.04; p<0.001) than those with no distant metastases. In the multivariable model, the hazard ratio associated with high sHsp70 was 3.508 (p<0.001) while high mortalin concentrations exhibited a weaker but still significant association with the mortality of patients independently of the sHSP70 levels (Table 3, below).

TABLE 3

Multivariate analysis by Cox-regression of the association between age, and sex of the patients as well as high baseline sHsp70 levels and high baseline mortalin levels on survival of the patients with colorectal cancer.				
Variable*	Significance (P)	Hazard Ratio	95.0% confidence interval	
Age at diagnosis	0.488	1.013	0.977	1.050
Sex	0.900	0.959	0.497	1.850
TNM-N	0.001	3.337	1.661	6.704
TNM-M	<0.001	4.044	2.014	8.118
mortalin	0.044	1.976	1.019	3.831
sHsp70	<0.001	3.508	1.753	7.019

*Age (years); Sex (female/male); TMN-N (1 or 2 metastasis in the regional lymph nodes vs. 0, yes/no); TNM-M (distant metastasis, yes/no); sHSP70 high: >median 1.67 ng/ml, low: <1.67 ng/ml); Mortalin (high: >median, 60 ng/ml, low: <60 ng/ml).

Thereafter the present inventors analyzed the survival of the patients stratified according to sHSP70 and mortalin levels (FIG. 4 and Table 4 below). In this type of multivariate analysis, high mortalin level with low sHsp70 or low mortalin with high sHsp70 did not affect the mortality of patients. In contrast, patients with concomitant high mortalin and high sHSP70 concentrations had a 4.2 times higher hazard of mortality than patients with both low sHSP70 and low mortalin levels, indicating the additive nature of these two biomarkers (FIG. 4). Median survival time of the patients with high mortalin/high sHSP70 and low mortalin/low sHSP70 levels were 27.5 (13.3-34.3) months and 57.0 (31.0-81.0) months, respectively. In addition, the mean survival time (from FIG. 4) of patients with high mortalin and high sHsp70 was: 24.9+/-14.0 months and that of patients with low mortalin and low sHsp70: 54.6+/-30.8 months.

TABLE 4

Provided are the survival data of patients at risk with low or high mortalin and Hsp70. Data is also presented in FIG. 4.						
Mortalin/Hsp70	Patients at risk (0-60 months)					
Low/Low	46	43	39	35	33	32
High/Low	43	40	35	31	29	28
Low/High	14	12	10	8	7	6
High/High	18	14	8	5	3	3

Finally, the present inventors examined the additive impact of serum concentrations of mortalin and sHSP70 on prediction of patients' survival, as compared to the baseline model that includes the basic demographic variables as well as the tumor stage (Table 5, below). According to the results of the likelihood-ratio test ($\chi^2=26.846$, $p<0.001$), addition of the biomarkers sHsp70 and mortalin to the baseline model significantly increased the survival-predicting value of the model. In the subgroup of patients with concomitantly high sHsp70 and mortalin levels, a hazard rate of 8.176 (95% CI 3.267-20.463, $P<0.001$) was observed, indicating that the baseline model (clinical stage, age and sex) and the biomarkers are independent and additive predictors of mortality in colorectal cancer. An attempt to include serum levels of CRP in the model did not result in an increase in the strength of association with the survival of the patients (data not shown).

TABLE 5

Additive effect of the high mortalin and high sHSP70 levels in serum on the age and tumor TNM-dependent mortality risk of patients with colorectal cancer. Analysis by model-building tool of Cox regression analysis.				
Variable*	Significance	Hazard	95.0% confidence interval	
	(P)	Ratio	Lower	Upper
Model 1				
Age at diagnosis	0.098	1.030	0.995	1.066
Sex	0.865	0.947	0.508	1.768
TNM-N	0.003	1.853	1.234	2.784
TNM-M	0.001	3.130	1.630	6.013
Model 2				
Age	0.323	1.017	0.983	1.052
Sex	0.571	1.218	0.617	2.404
TNM-N	0.003	1.879	1.244	2.840
TNM-M	<0.0001	5.000	2.423	10.318
Mortalin low & sHSP70 low	—	—	—	—
Mortalin high & sHSP70 low	0.223	0.390	0.086	1.772
Mortalin low & sHSP70 high	0.123	1.836	0.849	3.974
Mortalin high & sHSP70 high	<0.001	8.176	3.267	20.463

Likelihood-ratio-test as compared to model 1: $\chi^2 = 26.846$, $p < 0.0001$

*Age (years); Sex (female/male); TMN-N (1 or 2 metastasis in the regional lymph nodes vs. 0, yes/no); TNM-M (distant metastasis, yes/no); sHSP70 (high: $>median$ 1.67 ng/ml, low: <1.67 ng/ml); Mortalin (high: $>median$, 60 ng/ml, low: <60 ng/ml). Number of patients in the different (mortalin/sHsp70): low/low-46; high/low-43; low/high-14; high/high-18.

Analysis and Discussion

As shown here, colorectal cancer patients have elevated mortalin concentrations in their blood serum. This finding is surprising since mortalin is a mitochondrial protein and expected to be intracellular and not extracellular. Based on the in vitro study that demonstrated secretion of mortalin from cancer cells during an immune attack (16), the present inventors have hypothesized that such a release of mortalin may occur also in vivo during an immune attack. However,

finding of circulating mortalin in the absence of any therapy was surprising and unexpected.

Development of a novel and sensitive mortalin ELISA permitted the present inventors to quantify the soluble mortalin level in blood serum of colorectal cancer patients. The amount of circulating soluble mortalin was found to be highly variable (10-214 ng/ml) and was independent on the stage of the disease. This study raises additional intriguing questions regarding the function of extracellular mortalin, release of mortalin in other cancers and, in general, in disease, and the effect of therapy on serum mortalin levels. Here, the present inventors correlated the amount of circulating mortalin with survival of the cancer patients.

The cohort of cancer patients studied here was part of a larger group of 175 colorectal cancer patients studied earlier in which the level of soluble cytosolic Hsp70 was analyzed. Correlation was found between high sHSP70 and faster disease progression in colorectal cancer (20). A follow up study examined in the same patients several other potential prognostic bio-markers (21). High (above median) levels of CRP, C1-INH and sHSP70 were found to be independently associated with poor patient survival. The additive effect of high sHSP70, CRP and C1-INH levels on the survival of patients exceeded that of high sHSP70 alone (21). Updated survival information since the previous study was available on 163 patients. Serum sample of 121 patients were tested for soluble mortalin levels. Patients that exhibited a higher mortalin level (>60 ng/ml) also exhibited a significantly shorter median survival time (29 months) than patients that exhibited low mortalin level (113 months, $P=0.042$). The hazard value of high mortalin is 1.730. Therefore, without being bound by any theory, high circulating mortalin level is proposed as a risk factor in colorectal carcinoma. Multivariate analysis of survival data of 99 patients, for which age, sex, cancer stage, soluble HSP70 and mortalin information was available, indicated that patients with both high soluble HSP70 and high mortalin had more than 8-times higher risk of mortality (8.176, 3.267-20.463, $P<0.001$) as compared to those with low concentrations of both biomarkers. Without being bound by any theory, it is concluded that having concomitant high circulating HSP70 and mortalin levels indicates bad prognosis in colorectal cancer patients, thus measurement of these two biomarkers may be informative in this setting.

Immunohistochemical analysis demonstrated that mortalin is over-expressed in several tumor types, including colorectal adenocarcinoma and hepatocellular carcinoma (11, 14, 15, 22). Elevated mortalin in liver cancer was associated with metastasis and early cancer recurrence (22). Over-expression of mortalin in colorectal adenocarcinomas is also correlated with poor survival (15). Apparently, mortalin confers an advantage to cancer growth and metastasis, but the mechanism is still poorly characterized. In addition, mortalin promotes cell resistance to complement-dependent cytotoxicity (CDC) and may permit escape of cancer cells from antibody-based immunotherapy (16-18). Down regulation of mortalin, using siRNA, antibodies or MKT-077 enhance cells sensitivity to CDC, whereas over-expression of mortalin enhanced cell resistance to CDC (Pilzer et al. 2010, Int. J. Cancer 126:1428-1435). As shown here, serum levels of mortalin and HSP70 and the TNM stage are apparently independent determinants in survival of colorectal cancer patients. Without being bound by any theory, this may suggest that mortalin and HSP70 are secreted into the blood from different tissues and/or under distinct signals. The trigger that has induced in these patients mortalin release into the blood and the origin of this mortalin remain

to be identified. Without being bound by any theory, it is possible that inflammatory or other stress responses cause secretion of mortalin into the circulation from metastatic cells and/or non-cancerous affected tissues.

Cytosolic Hsp70 is also elevated in hepatocellular carcinoma compared with noncancerous liver tissue (23, 24). Expression level of Hsp70 in bladder cancer was significantly correlated with major prognostic indicators, including pathologic stage and tumor grade. Treatment of the bladder cancer cells with siRNA directed to Hsp70 sensitizes them to chemotherapy (25).

Extracellular Hsp70 was released from healthy cells (26) and was found in serum of normal individuals (27). Without being bound by any theory, the fact that they are both independent on tumor stage may propose that the main source for the two proteins in blood may not be the cancer cells themselves. Alternatively, it is possible that mortalin and Hsp70 are released to a similar extent from early stage and more advanced cancer cells but under distinct signals and are each associated with a more aggressive cancer phenotype.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

REFERENCES

ADDITIONAL REFERENCES ARE CITED IN TEXT

1. Kang, P. J., J. Ostermann, J. Shilling, W. Neupert, E. A. Craig, and N. Pfanner. 1990. Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature* 348:137-143.
2. Wiedemann, N., A. E. Frazier, and N. Pfanner. 2004. The protein import machinery of mitochondria. *The Journal of biological chemistry* 279:14473-14476.
3. Merrick, B. A., V. R. Walker, C. He, R. M. Patterson, and J. K. Selkirk. 1997. Induction of novel Grp75 isoforms by 2-deoxyglucose in human and murine fibroblasts. *Cancer Lett* 119:185-190.
4. Sadekova, S., S. Lehnert, and T. Y. Chow. 1997. Induction of PBP74/mortalin/Grp75, a member of the hsp70 family, by low doses of ionizing radiation: a possible role in induced radioresistance. *Int J Radiat Biol* 72:653-660.
5. Massa, S. M., F. M. Longo, J. Zuo, S. Wang, J. Chen, and F. R. Sharp. 1995. Cloning of rat grp75, an hsp70-family member, and its expression in normal and ischemic brain. *J Neurosci Res* 40:807-819.
6. Tsuchiya, T., J. M. Dhahbi, X. Cui, P. L. Mote, A. Bartke, and S. R. Spindler. 2004. Additive regulation of hepatic

gene expression by dwarfism and caloric restriction. *Physiol Genomics* 17:307-315.

7. Craig, E. A., J. Kramer, and J. Kosc-Smithers. 1987. SSC1, a member of the 70-kDa heat shock protein multigene family of *Saccharomyces cerevisiae*, is essential for growth. *Proc Natl Acad Sci USA* 84:4156-4160.
8. Liu, Y., W. Liu, X. D. Song, and J. Zuo. 2005. Effect of GRP75/mthsp70/PBP74/mortalin overexpression on intracellular ATP level, mitochondrial membrane potential and ROS accumulation following glucose deprivation in PC12 cells. *Mol Cell Biochem* 268:45-51.
9. Taurin, S., V. Seyrantepe, S. N. Orlov, T. L. Tremblay, P. Thibault, M. R. Bennett, P. Hamet, and A. V. Pshzhetsky. 2002. Proteome analysis and functional expression identify mortalin as an antiapoptotic gene induced by elevation of $[Na^+]/[K^+]_i$ ratio in cultured vascular smooth muscle cells. *Circ Res* 91:915-922.
10. Xu, L., L. A. Voloboueva, Y. Ouyang, J. F. Emery, and R. G. Giffard. 2009. Overexpression of mitochondrial Hsp70/Hsp75 in rat brain protects mitochondria, reduces oxidative stress, and protects from focal ischemia. *J Cereb Blood Flow Metab* 29:365-374.
11. Wadhwa, R., S. Takano, K. Kaur, C. C. Deocaris, O. M. Pereira-Smith, R. R. Reddel, and S. C. Kaul. 2006. Upregulation of mortalin/mthsp70/Grp75 contributes to human carcinogenesis. *Int J Cancer* 118:2973-2980.
12. Wadhwa, R., S. Takano, K. Taira, and S. C. Kaul. 2004. Reduction in mortalin level by its antisense expression causes senescence-like growth arrest in human immortalized cells. *J Gene Med* 6:439-444.
13. Ran, Q., R. Wadhwa, R. Kawai, S. C. Kaul, R. N. Sifers, R. J. Bick, J. R. Smith, and O. M. Pereira-Smith. 2000. Extramitochondrial localization of mortalin/mthsp70/PBP74/GRP75. *Biochem Biophys Res Commun* 275:174-179.
14. Takano, S., R. Wadhwa, Y. Yoshii, T. Nose, S. C. Kaul, and Y. Mitsui. 1997. Elevated levels of mortalin expression in human brain tumors. *Exp Cell Res* 237:38-45.
15. Dundas, S. R., L. C. Lawrie, P. H. Rooney, and G. I. Murray. 2005. Mortalin is over-expressed by colorectal adenocarcinomas and correlates with poor survival. *J Pathol* 205:74-81.
16. Pilzer, D., and Z. Fishelson. 2005. Mortalin/GRP75 promotes release of membrane vesicles from immune attacked cells and protection from complement-mediated lysis. *Int Immunol* 17:1239-1248.
17. Pilzer, D., M. Saar, K. Koya, and Z. Fishelson. 2010. Mortalin inhibitors sensitize K562 leukemia cells to complement-dependent cytotoxicity. *Int J Cancer* 126:1428-1435.
18. Pilzer, D., O. Gasser, O. Moskovich, J. A. Schifferli, and Z. Fishelson. 2005. Emission of membrane vesicles: roles in complement resistance, immunity and cancer. *Springer Semin Immunopathol* 27:375-387.
19. De Maio, A. 2011. Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: a form of communication during injury, infection, and cell damage. It is never known how far a controversial finding will go! Dedicated to Ferruccio Ritossa. *Cell Stress Chaperones* 16:235-249.
20. Kocsis, J., B. Madaras, E. K. Toth, G. Fust, and Z. Prohaszka. 2010. Serum level of soluble 70-kD heat shock protein is associated with high mortality in patients with colorectal cancer without distant metastasis. *Cell Stress Chaperones* 15:143-151.
21. Kocsis, J., T. Meszaros, B. Madaras, E. K. Toth, S. Kamondi, P. Gal, L. Varga, Z. Prohaszka, and G. Fust.

2011. High levels of acute phase proteins and soluble 70 kDa heat shock proteins are independent and additive risk factors for mortality in colorectal cancer. *Cell Stress Chaperones* 16:49-55.
22. Yi, X., J. M. Luk, N. P. Lee, J. Peng, X. Leng, X. Y. Guan, G. K. Lau, L. Beretta, and S. T. Fan. 2008. Association of mortalin (HSPA9) with liver cancer metastasis and prediction for early tumor recurrence. *Mol Cell Proteomics* 7:315-325.
23. Chuma, M., M. Sakamoto, K. Yamazaki, T. Ohta, M. Ohki, M. Asaka, and S. Hirohashi. 2003. Expression profiling in multistage hepatocarcinogenesis: identification of HSP70 as a molecular marker of early hepatocellular carcinoma. *Hepatology* 37:198-207.
24. Sakamoto, M. 2009. Early HCC: diagnosis and molecular markers. *J Gastroenterol* 44 Suppl 19:108-111.

25. Behnsawy, H. M., H. Miyake, Y. Kusuda, and M. Fujisawa. 2011. Small interfering RNA targeting heat shock protein 70 enhances chemosensitivity in human bladder cancer cells. *Urol Oncol*.
26. Hightower, L. E., and P. T. Guidon, Jr. 1989. Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins. *J Cell Physiol* 138:257-266.
27. Pockley, A. G., J. Shepherd, and J. M. Corton. 1998. Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals. *Immunol Invest* 27:367-377.
28. Rozenberg P, Kocsis J, Saar M, Prohaszka Z, Hist G, Fishelson Z. Elevated levels of mitochondrial mortalin and cytosolic HSP70 in blood as risk factors in patients with colorectal cancer. *Int. J. Cancer*. 2013 Jul. 15; 133(2):514-8. doi: 10.1002/ijc.28029. Epub 2013 Feb. 12.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 9

<210> SEQ ID NO 1

<211> LENGTH: 679

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

```

Met Ile Ser Ala Ser Arg Ala Ala Ala Arg Leu Val Gly Ala Ala
1           5           10           15

Ala Ser Arg Gly Pro Thr Ala Ala Arg His Gln Asp Ser Trp Asn Gly
20          25          30

Leu Ser His Glu Ala Phe Arg Leu Val Ser Arg Arg Asp Tyr Ala Ser
35          40          45

Glu Ala Ile Lys Gly Ala Val Val Gly Ile Asp Leu Gly Thr Thr Asn
50          55          60

Ser Cys Val Ala Val Met Glu Gly Lys Gln Ala Lys Val Leu Glu Asn
65          70          75          80

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

Gly Glu Arg Leu Val Gly Met Pro Ala Lys Arg Gln Ala Val Thr Asn
100         105         110

Pro Asn Asn Thr Phe Tyr Ala Thr Lys Arg Leu Ile Gly Arg Arg Tyr
115         120         125

Asp Asp Pro Glu Val Gln Lys Asp Ile Lys Asn Val Pro Phe Lys Ile
130         135         140

Val Arg Ala Ser Asn Gly Asp Ala Trp Val Glu Ala His Gly Lys Leu
145         150         155         160

Tyr Ser Pro Ser Gln Ile Gly Ala Phe Val Leu Met Lys Met Lys Glu
165         170         175

Thr Ala Glu Asn Tyr Leu Gly His Thr Ala Lys Asn Ala Val Ile Thr
180         185         190

Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln Ala Thr Lys Asp Ala
195         200         205

Gly Gln Ile Ser Gly Leu Asn Val Leu Arg Val Ile Asn Glu Pro Thr
210         215         220

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

Ala Val Tyr Asp Leu Gly Gly Gly Thr Phe Asp Ile Ser Ile Leu Glu
245         250         255

```

Ile	Gln	Lys	Gly	Val	Phe	Glu	Val	Lys	Ser	Thr	Asn	Gly	Asp	Thr	Phe		
			260					265					270				
Leu	Gly	Gly	Glu	Asp	Phe	Asp	Gln	Ala	Leu	Leu	Arg	His	Ile	Val	Lys		
			275					280					285				
Glu	Phe	Lys	Arg	Glu	Thr	Gly	Val	Asp	Leu	Thr	Lys	Asp	Asn	Met	Ala		
			290					295					300				
Leu	Gln	Arg	Val	Arg	Glu	Ala	Ala	Glu	Lys	Ala	Lys	Cys	Glu	Leu	Ser		
			305		310			315						320			
Ser	Ser	Val	Gln	Thr	Asp	Ile	Asn	Leu	Pro	Tyr	Leu	Thr	Met	Asp	Ser		
			325					330					335				
Ser	Gly	Pro	Lys	His	Leu	Asn	Met	Lys	Leu	Thr	Arg	Ala	Gln	Phe	Glu		
			340					345					350				
Gly	Ile	Val	Thr	Asp	Leu	Ile	Arg	Arg	Thr	Ile	Ala	Pro	Cys	Gln	Lys		
			355					360					365				
Ala	Met	Gln	Asp	Ala	Glu	Val	Ser	Lys	Ser	Asp	Ile	Gly	Glu	Val	Ile		
			370		375						380						
Leu	Val	Gly	Gly	Met	Thr	Arg	Met	Pro	Lys	Val	Gln	Gln	Thr	Val	Gln		
			385		390						395					400	
Asp	Leu	Phe	Gly	Arg	Ala	Pro	Ser	Lys	Ala	Val	Asn	Pro	Asp	Glu	Ala		
			405					410					415				
Val	Ala	Ile	Gly	Ala	Ala	Ile	Gln	Gly	Gly	Val	Leu	Ala	Gly	Asp	Val		
			420					425					430				
Thr	Asp	Val	Leu	Leu	Leu	Asp	Val	Thr	Pro	Leu	Ser	Leu	Gly	Ile	Glu		
			435					440					445				
Thr	Leu	Gly	Gly	Val	Phe	Thr	Lys	Leu	Ile	Asn	Arg	Asn	Thr	Thr	Ile		
			450		455						460						
Pro	Thr	Lys	Lys	Ser	Gln	Val	Phe	Ser	Thr	Ala	Ala	Asp	Gly	Gln	Thr		
			465		470						475					480	
Gln	Val	Glu	Ile	Lys	Val	Cys	Gln	Gly	Glu	Arg	Glu	Met	Ala	Gly	Asp		
			485					490					495				
Asn	Lys	Leu	Leu	Gly	Gln	Phe	Thr	Leu	Ile	Gly	Ile	Pro	Pro	Ala	Pro		
			500					505					510				
Arg	Gly	Val	Pro	Gln	Ile	Glu	Val	Thr	Phe	Asp	Ile	Asp	Ala	Asn	Gly		
			515					520					525				
Ile	Val	His	Val	Ser	Ala	Lys	Asp	Lys	Gly	Thr	Gly	Arg	Glu	Gln	Gln		
			530		535						540						
Ile	Val	Ile	Gln	Ser	Ser	Gly	Gly	Leu	Ser	Lys	Asp	Asp	Ile	Glu	Asn		
			545		550						555					560	
Met	Val	Lys	Asn	Ala	Glu	Lys	Tyr	Ala	Glu	Glu	Asp	Arg	Arg	Lys	Lys		
			565					570					575				
Glu	Arg	Val	Glu	Ala	Val	Asn	Met	Ala	Glu	Gly	Ile	Ile	His	Asp	Thr		
			580					585					590				
Glu	Thr	Lys	Met	Glu	Glu	Phe	Lys	Asp	Gln	Leu	Pro	Ala	Asp	Glu	Cys		
			595					600					605				
Asn	Lys	Leu	Lys	Glu	Glu	Ile	Ser	Lys	Met	Arg	Glu	Leu	Leu	Ala	Arg		
			610		615						620						
Lys	Asp	Ser	Glu	Thr	Gly	Glu	Asn	Ile	Arg	Gln	Ala	Ala	Ser	Ser	Leu		
			625		630						635					640	
Gln	Gln	Ala															

-continued

Ser Glu Arg Glu Gly Ser Gly Ser Ser Gly Thr Gly Glu Gln Lys Glu
660 665 670

Asp Gln Lys Glu Glu Lys Gln
675

<210> SEQ ID NO 2
<211> LENGTH: 3506
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

ttcctcccct ggactctttc tgagctcaga gccgccgcag ccgggacagg agggcaggct	60
ttctccaacc atcatgtgc ggagcatatt acctgtacgc cctggctccg ggagcggcag	120
tcgagtatcc tctggtcagg cggcgcgggc ggccctcag cggaagagcg ggcctctggg	180
ccgcagtgc caacccccgc cctcaccacc acgtgggttg aggtttccag aagcgtgcc	240
gccaccgcat cgcgcagctc ttgcccgcg gagecgtgt ttgtgcctc gtactcctcc	300
atttatccgc catgataagt gccagccgag ctgcagcagc ccgtctcgtg ggccgcgcag	360
cctcccgggg ccctacggcc gcccgccacc aggatagctg gaatggcctt agtcatgagg	420
cttttagact tgtttcaagg cgggattatg catcagaagc aatcaaggga gcagtgttg	480
gtattgattt gggctactacc aactcctgcg tggcagttat ggaaggtaaa caagcaaagg	540
tgctggagaa tgccgaaggt gccagaacca ccccttcagt tgtggccttt acagcagatg	600
gtgagcgact tgttggaatg ccggccaagc gacaggtgt caccaacca aacaatacat	660
tttatgttac caagcgtctc attggccggc gatatgatga tcctgaagta cagaaagaca	720
ttaaaaatgt tccctttaaa attgtccgtg cctccaatgg tgatgcctgg gttgaggctc	780
atgggaaatt gtattctccg agtcagattg gagcatttgt gttgatgaag atgaaagaga	840
ctgcagaaaa ttacttgggg cacacagcaa aaaatgctgt gatcacagtc ccagcttatt	900
tcaatgactc gcagagacag gccactaaag atgctggcca gatattctga ctgaatgtgc	960
ttcgggtgat taatgagccc acagctgctg ctcttgccca tggctagac aaatcagaag	1020
acaaagtcac tgctgtatat gatttaggtg gtggaacttt tgatatttct atcctggaaa	1080
ttcagaaagg agtatttgag gtgaaatcca caaatgggga taccttctta ggtggggaag	1140
actttgacca ggccttgcta cggcacattg tgaaggagtt caagagagag acaggggttg	1200
atttgactaa agacaacatg gcacttcaga gggtaaggga agctgctgaa aaggctaaat	1260
gtgaactctc ctcatctgtg cagactgaca tcaatttgcc ctatcttaca atggattctt	1320
ctggacccaa gcatttgaat atgaagtga cccgtgctca atttgaaggg attgtcactg	1380
atctaatacag aaggactatc gctccatgcc aaaaagctat gcaagatgca gaagtcagca	1440
agagtgcacat aggagaagtg attcttgttg gtggcatgac taggatgccc aaggttcagc	1500
agactgtaca ggatcttttt ggccagagccc caagtaaagc tgtcaatcct gatgaggctg	1560
tggccatttg agctgccatt caggagagtg tgttggccgg cgatgtcacg gatgtgctgc	1620
tccttgatgt cactcccctg tctctgggta ttgaaactct aggaggtgtc ttaccaaac	1680
ttattaatag gaataccact attccaacca agaagagcca ggtattctct actgccgctg	1740
atggtcaaac gcaagtggaa attaaagtgt gtcagggtga aagagagatg gctggagaca	1800
acaaactcct tggacagttt actttgattg gaattccacc agcccctcgt ggagttcctc	1860
agattgaagt tacatttgac attgatgcca atgggatagt acatgtttct gctaaagata	1920
aaggcacagg acgtgagcag cagattgtaa tccagtcttc tgggtggatta agcaaagatg	1980

-continued

```

atattgaaaa tatggttaaa aatgcagaga aatatgctga agaagaccgg cgaaagaagg 2040
aacgagttga agcagttaat atggctgaag gaatcattca cgacacagaa accaagattg 2100
aagaattcaa ggaccaatta cctgctgatg agtgcaacaa gctgaaagaa gagatttcca 2160
aaatgagggg gctcctggct agaaaagaca gcgaaacagg agaaaatatt agacaggcag 2220
catcctctct tcagcaggca tcaactgaagc tgttcgaaat ggcatacaaa aagatggcat 2280
ctgagcgaga aggctctgga agttctggca ctggggaaca aaaggaagat caaaaggagg 2340
aaaaacagta ataatagcag aaattttgaa gccagaagga caacatatga agcttaggag 2400
tgaagagact tcctgagcag aaatgggcca acttcagtct ttttactgtg tttttgcagt 2460
attctatata taatttcctt aattttgtaa tttagtgaac attagctagt gatcatttaa 2520
tggacagtga ttctaacagt ataaagtcca caatattcta tgtccctagc ctgtcatttt 2580
tcagctgcat gtaaaaggag gtaggatgaa ttgatcatta taaagattta actattttat 2640
gctgaagtga ccataatttc aaggggtgaa accatctcgc acacagcaat gaaggtagtc 2700
atccatagac ttgaaatgag accacatatg gggatgagat ccttctagtt agcctagtag 2760
tgctgtactg gcctgtatgt acatggggtc cttcaactga ggcttgcaa gtcaagctgg 2820
ctgtgccatg tttgtagatg gggcagagga atctagaaca atgggaaact tagctattta 2880
tattaggtac agctattaaa acaaggtagg aatgaggcta gacctttaac ttccctaagg 2940
catacttttc tagctacctt ctgccctgtg tctggcacct acatccttga tgattgttct 3000
cttaccatt ctggaatttt ttttttttta aataaataca gaaagcatct tgatctcttg 3060
tttgtgaggg gtgatgcct gagatttagc ttcaagaata tgccatggct catgcttccc 3120
atatttccca aagagggaaa tacaggattt gctaacactg gttaaaaatg caaattcaag 3180
atttggaagg gctgttataa tgaaataatg agcagtatca gcatgtgcaa atcttgtttg 3240
aaggatttta ttttctcccc ttagacctt ggtacattta gaatcttgaa agtttctaga 3300
tctctaacat gaaagtttct agatctctaa catgaaagtt tttagatctc taacatgaaa 3360
accaaggtgg ctattttcag gttgctttca gctccaagta gaaataacca gaattggctt 3420
acattaaaga aactgcattc agaaataagt cctaagatag tatttctatg gctcaaaaat 3480
aaaaggaacc cagatttctt tcccta 3506

```

<210> SEQ ID NO 3

<211> LENGTH: 641

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

```

Met Ala Lys Ala Ala Ile Gly Ile Asp Leu Gly Thr Thr Tyr Ser
1           5           10           15

Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile Ile Ala Asn Asp
          20           25           30

Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe Thr Asp Thr Glu
          35           40           45

Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala Leu Asn Pro Gln
          50           55           60

Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg Lys Phe Gly Asp
65           70           75           80

Pro Val Val Gln Ser Asp Met Lys His Trp Pro Phe Gln Val Ile Asn
          85           90           95

```

Asp	Gly	Asp	Lys 100	Pro	Lys	Val	Gln	Val 105	Ser	Tyr	Lys	Gly	Glu 110	Lys	
Ala	Phe	Tyr	Pro 115	Glu	Glu	Ile	Ser 120	Ser	Met	Val	Leu	Thr 125	Lys	Met	Lys
Glu	Ile	Ala	Glu 130	Ala	Tyr	Leu 135	Gly	Tyr	Pro	Val	Thr 140	Asn	Ala	Val	Ile
Thr 145	Val	Pro	Ala	Tyr	Phe 150	Asn	Asp	Ser	Gln	Arg 155	Gln	Ala	Thr	Lys	Asp 160
Ala	Gly	Val	Ile 165	Ala	Gly	Leu	Asn	Val 170	Leu	Arg	Ile	Ile	Asn	Glu	Pro
Thr	Ala	Ala	Ala 180	Ile	Ala	Tyr	Gly	Leu 185	Asp	Arg	Thr	Gly	Lys 190	Gly	Glu
Arg	Asn	Val	Leu 195	Ile	Phe	Asp	Leu 200	Gly	Gly	Gly	Thr	Phe 205	Asp	Val	Ser
Ile	Leu	Thr	Ile 210	Asp	Asp	Gly 215	Ile	Phe	Glu	Val	Lys 220	Ala	Thr	Ala	Gly
Asp 225	Thr	His	Leu	Gly	Gly 230	Glu	Asp	Phe	Asp	Asn	Arg 235	Leu	Val	Asn	His 240
Phe	Val	Glu	Glu 245	Phe	Lys	Arg	Lys	His 250	Lys	Lys	Asp	Ile	Ser	Gln	Asn 255
Lys	Arg	Ala	Val 260	Arg	Arg	Leu	Arg	Thr 265	Ala	Cys	Glu	Arg	Ala 270	Lys	Arg
Thr	Leu	Ser	Ser 275	Ser	Thr	Gln	Ala 280	Ser	Leu	Glu	Ile	Asp 285	Ser	Leu	Phe
Glu	Gly	Ile	Asp 290	Phe	Tyr	Thr 295	Ser	Ile	Thr	Arg	Ala 300	Arg	Phe	Glu	Glu
Leu 305	Cys	Ser	Asp	Leu	Phe 310	Arg	Ser	Thr	Leu	Glu 315	Pro	Val	Glu	Lys	Ala 320
Leu	Arg	Asp	Ala 325	Lys	Leu	Asp	Lys	Ala 330	Gln	Ile	His	Asp	Leu	Val	Leu
Val	Gly	Gly	Ser 340	Thr	Arg	Ile	Pro	Lys 345	Val	Gln	Lys	Leu 350	Leu	Gln	Asp
Phe	Phe	Asn	Gly 355	Arg	Asp	Leu	Asn 360	Lys	Ser	Ile	Asn	Pro 365	Asp	Glu	Ala
Val 370	Ala	Tyr	Gly	Ala	Ala 375	Val	Gln	Ala	Ala	Ile	Leu 380	Met	Gly	Asp	Lys
Ser 385	Glu	Asn	Val	Gln	Asp 390	Leu	Leu	Leu	Leu	Asp 395	Val	Ala	Pro	Leu	Ser 400
Leu	Gly	Leu	Glu 405	Thr	Ala	Gly	Gly	Val 410	Met	Thr	Ala	Leu	Ile	Lys	Arg 415
Asn	Ser	Thr	Ile 420	Pro	Thr	Lys	Gln	Thr 425	Gln	Ile	Phe	Thr 430	Thr	Tyr	Ser
Asp	Asn	Gln	Pro 435	Gly	Val	Leu	Ile 440	Gln	Val	Tyr	Glu	Gly 445	Glu	Arg	Ala
Met 450	Thr	Lys	Asp	Asn	Asn 455	Leu	Leu	Gly	Arg	Phe	Glu 460	Leu	Ser	Gly	Ile
Pro 465	Pro	Ala	Pro	Arg	Gly 470	Val	Pro	Gln	Ile	Glu 475	Val	Thr	Phe	Asp	Ile 480
Asp	Ala	Asn	Gly 485	Ile	Leu	Asn	Val	Thr 490	Ala	Thr	Asp	Lys	Ser	Thr	Gly 495
Lys	Ala	Asn	Lys 500	Ile	Thr	Ile	Thr	Asn 505	Asp	Lys	Gly	Arg 510	Leu	Ser	Lys

-continued

Glu Glu Ile Glu Arg Met Val Gln Glu Ala Glu Lys Tyr Lys Ala Glu
 515 520 525
 Asp Glu Val Gln Arg Glu Arg Val Ser Ala Lys Asn Ala Leu Glu Ser
 530 535 540
 Tyr Ala Phe Asn Met Lys Ser Ala Val Glu Asp Glu Gly Leu Lys Gly
 545 550 555 560
 Lys Ile Ser Glu Ala Asp Lys Lys Lys Val Leu Asp Lys Cys Gln Glu
 565 570 575
 Val Ile Ser Trp Leu Asp Ala Asn Thr Leu Ala Glu Lys Asp Glu Phe
 580 585 590
 Glu His Lys Arg Lys Glu Leu Glu Gln Val Cys Asn Pro Ile Ile Ser
 595 600 605
 Gly Leu Tyr Gln Gly Ala Gly Gly Pro Gly Pro Gly Gly Phe Gly Ala
 610 615 620
 Gln Gly Pro Lys Gly Gly Ser Gly Ser Gly Pro Thr Ile Glu Glu Val
 625 630 635 640

Asp

<210> SEQ ID NO 4
 <211> LENGTH: 641
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

Met Ala Lys Ala Ala Ala Ile Gly Ile Asp Leu Gly Thr Thr Tyr Ser
 1 5 10 15
 Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile Ile Ala Asn Asp
 20 25 30
 Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe Thr Asp Thr Glu
 35 40 45
 Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala Leu Asn Pro Gln
 50 55 60
 Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg Lys Phe Gly Asp
 65 70 75 80
 Pro Val Val Gln Ser Asp Met Lys His Trp Pro Phe Gln Val Ile Asn
 85 90 95
 Asp Gly Asp Lys Pro Lys Val Gln Val Ser Tyr Lys Gly Glu Thr Lys
 100 105 110
 Ala Phe Tyr Pro Glu Glu Ile Ser Ser Met Val Leu Thr Lys Met Lys
 115 120 125
 Glu Ile Ala Glu Ala Tyr Leu Gly Tyr Pro Val Thr Asn Ala Val Ile
 130 135 140
 Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln Ala Thr Lys Asp
 145 150 155 160
 Ala Gly Val Ile Ala Gly Leu Asn Val Leu Arg Ile Ile Asn Glu Pro
 165 170 175
 Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Arg Thr Gly Lys Gly Glu
 180 185 190
 Arg Asn Val Leu Ile Phe Asp Leu Gly Gly Gly Thr Phe Asp Val Ser
 195 200 205
 Ile Leu Thr Ile Asp Asp Gly Ile Phe Glu Val Lys Ala Thr Ala Gly
 210 215 220
 Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg Leu Val Asn His
 225 230 235 240

Phe	Val	Glu	Glu	Phe	Lys	Arg	Lys	His	Lys	Lys	Asp	Ile	Ser	Gln	Asn	
				245					250					255		
Lys	Arg	Ala	Val	Arg	Arg	Leu	Arg	Thr	Ala	Cys	Glu	Arg	Ala	Lys	Arg	
				260					265					270		
Thr	Leu	Ser	Ser	Ser	Thr	Gln	Ala	Ser	Leu	Glu	Ile	Asp	Ser	Leu	Phe	
				275					280					285		
Glu	Gly	Ile	Asp	Phe	Tyr	Thr	Ser	Ile	Thr	Arg	Ala	Arg	Phe	Glu	Glu	
				290					295					300		
Leu	Cys	Ser	Asp	Leu	Phe	Arg	Ser	Thr	Leu	Glu	Pro	Val	Glu	Lys	Ala	
				305					310					315		
Leu	Arg	Asp	Ala	Lys	Leu	Asp	Lys	Ala	Gln	Ile	His	Asp	Leu	Val	Leu	
				325					330					335		
Val	Gly	Gly	Ser	Thr	Arg	Ile	Pro	Lys	Val	Gln	Lys	Leu	Leu	Gln	Asp	
				340					345					350		
Phe	Phe	Asn	Gly	Arg	Asp	Leu	Asn	Lys	Ser	Ile	Asn	Pro	Asp	Glu	Ala	
				355					360					365		
Val	Ala	Tyr	Gly	Ala	Ala	Val	Gln	Ala	Ala	Ile	Leu	Met	Gly	Asp	Lys	
				370					375					380		
Ser	Glu	Asn	Val	Gln	Asp	Leu	Leu	Leu	Leu	Asp	Val	Ala	Pro	Leu	Ser	
				385					390					395		
Leu	Gly	Leu	Glu	Thr	Ala	Gly	Gly	Val	Met	Thr	Ala	Leu	Ile	Lys	Arg	
				405					410					415		
Asn	Ser	Thr	Ile	Pro	Thr	Lys	Gln	Thr	Gln	Ile	Phe	Thr	Thr	Tyr	Ser	
				420					425					430		
Asp	Asn	Gln	Pro	Gly	Val	Leu	Ile	Gln	Val	Tyr	Glu	Gly	Glu	Arg	Ala	
				435					440					445		
Met	Thr	Lys	Asp	Asn	Asn	Leu	Leu	Gly	Arg	Phe	Glu	Leu	Ser	Gly	Ile	
				450					455					460		
Pro	Pro	Ala	Pro	Arg	Gly	Val	Pro	Gln	Ile	Glu	Val	Thr	Phe	Asp	Ile	
				465					470					475		
Asp	Ala	Asn	Gly	Ile	Leu	Asn	Val	Thr	Ala	Thr	Asp	Lys	Ser	Thr	Gly	
				485					490					495		
Lys	Ala	Asn	Lys	Ile	Thr	Ile	Thr	Asn	Asp	Lys	Gly	Arg	Leu	Ser	Lys	
				500					505					510		
Glu	Glu	Ile	Glu	Arg	Met	Val	Gln	Glu	Ala	Glu	Lys	Tyr	Lys	Ala	Glu	
				515					520					525		
Asp	Glu	Val	Gln	Arg	Glu	Arg	Val	Ser	Ala	Lys	Asn	Ala	Leu	Glu	Ser	
				530					535					540		
Tyr	Ala	Phe	Asn	Met	Lys	Ser	Ala	Val	Glu	Asp	Glu	Gly	Leu	Lys	Gly	
				545					550					555		
Lys	Ile	Ser	Glu	Ala	Asp	Lys	Lys	Lys	Val	Leu	Asp	Lys	Cys	Gln	Glu	
				565					570					575		
Val	Ile	Ser	Trp	Leu	Asp	Ala	Asn	Thr	Leu	Ala	Glu	Lys	Asp	Glu	Phe	
				580					585					590		
Glu	His	Lys	Arg	Lys	Glu	Leu	Glu	Gln	Val	Cys	Asn	Pro	Ile	Ile	Ser	
				595					600					605		
Gly	Leu	Tyr	Gln	Gly	Ala	Gly	Gly	Pro	Gly	Pro	Gly	Gly	Phe	Gly	Ala	
				610					615					620		
Gln	Gly	Pro	Lys	Gly	Gly	Ser	Gly	Ser	Gly	Pro	Thr	Ile	Glu	Glu	Val	
				625					630					635		
				640					645					650		
				655												

-continued

```

<210> SEQ ID NO 5
<211> LENGTH: 639
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Met Ser Ala Arg Gly Pro Ala Ile Gly Ile Asp Leu Gly Thr Thr Tyr
 1             5             10             15

Ser Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile Ile Ala Asn
      20             25             30

Asp Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe Thr Asp Thr
      35             40             45

Glu Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala Met Asn Pro
 50             55             60

Thr Asn Thr Ile Phe Asp Ala Lys Arg Leu Ile Gly Arg Lys Phe Glu
 65             70             75             80

Asp Ala Thr Val Gln Ser Asp Met Lys His Trp Pro Phe Arg Val Val
      85             90             95

Ser Glu Gly Gly Lys Pro Lys Val Gln Val Glu Tyr Lys Gly Glu Thr
      100            105            110

Lys Thr Phe Phe Pro Glu Glu Ile Ser Ser Met Val Leu Thr Lys Met
      115            120            125

Lys Glu Ile Ala Glu Ala Tyr Leu Gly Gly Lys Val His Ser Ala Val
      130            135            140

Ile Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln Ala Thr Lys
      145            150            155            160

Asp Ala Gly Thr Ile Thr Gly Leu Asn Val Leu Arg Ile Ile Asn Glu
      165            170            175

Pro Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Lys Lys Gly Cys Ala
      180            185            190

Gly Gly Glu Lys Asn Val Leu Ile Phe Asp Leu Gly Gly Gly Thr Phe
      195            200            205

Asp Val Ser Ile Leu Thr Ile Glu Asp Gly Ile Phe Glu Val Lys Ser
      210            215            220

Thr Ala Gly Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg Met
      225            230            235            240

Val Ser His Leu Ala Glu Glu Phe Lys Arg Lys His Lys Lys Asp Ile
      245            250            255

Gly Pro Asn Lys Arg Ala Val Arg Arg Leu Arg Thr Ala Cys Glu Arg
      260            265            270

Ala Lys Arg Thr Leu Ser Ser Ser Thr Gln Ala Ser Ile Glu Ile Asp
      275            280            285

Ser Leu Tyr Glu Gly Val Asp Phe Tyr Thr Ser Ile Thr Arg Ala Arg
      290            295            300

Phe Glu Glu Leu Asn Ala Asp Leu Phe Arg Gly Thr Leu Glu Pro Val
      305            310            315            320

Glu Lys Ala Leu Arg Asp Ala Lys Leu Asp Lys Gly Gln Ile Gln Glu
      325            330            335

Ile Val Leu Val Gly Gly Ser Thr Arg Ile Pro Lys Ile Gln Lys Leu
      340            345            350

Leu Gln Asp Phe Phe Asn Gly Lys Glu Leu Asn Lys Ser Ile Asn Pro
      355            360            365

Asp Glu Ala Val Ala Tyr Gly Ala Ala Val Gln Ala Ala Ile Leu Ile
      370            375            380

```

-continued

Gly Asp Lys Ser Glu Asn Val Gln Asp Leu Leu Leu Leu Asp Val Thr
 385 390 395 400
 Pro Leu Ser Leu Gly Ile Glu Thr Ala Gly Gly Val Met Thr Pro Leu
 405 410 415
 Ile Lys Arg Asn Thr Thr Ile Pro Thr Lys Gln Thr Gln Thr Phe Thr
 420 425 430
 Thr Tyr Ser Asp Asn Gln Ser Ser Val Leu Val Gln Val Tyr Glu Gly
 435 440 445
 Glu Arg Ala Met Thr Lys Asp Asn Asn Leu Leu Gly Lys Phe Asp Leu
 450 455 460
 Thr Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln Ile Glu Val Thr
 465 470 475 480
 Phe Asp Ile Asp Ala Asn Gly Ile Leu Asn Val Thr Ala Ala Asp Lys
 485 490 495
 Ser Thr Gly Lys Glu Asn Lys Ile Thr Ile Thr Asn Asp Lys Gly Arg
 500 505 510
 Leu Ser Lys Asp Asp Ile Asp Arg Met Val Gln Glu Ala Glu Arg Tyr
 515 520 525
 Lys Ser Glu Asp Glu Ala Asn Arg Asp Arg Val Ala Ala Lys Asn Ala
 530 535 540
 Leu Glu Ser Tyr Thr Tyr Asn Ile Lys Gln Thr Val Glu Asp Glu Lys
 545 550 555 560
 Leu Arg Gly Lys Ile Ser Glu Gln Asp Lys Asn Lys Ile Leu Asp Lys
 565 570 575
 Cys Gln Glu Val Ile Asn Trp Leu Asp Arg Asn Gln Met Ala Glu Lys
 580 585 590
 Asp Glu Tyr Glu His Lys Gln Lys Glu Leu Glu Arg Val Cys Asn Pro
 595 600 605
 Ile Ile Ser Lys Leu Tyr Gln Gly Gly Pro Gly Gly Gly Ser Gly Gly
 610 615 620
 Gly Gly Ser Gly Ala Ser Gly Gly Pro Thr Ile Glu Glu Val Asp
 625 630 635

<210> SEQ ID NO 6

<211> LENGTH: 641

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

Met Ala Thr Ala Lys Gly Ile Ala Ile Gly Ile Asp Leu Gly Thr Thr
 1 5 10 15
 Tyr Ser Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile Ile Ala
 20 25 30
 Asn Asp Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe Thr Asp
 35 40 45
 Thr Glu Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala Met Asn
 50 55 60
 Pro Gln Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg Lys Phe
 65 70 75 80
 Asn Asp Pro Val Val Gln Ala Asp Met Lys Leu Trp Pro Phe Gln Val
 85 90 95
 Ile Asn Glu Gly Gly Lys Pro Lys Val Leu Val Ser Tyr Lys Gly Glu
 100 105 110
 Asn Lys Ala Phe Tyr Pro Glu Glu Ile Ser Ser Met Val Leu Thr Lys
 115 120 125

Leu 130	Glu	Thr	Ala	Glu	Ala	Phe	Leu	Gly	His	Pro	Val	Thr	Asn	Ala	
Val 145	Ile	Thr	Val	Pro	Ala	Tyr	Phe	Asn	Asp	Ser	Gln	Arg	Gln	Ala	Thr 160
Lys	Asp	Ala	Gly	Val	Ile	Ala	Gly	Leu	Asn	Val	Leu	Arg	Ile	Ile	Asn 175
Glu	Pro	Thr	Ala	Ala	Ala	Ile	Ala	Tyr	Gly	Leu	Asp	Lys	Gly	Gly	Gln
Gly	Glu	Arg	His	Val	Leu	Ile	Phe	Asp	Leu	Gly	Gly	Gly	Thr	Phe	Asp
Val	Ser	Ile	Leu	Thr	Ile	Asp	Asp	Gly	Ile	Phe	Glu	Val	Lys	Ala	Thr
Ala	Gly	Asp	Thr	His	Leu	Gly	Gly	Glu	Asp	Phe	Asp	Asn	Arg	Leu	Val 240
Ser	His	Phe	Val	Glu	Glu	Phe	Lys	Arg	Lys	His	Lys	Lys	Asp	Ile	Ser
Gln	Asn	Lys	Arg	Ala	Val	Arg	Arg	Leu	Arg	Thr	Ala	Cys	Glu	Arg	Ala
Lys	Arg	Thr	Leu	Ser	Ser	Ser	Thr	Gln	Ala	Asn	Leu	Glu	Ile	Asp	Ser
Leu	Tyr	Glu	Gly	Ile	Asp	Phe	Tyr	Thr	Ser	Ile	Thr	Arg	Ala	Arg	Phe
Glu	Glu	Leu	Cys	Ala	Asp	Leu	Phe	Arg	Gly	Thr	Leu	Glu	Pro	Val	Glu 320
Lys	Ala	Leu	Arg	Asp	Ala	Lys	Met	Asp	Lys	Ala	Lys	Ile	His	Asp	Ile
Val	Leu	Val	Gly	Gly	Ser	Thr	Arg	Ile	Pro	Lys	Val	Gln	Arg	Leu	Leu
Gln	Asp	Tyr	Phe	Asn	Gly	Arg	Asp	Leu	Asn	Lys	Ser	Ile	Asn	Pro	Asp
Glu	Ala	Val	Ala	Tyr	Gly	Ala	Ala	Val	Gln	Ala	Ala	Ile	Leu	Met	Gly
Asp	Lys	Ser	Glu	Lys	Val	Gln	Asp	Leu	Leu	Leu	Leu	Asp	Val	Ala	Pro 400
Leu	Ser	Leu	Gly	Leu	Glu	Thr	Ala	Gly	Gly	Val	Met	Thr	Ala	Leu	Ile
Lys	Arg	Asn	Ser	Thr	Ile	Pro	Thr	Lys	Gln	Thr	Gln	Ile	Phe	Thr	Thr
Tyr	Ser	Asp	Asn	Gln	Pro	Gly	Val	Leu	Ile	Gln	Val	Tyr	Glu	Gly	Glu
Arg	Ala	Met	Thr	Lys	Asp	Asn	Asn	Leu	Leu	Gly	Arg	Phe	Asp	Leu	Thr
Gly	Ile	Pro	Pro	Ala	Pro	Arg	Gly	Val	Pro	Gln	Ile	Glu	Val	Thr	Phe 480
Asp	Ile	Asp	Ala	Asn	Gly	Ile	Leu	Asn	Val	Thr	Ala	Thr	Asp	Lys	Ser
Thr	Gly	Lys	Val	Asn	Lys	Ile	Thr	Ile	Thr	Asn	Asp	Lys	Gly	Arg	Leu
Ser	Lys	Glu	Glu	Ile	Glu	Arg	Met	Val	Leu	Asp	Ala	Glu	Lys	Tyr	Lys
Ala	Glu	Asp	Glu	Val	Gln	Arg	Glu	Lys	Ile	Ala	Ala	Lys	Asn	Ala	Leu

-continued

Glu Ser Tyr Ala Phe Asn Met Lys Ser Val Val Ser Asp Glu Gly Leu
 545 550 555 560
 Lys Gly Lys Ile Ser Glu Ser Asp Lys Asn Lys Ile Leu Asp Lys Cys
 565 570 575
 Asn Glu Leu Leu Ser Trp Leu Glu Val Asn Gln Leu Ala Glu Lys Asp
 580 585 590
 Glu Phe Asp His Lys Arg Lys Glu Leu Glu Gln Met Cys Asn Pro Ile
 595 600 605
 Ile Thr Lys Leu Tyr Gln Gly Gly Cys Thr Gly Pro Ala Cys Gly Thr
 610 615 620
 Gly Tyr Val Pro Gly Arg Pro Ala Thr Gly Pro Thr Ile Glu Glu Val
 625 630 635 640
 Asp

<210> SEQ ID NO 7
 <211> LENGTH: 643
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

Met Gln Ala Pro Arg Glu Leu Ala Val Gly Ile Asp Leu Gly Thr Thr
 1 5 10 15
 Tyr Ser Cys Val Gly Val Phe Gln Gln Gly Arg Val Glu Ile Leu Ala
 20 25 30
 Asn Asp Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe Thr Asp
 35 40 45
 Thr Glu Arg Leu Val Gly Asp Ala Ala Lys Ser Gln Ala Ala Leu Asn
 50 55 60
 Pro His Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg Lys Phe
 65 70 75 80
 Ala Asp Thr Thr Val Gln Ser Asp Met Lys His Trp Pro Phe Arg Val
 85 90 95
 Val Ser Glu Gly Gly Lys Pro Lys Val Arg Val Cys Tyr Arg Gly Glu
 100 105 110
 Asp Lys Thr Phe Tyr Pro Glu Glu Ile Ser Ser Met Val Leu Ser Lys
 115 120 125
 Met Lys Glu Thr Ala Glu Ala Tyr Leu Gly Gln Pro Val Lys His Ala
 130 135 140
 Val Ile Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln Ala Thr
 145 150 155 160
 Lys Asp Ala Gly Ala Ile Ala Gly Leu Asn Val Leu Arg Ile Ile Asn
 165 170 175
 Glu Pro Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Arg Arg Gly Ala
 180 185 190
 Gly Glu Arg Asn Val Leu Ile Phe Asp Leu Gly Gly Gly Thr Phe Asp
 195 200 205
 Val Ser Val Leu Ser Ile Asp Ala Gly Val Phe Glu Val Lys Ala Thr
 210 215 220
 Ala Gly Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg Leu Val
 225 230 235 240
 Asn His Phe Met Glu Glu Phe Arg Arg Lys His Gly Lys Asp Leu Ser
 245 250 255
 Gly Asn Lys Arg Ala Leu Arg Arg Leu Arg Thr Ala Cys Glu Arg Ala
 260 265 270

-continued

Lys Arg Thr Leu Ser Ser Ser Thr Gln Ala Thr Leu Glu Ile Asp Ser
 275 280 285
 Leu Phe Glu Gly Val Asp Phe Tyr Thr Ser Ile Thr Arg Ala Arg Phe
 290 295 300
 Glu Glu Leu Cys Ser Asp Leu Phe Arg Ser Thr Leu Glu Pro Val Glu
 305 310 315 320
 Lys Ala Leu Arg Asp Ala Lys Leu Asp Lys Ala Gln Ile His Asp Val
 325 330 335
 Val Leu Val Gly Gly Ser Thr Arg Ile Pro Lys Val Gln Lys Leu Leu
 340 345 350
 Gln Asp Phe Phe Asn Gly Lys Glu Leu Asn Lys Ser Ile Asn Pro Asp
 355 360 365
 Glu Ala Val Ala Tyr Gly Ala Ala Val Gln Ala Ala Val Leu Met Gly
 370 375 380
 Asp Lys Cys Glu Lys Val Gln Asp Leu Leu Leu Leu Asp Val Ala Pro
 385 390 395 400
 Leu Ser Leu Gly Leu Glu Thr Ala Gly Gly Val Met Thr Thr Leu Ile
 405 410 415
 Gln Arg Asn Ala Thr Ile Pro Thr Lys Gln Thr Gln Thr Phe Thr Thr
 420 425 430
 Tyr Ser Asp Asn Gln Pro Gly Val Phe Ile Gln Val Tyr Glu Gly Glu
 435 440 445
 Arg Ala Met Thr Lys Asp Asn Asn Leu Leu Gly Arg Phe Glu Leu Ser
 450 455 460
 Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln Ile Glu Val Thr Phe
 465 470 475 480
 Asp Ile Asp Ala Asn Gly Ile Leu Ser Val Thr Ala Thr Asp Arg Ser
 485 490 495
 Thr Gly Lys Ala Asn Lys Ile Thr Ile Thr Asn Asp Lys Gly Arg Leu
 500 505 510
 Ser Lys Glu Glu Val Glu Arg Met Val His Glu Ala Glu Gln Tyr Lys
 515 520 525
 Ala Glu Asp Glu Ala Gln Arg Asp Arg Val Ala Ala Lys Asn Ser Leu
 530 535 540
 Glu Ala His Val Phe His Val Lys Gly Ser Leu Gln Glu Glu Ser Leu
 545 550 555 560
 Arg Asp Lys Ile Pro Glu Glu Asp Arg Arg Lys Met Gln Asp Lys Cys
 565 570 575
 Arg Glu Val Leu Ala Trp Leu Glu His Asn Gln Leu Ala Glu Lys Glu
 580 585 590
 Glu Tyr Glu His Gln Lys Arg Glu Leu Glu Gln Ile Cys Arg Pro Ile
 595 600 605
 Phe Ser Arg Leu Tyr Gly Gly Pro Gly Val Pro Gly Gly Ser Ser Cys
 610 615 620
 Gly Thr Gln Ala Arg Gln Gly Asp Pro Ser Thr Gly Pro Ile Ile Glu
 625 630 635 640
 Glu Val Asp

<210> SEQ ID NO 8

<211> LENGTH: 646

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

-continued

<400> SEQUENCE: 8

```

Met Ser Lys Gly Pro Ala Val Gly Ile Asp Leu Gly Thr Thr Tyr Ser
1      5      10      15
Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile Ile Ala Asn Asp
20      25      30
Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe Thr Asp Thr Glu
35      40      45
Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala Met Asn Pro Thr
50      55      60
Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg Arg Phe Asp Asp
65      70      75      80
Ala Val Val Gln Ser Asp Met Lys His Trp Pro Phe Met Val Val Asn
85      90      95
Asp Ala Gly Arg Pro Lys Val Gln Val Glu Tyr Lys Gly Glu Thr Lys
100     105     110
Ser Phe Tyr Pro Glu Glu Val Ser Ser Met Val Leu Thr Lys Met Lys
115     120     125
Glu Ile Ala Glu Ala Tyr Leu Gly Lys Thr Val Thr Asn Ala Val Val
130     135     140
Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln Ala Thr Lys Asp
145     150     155     160
Ala Gly Thr Ile Ala Gly Leu Asn Val Leu Arg Ile Ile Asn Glu Pro
165     170     175
Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Lys Lys Val Gly Ala Glu
180     185     190
Arg Asn Val Leu Ile Phe Asp Leu Gly Gly Gly Thr Phe Asp Val Ser
195     200     205
Ile Leu Thr Ile Glu Asp Gly Ile Phe Glu Val Lys Ser Thr Ala Gly
210     215     220
Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg Met Val Asn His
225     230     235     240
Phe Ile Ala Glu Phe Lys Arg Lys His Lys Lys Asp Ile Ser Glu Asn
245     250     255
Lys Arg Ala Val Arg Arg Leu Arg Thr Ala Cys Glu Arg Ala Lys Arg
260     265     270
Thr Leu Ser Ser Ser Thr Gln Ala Ser Ile Glu Ile Asp Ser Leu Tyr
275     280     285
Glu Gly Ile Asp Phe Tyr Thr Ser Ile Thr Arg Ala Arg Phe Glu Glu
290     295     300
Leu Asn Ala Asp Leu Phe Arg Gly Thr Leu Asp Pro Val Glu Lys Ala
305     310     315     320
Leu Arg Asp Ala Lys Leu Asp Lys Ser Gln Ile His Asp Ile Val Leu
325     330     335
Val Gly Gly Ser Thr Arg Ile Pro Lys Ile Gln Lys Leu Leu Gln Asp
340     345     350
Phe Phe Asn Gly Lys Glu Leu Asn Lys Ser Ile Asn Pro Asp Glu Ala
355     360     365
Val Ala Tyr Gly Ala Ala Val Gln Ala Ala Ile Leu Ser Gly Asp Lys
370     375     380
Ser Glu Asn Val Gln Asp Leu Leu Leu Leu Asp Val Thr Pro Leu Ser
385     390     395     400
Leu Gly Ile Glu Thr Ala Gly Gly Val Met Thr Val Leu Ile Lys Arg
405     410     415

```

-continued

Asn	Thr	Thr	Ile	Pro	Thr	Lys	Gln	Thr	Gln	Thr	Phe	Thr	Thr	Tyr	Ser
			420					425					430		
Asp	Asn	Gln	Pro	Gly	Val	Leu	Ile	Gln	Val	Tyr	Glu	Gly	Glu	Arg	Ala
		435					440					445			
Met	Thr	Lys	Asp	Asn	Asn	Leu	Leu	Gly	Lys	Phe	Glu	Leu	Thr	Gly	Ile
	450					455					460				
Pro	Pro	Ala	Pro	Arg	Gly	Val	Pro	Gln	Ile	Glu	Val	Thr	Phe	Asp	Ile
465					470					475					480
Asp	Ala	Asn	Gly	Ile	Leu	Asn	Val	Ser	Ala	Val	Asp	Lys	Ser	Thr	Gly
				485					490					495	
Lys	Glu	Asn	Lys	Ile	Thr	Ile	Thr	Asn	Asp	Lys	Gly	Arg	Leu	Ser	Lys
			500					505					510		
Glu	Asp	Ile	Glu	Arg	Met	Val	Gln	Glu	Ala	Glu	Lys	Tyr	Lys	Ala	Glu
		515					520					525			
Asp	Glu	Lys	Gln	Arg	Asp	Lys	Val	Ser	Ser	Lys	Asn	Ser	Leu	Glu	Ser
	530					535					540				
Tyr	Ala	Phe	Asn	Met	Lys	Ala	Thr	Val	Glu	Asp	Glu	Lys	Leu	Gln	Gly
545					550					555					560
Lys	Ile	Asn	Asp	Glu	Asp	Lys	Gln	Lys	Ile	Leu	Asp	Lys	Cys	Asn	Glu
				565					570					575	
Ile	Ile	Asn	Trp	Leu	Asp	Lys	Asn	Gln	Thr	Ala	Glu	Lys	Glu	Glu	Phe
			580					585					590		
Glu	His	Gln	Gln	Lys	Glu	Leu	Glu	Lys	Val	Cys	Asn	Pro	Ile	Ile	Thr
		595					600					605			
Lys	Leu	Tyr	Gln	Ser	Ala	Gly	Gly	Met	Pro	Gly	Gly	Met	Pro	Gly	Gly
	610					615					620				
Phe	Pro	Gly	Gly	Gly	Ala	Pro	Pro	Ser	Gly	Gly	Ala	Ser	Ser	Gly	Pro
625					630					635					640
Thr	Ile	Glu	Glu	Val	Asp										
					645										

<210> SEQ ID NO 9

<211> LENGTH: 493

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

Met	Ser	Lys	Gly	Pro	Ala	Val	Gly	Ile	Asp	Leu	Gly	Thr	Thr	Tyr	Ser
1				5					10					15	
Cys	Val	Gly	Val	Phe	Gln	His	Gly	Lys	Val	Glu	Ile	Ile	Ala	Asn	Asp
		20					25						30		
Gln	Gly	Asn	Arg	Thr	Thr	Pro	Ser	Tyr	Val	Ala	Phe	Thr	Asp	Thr	Glu
		35				40						45			
Arg	Leu	Ile	Gly	Asp	Ala	Ala	Lys	Asn	Gln	Val	Ala	Met	Asn	Pro	Thr
	50					55					60				
Asn	Thr	Val	Phe	Asp	Ala	Lys	Arg	Leu	Ile	Gly	Arg	Arg	Phe	Asp	Asp
65					70					75					80
Ala	Val	Val	Gln	Ser	Asp	Met	Lys	His	Trp	Pro	Phe	Met	Val	Val	Asn
				85					90					95	
Asp	Ala	Gly	Arg	Pro	Lys	Val	Gln	Val	Glu	Tyr	Lys	Gly	Glu	Thr	Lys
			100					105					110		
Ser	Phe	Tyr	Pro	Glu	Glu	Val	Ser	Ser	Met	Val	Leu	Thr	Lys	Met	Lys
		115						120					125		
Glu	Ile	Ala	Glu	Ala	Tyr	Leu	Gly	Lys	Thr	Val	Thr	Asn	Ala	Val	Val
	130						135					140			

-continued

Thr	Val	Pro	Ala	Tyr	Phe	Asn	Asp	Ser	Gln	Arg	Gln	Ala	Thr	Lys	Asp	145	150	155	160
Ala	Gly	Thr	Ile	Ala	Gly	Leu	Asn	Val	Leu	Arg	Ile	Ile	Asn	Glu	Pro	165	170	175	
Thr	Ala	Ala	Ala	Ile	Ala	Tyr	Gly	Leu	Asp	Lys	Lys	Val	Gly	Ala	Glu	180	185	190	
Arg	Asn	Val	Leu	Ile	Phe	Asp	Leu	Gly	Gly	Gly	Thr	Phe	Asp	Val	Ser	195	200	205	
Ile	Leu	Thr	Ile	Glu	Asp	Gly	Ile	Phe	Glu	Val	Lys	Ser	Thr	Ala	Gly	210	215	220	
Asp	Thr	His	Leu	Gly	Gly	Glu	Asp	Phe	Asp	Asn	Arg	Met	Val	Asn	His	225	230	235	240
Phe	Ile	Ala	Glu	Phe	Lys	Arg	Lys	His	Lys	Lys	Asp	Ile	Ser	Glu	Asn	245	250	255	
Lys	Arg	Ala	Val	Arg	Arg	Leu	Arg	Thr	Ala	Cys	Glu	Arg	Ala	Lys	Arg	260	265	270	
Thr	Leu	Ser	Ser	Ser	Thr	Gln	Ala	Ser	Ile	Glu	Ile	Asp	Ser	Leu	Tyr	275	280	285	
Glu	Gly	Ile	Asp	Phe	Tyr	Thr	Ser	Ile	Thr	Arg	Ala	Arg	Phe	Glu	Glu	290	295	300	
Leu	Asn	Ala	Asp	Leu	Phe	Arg	Gly	Thr	Leu	Asp	Pro	Val	Glu	Lys	Ala	305	310	315	320
Leu	Arg	Asp	Ala	Lys	Leu	Asp	Lys	Ser	Gln	Ile	His	Asp	Ile	Val	Leu	325	330	335	
Val	Gly	Gly	Ser	Thr	Arg	Ile	Pro	Lys	Ile	Gln	Lys	Leu	Leu	Gln	Asp	340	345	350	
Phe	Phe	Asn	Gly	Lys	Glu	Leu	Asn	Lys	Ser	Ile	Asn	Pro	Asp	Glu	Ala	355	360	365	
Val	Ala	Tyr	Gly	Ala	Ala	Val	Gln	Ala	Ala	Ile	Leu	Ser	Gly	Asp	Lys	370	375	380	
Ser	Glu	Asn	Val	Gln	Asp	Leu	Leu	Leu	Leu	Asp	Val	Thr	Pro	Leu	Ser	385	390	395	400
Leu	Gly	Ile	Glu	Thr	Ala	Gly	Gly	Val	Met	Thr	Val	Leu	Ile	Lys	Arg	405	410	415	
Asn	Thr	Thr	Ile	Pro	Thr	Lys	Gln	Thr	Gln	Thr	Phe	Thr	Thr	Tyr	Ser	420	425	430	
Asp	Asn	Gln	Pro	Gly	Val	Leu	Ile	Gln	Val	Tyr	Glu	Gly	Glu	Arg	Ala	435	440	445	
Met	Thr	Lys	Asp	Asn	Asn	Leu	Leu	Gly	Lys	Phe	Glu	Leu	Thr	Gly	Met	450	455	460	
Pro	Gly	Gly	Met	Pro	Gly	Gly	Phe	Pro	Gly	Gly	Gly	Ala	Pro	Pro	Ser	465	470	475	480
Gly	Gly	Ala	Ser	Ser	Gly	Pro	Thr	Ile	Glu	Glu	Val	Asp				485	490		

What is claimed is:

1. A method of measuring the level of soluble mortalin in a body fluid of a human subject diagnosed with a solid cancerous tumor, comprising:

(a) obtaining a body fluid sample from the human subject, said body fluid is selected from the group consisting of: blood, urine, tear, saliva, stool, cerebrospinal fluid, synovial fluid, lymph fluids, external secretions of a respiratory tract, external secretions of an intestinal tract external secretions of a genitourinary tract, milk and amniotic fluid,

(b) contacting said body fluid sample with a monoclonal anti-mortalin antibody immobilized to a solid support, thereby producing an immobilized mortalin-monoclonal antibody complex,

(c) contacting said immobilized mortalin-monoclonal antibody complex with a second, free anti-mortalin antibody which binds to a different epitope of said mortalin than said monoclonal anti-mortalin antibody, and specifically binds said immobilized mortalin-monoclonal antibody complex, thereby producing an

63

immune complex comprising said second anti-mortalin antibody bound to said immobilized mortalin-monoclonal antibody complex;

(d) producing a linear dose-dependent calibration curve for detecting mortalin by sequentially:

(i) contacting a range of concentrations of purified recombinant mortalin with said immobilized monoclonal anti-mortalin antibody to produce immobilized purified mortalin-monoclonal antibody complex;

(ii) contacting said immobilized purified mortalin-monoclonal antibody complex with said second, free antibody to form an immune complex comprising said second antibody bound to said immobilized purified mortalin-monoclonal antibody complex,

(iii) washing the immune complex of (ii), and

(iv) detecting amounts of said purified recombinant mortalin in said immune complex of (ii),

thereby producing a calibration curve, and

(v) selecting a linear portion of the calibration curve of (iv), and

(e) detecting the presence of said immune complex comprising said second anti-mortalin antibody of (c), within the linear range of the calibration curve of (d)(v),

thereby measuring the level of the soluble mortalin in the body fluid of the human subject,

and wherein said method gives a reproducible and sensitive detection of ≥ 0.25 ng of mortalin in human serum.

2. The method of claim 1, wherein said second, free antibody comprises a detectable moiety not found on said immobilized monoclonal anti-mortalin antibody, for detecting the presence of the immune complex comprising said second anti-mortalin antibody.

3. The method of claim 1, wherein said level of said second immune complex of claim 1(c) and 1(d)(ii) is measured by surface plasmon resonance.

4. The method of claim 1, wherein said level of said second immune complex of claim 1(c) and 1(d)(ii) is measured by capture Enzyme-linked immunosorbent assay (ELISA) or capture immunofluorescence.

5. The method of claim 1, wherein said presence of said immune complex of claim 1(c) comprising said second anti-mortalin antibody bound to said immobilized mortalin-monoclonal antibody complex or said immune complex of claim 1(d)(ii) comprising said second antibody bound to said immobilized purified mortalin-monoclonal antibody complex is detected by contacting with a secondary antibody specific for said second antibody and comprising a detectable moiety.

6. The method of claim 1, wherein said body fluid is a blood sample.

7. The method of claim 1, wherein said body fluid is a serum sample.

64

8. The method of claim 1, wherein said mortalin comprises the amino acid sequence set forth by SEQ ID NO:1.

9. The method of claim 1, wherein said detection of said second immune complex of claim 1(c) is followed by mass spectroscopy or nephelometry.

10. A method of measuring the level of soluble mortalin in a body fluid of a human subject, comprising:

(a) obtaining a body fluid sample from the human subject, said body fluid is selected from the group consisting of: blood, urine, tear, saliva, stool, cerebrospinal fluid, synovial fluid, lymph fluids, external secretions of a respiratory tract, external secretions of an intestinal tract external secretions of a genitourinary tract, milk and amniotic fluid,

(b) contacting said body fluid sample with a monoclonal anti-mortalin antibody immobilized to a solid support, thereby producing an immobilized mortalin-monoclonal antibody complex,

(c) contacting said immobilized mortalin-monoclonal antibody complex with a second, free anti-mortalin antibody which binds to a different epitope of said mortalin than said monoclonal anti-mortalin antibody, and specifically binds said immobilized mortalin-monoclonal antibody complex, thereby producing an immune complex comprising said second anti-mortalin antibody bound to said immobilized mortalin-monoclonal antibody complex;

(d) producing a linear dose-dependent calibration curve for detecting mortalin by sequentially:

(i) contacting a range of concentrations of purified recombinant mortalin with said immobilized monoclonal anti-mortalin antibody to produce immobilized purified mortalin-monoclonal antibody complex;

(ii) contacting said immobilized purified mortalin-monoclonal antibody complex with said second, free antibody to form an immune complex comprising said second antibody bound to said immobilized purified mortalin-monoclonal antibody complex,

(iii) washing the immune complex of (ii), and

(iv) detecting amounts of said purified recombinant mortalin in said immune complex of (ii),

thereby producing a calibration curve, and

(v) selecting a linear portion of the calibration curve of (iv) which gives a reproducible and sensitive detection of ≥ 0.25 ng of mortalin in human serum, and

(e) detecting the presence of said immune complex comprising said second anti-mortalin antibody of (c), within the linear range of the calibration curve of (d)(v),

thereby measuring the level of the soluble mortalin in the body fluid of the human subject.

11. The method of claim 10, wherein said subject is a subject diagnosed with a solid cancerous tumor.

* * * * *