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(54) **Title**: MATERIALS AND METHODS FOR TREATMENT OF LIVER CANCER BACKGROUND OF THE INVENTION

(57) **Abstract**: Granulin-epithelin precursor (GEP), a pluripotent growth factor, is a hepatic oncofetal protein defining a cancer stem cell (CSC) population in liver cancer. The present invention provides the use of GEP inhibitors (including anti-GEP antibody) for inhibiting immune evasion by liver cancer cells and for eliminating liver cancer stem cells.

# DESCRIPTION

# MATERIALS AND METHODS FOR TREATMENT OF LIVER CANCER

## BACKGROUND OF THE INVENTION

Liver cancer is the third leading cancer killer in the world, with more than half a million individuals dying globally each year. In China, liver cancer is the second major cause of cancer death. Surgical resection, in the form of a partial hepatectomy or a liver transplant, is the mainstay of curative treatment. Nonetheless, cancer recurrence is still common after curative surgery. In addition, liver cancer is frequently diagnosed at an advanced stage, which precludes curative treatment. No effective therapeutic option exists for the treatment of the majority of liver cancer patients.

Granulin-epithelin precursor (GEP) is a pluripotent growth factor regulating fetal development, tissue repair and tumorigenesis in various cancers. GEP is over-expressed in more than 70% of human hepatocellular carcinoma (HCC). GEP expression has been shown to associate with aggressive HCC features. Functional studies demonstrate that GEP plays a role in regulating HCC cell proliferation, invasion, tumorigenicity, and chemoresistance. Moreover, neutralization of GEP could inhibit the growth of established HCC in mouse model.

Cancer stem cells (CSCs) are considered as the "root" of cancers. CSCs are not only responsible for tumor initiation and progression, but also endowed with stem cell properties, including self-renewal, differentiation capacity and chemoresistance. Although existing therapies can initially eliminate the tumor bulk, stem cell properties of CSCs enable them to survive and repopulate the tumor, resulting in disease relapse. Thus, CSC eradication may be the key for curing aggressive malignancies including HCC.

The immune system has the ability to identify and eliminate tumor cells before they develop into malignancy. NK cells are the major component of the innate immune system and represent the first line of defense against tumors. Anti-tumor cytotoxicity of NK cells is mediated by direct lysis or induction of IFN-γ. However, it was reported that cytotoxicity of NK cells was impaired in HCC patients and the reduced activities of NK cells are associated with HCC progression.

NK cytotoxicity is regulated through integrated signaling from their cell surface receptors that interact with ligands expressed on target cells. NKG2D (natural killer group 2, member D) is a stimulatory receptor expressed on the surface of all NK cells and its recognition is crucial for tumor immunosurveillance. MHC class I chain-related molecule A (MICA), a ligand of human NKG2D receptor, is frequently expressed in tumors, but not in normal tissues. Engagement of MICA and NKG2D strongly activates NK cells, enhancing their cytotoxicity and cytokine production. Thus, the NKG2D-MICA pathway is an important mechanism by which host immune system recognizes and eliminates tumor cells. CD94/NKG2A is an inhibitory receptor that controls the activity of human NK cells following interactions with the non-classic class I human leukocyte antigen E (HLA-E) on target cells. HLA-E is ubiquitously expressed by nearly all cells in the body, but is over-expressed by tumor cells and is likely to protect the tumor cells from NK cytotoxic activity through inhibition via interactions with the CD94/NKG2A receptor.

Immune evasion is one of the important properties of CSCs that enable them to survive better in the host. Recently, ABCB5+ malignant melanoma initiating cells were found to possess the capacity to modulate anti-tumor immunity by preferentially inhibiting IL-2-dependent T-cell activation and to support induction of regulatory T cells. Moreover, CD200+ CSC cells were found to suppress anti-tumor immunity by down-regulating the expression of Thl cytokines and co-stimulatory molecules in ovarian cancer, melanoma and leukemia. However, evaluation of CSCs in the context of host immune responses has largely been disregarded in HCC. Therapeutic regimes that inhibit tumor cells from escaping from immunosurveillance can be used to treat hepatic CSCs.

## BRIEF SUMMARY OF THE INVENTION

The present invention provides a method for suppressing immune evasion and for eradicating GEP-expressing CSCs in hepatocellular carcinoma (HCC). The present invention is based on the surprising discovery that granulin-epithelin precursor (GEP) expression is associated with the immune evasion ability of human liver cancer; GEP is associated with the natural killer (NK) cell activity in human liver cancer; and anti-GEP antibody treatment enhances the NK cell cytotoxic activities against liver cancer cells.

The present invention provides a method for treating liver cancer, wherein the method comprises administering to a subject in need of such treatment an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP) and, optionally, NK cells and/or an agent that promotes natural killer (NK) cell activity.

In one embodiment, the agent that promotes NK cell activity enhances cytotoxicity of NK cells.

In one specific embodiment, the present invention provides a method for treating liver cancer, wherein the method comprises administering to a subject in need of such treatment an antibody that binds to granulin-epithelin precursor (GEP) and, optionally, NK cells and/or an agent that promotes natural killer (NK) cell activity.

In one embodiment, the subject had underwent partial or total hepatectomy and/or liver transplantation, and the present invention can be used to prevent or reduce the likelihood of liver tumor or cancer recurrence.

The present invention also provides a method of treating liver cancer in a subject, the method comprising administering to a subject an effective amount of an agent that increases the activity or expression of MICA present on a liver cancer cell.

The present invention also provides a method of treating liver cancer in a subject with increased levels of soluble MICA compared to a subject without liver cancer, the method comprising administering to the subject an effective amount of an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP).

The present invention also provides a method of determining the suitability of a subject with liver cancer for treatment with an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP), the method comprising i) obtaining a sample from the subject and ii) measuring the amount of soluble MICA in the sample. Preferably the sample is a blood sample, preferably a serum sample. The method may further comprise comparing the amount of soluble MICA in the sample to a chart correlating a quantity of soluble MICA and the presence or progression of liver cancer, wherein an amount of soluble MICA which correlates with presence or progression of liver

cancer indicates suitability of the subject for treatment with an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP).

The present invention also provides a method of increasing MICA expression on a cancer cell comprising administering an effective amount of an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP).

The present invention also provides a method of assessing or monitoring the extent or progression of liver cancer in a subject, the method comprising analysing a sample from the subject for a quantity of soluble GEP.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** shows that GEP expression of HCC cells modulates anti-tumor cytotoxic activity of immune cells. (**A**) GEP modulation in Hep3B (high endogenous GEP cell line) and HepG2 (low endogenous GEP cell line). Hep3B cells were stably transfected for GEP suppression by shRNA, while HepG2 cells were stably transfected for over-expression by full-length GEP cDNA. Protein expression of GEP in controls and transfectants was measured by flow cytometry. Grey line: isotype control staining. (**B**) GEP suppression increased, while over-expression decreased cell cytotoxicity of human PBMC against HCC cells. HCC cells (target cells) labeled with green fluorescence CFSE were co-cultured with human PBMC (effector cells) at effector/target ratio (E/T ratio) of 25 : 1 for 5 h. Cells were harvested and stained with propidium iodide (PI), with lysed HCC cells recognized as $CFSE^+PI^+$ and quantified by flow cytometry. Cell cytotoxicity level was calculated as follows: [cell cytotoxicity (% ) = (Percentage of $CFSE^+PI^+$ cells / (Percentage of $CFSE^+PI^+$ cells plus percentage of $CFSE+PI^-$ cells) x 100). $*P < 0.05$ when compared with control cells. (**C**) Depletion of NK cells markedly abolished the GEP-mediated cell cytotoxicity. $CD56^+$ NK cells were depleted from human PBMC by magnetic sorting. NK cells-depleted PBMC were then cultured with HCC cells for 5 hr at E/T ratio of 25:1. Cell cytotoxicity was then measured by flow cytometry. (**D**) GEP suppression increased, while over-expression decreased cell cytotoxicity of NK cells against HCC cells. $CD56^+$ NK cells were

isolated from human PBMC using magnetic sorting and then cultured with HCC cells for 5 hours at indicated E/T ratio. * $P < 0.05$, ** $P < 0.01$ when compared with control cells at respective E/T ratio.

**Figure 2** shows that GEP differentially regulates the expression of MICA and HLA-E of HCC cells. (**A**) GEP suppression up-regulated membrane-bound MICA, and suppressed soluble MICA release and membrane-bound HLA-E expression. Hep3B cells (high endogenous GEP cell line) were stably transfected for GEP suppression by shRNA. Membrane-bound MICA and HLA-E expression was measured by surface immunofluorescence staining and flow cytometry. Gray line: isotype control staining. Soluble MICA in culture supernatant was measured by ELISA. (**B**) GEP over-expression down-regulated membrane-bound MICA, and increased soluble MICA production and membrane-bound HLA-E expression. HepG2 cells (low endogenous GEP cell line) were stably transfected for over-expression by full-length GEP cDNA. Gray line: isotype control staining.

**Figure 3** shows that GEP blockage by anti-GEP antibody A23 modulates the expression of MICA and HLA-E of HCC cells. Hep3B cells were treated with anti-GEP monoclonal antibody (A23), mouse IgG isotype antibody (IgG) (antibodies at 50 J.Lg/ml) or without antibody (control) for 24h. (**A**) A23 significantly reduced GEP expression of Hep3B cells. (**B**) A23 induced surface MICA expression, and suppressed soluble MICA release and surface HLA- E expression in Hep3B cells. Dotted line: isotype control staining. *$P < 0.05$, **$P < 0.01$ when compared with control.

**Figure 4** shows that GEP blockage by anti-GEP antibody A23 enhances NK cell cytotoxicity against HCC cells via MICA. (**A**) GEP blockage by anti-GEP antibody A23 enhances PBMC-mediated cytotoxicity against HCC cells. The cytotoxicity is suppressed by anti-MICA neutralizing antibody. Hep3B and HepG2 cells were treated with anti-GEP monoclonal antibody (A23), mouse IgG isotype control (migG) (50 J.Lg/ml) or without antibody (control) for 24h prior to co-culture with PBMC at E/T ratio of 25: 1 for 5h. For A23 treatment, cells were co-cultured with PBMC with or without neutralizing MICA antibody or mouse IgG2a isotype control (2 μg/ml) for 5h. * $P< 0.05$ when compared with control; #$P < 0.05$ between groups denoted by horizontal lines. (**B**) A23-enhanced cell cytotoxicity of NK cells against Hep3B and HepG2 cells was suppressed by anti-MICA neutralizing antibody. * $P < 0.05$, * *$P$ <0.0 1, ** $P < 0.001$ when

compared with control; #$P < 0.05$, ## $P < 0.001$ when compared with A23 alone treatment at respective E/T ratio.

**Figure 5** shows co-expression of GEP with (**A**) MICA and (**B**) HLA-E in HCC clinical specimens. Liver tissues were digested into disaggregated cell suspension, and assessed for the expression of GEP and MICA or HLA-E. Co-expression was performed by triple-color flow cytometry, gating on the albumin+ hepatocytes of HCC specimens. Protein expression of GEP was measured by intracellular staining, while those of membrane-bound MICA and HLA-E were assessed by surface staining. Cells co-expressing the respective markers were shown in the upper right quadrant of dot plots. Mean percentage of cells ± SD from 3 HCC clinical specimens were shown. Table summarizes the mean percentage of positive cells ± SD from 3 samples.

**Figure 6** shows antibody-dependent cellular cytotoxicity mediated by anti-GEP monoclonal antibody A23. A23 mediated ADCC against HCC cells dose-dependently. Hep3B or HepG2 cells labeled with CFSE were incubated with or without anti-GEP antibody A23 (A23) or mouse isotype control (migG) of the indicated concentrations of the antibodies for 30 min prior to co-culture with or without PBMC at E/T ratio of 25 : 1 for 5h. * $P < 0.05$, * * $P < 0.01$ when compared with control cells without antibody. (**B**) Depletion of CD56+ NK cells from human PBMC markedly abolished A23-mediated ADCC. Hep3B or HepG2 cells labeled with CFSE were incubated with or without anti-GEP antibody A23 of indicated concentrations for 30 min prior to co-culture with PBMC or NK cell-depleted PBMC at E/T ratio of 25:1 for 5h. * $P<0.05$, ** $P < 0.01$ when compared with control cells without antibody. (**C**) A23-mediated ADCC against HCC cells was dependent on NK cells. CD56+ NK cells were isolated and cultured with HCC cells for 5 hr at indicated E/T ratio. *$P < 0.05$, **$P < 0.01$, ***P<0.001 when compared with control cells without antibody.

**Figure 7** shows the effect of Natural Killer (NK) cells isolated from liver cancer patients against liver cancer cells. A. NK cells showed enhanced cytotoxicity in liver cancer cells with GEP suppression (hep3B cells transfected with GEP shRNA, 3B-sh1). B. NK cells showed decreased cytoxicity in liver cancer cells with GEP over-expression (HepG2 transfected with GEP full length, G2-FL).

## BRIEF DESCRIPTION OF THE SEQUENCES

**SEQ ID NO:1** is a cDNA sequence of a granulin-epithelin precursor (GEP).

**SEQ ID NO:2** is the amino acid sequence of a granulin-epithelin precursor.

**SEQ ID NO:3** is the amino acid sequence of part of GEP (GEP amino acids 578-593).

**SEQ ID NO:3a** is the nucleic acid sequence encoding amino acids 578-593 of GEP.

**SEQ ID NO:4** is the amino acid sequence of part of GEP (GEP amino acids 351-365).

**SEQ ID NO:4a** is the nucleic acid sequence encoding amino acids 351-365 of GEP.

**SEQ ID NO:5** is the amino acid sequence of part of GEP (GEP amino acids 574-593).

**SEQ ID NO: 5a** is the nucleic acid sequence encoding amino acids 574-593 of GEP.

**SEQ ID NO:6** is the amino acid sequence of part of GEP (GEP amino acids 337-363).

**SEQ ID NO:6a** is the nucleic acid sequence encoding amino acids 337-363 of GEP.

**SEQ ID NO:7** is the amino acid sequence of part of GEP signaling peptide (GEP amino acids 1-17).

**SEQ ID NO:7a** is the nucleic acid sequence encoding amino acids 1-17 of GEP.

**SEQ ID NO:8** is the amino acid sequence of part of the GEP linker (GEP amino acids

18-57).

**SEQ ID NO: 8a** is the nucleic acid sequence encoding amino acids 18-57 of GEP.

**SEQ ID NO:9** is the amino acid sequence of part of the GEP linker (GEP amino acids 114-122).

**SEQ ID NO: 9a** is the nucleic acid sequence encoding amino acids 114-122 of GEP.

**SEQ ID NO:10** is the amino acid sequence of part of the GEP linker (GEP amino acids 180-205).

**SEQ ID NO:10a** is the nucleic acid sequence encoding amino acids 180-205 of GEP.

**SEQ ID NO:11** is the amino acid sequence of part of the GEP linker (GEP amino acids 262-280).

**SEQ ID NO:11a** is the nucleic acid sequence encoding amino acids 262-280 of GEP.

**SEQ ID NO:12** is the amino acid sequence of part of the GEP linker (GEP amino acids 418-441).

**SEQ ID NO: 12a** is the nucleic acid sequence encoding amino acids 418-441 of GEP.

**SEQ ID NO:13** is the amino acid sequence of part of the GEP linker (GEP amino acids 497-517).

**SEQ ID NO: 13a** is the nucleic acid sequence encoding amino acids 497-517 of GEP.

## DETAILED DISCLOSURE OF THE INVENTION

The present invention provides a method for suppressing immune evasion and for eradicating the GEP-expressing CSCs in hepatocellular carcinoma (HCC). The present invention is based on the surprising discovery that granulin-epithelin precursor (GEP) expression is associated with the immune evasion ability of human liver cancer; GEP is associated with the natural killer (NK) cell activity in human liver cancer; and anti-GEP antibody treatment enhances the NK cell cytotoxic activities against liver cancer cells.

The present invention is also based upon the surprising discovery that serum GEP and MICA levels are elevated in subjects with HCC, and high levels of soluble GEP and/or soluble MICA are associated with poor recurrence-free survival. The inventors have shown that GEP down regulates MICA on the HCC cell surface, thereby rendering the HCC cells less susceptible to NK cell cytotoxicity.

In addition, the present inventors have shown soluble GEP levels to correlate with tumour size.

Effect of GEP on Immune Evasion and Cancer Stem Cell Population

The present invention shows that granulin-epithelin precursor (GEP) rendered hepatocellular carcinoma (HCC) cells resistant to cytotoxic activity of NK cells, an important mechanism of anti-tumor responses. The alteration in immune response induced by GEP would cause an imbalance in the host's immune responses, allowing the subsequent growth of a tumor. Thus, GEP provides a selective advantage to cancer stem cells (CSCs) by enabling them to proliferate in hosts.

GEP blockage by monoclonal antibody A23 significantly suppresses GEP level in HCC cells, and the GEP-mediated regulation on MHC class I chain-related molecule A (MICA) and HLA-E expression on HCC cells could be reversed. As a result, the HCC cells can be sensitized to NK cytotoxic activity. In addition, the present invention shows that anti-GEP monoclonal antibody A23 induces NK cell-mediated ADCC against HCC cells, and therefore further amplifies the anti-tumor response of the antibody. Therefore, targeting antibody that binds specific to GEP (such as A23 antibody) can be used for suppressing immune evasion and eradicating the GEP-expressing CSCs in HCC.

GEP defines a hepatic CSC population with greater chemoresistance, self-renewal and tumor-initiating ability. The present inventors discovered that GEP conferred the CSCs' ability to escape from NK immunosurveillance, giving a selective advantage to the $GEP^{+}$ cells and enabling them to survive and proliferate in the host.

As a hallmark of tumor progression and recurrence, tumors have developed diverse mechanisms to evade the immune system. It is postulated that tumor cells have evolved to down-regulate their surface MICA by proteolytic shedding, therefore reducing their susceptibility to NKG2D-mediated NK cytotoxicity. Also, the soluble MICA (sMICA) released from tumor cell surface can not only block the NKG2D-binding site for other NKG2D ligands, but also suppress NKG2D expression on NK cells. Production of sMICA therefore represents one of the mechanisms in tumor immune evasion. It was reported that sMICA were frequently elevated in advanced HCC patients and such elevation was associated with down-regulated NKG2D expression and impaired NK cell activation in HCC. Significance of sMICA as predictive and prognostic marker has been reported in HBV-induced HCC and advanced HCC.

The present invention shows that GEP suppression significantly up-regulated, while GEP over-expression reduced surface MICA level on HCC cells. GEP-expressing cells might therefore evade NK cytotoxicity by reducing surface MICA level and become less susceptible to NK cell responses. In addition, sMICA level decreased significantly in the culture supernatant of GEP-suppressed HCC cells, but increased significantly when GEP was over-expressed. The results show that GEP could confer HCC cells immune evasion ability by promoting shedding of MICA from cell surface and promote the release of sMICA which disturbs the NKG2D-mediated NK cytotoxicity.

Up-regulation of HLA-E, a ligand for the NK inhibitory immunoreceptor CD94/NKG2A9, has been suggested to represent a mechanism of tumor escape from NK cell immunosurveillance in ovarian cancer and melanoma. It was previously reported that HLA-E mRNA was up-regulated in HCC specimens in comparison with their adjacent normal counterpart. Also, HLA-E polymorphism was also associated with risk of hepatitis B or HCC. Nevertheless, before the present invention, the role of HLA-E in HCC pathogenesis has not been reported. In addition, it was not elucidated that HCC cells over-express HLA-E.

The present invention shows that GEP expression up-regulated HCC cells' surface expression of HLA-E, which interacts with NK cell inhibitory immunoreceptor CD94/NKG2A and suppresses NK cell activity.

HLA-E could play a role in HCC pathogenesis by promoting the immune evasion ability of GEP-expressing CSCs. GEP blockage using anti-GEP monoclonal antibody A23 could significantly

suppress GEP level and sensitize the $GEP^+$ cells to NK cytotoxic activity. The results show that antibodies that bind to GEP have antitumor effect against HCC cells once NK cells are efficiently activated during A23 treatment. The combination therapy of anti-HCC molecular targeted therapy and immunotherapy targeting activation of NK cells could improve the antitumor effect against unresectable HCC and the prognosis of patients with HCC. Also, as the present inventors have shown that GEP expression would induce shedding of MICA from HCC cell surface and increase soluble MICA, patients with higher soluble MICA level in their serum would be more responsive to the anti-GEP monoclonal antibody treatment. The present invention can be used to define criteria to select patients who are potentially responsive to anti- GEP antibody treatment.

In addition to immune escape, another immunologic mechanism known to play a role in the anti-tumor activity of antibodies is their ability to induce antibody-dependent cell-mediated cytotoxic activity (ADCC) mediated by cytotoxic immune cells (such as NK cells) to lyse the tumor cells. ADCC is an innate immune effector mechanism in which antibodies evoke tumor cell death when antibodies bind to antigen on tumor cells and the antibody Fc domains engage Fc receptors on the surface of immune effector cells.

The present invention shows that anti-GEP monoclonal antibody A23 could induce NK cell-mediated ADCC against HCC cells. Therefore, the anti-tumor effect of anti-GEP monoclonal antibody A23 can be further amplified by inducing destruction of the A23-coated HCC cells by NK cells. Targeting GEP by antibody A23 represents a novel therapeutic tool for suppressing immune evasion and for eradicating the GEP-expressing CSCs in HCC.

Liver Cancer or Tumor Therapy

In one embodiment, the present invention provides a method for treating liver cancer, wherein the method comprises administering to a subject in need of such treatment an effective amount of an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP) and, optionally, natural killer (NK) cells and/or an agent that promotes NK cell activity.

In one embodiment, the agent that promotes NK cell activity enhances cytotoxicity of NK cells.

In one specific embodiment, the present invention provides a method for treating liver cancer, wherein the method comprises administering to a subject in need of such treatment an antibody that binds to granulin-epithelin precursor (GEP) and, optionally, NK cells and/or an agent that promotes natural killer (NK) cell activity.

In another specific embodiment, the present invention provides a method for treating liver cancer, wherein the method comprises administering to a subject in need of such treatment, a GEP antisense polynucleotide and, optionally, NK cells and/or an agent that promotes natural killer (NK) cell activity. In one specific embodiment, the GEP antisense polynucleotide is administered to a liver tumor of the subject.

In one embodiment, the agent that inhibits the expression or activity of granulin-epithelin precursor (GEP) is administered before, during, or after NK cells and/or an agent that promotes NK cell activity. In one embodiment, the method comprises administering a therapeutic composition comprising an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP), NK cells, and/or an agent that promotes NK cell activity.

In one embodiment, the present invention provides a method of treating liver cancer in a subject, the method comprising administering to a subject an effective amount of an agent that increases the activity or expression of MICA present on a liver cancer cell.

In another embodiment, the present invention provides a method of treating liver cancer in a subject with increased levels of soluble MICA compared to a subject without liver cancer, the method comprising administering to the subject an effective amount of an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP).

In another embodiment, the present invention provides a method of increasing MICA expression on a cancer cell comprising administering an effective amount of an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP).The term "treatment" or any grammatical variation thereof (*e.g.*, treat, treating, and treatment *etc.*), as used herein, includes but is not limited to, ameliorating or alleviating a symptom of a disease or condition, reducing, suppressing, inhibiting, lessening, or affecting the progression, severity, and/or scope of a condition.

The term "prevention" or any grammatical variation thereof (*e.g.*, prevent, preventing, and prevention *etc.*), as used herein, includes but is not limited to, reducing the likelihood of

developing a disease, delaying the onset of symptoms, preventing relapse to a disease, increasing latency between symptomatic episodes, or a combination thereof. Prevention, as used herein, does not require the complete absence of symptoms.

The term "effective amount," as used herein, refers to an amount that is capable of treating or ameliorating a disease or condition or otherwise capable of producing an intended therapeutic effect.

The term "subject," as used herein, describes an organism, including mammals such as primates, to which treatment with the compositions according to the subject invention can be provided. Mammalian species that can benefit from the disclosed methods of treatment include, but are not limited to, apes, chimpanzees, orangutans, humans, monkeys; and other animals such as dogs, cats, horses, cattle, pigs, sheep, goats, chickens, mice, rats, guinea pigs, and hamsters. In one embodiment, the subject has, or is diagnosed with, liver tumor or cancer. In one embodiment, the subject had underwent partial or total hepatectomy and/or liver transplantation.

The term "partial hepatectomy," refers to the surgical resection of less than the entire, but more than 5% (including, any percent higher than 5%, including but not limited to, more than 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%) of the liver.

In one embodiment, the present invention can be used to eliminate or reduce liver cancer stem cells. In one embodiment, the administration of one or more GEP inhibitors (such as, anti-GEP antibody) to a subject who had surgical resection of a primary liver tumor can be used to prevent the recurrence of liver tumor or cancer. In one embodiment, the administration of one or more GEP inhibitors (such as, anti-GEP antibody or aptamer) to a subject who had surgical resection of a primary liver tumor can be used to treat liver tumor or cancer.

In one embodiment, one or more GEP inhibitors (such as, anti-GEP antibody or aptamer) to a subject who had partial hepatectomy or liver transplantation. In one embodiment, the present invention can be used to prevent or reduce the likelihood of liver tumor or cancer recurrence.

In one embodiment, the subject has undergone surgical resection of an entire primary liver tumor. In one embodiment, the subject has undergone surgical resection of part of a primary liver tumor.

In one embodiment, one or more GEP inhibitors are administered before or after the subject receives surgical resection of the liver tumor.

The present application incorporates by reference WO2005/106019, WO2008/064570 and WO2011/140828 in their entity. In certain embodiments, the present invention does not encompass the treatment of liver cancer described in WO2005/106019, WO2008/064570 and WO2011/140828.

GEP Inhibitors

GEP inhibitors useful according to the present invention include, but are not limited to, agents that inhibit GEP activity; and agents that reduce or inhibit the expression of GEP, such as agents that inhibit the transcription and/or translation of GEP.

Agents that inhibit GEP activity include, but are not limited to, anti-GEP antibodies, aptamers, GEP binding partners, and small molecule inhibitors of GEP.

In one embodiment, the GEP inhibitor is an antibody, aptamer, or binding partner that binds to GEP. In a specific embodiment, the GEP inhibitor is an antibody, aptamer, or binding partner that binds specifically to GEP. In a further specific embodiment, the GEP inhibitor is an antibody, aptamer, or binding partner that binds specifically to human GEP.

In one embodiment, the cDNA sequence of a GEP useful according to the present invention is SEQ ID NO. 1. In one embodiment, the GEP protein useful according to the present invention comprises an amino acid sequence of SEQ ID NO.2.

In a further specific embodiment, the GEP inhibitor is an antibody, aptamer, or binding partner that binds specifically to a GEP of SEQ ID NO:2.

Embodiments of anti-GEP antibodies are also described in WO2008/064570, which is herein incorporated by reference in its entirety.

In one specific embodiment, the present invention provides a method for treating liver cancer, wherein the method comprises administering, to a subject that has liver cancer, a composition comprising an antibody that binds to granulin-epithelin precursor (GEP), and, optionally, NK cells and/or an agent that promotes natural killer (NK) cell activity.

In certain embodiments, the GEP inhibitor is an antibody, aptamer, or binding partner that binds specifically to a GEP protein of non-human animal species including, but not limited to, dogs, cats, horses, pigs, sheep, goats, chickens, mice, rats, and guinea pigs. The skilled artisan can readily make antibodies, aptamers, or binding partners that specifically bind to GEP proteins that are publically known. In another embodiment, the GEP inhibitor is a fusion construct comprising the antibody, aptamer, or binding partner that binds specifically to a GEP protein (such as human GEP).

In one embodiment, GEP-specific antibodies can be generated by immunizing BALB/c mice or New Zealand white rabbits with GEP specific peptide sequences, as described in WO2008/064570, which is herein incorporated by reference in its entirety.

"Specific binding" or "specificity" refers to the ability of a protein to detectably bind an epitope presented on a protein or polypeptide molecule of interest, while having relatively little detectable reactivity with other proteins or structures. Specificity can be relatively determined by binding or competitive binding assays, using, *e.g.*, Biacore instruments. Specificity can be exhibited by, *e.g.*, an about 10:1, about 20:1, about 50:1, about 100:1, 10.000:1 or greater ratio of affinity/avidity in binding to the specific target molecule versus nonspecific binding to other irrelevant molecules.

Anti-GEP antibodies of the present invention can be in any of a variety of forms, including intact immunoglobulin molecules, fragments of immunoglobulin molecules such as Fv, Fab and similar fragments; multimers of immunoglobulin molecules (*e.g.*, diabodies, triabodies, and bi-specific and tri-specific antibodies, as are known in the art; *see, e.g.*, Hudson and Kortt, J. Immunol. Methods 231:177 189, 1999); fusion constructs containing an antibody or antibody fragment; and human or humanized immunoglobulin molecules or fragments thereof.

Antibodies within the scope of the invention can be of any isotype, including IgG, IgA, IgE, IgD, and IgM. IgG isotype antibodies can be further subdivided into IgG1, IgG2, IgG3, and IgG4 subtypes. IgA antibodies can be further subdivided into IgA1 and IgA2 subtypes.

Antibodies of the present invention include polyclonal and monoclonal antibodies. The term "monoclonal antibody," as used herein, refers to an antibody or antibody fragment obtained from a substantially homogeneous population of antibodies or antibody fragments (*i.e.* the individual

antibodies within the population are identical except for possible naturally occurring mutations that may be present in a small subset of the antibody molecules).

A monoclonal antibody composition is typically composed of antibodies produced by clones of a single cell called a hybridoma that secretes (produces) only one type of antibody molecule. The hybridoma cell is formed by fusing an antibody-producing cell and a myeloma or other self-perpetuating cell line. Such antibodies were first described by Kohler and Milstein, *Nature*, 1975, 256:495-497, the disclosure of which is herein incorporated by reference. An exemplary hybridoma technology is described by Niman et *al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1983, 80:4949-4953. Other methods of producing monoclonal antibodies, a hybridoma cell, or a hybridoma cell culture are also well known. See *e.g.*, Antibodies: A Laboratory Manual, Harlow *et al.*, Cold Spring Harbor Laboratory, 1988; or the method of isolating monoclonal antibodies from an immunological repertoise as described by Sasatry, *et al.*, *Proc. Natl. Acad. Sci. USA*, 1989, 86:5728-5732; and Huse *et al.*, *Science*, 1981, 246:1275-1281. The references cited are hereby incorporated herein by reference.

In an embodiment, the GEP inhibitor is a GEP antisense polynucleotide. In an embodiment, the GEP inhibitor is an antisense polynucleotide that targets human GEP mRNA.

In some embodiments, the GEP antisense polynucleotides target GEP mRNAs of non-human animals including, but not limited to, dogs, cats, horses, pigs, sheep, goats, chickens, mice, rats, and guinea pigs. The skilled artisan would readily appreciate that the antisense polynucleotides can be designed to target any GEP mRNAs publically known.

In some embodiments, the GEP inhibitor is a siRNA having a sequence sufficiently complementary to a target GEP mRNA sequence to direct target-specific RNA interference (RNAi).

In some embodiments, the GEP inhibitor is siRNA having a sequence sufficiently complementary to a target human GEP mRNA sequence to direct target-specific RNA interference.

Examples of antisense polynucleotides include, but are not limited to, single-stranded DNAs and RNAs that bind to complementary target GEP mRNA and inhibit translation and/or induce RNaseH-mediated degradation of the target transcript; siRNA oligonucleotides that target or mediate GEP mRNA degradation; ribozymes that cleave GEP mRNA transcripts; and nucleic acid

aptamers and decoys, which are non-naturally occurring oligonucleotides that bind to and block GEP protein targets in a manner analogous to small molecule drugs.

The term "nucleotide" refers to a nucleoside having one or more phosphate groups joined in ester linkages to the sugar moiety. Exemplary nucleotides include nucleoside monophosphates, diphosphates and triphosphates. The terms "polynucleotide" and "nucleic acid molecule" are used interchangeably herein and refer to a polymer of nucleotides joined together by a phosphodiester linkage between 5' and 3' carbon atoms. The terms "nucleic acid" or "nucleic acid sequence" encompass an oligonucleotide, nucleotide, polynucleotide, or a fragment of any of these, DNA or RNA of genomic or synthetic origin, which may be single-stranded or double-stranded and may represent a sense or antisense strand, peptide nucleic acid (PNA), or any DNA-like or RNA-like material, natural or synthetic in origin. As will be understood by those of skill in the art, when the nucleic acid is RNA, the deoxynucleotides A, G, C, and T are replaced by ribonucleotides A, G, C, and U, respectively.

As used herein, the term "RNA" or "RNA molecule" or "ribonucleic acid molecule" refers generally to a polymer of ribonucleotides. The term "DNA" or "DNA molecule" or deoxyribonucleic acid molecule" refers generally to a polymer of deoxyribonucleotides. DNA and RNA molecules can be synthesized naturally *(e.g.*, by DNA replication or transcription of DNA, respectively). RNA molecules can be post-transcriptionally modified. DNA and RNA molecules can also be chemically synthesized. DNA and RNA molecules can be single-stranded (*i.e.*, ssRNA and ssDNA, respectively) or multi-stranded (*e.g.*, double stranded, *i.e.*, dsRNA and dsDNA, respectively). Based on the nature of the invention, however, the term "RNA" or "RNA molecule" or "ribonucleic acid molecule" can also refer to a polymer comprising primarily (*i.e.*, greater than 80% or, preferably greater than 90%) ribonucleotides but optionally including at least one non-ribonucleotide molecule, for example, at least one deoxyribonucleotide and/or at least one nucleotide analog.

As used herein, the term "nucleotide analog," also referred to herein as an "altered nucleotide" or "modified nucleotide," refers to a non-standard nucleotide, including non- naturally occurring ribonucleotides or deoxyribonucleotides. Preferred nucleotide analogs are modified at any position so as to alter certain chemical properties of the nucleotide yet retain the ability of the nucleotide analog to perform its intended function.

As used herein, the term "RNA interference" ("RNAi") refers to a selective intracellular degradation of RNA. RNAi occurs in cells naturally to remove foreign RNAs (*e.g.*, viral RNAs). Natural RNAi proceeds via fragments cleaved from free dsRNA which direct the degradative mechanism to other similar RNA sequences. Alternatively, RNAi can be initiated by the hand of man, for example, to silence the expression of endogenous target genes.

As used herein, the term "small interfering RNA" ("siRNA") (also referred to in the art as "short interfering RNAs") refers to an RNA (or RNA analog) comprising between about 10-50 nucleotides (or nucleotide analogs) which is capable of directing or mediating RNA interference.

As used herein, a siRNA having a "sequence sufficiently complementary to a target mRNA sequence to direct target-specific RNA interference (RNAi)" means that the siRNA has a sequence sufficient to trigger the destruction of the target mRNA (*e.g.*, GEP mRNA) by the RNAi machinery or process. "mRNA" or "messenger RNA" or "transcript" is single-stranded RNA that specifies the amino acid sequence of one or more polypeptides. This information is translated during protein synthesis when ribosomes bind to the mRNA.

The present invention also contemplates vectors (*e.g.*, viral vectors) and expression constructs comprising the nucleic acid molecules useful for inhibiting GEP expression and/or activity. In an embodiment, the vector comprises a siRNA that targets GEP mRNA. In another embodiment, the vector comprises a nucleic acid molecule encoding an anti-GEP A antibody.

As used herein, the term "expression construct" refers to a combination of nucleic acid sequences that provides for transcription of an operably linked nucleic acid sequence. As used herein, the term "operably linked" refers to a juxtaposition of the components described, wherein the components are in a relationship that permits them to function in their intended manner. In general, operably linked components are in contiguous relation.

Expression constructs of the invention will also generally include regulatory elements that are functional in the intended host cell in which the expression construct is to be expressed. Thus, a person of ordinary skill in the art can select regulatory elements for use in, for example, bacterial host cells, yeast host cells, mammalian host cells, and human host cells. Regulatory elements include promoters, transcription termination sequences, translation termination sequences, enhancers, and polyadenylation elements.

An expression construct of the invention can comprise a promoter sequence operably linked to a polynucleotide sequence encoding a peptide of the invention. Promoters can be incorporated into a polynucleotide using standard techniques known in the art. Multiple copies of promoters or multiple promoters can be used in an expression construct of the invention. In a preferred embodiment, a promoter can be positioned about the same distance from the transcription start site as it is from the transcription start site in its natural genetic environment. Some variation in this distance is permitted without substantial decrease in promoter activity. A transcription start site is typically included in the expression construct.

Agents That Promote NK Cell Activity

In certain embodiments, agents that promote NK cell activity include, but are not limited to, agents that promote expansion and maturation of NK cells, and agents that enhance cytotoxicity of NK cells. Various agents that enhance NK cell activity are known in the art. In certain embodiments, agents that enhance NK cell activity include, but are not limited to, IL-15, IL-12, pectin, interferons (such as IFN-gamma), IL-2, and IL-21.

Interleukin-12 (IL-12) is a cytokine that promotes and stimulates activity of NK cells and T lymphocytes, and has antitumor activity (U.S. Patent Application Publication No. 2012/0251441).

Interleukin 15 (IL-15) is a cytokine that stimulates NK cells [Fehniger T A, Caligiuri M A. Blood 97(1):14-32 (2001)]. It has become apparent that IL-15 presented through cell-to-cell contact has a higher NK stimulating activity than soluble IL-15 [Dubois S, et al., Immunity 17(5):537-547 (2002); Kobayashi H, et al., Blood (2004) PMID: 15367431; Koka R, et al., J Immunol 173(6):3594-3598 (2004); Burkett P R, et al., J Exp Med 200(7):825-834 (2004)]. (U.S. Patent Application Publication No. 2012/0015434).

Pectin is generally a natural macromolecular polysaccharide polymer, widely distributed in plant cell walls (Hyunjo Kim, et al. *International Journal of Pharmaceutics,* 1998, 161:149-159). Pectin can improve the immune function of hosts by enhancing the mononuclear phagocyte system, activating macrophages, T cells, B cells, NK cells and complement system; promoting cytokine secretion; enhancing immunity of erythrocytes; and exerting direct anticancer effects by changing the growth characteristics of solid cancer cell membrane, affecting signal transmission

path in solid cancer cells and anti-free radical effect, inducing differentiation and apoptosis, inhibiting synthesis of nucleic acid and protein of the solid cancer cells, affecting ultrastructure of the solid cancer cells, affecting oncogenes and antimutation effect, and inhibiting vascularization of solid cancer (*Chinese Journal of Information on Traditional Chinese Medicine,* 1999, 5:64) (U.S. Patent Application Publication No. 2012/0244193).

Interleukin 21 (IL-21) promotes the expansion and maturation of NK cells. (U.S. Patent Application Publication No. 2011/0268766).

Interferons (such as, IFN-gamma) and IL-2 have been shown to promote NK cell activity.

Agents that increase MICA expression on a liver cancer cell.

In certain embodiments, agents that promote MICA expression on a liver cancer cell include, but are not limited to, agents which inhibit the expression or activity of GEP, for example as described herein.

Therapeutic Compositions and Routes of Administration

The subject invention further provides for therapeutic or pharmaceutical compositions.

In an embodiment, the composition comprises a therapeutically effective amount of a protein, nucleic acid molecule, and/or compound of the subject invention and, optionally, a pharmaceutically acceptable carrier.

The subject invention contemplates therapeutic compositions useful for practicing the therapeutic methods described herein. The therapeutic composition can be any form of pharmaceutical format, including injectable formulations such as liquid and lyophilized injections.

Suitable non-toxic pharmaceutically acceptable carriers for use with the agent will be apparent to those skilled in the art of pharmaceutical formulation. See, for example, *Remington's Pharmaceutical Sciences*, seventeenth edition, ed. Alfonso R. Gennaro, Mack Publishing Company, Easton, Pa. (1985). Suitable carriers include ethanol, dimethyl sulfoxide, glycerol, silica, alumina, starch, sorbitol, inosital, xylitol, D-xylose, manniol, powdered cellulose, microcrystalline cellulose, talc, colloidal silicon dioxide, calcium carbonate, magnesium carbonate, calcium phosphate, calcium

aluminum silicate, aluminum hydroxide, sodium starch phosphate, lecithin, and equivalent carriers and diluents. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions.

Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, and the like. The therapeutic composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary, depending on the type of the condition and the subject to be treated. In general, a therapeutic composition contains from about 5% to about 95% active ingredient (w/w). More specifically, a therapeutic composition contains from about 20% (w/w) to about 80%, or about 30% to about 70%, active ingredient (w/w).

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as injectables either as liquid solutions or suspensions; however, solid forms suitable for solution, or suspensions, in liquid prior to use also can be prepared. The preparation also can be emulsified.

The therapeutic composition of the subject invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of a polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups also can be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients, e.g., compound, carrier suitable for administration.

The compositions of the subject invention can be administered to the subject being treated by standard routes, including oral, inhalation, or parenteral administration including intravenous, subcutaneous, topical, transdermal, intradermal, transmucosal, intraperitoneal, intramuscular, intracapsular, intraorbital, intracardiac, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection, infusion, and electroporation, as well as co-administration as a component of any medical device or object to be inserted (temporarily or permanently) into a subject.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Sterile injectable solutions are prepared by incorporating the active ingredients in the required amount in the appropriate solvent followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying techniques, which yield a powder of the active ingredient, plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Soluble GEP and soluble MICA as biomarkers for liver cancer

In an embodiment, the present invention provides a method of determining the suitability of a subject with liver cancer for treatment with an agent that inhibits the expression or activity of

granulin-epithelin precursor (GEP), the method comprising i) obtaining a sample from the subject and ii) measuring the amount of soluble MICA in the sample.

In a specific embodiment, the method may further comprise comparing the amount of soluble MICA in the sample to a chart correlating a quantity of soluble MICA and the presence or progression of liver cancer, wherein an amount of soluble MICA in the sample which correlates with presence or progression of liver cancer indicates suitability of the subject for treatment with an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP).

In an embodiment, the present invention also provides a method of assessing or monitoring the extent or progression of liver cancer in a subject, the method comprising analysing a sample from the subject for a quantity of soluble GEP.

A method of the present invention may be performed *in vitro*, on a sample removed from the subject. Preferably, the sample is a circulatory sample, i.e. a blood sample, a plasma sample or a serum sample.

In an embodiment of the present invention, the method may comprise a binding assay, comprising i) contacting a sample from a subject with a support; ii) contacting the sample with a detection binding partner which is specific for soluble GEP or soluble MICA; and iii) detecting the fraction of detecting binding partner bound to the support. Optionally, the method may comprise washing the support after incubation with a detection binding partner to remove any unbound or non-specifically bound binding partner. In an embodiment, a detection binding partner may be linked to a signal (e.g. an enzyme) or can itself be detected by a secondary antibody that is linked to a signal for example through bioconjugation (e.g. in an indirect immunoassay format). In an embodiment, the method may comprise the step of developing and/or measuring the signal.

The method of the invention may be any suitable method for determining a quantity of a soluble GEP or MICA in a sample. The method may be qualitative or quantitative. Suitable methods include a binding assay, for example an immunoassay, such as an ELISA, a Western blot, or an RIA assay, spectrometric methods for example mass spectrometry, nuclear magnetic resonance spectrometry, and combinations thereof; mass spectrometry assays coupled to immunaffinity assays; matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass mapping and liquid chromatography/quadrupole time-of-flight electrospray ionization tandem

mass spectrometry (LC/Q-TOF-ESI-MS/MS) sequence tag of proteins separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Kiernan et al., *Anal. Biochem.,* 301: 49-56, 2002; Poutanen et al., *Mass Spectrom.,* 15: 1685-1692, 2001); quantitative mass spectroscopic methods, such as SELDI; MRM mass spectrometry or tandem mass spectrometry (such as LC-MRM-MS); flow cytometry; and immunohistochemical techniques (see for example Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1998).

The assay may be based upon measurement of direct signal (e.g. where signal is not amplified, such as coloured particles or fluorescence based assays) or measurement of generated signal, e.g. where signal is developed and/or amplified, for example by a catalyst or enzyme. The assay is preferably an ELISA assay, for example a direct, sandwich, indirect or competition ELISA. Such an assay preferably uses a binding partner for GEP, sich as an anti-GEP antibody, for example a monoclonal antibody or polyclonal antibody, preferably A23 as described herein. In such an embodiment, the signal is an enzyme. Similar formats of assay are also within the scope of the invention, where the signal is not an enzyme. Variations and formats of immunoassays and ELISA assays are included within the scope of this invention, where appropriate, and are known in the art.

The method may comprise the step of comparison to a control sample. This may be particularly relevant for quantitative assays. Where a qualitative result is suitable, comparison to a control may not be required. A control is a sample or standard used for comparison with a test sample. It may be derived from a healthy patient, such as one that does not have liver cancer. It will be appreciated that various different forms of controls may be used in such methods, depending upon the condition being assessed. A control sample may be an average value, obtained from a plurality of control subjects as defined above, and represented on a chart. A control can also be represented by a reference value or range of values representing an amount of activity or expression determined to be representative of a given condition. Reference values can include a range of values, real or relative expected to occur under certain conditions. These values can be compared with experimental values to determine if a biomarker is up-regulated or down-regulated in a particular sample for instance.

A quantity indicative of liver cancer or suitability for treatment with an agent which inhibits the activity or expression of GEP may be elevated levels of soluble GEP or soluble MICA compared

to levels in a control sample or compared to a reference value, or within a margin of 20%, 15%, 10%, 5% or less. By "elevated" may mean at least 1.5 fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 8-fold, or greater increase or decrease compared to a normal subject or a control.

The method may be qualitative, providing a binary positive/negative indication, or may be quantitative. In the latter embodiment, the quantity may be indicated by the strength of signal provided in a Western blot or immunoassay including RIA and ELISA. Signal intensity in an immunoassay may be measured, for example, by determining the absorbance or reflection of a light, including fluorescent signals passed through the sample. Preferably, a step of quantification may be performed prior to comparison with a control sample or reference value.

The method may further comprise providing a result to the subject, a clinician or other caregiver. An output may show a cut-off value or level that indicates presence or progression of liver cancer. The output may be a paper output (for example, a written or printed output), a display on a screen, a graphical output (for example, a graph, chart, voltammetric trace, or other diagram), or an audible output. The output may be communicated to the user, for example by providing an output via physical, audible, or electronic means (for example by mail, telephone, facsimile transmission, email, or communication to an electronic medical record). The output may be a diagnosis, prognosis or an indication that further tests may be required. The output may provide a recommended therapeutic regimen. Preferably, the output is adjusted to account for any background signal which may be measured prior to, or during the assay.

The calculated result can then be transmitted to a display device, which will present the signal is a readable format. This may be a yes/no type result, in the form of words or signs, or may be a quantitative result providing a value which is indicative of the amount of analyte present. Alternatively a result decision and raw data may be transmitted by wired, wireless far field or wireless near field communication techniques to a receiving "reader" docking device. A reader would be capable of relaying the information to a computer or through a computer network to a remote computer or to a hand held computing device (e.g. smart phone or tablet computer). Such a computing device could provide electronic storage and also permit more detailed analysis such as but not limited to trend analysis. The results could also be made available to a remote clinician.

In some examples, the methods further include a care pathway involving further diagnostic tests, treatment or preventative therapy, for example if the subject is diagnosed as being suitable for treatment with an agent which inhibits activity or expression of GEP.

Thus, a method of the invention may further comprise, depending on the subject's results, a) additional diagnostic tests; b) prescribing a treatment regimen for the subject (e.g an agent which inhibits activity or expression of GEP or increases MICA on cancer cells); c) not prescribing a treatment regimen for the subject; or d) administering a treatment (such as surgery, chemotherapy, radiotherapy, to the subject if the subject's determined diagnosis is positive; or e) further monitoring using a method of the invention after a suitable time interval. The treatment regime may be any suitable treatment known to be suitable for preventing or reducing the cause and/or symptoms associated with the liver cancer.

## Materials and Methods

### Cell culture and assays

Human HCC cell lines, Hep3B and HepG2, were purchased from American Type Culture Collection (Manassas, VA) and were cultured; while Huh7 was purchased from Health Science Research Resources Bank (Osaka, Japan) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA).

Stable transfectant for GEP over-expression was established by transfecting GEP full-length cDNA into HepG2, a cell line with low endogenous GEP level; while GEP suppression was performed by transfecting GEP shRNA into Hep3B, a cell line with high endogenous GEP level. Both GEP over-expression and suppression transfectants were maintained in 10% AMEM with 0.4 mg/ml G418.

GEP blockage in Hep3B was performed by incubating the cells with or without 50 μg/ml anti-GEP monoclonal antibody A23 or mouse IgG isotype control antibody (Sigma-Aldrich, St. Louis, MO) for 24 h. GEP stimulation in HepG2 cells was performed by incubating the cells with or without 0.8 μg/ml recombinant GEP for 24 h.

Clinical specimens

The study protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB). Five patients who underwent curative partial hepatectomy or liver transplantation for HCC at Queen Mary Hospital, Hong Kong, were recruited with written informed consent to the study. The patients had been diagnosed with primary HCC and confirmed by pathological examinations. Ethics approval for the use of archived fetal tissues identified from a computer database has been obtained from the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB).

Immunofluorescence staining and flow cytometric analysis

For the surface expression of MICA and HLA-E, cells were stained with APC-conjugated mouse anti-human MICA, PE-conjugated mouse anti-human HLA-E antibodies or equal amount of corresponding isotype control antibodies (BD Biosciences).

For co-expression with GEP, cells were stained with the above surface markers, followed by permeabilization with ice-cold 0.1% saponin and then incubated with FITC-conjugated mouse anti-human GEP antibody (homemade, described previously) or equal amount of FITC- conjugated mouse IgG isotype antibody (Sigma).

Preparation of human effector cells

Human peripheral blood mononuclear cells (PBMC) were freshly isolated from the huffy coats of healthy donors by density gradient centrifugation using Ficoll-Paque Plus. NK cells were isolated from PBMC using magnetic cell sorting by positive selection with anti-CD56 microbeads, while the unlabeled flow-through were the NK cell-depleted PBMCs, according to the manufacturer's instructions (Miltenyi Biotech). The resulting PBMCs, NK cells, and NK cell-depleted PBMCs were washed with RPMI 1640 with 10% FBS and used as human effector cells in the cell cytotoxicity assay.

Cell cytotoxicity assay

Cell cytotoxicity was determined by dual-color flow cytometry using the HCC cell lines Hep3B or HepG2 as target cells, while PBMC, NK cells, or NK cell-depleted PBMCs were used as effector cells. Briefly, HCC cells ($1x10^6$ cells/ml) were labeled with the green fluorescence cytoplasmic dye 5- and (6)-carboxyfluorescein diacetate,succinimidylester (CSFE) at 0.1μM for 15 min at 37°C in dark. After washing, CFSE-labeled HCC cells were then co-cultured with or without effector cells at indicated effector to target cells ratio (E/T ratio) in RPMI supplemented with 10% FBS for additional 5 hr at 37°C in 5% C02. Cells were then collected and stained with 10 μg/ml propidium iodide (PI) for 15 min at room temperature in dark. Cells were subject to dual-color flow cytometric analysis, with lysed target cells recognized as $CFSE^+PI^+$, while viable target cells as $CFSE^+PI^-$. Cell cytotoxicity level was expressed as percentage of lysed cells within the $CFSE^+$ target cells (Hep3B or HepG2), and calculated as follows:

Percentage of $CFSE^+PI^+$cells *I* (Percentage of $CFSE^+PI^+$ cells plus percentage of $CFSE^+PI^-$ cells) x 100.

For blocking experiments, neutralization of MICA was performed by incubating the CFSE-labeled HCC cells with or without anti-MICA neutralizing antibody or mouse IgG2a isotype control antibodies (2 ug/ml, Biolegend) and co-cultured with effector cells.

For antibody-dependent cytotoxicity (ADCC), CFSE-labeled HCC cells were stained with or without indicated concentrations of anti-GEP antibody A23, or mouse IgG isotype antibody for 30 min at 4°C. Labeled target cells were then co-cultured with effector cells at indicated E/T ratio.

Statistical analyses

All data were expressed as mean values ± standard deviation (SD) from at least three independent experiments. Differences between groups were assessed by the Student's t test. A

probability (p) < 0.05 was considered significantly different. All analyses were performed using the statistical software GraphPad Prism for Windows, Version 3.00 (GraphPad Software, CA, USA).

## EXAMPLES

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting.

### EXAMPLE 1 - GEP EXPRESSION OF HCC CELLS MODULATES ANTI-TUMOR CYTOTOXIC ACTIVITY OF IMMUNE CELLS

It is postulated that only a restricted minority of tumorigenic malignant cells may possess the characteristics needed to modulate tumor-directed immune activation. To examine the effect of GEP on anti-tumor immunity, stable transfectant of GEP suppression was established using shRNA in Hep3B, a HCC cell line with high endogenous GEP; stable transfectant of GEP over-expression was established using GEP full-length cDNA in HepG2, a HCC cell line with low endogenous GEP level (Fig. 1A).

Cytotoxic activity of human PBMC against the HCC cells and their GEP transfectants was examined by dual-color flow cytometry. Cytotoxic activity of human PBMC against GEP-suppressed Hep3B cells was significantly higher than control Hep3B cells. On the contrary, cytotoxic activity of PBMC against HepG2 cells was significantly reduced when GEP was over-expressed.

The results show that GEP expression is crucial for rendering HCC cells resistant to anti-tumor cytotoxic activity of PBMC (Fig. 1B).

PBMC encompasses a variety of immune cells including T and B lymphocytes, monocytes, natural killer (NK) cells, and dendritic cells. The anti-tumor activity of NK cells in liver has been well documented. Clinical studies also suggest that NK cells contribute to innate defenses against primary liver tumors and liver metastases in patients.

To evaluate whether NK cells are involved in the GEP-modulated cytotoxic activity, $CD56^+$ NK cells were depleted from human PBMC by magnetic sorting. Upon NK cell depletion, the increased cytotoxic activity against GEP-suppressed Hep3B cells was abolished; while the reduction of cytotoxic activity against GEP over-expressed HepG2 was restored (Fig. 1C).

To further confirm the role of NK cells, HCC cells and their GEP transfectants were co-cultured with NK cells at different E/T ratio. Cytotoxic activity of NK cells against GEP-suppressed Hep3B cells was significantly higher than that against control Hep3B cells; while significant reduction in NK cytotoxic activity was demonstrated in GEP over-expressed HepG2 cells when compared with control HepG2 cells (Fig. 1D). The results show that NK cells play a role in the GEP-regulated anti-tumor cytotoxic activity.

## EXAMPLE 2 - GEP DIFFERENTIALLY REGULATES THE EXPRESSION OF MICA AND HLA-E OF HCC CELLS

NK cell activity is regulated through the integrated signaling from stimulatory and inhibitory cell surface receptors that interact with ligands expressed on target cells. To elucidate the mechanism for GEP-regulated NK cell cytotoxicity, we determined the effect of GEP on the expression of ligands for activating immunoreceptor NKG2D and the stimulatory killer-cell immunoglobulin-like receptors (KIR), and inhibiting immunoreceptor CD94/NKG2A and inhibitory KIR2DL4 on HCC cells.

To delineate the effect of GEP, we measured the surface expression of MICA and HLA-E on Hep3B and HepG2 cells, and their GEP transfectants by flow cytometry. To further study the effect of GEP on the expression of MICA, we also analyzed the culture supernatants of the HCC cells and their GEP transfectants and measured the soluble MICA by ELISA.

Our results showed that upon GEP suppression, the expression of surface MICA significantly increased, while soluble MICA production and surface HLA-E expression significantly decreased when compared with control Hep3B cells, cell line with high endogenous GEP level (Fig. 2A). When GEP was over-expressed in HepG2, cell line with low endogenous GEP level,

expression of surface MICA significantly decreased, while soluble MICA release and surface HLA-E level significantly increased when compared with control cells (Fig. 2B).

All these findings confirmed the regulatory role of GEP in the expression of MICA and HLA-E, rendering HCC cells resistant to NK cell cytotoxic activity.

## EXAMPLE 3 - GEP BLOCKAGE BY ANTI-GEP ANTIBODY A23 MODULATES THE EXPRESSION OF MICA AND HLA-E OF HCC CELLS

To further validate the regulatory role of GEP on the expression of MICA and HLA-E, GEP blockage by anti-GEP monoclonal antibody A23 was performed in Hep3B cells. A23, but not mouse IgG, significantly suppressed GEP expression of Hep3B cells (Fig. 3A), demonstrating the suppressing effect was specific. A23 was found to significantly increase the expression of surface MICA, while decrease soluble MICA release and surface HLA-E expression of Hep3B when compared with control and isotype control treatment (Fig. 3B).

## EXAMPLE 4 - GEP BLOCKAGE BY ANTI-GEP ANTIBODY A23 ENHANCES NK CELL CYTOTOXICITY AGAINST HCC CELLS VIA MICA

Confirming the role of GEP in tumor immune evasion, we proceeded to study the therapeutic potential of anti-GEP monoclonal antibody A23 in anti-tumor immunity in HCC.

HCC cells were treated with A23 or isotype control antibody for 24h prior to co-culture with PBMC to assess the effect of A23 on anti-tumor cytotoxic activity against HCC cells. Results showed that cytotoxic acitivities of PBMC against A23-treated Hep3B and HepG2 cells were significantly higher than those of control and isotype control antibody-treated cells (Fig. 4A).

To confirm the role of MICA in A23-induced NK cell cytotoxicity against HCC cells, blocking antibody of MICA was used. Hep3B and HepG2 cells were treated with A23 for 24h, and then incubated with anti-MICA neutralizing antibody or isotype control antibody and co- cultured with PBMC. Results showed that the addition of anti-MICA blocking antibody could markedly abrogate the A23-induced cytotoxic activity against Hep3B and HepG2 cells, whereas there was no

effect exerted by the mouse $IgG_{2a}$ isotype control antibody. The results suggested that the A23-induced cytotoxic activity against HCC cells was dependent on MICA. (Fig. 4A)

Since MICA-NKG2D signal is an important inhibitory signal to NK cells, we proceeded to study the effect of MICA neutralization on A23-induced NK sensitivity of Hep3B and HepG2 cells. Results showed that blocking MICA could significantly abolish the A23-induced NK sensitivity of HCC cells, therefore confirming that A23-induced NK cell cytotoxicity against HCC cells was dependent on the MICA-NKG2D (Fig. 4B).

## EXAMPLE 5 - CO-EXPRESSION OF GEP WITH MICA AND HLA-E IN HCC CLINICAL SPECIMENS

The present inventors have shown that GEP conferred HCC cells resistance to NK cell cytotoxic activity by differentially regulated surface MICA and HLA-E expression in HCC cell line models. To confirm the immune evasion ability of GEP-expressing cells in clinical settings, GEP was co-stained with surface MICA and HLA-E in HCC clinical specimens by immunofluorescence staining and flow cytometry. Co-expression of GEP and MICA or HLA-E was performed by triple-color flow cytometry, gating on the $albumin^+$ hepatocytes isolated from HCC tissues.

The results showed that $GEP^+$ cells always expressed lower level of surface MICA than $GEP^-$ cells (Fig. 5A). However, GEP was found to co-express with surface HLA-E in the HCC clinical specimens (Fig. 5B). The results are consistent with our cell line model that GEP down-regulated MICA, but up-regulated HLA-E on the surface of HCC cells, making the cells less susceptible to NK anti-tumor cytotoxic activity.

## EXAMPLE 6 - ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY MEDIATED BY ANTI-GEP MONOCLONAL ANTIBODY A23

To further characterize the immunomodulatory mechanism of anti-GEP monoclonal antibody A23, antibody-dependent cellular cytotoxicity (ADCC) of A23 was assessed. ADCC occurs when antibodies bind to antigen on tumor cells and the antibody Fc domains engage Fc

receptors on the surface of immune effector cells. Hep3B or HepG2 cells were stained with or without anti-GEP antibody A23 or mouse isotype control antibody for 30 min and the antibody-labeled cancer cells were then co-cultured with PBMC. A23, but not isotype control antibody, significantly induced ADCC of human PBMC against both Hep3B and HepG2 cells in a dose-dependent manner (Fig. 6A). The results show that the anti-tumor activity of A23 was at least partly attributable to ADCC.

In addition, this Example further elucidates which effector cells are important for the ADCC activity of A23 in human. NK cells were depleted from PBMC and the A23-mediated ADCC in both cell lines was markedly abolished upon depletion of NK cells from PBMC (Fig. 6B). A23-labeled cancer cells were then co-cultured with NK cells and the A23-mediated ADCC against the HCC cells increased along the E/T ratio (Fig. 6C). Therefore, it was confirmed that NK cells also play an important role as effector cells for the A23-induced ADCC in human.

## EXAMPLE 7 –GEP AND IMMUNE ACTIVITIES OF HUMAN LIVER CANCER

### Association of GEP and immune evasion ability in human liver cancer

Liver tissue specimens:

The expression levels and patterns of GEP and immune evasion related molecules in human liver cancer specimens are examined, including MICA (ligand for NK cell activating immunoreceptor) and HLA-E (ligand for NK cell inhibitory immunoreceptor). Transcript and protein expression levels of GEP, MICA and HLA-E are examined in tumor tissue and adjacent non-tumor liver tissues of liver cancer patients and in non-cancer liver tissues (including normal, hepatitis and cirrhosis) by real-time qPCR, immunohistochemical staining, and western blot analysis. The clinico-pathological features including survival outcomes with these molecules are analyzed. GEP modulated the expression of MICA and HLA-E, and the expression levels of GEP in patient specimens can be used to determine the role of GEP on immune evasion ability in clinical settings.

Blood specimens:

The levels of soluble MICA and GEP in the sera of liver cancer patients, patients with cirrhosis / hepatitis and healthy individuals are measured by enzyme-linked immunosorbent assay (ELISA). Since GEP expression induces shedding of MICA from HCC cells, soluble MICA and GEP levels in the serum sample of liver cancer patient can be used as a predictive marker for corresponding immunotherapy.

Association of GEP expression in human liver cancer and NK cell infiltration

Liver tissue specimens:

The GEP expression levels in liver cancer cells and the abundance of NK cell infiltration into the tumor bulk are investigated. The number of infiltrating NK cells is assessed in the tumor tissues and adjacent non-tumor liver tissues of liver cancer patients and in non-cancer liver tissues by immunohistochemical staining.

GEP expression rendered liver cancer cells resistance to NK cell cytotoxic activities.

GEP antibody treatment enhanced the NK cell cytotoxic activities against liver cancer cells

Animal models:

The effect of GEP monoclonal antibody A23 treatment is examined in the human liver cancer xenografts in the mice models. To simulate the clinical situation of intervention on advanced liver cancer, treatment is started after large tumor bulk has been established in the mice. Mice receive GEP monoclonal antibody A23 or control treatment by intraperitoneal injection over extended time periods, and the xenografts are dissected and examined for transcript and protein expression of GEP, MICA and HLA-E by real-time qPCR and immunohistochemical staining. Infiltration of NK cells into the tumor bulk is examined by immunohistochemical staining. The role of GEP antibody on the tumor expressions of MICA and HLA-E, and the cytotoxic activities of NK cells in the tumor bulk is investigated.

Above, it has been demonstrated that granulin-epithelin precursor (GEP) protected liver cancer cells from cytotoxic activities of natural killer (NK) cells. These NK cells were isolated lymphocytes from healthy individuals. Using NK cells were isolated from liver cancer patients; it has been

shown that these NK cells demonstrated cytotoxic activities against liver cancer cells (Figure 7). Thus, activation of NK cells in liver cancer patients, by GEP antibody or inhibitors as described herein, could result in control of tumor growth. Transfusion of NK cells to liver cancer patients would not be necessary.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated with the scope of the invention without limitation thereto.

CLAIMS

We claim:

1. A method of treating liver cancer, comprising administering to a subject that has liver cancer an effective amount of:

1) an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP); and

2) natural killer (NK) cells or an agent that promotes NK cell activity

2. The method according to claim 1, comprising administering an agent that inhibits the activity of GEP.

3. The method according to claim 1 or 2, wherein the agent that inhibits the activity of GEP is an anti- GEP antibody.

4. The method according to claim 3, wherein the anti-GEP antibody is a monoclonal antibody.

5. The method according to claim 3, wherein the anti-GEP antibody is a polyclonal antibody.

6. The method according to any one of claims 1 to 5, comprising administering an effective amount of NK cells.

7. The method according to any one of claims 1 to 5, comprising administering an effective amount of an agent that promotes NK cell activity.

8. The method according to claim 7, wherein the agent that promotes NK cell activity is selected from IL-15, IL-12, pectin, IL-2, IFN-gamma, and IL-21.

9. The method according to any one of the previous claims, wherein the subject is a human.

10. A method of preventing recurrence of or treating liver cancer in a subject, wherein the method comprises administering to a subject, an effective amount of an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP), wherein the subject had surgical resection of a primary liver tumor, had underwent hepatectomy, and/or had liver transplantation.

11. The method according to claim 10, comprising administering an agent that inhibits the activity of GEP.

12. The method according to claim 10 or 11, wherein the agent that inhibits the activity of GEP is an anti-GEP antibody.

13. The method according to claim 12, wherein the anti-GEP antibody is a monoclonal antibody.

14. The method according to claim 12, wherein the anti-GEP antibody is a polyclonal antibody.

15. The method according to any one of claims 10 to 14, further comprising administering an effective amount of NK cells.

16. The method according to any one of claims 10 to 14, further comprising administering an effective amount of an agent that promotes NK cell activity.

17. The method according to claim 16, wherein the agent that promotes NK cell activity is selected from IL-15, IL-12, pectin, IFN-gamma, IL-2, and IL-21.

18. The method according to any one of claims 10 to 17, wherein the subject is a human.

19. A method of treating liver cancer in a subject, the method comprising administering to the subject an effective amount of an agent that increases the activity or expression of MICA present on a liver cancer cell.

20. A method according to claim 19 wherein the agent inhibits the expression or activity of granulin-epithelin precursor (GEP).

21. A method according to claim 20 wherein the wherein the agent that inhibits the activity of GEP is an anti- GEP antibody.

22. The method according to claim 21, wherein the anti-GEP antibody is a monoclonal antibody.

23. The method according to claim 21, wherein the anti-GEP antibody is a polyclonal antibody.

24. A method of treating liver cancer in a subject with increased levels of soluble MICA compared to a subject without liver cancer, the method comprising administering to the subject an effective amount of an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP).

25. The method according to claim 24, comprising first measuring the amount of soluble MICA present in a serum sample from the subject.

26. The method according to claim 24 or 25, comprising administering an agent that inhibits the activity of GEP.

27. The method according to claim 26, wherein the agent that inhibits the activity of GEP is an anti- GEP antibody.

28. The method according to claim 27, wherein the anti-GEP antibody is a monoclonal antibody.

29. The method according to claim 27, wherein the anti-GEP antibody is a polyclonal antibody.

30. A method of determining the suitability of a subject with liver cancer for treatment with an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP), the method

comprising i) obtaining a serum sample from the subject and ii) measuring the amount of soluble MICA in the sample.

31. The method according to claim 30 further comprising comparing the amount of soluble MICA in the serum sample to a chart correlating a quantity of soluble MICA and the presence or progression of liver cancer, wherein an amount of soluble MICA in the serum which correlates with presence or progression of liver cancer indicates suitability of the subject for treatment with an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP).

32. The method according to claim 30 or 31, comprising administering an agent that inhibits the activity of GEP.

33. The method according to claim 32, wherein the agent that inhibits the activity of GEP is an anti- GEP antibody.

34. The method according to claim 33, wherein the anti-GEP antibody is a monoclonal antibody.

35. The method according to claim 33, wherein the anti-GEP antibody is a polyclonal antibody

36. A method of increasing MICA expression on a cancer cell comprising administering an effective amount of an agent that inhibits the expression or activity of granulin-epithelin precursor (GEP).

37. The method according to claim 36, comprising administering an agent that inhibits the activity of GEP.

38. The method according to claim 37, wherein the agent that inhibits the activity of GEP is an anti- GEP antibody.

39. The method according to claim 38, wherein the anti-GEP antibody is a monoclonal antibody.

40. The method according to claim 38, wherein the anti-GEP antibody is a polyclonal antibody

41. A method of assessing or monitoring the extent or progression of liver cancer in a subject, the method comprising analysing a sample from the subject for a quantity of soluble GEP.

42. The method according to claim 41 wherein the sample is a serum sample.

43. The method according to claim 41 or 42 further comprising the amount of soluble GEP in the sample to a chart correlating a quantity of soluble GEP and tumour size.

44. The method according to any one of claims 41 to 43 wherein the method comprises i) obtaining a sample from the subject; and ii) contacting the sample with a detectable binding partner which is specific for soluble GEP.

45. The method according to claim 44 wherein the detectable binding partner is an anti-GEP antibody.

46. The method according to claim 45 wherein the antibody is a monoclonal antibody.

47. The method according to claim 45 wherein the antibody is a polyclonal antibody.

48. The method according to claim 43 wherein the detectable binding partner is a nucleic acid molecule which binds to a nucleic acid molecule encoding GEP.

49. The method according to claim 48 wherein the nucleic acid molecule is an siRNA or an shRNA.

# INTERNATIONAL SEARCH REPORT

International application No.
**PCT/CN2014/078740**

## A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/395(2006.01)i; A61P 35/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K39; A61P35

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI; EPODOC; CNPAT; CNKI; PubMed; ISI; granulin-epithelin precursor, GEP, hepatocarcinoma, liver cancer, hepatocellular carcinoma, natural killer cell, nk cell, MICA, IL-15, IL-12, pectin, IFN-gamma, IL-2, IL-21

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2005/106019 A1 (THE UNIVERSITY OF HONG KONG) 10 November 2005 (2005-11-10)<br>description, page 3, line 30 to page 4, line 2, page 5, lines 18-25, page 8, line 10 to page 9, line 18 and page 13, lines 9-12 | 10-14, 18 and 41-49 |
| X | MASAHISA, J. et al. "Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid."<br>*Int. J. Cancer.*, Vol. vol. 104, 31 December 2003 (2003-12-31),<br>pages 354-361 | 19 |
| X | CN 102985111 A (THE UNIVERSITY OF HONG KONG) 20 March 2013 (2013-03-20)<br>claims 1-14 | 10-14, 18 and 41-49 |
| X | CN 101553728 A (THE UNIVERSITY OF HONG KONG) 07 October 2009 (2009-10-07)<br>claims 11-22 | 10-14, 18 and 41-49 |
| A | WO 98/19167 A2 (FRED HUTCHINSON CANCER RESEARCH CENTER, INC.) 07 May 1998 (1998-05-07)<br>the whole document | 1-49 |

☐ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| **05 August 2014** | **27 August 2014** |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| **STATE INTELLECTUAL PROPERTY OFFICE OF THE P.R.CHINA(ISA/CN)**<br>**6,Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088 China** | **PAN,Hao** |
| Facsimile No. **(86-10)62019451** | Telephone No. **(86-10)82245455** |

**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/CN2014/078740**

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claims Nos.: **1-29**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] The subject matter of claims 1-29 relates to a method of treating liver cancer or preventing recurrence of liver cancer in a subject, and therefore, according to the criteria set out in Rule 39.1(iv), relates to subject matter for which an international search is not required. However, the search has been carried out and based on the use of the agents and the cells for the manufacturing of a medicament.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**INTERNATIONAL SEARCH REPORT**
Information on patent family members

International application No.
**PCT/CN2014/078740**

| Patent document cited in search report | Publication date (day/month/year) | Patent family member(s) | Publication date (day/month/year) |
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