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(54) METHOD AND KIT FOR DIAGNOSING AND FOR TREATMENT OF A CANCER BASED ON THE OVEREXPRESSION OF THE ADAMTSL5 GENE

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(57) **ABSTRACT**

The invention relates to a method for diagnosing a cancer in a mammal in a need thereof. The method comprises the following steps: collecting a biological sample from said mammal; determining, from said biological sample, if the ADAMTSL5 gene is overexpressed; diagnosing a cancer from the determination of the overexpression of said gene. It also relates to a kit for determining an overexpression of the ADAMTSL5 gene in a biological sample obtained from a mammal, to a pharmaceutical composition comprising an agent targeting ADAMTSL5 or the ADAMTSL5 pathway as well as a pharmaceutically acceptable carrier for use in the treatment of a cancer, and to a use of an ADAMTSL5 mRNA or protein as a biomarker of cancer.

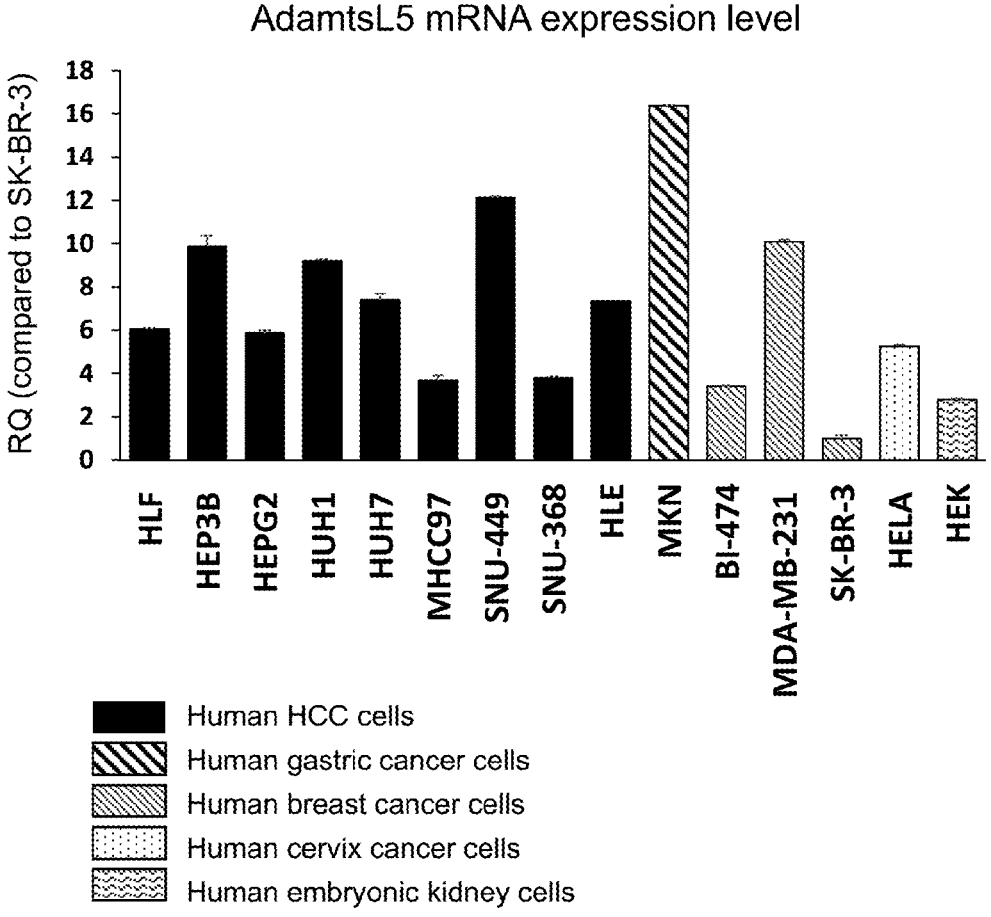


FIG. 1A

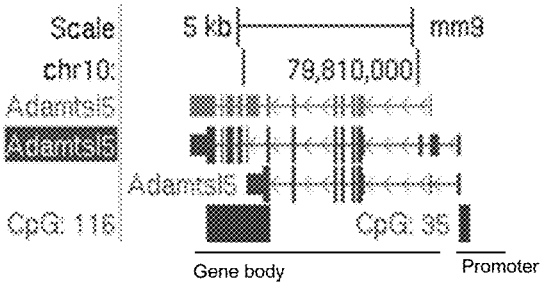


FIG. 1B

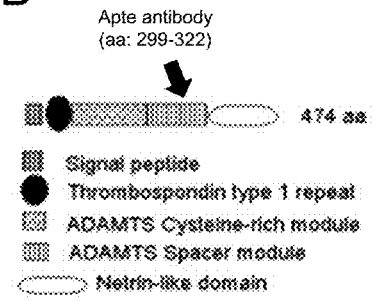


FIG. 1C

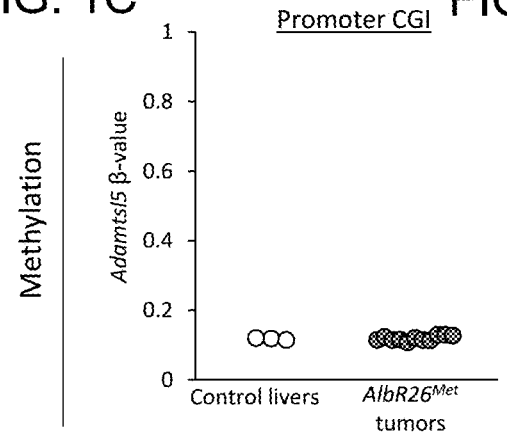


FIG. 1D

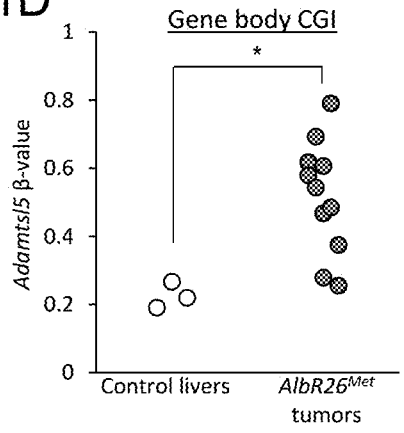


FIG. 1E

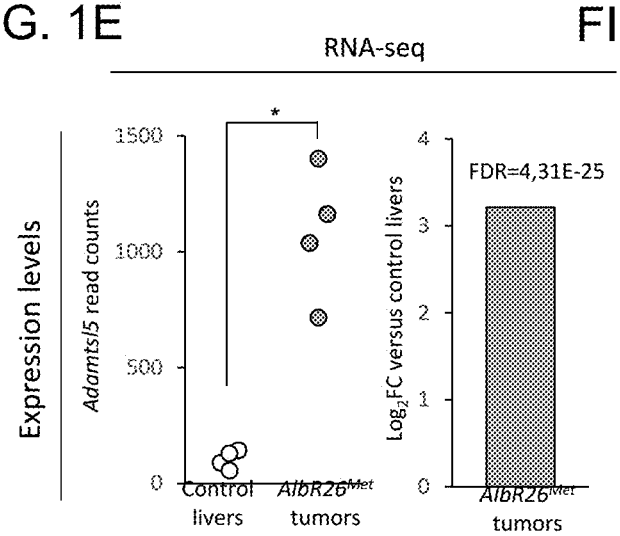


FIG. 1F

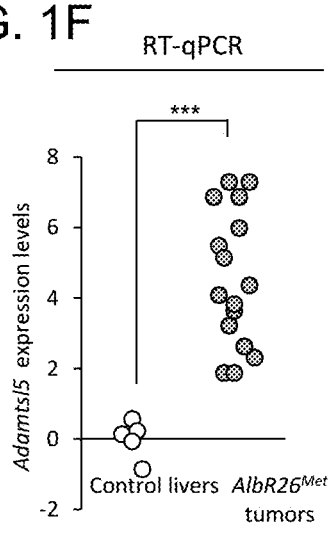


FIG. 2A

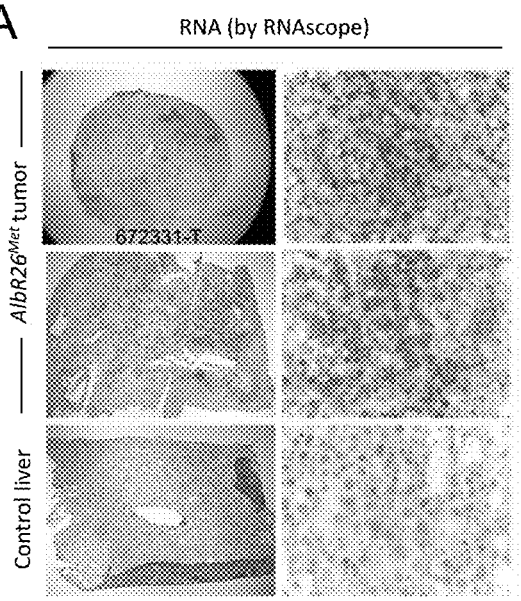

RNA (by RNAscope)
AlbR26Met tumor
Control liver

FIG. 2B

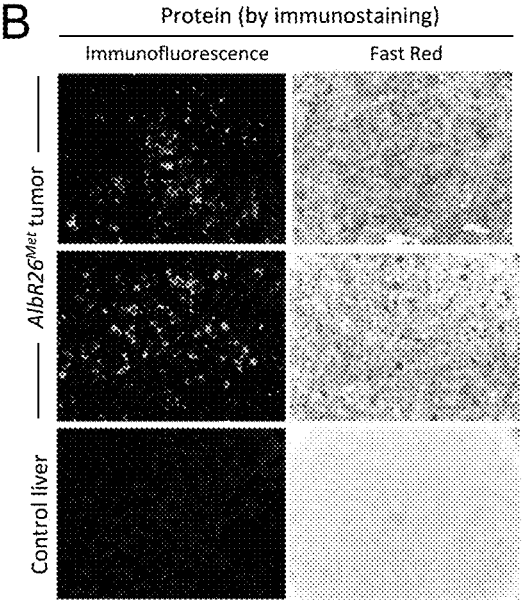

Protein (by immunostaining)
Immunofluorescence
Fast Red
AlbR26Met tumor
Control liver

FIG. 3A

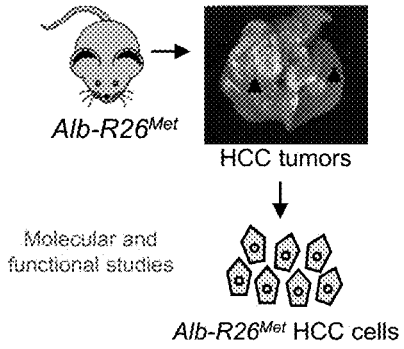


FIG. 3B

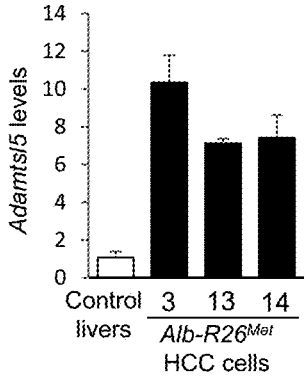


FIG. 3C

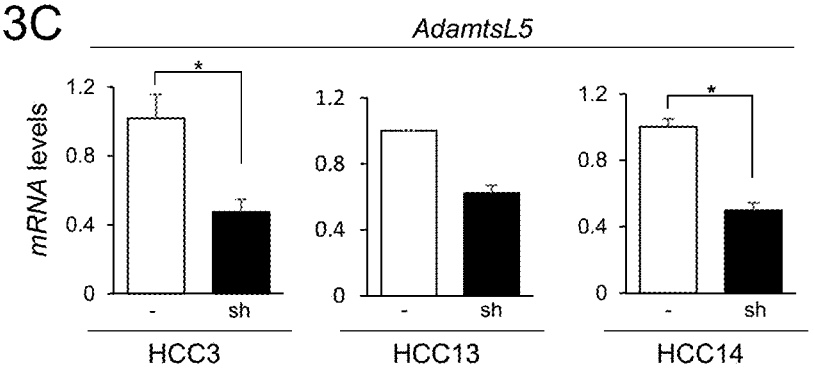


FIG. 3D

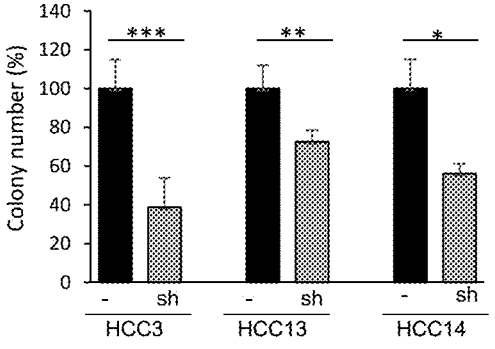


FIG. 3E

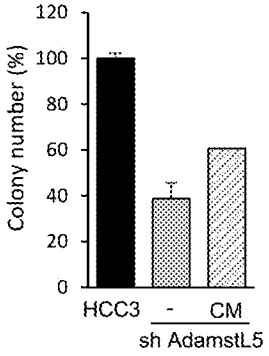


FIG. 3F

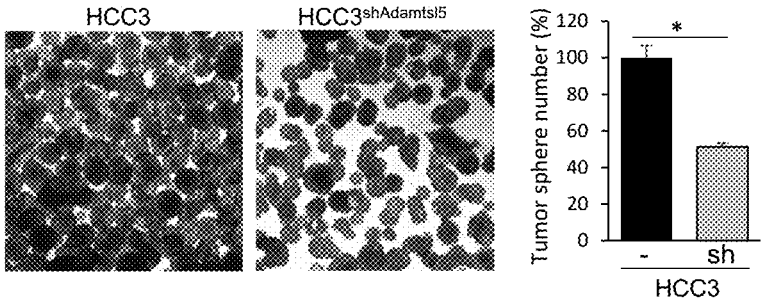


FIG. 4A

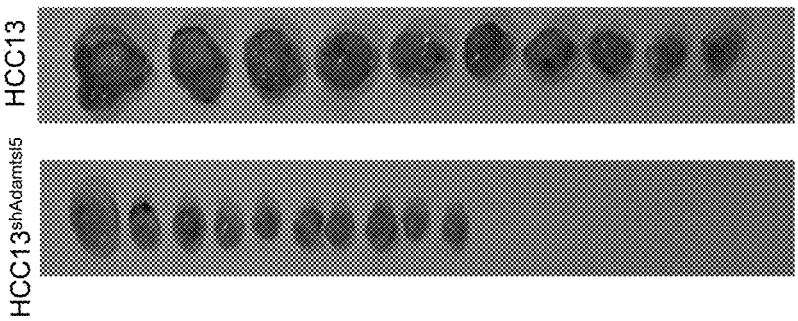

HCC13
HCC13shAdamtsl5

FIG. 4B

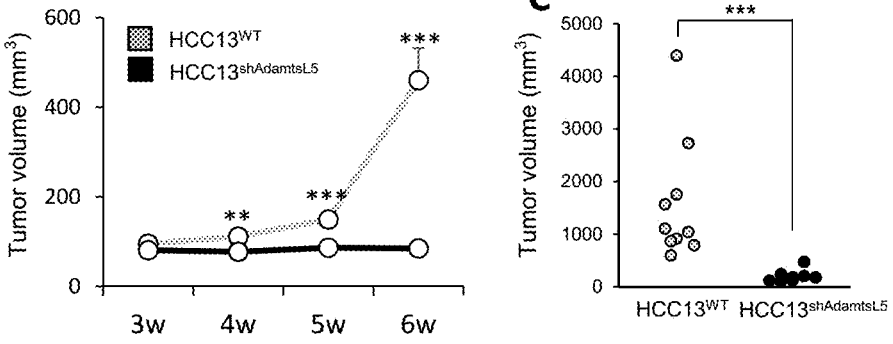

Tumor volume (mm3)
600
400
200
0
HCC13WT
HCC13shAdamtsL5
**
***
***
3w
4w
5w
6w
C
Tumor volume (mm3)
5000
4000
3000
2000
1000
0
***
HCC13WT
HCC13shAdamtsL5

FIG. 5A

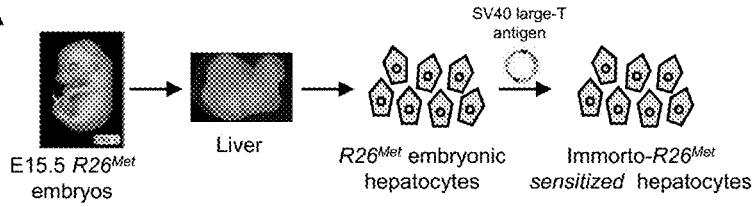


FIG. 5B

FIG. 5C

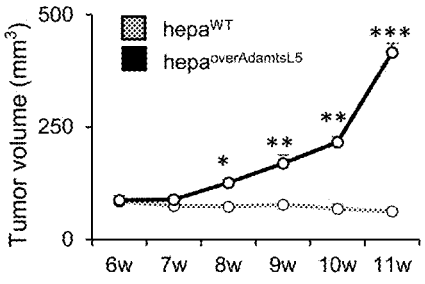


FIG. 5D

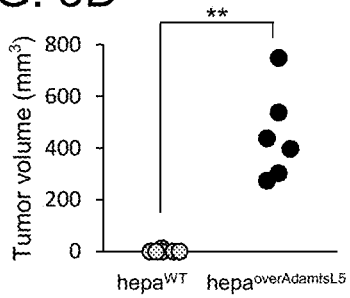


FIG. 6A

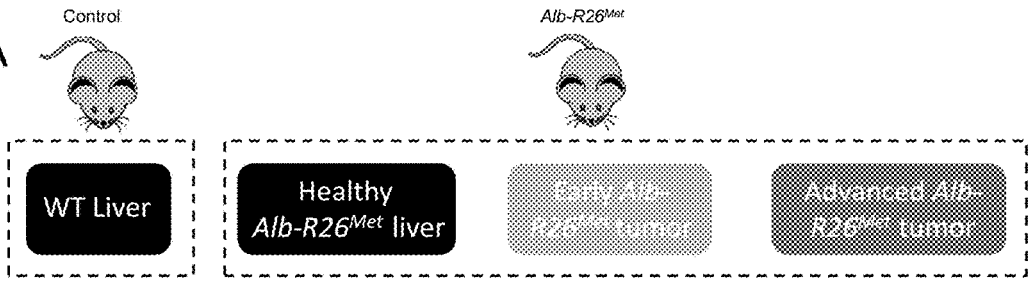


FIG. 6B

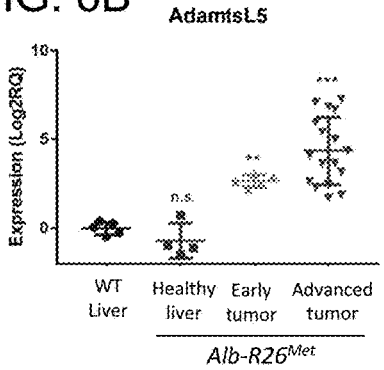


FIG. 6C

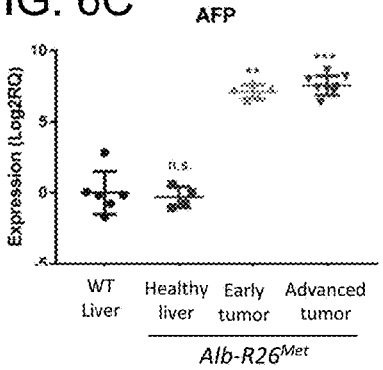


FIG. 6D

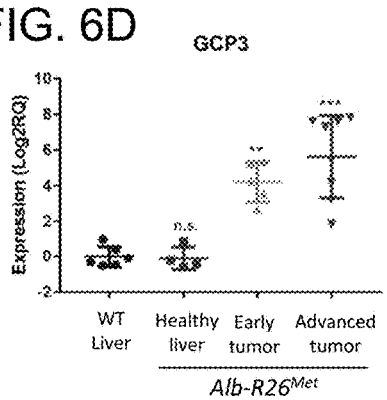


FIG. 6E

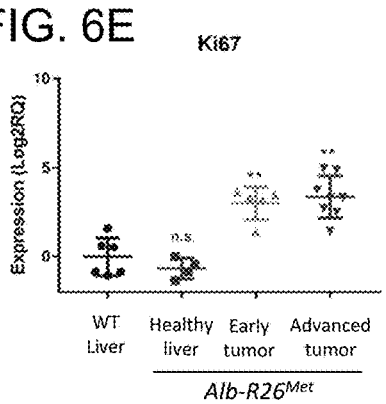


FIG. 7A

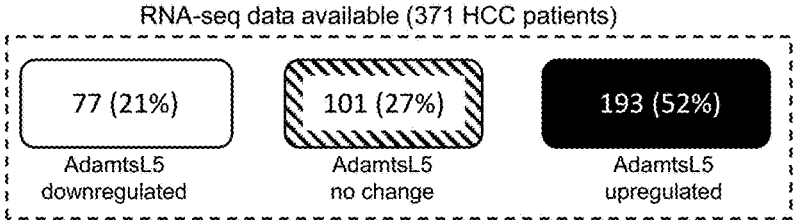


FIG. 7B

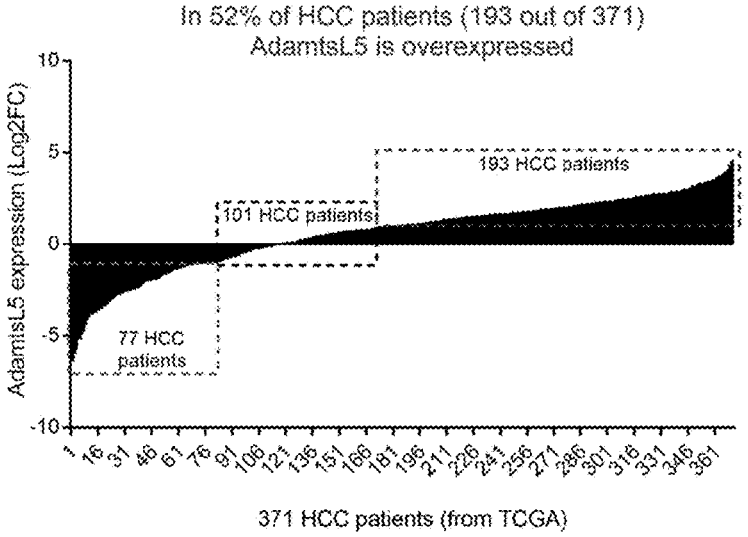
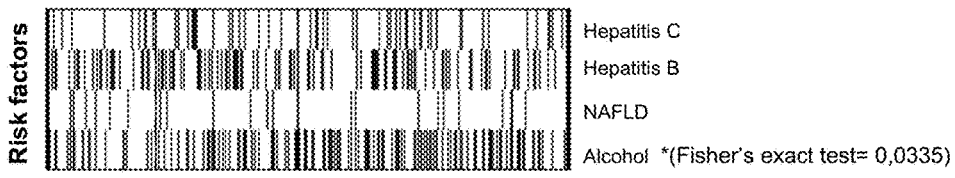


FIG. 7C



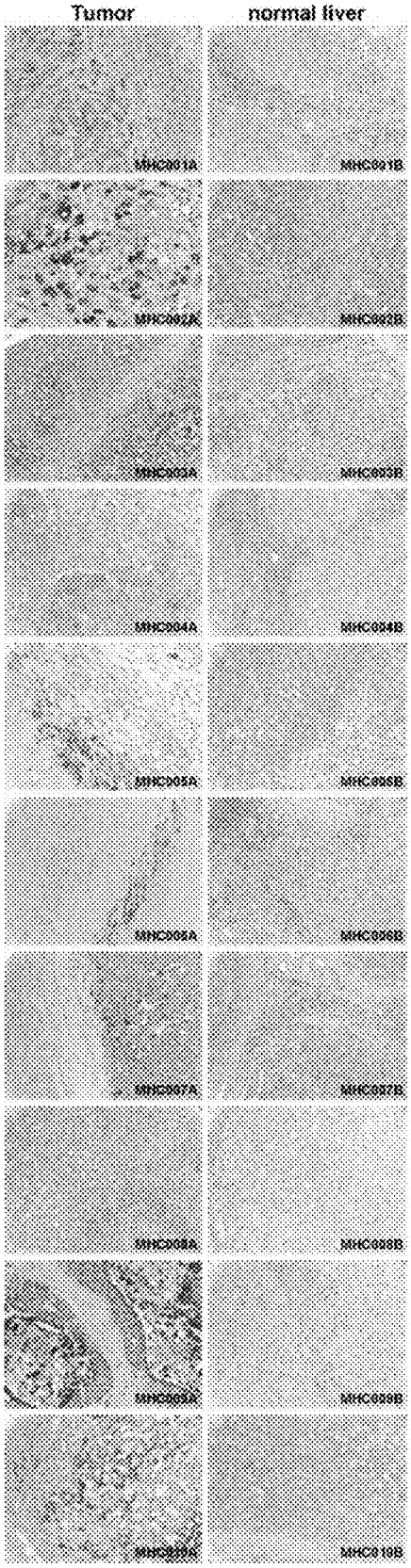


FIG. 8

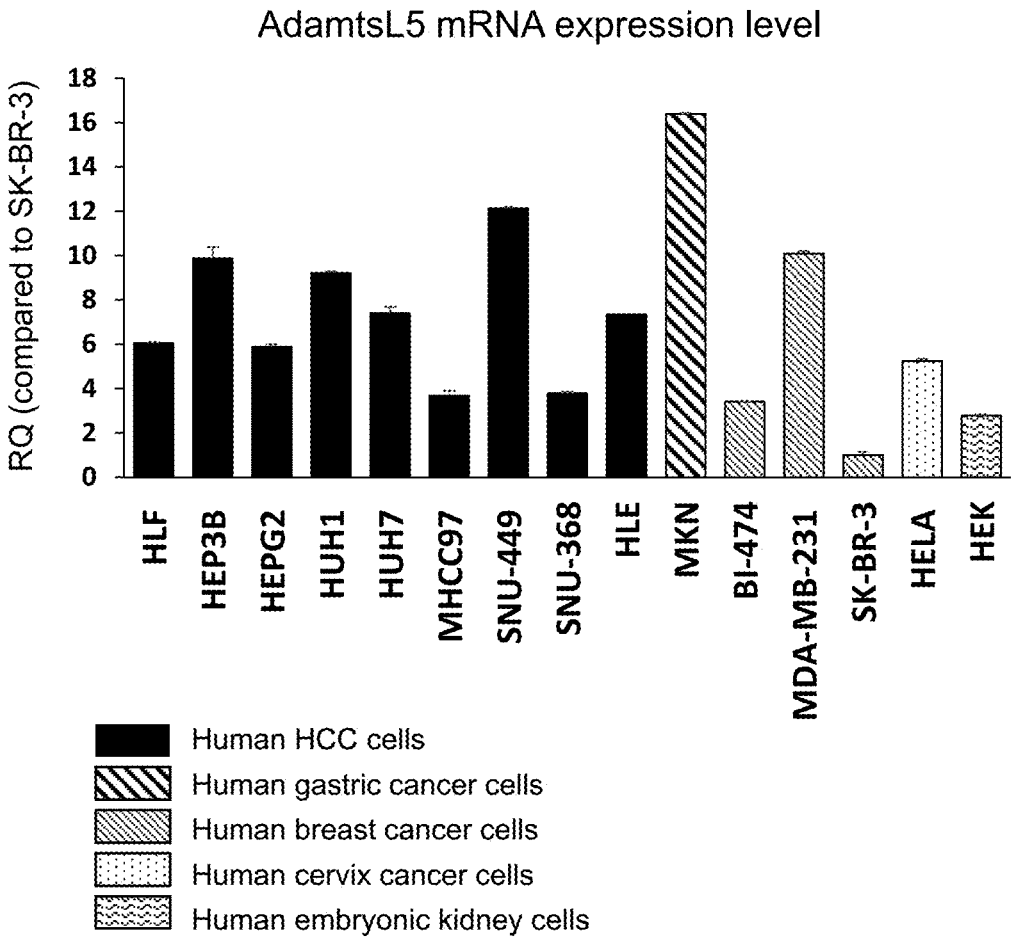

AdamtsL5 mRNA expression level
RQ (compared to SK-BR-3)
18
16
14
12
10
8
6
4
2
0
HLF
HEP3B
HEPG2
HUH1
HUH7
MHCC97
SNU-449
SNU-368
HLE
MKN
BI-474
MDA-MB-231
SK-BR-3
HELA
HEK
Human HCC cells
Human gastric cancer cells
Human breast cancer cells
Human cervix cancer cells
Human embryonic kidney cells

FIG. 9

METHOD AND KIT FOR DIAGNOSING AND FOR TREATMENT OF A CANCER BASED ON THE OVEREXPRESSION OF THE ADAMTSL5 GENE

TECHNICAL FIELD OF THE INVENTION

[0001] The invention relates to a method for diagnosing a cancer in a mammal in need thereof. The method according to the invention comprises a step of determining the expression level of specific biomarker in a biological sample obtained from said mammal. The invention also relates to a kit for determining such an expression level, and to a pharmaceutical composition for inhibiting the specific biomarker pathway.

BACKGROUND OF THE INVENTION

[0002] Cancer is the second leading cause of death in world. Nearly one in six deaths worldwide is due to cancer. For example in 2015, 8.8 million people died of cancer. Therefore, it is important to develop new methods for improving the diagnostic of cancer in patients in need thereof, and notably for predicting their susceptibility to the treatments so as to increase their therapeutic responses, which will results in increased survival expectancies.

[0003] Epigenetics is the study of changes in gene activity, which does not involve the modifications of the DNA. sequence and which can be transmitted in cell divisions. Unlike mutations that affect the DNA sequence, epigenetic modifications are reversible. It is of common knowledge that epigenetic abnormalities lead to the development and progression of human diseases, especially cancers. Epigenetic processes intervene in the regulation of many events such as cell division, differentiation, survival, and mobility. The alteration of these mechanisms promotes the transformation of healthy cells into cancer cells, and so any epigenetic aberration may be involved in tumorigenicity. Among consequences linked to epigenetic abnormalities in cells, there is silencing of tumor suppressor genes or overexpression of oncogenes such as proto-oncogenes, which involve aberrant DNA methylation or mutations of genes encoding enzymes responsible for chromatin modifications. DNA methylation is an essential epigenetic mechanism influencing gene expression levels in cells. These alterations may lead to dramatic biological changes as well as acquisition of malignant properties. The cancer landscape is generally characterized by a diffuse DNA hypomethylation and by focal hypermethylation in CpG-rich regions known as CpG islands (CGI). Generally, CGI hypermethylation at promoters represses transcription of genes acting as tumor suppressors. Nevertheless, a large fraction of DNA methylation is also observed in gene body CGIs, with a positive correlation between methylation and upregulation of gene expression. This implies that DNA hypermethylation in gene body CGIs is linked to the upregulations of genes that would act as positive regulators of tumorigenicity, which is coherent with the reported function of some of them in cancer cells. However, the implication of several others hypermethylated and upregulated genes in cell tumorigenicity still remain unknown.

[0004] These findings have busted the development of drugs targeting epigenetic regulators, which are called epi-drugs or epi-medicines. Two main families of compounds have been notably developed until now: (i) compounds that inhibit the DNA methylation, and (ii) compounds that target the histones modifications. However, at present these types of compounds lack of specific action.

[0005] Based on the above, there remains a need for identifying genes that can be used as new specific biomarkers and/or targets to modulate with bioactive agents for cancer treatment. In particular, there remains a need to develop new methods for diagnosis, prognosis, and treatment of cancer.

SUMMARY OF THE INVENTION

[0006] In accordance with a first aspect, the invention relates to a method for diagnosing a cancer in a mammal in a need thereof. The method comprises the following steps:

[0007] collecting a biological sample from said mammal;

[0008] determining, from said biological sample, if the ADAMTSL5 gene is overexpressed;

[0009] diagnosing a cancer from the determination of the overexpression of said gene.

[0010] According to a second aspect, the invention concerns a kit for determining an overexpression of the ADAMTSL5 gene in a biological sample obtained from a mammal. The kit comprises at least one antibody anti-ADAMTSL5 type, a container for holding the biological sample, and a protocol for measuring an overexpression of the ADAMTSL5 cancer marker gene, preferably hepatocellular carcinoma marker gene, in a biological sample obtained from a mammal.

[0011] According to a third aspect, the invention relates to a pharmaceutical composition. The pharmaceutical composition comprises an agent targeting the ADAMTSL5 gene itself and/or the pathway in which ADAMTSL5 acts, and a pharmaceutically acceptable carrier for use in the treatment of a cancer.

[0012] According to a fourth aspect, the invention concerns a use of an ADAMTSL5 protein as a biomarker of cancer.

[0013] In a fifth aspect, the invention relates to method for treating cancer in a mammal in a need thereof. The cancers are the same as those above-described. The method comprises a first step of diagnosing the cancer in a mammal in a need thereof, and a second step of treating the cancer by administering an inhibitor of ADAMTLS5. The step of diagnosing the cancer involves the same steps as these above-described in the method for diagnosing a cancer in a mammal in a need thereof.

[0014] In a sixth aspect, the invention relates to an in vitro method for monitoring the response to an anticancer treatment of a mammal suffering from cancer comprising determining the ADAMTSL5 level of expression in a biological sample of said mammal at two or more time points during said anticancer treatment, wherein an equal or higher ADAMTSL5 level of expression in a biological sample of the subject at a later time point, compared to a reference value obtained in a biological sample of the subject at an earlier time point, is indicative of a resistance of the subject to said anticancer treatment whereas a lower ADAMTSL5 level is indicative of a response of the subject to said anticancer treatment.

[0015] Advantageously, the method for diagnosing a cancer in a mammal in a need thereof according to the invention is characterized in that: the cancer is selected from the group consisting of brain cancer, CNS cancer, colorectal cancer,

breast cancer, lung cancer, skin cancer, kidney cancer, gastrointestinal cancer, myeloma, lymphoma, leukemia, cervix cancer, liver cancer, and hepatocellular carcinoma, preferably the cancer is an hepatocellular carcinoma; the biological sample is selected from the group consisting of blood, biopsy tissue, blood serum, blood plasma, urine, stool, sputum, cerebrospinal fluid, or supernatant from cell lysate, preferably the biological sample is tissue biopsy, blood, blood plasma, blood serum, or urine; the overexpression of ADAMTSL5 gene in the biological sample is determined by measuring the ADAMTSL5 protein levels or mRNA levels in said biological sample; the ADAMTSL5 protein levels in the biological sample are measured by adding at least one antibody anti-ADAMTSL5 type to said biological sample; the antibody anti-ADAMTSL5 type is selected from the group consisting of uncoupled or coupled with alkaline phosphatase horse-radish peroxidase, or with fluorescent dyes; the ADAMTSL5 protein levels in the biological sample are measured by using immunostaining, immunofluorescence, western blot, or ELISA.

[0016] Advantageously, the kit for determining an overexpression of the ADAMTSL5 gene according to the invention is characterized in that: the antibody anti-ADAMTSL5 type is selected from the group consisting of uncoupled or coupled/conjugated with alkaline phosphatase, horse-radish peroxidase, or with fluorescent dyes.

[0017] Advantageously, the pharmaceutical composition according to the invention is characterized in that: the agents targeting ADAMTSL5 or the ADAMTSL5 pathway are selected from the group consisting of blocking antibodies, peptides, sh-RNA, si-RNA, micro-RNA, antisense RNA, chemical drugs, a demethylating agent and an agent modulating glycosylation and/or heparin binding.

BRIEF DESCRIPTION OF DRAWINGS

[0018] Other features and aspects of the present invention will be apparent from the following description and the accompanying drawings, in which:

[0019] FIG. **1** illustrates hypermethylation in the gene body CGI of ADAMTSL5 and overexpression of ADAMTSL5 in a clinically relevant cancer mouse model (Alb-R26^{Met} mice):

[0020] FIG. **1**(A) shows a schematic representation of mouse ADAMTSL5 transcripts and CGIs from the UCSC genome browser, and Refseq gene annotations based on the NCBI37/mm9 mouse reference,

[0021] FIG. **1**(B) is a schematic representation of the human ADAMTSL5 protein from Badel et al. Matric Biology, 2012,

[0022] FIGS. **1**(C) and (D) illustrate the methylation levels (β-value) of ADAMTSL5 in the promoter CGI (FIG. **1**(C)) and in the gene body CGI (FIG. **1**(D)) in control livers (n=3) and Alb-R26^{Met} tumors (n=10),

[0023] FIG. **1**(E) illustrates the ADAMTSL5 mRNA expression levels in Alb-R26^{Met} tumors compared to control livers by RNA-seq,

[0024] FIG. **1**(F) illustrates the ADAMTSL5 expression levels (by RT-qPCR) in Alb-R26^{Met} tumors (n=16) relative to control livers (n=5);

[0025] FIG. **2** illustrates overexpression of ADAMTSL5 mRNA and protein in a clinically relevant cancer mouse model (Alb-R26^{Met} mice):

[0026] FIG. **2**(A) shows representative images of the ADAMTSL5 mRNA expression levels (by RNA-scope) in tumors (dissected from Alb-R26^{Met} mice) and in control liver (dissected from control mice),

[0027] FIG. **2**(B) shows representative images of the ADAMTSL5 protein levels by immunofluorescence (left) and immunostaining (right) in tumors (dissected from Alb-R26^{Met} mice) and in control liver (dissected. from control mice);

[0028] FIG. **3** illustrates the in vitro tumorigenic properties of hepatocellular carcinoma (HCC) cells established from a clinically relevant cancer mouse model (Alb-R26^{Met} mice) with high expression levels of ADAMTSL5 compared to cells with reduced ADAMTSL5 expression levels:

[0029] FIG. **3**(A) is a schematic representation of the establishment of Alb-R26^{Met} HCC cell lines (Fan et al. Hepatology, 2017) used for molecular and functional studies,

[0030] FIG. **3**(B) is a graph reporting the ADAMTSL5 expression levels (by RT-qPCR) in three Alb-R26^{Met} HCC cell lines (HCC3, HCC13, and HCC14) relative to control livers,

[0031] FIG. **3**(C) shows the mRNA expression levels of ADAMTSL5 in Alb-R26^{Met} HCC cells stably transfected with a plasmid carrying a shRNA targeting sequence (carrying also the puromycin gene for selection of stable clones) versus controls,

[0032] FIG. **3**(D) illustrates results of anchorage independent growth assay (soft agar assay) performed using either HCC control cells or HCC cells carrying the shRNA sequence targeting ADAMTSL5,

[0033] FIG. **3**(E) is descriptive of the anchorage independent growth assay (soft agar assay) showing partially rescue of in vitro tumorigenic properties of ADAMTSL5-targeted Alb-R26^{Met} HCC cells with condition media from control cells,

[0034] FIG. **3**(F) are representative images (left) and quantification (right) of tumor spheres derived from control and ADAMTSL5-targeted Alb-R26^{Met} HCC cells (right);

[0035] FIG. **4** illustrates the in vivo loss of tumorigenic properties of HCC cells following ADAMTSL5 downregulation:

[0036] FIG. **4**(A) contains images of dissected tumors from xenografts in nude mice injected either with Alb-R26^{Met} HCC cells (top) or with Alb-R26^{Met}-shADAMTSL5 HCC cells (bottom),

[0037] FIG. **4**(B) contains xenograft growth curves reporting the mean tumor volume per group measured every week,

[0038] FIG. **4**(C) shows a quantitative analysis of the volume of tumors dissected 8 weeks after cell injection;

[0039] FIG. **5** illustrates the in vivo acquisition of tumorigenic properties of immortalized hepatocytes from a clinically relevant mouse model (R26^{Met} mice) following ADAMTSL5 overexpression:

[0040] FIG. **5**(A) is a schematic representation of the establishment of immorto-R26^{Met} sensitized hepatocytes (embryonic hepatocytes carrying increased levels of the Met RTK and immortalized with the SV40 large-T antigen),

[0041] FIG. **5**(B) comprises images of mice after 11 weeks of xenografts establishment,

[0042] FIG. **5**(C) contains xenograft growth curves reporting the mean tumor volume per group measured every week,

[0043] FIG. **5**(D) shows a quantitative analysis of the volume of tumors dissected 11 weeks after cell injection;

[0044] FIG. **6** illustrates ADAMTSL5 expression levels in a clinically relevant cancer mouse model (Alb-R26^{Met} mice) at early and latest stages of tumorigenesis:

[0045] FIG. **6**(A) is a schematic representation of the tissue samples used for RT-qPCR analysis of ADAMTSL5 levels,

[0046] FIG. **6**(B), FIG. **6**(C), FIG. **6**(D), and FIG. **6**(E) are graphs reporting the expression levels (by RT-qPCR) of ADAMTSL5, AFP (alpha-fetoprotein), GPC3 (Glypican-3) (two HCC markers), and Ki67 (a proliferative marker), respectively;

[0047] FIG. **7** illustrates high ADAMTSL5 mRNA levels in 52% of HCC patients, with a predominance in those associated to alcohol taken:

[0048] FIG. **7**(A) is a chart reporting the cohort of HCC patients (371 patients) with ADAMTSL5 mRNA levels,

[0049] FIG. **7**(B) is a graph reporting the three subgroup of HCC patients according to low, unchanged, and high ADAMTSL5 expression levels (numbers and percentages are indicated),

[0050] FIG. **7**(C) is a table reporting the presence (black line) or the absence of major HCC risk factors in all 371 analyzed patients;

[0051] FIG. **8** illustrates overexpression of ADAMTSL5 protein levels in 9/10 HCC patients compared to the adjacent liver; and

[0052] FIG. **9** illustrates ADAMTSL5 mRNA levels in human cancer cell lines.

DETAILLED DESCRIPTION OF THE INVENTION

[0053] The invention relates to a method for diagnosing and prognosis a cancer in a mammal in a need thereof. First of all, the method comprises a step of collecting a biological sample from said mammal, followed by a step of determining, from said biological sample, if the ADAMTSL5 gene/protein is overexpressed, and then, according to a third step, diagnosing/prognosis a cancer from the determination of the overexpression of said gene or protein.

[0054] According to the invention, the mammal is in particular a human. However, all mammals are concerned including even cat, dog, horse or rodents such as mice and rats.

[0055] According to a preferred embodiment of the invention, the cancer is a brain cancer, a cancer in the central nervous system (CNS), a colorectal cancer, a breast cancer, a lung cancer, a skin cancer, a gastrointestinal cancer, a kidney cancer, myeloma, lymphoma, leukemia, cervix cancer, liver cancer such as an hepatocellular carcinoma (HCC). The cancer, which is in particular diagnosed according to the invention, is the HCC.

[0056] According to the invention, the biological sample is selected from the group consisting of blood, tissue biopsy, blood serum, blood plasma, urine, stool, sputum, cerebrospinal fluid, and supernatant from cell lysate. The biological sample that is in particular used is tissue biopsy, blood, blood plasma, blood serum, or urine.

[0057] According to a preferred embodiment of the invention, the overexpression of ADAMTSL5 gene in the biological sample obtained from the mammals in need thereof is determined by measuring the ADAMTSL5 protein levels or RNA levels in said biological sample. The term "up-regulated", "up-regulation", "overexpressed", or "overexpression" is used to mean that the expression, activity, or level of a gene, or RNA transcripts or protein products of the gene, is greater than relative to one or more controls, such as, for example, one or more positive and/or negative controls. In particular, increased levels are considered when levels are higher than those in control healthy tissues.

[0058] Practically, mammalian genomes contain 19 ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin) genes numbered 1 to 20. Like their relatives, the matrix metalloproteinases (MMPs) and the ADAMs, the ADAMTSs belong to the metzincin protease superfamily, named for the conserved methionine residue close to the zinc ion-dependent metalloproteinase active site. Representatives of the ADAMTS family are found in all metazoans, although, to date, they have not been identified in single-cell organisms or in plants. All ADAMTSs are secreted, extracellular enzymes that have a compound domain organization, comprising, from the amino-terminus: a signal peptide followed by a pro-region of variable length; a metalloproteinase domain; a disintegrin-like domain; a central thrombospondin type 1 sequence repeat (TSR) motif; and a cysteine-rich domain followed by a spacer region. Separate from the ADAMTSs, another family of seven ADAMT5-like genes (ADAMTSL) encode proteins that resemble the ancillary domains of ADAMTS, although lack their catalytic domains. These ADAMTSL proteins, which include ADAMTSL 1 to 6 and papilin, may function to modulate the activities of the ADAMTSs. ADAMTSL5 is a protein that has been discovered in the late 2000s and is described to bind to fibrillin-1 and to promote fibril formation. The role of ADAMTSL5 in microfibril formation is of considerable interest as a crucial mechanism for growth factor regulation in extracellular matrix. ADAMTSL5 is more particularly a secreted protein with a unique domain composition, comprising an N-terminal thrombospondin type 1 repeat, a cystein-rich module, a spacer module, and a C-terminal netrin-like module, which is connected to the spacer by a proline-rich segment. ADAMTSL5 is known as already involved in some disease such as psoriasis but, to date, ADAMTSL5 has not been linked to cancer, as potential biomarker or as a target for molecular therapies.

[0059] In the context of the present invention and in a preferred embodiment, the ADAMTSL5 protein level is measured by adding at least one antibody to said biological sample. The antibody, which is in particular used for measuring the ADAMTSL5 protein levels, is of an anti-ADAMTSL5 type. The antibody anti-ADAMTSL5 is in particular selected from the group consisting of uncoupled or coupled or conjugated with alkaline phosphatase, horseradish peroxidase (HRP), or with fluorescent dyes.

[0060] According to the invention, the ADAMTSL5 protein levels present in the biological sample of the mammals in need thereof is in particular measured by using immunostaining, immunofluorescence, western blot, or ELISA.

[0061] In biochemistry, immunostaining is known as use of an antibody-based method to detect a specific protein in a biological sample. Immunostaining encompasses a broad range of techniques used in histology, cell biology, and molecular biology, which use antibody-based staining methods. Immunofluorescence is a technique used for light microscopy with a fluorescence microscope and is used primarily on microbiological samples. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell,

and therefore allows visualization of the distribution of the target molecule through the sample.

[0062] According to a second aspect, the invention provides a kit for determining an overexpression of the ADAMTSL5 gene in a biological sample obtained from a mammal. The kit comprises at least one antibody anti-ADAMTSL5 type and a container for holding the biological sample. The antibodies anti-ADAMTSL5 type used in the kit are the same as those above-described.

[0063] According to the invention, ADAMTSL5 levels present in the biological sample of the mammals in need thereof can be also determined by measuring ADAMTSL5 mRNA levels using, for example, microarray, RNA-seq, in situ hybridization, RNA-scope, as well as regular, semi-quantitative, or quantitative RT-PCRs.

[0064] According to a third aspect, the invention relates to a pharmaceutical composition. The pharmaceutical composition comprises an agent targeting ADAMTSL5 itself or ADAMTSL5 pathway and a pharmaceutically acceptable carrier for use in the treatment of a cancer. The terms "ADAMTSL5 pathway" and grammatical variations thereof, refer to a pathway wherein the ADAMTSL5 gene is involved.

[0065] The agent targeting the ADAMTSL5 pathway that is used is, in particular, blocking antibodies, peptides, sh-RNA, si-RNA, micro-RNA, antisense RNA, and chemical drugs. It also includes a demethylating agent, such as for example Decitabine, or agents modulating glycosylation and/or heparin binding. A demethylating agent, according to the invention, is a compound that leads to genomic DNA hypomethylation by inhibiting the DNA methyltransferase.

[0066] In a fourth aspect, the invention concerns a use of an ADAMTSL5 protein as a biomarker of cancer. The cancers are the same as those above-described.

[0067] In a fifth aspect, the invention relates to method for treating cancer in a mammal in a need thereof. The cancers are the same as those above-described. The method comprises a first step of diagnosing the cancer in a mammal in a need thereof, and a second step of treating the cancer by administering an inhibitor of ADAMTLS5. The step of diagnosing the cancer involves the same steps as these above-described in the method for diagnosing a cancer in a mammal in a need thereof.

[0068] In a sixth aspect, the invention relates to an in vitro method for monitoring the response to an anticancer treatment of a mammal suffering from cancer comprising determining the ADAMTSL5 level of expression in a biological sample of said mammal at two or more time points during said anticancer treatment, wherein an equal or higher ADAMTSL5 level of expression in a biological sample of the subject at a later time point, compared to a reference value obtained in a biological sample of the subject at an earlier time point, is indicative of a resistance of the subject to said anticancer treatment whereas a lower ADAMTSL5 level is indicative of a response of the subject to said anticancer treatment.

EXAMPLES

Materials and Methods

[0069] In the following examples, genetically modified mice where used. In these mice, the expression levels of Met, a receptor tyrosine kinase (RTK) activated in about 50% of human HCCs, is slightly enhanced above the endogenous level, based on a genetic approach allowing modulation of gene expression in a tissue/temporal-specific manner ($R26^{stopMet}$ mice; Fan et al. PLoS Genetics 2015; Fan et al. Hepatology 2017). It was recently demonstrated that enhanced methylation (Met) RTK expression levels in the liver perturbs tissue homeostasis, leading to tumor initiation and evolution into HCC (Alb-$R26^{Met}$). Based on comparisons of Met expression levels in Alb-$R26^{Met}$ (n=32) and human (n=249) liver tumors, it was shown that Met levels in the Alb-$R26^{Met}$ genetic setting (3.16±0.06 versus control livers) correspond to those found in about 20% of HCC patients (48/249). By analyzing 96 different genes in a panel of tumor samples (n=32), it was shown that liver tumorigenesis modelled by the Alb-$R26^{Met}$ mice corresponds to a subset of HCC patients, thus establishing the clinical relevance of the Alb-$R26^{Met}$ HCC mouse model. It was used the Alb-$R26^{Met}$ mouse model to explore the impact of DNA methylation on transcriptional switches associated with tumorigenesis. It was identified a striking enrichment in genes simultaneously hypermethylated in CpG islands (CGIs) and overexpressed. Among them, it is found ADAMTSL5.

Example 1

Hypermethylation in the Gene Body CGI of ADAMTSL5 and Overexpression of mRNA ADAMTSL5 in the Alb-$R26^{Met}$ Tumor Model Compared with Control Livers

[0070] FIG. **1**(A) shows a schematic representation of mouse ADAMTSL5 transcripts and CGIs from the UCSC genome browser, and Refseq gene annotations based on the NCBI37/mm9 mouse reference. The scheme allows visualization of exons, introns and CGIs of AdamtsL5, highlighting with a square the gene body CGI found hypermethylated in tumors.

[0071] FIG. **1**(B) is a schematic representation of the human ADAMTSL5 protein from Badel et al. Matric Biology, 2012. The different domains present in the ADAMTSL5 protein, which could be involved in the modulation of ADAMTSL5 interactions with other protein and in ADAMTSL5 biological functions, are reported.

[0072] FIGS. **1**(C) and (D) illustrate the methylation levels (β-value) of ADAMTSL5 in the promoter CGI (FIG. **1**(C)) and in the gene body CGI (FIG. **1**(D)) in control livers (n=3) and Alb-$R26^{Met}$ tumors (n=10). It is noted that the methylation levels of ADAMTSL5 in the promoter CGI is similar in control and tumor samples, whereas the methylation levels in gene body CGI are significantly higher in Alb-$R26^{Met}$ tumors compared to control livers. It is concluded that tumors are characterized by a hypermethylation of ADAMTSL5 in the gene body CGI.

[0073] FIG. **1**(E) illustrates the ADAMTSL5 mRNA expression levels in Alb-$R26^{Met}$ tumors compared to control livers by RNA-seq. The graphs show the read counts (on the right) and Log_2FC (fold-change) (on the left). Significance: *: $P<0.05$; FDR (p-value adjusted)=4.31^{E-15}. It is concluded that tumors are characterized by an overexpression of ADAMTSL5 mRNA.

[0074] FIG. **1**(F) illustrates the ADAMTSL5 expression levels (by RT-qPCR) in Alb-$R26^{Met}$ tumors (n=16) relative to control livers (n=5). It is noted high expression levels of ADAMTSL5 in Alb-$R26^{Met}$ tumors compared to control livers. It is concluded that tumors are characterized by a consistent overexpression of ADAMTSL5 mRNA.

Example 2

Overexpression of ADAMTSL5 mRNA and Protein in a Alb-R26^{Met} Tumor Model

[0075] FIG. **2**(A) shows representative images of the ADAMTSL5 mRNA expression levels (by RNA-scope) in tumors (dissected from Alb-R26^{Met} mice) and in control liver (dissected from control mice). Strong staining is observed in Alb-R26^{Met} tumors, but not in control livers. It is concluded that, similar to data from RNA-seq and RT-qPCR analyses, tumors are characterized by a consistent overexpression of ADAMTSL5 mRNA.

[0076] FIG. **2**(B) shows representative images of the ADAMTSL5 protein levels by immunofluorescence (left) and immunostaining (right) in tumors (dissected from Alb-R26^{Met} mice) and in control liver (dissected from control mice). It is noted that ADAMTSL5 is overexpressed in Alb-R26^{Met} tumors compared to control livers. It is concluded that, tumors are characterized by a consistent overexpression of ADAMTSL5 protein, coherent with high mRNA levels.

Example **3**

In Vitro Tumorigenic Properties of Alb-R26^{Met} HCC Cells, in which ADAMTSL5 is Overexpressed. *: P<0.05; **: P<0.01; ***: P<0.001

[0077] FIG. **3**(A) is a schematic representation of the establishment of Alb-R26^{Met} HCC cell lines (Fan et al. Hepatology, 2017) used for molecular and functional studies. The scheme recapitulates how HCC cell lines have been generated for their molecular characterization and for their use in biological assays. Following the protocol, it is established and reported in Fan et al. Hepatology, 2017, that HCC cell lines were generated from liver tumors dissected from different Alb-R26^{Met} mice. Briefly, dissected tumors were minced, incubated in a solution containing collagenase and DNAse, and tissue debris were removed using a 100 μm sterile filter. Cells were then cultured in William E medium with supplements either directly in adherent conditions or for a week in non-adherent conditions to enrich cells with tumorigenic properties. After expansion of cultured cells, a proportion of them was frozen and kept as stocks, whereas another proportion was characterized molecularly (biochemistry, RT-qPCR) and functionally (for their capacity to form tumors in nude mice).

[0078] FIG. **3**(B) is a graph reporting the ADAMTSL5 expression levels (by RT-qPCR) in three Alb-R26^{Met} HCC cell lines (HCC3, HCC13, and HCC14) relative to control livers. It is concluded that HCC cells are characterized by a consistent overexpression of ADAMTSL5 mRNA, as shown in tumors.

[0079] FIG. **3**(C) shows the mRNA expression levels of ADAMTSL5 in Alb-R26^{Met} HCC cells stably transfected with a plasmid carrying a shRNA targeting sequence (carrying also the puromycin gene for selection of stable clones) versus controls. Cells are transfected with the plasmid of interest, then exposed after 48 hours to puromycin for 7 days in order to select cells in which the plasmid has been stably integrated. Clones in which the expression levels of ADAMTSL5 are downregulated by the shRNA targeting sequence are identified by RT-qPCR. It is concluded that the shRNA targeting sequence against ADAMTSL5 leads to an efficient downregulation of ADAMTSL5 mRNA levels in HCC cells, which interfere with ADAMTSL5 protein expression levels.

[0080] FIG. **3**(D) illustrates results of anchorage independent growth assay (soft agar assay) performed using either HCC control cells or HCC cells carrying the shRNA sequence targeting ADAMTSL5. This assay exemplifies the capacity of cells to form colonies in non-adherent condition, therefore revealing their in vitro tumorigenic properties. Results show that the number of colonies formed by the Alb-R26^{Met} HCC cells carrying a shRNA sequence targeting ADAMTSL5 is reduced compared with control cells. It is concluded that high levels of ADAMTSL5 in HCC cells is required for their in vitro tumorigenic properties.

[0081] FIG. **3**(E) is descriptive of the anchorage independent growth assay (soft agar assay) showing partially rescue of in vitro tumorigenic properties of ADAMTSL5-targeted Alb-R26^{Met} HCC cells with condition media from control cells. The results indicate that extracellular ADAMTSL5 confers tumorigenicity to cells expressing low levels of ADAMTSL5. It is concluded that ADAMTSL5, which is a secreted protein, elicits its function in the extracellular environment and can act as well in a cell non-autonomous manner.

[0082] FIG. **3**(F) are representative images (left) and quantification (right) of tumor spheres derived from control and ADAMTSL5-targeted Alb-R26^{Met} HCC cells (right). It is noted higher numbers and size of tumor sphere generated from control cells compared with Alb-R26^{Met} HCC^{shAdamts15} cells (carrying the shRNA sequence targeting ADAMTSL5). It is conclude that high levels of ADAMTSL5 in HCC cells confers self-renewal capabilities.

Example **4**

Loss of in Vivo Tumorigenic Properties of HCC Cells Following ADAMTSL5 Downregulation in Alb-R26^{Met} HCC Cells. *: P<0.05; **: P<0.01; ***: P<0.001

[0083] FIG. **4**(A) contains images of dissected tumors from xenografts in nude mice injected either with Alb-R26^{Met} HCC cells (top) or with Alb-R26^{Met}-shADAMTSL5 HCC cells (bottom).

[0084] Xenograft studies were performed by subcutaneous injection of Alb-R26^{Met} or Alb-R26^{Met}-shAdamtsL5 HCC cells (5×10^6 cells) in the flank of nude mice. FIG. **4**(A) comprises images of dissected tumors after 8 weeks of xenografts establishment. It is noted that tumors are formed in nude mice wherein the Alb-R26^{Met} HCC cells were subcutaneous injected, whereas tumors from mice wherein Alb-R26^{Met}-shAD shADAMTSL5 HCC cells were subcutaneous injected are drastically reduced, illustrating impaired cell tumorigenic properties in vivo.

[0085] FIG. **4**(B) contains xenograft growth curves reporting the mean tumor volume per group measured every week. It is concluded that downregulation of ADAMTSL5 levels in HCC cells (achieved by stable transfection of a shRNA targeting sequence) interferes with in vivo tumorigenic properties of HCC cells. Collectively, these results show that downregulation of ADAMTSL5 expression levels interfere with tumor establishment/evolution.

[0086] In FIG. **4**(C), it is shown a quantitative analysis of the volume of tumors dissected 8 weeks after cell injection.

Each dot corresponds to the volume of each tumor. It is concluded that downregulation of AdamtsL5 levels in HCC cells (achieved by stable transfection of a shRNA targeting sequence) interferes with in vivo tumorigenic properties.

Example 5

Acquisition of In Vivo Tumorigenic Properties Following ADAMTSL5 Overexpression by Immorto-R26^{Met} Sensitized Hepatocytes

[0087] FIG. **5**(A) is a schematic representation of the establishment of immorto-R26^{Met} sensitized hepatocytes (embryonic hepatocytes carrying increased levels of the Met RTK and immortalized with the SV40 large-T antigen). Briefly, cultured hepatocytes from E15.5 mouse R26^{Met} embryonic livers were infected with a retrovirus carrying the SV40 large-T antigen for immortalization (plus the neomycin gene for selection of stable clones), then subsequently treated for 7 days with a media permissive for hepatocytes to deplete other cell types. The immorto-R26^{Met} hepatocytes are sensitized because of 3-folds increased of wild-type Met levels, although not tumorigenic as incompetent to form tumors in xenografts.

[0088] FIG. **5**(B) comprises images of mice after 11 weeks of xenografts establishment. It is noted that nude mice wherein subcutaneous injection of immorto-R26^{Met} hep^{over_Adams15} (cells overexpressing ADAMTSL5) develop tumors in both flanks.

[0089] Xenograft studies were performed by subcutaneous injection of immorto-R26^{Met} control (immorto-R26^{Met} hepa^{WT}) or overexpressing ADAMTSL5 ($_{immorto\text{-}R}$26^{Met} hepa^{overAdamts15}) hepatocytes (5×10^6 cells) in both flanks of nude mice.

[0090] FIG. **5**(C) contains xenograft growth curves reporting the mean tumor volume per group measured every week.

[0091] In FIG. **5**(D), it is shown a quantitative analysis of the volume of tumors dissected 11 weeks after cell injection. Each dot corresponds to the volume of each tumor. It is noted that all mice injected with immorto-R26^{Met} hepa^{over_Adamts15} formed tumors in contrast to mice injected with immorto-R26^{Met} hepa^{WT}. It is concluded that overexpression of ADAMTSL5 levels in immorto-hepatocytes, which are not tumorigenic as incompetent to form tumors in xenografts, is sufficient to confer to them in vivo cell tumorigenic properties.

Example **6**

ADAMTSL5 Expression Level in Alb-R26^{Met} Tumors at Early and Latest Stages

[0092] FIG. **6**(A) is a schematic representation of the tissue samples used for RT-qPCR analysis of ADAMTSL5 levels. The samples used are: control wild-type livers (WT), Alb-R26^{Met} healthy livers, Alb-R26^{Met} tumors at early and advanced stages.

[0093] In FIG. **6**(B) to FIG. **6**(E), the graphs report the expression levels (by RT-qPCR) of ADAMTSL5, AFP (alpha-fetoprotein), GPC3 (Glypican-3) (two HCC markers), and Ki67 (a proliferative marker), respectively. It is noted comparable low expression levels of ADAMTSL5 as well as for the other analyzed markers in wild-type and Alb-R26^{Met} healthy livers. In contrast, transcript levels are already increased in Alb-R26^{Met} tumors at early stages. It is concluded that ADAMTSL5 transcript levels can be used to discriminate healthy from neoplastic samples already at early tumorigenic state.

Example **7**

High ADAMTSL5 mRNA Levels are Present in 52% of HCC Patients, with a Predominance in those Associated to Alcohol Taken

[0094] In FIG. **7**(A), the cohort of HCC patients (371 patients) with ADAMTSL5 mRNA levels is reported: 21% are characterized by ADAMTSL5 downregulation (white, left), 27% by no changes (white with black lines, center), and 52% of HCC patients with upregulation of ADAMTSL5 mRNA levels (black, right). FIG. **7**(B) is a graph reporting the three subgroup of HCC patients according to low, unchanged, and high ADAMTSL5 expression levels (numbers and percentages are indicated). Data correspond to RNA-seq studies of a cohort of 371 HCC patients (from TCGA). Note that ADAMTSL5 is overexpressed in 193 out of 371 HCC patients (52%; $Log_2FC>1$; $FDR<0.05$). It is concluded that high ADAMTSL5 transcript levels can be detected in more than 50% of HCC patients.

[0095] In FIG. **7**(C), the table reports the presence (black line) or the absence of major HCC risk factors in all 371 analyzed patients. Note that HCC patients with increased ADAMTSL5 levels are significantly associated, although not exclusively, to alcohol taken. It is concluded that high mRNA levels of ADAMTSL5 is present in a large proportion of patients characterized by several risk factors, with a predominance for those associated with alcohol taken.

Example **8**

ADAMTSL5 Protein Levels are Overexpressed in a Vast Majority of HCC Analyzed Patients

[0096] The figure reports immunostaining with anti-ADAMTSL5 antibodies in tumor samples compared to the adjacent liver. Note strong ADAMTSL5 protein levels (dark staining; by Fast Red) in all analyzed HCC patients (except #8 patient). It is concluded that high ADAMTSL5 protein levels characterize a vast majority of HCC patients.

Example **9**

ADAMTSL5 Overexpression in a Panel of Human Cancer Cell Lines

[0097] The graph shows the mRNA expression levels of ADAMTSL5 in a panel of human HCC cell lines, in MKN (gastric cancer) cell line, in human breast cell lines, in HELA (human cervix) cancer cell line, and in HEK (human embryonic kidney) cell line. Mean value of three independent experiments. It is concluded that high mRNA levels of ADAMTSL5 are present in cancer cells with different origin.

1. A method for diagnosing an hepatocellular carcinoma in a mammal in a need thereof, comprising:

collecting a biological sample from the mammal;

determining, from the biological sample, if the ADAMTSL5 gene is overexpressed;

diagnosing an hepatocellular carcinoma from the determination of the overexpression of the gene.

2. The method according to claim 1, wherein the biological sample is selected from the group consisting of blood, biopsy tissue, blood serum, blood plasma, urine, stool, sputum, cerebrospinal fluid, and supernatant from cell lysate.

3. The method according to claim 1, wherein the overexpression of ADAMTSL5 gene in the biological sample is determined by measuring the ADAMTSL5 protein levels or mRNA levels in the biological sample.

4. The method according to claim 3, wherein the ADAMTSL5 protein levels in the biological sample are measured by adding at least one antibody anti-ADAMTSL5 type to the biological sample.

5. The method according to claim 4, wherein the antibody anti-ADAMTSL5 type is selected from the group consisting of uncoupled, coupled with alkaline phosphatase horse-radish peroxidase, and coupled with fluorescent dyes.

6. The method according to claim 3, wherein the ADAMTSL5 protein levels in the biological sample are measured by using immunostaining, immunofluorescence, western blot, or ELISA.

7. A kit for diagnosing an hepatocellular carcinoma in a mammal in a need thereof comprising:

at least one antibody anti-ADAMTSL5 type,

a container for holding the biological sample, and

a protocol for measuring an overexpression of the ADAMTSL5 hepatocellular carcinoma marker gene, in a biological sample obtained from the mammal.

8. The kit according to claim 7, wherein the antibody anti-ADAMTSL5 type is selected from the group consisting of uncoupled, coupled/conjugated with alkaline phosphatase, coupled/conjugated with horse-radish peroxidase, and coupled/conjugated with fluorescent dyes.

9. The method according to claim 3, wherein the ADAMTSL5 mRNA levels in the biological sample are measured by at least one methodology selected from the group consisting of microarray, RNA-seq, in situ hybridization, RNA-scope, and regular, semi-quantitative, or quantitative RT-PCRs.

10. A pharmaceutical composition comprising:

an agent or agents targeting ADAMTSL5 or the ADAMTSL5 pathway, and

a pharmaceutically acceptable carrier,

wherein the composition is adapted for the treatment of an hepatocellular carcinoma,

wherein the agent or agents targeting ADAMTSL5 or the ADAMTSL5 pathway is or are selected from the group consisting of blocking antibodies, peptides, sh-RNA, si-RNA, micro-RNA, antisense RNA, chemical drugs, demethylating agents, agents modulating glycosylation, and agents modulating heparin binding.

11. (canceled)

12. An in vitro method for monitoring the response to an anticancer treatment of a mammal suffering from hepatocellular carcinoma comprising:

determining the ADAMTSL5 level of expression in a biological sample of said mammal at two or more time points during the anticancer treatment,

wherein an equal or higher ADAMTSL5 level of expression in a biological sample of the subject at a later time point, compared to a reference value obtained in a biological sample of the subject at an earlier time point, is indicative of a resistance of the subject to the anticancer treatment whereas a lower ADAMTSL5 level is indicative of a response of the subject to the anticancer treatment.

13. The method according to claim 2, wherein the biological sample is tissue biopsy, blood, blood plasma, blood serum, or urine.

14. The method according to claim 2, wherein the overexpression of ADAMTSL5 gene in the biological sample is determined by measuring the ADAMTSL5 protein levels or mRNA levels in the biological sample.

15. The method according to claim 14, wherein the ADAMTSL5 protein levels in the biological sample are measured by adding at least one antibody anti-ADAMTSL5 type to the biological sample.

16. The method according to claim 15, wherein the antibody anti-ADAMTSL5 type is selected from the group consisting of uncoupled, coupled with alkaline phosphatase horse-radish peroxidase, and coupled with fluorescent dyes.

17. The method according to claim 4, wherein the ADAMTSL5 protein levels in the biological sample are measured by using immunostaining, immunofluorescence, western blot, or ELISA.

18. The method according to claim 5, wherein the ADAMTSL5 protein levels in the biological sample are measured by using immunostaining, immunofluorescence, western blot, or ELISA.

19. The method according to claim 14, wherein the ADAMTSL5 protein levels in the biological sample are measured by using immunostaining, immunofluorescence, western blot, or ELISA.

20. The method according to claim 15, wherein the ADAMTSL5 protein levels in the biological sample are measured by using immunostaining, immunofluorescence, western blot, or ELISA.

21. The method according to claim 16, wherein the ADAMTSL5 protein levels in the biological sample are measured by using immunostaining, immunofluorescence, western blot, or ELISA.

* * * * *