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(54) **Title:** USE OF GLYPICAN-3-TARGETING MICRORNAS FOR TREATING LIVER CANCER

(57) **Abstract:** The present invention relates to miRNAs targeting Glypican-3 for use in the treatment of liver in particular hepatocellular carcinoma and hepatoblastoma.



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Use of Glypican-3-targeting microRNAs for treating liver cancer

Field of the Invention

The present invention relates to the field of oncology. In particular, it provides miRNAs useful for
5 detecting and/or treating cancer.

Background of the Invention

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and is one of the most frequent tumors worldwide (7% of all cancers and 750,000 new cases by year worldwide). It is the second highest
10 cause of mortality from any type of cancerous malignancies worldwide and the most deadly cancer in terms of increased mortality in US during the last two decades. Most cases of HCC generally develop on a cirrhotic liver, resulting from a chronic viral hepatitis infection (HBV, HCV) or some metabolic diseases. The usual outcome is very poor with solely 7% of 10- year survival.

Another type of liver cancer is Hepatoblastoma (HBL) which is an uncommon malignant liver neoplasm
15 occurring in infants and children (1% of pediatric cancers, 0.02% of all cancers and around 3,500 new cases by year worldwide) with a 10-year survival of 61%.

Several therapeutic strategies are available, the most effective being the surgical resection of the tumor or liver transplantation. However, the surgical resection or liver transplantation is possible only in about 30% of HCC and 70% of HBL.

Therefore, many new strategies are under development. For instance, numerous teams around the world keep trying to identify miRNAs which could be used for treating liver cancers. At this time, only a single one reaches the phase I of a clinical trial. More particularly, this miRNA called miR-34a (recently renamed miR-34a-5p in the last version of miRBase) is currently tested in unresectable primary liver cancer or solid cancers with liver metastasis. miR-34a regulates several key oncogenic targets including
25 CTNNB1, BCL2, E2F3, HDAC1, MET, MAK1, CDK4/6, PDGFR- α , WINT1/3 and NOTCH-1 (Bader, Front. Genet., **120**, 1-9).

A therapeutic solution remains urgently needed for these two cancers, in particular for patients who are unable to benefit from treatment by surgery or liver transplantation, fail to properly respond to first-line treatments, already have unresectable metastasis or relapse.

Summary of the Invention

The inventors identified 5 new miRNAs decreasing the level of the oncoprotein glypican-3 (GPC-3) in liver cancer cells. These miRNAs are down-regulated in liver tumors and are capable of inhibiting HCC cell growth and inducing HCC cell cycle arrest. Some of them are even capable of inducing tumor cell
35 apoptosis. They especially showed that *in vitro*, miR-4510 is more effective than miR-34a (the miRNA currently tested in clinical trials for the treatment of patients with liver cancer or liver tumor involvement) for blocking the growth of HCC and HBL cells and for inducing their apoptosis.

The present invention relates to a molecule selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or any combination thereof or a DNA or RNA encoding for said miRNA for use for treating a liver cancer. The present also relates to the use of a molecule selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or any combination thereof or a DNA or RNA encoding for said miRNA for the manufacture of a medicament for treating a liver cancer. It further relates to a method for treating a liver cancer in a subject, comprising administering a therapeutically effective amount of a molecule selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or any combination thereof or a DNA or RNA encoding for said miRNA to said patient.

The liver cancer can be a hepatocellular carcinoma. Alternatively, the liver cancer can be a hepatoblastoma.

Optionally, the molecule is to be used in combination with one or more therapeutic agents, preferably another antitumor therapy, and in particular with sorafenib, doxorubicin, cisplatin, 5-Fluoro-uracil, gemcitabine, oxaliplatin, mitomycin C, tamoxifen, MSC2156119J, foretinib, refametinib, cabozantinib, tivantinib, or any combination thereof, preferably with doxorubicin, cisplatin, gemcitabine, oxaliplatin, mitomycin C, tamoxifen, sorafenib or any combination thereof. In a preferred embodiment, the molecule is to be used in combination with sorafenib, in particular for use in the treatment of HCC. In another preferred embodiment, the molecule is to be used in combination with cisplatin and/or doxorubicin, in particular for use in the treatment of HBL. Optionally, the molecule is to be used in combination with another antitumor therapy and a drug lowering the toxicity and side effects of the antitumor therapy. For instance, drug lowering the toxicity and side effects of the antitumor therapy can be sodium thiosulfate or N-acetyl cysteine. Optionally, the molecule is to be used in combination with one or more immunotherapeutic agents, and in particular with monoclonal antibodies binding antigens on cancer cells or targeting immune system checkpoints (e.g. immune checkpoint inhibitors) and especially drugs targeting PD-1 or PD-L1 such as for example pembrolizumab, nivolumab, atezolizumab. Optionally, the molecule is to be used in combination with resection, radiofrequency ablation and/or percutaneous ethanol injection. Optionally, the molecule is to be used after or before resection, radiofrequency ablation and/or percutaneous ethanol injection. Optionally, the molecule is for use as neo-adjuvant therapy or adjuvant therapy.

Optionally, the subject has liver metastasis, and/or does not respond to the first line treatment and/or is not suitable for tumor resection or ablation. Optionally, the subject has a miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or combination thereof which is under-expressed in comparison with a healthy or non-tumoral control.

Optionally, the subject has liver tumors or liver metastasis expressing the glypican-3 oncoprotein, called GPC-3.

The present invention further relates to a method for selecting a subject suitable for a treatment by miRNA as disclosed herein comprising determining the level of a miRNA selected from the group

consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or of a combination thereof in a biological sample from the subject, and selecting the subject if at least one of the miRNA is under-expressed in comparison with a healthy or non-tumoral control.

The present invention further relates to a method for selecting a subject suitable for a treatment by miRNA as disclosed herein comprising determining the level of GPC3 thereof in a biological sample from the subject, and selecting the subject if GPC3 is upper-expressed or overexpressed in comparison with a healthy or non-tumoral control.

The present invention also relates to the use of a miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or of any combination thereof as a marker for detecting a liver cancer or a susceptibility to develop a liver cancer. Preferably, the liver cancer is a hepatocellular carcinoma or a hepatoblasma.

The present invention relates to a method for detecting a liver cancer or a susceptibility to develop a liver cancer in a subject, comprising determining the level of a miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or of any combination thereof in a biological sample from the subject, an under-expression of at least one of the miRNA in comparison with a healthy or non-tumoral control being indicative of a liver cancer or a susceptibility to develop a liver cancer. Preferably, the liver cancer can be a hepatocellular carcinoma.

The present invention also relates to the use of a miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or of any combination thereof as a marker for the prognosis in a subject having a liver cancer, preferably a hepatocellular carcinoma. The present invention also relates to a method for determining the prognosis in a subject having a liver cancer, preferably a hepatocellular carcinoma, comprising determining the level of a miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or of a combination thereof in a biological sample from the subject, the level of expression of said at least one of the miRNA being correlated with the clinical prognosis. More preferably, the miRNA is selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, and any combination thereof.

Finally, the present invention relates to a kit for detecting a liver cancer or a susceptibility to develop a liver cancer in a subject or for selecting a subject suitable for a treatment by a molecule as disclosed herein or for determining the prognosis in a subject having a liver cancer, the kit comprising detection means specific for at least one miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or for any combination thereof. It relates to the use of the kit for detecting a liver cancer or a susceptibility to develop a liver cancer in a subject or for selecting a subject suitable for a treatment by a miRNA as disclosed herein or for determining the prognosis in a subject having a liver cancer. Preferably, the liver cancer is a hepatocellular carcinoma.

Brief Description of the Drawings

Figure 1: Ten new miRNAs regulate GPC3 expression.

Figure 1 A: Relative expression of total GPC3 protein in Huh7 cells following transfection with small RNAs. Bars show control (black), si-GPC3 (white), positive control miRNAs (in dark grey) and tested miRNAs (light grey). Bars represent means + SEM (n=5, ANOVA $p<0.0001$). Western blots on the top show representative results obtained with control RNAs (left) and ineffective miRNAs (right). The bottom blots show miRNAs inhibiting (left) or increasing (right) the amount of GPC3. Protein size is shown in brackets on the left of the blot. All cropped blots retained at least 6 bandwidths above and below the bands.

Figure 1B: Relative expression of membrane-anchored GPC3 protein in Huh7 cells transfected with the indicated small RNAs. Results are shown as Mean Fluorescence Intensity (MFI) ratios. Bars represent means + SEM (n=4, ANOVA $p<0.0001$)

Figure 1C: Relative expression of *GPC3* mRNA in Huh7 cells following small RNA transfection. Bars represent means + SEM (n=3, ANOVA $p<0.0001$). The ANOVA test was followed by a multiple comparison post-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 2: Relative expression of 5 GPC3-regulating miRNAs in 19 NTL (Non-tumoral liver) and 98 HCC samples. Non-parametric Mann-Whitney test for unpaired samples: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 3: Expression ratio of 5 GPC3-regulating miRNAs in HCC in each 19 pairs of tumor and adjacent non-tumoral liver. Results are presented as HCC/NTL expression ratios. The median is shown as a full line and the reference ratio value “1” is shown as a dotted line. The statistical analyses were done with the Wilcoxon matched-pairs signed rank test: * $p<0.05$; *** $p<0.001$.

Figure 4: Expression of miR-4510 and miR-548aa in HCC tumors with a good or poor prognosis (see p-value above the box and whiskers graph).

Figure 5A: Cell growth of Huh7 cells transfected with miRNAs, si-GPC3 or controls was assessed by Sulforhodamine B colorimetric assay (Abs 492) at the indicated time points. Results are presented as mean +/- SEM (n=5, ANOVA $p<0.0001$).

Figure 5B: Three days after Huh7 cells transfection, cell proliferation was determined by cell counting.

Figure 6: Growth of Huh7 HCC cells at day 6 following transfection by the corresponding small RNAs. ANOVA test: *** $p<0.0001$; Holm-Sidak's multiple comparisons test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 7: Apoptosis of Huh7 cells was determined by annexin/7-ADD staining at day 3 following transfection by the corresponding small RNAs. Bars represent means + SEM (n=3, ANOVA test: *** $p<0.0001$; Holm-Sidak's multiple comparisons test: ** $p<0.01$; *** $p<0.001$).

Figure 8: Apoptosis of Huh7 cells determined by caspase 3/7 activity was measured by a luminescent assay. Bars represent means + SEM (n=3, ANOVA $p<0.0001$). Holm-Sidak's multiple comparisons test: *** $p<0.001$.

Figure 9: Cycling of Huh7 cells at day 3 following transfection by the corresponding small RNAs. ANOVA test: *** $p < 0.0001$; Holm-Sidak's multiple comparisons test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 10: (Figure 10A) Six days after transfection, the effect of miR-4510 and miR-34a-5p on the growth of Huh7, Hep3B, Huh6 and HepG2 cells was compared as described in Fig. 5. Bars represent means + SEM ($n=4$, ANOVA $p < 0.01$). (Figure 10B) Three days after transfection, the effect of miR-4510 and miR-34a-5p on the apoptosis of the indicated cell lines was compared by Annexin V/7-ADD staining. Bars represent means + SEM ($n=3$, ANOVA $p < 0.01$). Holm-Sidak's multiple comparisons test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (Figure 10C) Apoptosis was assessed in Huh7 and Huh6 cells by Annexin V/7-ADD 3 days after transfection with miR-4510 or Ctrl in presence or absence of sorafenib (S, left panel) or cisplatin (C, right panel), respectively. Bars represent means + SEM ($n=3$, ANOVA $p < 0.0001$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 11: Number of Huh7 cells at day 6 following transfection by the corresponding small RNAs. "si-ctl": control RNA; "AM": anti-miRNA; "miR": miR-4510. ANOVA test: *** $p < 0.0001$; Holm-Sidak's multiple comparisons test: * $p < 0.05$; *** $p < 0.001$.

Figure 12: Apoptosis of Huh7 cells was determined by Annexin V/7-ADD staining at day 3 following transfection by the corresponding small RNAs and/or incubation with Sorafenib. "siCtl": control RNA; "AM": anti-miRNA. ANOVA test: *** $p < 0.0001$; Holm-Sidak's multiple comparisons test: * $p < 0.05$; *** $p < 0.001$.

Figure 13: Apoptosis of Hep3B HCC cells at day 3 following transfection by the corresponding small RNAs. "si-ctl": control RNA; "AM": anti-miRNA; "miR": miR-4510. ANOVA test: *** $p < 0.0001$; Holm-Sidak's multiple comparisons test: *** $p < 0.001$.

Figure 14: Apoptosis of HuH6 HBL cells at day 3 following transfection by the corresponding small RNAs. "si-ctl": control RNA; "AM": anti-miRNA; "miR": miR-4510. ANOVA test: *** $p < 0.0001$; Holm-Sidak's multiple comparisons test: *** $p < 0.001$.

Figure 15. Number of Huh7 cells 4 days after transfection with miR-4510 or a control RNA (Ctrl) and treatment or not with 0.1 $\mu\text{g/mL}$ of doxorubicin (D). Bars represent means + SEM of triplicate data ($n=1$)

Figure 16: Effect of control RNA (Ctrl) and miR-4510 on gene targets and Wnt/ β -catenin pathway in Huh7 cells 24hr after transfection. The expression of several targets of miR-4510 (Figure16A) and effectors of the TGF- β 1/SMAD3 (Figure16B) and Wnt/ β -catenin pathways (Figure16C, left panel) was assessed by Western blotting (one representative blot of 3 independent experiments is shown). Protein size is shown in brackets on the left of the blot. All cropped blots retained at least 6 bandwidths above and below the bands. The transcriptional activity of β -catenin was measured by TOPflash/FOPflash assay 72hr after transfection (Figure16C, right panel). Bars represent means + SEM ($n=3$, ANOVA $p < 0.0001$).

Figure 17: Kinetic growth of HBL-derived HuH6 cells following transfection by the corresponding small RNAs. ANOVA test: *** $p < 0.0001$; Holm-Sidak's multiple comparisons test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 18: Growth of Huh7 cells transfected by a control RNA (siCtrl) or miR-4510 or not transfected (NT) at days 3 and 6 on the chick chorioallantoic membrane following transfection by the corresponding small RNAs. (A) Representative pictures of tumors grown on the chick chorioallantoic membrane at D3 and D6. (B) Percentage of tumors with bleeding in each experimental group at D3 (on the left) and at D6 (on the right). The number of tumors analyzed is depicted in brackets in each bar. ANOVA test: *** $p < 0.0001$; Holm-Sidak's multiple comparisons test: * $p < 0.05$; ** $p < 0.01$.

Figure 19: Relative expression of miR-4510 in HCC subgroups. Data are presented as box and whiskers with minimal and maximal values (ANOVA $p < 0.0001$). NTL: Non-tumoral liver ($n=19$). HCC: Hepatocellular Carcinomas ($n=98$). Dunnett's multiple comparisons test. ** $p < 0.01$, *** $p < 0.001$.

Figure 20: Relative expression of miR-4510 in 24 pairs of HBL and adjacent normal liver samples. Results are presented as HBL/NTL expression ratios. The median is shown as a full line and the reference ratio value "1" is shown as a dotted line. Two-tailed Wilcoxon matched-pairs signed ranked test. *** $p < 0.001$.

Figure 21: Assessment of miR-4510 regulatory activity following encapsulation in stable nucleic acid lipid particles (SNALPs). The SNALPs used is a combination of a KAUDO nucleolipid and a dioleoylphosphatidylethanolamine (DOPE) lipid. Huh7 cells expressing the reference Tomato transgene and the test eGFP transgene carrying the wild-type *GPC-3* 3'-untranslated region were incubated with miR-4510 or a control RNA encapsulated in SNALPs. Three days later, the red and green fluorescence signals were measured and the ratios were calculated using the Dual Fluorescence-FunREG system. As positive control, TGG Huh7 cells were transfected with lipofectamine and 15nM of miR-4510 or control RNA. Bars represent means + SEM of triplicate values. Two independent experiments are shown.

Figure 22: Inverse correlation between *GPC3* mRNA and miR-4510 expressions in HCC samples. Spearman r correlation = -0.3243, *** $p < 0.001$.

Figure 23: MiR-4510 inhibits HCC tumor development in vivo.

(A) Twenty-four hours after transfection with miR-4510 or Ctrl miRNA, Huh7 cells were collected and grafted on the chicken chorioallantoic membrane (CAM). Tumor growth was monitored from day 1 to day 6. Tissue fixation was done at day 3 and day 6. (B) Photographs of tumors (top panels) and hematoxylin and eosin (H&E) staining (bottom panels) were performed 3 and 6 days after cells implantation. (C) Hematoxylin and eosin (H&E), Ki67 and activated Caspase-3-staining was performed on sections of tumors treated with miR-4510 or Ctrl. Magnification scale bars are as indicated on each microscopic image. (D) The number of CAMs with tumor presenting or not bleeding in Ctrl versus miR-4510 at Day 3 (left panel) and Day 6 (right panel) is shown as bars. Two-sided Fisher's exact test, * $p < 0.05$, ** $p < 0.01$.

Detailed Description of the Invention

The present invention relates to the identification of miRNAs decreasing the level of glypican-3 (GPC-3) expression which are useful for the treatment of liver cancer and/or for diagnosis of liver cancer and/or for prognosis of liver cancer.

Definition

Glypican-3 (GPC-3) is described in Uniprot under ID P51654 and has a Reference Sequence of mRNA NM_001164617 and a Reference Sequence of protein NP_001158089.

The term “identity” refers to a relationship between the sequences of two or more nucleic acid molecules, as determined by comparing the sequences. In the art, “identity” also means the degree of sequence relatedness between amino acid or nucleic acid molecule sequences, as the case may be, as determined by the match between strings of nucleotide or amino acid sequences. “Identity” measures the percent of identical matches between two or more sequences with gap alignments addressed by a particular mathematical model or computer programs (i.e., “algorithms”).

Identity of related nucleic acid molecules can be readily calculated by known methods, including but not limited to those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48:1073 (1988).

Non-limiting methods for determining identity are designed to give the largest match between the sequences tested. Methods to determine identity are codified in publicly available computer programs.

Preferred computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux, et al., Nucleic Acids Research 12:387 [1984]; Genetics Computer Group, University of Wisconsin, Madison, Wis.), BLASTN, and FASTA (Atschul et al., J. Molec. Biol. 215:403-410 [1990]). The BLAST X program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (BLAST Manual, Altschul et al., NCB NLM NIH Bethesda, Md. 20894; Altschul et al., J. Mol. Biol. 215:403-410 [1990]). The well-known Smith Waterman algorithm may also be used to determine identity.

Exemplary parameters for nucleic acid molecule sequence comparison include the following: Algorithm: Needleman and Wunsch, J. Mol Biol. 48:443-453 (1970); Comparison matrix: matches=+10, mismatch=0; Gap Penalty: 50; Gap Length Penalty: 3. The GAP program is also useful with the above parameters. The aforementioned parameters are the default parameters for nucleic acid molecule comparisons.

Other exemplary algorithms, gap opening penalties, gap extension penalties, comparison matrices, thresholds of similarity, etc. can be used by those of skill in the art, including those set forth in the Program Manual, Wisconsin Package, Version 9, September 1997. The particular choices to be made will depend on the specific comparison to be made, such as DNA to DNA or RNA to DNA; and additionally, whether the comparison is between given pairs of sequences (in which case GAP or BestFit are generally preferred) or between one sequence and a large database of sequences (in which case FASTA or BLASTA are preferred).

Identified miRNAs

10 *hsa-miR-4510*

The inventors identified miR-4510 as a therapeutic agent against cancer, especially liver cancer.

Mature sequence of miR-4510: U**GAGGGAGU**AGGAUGUAUGGUU (MIMAT0019047) (SEQ ID No 1)

Stem-loop pre-miRNA of miR-4510:

15 GUGUAUGU**GAGGGAGU**AGGAUGUAUGGUUGUUAGAUAGACAACUACAAUCUUUUCUC
ACAACAGACAG (MI0016876) (SEQ ID No 2)

The seed sequence of miR-4510 is encompassed in the sequence shown in bold highlighting. The mature miR-4510 is underlined.

hsa-miR-548aa

20 The inventors identified miR-548aa as a therapeutic agent against cancer, especially liver cancer.

Mature sequence of miR-548aa: A**AAAACCACA**AUUACUUUUGCACCA (MIMAT0018447) (SEQ ID No 3)

Stem-loop pre-miRNA of miR-548aa:

CUUUAUUAGUCUGGUGCAAAAGAAACUGUGGUUUUUGCCAUUACUUUACAGGCAAA
25 AACCACAAUUACUUUUGCACCAACCUAUAUAACUUGUUU (MI0016689) (SEQ ID No 4)

or

UUUUAUUAGGUUGGUGCAAAAGAAACUGUGGUUUUUGCCAUUACUUCAAUGGC**AAA**
AACCACAAUUACUUUUGCACCAACCUAUAUCUCCUCUC (MI0016690) (SEQ ID No 5)

30 The seed sequence of miR-548aa is encompassed in the sequence shown in bold highlighting. The mature miR-548aa is underlined.

hsa-miR-548v

The inventors identified miR-548v as a therapeutic agent against cancer, especially liver cancer.

Mature sequence of miR-548v: A**GCUACAGU**UACUUUUGCACCA (MIMAT0015020) (SEQ ID No 6)

35 Stem-loop pre-miRNA of miR-548v:

AAUACUAGGUUUGAGCAAAAGUAAUUGCGGUUUUGCCAUCAUGCCAAA**AGCUACAGU**
UACUUUUGCACCAGCCUAAUAUU (MI0014174) (SEQ ID No 7)

The seed sequence of miR-548v is encompassed in the sequence shown in bold highlighting. The mature miR-548v is underlined.

hsa-miR-376b-3p

The inventors identified miR-376b-3p as a therapeutic agent against cancer, especially liver cancer.

- 5 Mature sequence of miR-376b-3p: **AUCAUAGAGG**AAAAUCCAUGUU (MIMAT0002172) (SEQ ID No 8)

Stem-loop pre-miRNA of 376b-3p:

- 10 CAGUCCUUCUUUGGUAUUUAAAACGUGGAUAUCCUUCUAUGUUUACGUGAUUCCUG
GUUAA**AUCAUAGAGG**AAAAUCCAUGUUUUCAGUAUCAAAGCUG (MI0002466) (SEQ ID No 9)

The seed sequence of miR-376b-3p is encompassed in the sequence shown in bold highlighting. The mature miR-376b-3p is underlined.

hsa-miR-203a-3p

The inventors identified miR-203a-3p as a therapeutic agent against cancer, especially liver cancer.

- 15 Mature sequence of miR-203a-3p: **GUGAAAUGUU**UAGGACCACUAG (MIMAT0000264) (SEQ ID No 10)

Stem-loop pre-miRNA of 203a-3p:

- 20 GUGUUGGGGACUCGCGCGCUGGGUCCAGUGGUUCUUAACAGUUCAACAGUUCUGUAGC
GCAAUUG**GUGAAAUGUU**UAGGACCACUAGACCCGGCGGGCGCGGCGACAGCGA
(MI0000283) (SEQ ID No 11)

The seed sequence of miR-203a-3p is encompassed in the sequence shown in bold highlighting. The mature miR-203a-3p is underlined.

The Accession numbers MI and MIMAT make reference to the miRBASE (www.mirbase.org/).

- 25 The microRNAs are well-known in the art and a person skilled in the art would understand that they include the conventional naturally occurring sequences (provided herein) but also any chemically modified versions and sequence homologues thereof. Chemically modified versions and sequence homologues of miRNAs are generally called miRNA mimic, analog or derivative. The miRNA mimic, analog or derivative has retained or enhanced activity of the original miRNA.

- 30 In one embodiment, the miRNA can be mature miRNA, precursor (pre)-miRNA, primary (pri)-miRNA, a miRNA mimic, analog or derivative thereof. As used herein, the prefix “hsa” indicates Homo sapiens or human. Even in its absence, all miRNA of the invention are human. Similarly, “miRNA” and “microRNA” can be identical and are substitutable.

- 35 The miRNA is a single-stranded nucleic acid molecule, especially a RNA molecule, of no more than 30 nucleotides in length, preferably no more than 25 nucleotides in length, and generally about 21-23 nucleotides in length. It comprises a sequence which is identical or substantially identical to the seed sequence. By “substantially identical” is meant that at most 1 or 2 substitutions or deletions are allowed. In a preferred embodiment, it comprises a sequence identical to the seed sequence. The seed sequence

usually corresponds to a sequence located between position 2 and position 9 of the mature miRNA. For instance, the seed sequence may consist in the sequence between position 2 and position 7, 8 or 9 of the mature miRNA.

Preferably, the miRNA comprises, essentially consists in or consists in a sequence which is at least 80%, 85%, 90%, 95% or 99% identical to the respective full length sequence of the mature miRNA. In particular, the mature miRNA sequence is selected in the group consisting of SEQ ID Nos 1, 3, 6, 8 and 10, preferably selected in the group consisting of SEQ ID Nos 1, 3, 6, and 8, preferably consisting of SEQ ID No 1. Optionally, the miRNA comprises, essentially consists in or consists in a sequence which is at least 80%, 85%, 90%, 95% or 99% identical to the respective full length sequence of the mature miRNA and comprises a sequence identical to the seed sequence.

The miRNAs as pre-miRNA, a precursor of mature miRNA, has a stem-loop sequence and comprises a guide strand comprising the mature miRNA, and more specifically the seed sequence, and a passenger strand which is complementary or substantially complementary to the seed sequence of the guide strand. Optionally, an alternative miRNA can be a double-stranded molecule comprising two separate strands as defined before instead of the stem-loop structure.

More particularly, the guide strand comprises a sequence which is identical or substantially identical to the seed sequence. By “substantially identical” is meant that at most 1 or 2 substitutions or deletions are allowed. Preferably, the guide strand comprises a sequence which is at least 80%, 85%, 90%, 95% or 99% identical to the respective full length sequence of the mature miRNA. In particular, the mature miRNA sequence is selected in the group consisting of SEQ ID Nos 1, 3, 6, 8 and 10, preferably selected in the group consisting of SEQ ID Nos 1, 3, 6, and 8, preferably consisting of SEQ ID No 1. Optionally, the guide strand of the miRNA comprises a sequence which is at least 80%, 85%, 90%, 95% or 99% identical to the guide strand of pre-miRNA as disclosed in a sequence selected in the group consisting of SEQ ID Nos 2, 4-5, 7, 9 and 11, preferably selected in the group consisting of SEQ ID Nos 2, 4-5, 7, and 9, preferably consisting of SEQ ID No 2. In a particular embodiment, the guide strand of the miRNA comprises, essentially consists in or consists in a sequence of the guide strand of pre-miRNA as disclosed in a sequence selected in the group consisting of SEQ ID Nos 2, 4-5, 7, 9 and 11, preferably selected in the group consisting of SEQ ID Nos 2, 4-5, 7, and 9, preferably consisting of SEQ ID No 2. By “substantially complementary” is intended that at most 1 or 2 mismatches and/or deletions are allowed. Preferably, the passenger strand comprises a sequence which is at least 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% identical to the complement of the respective full length sequence of the mature miRNA. In particular, the mature miRNA sequence is selected among the SEQ ID Nos 1, 3, 6, 8 and 10, preferably selected among the SEQ ID Nos 1, 3, 6, and 8, preferably consisting of SEQ ID No 1. Optionally, the passenger strand of the miRNA comprises a sequence which is at least 80%, 85%, 90%, 95% or 99% identical to the passenger strand of pre-miRNA as disclosed in a sequence selected in the group consisting of SEQ ID Nos 2, 4-5, 7, 9 and 11, preferably selected in the group consisting of SEQ ID Nos 2, 4-5, 7, and 9, preferably consisting of SEQ ID No 2. In a particular embodiment, the passenger

strand of the miRNA comprises, essentially consists in or consists in a sequence of the passenger strand of pre-miRNA as disclosed in a sequence selected in the group consisting of SEQ ID Nos 2, 4-5, 7, 9 and 11, preferably selected in the group consisting of SEQ ID Nos 2, 4-5, 7, and 9, preferably consisting of SEQ ID No 2.

- 5 In some embodiments, the miRNA is between 17 and 30 nucleotides in length, preferably 22-23 nucleotides in length, and comprises (i) a microRNA region having a sequence from 5' to 3' that is at least 80 % identical to at least one of SEQ ID Nos 1, 3, 6, 8 and 10, preferably SEQ ID Nos 1, 3, 6, and 8; and (ii) a complementary region having a sequence from 5' to 3' that is 60-100 % complementary to the microRNA region. Preferably, the microRNA region has a sequence that is at least 80, 85, 90, 95 or 100 % identical to at least one of SEQ ID Nos 1, 3, 6, 8 and 10, preferably SEQ ID Nos 1, 3, 6, and 8. Preferably, the miRNA comprises a hairpin structure.

- Alternatively, the miRNA is between 17 and 30 nucleotides in length, preferably 22-23 nucleotides in length, and comprises (i) a first polynucleotide having a sequence from 5' to 3' that is at least 80 % identical to at least one of SEQ ID Nos 1, 3, 6, 8 and 10, preferably SEQ ID Nos 1, 3, 6, and 8; and (ii) 15 a second separate polynucleotide having a sequence that is 60-100 % complementary to the first polynucleotide. Preferably, the microRNA region has a sequence that is at least 80, 85, 90, 95 or 100 % identical to at least one of SEQ ID Nos 1, 3, 6, 8 and 10, preferably SEQ ID Nos 1, 3, 6, and 8.

The miRNA can include some chemical modifications, in particular for increasing its stability, resistance to degradation and/or its cellular uptake.

- 20 If desired, microRNA molecules may be modified to stabilize the miRNAs against degradation, to enhance half-life, or to otherwise improve efficacy. Desirable modifications are described, for example, in US20070213292, US20060287260, US20060035254, US20060008822, WO2015131115, US2016053264, WO2010144485 and US20050288244, each of which is hereby incorporated by reference in its entirety. For increased nuclease resistance and/or binding affinity to the target, the 25 miRNA can include 5' cap, 3' cap, backbone modifications, ribose modifications, mismatch, as well as nucleobase modifications.

Ribose modifications include 2'-O-methyl, 2'-O-methoxy, 2'-O-fluorine, 2'-O-methoxyethyl, 2'-O-aminopropyl, 2'-amino. Backbone modifications include phosphorothioate linkages or morpholinos.

- The inclusion of pyranose sugars in the oligonucleotide backbone can also decrease endonucleolytic cleavage. In another alternative, the 3'-terminus can be blocked with an aminoalkyl group. Other 3' 30 conjugates can inhibit 3'-5' exonucleolytic cleavage. While not being bound by theory, a 3' may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 3' end of the oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

- 35 The 5' cap refers to at least one modified nucleotide that block 5' OH or 5' phosphate at the 5' terminus. Preferably, the modification can be selected among an amine group, biotin, a lower alkylamine group, NHCOCH_3 , an acetyl group, 2' oxygen-methyl (2'OMe), 4' thionucleotide, phosphorothioate linkage,

abasic residue, inverted nucleotide or inverted abasic moiety, phosphorodithioate monophosphate and methylphosphonate moiety.

Modified bases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo (including 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines), 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

More preferably, the guide strand of pre-miRNA or mature miRNA can include 2'-fluorine modifications while the passenger strand can include 2'-O-methyl modifications.

In some embodiments, the miRNA comprises one or more of the following (i) a replacement group for phosphate or hydroxyl of the nucleotide at the 5' terminus of the complementary strand or passenger strand (5' cap); (ii) one or more sugar modifications in the first or last 1-6 residues of the complementary strand or passenger strand; or (iii) non-complementarity between one or more nucleotides in the last 1-5 residues at the 3' end of the complementary strand or passenger strand and the corresponding nucleotides of the microRNA region or guide strand.

In some specific embodiments, the miRNA comprises a fully complementary passenger strand comprising (i) modified nucleotides in the first and last two nucleotides of the passenger strand, and/or (ii) a terminal modification of the nucleotide at the 5' end.

In some specific embodiments, the passenger strand comprises modified nucleotides and fewer than half of the total number of nucleotides in the passenger are modified nucleotides. For instance, 2-10, 4-8 or 5-7 nucleotides in the passenger are modified nucleotides. In a particular embodiment, the modified nucleotides are selected from the group consisting of the two-three first and the two last nucleotides of the passenger strand.

In some specific embodiments, the guide strand comprise at least one or two modified nucleotides. Preferably, the guide strand does not comprise modified nucleotides in the first two positions at the 5' end of the guide strand and/or in the last two positions at the 3' end of the guide strand.

In a particular embodiment of the present invention, the microRNA molecules may comprise alternate stretches or portions of nucleotides with 2'-O-methyl modifications and stretches or portions of nucleotides without the modification. By "alternate stretches or portions" it is meant that, when considering the double-stranded RNA molecule, for each pair of nucleotides, at least one nucleotide of the pair, preferably only one, has a 2'-O-methyl modification. The length of the stretches/portions can vary from 1 to 7 consecutive nucleotides. Accordingly, just for illustrating this aspect, the mature miRNA may present one of the following structures:

Sens 5' NNNNNNNNNNNNNNNNNNNNN 3'
 Antisense 3' NNNNNNNNNNNNNNNNNNNN 5'

or

5 Sens 5' NNNNNNNNNNNNNNNNNNNNNNNNN 3'
 Antisense 3' NNNNNNNNNNNNNNNNNNNNNNNNN 5'

or

Sens 5' NNNNNNNNNNNNNNNNNNNNNNNNN 3'
 Antisense 3' NNNNNNNNNNNNNNNNNNNNNNNNN 5'

wherein N refers to a nucleotide having 2'-O-methyl modification.

- 10 In an alternative embodiment of the present invention, the microRNA molecules may comprise stretches or portions of nucleotides with 2'-O-methyl modifications. In this embodiment, when considering the double-stranded RNA molecule, both nucleotide of the pair have 2'-O-methyl modifications. The length of the stretches/portions can vary from 1 to 7 consecutive nucleotides. Accordingly, just for illustrating this aspect, the mature miRNA may present one of the following structures:

15 Sens 5' NNNNNNNNNNNNNNNNNNNNN 3'
 Antisense 3' NNNNNNNNNNNNNNNNNNNNN 5'

or

Sens 5' NNNNNNNNNNNNNNNNNNNNNNNNN 3'
 Antisense 3' NNNNNNNNNNNNNNNNNNNNNNNNN 5'

20 or

Sens 5' NNNNNNNNNNNNNNNNNNNNNNNNN 3'
 Antisense 3' NNNNNNNNNNNNNNNNNNNNNNNNN 5'

wherein N refers to a nucleotide having 2'-O-methyl modification.

- For instance, when considering the specific example of miR-4510, the miRNA may present one of the following structures:

V1

Sens 5' AACCAUACAUCCUACUCCCUCA 3'
 Antisense 3' UUGGUAUGUAGGAUGAGGGAGU 5'

V2

30 Sens 5' AACCAUACAUCCUACUCCCUCA 3'
 Antisense 3' UUGGUAUGUAGGAUGAGGGAGU 5'

V3

Sens 5' AACCAUACAUCCUACUCCCUCA 3'
 Antisense 3' UUGGUAUGUAGGAUGAGGGAGU 5'

- 35 wherein the bold underlined nucleotide have 2'-O-methyl modification.

In addition, the miRNA can present a modification at one or both 3' ends, preferable a modified sugar.

A specific example of modified sugar is Triantennary N-acetyl galactosamine (GalNAC₃).

For instance, when considering the specific example of miR-4510, the miRNA may present the following structure:

V4

Sens 5' **AACCAUACAUCUACUCCUCA** (GalNAC₃) 3'
 Antisense 3' **UUGUAUGUAGGAUGAGGAGU** 5'

Preferably, when a molecule increasing the cellular uptake such as cholesterol or tocopherol is linked to the miRNA, the molecule is linked to the passenger strand of the pre-miRNA. In addition, the miRNA may be linked to a moiety allowing the targeting of the liver.

In another aspect, the disclosure provides a nucleic acid molecule or any modified molecule derivatives encoding or leading to a miRNA as disclosed above and a recombinant expression vector comprising a recombinant nucleic acid sequence operatively linked to an expression control sequence, wherein expression of the recombinant nucleic acid sequence provides a miRNA sequence, a precursor miRNA sequence, or a primary miRNA sequence as described herein. The resulting sequence (e.g., primary or precursor miRNAs) can optionally be further processed to provide the miRNA sequence. In embodiments, the recombinant expression vector comprises at least one sequence selected from the group consisting of SEQ ID Nos 1-11, preferably of SEQ ID Nos 1-9, more preferably of SEQ ID Nos 1-2. Any suitable expression vector can be used such as, for example, a DNA vector (e.g., viral vector, plasmid, etc.). In some embodiments the expression vector is selected for expression in a eukaryotic cell such as, for example, a mammalian cell. One of skill in the art will be able to select an appropriate vector based on the particular application and/or expression system to be employed. In a further aspect, the expression cassette is comprised in a viral vector, or plasmid DNA vector or other therapeutic nucleic acid vector or delivery vehicle, including liposomes and the like.

miRNA therapeutic uses.

The miRNA miR-4510, miR-548aa, miR-548v, miR-376b-3p, and any combination thereof as disclosed above can be for use for treating a solid cancer in a subject, preferably a liver cancer or a solid cancer with liver metastasis. The present disclosure also relates a pharmaceutical composition comprising miR-4510, miR-548aa, miR-548v, miR-376b-3p, and any combination thereof.

For instance, the miRNA can be selected in the group consisting of miR-4510, miR-548aa, miR-548v, miR-376b-3p, and any combination of two, three, four or five miRNAs.

In particular, the combination may include at least miR-4510 and 1-4 miRNAs selected among miR-548aa, miR-548v, miR-376b-3p, and miR-203a-3p, e.g., miR-4510 and miR-548aa; miR-4510 and miR-548v; miR-4510 and miR-376b-3p; miR-4510 and miR-203a-3p; miR-4510, miR-548aa, and miR-548v; miR-4510, miR-548aa, and miR-376b-3p; miR-4510, miR-548aa, and miR-203a-3p; miR-4510, miR-548v, and miR-376b-3p; miR-4510, miR-548v, and miR-203a-3p; miR-4510, miR-376b-3p and miR-203a-3p; miR-4510, miR-548aa, miR-548v and miR-376b-3p; miR-4510, miR-548aa, miR-548v and miR-203a-3p; miR-4510, miR-548v, miR-376b-3p and miR-203a-3p; and miR-4510, miR-548aa, miR-548v, miR-376b-3p and miR-203a-3p. The combination can further comprise an additional miRNA, for instance miR-34a.

In another particular aspect, the combination may include at least miR-548aa and 1-4 miRNAs selected among miR-4510, miR-548v, miR-376b-3p, and miR-203a-3p, e.g., miR-548aa and miR-548v; miR-548aa and miR-376b-3p; miR-548aa and miR-203a-3p; miR-548aa, miR-548v and miR-376b-3p; miR-548aa, miR-548v and miR-203a-3p; miR-548aa, miR-376b-3p and miR-203a-3p; and miR-548aa, miR-548v, miR-376b-3p and miR-203a-3p. The combination can further comprise an additional miRNA, for instance miR-34a.

In an additional particular aspect, the combination may include at least miR-548v and 1-4 miRNAs selected among miR-4510, miR-548aa, miR-376b-3p, and miR-203a-3p, e.g., miR-548v and miR-376b-3p; miR-548v and miR-203a-3p; and miR-548v, miR-376b-3p and miR-203a-3p. The combination can further comprise an additional miRNA, for instance miR-34a-5p.

In a further particular aspect, the combination may include at least miR-376b-3p and 1-4 miRNAs selected among miR-4510, miR-548aa, miR-548v, and miR-203a-3p, e.g., miR-376b-3p and miR-203a-3p. The combination can further comprise an additional miRNA, for instance miR-34a.

The miRNA and any combination thereof can be used for treating liver cancer or a solid cancer with liver involvement (e.g. metastasis). Their use for the treatment of other specific solid cancers with or without liver involvement can also be contemplated, in particular breast, colorectal, esophageal, lung, melanoma, pancreatic, stomach, ovaries, neuroendocrine, uterus, CNS (central nervous system) and brain cancer.

In a preferred aspect, the liver cancer can be a hepatocellular carcinoma and a hepatoblastoma.

The subject can be an adult or a child. In a particular aspect, the subject has liver metastasis, and/or does not respond to the first line treatment and/or is not suitable for tumor resection or ablation.

Optionally, the miRNA or combination thereof can be used in combination with one or more therapeutic agents, especially any antitumor treatment. Optionally, the miRNA is to be used in combination with resection, radiofrequency ablation and/or percutaneous ethanol injection. Optionally, the miRNA is to be used after or before resection, radiofrequency ablation and/or percutaneous ethanol injection. Optionally, the miRNA is to be used in combination with a chemotherapy. Accordingly, the present invention relates to a pharmaceutical composition comprising one or several miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or any combination thereof and another drug, in particular an antitumor drug. It also relates to a product comprising one or several miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or any combination thereof and another drug, in particular an antitumor drug, as a combined preparation for simultaneous, separate or sequential use, preferably for treating a solid cancer, in particular a liver cancer or a solid tumor with liver metastasis.

The antitumor drug can be selected from the group consisting of an inhibitor of topoisomerases I or II, a DNA crosslinker, a DNA alkylating agent, an anti-metabolic agent and inhibitors of the mitotic spindles. It can also be an immunotherapy. It can be selected in the group consisting of MSC2156119J, foretinib, refametinib, cabozantinib, tivantinib, 5-fluoro-uracil, doxorubicin, cisplatin, carboplatin,

gemcitabine, oxaliplatin, mitomycin C, tamoxifen, paclitaxel, larotaxel, taxol, lapatinib, docetaxel, methotrexate, capecitabine, vinorelbine, cyclophosphamide, gemcitabine, amrubicin, cytarabine, etoposide, camptothecin, dexamethasone, dasatinib, tipifarnib, bevacizumab, sirolimus, temsirolimus, everolimus, lonafernib, cetuximab, erlotinib, gefitinib, imatinib mesylate, rituximab, trastuzumab, 5 nocodazole, sorafenib, sunitinib, bortezomib, alemtuzumab, gemtuzumab, tositumomab or ibritumomab or any combination thereof. In a preferred embodiment, the antitumor drug is sorafenib, in particular for use in the treatment of hepatocellular carcinoma. In an alternative preferred embodiment, the antitumor drug is cisplatin or doxorubicin, in particular for use in the treatment of a hepatoblastoma.

Inhibitors of topoisomerases I and/or II include, but are not limited to, etoposide, topotecan, 10 camptothecin, irinotecan, amsacrine, intoplicin, anthracyclines such as doxorubicin, epirubicin, daunorubicin, idarubicin and mitoxantrone. Inhibitors of Topoisomerase I and II include, but are not limited to intoplicin.

DNA crosslinkers include, but are not limited to, cisplatin, carboplatin and oxaliplatin. In a preferred embodiment, the DNA crosslinker is cisplatin.

15 Anti-metabolic agents block the enzymes responsible for nucleic acid synthesis or become incorporated into DNA, which produces an incorrect genetic code and leads to apoptosis. Non-exhaustive examples thereof include, without limitation, folic acid antagonists, pyrimidine analogs, purine analogs and adenosine deaminase inhibitors, and more particularly Methotrexate, Floxuridine, Cytarabine, 6-Mercaptopurine, 6- Thioguanine, Fludarabine phosphate, Pentostatine, 5-fluorouracil, gemcitabine and 20 capecitabine.

The DNA-damaging anti-tumoral agent can be alkylating agents including, without limitation, nitrogen mustards, ethylenimine derivatives, alkyl sulfonates, nitrosoureas, metal salts and triazenes. Non-exhaustive examples thereof include Uracil mustard, Chlormethine, Cyclophosphamide (CYTOXAN(R)), Ifosfamide, Melphalan, Chlorambucil, Pipobroman, Triethylenemelamine, 25 Triethylenethiophosphoramine, Busulfan, Carmustine, Lomustine, cisplatin, carboplatin, oxaliplatin, thiotepa, Streptozocin, Dacarbazine, and Temozolomide.

The therapeutic agent can also be an immunotherapeutic drug. The term “immunotherapy” or “immunotherapeutic drug” refers to a cancer therapeutic treatment with therapeutic antibodies. In particular, antibodies are directed against specific antigens such as the unusual antigens that are 30 presented on the surface of tumors or targeting immune system checkpoints (e.g. immune checkpoint inhibitors). Preferably, therapeutic antibodies functions to deplete tumor cells in a patient. In particular, therapeutic antibodies specifically bind to antigens present on the surface of the tumor cells, e.g. tumor specific antigens present predominantly or exclusively on tumor cells. Alternatively, therapeutic antibodies may also prevent tumor growth by blocking specific cell receptors.

35 The immunotherapeutic drug may target multiple elements of the immune pathway: a therapy that enhances tumor antigen presentation; a therapy that inhibits negative immune regulation e.g., by inhibiting CTLA-4 and/or PD1/PD-L1/PD-L2 pathway and/or depleting or blocking Tregs or other

immune suppressing cells; a therapy that stimulates positive immune regulation, e.g., with agonists that stimulate the CD- 137, OX-40, and/or GITR pathway and/or stimulate T cell effector function; a therapy that increases systemically the frequency of anti-tumor T cells; a therapy that depletes or inhibits Tregs, such as Tregs in the tumor, e.g., using an antagonist of CD25 (e.g., daclizumab) or by ex vivo anti-CD25 bead depletion; a therapy that impacts the function of suppressor myeloid cells in the tumor; a therapy that enhances immunogenicity of tumor cells (e.g., anthracyclines); adoptive T cell or NK cell transfer including genetically modified cells, e.g., cells modified by chimeric antigen receptors (CAR-T therapy); a therapy that inhibits a metabolic enzyme such as indoleamine dioxigenase (IDO), dioxigenase, arginase, or nitric oxide synthetase; a therapy that reverses/prevents T cell anergy or exhaustion; a therapy that triggers an innate immune activation and/or inflammation at a tumor site; administration of immune stimulatory cytokines; or blocking of immunorepressive cytokines.

In a preferred embodiment, the immunotherapeutic drug is a drug targeting PD-1 or PD-L1. The PD-1/PD-L1 agent is preferably selected from the group consisting of Nivolumab (Opdivo, Bristol-Myers Squibb), Pembrolizumab (Keytruda, MK-3475, Merck), Pidilizumab (CT-011, Cure Tech), BMS 936559 (Bristol Myers Squibb), atezolizumab or MPDL3280A (Roche), and a combination thereof.

In a particular aspect of the present invention, the combined association of one or several miRNA as disclosed herein with another antitumor drug can allow the use of a lower/decreased amount of the other antitumor drug that could result in a reduction of the adverse effects and toxicity. In particular, the amount of the other antitumor drug can be a sub-therapeutic amount. More specifically, the other antitumor drug is used at lower dosage than the conventional dosage used in chemotherapy for the same indication and the same administration route when it is used alone (i.e., an amount equal to or preferably lower than the one used in conventional chemotherapy), also called herein a sub-therapeutic amount. More particularly, the amount can be for instance 90, 80, 70, 60, 50, 40, 30, 20 or 10 % of the conventional therapeutic dosage (in particular for the same indication and the same administration route). The conventional therapeutic dosages are those acknowledged by the drug approvals agencies (e.g., FDA or EMEA) and can be found in reference Manuals such as Merck Manuals (www.merck.com/mmpe/lexicomp/). Alternatively, instead of lowering the amount or dosage of the other antitumor drug, the administration frequency of the other antitumor drug or its treatment period can be reduced. For instance, the treatment period may be reduced, for instance by 90, 80, 70, 60 or 50%. Alternatively, the interval between treatments with the other antitumor drug can be increased, for instance by 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% or by 1.5, 2, 2.5 or 3 fold.

The present invention relates to a method of treating a patient with a cancer, in particular a liver cancer comprising (a) administering to the patient a therapeutically effective amount of a molecule selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or any combination thereof or a DNA or RNA encoding for said miRNA; and (b) administering a second therapy, wherein the molecule sensitizes the patient to the second therapy. Preferably, the second therapy

is another antitumor drug. Preferably, the other antitumor drug is cisplatin or doxorubicin or sorafenib. Optionally, the other antitumor drug is administered in a sub-therapeutic amount.

Optionally, the molecule is to be used in combination with another antitumor therapy and a drug lowering/decreasing the toxicity and side effects of the antitumor therapy. For instance, the drug lowering the toxicity and side effects of the antitumor therapy can be sodium thiosulfate or N-acetyl cysteine. In a specific aspect, the molecule is to be used in combination with cisplatin and, sodium thiosulfate or N-acetyl cysteine.

miRNAs, pharmaceutical compositions, or products of the invention can be used in humans with existing cancer or tumour, including at early or late stages of progression of the cancer. The miRNAs, pharmaceutical compositions, or products of the invention will not necessarily cure the patient who has the cancer but will delay or slow the progression or prevent further progression of the disease, ameliorating thereby the patients' condition or survival. In particular, the miRNAs, pharmaceutical compositions, or products of the invention reduce the development of tumors, reduce tumor burden, produce tumor regression in a mammalian host and/or prevent metastasis occurrence and cancer relapse.

In treating the cancer, the pharmaceutical composition of the invention is administered in a therapeutically effective amount. Accordingly, as used herein, the term "treatment", "treat" or "treating" refers to any act intended to ameliorate the health status of patients such as therapy, prevention, prophylaxis and retardation of the disease. In certain embodiments, such term refers to the amelioration or eradication of a disease or symptoms associated with a disease. In other embodiments, this term refers to minimizing the spread or worsening of the disease resulting from the administration of one or more therapeutic agents to a subject with such a disease. More particularly, the treatment may reduce the development of tumors, reduce tumor burden, produce tumor regression in a mammalian host and/or prevent metastasis occurrence and cancer relapse.

By "effective amount" or "therapeutically effective" it is meant the quantity of the pharmaceutical composition of the invention which prevents, removes or reduces the deleterious effects of the treated disease in mammals, including humans. It is understood that the administered dose may be adapted by those skilled in the art according to the patient, the pathology, the mode of administration, etc. For instance, the effective amount can be the amount necessary for decreasing or repressing the expression of Glypican-3 gene, e.g., by at least 10, 20, 30, 40 or 50 % in comparison to the expression in a normal tissue. Alternatively, the effective amount can be the amount necessary for decreasing the tumor growth, inducing tumor regression, decreasing, slowing or preventing the occurrence of metastasis and/or cancer relapse, and/or reducing the development of tumors. For example, a miRNA may be administered in dosages between about 1 and 100 mg/kg (e.g., 1, 5, 10, 20, 25, 50, 75, and 100 mg/kg). In other embodiments, the dosage ranges from between about 10 and 500 mg/m²/day. The miRNA can be administered 1, 2, 3, 4, 5, 6, or 7 times by week.

The pharmaceutical composition of the invention can comprise a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are covalently or non-covalently bound, admixed, encapsulated,

conjugated, operably-linked, or otherwise associated with the miRNA such that the pharmaceutically acceptable carrier increases the cellular uptake, stability, solubility, half-life, binding efficacy, specificity, targeting, distribution, absorption, or renal clearance of the miRNA. Pharmaceutically acceptable carriers of the invention are viral and non-viral miRNA delivery systems/mechanisms that increase uptake of the miRNA by targeted cells. For example, pharmaceutically acceptable carriers of the invention are liposomes, lipids, for example cationic lipids, anionic lipids, amphoteric lipids or uncharged lipids, cationic polymers, polymers, hydrogels, micro- or nano-capsules (biodegradable), microspheres (optionally bioadhesive), cyclodextrins, proteinaceous vectors, or any combination of the preceding elements. Moreover, pharmaceutically acceptable carriers that increase cellular uptake can be modified with cell-specific proteins or other elements such as receptors, ligands, antibodies to specifically target cellular uptake to a chosen cell type. The person skilled in the art has several delivery means available as shown for instance by Zhang et al (2013, *J Control Release*, **172**, 962-974), Garzon et al (2010, *Nat Rev Drg Discov*, **9**, 775-789), and Zhao et al (2009, *Exp. Opin. Drug Deliv.* **6**:673-686). More preferably, the delivery system can be selected among the lipid-based delivery system, the PEI (polyethylenimine)-based delivery system, dendrimers, PLGA (poly(lactide-co-glycolide)) particles, WO15023775, and the like.

In one aspect, the pharmaceutically acceptable carriers will protect the miRNA against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Examples of materials which can form hydrogels include polylactic acid, polyglycolic acid, PLGA polymers, alginates and alginate derivatives, gelatin, collagen, agarose, natural and synthetic polysaccharides, polyamino acids such as polypeptides particularly poly(lysine), polyesters such as polyhydroxybutyrate and poly-epsilon.-caprolactone, polyanhydrides; polyphosphazines, poly(vinyl alcohols), poly(alkylene oxides) particularly poly(ethylene oxides), poly(allylamines)(PAM), poly(acrylates), modified styrene polymers such as poly(4-aminomethylstyrene), pluronic polyols, polyoxamers, poly(uronic acids), poly(vinylpyrrolidone) and copolymers of the above, including graft copolymers.

Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

Pharmaceutically acceptable carriers are cationic lipids that are bound or associated with miRNA. Alternatively, or in addition, miRNAs are encapsulated or surrounded in cationic lipids, e.g. liposomes, for in vivo delivery. Exemplary cationic lipids include, but are not limited to, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA); 1,2-bis(oleoyloxy)-3-3-

(trimethylammonium)propane (DOTAP), 1,2-bis(dimyristoyloxy)-3-3-(trimethylammonia)propane (DMTAP); 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide (DMRIE); dimethyldioctadecylammonium bromide (DDAB); 3-(N-(N',N'-dimethylarninoethane)carbamoyle)cholesterol (DC-Choi); 3 beta-[N',N'-diguandinoethyl-aminoethane)carbamoyle cholesterol (BGTC); 2-(2-(3-(bis(3-aminopropyl)amino)propylamino)acetamido)-N,N-ditetradecylacetamide (RPR209120); pharmaceutically acceptable salts thereof, and mixtures thereof. Further exemplary cationic lipids include, but are not limited to, 1-dialkenoyl-sn-glycero-3-ethylphosphocholines (EPCs), such as 1-dioleoyl-sn-glycero-3-ethylphosphocholine, 1,2-distearoyl-sn-glycero-3-ethylphosphocholine, 1,2-dipalmitoyl-sn-glycero-3-ethylphosphocholine, pharmaceutically acceptable salts thereof, and mixtures thereof.

Exemplary polycationic lipids include, but are not limited to, tetramethyltetrapalmitoyl spermine (TMTPS), tetramethyltetraoleyl spermine (TMTOS), tetramethyltetralauryl spermine (TMTLS), tetramethyltetramyristyl spermine (TMTMS), tetramethyldioleoyl spermine (TMDOS), pharmaceutically acceptable salts thereof, and mixtures thereof. Further exemplary polycationic lipids include, but are not limited to, 2,5-bis(3-aminopropylamino)-N-(2-(dioctadecylamino)-2-oxoethyl)pentanamide (DOGS); 2,5-bis(3-aminopropylamino)-N-(2-(di(Z)-octadeca-9-dienylamino)-2-oxoethyl) pentanamide (DOGS-9-en); 2,5-bis(3-aminopropylamino)-N-(2-(di(9Z,12Z)-octadeca-9,12-dienylamino)-2-oxoethyl)pentanamide (DLinGS); 3-beta-(N4-(N1,Nd-dicarbobenoxyspermidine)carbamoyle)cholesterol (GL-67); 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propyl amide (DOSPER); N-(2-([N(2),N(5)-bis(3-aminopropyl)ornithyl]amino)ethyl)-N,N-dimethyl-2,3-bis[(9Z)-octadec-9-en-1-yloxy]propan-1-aminium trifluoroacetate (DOSPA); pharmaceutically acceptable salts thereof, and mixtures thereof. Examples of cationic lipids are described in U.S. Pat. Nos. 4,897,355; 5,279,833; 6,733,777; 6,376,248; 5,736,392; 5,334,761; 5,459,127; 2005/0064595; U.S. Pat. Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992.

Pharmaceutically acceptable carriers of the invention also include non-cationic lipids, such as neutral, zwitterionic, and anionic lipids. Exemplary non-cationic lipids include, but are not limited to, 1,2-Dilauroyl-sn-glycerol (DLG); 1,2-Dimyristoyl-snglycerol (DMG); 1,2-Dipalmitoyl-sn-glycerol (DPG); 1,2-Distearoyl-sn-glycerol (DSG); 1,2-Dilauroyl-sn-glycero-3-phosphatidic acid (sodium salt; DLPA); 1,2-Dimyristoyl-snglycero-3-phosphatidic acid (sodium salt; DMPA); 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid (sodium salt; DPPA); 1,2-Distearoyl-sn-glycero-3-phosphatidic acid (sodium salt; DSPA); 1,2-Diarachidoyl-sn-glycero-3-phosphocholine (DAPC); 1,2-Dilauroyl-sn-glycero-3-phosphocholine (DLPC); 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC); 1,2-Dipalmitoyl-sn-glycero-0-ethyl-3-phosphocholine (chloride or triflate; DPePC); 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC); 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC); 1,2-Dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE); 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE); 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE); 1,2-Distearoylsn-glycero-3-

phosphoethanolamine (DSPE); 1,2-Dilauroyl-sn-glycero-3-phosphoglycerol (sodium salt; DLPG); 1,2-Dimyristoyl-sn-glycero-3-phosphoglycerol (sodium salt; DMPG); 1,2-Dimyristoyl-sn-glycero-3-phospho-sn-l-glycerol (ammonium salt; DMP-sn-l-G); 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol (sodium salt; DPPG); 1,2-Distearoyl-sn-glycero-3-phosphoglycerol (sodium salt; DSPG); 1,2-Distearoyl-snglycero-3-phospho-sn-l-glycerol (sodium salt; DSP-sn-l-G); 1,2-Dipalmitoyl-snglycero-3-phospho-L-serine (sodium salt; DPP S); 1-Palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLinoPC); 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC); 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (sodium salt; POPG); 1-Palmitoyl-2-oleoyl-snglycero-3-phosphoglycerol (sodium salt; POPG); 1-Palmitoyl-2-oleoyl-snglycero-3-phosphoglycerol (ammonium salt; POPG); 1-Palmitoyl-2-4o-sn-glycero-3-phosphocholine (P-lyso-PC); 1-Stearoyl-2-lyso-sn-glycero-3-phosphocholine (S-lysoPC); and mixtures thereof. Further exemplary non-cationic lipids include, but are not limited to, polymeric compounds and polymer-lipid conjugates or polymeric lipids, such as pegylated lipids, including polyethyleneglycols, N-(Carbonylmethoxypolyethyleneglycol-2000)-1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (sodium salt; DMPE-MPEG-2000); N-(Carbonylmethoxypolyethyleneglycol-5000)-1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (sodium salt; DMPE-MPEG-5000); N-(Carbonylmethoxypolyethyleneglycol 2000)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (sodium salt; DPPE-MPEG-2000); N-(Carbonylmethoxypolyethyleneglycol 5000)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (sodium salt; DPPE-MPEG-5000); N-(Carbonylmethoxypolyethyleneglycol 750)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (sodium salt; DSPE-MPEG-750); N-(Carbonylmethoxypolyethyleneglycol 2000)-1,2-distearoyl-snglycero-3-phosphoethanolamine (sodium salt; DSPE-MPEG-2000); N-(Carbonylmethoxypolyethyleneglycol 5000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (sodium salt; DSPE-MPEG-5000); sodium cholesteryl sulfate (SCS); pharmaceutically acceptable salts thereof, and mixtures thereof. Examples of non-cationic lipids include, but are not limited to, dioleoylphosphatidylethanolamine (DOPE), diphytanoylphosphatidylethanolamine (DPhPE), 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC), 1,2-Diphytanoyl-sn-Glycero-3-Phosphocholine (DPhPC), cholesterol, and mixtures thereof.

Pharmaceutically-acceptable carriers of the invention further include anionic lipids. Exemplary anionic lipids include, but are not limited to, phosphatidylserine, phosphatidic acid, phosphatidylcholine, platelet-activation factor (PAF), phosphatidylethanolamine, phosphatidyl-DL-glycerol, phosphatidylinositol, phosphatidylinositol (pi(4)p, pi(4,5)p2), cardiolipin (sodium salt), lysophosphatides, hydrogenated phospholipids, sphingolipids, gangliosides, phytosphingosine, sphinganine, pharmaceutically acceptable salts thereof, and mixtures thereof. [107] Supplemental or complementary methods for delivery of nucleic acid molecules for use herein are described, e.g., in Akhtar, et al., Trends Cell Bio. 2:139, 1992; Delivery Strategies for Antisense Oligonucleotide Therapeutics, ed. Akhtar, 1995; Maurer, et al., Mol. Membr. Biol. 16: 129-140, 1999; Hofland and Huang, Handb. Exp. Pharmacol. 137:165-192, 1999; and Lee, et al., ACS Symp. Ser. 752:184-192,

2000. Sullivan, et al., International PCT Publication No. WO 94/02595, further describes general methods for delivery of enzymatic nucleic acid molecules.

Amphoteric liposomes can also be used as pharmaceutically acceptable carriers such as those disclosed in US 8,580,297 (the disclosure thereof being incorporated herein by reference). The materials can also
5 be obtained commercially from Marina Biotech (Smarticles®).

The miRNA and pharmaceutical composition can be administered by local or systemic routes. The miRNA and pharmaceutical composition can be administered or suitable for being administered by enteral routes, parenteral routes (including subcutaneous, intravenous, intramuscular intratumoral or intraperitoneal), or by rectal, topical, transdermal, or oral routes.

10 The nucleic acid molecules of the present invention may be alternatively delivered into a target cell using a viral vector. The viral vector may be any virus which can serve as a viral vector. Suitable viruses are those which infect the target cells, can be propagated *in vitro*, and can be modified by recombinant nucleotide technology known in the art. Viral vectors expressing nucleic acids of the invention can be constructed based on viral backbones including, but not limited to, a retrovirus, lentivirus, adenovirus,
15 adeno-associated virus, pox virus or alphavirus. Adenovirus-associated vectors (AAV) are an appealing method since they have acceptable toxicity profiles and have been successfully used to restore miRNA expression. Different AAV serotypes can successfully target different neuronal tissue. In a preferred embodiment, the viral vector is a non-replicating viral vector. In a preferred embodiment, the viral vector is a non-integrative viral vector, in particular for preventing any oncogenic effect associated with the
20 knock-down of tumor suppressor gene by insertional mutation. In a most preferred embodiment, the viral vector is a non-replicating non-integrative viral vector. In one embodiment, the non-replicating poxvirus vector is selected from: a Modified Vaccinia virus Ankara (MVA) vector, a NYVAC vaccinia virus vector, a canarypox (ALVAC) vector, and a fowlpox (FPV) vector. MVA and NYVAC are both attenuated derivatives of vaccinia virus. In another embodiment, the adenovirus vector is a non-
25 replicating adenovirus vector (wherein non-replicating is defined as above). Adenoviruses can be rendered non-replicating by deletion of the E1 or both the E1 and E3 gene regions. Alternatively, an adenovirus may be rendered non-replicating by alteration of the E1 or of the E1 and E3 gene regions such that said gene regions are rendered non-functional. For example, a non-replicating adenovirus may lack a functional E1 region or may lack functional E1 and E3 gene regions. In this way the adenoviruses
30 are rendered replication incompetent in most mammalian cell lines and do not replicate in immunized mammals. Most preferably, both E1 and E3 gene region deletions are present in the adenovirus, thus allowing a greater size of transgene to be inserted. This is particularly important to allow larger antigens to be expressed, or when multiple antigens are to be expressed in a single vector, or when a large promoter sequence, such as the CMV promoter, is used. Deletion of the E3 as well as the E1 region is
35 particularly favored for recombinant Ad5 vectors. Optionally, the E4 region can also be engineered. In one embodiment, the adenovirus vector is selected from: a human adenovirus vector, a simian adenovirus vector, a group B adenovirus vector, a group C adenovirus vector, a group E adenovirus

vector, an adenovirus 6 vector, a PanAd3 vector, an adenovirus C3 vector, a ChAdY25 vector, an AdC68 vector, and an Ad5 vector.

Diagnostic and/or prognostic uses of miRNA.

- 5 One or several miRNA selected from the group consisting of miR-4510, miR-548aa, miR-548v, and miR-376b-3p can be used as a biomarker. More specifically, they can be used as a biomarker of a liver cancer, including hepatocellular carcinoma and hepatoblastoma, preferably a hepatocellular carcinoma. In addition, they can be used as a biomarker of the outcome of a liver cancer, in particular of a hepatocellular carcinoma. Their expression can be correlated with the good or bad prognosis. Therefore,
- 10 they can be used for detecting a liver cancer or a predisposal or susceptibility to develop a liver cancer or for predicting clinical prognosis or outcome of a liver cancer. They can also be used for selecting patient suitable for a treatment by one of these miRNA or any combination thereof. More generally, the present invention relates to kits and methods for providing information useful for detecting a liver cancer or a predisposal or susceptibility to develop a liver cancer, or for predicting clinical prognosis or
- 15 outcome of a liver cancer or for selecting a subject suitable for a treatment by a miRNA as disclosed above.

The present invention also relates to a kit for detecting a liver cancer or a susceptibility to develop a liver cancer in a subject or for selecting a subject suitable for a treatment by a miRNA as disclosed above or for determining the prognosis of a subject having a liver cancer, in particular a hepatocellular

20 carcinoma, the kit comprising detection means for at least one miRNA selected from the group consisting of miR-4510, miR-548aa, miR-548v, and miR-376b-3p or for any combination thereof. Preferably, the kit comprises detection means specific for at least 2, 3 or 4 of miR-4510, miR-548aa, miR-548v, and miR-376b-3p. Optionally, the kit does not comprises detection means specific for more than 10 miRNAs.

- 25 Detection means are preferably primers or probes specific for miR-4510, miR-548aa, miR-548v, or miR-376b-3p. Preferably the one or several miRNAs are selected from the group consisting of miR-4510, miR-548aa, miR-548v, and miR-376b-3p. Preferably the one or several miRNA are selected from the group consisting of miR-4510 and miR-548aa, more preferably miR-4510. The kit may comprises detection means specific for one of the following combination: miR-548aa, miR-548v, miR-376b-3p,
- 30 and miR-203a-3p, e.g., miR-4510 and miR-548aa; miR-4510 and miR-548v; miR-4510 and miR-376b-3p; miR-4510 and miR-203a-3p; miR-4510, miR-548aa, and miR-548v; miR-4510, miR-548aa, and miR-376b-3p; miR-4510, miR-548aa, and miR-203a-3p; miR-4510, miR-548v, and miR-376b-3p; miR-4510, miR-548v, and miR-203a-3p; miR-4510, miR-376b-3p and miR-203a-3p; miR-4510, miR-548aa, miR-548v and miR-376b-3p; miR-4510, miR-548aa, miR-548v and miR-203a-3p; miR-4510, miR-
- 35 548v, miR-376b-3p and miR-203a-3p; miR-4510, miR-548aa, miR-548v, miR-376b-3p and miR-203a-3p; miR-4510, miR-548v, miR-376b-3p, and miR-203a-3p, e.g., miR-548aa and miR-548v; miR-548aa and miR-376b-3p; miR-548aa and miR-203a-3p; miR-548aa, miR-548v and miR-376b-3p; miR-548aa,

miR-548v and miR-203a-3p; miR-548aa, miR-376b-3p and miR-203a-3p; miR-548aa, miR-548v, miR-376b-3p and miR-203a-3p; miR-4510, miR-548aa, miR-376b-3p, and miR-203a-3p, e.g., miR-548v and miR-376b-3p; miR-548v and miR-203a-3p; miR-548v, miR-376b-3p and miR-203a-3p; miR-4510, miR-548aa, miR-548v, and miR-203a-3p, e.g., miR-376b-3p and miR-203a-3p.

- 5 The present invention also relates to the use of the kit for detecting a liver cancer or a susceptibility to develop a liver cancer in a subject or for selecting a subject suitable for a treatment by a miRNA as disclosed above or for determining the prognosis or clinical outcome in a subject having a liver cancer, especially a hepatocellular carcinoma.

10 An under-expression of the miRNAs as disclosed herein is indicative of a cancer, especially a liver cancer or liver metastasis, a predisposition to develop a cancer, especially a liver cancer or liver metastasis or a suitability to be treated with the miRNA as disclosed herein. In a preferred embodiment, the under-expression of the miRNAs as disclosed herein is indicative of a hepatocellular carcinoma. In a preferred embodiment, the miRNA is selected from the group consisting of miR-4510, miR-548aa, miR-548v and miR-376b.

- 15 The present invention relates to a method for determining if a subject has or is predisposed to a liver cancer or liver metastasis, comprising determining the level of one or several miRNA selected from the group consisting of miR-4510, miR-548aa, miR-548v, and miR-376b-3p in the biological sample from the subject, and wherein the subject has or is predisposed to a liver cancer or liver metastasis if the level of one of said one or several miRNA is decreased when compared to a non-tumoral control or healthy
20 subject. Optionally, the method may further determine the level of miR-203a-3p. Preferably, the liver cancer is a hepatocellular carcinoma. Optionally, the method may further comprise determining the expression level of GPC-3, an increased level of expression when compared to a healthy or non-tumoral control being indicative of a cancer, especially a liver cancer or liver metastasis, a predisposition to develop a cancer, especially a liver cancer or liver metastasis. Optionally, the method may comprise an
25 initial step of providing a biological sample from the subject. In a preferred embodiment, the miRNA is selected from the group consisting of miR-4510, miR-548aa, miR-548v and miR-376b.

The present invention also relates to a method for selecting a subject suitable for a treatment by a miRNA selected from the group consisting of miR-4510, miR-548aa, miR-548v, and miR-376b-3p or any combination thereof, comprising determining the level of one or several miRNA selected from the group
30 consisting of miR-4510, miR-548aa, miR-548v, and miR-376b-3p in the biological sample from the subject, and selecting the subject if at least one of said miRNAs is under-expressed in comparison with a healthy or non-tumoral control. Optionally, the method may further determine the level of miR-203a-3p. Preferably, the liver cancer is a hepatocellular carcinoma. Optionally, the method may comprise an initial step of providing a biological sample from the subject.

- 35 Alternatively or in combination with the previous method, the present invention also relates to a method for selecting a subject suitable for a treatment by a miRNA selected from the group consisting of miR-4510, miR-548aa, miR-548v, and miR-376b-3p or any combination thereof, determining the level of

GPC3 thereof in a biological sample from the subject, and selecting the subject if GPC3 is upper-expressed or overexpressed in comparison with a healthy or non-tumoral control.

The method may further comprise administering an effective amount of a miRNA selected from the group consisting of miR-4510, miR-548aa, miR-548v, miR-376b-3p and miR-203a-3p or any combination thereof to said subject.

The present invention further relates to a method for determining a clinical prognosis or outcome in a subject having a liver cancer, especially a hepatocellular carcinoma. The method comprises determining the level of one or several miRNA selected from the group consisting of miR-4510, miR-548aa, miR-548v, and miR-376b-3p in the biological sample from the subject. More preferably, the miRNA is selected from the group consisting of miR-4510, miR-548aa, and combination thereof. The clinical prognosis is correlated with the level of expression of the miRNA. More particularly, when compared with a non-tumoral control or healthy subject, an under-expression of one of several of these miRNA is associated with a poor prognosis. Preferably, the liver cancer is a hepatocellular carcinoma. A patient may be considered to have a "poor prognosis" or "bad prognosis" where, for example, the survival rate associated with the cancer subtype is less than the survival rate associated with other related cancer subtypes.

The level of miRNA can be determined by any method available to the one skilled in the art such as Northern blot analysis, RT-PCR, quantitative RT-PCR, microarray, in situ hybridization, RNA sequencing. miRNA expression can be quantified in a two-step polymerase chain reaction process of modified RT-PCR followed by quantitative PCR. miRNA expression can be quantified by hybridization on a microarray, RNA sequencing, slides or chips. For instance, probes or primers may be coupled to a support. Such supports are well known to those of ordinary skill in the art and include, but are not limited to glass, plastic, metal, or latex. The support can be planar or in the form of a bead or other geometric shapes or configurations known in the art.

For instance, the determination of the expression level can be carried out by forming a preparation comprising nucleic acid from said biological samples, an oligonucleotide probe or probes adapted to anneal to one or several miRNAs selected from the group consisting of miR-4510, miR-548aa, miR-548v, miR-376b-3p or miR-203a-3p, a thermostable DNA polymerase, deoxynucleotide triphosphates and co-factors; providing polymerase chain reaction conditions sufficient to amplify all or part of said nucleic acid molecule; analyzing the amplified products of said polymerase chain reaction for the presence of miRNA; and optionally comparing the amplified product with a normal matched control. Alternatively, the method can further comprise one or more of the steps including: (a) obtaining a sample from the patient, (b) isolating nucleic acids from the sample, (c) labeling the nucleic acids isolated from the sample, and (d) hybridizing the labeled nucleic acids to one or more probes.

The levels of miRNA are considered as under-expressed when decreased by at least 1.5 fold when compared to a normal control. More particularly, the levels are decreased by about 2, 3, 4, 5, 6, 7, 8, 9 or at least 10-fold compared to a normal control level.

The biological sample from the subject can be a sample from blood, blood plasma or serum, lymph fluid, spinal or cerebrospinal fluid, saliva, sputum, lavage, urine, feces, bronchoalveolar lavage, or human tissue biopsy, especially a tumor sample. Preferably, the sample is a blood sample, a liver sample or a liver tumor sample.

- 5 A normal or non-tumoral or healthy control is the miRNA in a sample from a histologically matched sample, for instance a subject which has no cancer or the miRNA in a normal or non-tumoral or healthy tissue taken at a reasonable distance of the tumor in a patient with a cancer.

10 Further aspects and advantages of the present invention will be disclosed in the following experimental section, which should be regarded as illustrative and not limiting the scope of the present application. A number of references are cited in the present specification; each of these cited references is incorporated herein by reference.

Examples

- 15 The inventors worked on the regulation of genes by miRNAs and the role of these post-transcriptional regulations in two primary liver cancers: the hepatocellular carcinoma (HCC), and the hepatoblastoma (HBL). They focused their study on Glypican-3 (*GPC3*), a Wnt signaling pathway-associated gene. *GPC3* is an oncogene in liver and actively participates in hepatocarcinogenesis by sustaining tumoral cell proliferation, dedifferentiation and stemness. Their work aimed at identifying miRNAs negatively
20 regulating *GPC3* in tumoral hepatic cells and at determining those that are involved in liver carcinogenesis by facilitating *GPC3* overexpression and its oncogenic effect.

Results

Ten new miRNAs regulate *GPC3* expression in HCC cells

- 25 To exhaustively identify miRNAs targeting *GPC3*, the inventors used a screening approach, named Dual Fluorescence-FunREG (DF-FunREG) (Maurel et al, 2013, *Hepatology* **57**, 195-204). Using a HCC-derived Huh7 cell line expressing an eGFP transgene carrying the *GPC3* 5'+3'-untranslated regions (UTRs) and the Tomato transgene as reference, they screened a library of 1712 miRNA mimics (Qiagen, miRBase V17.0). 28 miRNAs modulating the eGFP/Tomato ratio above or below arbitrary threshold
30 values were pre-selected as candidates. The previously identified *GPC3*-repressing miRNAs miR-96 and miR-1271 were amongst the selected candidates [Maurel et al, 2013, *Hepatology* **57**, 195-204; Jalvy-Delvaile et al, 2012, *Nucleic Acid Res* **40**, 1356-1365].

- A secondary screen using the corresponding Huh7 cells expressing an eGFP transgene lacking *GPC3* 5'+3'-UTRs and the Tomato was performed with the 28 pre-selected miRNA candidates. Following this
35 step, 10 false positive hits were eliminated and 18 miRNA candidates including miR-96 and miR-1271 were retained (Table 1). Results from western blot experiments showed that 10 new miRNAs significantly increase or decrease *GPC3* expression in HCC cells (Table 1, Figure 1A).

As GPC3 is a protein anchored at the external side of the membrane, the inventors also measured its membrane expression after miRNA transfection. The regulatory effect of the previous miRNAs on membrane GPC3 expression was confirmed, except for miR-203a-3p and the control miR-219-5p (Huang, N et al, FEBS Lett 2012, 586(6):884-91), which had no significant inhibitory effect (Fig. 1B).

Moreover, miR-548v decreased GPC3 expression at the membrane (Fig. 1B) confirming the trend observed with the endogenous protein (Fig. 1A). Together, these investigations validated ten miRNAs regulating GPC3 expression in Huh7 cells. Finally, the inventors assessed the effect of these ten miRNAs on *GPC3* mRNA expression. MiR-4510, which exerted the strongest inhibitory effect on GPC3 protein (Fig. 1A-B), also decreased *GPC3* mRNA expression (Fig. 1C). Surprisingly, a slight increase in *GPC3* mRNA expression was observed with miR-203a-3p (Fig. 1C). Altogether, it was noticed few discrepancies between the screening results and GPC3 expression following miRNA transfection (Fig. 1). Those discrepancies may be due to the pleiotropic effects of miRNAs on multiple targets or the involvement of other post-transcriptional regulators. To summarize, the inventors' investigations led to the validation of ten miRNAs regulating the *GPC3* in HCC cells. Some miRNAs (e.g. miR-4510 and miR-135b-3p) may function through a direct miRNA-*GPC3* mRNA interaction.

Downregulation of five miRNAs correlates with GPC3 overexpression in HCC

To evaluate the relevance of these 10 miRNAs in *GPC3*-associated liver carcinogenesis, the inventors measured their expression by RT-qPCR in 98 HCC and in 19 non-tumoral livers (NTL). 5 (miR-4510, miR-548aa, miR-548v, miR-376b-3p, miR-203a-3p) out of 10 *GPC3*-regulating miRNAs were significantly decreased in tumors compared to NTL (Figure 2; Table 1). The others were not expressed in liver tissue or not deregulated in HCC. All these results were further validated in an independent set of 19 pairs of HCC/non-tumoral liver (Figure 3) demonstrating the strong relevance of these data.

At a clinical level, the down-regulation of miR-4510 and miR-548aa was associated with a poor prognosis (Figure 4) stressing that these two miRNAs also constitute prognostic biomarkers in HCC.

Noticeably, miR-4510 was constantly decreased in HCC tumors (Fig. 3) and was strongly inversely correlated with *GPC3* mRNA in the 98 HCC samples (Fig. 22) suggesting a possible role of this miRNA in *GPC3* overexpression and in HCC. MiR-4510 decrease was independent of HCC subgroup clustering (Figure 19) and was also observed in HBL tumors (Figure 20). Thus a decrease of this miRNA constitutes a good indicator of liver tumorigenesis. Finally, the expression of each miRNA was correlated to HCC patients' clinical parameters. While miR-548aa downregulation in HCC was associated with the presence of satellite nodules ($p=0.05$), miR-376b-3p downregulation was associated with tumors harboring p53 mutations ($p=0.02$).

MiR-4510, miR-203a-3p, miR-548aa, miR-376b-3p and miR-548v exert antitumor effects

The capacity of these 5 miRNAs to block HCC cell growth *in vitro* was then investigated by cell transfection using small synthetic RNAs.

The inventors investigated the effect of the five *GPC3*-regulating miRNAs on tumor cells *in vitro*. All miRNAs significantly inhibited Huh7 cell growth and proliferation (Figure 5). Results showed that the 5 *GPC3*-regulating miRNAs significantly inhibited HCC Huh7 cell growth at Day 3 and Day 6 (Figures 5A, 5B and 6; Table 1); miR-4510 and miR-548aa being the most potent (> 35% inhibition and even > 40% inhibition) at Day 6. Interestingly these two miRNAs were significantly more active to reduce HCC cell growth than a small interfering RNA targeting *GPC3* or than miR-34a-5p (Figure 6), the miRNA currently tested through a phase-I clinical trial in liver cancer.

Table 1: Main results summary

	Regulation of <i>GPC3</i> in HCC Huh7 cells
<i>miRNAs selected through the primary + secondary screens</i>	18 (including miR-96 and miR-1271)
<i>New miRNAs validated as regulators of Glypican-3 in HCC cells</i>	10 (Figure 1)
<i>miRNAs significantly down-regulated in patients' tumors</i>	5 (Figures 2 and 3)
<i>miRNAs constituting prognostic biomarkers in HCC</i>	2 (miR-4510 and miR-548aa; Figure 4)
<i>miRNAs inhibiting HCC cell growth in vitro</i>	5 (Figure 6 with miR-4510 and miR-548aa being the most potent)
<i>miRNAs blocking hepatoma cell proliferation more effectively than miR-34a-5p in vitro</i>	2 (Figure 6 with miR-4510 and miR-548aa)
<i>miRNAs inducing apoptosis</i>	2 (Figure 7 with miR-4510 being the most potent)
<i>miRNAs blocking HCC cell cycling in vitro</i>	5 (Figure 9 with miR-4510 and miR-548aa being the most potent)

To explain the negative effect of these miRNAs on Huh7 cell growth, the inventors first measured the rate of cell death. Data in Figure 7 show that miR-4510 and miR-203a-3p induced apoptosis (Annexin V-based test); miR-4510 being the most potent and acting through caspase 3/7 activation (Figure 8). They then measured the number of actively proliferating cells. As shown in Figure 9, the 5 miRNAs inhibited Huh7 cell cycling by prolonging G2/M phase, lengthening G0/G1 phase and/or shortening S phase. Here again miR-4510 and miR-548aa were the most potent (Figure 9), thereby explaining the strong inhibitory effect observed with these two miRNAs on HCC cell growth *in vitro*.

In conclusion, the inventors identified 5 miRNAs, namely miR-4510, miR-548aa, miR-548v, miR-376b-3p and miR-203a-3p, that down-regulate *GPC3* expression in Huh7 cells and inhibit HCC cell growth *in vitro* (Table 1). In HCC, the down-regulation of miR-4510, miR-548aa, miR-548v, miR-376b-3p and

miR-203a-3p constitutes a diagnostic biomarker and the decreased expression of miR-4510 and miR-458aa also constitutes a prognosis biomarker.

MiR-4510 and with a lesser extend miR-203-3p induced apoptosis in Huh7 cells, while the other miRNAs had not pro-apoptotic effect, suggesting the regulation of a different set of genes by these 5 miRNAs with the exception of *GPC3* being a common target. All these miRNAs are significantly less expressed in HCC tumors compared to NTL suggesting their involvement in the abnormal expression of *GPC3* and its oncogenic effect in HCC. Interestingly miR-4510 and miR-548aa inhibited HCC cell growth more effectively than a small interfering-RNA targeting *GPC3* or than miR-34a-5p (Figure 6), the miRNA currently tested in clinic trial. Altogether these data show that miR-4510, miR-548aa, miR-548v, miR-376b, miR-203a-3p act as tumor suppressors in HCC and could mediate their antitumor effect through the down-regulation of *GPC3* oncogene and other target genes that remain to be identified. These 5 miRNAs therefore act as tumor inhibitors in HCC. Finally inventors clearly showed that miR-4510 and miR-548aa display higher antitumoral properties *in vitro* than miR-34a.

Based on the results summarized in Table 2, the inventors more particularly focused their subsequent work on miR-4510 and its antitumor character in liver cancer. miR-1271, a previously reported *GPC3*-regulating miRNA (Maurel et al, 2013, *Hepatology* **57**, 195-204), was used as a comparison.

Table 2: *In vitro* data summary

	miR-4510	miR-548v	miR-376b-3p	miR-548aa	miR-203a-3p	miR-1271
Growth inhibition	++	+	+	++	++	++
Apoptosis	++	-	-	-	+	+
Cell Cycle arrest	+++	+	+	+++	++	++
Specificity	3' UTR	5'+3' UTRs	3' UTR	5' UTR	5'+3' UTRs	3' UTR

To summarize, previous data showed that miR-4510 is one of the most effective miRNAs for inhibiting the growth of HCC Huh7 cells (Figure 6), the most effective for inducing cell apoptosis (Figure 7) and one of the two most effective for triggering cell cycle arrest through G0 phase entering and S phase decreasing (Figure 9). Inventors showed that miR-4510 down-regulates Glypican-3 through its 3'UTR.

MiR-4510 is a potent tumor suppressor in liver cancer and directly interacts with GPC3 3'-UTR

By transfecting Huh7 cells with a specific inhibitor of miR-4510 (AM4510) in association or not with miR-4510, inventors demonstrated the specificity of the antitumoral properties displayed by miR-4510 in HCC (Figures 11 and 12). Compared to control cells, blocking the endogenous miR-4510 by AM4510 led to an increased proliferation of Huh7 cells (Figure 11) further underlining the anti-proliferative role of this miRNA in HCC cells.

The effect of miR-4510 on hepatoma cells was compared with that of miR-34a-5p, a miRNA currently tested in clinic. MiR-4510 inhibited the growth of HCC-derived Huh7 and Hep3B cells and of HBL-derived Huh6 and HepG2 cells and it was more effective than miR-34a-5p in Hep3B cells (Figures 10A and 17). Moreover, it significantly induced apoptosis in three hepatoma cell lines (Figures 10B, 12, 13 and 14). MiR-4510 specifically induced the apoptosis of Hep3B, another HCC cell line (Figure 13), and of the HBL-derived HuH6 cell line (Figure 14). Moreover it blocked the growth of these two cell lines at Day 6 more effectively than miR-34a-5p (Figures 10A).

Altogether these data demonstrate that miR-4510 is a powerful tumor suppressor and a relevant antitumoral agent in both HCC and HBL cells. Interestingly miR-4510 significantly sensitized Huh7 cells and Huh6 cells to Sorafenib- and Cisplatin- mediated cell death, respectively (Figure 10C, Figure 12). Sorafenib is the current treatment of unresectable and metastatic liver cancer. Moreover, miR-4510 further decreased Huh7 cell growth inhibition mediated by doxorubicin (Fig. 15).

Using Ingenuity and bioinformatic tools, the inventors also found that most of predicted targets of miR-4510 are associated with cancerous processes and liver tumorigenesis suggesting its role as a central tumor suppressor in liver.

MiR-4510 is a central multifunctional regulator in liver

MiRNAs are pleiotropic regulators that target many genes in cells. The differences in the effect of miR-4510 and si-GPC3 on Huh7 cell growth (Fig. 5) suggested that miR-4510 targets additional genes in hepatoma cells. MiRNA:targets prediction tools showed that miR-4510 could potentially interact with around 700 genes. Using Ingenuity Pathway Analysis program, we found that 82% of these genes are associated with cancer and 34% are related to HCC and liver hyperplasia/hyperproliferation. Moreover, numerous predicted genes are involved in cell survival and cell cycle progression. Among them is the pro-survival *B-cell lymphoma-extra large (BCL-XL)* protein which is upregulated in HCC. Western blot analyses showed that miR-4510 inhibits BCL-XL expression (Fig. 16A), a result in agreement with the pro-apoptotic effect of miR-4510 (Figures 7-8, 10B, 12, 13 and 14). Similarly, the inventors observed a decrease of cyclin-dependent kinases (CDK) 1, 2 and 6 (Fig. 16A). Because CDKs are all involved in proper cell cycle progression and CDK1 is a hub gene in HCC, the strong cell division arrest mediated by miR-4510 in Huh7 cells (Fig. 9) is likely the consequence of the simultaneous repression of multiple CDKs by this miRNA. MiR-4510 was also predicted to interact with upstream and downstream regulators of the transforming growth factor-beta 1 (TGF- β 1)/SMAD pathway including *ACVR2A*, *TAB1* and *SMAD3*. TGF- β -pathway signaling activity has been associated with several features of HCC tumors. The inventors showed here that the levels of TAB1 and SMAD3 decrease following miR-4510 treatment (Fig. 16B). Finally, miR-4510 was predicted to target *TCF4*, which is one of the main transcriptional effectors of the Wnt pathway. MiR-4510 decreased TCF4 expression and inhibited Wnt activity without affecting β -catenin expression in Huh7 cells (Fig. 16C).

MiR-4510 inhibits HCC tumor growth and induces HCC cell apoptosis in vivo

Finally the ability of miR-4510 to block the growth of a hepatic tumor *in vivo* was investigated by inventors using the chick chorioallantoic membrane (CAM) (Hagedorn M et al, PNAS 2005; Dumartin L et al, Gastroenterology 2010). Critical biological features of human tumor progression such as cell proliferation, angiogenesis, normal tissue invasion, and tumor cell-host interactions have been previously successfully reproduced in this model. Moreover, the CAM model is useful for testing small non-coding RNA-mediated gene knockdown on tumor growth and angiogenesis [Asangani IA et al, Mol Cell 2009]. Huh7 cells transfected with miR-4510 or Ctrl were deposited on the CAM and tumor growth was monitored on days 3 and 6. As shown in Figures 18 and 23, miR-4510 dramatically inhibited the growth of Huh7 cell-mediated tumors (tumor aggressiveness and active growth being characterized by the presence of bleeding) *in vivo* further stressing the tumor suppressive properties of this miRNA in liver cancer. Control experiments validated the inhibition of GPC3 by miR-4510 in Huh7 cell before CAM implantation (data not shown). At day 3, no obvious difference was observed between the control RNA (Ctrl) and miR-4510 in tumor appearance or size, nor in tissue cross-sections stained with Hematoxylin and Eosin (Fig. 23B and C, upper panels), with the exception of a significantly lower number of tumors with bleeding and bloody areas in presence of miR-4510 (Fig. 23D, left panel). GPC3 protein expression was also decreased in tumors transfected with miR-4510 compared to control (data not shown). At day 6, the growth of miR-4510 tumors was noticeably impeded compared to Ctrl tumors (Fig. 23B-C, upper panels), as assessed by a disappearance of yellowish, bloody and coagulation areas (Fig. 23B, upper panels) and of blood cells and large vessels in tumoral tissue (Fig. 23C, upper panels). As shown at day 6 in Fig. 23D, 80% of Ctrl tumors were characterized by bleeding, while only 30 % of miR-4510 tumors presented this macroscopic feature suggesting a reduction of the aggressiveness of miR-4510-transfected HCC cells during tumor development. Because miR-4510 tumors seemed to hardly develop, Ki67 and Caspase 3 staining was performed. The decrease of the proliferative marker Ki67 in miR-4510 tumors was visible both at day 3 and day 6 of tumor growth demonstrating the inhibition of HCC cells proliferation by miR-4510. While no Caspase-3 staining was visible at day 3 and at day 6 in Ctrl tumors, miR-4510 tumors were markedly stained at day 6 showing that miR-4510 induces HCC cell apoptosis at later stages of tumor development (Fig. 23C, lower panels). Altogether these results showed that miR-4510 induces HCC cell apoptosis and inhibits the growth and angiogenesis of HCC tumors *in vivo*.

Altogether these data strongly support the finding that miR-4510 acts as a tumor suppressor in liver and constitutes one of the most relevant candidates (amongst the 5 identified Glypican-3-targeting miRNAs) for a therapeutic use in HCC and in HBL.

The regulatory activity of miR-4510 is maintained when encapsulated in stable nucleic acid lipid particles (SNALPs). To assess if miR-4510 maintains its regulatory effect when formulated with

liposomal nanoparticles, we used the Dual Fluorescence-FunREG system and TGG cells which stably express the reference Tomato transgene and the test eGFP transgene carrying the wild-type *GPC-3* 3'-untranslated region (Maurel et al, 2013, *Hepatology* **57**, 195-204). MiR-4510 was encapsulated in SNALPs using a combination of KAUDO nucleolipid + dioleoylphosphatidylethanolamine (DOPE) lipid and then, incubated with TGG cells at a final concentration of 15nM for three days. Compared to the control RNA, miR-4510 decreased the eGFP/Tomato ratio and it was as efficient as lipofectamine reagent (Figure 21) demonstrating its ability to interact with its target genes *in cellulo* when nanoformulated with liposomes.

Materials and Methods

Plasmids construction

The lentiviral pTRIP-eGFP-GLO, pTRIP-eGFP-GPC3, pL-GFP, pL-GFP-GPC3 (bearing the GPC3 3'UTR) and pL-Tomato plasmids were as previously described (Laloo, B., et al, MCP 2009; Jalvy-Delvalle, S., et al, 2012; Maurel, M., et al, 2013). The lentiviral pL-5'UTR-GPC3-GFP-3'UTR-GPC3 and pL-GFP-5'UTR-GPC3 constructs were obtained by inserting the GPC3 5'UTR in the pL-GFP-3'UTR-GPC3 and pL-GFP plasmids, respectively. The pGEM-T-hGPC3 plasmid was constructed as follow. The *GPC3* Open Reading Frame (ORF) was PCR amplified using the following primers: ATTCTCTAGAGAATTTCGGATCCATGGCCGGGACCGTGCGC (SEQ ID No 12) on the 5' end and CTCACCTCTAGAGCGGCCGCTCAGTGCACCAGGAAG (SEQ ID No 13) on the 3' end. As matrix we used the pEF-BOS plasmid which contains the human GPC3 cDNA (kindly provided by Jorge Filmus; Duenas Gonzales A, et al. J Cell Biol 1998; 141:1407–14). After adenylation the PCR fragment was subcloned into the pGEM®-T vector from Promega and the integrity of the insert sequence was assessed by DNA sequencing. The lentiviral pL-hGPC3 was constructed by subcloning the human GPC3 ORF of the pGEM-T-hGPC3 plasmid in the pL-GFP plasmid using the BamH I-Xba I restriction sites.

Cell lines

The hepatocellular carcinoma (HCC)-derived Huh7 and Hep3B and the hepatoblastoma (HB)-derived HepG2 cell lines were grown in DMEM medium (Invitrogen) containing 4.5 g/L of D-glucose supplemented with 10% FCS and penicillin/streptomycin antibiotics. The hepatoblastoma-derived HuH6 cell line was grown in DMEM medium (Invitrogen) containing 1 g/L of D-glucose supplemented with 10% FCS and penicillin/streptomycin antibiotics. Huh7 stable cell lines co-expressing the pL-Tomato and pL-GFP transgenes, as well as the pL-Tomato and pL-GFP transgenes bearing either the GPC3 3'UTR, GPC3 5'UTR or both respectively, were developed by lentiviral transduction (multiplicity(ies) of infection [m.o.i.] = 1) and cell sorting.

Lentiviral production, Titration, Cell transduction and Cytometry analysis

Production and titration of infectious lentiviral particles, as well as biosafety considerations, procedures and policies have been described previously (Laloo, B., et al, 2009). Lentiviral particles were added to the target cells and incubated for 72 h. Then the cells were washed twice in PBS and grown in the presence of complete medium for a week before use. Cells expressing Tomato and eGFP were washed in PBS, detached with trypsin/EDTA, collected and analyzed by FACS using a BD FACS Canto II (BD Biosciences, San Jose, CA, USA) and the BD FACS Diva software as described previously (Laloo, B., et al, 2009). Cell sorting was performed using the BD FACS Aria cell sorter.

Small RNAs, miRNA mimic library, Cell transfection and Sorafenib or Doxorubicin or Cisplatin treatment

The miRNA mimics were from Qiagen, Sigma and Exiqon. Hairpin inhibitors were from Thermo-Scientific-Dharmacon Products. The Human miScript miRNA Mimic 96 Set (miRBase V17.0) and the 1027281 negative siRNA control (Ctrl) were from Qiagen. Small non-coding RNAs or hairpin inhibitors were transferred into the target cells by reverse transfection using Lipofectamine RNAi Max (Invitrogen) according to manufacturer's instructions at a final concentration of 12 nM and cells were grown for 3 days before analysis. 24 hr after miR-4510 transfection, sorafenib (Selleckchem) at a final concentration of 10 μ M or cisplatin at a final concentration of 3.8 μ M or doxorubicin at a final concentration of 0.1 μ g/ml were added to the cells during 48 hr or 72 hr, respectively.

DF-FunREG screening

FunREG and DF-FunREG analyses were performed three days after transfection as previously described (Jalvy-Delvaile S et al, *Nucleic Acids res* 2012; Maurel M et al, *Hepatology* 2013; Maurel M et al, *RNA* 2013) with few modifications including fluorescence measurements using the Envision multiplate reader (Perkin Elmer).

For the DF-FunREG screening, 15 000 Tomato/eGFP Huh7 cells were plated per well of 96-well microplates and reverse transfected by each miRNA mimic of the library. Three days after transfection, cells were washed in PBS and fluorescence signals were measured using an Envision multiplate reader (Perkin Elmer). Then eGFP/Tomato ratios were calculated. For liposomal nanoformulations testing, Huh7 cells were incubated with small RNA-containing SNALPs at a final concentration of 15 nM for three days at 37°C and 5 CO₂. Then, fluorescence signals were measured as described above.

Liver Samples and Clinical Data

Liver tissues were immediately frozen in liquid nitrogen and stored at -80°C until used for molecular studies. All patients were recruited in accordance with French law and institutional ethical guidelines. Liver samples were clinically, histologically, and genetically characterized as previously described (Maurel, M., et al, 2013). A first set of 133 liver samples (112 HCC and 21 non tumorous liver [NTL] samples) was collected from 118 patients surgically treated at French University Hospitals. A second

set of 38 liver paired samples (19 HCC and their corresponding NTL samples) was collected from 19 patients surgically treated at French University Hospitals.

miRNA quantification

5 Taqman microRNA assays (Applied Biosystems) were used to quantify the relative expression levels of mature miRNAs in the first set of 133 liver samples. Sybergreen microRNA assays (Qiagen) were used to quantify the absolute expression of mature miRNAs in the second kit of 38 liver paired patients or in cell lines.

10 *Quantification of GPC3 protein*

Western blotting: Whole cell extracts were prepared by treating cells with RIPA buffer (Sigma). Proteins were separated by 10% SDS-PAGE and blotted onto nitrocellulose membrane (Protran, Whatman). After blotting, total loaded proteins were quantified with SYPRO Ruby following manufacturer's instructions (Invitrogen). Stained membranes were imaged with the Molecular Imager PharosFX Plus
15 System (Biorad) and proteins were analyzed with the Quantity One (Biorad) basic software. Then membranes were saturated in Odyssey Blocker and successively incubated with the indicated primary antibodies and adequate InfraRed-labeled secondary antibody (either IRDye-680 or -800 conjugated secondary antibodies) following manufacturer's instructions. Fluorescence signals were detected and quantified using the Odyssey infrared imaging system. Blocker and Odyssey infrared imaging system
20 were from LI-COR Biosciences (ScienceTec, Les Ulis, France). Specific protein signal was normalized to the house-keeping protein GAPDH and total proteins (SYPRO Ruby). The rabbit monoclonal anti-GPC3 (EPR5547) antibody was from Abcam and the rabbit polyclonal anti-GAPDH (FL-335) antibody was from Santa Cruz.

Specific protein signal was normalized to the house-keeping GAPDH protein or total proteins (SYPRO
25 Ruby, Sigma-Aldrich, Lyon, France) as indicated on each figure. The rabbit monoclonal anti-ACVR2A (EPR7407, 1:2000), anti-BCL-XL (E18, 1:1000), anti-CDK1 (E161, 1:2000), anti-GPC3 (EPR5547, 1:5000), anti-SMAD3 (EP568Y, 1:2000) and rabbit polyclonal anti-TAB1 (1:200) antibodies were purchased from Abcam. The mouse monoclonal anti-CDK2 (D-12, 1:200), rabbit polyclonal anti-CDK6 (C-21, 1:200), anti-GAPDH (FL-335, 1:2000) and anti-TCF4 (H-125, 1:200) antibodies were from
30 Santa Cruz and the mouse monoclonal anti- β -Catenin (C-14, 1:4000) was from BD Biosciences.

The anti-human GPC3-Allophycocyanin (APC) monoclonal antibody and IgG2a-APC isotype control were from R&D systems. Huh7 cells were washed in PBS, detached with PBS/EDTA, collected and incubated with the fluorescent anti-GPC3 or control antibody. Expression of the membrane GPC3 protein was analyzed by FACS. Cells incubated with the IgG2a-APC isotype control were used as
35 negative control to gate the eGFP-positive cell populations and to measure the basal mean fluorescence intensity of the whole cell population.

Flow cytometry

Huh7 cells were washed in PBS, detached with PBS/5mM EDTA, collected and stained with a fluorescent anti-GPC3 antibody. Expression of membranous GPC3 protein was measured by FACS using a BD FACS Canto II and the BD FACS Diva software as described previously (Laloo, B., et al, 2009). The anti-human GPC3-Allophycocyanin (APC) monoclonal antibody and IgG2a-APC isotype control were from R&D systems.

Cell growth and proliferation assays

Cell growth was measured using the In vitro Toxicology assay kit (Sigma), which measures the total cellular proteins, according to the manufacturer's instructions. Briefly, 3,500 cells were transfected and seeded into 96-well microplates in a volume of 100 µl. One day, three days and six days later, cell growth was stopped by the addition of cold trichloroacetic acid, then Sulforhodamine B staining was performed and absorbance was measured at 565 nm using the CLARIOstar multiplate reader (BMG labtech). For proliferation assay, 200 000 cells were transfected and seeded into 6-well plates in a volume of 2.5 ml. Three days later, total cells were counted with Malassez cell.

Cell death assays and caspase assays

Prior to cell apoptosis detection, 200 000 cells were transfected with small RNAs at a concentration of 15 nM and seeded into 6-well plates in a volume of 2.5 ml. Three days later, total cells were collected and cell apoptosis was analysed using the Annexin V-PE/7-Amino-Actinomycin (AAD) apoptosis detection kit (BD Pharmingen). Viable cells with intact membranes exclude 7-ADD and are Annexin V-PE negative. Fluorescence generated by the cell-bound Annexin V-PE, which measure the percentage of early apoptotic cells, and the 7AAD, which measure the percentage of late apoptotic cells, were analyzed by the BD FACS CANTO II. Activities of Caspases 3 and 7 were measured using the Luminescent Caspase-Glo 3/7 assay from Promega, except that luminescence was measured using the CLARIOstar multiplate reader (BMG labtech).

Cell cycle assays

Cell cycle was studied with the APC/BrdU flow kit from BD Pharmingen according to manufacturer's instructions. Briefly, 200 000 cells were transfected and seeded into 6-well plates in a volume of 2.5 ml. Three days later, BrdU was added in each well and incorporated into newly synthesized DNA by cells entering and progressing through the S phase of the cell cycle. The incorporated BrdU was stained with an APC anti-BrdU fluorescent antibody and the levels of cell-associated BrdU were then measured by flow cytometry on the BD FACS CANTO II.

Wnt transcriptional activity and associated reagents

Wnt transcriptional activity was assessed using the TOPflash/FOPflash assay. First, 200 000 cells were transfected with Ctrl or miR-4510 and seeded into 6-well plates in a volume of 2.5 ml. Two days later cells were collected, 10 000 cells were seeded into 96-well plates in a volume of 100 µL and transfected with the control plasmid pRL-TK-Renilla (Promega) and either the TOPFLASH or FOPFLASH plasmids kindly provided by Hans Clevers (Korinek V et al, *Science* 1997). Twenty-four hours later, cells were lysed and luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's instructions. The expression of 84 genes related to WNT-mediated signal transduction was estimated using Human Wnt Signaling Targets RT² Profiler PCR Array (Qiagen).

In vivo Chick Chorioallantoic Membrane (CAM) assays and immunohistological studies

Eggs opening: All the eggs were received at the stage of segmentation and then incubated at 37.4°C at 70% humidity for recovery development. Three days after developing, the eggshell were opened on the top and a plaster was placed on the gap.

Huh7 cells transfection and eggs implantation: A week after eggs opening, Huh7 cells were transfected with either 1027281 negative siRNA control from Qiagen or miR-4510 from Sigma at a final concentration of 12 nM by forward transfection using Lipofectamine RNAi Max (Invitrogen) according to manufacturer's instructions. One day later, cells were washed in PBS, detached with trypsin/EDTA and collected. Meanwhile, the egg plaster was removed, a plastic ring was added in the gap on the top of each chick CAM and 2 million transfected Huh7 cells were seeded on the CAM in the center of the ring. Finally the plaster was put back on the gap and eggs were re-incubated.

Tumour growth monitoring, fixation and CAM collection

Pictures of the growth tumour were done every day until 6 days using the stereomicroscope (SMZ745T) and camera (DS-Fi2) from Nikon and then analysed with the NSI Element D software. Three and 6 days after deposition of tumor cells, CAMs were fixed and processed for histology. Four µm-cut-sections of the tumor-containing CAM were performed and stained with Eosin-hematoxylin or rabbit polyclonal anti-cleaved-Caspase-3 antibody (AF835) (R&D systems), mouse monoclonal anti-GPC3 (C-1G12) antibody (Zytomed) systems or a mouse monoclonal anti-Ki-67 (MIB-1) antibody (Dako). Finally, tumor-CAM sections were scanned using a Hamamatsu Nanozoomer 2.0HT (Bordeaux Imaging Centre, Bordeaux University).

Bioinformatic tools

Different algorithms of prediction were used to investigate target:miRNA interactions including TargetScan, miRDB, TargetMiner, miRanda, RNA Hybrid, PICTAR5, DIANAmt and Diana lab. The

list of miR-4510-target genes generated from miRDB was imported to Ingenuity Pathways Analysis (IPA) to investigate the cellular functions and molecular pathways of miR-4510 target genes.

Statistical analyses

- 5 Graphs and statistical analyses were done using GraphPad Prism 5.0 or 6.03 software. When experiment contained two unmatched groups of values, the non-parametric Mann-Whitney test was used for the comparison of means. When experiment contained two matched groups of values, the parametric Wilcoxon matched-pairs signed rank test was used for the comparison of means. When experiment contained three groups of values or more, regular one-way analysis of variance (ANOVA) was used for
- 10 the comparison of multiple means. For each experiment, the ANOVA P value is as indicated in brackets. The ANOVA test was followed by a Bonferroni's or Holm-Sidak's multiple-comparison post-test and selected pairs of data were compared. For each figure, the number of independent experiments (n). The P value is indicated at the bottom of each Figure legend. Means were considered significantly different when $P < 0.05$. Significant variations were represented by asterisks above the corresponding bar when
- 15 comparing the test with the control condition or above the line when comparing the two indicated conditions.

Claims

- 1- A molecule selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or combination thereof or a DNA or RNA encoding for said miRNA
5 for use for treating a liver cancer.
- 2- The miRNA for use according to claim 1, wherein the liver cancer is a hepatocellular carcinoma.
- 3- The miRNA for use according to claim 1, wherein the liver cancer is a hepatoblastoma.
- 4- The miRNA for use according to any one of claims 1 to 3, wherein the molecule is to be used in combination with one or more therapeutic agents.
- 10 5- The miRNA for use according to claim 4, wherein the therapeutic agent is another antitumor therapy, in particular with doxorubicin, cisplatin, gemcitabine, oxaliplatin, mitomycin C, tamoxifen, sorafenib or any combination thereof.
- 6- The miRNA for use according to claim 4, wherein the therapeutic agent is an immunotherapeutic agent such as drugs targeting immune system checkpoints such as PD-1 or PD-L1.
15
- 7- The miRNA for use according to any one of claims 1 to 6, wherein the molecule is to be used before or after resection, radiofrequency ablation and/or percutaneous ethanol injection.
- 8- The miRNA for use according to any one of claims 1 to 5, wherein the molecule is for use as neo-adjuvant therapy or adjuvant therapy.
- 20 9- The miRNA for use according to any one of claims 1 to 7, wherein the subject has metastasis, and/or does not respond to the first line treatment and/or is not suitable for tumor resection or ablation.
- 10- The miRNA for use according to any one of claims 1 to 9, wherein the subject has a miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or combination thereof which is under-expressed in comparison with a healthy or non-tumoral control.
25
- 11- Use of a miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or of a combination thereof as a marker for detecting a liver cancer or a susceptibility to develop a liver cancer.
- 30 12- The use according to claim 11, wherein the liver cancer is a hepatocellular carcinoma or a hepatoblastoma.
- 13- A method for detecting a liver cancer or a susceptibility to develop a liver cancer in a subject, comprising determining the level of a miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or of a combination thereof in a
35 biological sample from the subject, an under-expression of at least one of the miRNA in comparison with a healthy or non-tumoral control being indicative of a liver cancer or a susceptibility to develop a liver cancer, preferably a hepatocellular carcinoma.

- 14- A method for selecting a subject suitable for a treatment by a molecule as disclosed in claim 1 comprising determining the level of a miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or of a combination thereof in a biological sample from the subject, and selecting the subject if at least one of the miRNA is under-expressed in comparison with a healthy or non-tumoral control.
- 5
- 15- A method for determining the prognosis in a subject having a liver cancer comprising determining the level of a miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or of a combination thereof in a biological sample from the subject, the level of expression of said at least one of the miRNA being correlated with the clinical prognosis.
- 10
- 16- Use of a kit for detecting a liver cancer or a susceptibility to develop a liver cancer in a subject or for selecting a subject suitable for a treatment by a molecule as disclosed in claim 1, or for determining the prognosis in a subject having a liver cancer, wherein the kit comprises a detection means for at least one miRNA selected from the group consisting of hsa-miR-4510, hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or for a combination thereof.
- 15

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FIGURE 1A

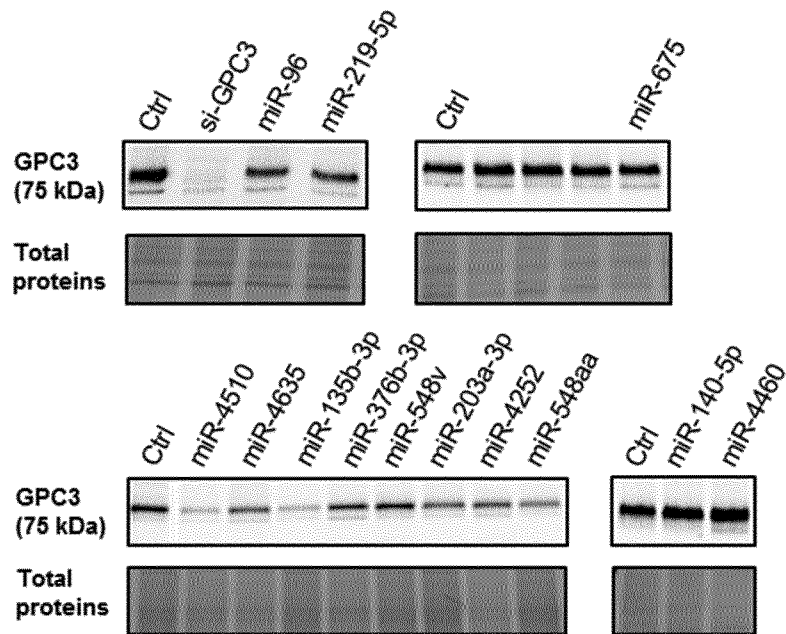
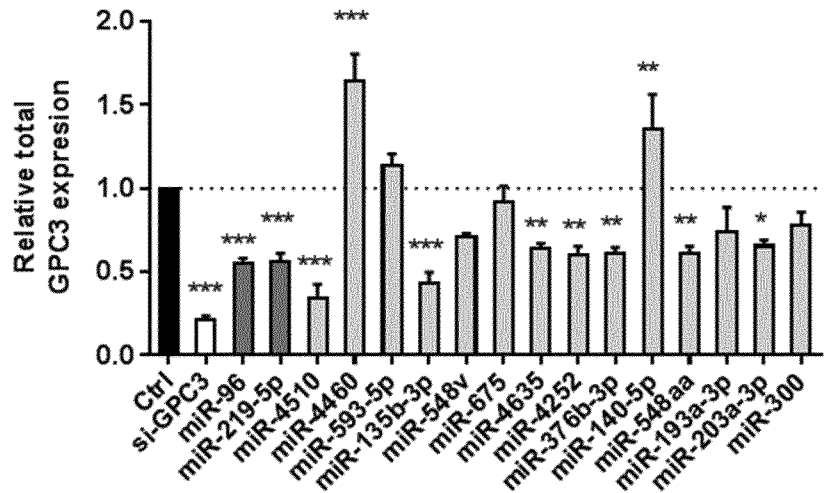
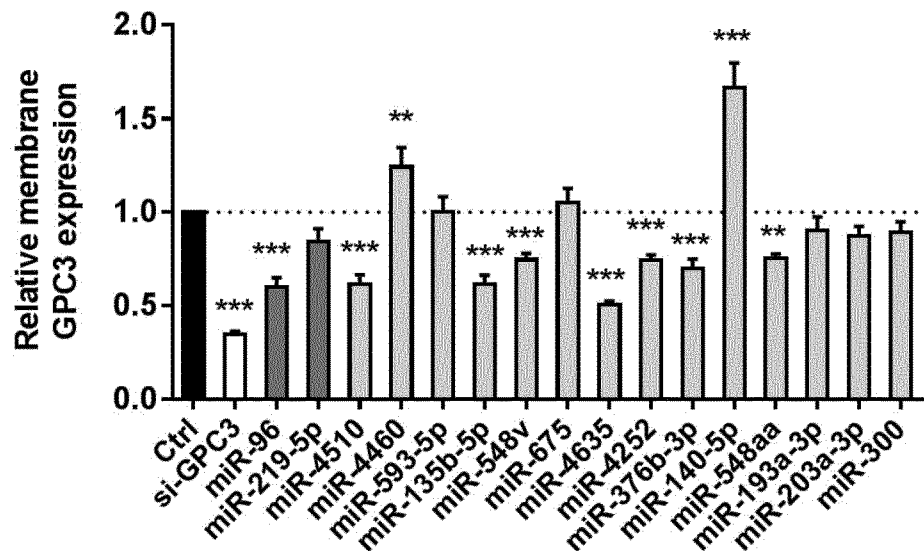
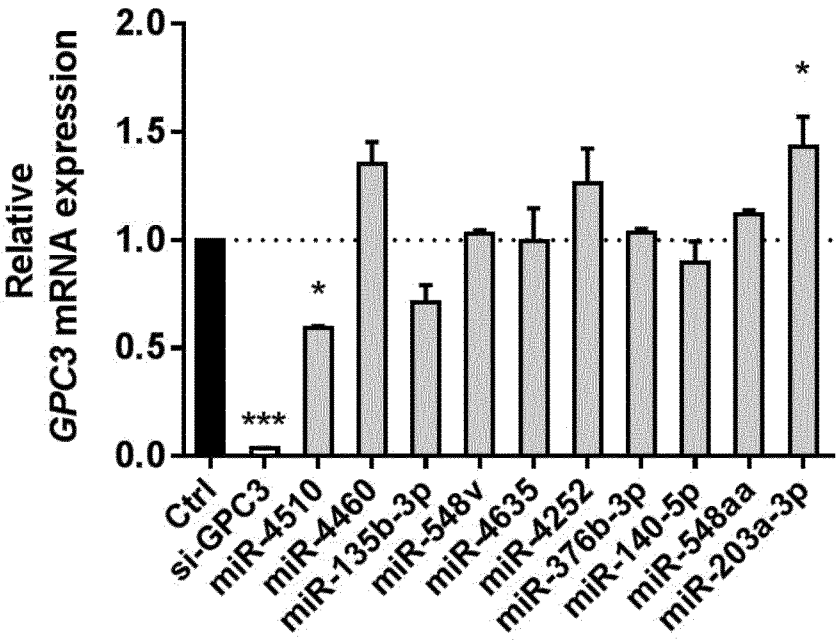


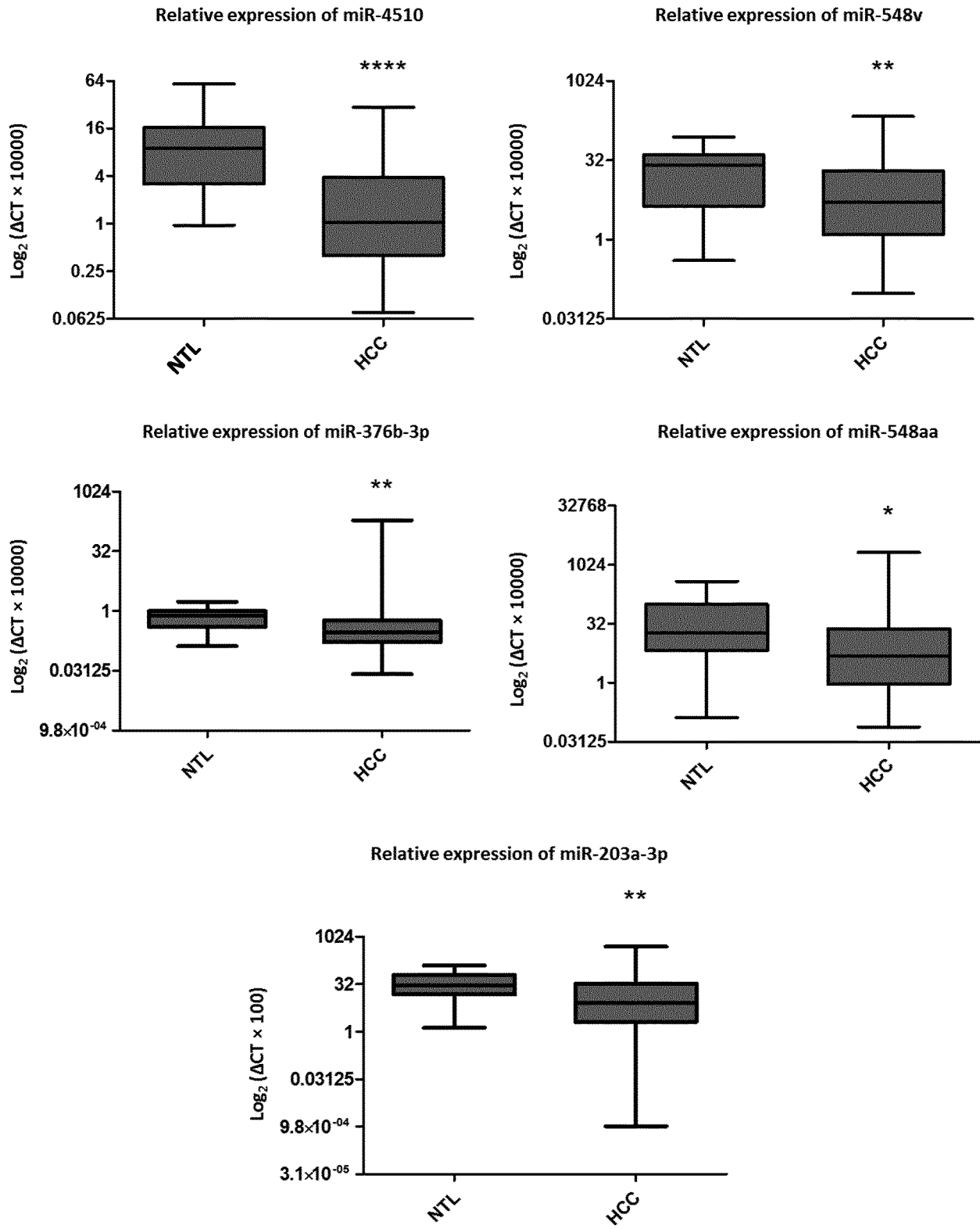
FIGURE 1B



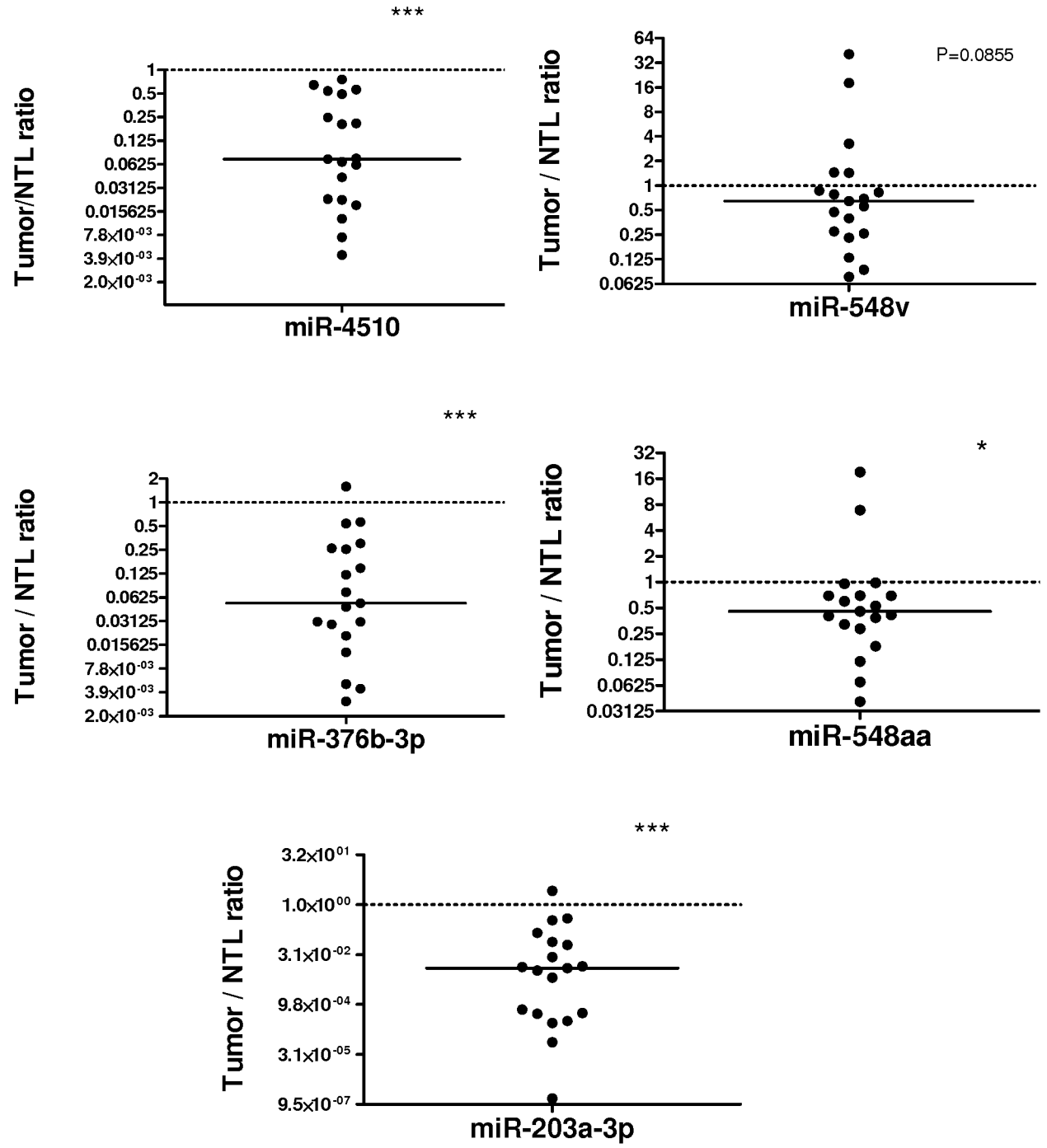
2/14
FIGURE 1C



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FIGURE 2



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FIGURE 3



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FIGURE 4

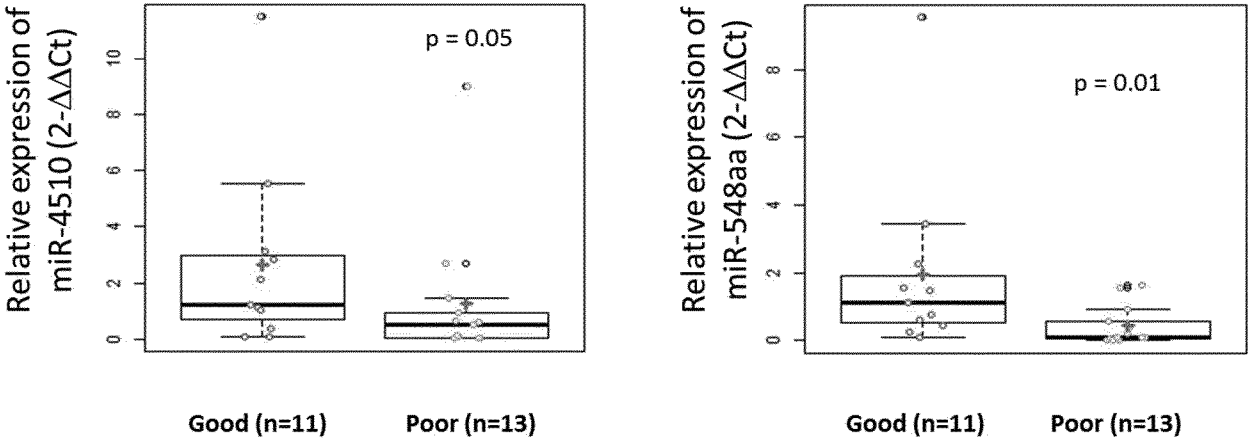
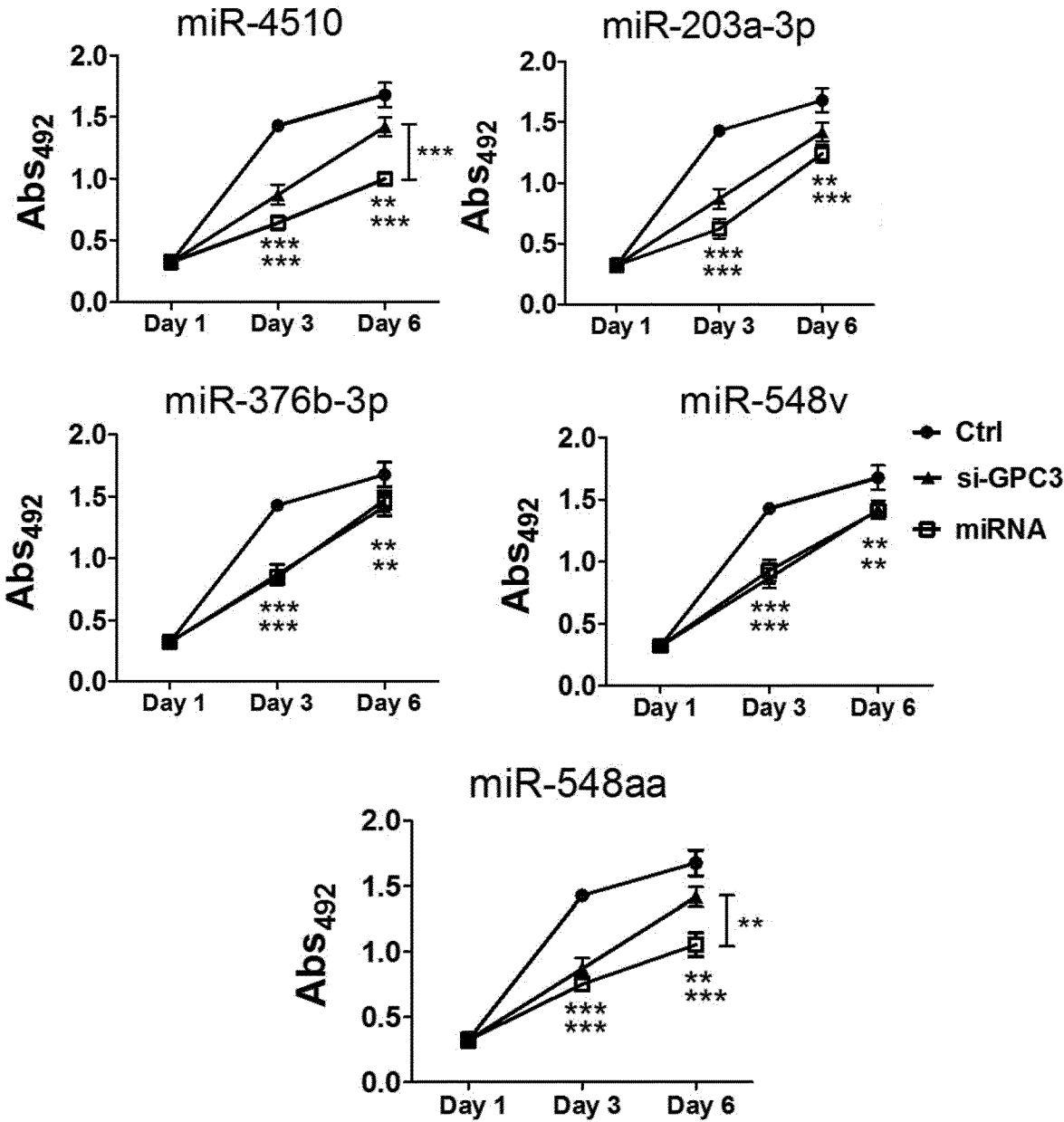


FIGURE 5A



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FIGURE 5B

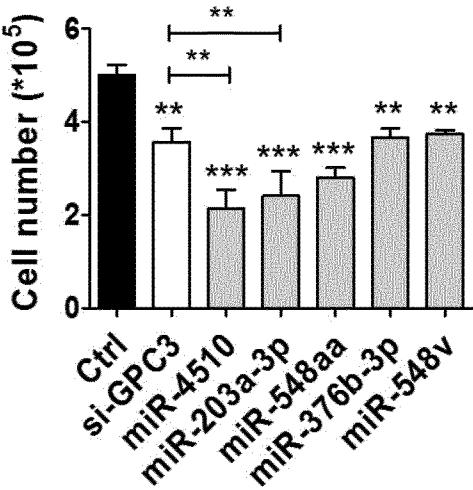


FIGURE 6

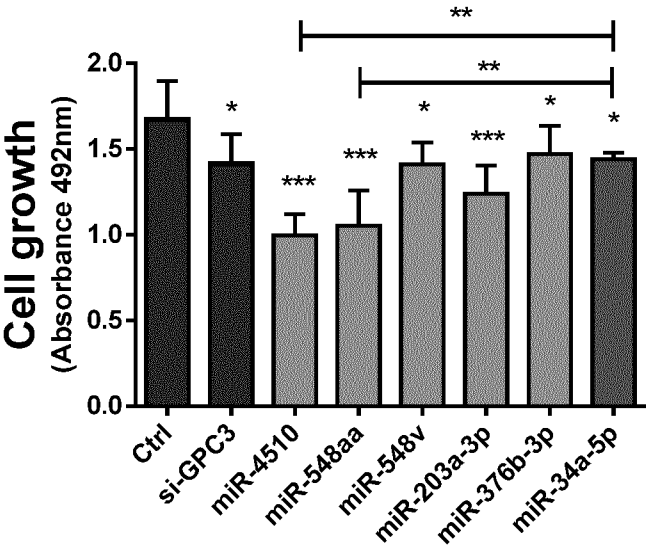
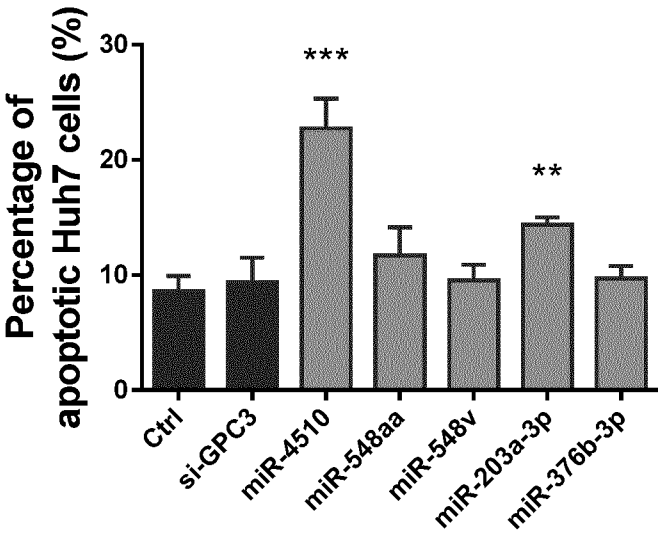


FIGURE 7



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FIGURE 8

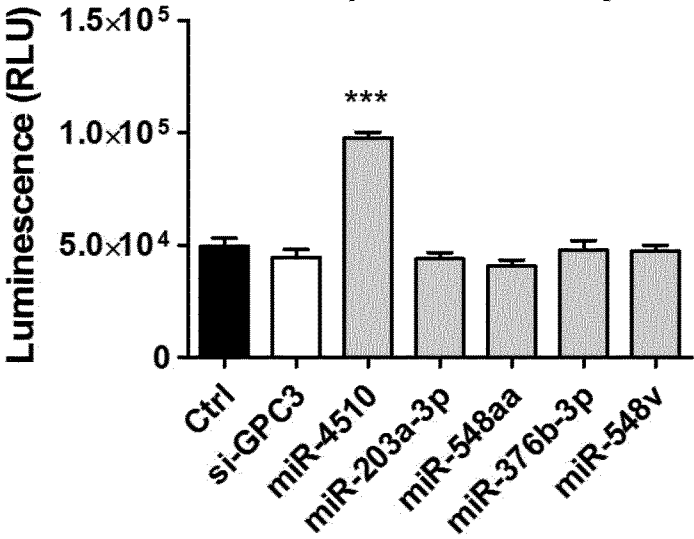
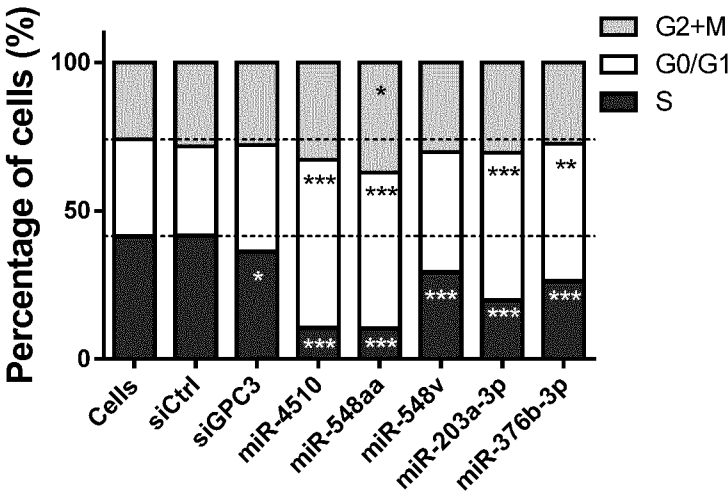
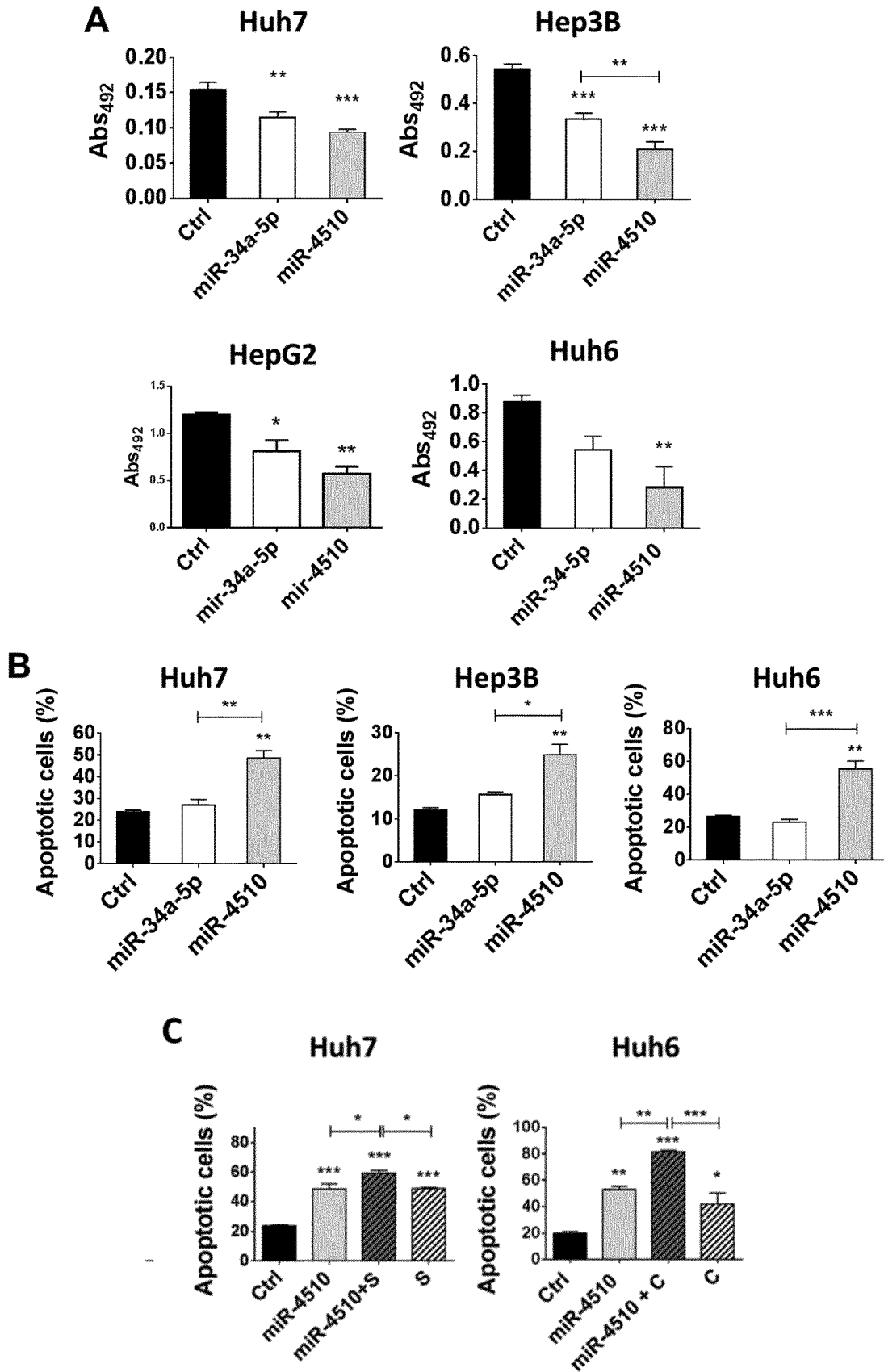


FIGURE 9



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FIGURE 10



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FIGURE 11

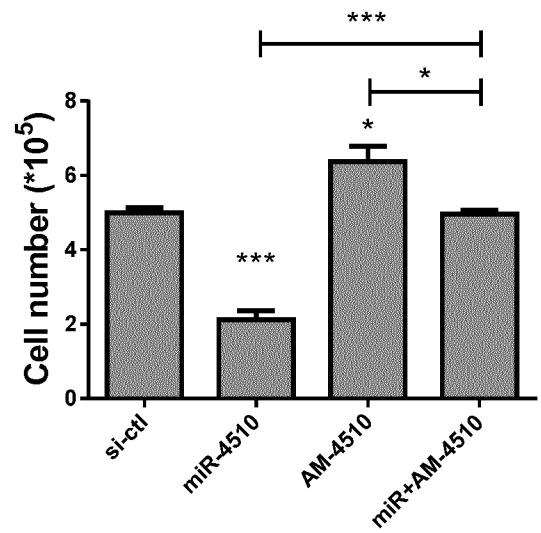


FIGURE 12

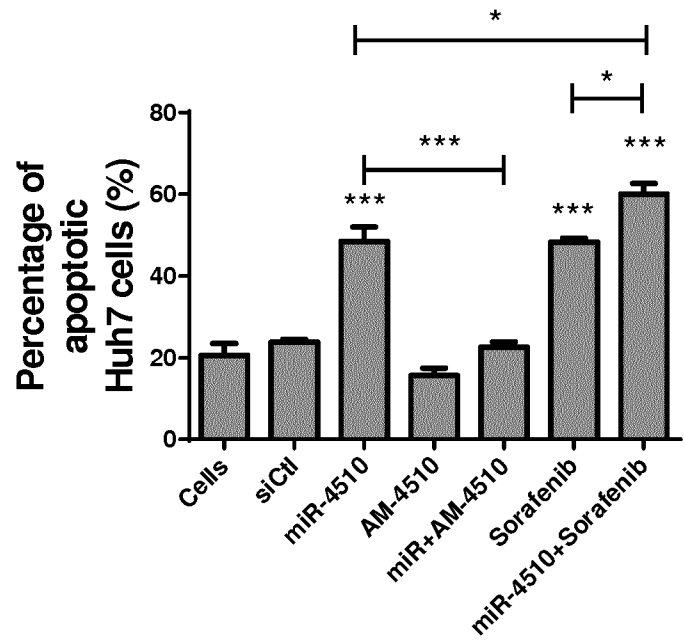
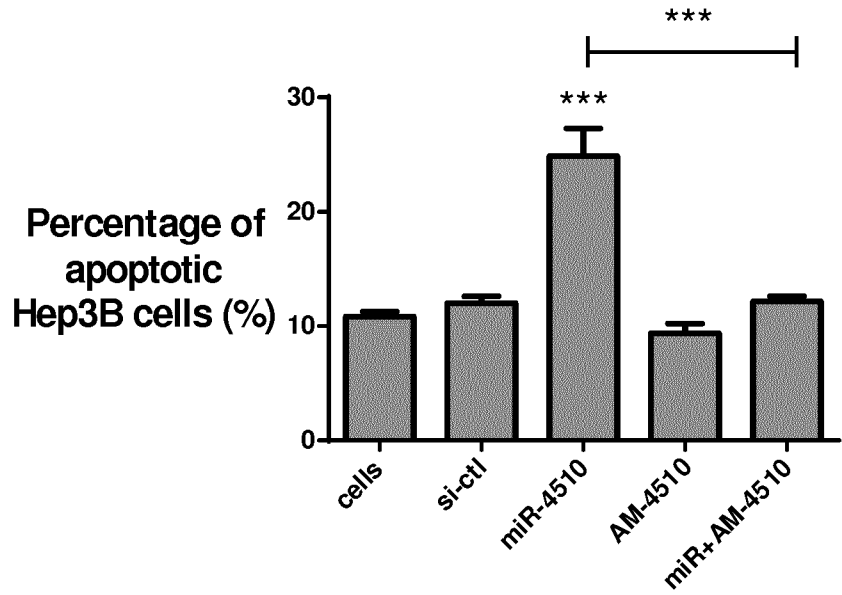


FIGURE 13



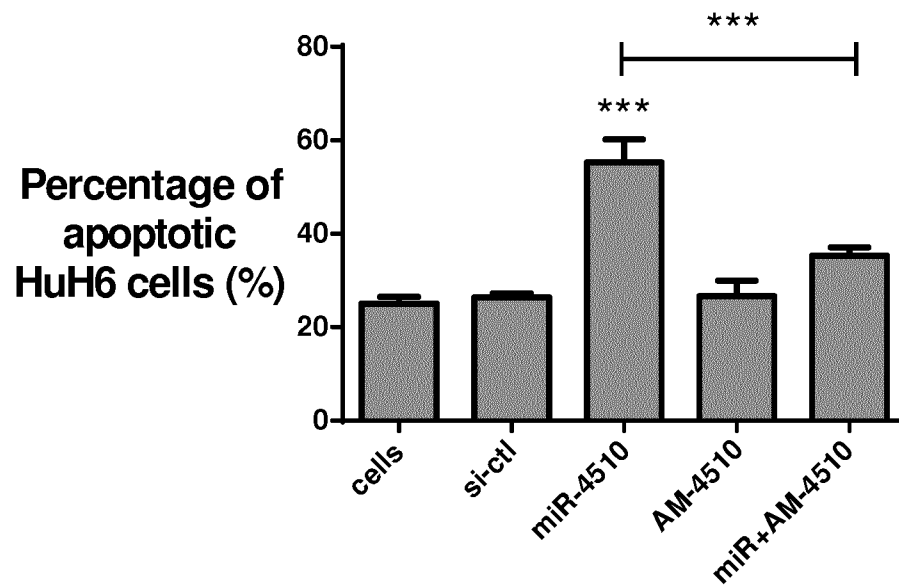
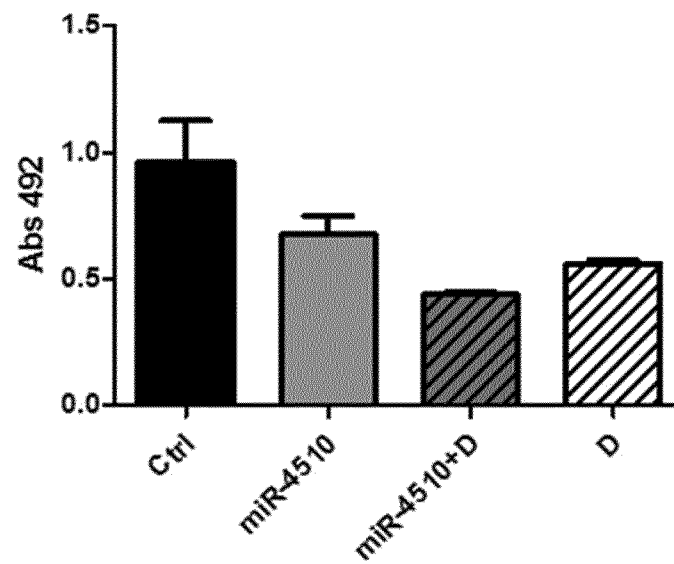
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FIGURE 14

FIGURE 15

Doxorubicin - Huh7



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FIGURE 16

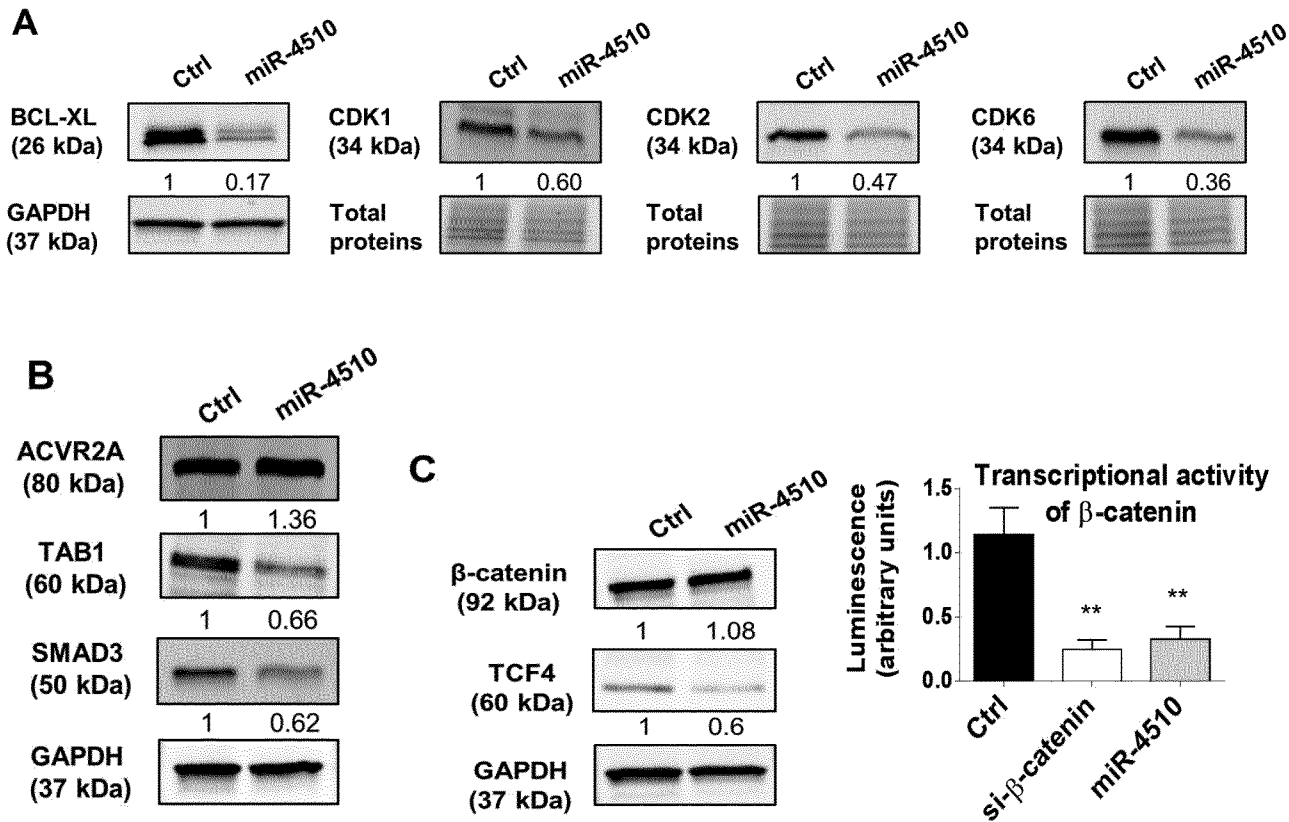
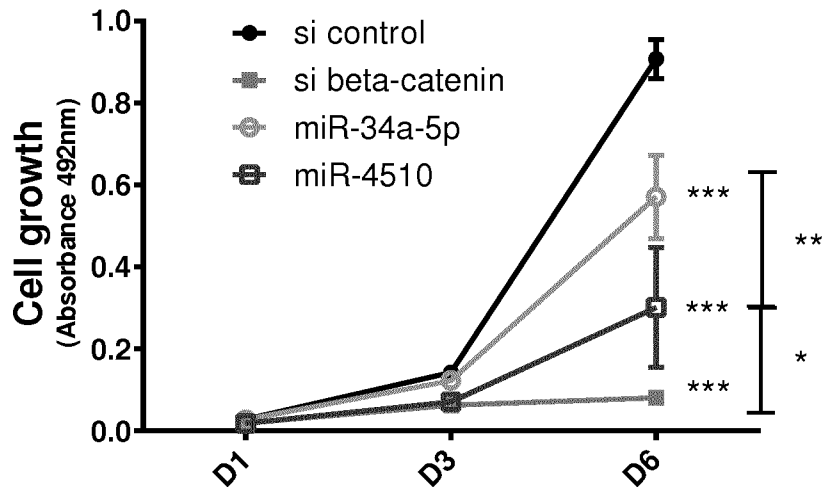


FIGURE 17



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FIGURE 18

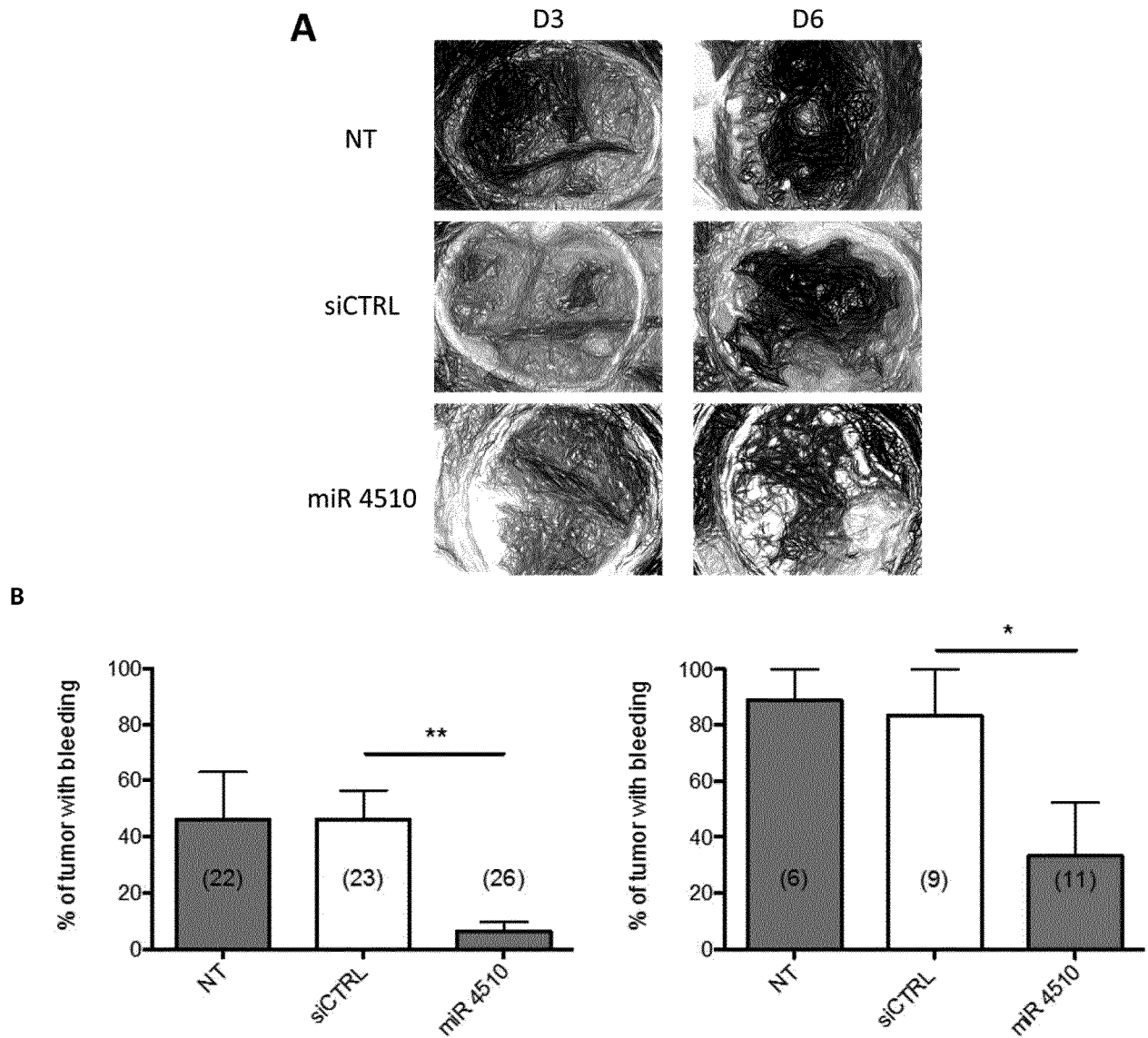


FIGURE 19

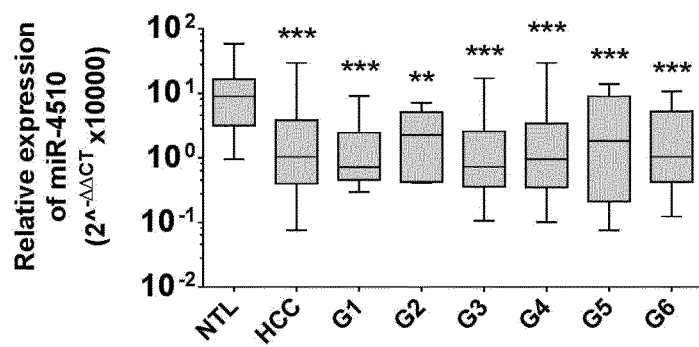
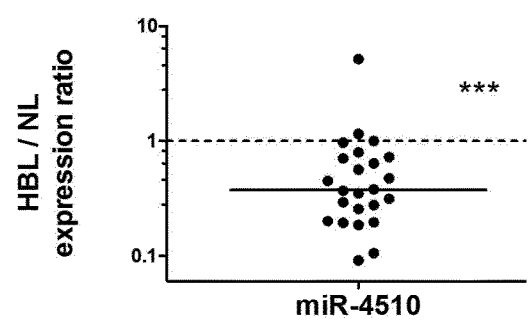


FIGURE 20



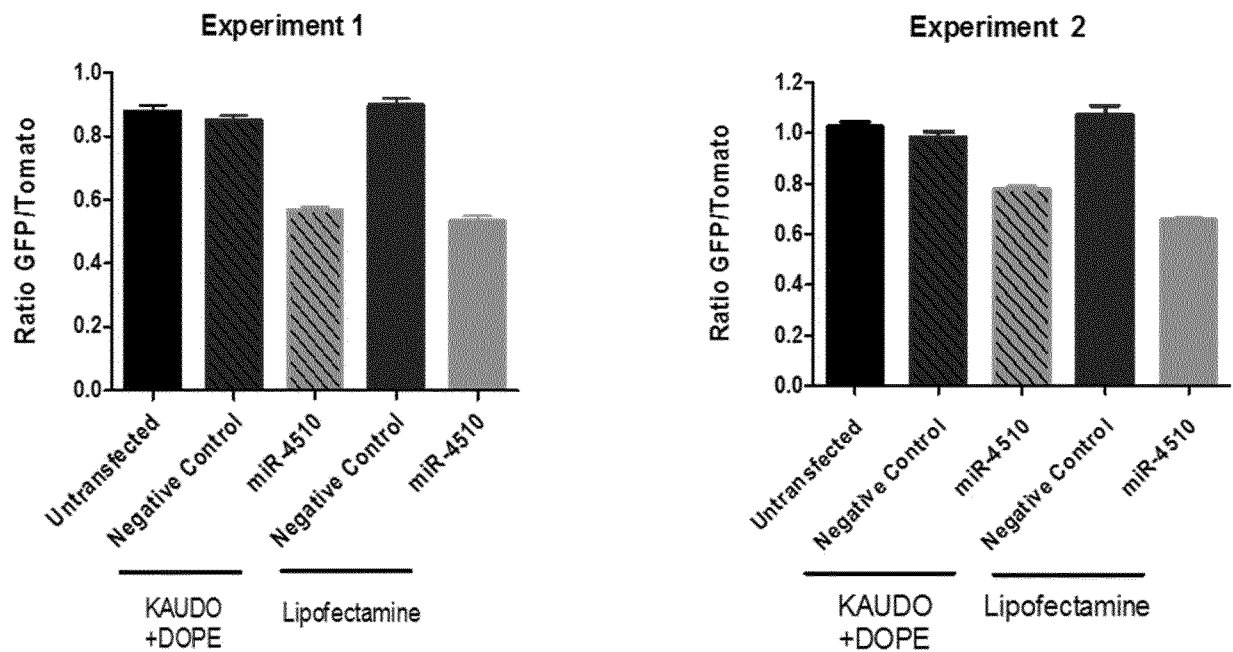
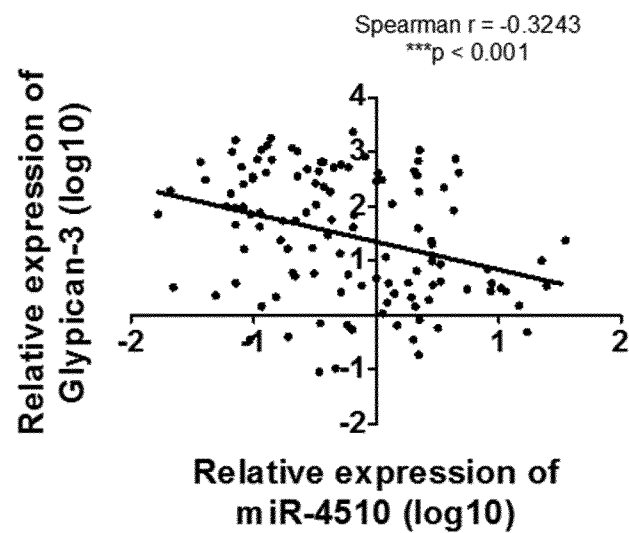
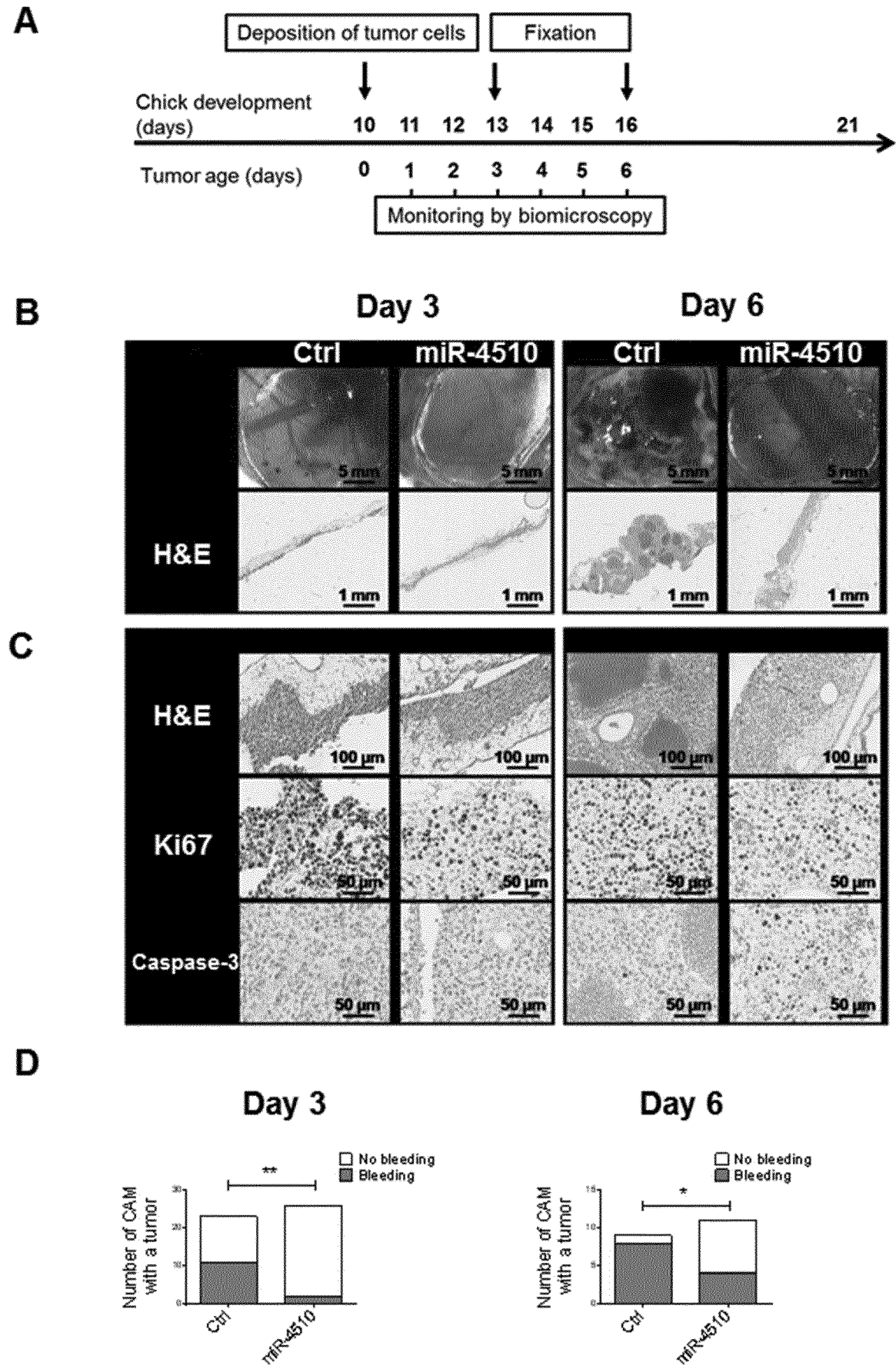
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FIGURE 21

FIGURE 22



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FIGURE 23



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/065933

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/11 A61K31/7088
ADD.

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)
C12N A61K

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)

EPO-Internal, EMBASE, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NA HUANG ET AL: "MiR-219-5p inhibits hepatocellular carcinoma cell proliferation by targeting glypican-3", FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 586, no. 6, 11 February 2012 (2012-02-11), pages 884-891, XP028472089, ISSN: 0014-5793, DOI: 10.1016/J.FEBSLET.2012.02.017 [retrieved on 2012-02-20] the whole document ----- -/--	1-16



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

5 October 2016

Date of mailing of the international search report

05/12/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Franz, Cerstin

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/065933

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MARION MAUREL ET AL: "A functional screening identifies five micrornas controlling glypican-3: role of mir-1271 down-regulation in hepatocellular carcinoma", HEPATOLOGY, vol. 57, no. 1, 7 January 2013 (2013-01-07), pages 195-204, XP055238650, USA ISSN: 0270-9139, DOI: 10.1002/hep.25994 the whole document	1-16
A	----- HUI-LAI MIAO ET AL: "MicroRNA-520c-3p inhibits hepatocellular carcinoma cell proliferation and invasion through induction of cell apoptosis by targeting glypican-3", HEPATOLOGY RESEARCH, vol. 44, no. 3, 10 March 2014 (2014-03-10), pages 338-348, XP055238653, NL ISSN: 1386-6346, DOI: 10.1111/hepr.12121 the whole document	1-16
A	----- X. LIU ET AL: "Expression of glypican 3 enriches hepatocellular carcinoma development-related genes and associates with carcinogenesis in cirrhotic livers", CARCINOGENESIS., vol. 36, no. 2, 1 February 2015 (2015-02-01), pages 232-242, XP055239027, GB ISSN: 0143-3334, DOI: 10.1093/carcin/bgu245 the whole document	1-16
A	----- S. JALVY-DELVAILLE ET AL: "Molecular basis of differential target regulation by miR-96 and miR-182: the Glypican-3 as a model", NUCLEIC ACIDS RESEARCH, vol. 40, no. 3, 1 February 2012 (2012-02-01), pages 1356-1365, XP055238778, GB ISSN: 0305-1048, DOI: 10.1093/nar/gkr843 the whole document	1-16
A	----- WO 2007/053659 A2 (US GOV HEALTH & HUMAN SERV [US]; ZHONGSHAN HOSPITAL [CN]; WANG XIN WEI) 10 May 2007 (2007-05-10) claims 1-51 ----- -/--	1-16

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/065933

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/022852 A1 (APTAMIR THERAPEUTICS INC [US]) 6 February 2014 (2014-02-06) the whole document -----	1-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/065933

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.:
because they relate to subject matter not required to be searched by this Authority, namely:
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).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-16(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-16(partially)

A molecule hsa-miR-4510 or combinations thereof comprising hsa-miR-548aa, hsa-miR-548v, hsa-miR-376b-3p or a DNA or RNA encoding for said miRNA for use in treating liver cancer, use of said miRNA or combinations thereof as marker for detecting liver cancer or susceptibility to develop a liver cancer, methods for detecting a liver cancer or a susceptibility to develop a liver cancer or for selecting a subject suitable for a treatment or determining the prognosis of a subject having liver cancer by determining the expression level of hsa-miR-4510 or combinations thereof, a kit comprising means for detecting said miRNA(s) and use of said kit for determining the prognosis in a subject having a liver cancer.

2. claims: 1-16(partially)

A molecule hsa-miR-548aa or combinations thereof comprising hsa-miR-4510, hsa-miR-548v, hsa-miR-376b-3p or a DNA or RNA encoding for said miRNA for use in treating liver cancer, use of said miRNA or combinations thereof as marker for detecting liver cancer or susceptibility to develop a liver cancer, methods for detecting a liver cancer or a susceptibility to develop a liver cancer or for selecting a subject suitable for a treatment or determining the prognosis of a subject having liver cancer by determining the expression level of hsa-miR-4510 or combinations thereof, a kit comprising means for detecting said miRNA(s) and use of said kit for determining the prognosis in a subject having a liver cancer.

3. claims: 1-16(partially)

A molecule hsa-miR-548v or combinations thereof comprising hsa-miR-548aa, hsa-miR-4510, hsa-miR-376b-3p or a DNA or RNA encoding for said miRNA for use in treating liver cancer, use of said miRNA or combinations thereof as marker for detecting liver cancer or susceptibility to develop a liver cancer, methods for detecting a liver cancer or a susceptibility to develop a liver cancer or for selecting a subject suitable for a treatment or determining the prognosis of a subject having liver cancer by determining the expression level of hsa-miR-4510 or combinations thereof, a kit comprising means for detecting said miRNA(s) and use of said kit for determining the prognosis in a subject having a liver cancer.

4. claims: 1-16(partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A molecule hsa-miR-376b-3p or combinations thereof comprising hsa-miR-548aa, hsa-miR-548v, hsa-miR-4510 or a DNA or RNA encoding for said miRNA for use in treating liver cancer, use of said miRNA or combinations thereof as marker for detecting liver cancer or susceptibility to develop a liver cancer, methods for detecting a liver cancer or a susceptibility to develop a liver cancer or for selecting a subject suitable for a treatment or determining the prognosis of a subject having liver cancer by determining the expression level of hsa-miR-4510 or combinations thereof, a kit comprising means for detecting said miRNA(s) and use of said kit for determining the prognosis in a subject having a liver cancer.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/065933

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007053659	A2	10-05-2007	NONE

WO 2014022852	A1	06-02-2014	CA 2880833 A1 06-02-2014
			EP 2880161 A1 10-06-2015
			HK 1211317 A1 20-05-2016
			US 2015216892 A1 06-08-2015
			WO 2014022852 A1 06-02-2014
