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(54) **Title**: DIAGNOSTIC KITS COMPRISING MICRORNA BIOMARKERS AND METHODS FOR DIAGNOSIS OF HEPATOCELLULAR CANCER

FIGURE 2

Name	Expression	Accuracy (%)
Tumor-related miRNA		
hsa-miR-122	UP	80
hsa-miR-199b-3p	UP	73
hsa-miR-10a	UP	72
hsa-miR-192	UP	65
hsa-miR-103	UP	68
hsa-miR-21	UP	65
hsa-miR-139-3p	DOWN	77
Plasma-specific miRNA		
hsa-miR-34a	UP	75
hsa-miR-136	UP	70
hsa-miR-151-5p	UP	70
hsa-miR-193b*	DOWN	76
hsa-miR-124	DOWN	74
hsa-miR-936	DOWN	75
hsa-miR-198	DOWN	73
Combination		
hsa-miR-34a/hsa-miR-193b*	UP	94
hsa-miR-34a/hsa-miR-198	UP	89
hsa-miR-192/hsa-miR-124	UP	87
hsa-miR-122/hsa-miR-193b*	UP	87
hsa-miR-122/hsa-miR-198	UP	87

(57) **Abstract**: The present invention provides microRNA biomarkers and diagnostic kits comprising a plurality of nucleic acid molecules encoding said microRNA biomarkers for identifying one or more target plasma exhibiting hepatocellular cancer. The invention also provides methods for diagnosis and treatment of hepatocellular cancer, and pharmaceutical compositions for prevention and treatment of hepatocellular cancer.

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COMPOSITIONS AND METHODS FOR MICRORNA EXPESSION PROFILING IN PLASMA OF HEPATOCELLULAR CANCER

FIELD OF THE INVENTION

The present invention relates to compositions and methods for microRNA expression profiling in plasma of hepatocellular cancer.

BACKGROUND OF THE INVENTION

Hepatocellular cancer (also referred to as hepatocellular carcinoma, HCC) is one of the most common and rapidly fatal human malignancies worldwide. It represents the major histological type of liver cancer and likely accounts for 70%-85% of all cases of liver cancer. Approx. 500,000 new cases occur worldwide every year, with almost the same number of fatalities, reflecting the lack of effective early detection and treatment options (Thorgeirsson, S.S. and Grisham, J.W. (2002) *Nat Genet* 31, 339-346; Parkin, D.M. et al. (2005) *CA Cancer J Clin* 55, 74-108; Bosch F.X. et al (2004) *Gastroenterology* 127, S5-S16; Perz J.F. et al. (2006) *J Hepatol* 45, 529-538).

Hepatocellular cancer thus represents a type of an extremely poor prognostic cancer. The prognosis of patients depends on the stage when the disease is diagnosed. The 5-year survival in HCC patients without operation is < 5%, while the postoperative 5-year survival is 60%-70%. When tumor size is < 2 cm with surgical removal, the 5-year survival can be reached to 86%. However, the 3-year survival in early cancer patients (tumor size < 5 cm) without any treatment is only 17-21%. This illustrates the early cancer detection is critical for the treatment and the patient survival (Tang, Z.Y. (2001) *World J Gastroenterol* 7, 445-454; Chambers, A.F. et al. (2002) *Nat Rev Cancer* 2, 563-572; Motola-Kuba D. et al. (2006) *Annals of Hepatology* 5, 16-24).

Although early detection and surgical removal of hepatocellular tumors have significantly improved the patient survival in recent years, the majority of tumors are still not detected early in tumor progression, that is, in a non-fatal stage. Only about 10%-20% of patients with hepatocellular cancer, defined by parameters of relatively normal liver function and a manageable tumor lesion as determined by the available

clinical staging systems, are currently eligible for surgical intervention. Moreover, patients who were resected often have a high frequency of metastasis/recurrence, and postoperative 5-year survival is only 30%-40%.

A definitive diagnosis of liver cancer is always based on histological confirmation. Tissue can be sampled with a needle aspiration or biopsy. However, some liver cancers are well differentiated, which means they are made up of nearly fully developed, mature hepatocytes. Therefore, these cancers can look very similar to non-cancerous liver tissue under a microscope. Moreover, not all pathologists are trained to recognize the subtle differences between well-differentiated liver cancer and normal liver tissue. Also, some pathologists can mistake liver cancer for adenocarcinoma in the liver. An adenocarcinoma is a different type of cancer, and it originates from outside of the liver. Most importantly, a metastatic adenocarcinoma would be treated differently from a primary liver cancer. Therefore, early detection of such tumors would be desirable in order to discriminate these different types of tumor and to guide the therapy decision in patients exhibiting a type of hepatocellular cancer and thus can markedly help to improve long-term survival.

The most common risk of the aspiration or biopsy in liver tissue is bleeding, especially because liver cancer is a tumor that is very vascular (contains many blood vessels). In many instances, there is probably no need for a tissue diagnosis by biopsy or aspiration. If a patient has a risk factor for liver cancer (for example, cirrhosis, chronic hepatitis B, or chronic hepatitis C) and a significantly elevated alpha-fetoprotein (AFP) blood level, the doctor can be almost certain that the patient has liver cancer without doing a biopsy. Currently, AFP is only serum marker used for the early detection of hepatocellular cancer (Mizejewski, G.J. (2003) Expert Rev Anticancer Ther 2, 709-735; Paul, S.B. et al. (2007) Oncology 72, Suppl. 1, 117-123). However, this single marker has a low specificity and is frequently inadequate because of false-positive results. The serum AFP test can readily detect hepatocellular tumors in only 60% of the patients. On the other hand, in a large number of cirrhosis patients, AFP can be elevated in the absence of cancerous states. Therefore, there is still a continuing need for the identification of alternative molecular markers and development of sensitive blood-based tests for early detection and differential diagnosis of hepatocellular cancer.

One approach to address this issue might be based on small regulatory RNA molecules, in particular on microRNAs (miRNAs) which, constitute an evolutionary conserved class of endogenously expressed small non-coding RNAs of 20-25 nucleotides (nt) in size that can mediate the expression of target mRNAs and thus – since their discovery about ten years ago – have been implicated with critical functions in cellular development, differentiation, proliferation, and apoptosis (Bartel, D.P. (2004) *Cell* 116, 281-297, Ambros, V. (2004) *Nature* 431, 350-355; He, L. et al. (2004) *Nat Rev Genet* 5, 522-531). Furthermore, miRNAs have advantages over mRNAs as cancer biomarkers, since they are very stable *in vitro* and long-lived *in vivo* (Lu, J. et al., (2005) *Nature* 435, 834-838; Lim, L.P. et al., (2005) *Nature* 433, 769-773).

MiRNAs are produced from primary transcripts that are processed to stem-loop structured precursors (pre-miRNAs) by the RNase III Drosha. After transport to the cytoplasm, another RNase III termed Dicer cleaves of the loop of the pre-miRNA hairpin to form a short double-stranded (ds) RNA, one strand of which is incorporated as mature miRNA into a miRNA-protein (miRNP). The miRNA guides the miRNPs to their target mRNAs where they exert their function (Bartel, D.P. (2004) *Cell* 23, 281-292; He, L. and Hannon, G.J. (2004) *Nat Rev Genet* 5, 522-531).

Depending on the degree of complementarity between the miRNA and its target, miRNAs can guide different regulatory processes. Target mRNAs that are highly complementary to miRNAs are specifically cleaved by mechanisms identical to RNA interference (RNAi). Thus, in such scenario, the miRNAs function as short interfering RNAs (siRNAs). Target mRNAs with less complementarity to miRNAs are either directed to cellular degradation pathways or are translationally repressed without affecting the mRNA level. However, the mechanism of how miRNAs repress translation of their target mRNAs is still a matter of controversy.

High-throughput miRNA quantification technologies, such as miRNA microarray, real-time RT-PCR-based TaqMan miRNA assays, have provided powerful tools to study the global miRNA profile in whole cancer genome. Emerging data available indicate that dysregulation of miRNA expression may inter alia be associated with the development and/or progression of certain types of cancer. For example, two miRNAs, miR-15 and miR-16-1, were shown to map to a genetic locus that is deleted in

chronic lymphatic leukemia (CLL) and it was found that in about 70% of the CLL patients, both miRNA genes are deleted or down-regulated. Furthermore, down-regulation of miR-143 and miR-145 was observed in colorectal neoplasia, whereas expression of the miRNA let-7 is frequently reduced in lung cancers (Michael, M.Z. et al. (2003) *Mol Cancer Res* 1, 882-891; Mayr, C. et al. (2007) *Science* 315, 1576-1579). In fact, it has been speculated based on cancer-associated alterations in miRNA expression and the observation that miRNAs are frequently located at genomic regions involved in cancers that miRNAs may act both as tumor suppressors and as oncogenes (Esquela-Kerscher, A. and Slack, F.J (2006) *Nat Rev Cancer* 6, 259-269; Calin, G.A. and Croce, C.M. (2007) *J Clin Invest* 117, 2059-2066; Blenkiron, C. and Miska, E.A. (2007) *Hum Mol Genet* 16, R106-R113). Demonstrated abnormal expression patterns of miRNAs in human cancers highlight their potential use as diagnostic and prognostic biomarkers.

Several studies have reported miRNA expression profiling in human hepatocellular cancer (Murakami, Y. et al. (2006) *Oncogene* 25, 2537-2545; Li, W. et al. (2008) *Int J Cancer* 123, 1616-1622; Huang, Y.S. et al. (2008) *Hepatology* 23, 87-94; Ladeiro, Y. et al. (2008) *Hepatology* 47, 1955-1963; Jiang, J. et al. (2008) *Clin Cancer Res* 14, 419-427). Consistently, these studies have shown that specific miRNAs are aberrantly expressed in malignant cells or tissues as compared to nonmalignant hepatocytes or tissue. Thus, such miRNAs may provide insights into cellular processes involved in malignant transformation and progression.

Among the many possible types of samples, blood is thought to be ideal for screening high risk individuals, leading to early detection, diagnosis, monitoring and efficient treatment of cancers– since blood can be collected easily in a minimally invasive manner. It has been demonstrated that tumor-derived miRNAs are present in human plasma or serum in a remarkably stable form that is protected from endogenous RNase activity. These tumor-derived miRNAs in serum or plasma are at levels sufficient to be measurable as biomarkers for cancer detection. Moreover, the levels of plasma and serum miRNAs correlate strongly, suggesting that either plasma or serum samples will be suitable for clinical applications using miRNAs as cancer diagnostic biomarkers (Mitchell, P.S. et al. (2008) *Proc Natl Acad Sci USA* 105, 10513–10518;

Gilad, S. et al. (2008) *PLoS ONE* 3, e3148; Chen, X. et al. (2008) *Cell Res* 18, 997-1006). To date, no blood-based miRNA biomarker has been identified in plasma or serum of hepatocellular cancer patients.

Thus, there is urgently needed for blood-based diagnostic markers, particularly in form of a "expression signature" or a "molecular footprint", that enable the rapid, reliable and cost-saving detection of hepatocellular cancer. In addition, there is also a continuing need for corresponding methods for early stage-HCC screening in high risk individuals, differential HCC diagnosis, early detection of the cancer recurrence, and/or monitoring the cancer therapy.

OBJECT AND SUMMARY OF THE INVENTION

It is an objective of the present invention to provide novel approaches for diagnosing hepatocellular cancer, monitoring the cancer therapy and/or treating the cancer by determining a plurality of nucleic acid molecules in blood, each nucleic acid molecule encoding a microRNA (miRNA) sequence, wherein one or more of the plurality of nucleic acid molecules are differentially expressed in plasma of hepatocellular cancer, analyzed as compared to healthy controls, and/or as compared to healthy individuals, colorectal cancer and lung cancer, wherein the one or more differentially expressed nucleic acid molecules together represent a nucleic acid expression signature that is indicative for the presence of hepatocellular cancer, wherein the nucleic acid expression signatures include tumor-related signatures and plasma-specific signatures.

Furthermore, it is an object of the invention to provide corresponding methods for identifying one or more nucleic acid expression signatures in blood exhibiting hepatocellular cancer. More specifically, it is an object of the invention to provide methods for differentiating hepatocellular cancer as compared to healthy control, and/or as compared to healthy individuals, colorectal cancer and lung cancer.

These objectives as well as others, which will become apparent from the ensuing description, are attained by the subject matter of the independent claims. Some of the preferred embodiments of the present invention are defined by the subject matter of the dependent claims.

In a first aspect, the present invention relates to a diagnostic kit of molecular markers in blood for identifying hepatocellular cancer, the kit comprising a plurality of nucleic acid molecules, each nucleic acid molecule encoding a microRNA sequence, wherein one or more of the plurality of nucleic acid molecules are differentially expressed in the target plasma as compared to healthy controls, and wherein the differentially expressed signatures are derived from tumor-related or plasma-specific signatures, and wherein the one or more differentially expressed nucleic acid molecules together represent a nucleic acid expression signature that is indicative for the presence of hepatocellular cancer.

The nucleic acid expression signature, as defined herein, may comprise at least thirty-twonucleic acid molecules, preferably at least twelve nucleic acid molecules, and particularly preferably at least sixnucleic acid molecules.

In preferred embodiments, the nucleic acid expression signature comprises at least one nucleic acid molecule encoding a microRNA sequence whose expression is up-regulated in the one or more target plasma compared to the one or more healthy controls and at least one nucleic acid molecule encoding a microRNA sequence whose expression is down-regulated in the one or more target plasma compared to the one or more healthy controls.

In preferred embodiments, the nucleic acid expression signature comprises any one or more of the nucleic acid molecules encoding tumor-related signatures hsa-miR-122, hsa-miR-199b-3p, hsa-miR-192, hsa-miR-21, hsa-miR-139-3p, hsa-miR-10a, hsa-miR-103, hsa-miR-181d, hsa-miR-125b-2*, hsa-miR-125a-3p; plasma-specific signatures hsa-miR-34a, hsa-miR-136, hsa-miR-151-5p, hsa-miR-193b*, hsa-miR-124, hsa-miR-936, hsa-miR-198, hsa-miR-149*, hsa-miR-138, hsa-miR-601, hsa-miR-769-3p, hsa-miR-513c, hsa-miR-525-5p, hsa-miR-654-5p, hsa-miR-518c*, hsa-miR-500, hsa-miR-181c*, hsa-miR-1226*, hsa-miR-202, hsa-miR-629* and internal stable controls hsa-miR-1238 and hsa-miR-1228.

Particularly preferably, the expression of any one or more of the nucleic acid molecules encoding hsa-miR-122, hsa-miR-199b-3p, hsa-miR-192, hsa-miR-21, hsa-miR-10a, hsa-miR-103, hsa-miR-34a, hsa-miR-136, hsa-miR-151-5p is up-regulated and the expression of any one or more of the nucleic acid molecules encoding hsa-miR-

139-3p, hsa-miR-193b*, hsa-miR-124, hsa-miR-181d, hsa-miR-125b-2*, hsa-miR-125a-3p, hsa-miR-936, hsa-miR-198, hsa-miR-149*, hsa-miR-138, hsa-miR-601, hsa-miR-769-3p, hsa-miR-513c, hsa-miR-525-5p, hsa-miR-654-5p, hsa-miR-518c*, hsa-miR-500, hsa-miR-181c*, hsa-miR-1226*, hsa-miR-202, hsa-miR-629* is down-regulated; hsa-miR-1238 and hsa-miR-1228 is un-changed in the one or more target plasma compared to the one or more healthy controls.

In more preferred embodiments, the nucleic acid expression signature comprises any one or more of the nucleic acid molecules encoding tumor-related signatures hsa-miR-122, hsa-miR-199b-3p, hsa-miR-192, hsa-miR-21, hsa-miR-139-3p, hsa-miR-10a, hsa-miR-103 and plasma-specific signatures hsa-miR-34a, hsa-miR-136, hsa-miR-151-5p, hsa-miR-193b*, hsa-miR-124.

Particularly preferably, the expression of any one or more of the nucleic acid molecules encoding hsa-miR-122, hsa-miR-199b-3p, hsa-miR-192, hsa-miR-21, hsa-miR-10a, hsa-miR-103, hsa-miR-34a, hsa-miR-136, hsa-miR-151-5p is up-regulated and the expression of any one or more of the nucleic acid molecules encoding hsa-miR-139-3p, hsa-miR-193b*, hsa-miR-124 is down-regulated in the one or more target plasma compared to the one or more healthy controls.

In further preferred embodiments, the nucleic acid expression signature comprises any one or more of the nucleic acid molecules encoding tumor-related signatures hsa-miR-122, hsa-miR-199b-3p, hsa-miR-192, hsa-miR-21, hsa-miR-139-3p and plasma-specific signatures hsa-miR-34a.

Particularly preferably, the expression of any one or more of the nucleic acid molecules encoding hsa-miR-122, hsa-miR-199b-3p, hsa-miR-192, hsa-miR-21, hsa-miR-34a is up-regulated and the expression of any one or more of the nucleic acid molecules encoding hsa-miR-139-3p is down-regulated in the one or more target plasma compared to the one or more healthy controls.

In particularly preferred embodiments, the nucleic acid expression signature comprises any one or more nucleic acid combinations encoding hsa-miR-34a/hsa-miR-193b*, hsa-miR-21/hsa-miR-936, hsa-miR-192/hsa-miR-124, hsa-miR-122/hsa-miR-193b*, hsa-miR-34a/hsa-miR-138, hsa-miR-34a/hsa-miR-198, hsa-miR-122/hsa-miR-

124, hsa-miR-103/hsa-miR-139-3p, hsa-miR-192/hsa-miR-193b*, hsa-miR-122/hsa-miR-138, hsa-miR-34a/hsa-miR-139-3p, hsa-miR-122/hsa-miR-198, hsa-miR-122/hsa-miR-769-3p, hsa-miR-103/hsa-miR-193b*, hsa-miR-34a/hsa-miR-124, hsa-miR-192/hsa-miR-139-3p, hsa-miR-34a/hsa-miR-769-3p, hsa-miR-192/hsa-miR-936, hsa-miR-10a/hsa-miR-193b*, hsa-miR-122/hsa-miR-601, hsa-miR-21/hsa-miR-769-3p, hsa-miR-199b-3p/hsa-miR-193b*, hsa-miR-103/hsa-miR-138, hsa-miR-103/hsa-miR-198, hsa-miR-199b-3p/hsa-miR-139-3p, hsa-miR-21/hsa-miR-139-3p and hsa-miR-21/hsa-miR-193b*.

In a second aspect, the present invention relates to a diagnostic kit of molecular markers for discriminating hepatocellular cancer from healthy individuals, colorectal cancer and lung cancer, the kit comprising a plurality of nucleic acid molecules, each nucleic acid molecule encoding a microRNA sequence, wherein one or more of the plurality of nucleic acid molecules are differentially expressed in the target plasma and in one or more healthy individuals, colorectal cancer and lung cancer, and wherein the one or more differentially expressed nucleic acid molecules together represent a nucleic acid expression signature that is indicative for the presence of hepatocellular cancer.

The nucleic acid expression signature, as defined herein, may comprise at least sixteen nucleic acid molecules, preferably at least six nucleic acid molecules.

In preferred embodiments, the nucleic acid expression signature comprises at least one nucleic acid molecule encoding a microRNA sequence whose expression is up-regulated in the one or more target plasma compared to the one or more healthy individuals, colorectal cancer and lung cancer, and at least one nucleic acid molecule encoding a microRNA sequence whose expression is down-regulated in the one or more target plasma compared to the one or more healthy individuals, colorectal cancer and lung cancer.

In preferred embodiments, the nucleic acid expression signature comprises any one or more of the nucleic acid molecules encoding tumor-related signatures: hsa-miR-122, hsa-miR-192, hsa-miR-215, hsa-let-7c, hsa-miR-103, hsa-miR-139-3p, hsa-miR-26b; plasma-specific signatures: hsa-miR-936, hsa-miR-193b*, hsa-miR-124, hsa-miR-34a, hsa-miR-198, hsa-let-7g and hsa-miR-363 and internal stable controls: has-miR-1228 and hsa-miR-1238.

Particularly preferably, the expression of any one or more of the nucleic acid molecules encoding hsa-miR-122, hsa-miR-192, hsa-miR-215, hsa-let-7c, hsa-miR-103, hsa-miR-26b, hsa-miR-34a, hsa-let-7g, hsa-miR-363 is up-regulated and the expression of any one or more of the nucleic acid molecules encoding hsa-miR-936, hsa-miR-193b*, hsa-miR-124, hsa-miR-139-3p, hsa-miR-198 is down-regulated; hsa-miR-1238 and hsa-miR-1228 is un-changed in the one or more target plasma compared to the one or more healthy individuals, colorectal cancer and lung cancer.

In more preferred embodiments, the nucleic acid expression signature comprises any one or more of the nucleic acid molecules encoding tumor-related signatures: hsa-miR-122, hsa-miR-192, hsa-miR-215 and plasma-specific signatures: hsa-miR-936, hsa-miR-193b* and hsa-miR-124.

Particularly preferably, the expression of any one or more of the nucleic acid molecules encoding hsa-miR-122, hsa-miR-192, hsa-miR-215 is up-regulated and the expression of any one or more of the nucleic acid molecules encoding hsa-miR-936, hsa-miR-193b* and hsa-miR-124 is down-regulated in the one or more target plasma compared to the one or more healthy individuals, colorectal cancer and lung cancer.

In particularly preferred embodiments, the nucleic acid expression signature comprises any one or more nucleic acid combinations encoding hsa-miR-122/hsa-miR-936, hsa-miR-34a/hsa-miR-193b*, hsa-miR-34a/hsa-miR-198, hsa-miR-192/hsa-miR-936, hsa-miR-122/hsa-miR-193b*, hsa-miR-122/hsa-miR-198, hsa-miR-192/hsa-miR-124, hsa-miR-192/hsa-miR-193b*, hsa-miR-122/hsa-miR-124, hsa-miR-192/hsa-miR-198, hsa-miR-363/hsa-miR-936, hsa-miR-215/hsa-miR-193b*, hsa-miR-103/hsa-miR-936, hsa-miR-122/hsa-miR-139-3p, hsa-let-7c/hsa-miR-936, hsa-miR-215/hsa-miR-198, hsa-miR-192/hsa-miR-139-3p, hsa-miR-27b/hsa-miR-198, hsa-miR-26b/hsa-miR-936, hsa-let-7g/hsa-miR-936, hsa-miR-103/hsa-miR-198, hsa-let-7c/hsa-miR-193b*, hsa-miR-103/hsa-miR-193b*, hsa-miR-26b/hsa-miR-139-3p, hsa-let-7c/hsa-miR-198, hsa-miR-27b/hsa-miR-193b*, hsa-let-7g/hsa-miR-193b*, hsa-miR-363/hsa-miR-139-3p, hsa-let-7g/hsa-miR-198, hsa-miR-363/hsa-miR-198, hsa-miR-26b/hsa-miR-198, hsa-miR-103/hsa-miR-139-3p, hsa-miR-301a/hsa-miR-198, hsa-miR-26b/hsa-miR-193b*, hsa-let-7g/hsa-miR-139-3p, hsa-miR-363/hsa-miR-124, hsa-let-7c/hsa-miR-139-3p, hsa-miR-301a/hsa-miR-193b*, hsa-miR-301a/hsa-miR-139-3p, hsa-miR-26b/hsa-miR-

124, hsa-let-7d/hsa-miR-198, hsa-let-7d/hsa-miR-139-3p, hsa-miR-103/hsa-miR-124, hsa-miR-363/hsa-miR-193b* and hsa-let-7d/hsa-miR-193b*.

In a third aspect, the present invention relates to a method for identifying one or more target plasma exhibiting hepatocellular cancer, the method comprising: (a) determining in the one or more target plasma the expression levels of a plurality of nucleic acid molecules, each nucleic acid molecule encoding a microRNA sequence; (b) determining the expression levels of the plurality of nucleic acid molecules in one or more healthy control plasma; and (c) identifying from the plurality of nucleic acid molecules one or more nucleic acid molecules that are differentially expressed in the target and control plasma by comparing the respective expression levels obtained in steps (a) and (b), wherein the one or more differentially expressed nucleic acid molecules together represent a nucleic acid expression signature, as defined herein, that is indicative for the presence of hepatocellular cancer.

In preferred embodiments of the invention, the method comprising: (a) determining in the one or more target plasma the expression levels of a combination of nucleic acid molecules, each nucleic acid molecule encoding a microRNA sequence, and calculate with certain formula, then; (b) determining the expression levels of the combination of nucleic acid molecules in healthy control plasma, and calculate with certain formula; and (c) identifying the difference of the combination in the one or more target and control plasma by comparing the respective calculation results obtained in steps (a) and (b), wherein the one or more differentially expressed combinations together represent a signature, as defined herein, that is indicative for the presence of hepatocellular cancer.

In more preferred embodiments of the invention, the method is for the further use of discriminating hepatocellular cancer from healthy individuals, colorectal cancer and lung cancer.

In a fourth aspect, the present invention relates to a method for monitoring treatment of hepatocellular cancer, the method comprising: (a) identifying in the one or more target plasma a nucleic acid expression signature by using a method, as defined herein; and (b) monitoring in blood the expression of one or more nucleic acid molecules encoding a microRNA sequence that is/are comprised in the nucleic acid

expression signature in such way that the expression of a nucleic acid molecule whose expression in plasma is up-regulated before treatment but is down-regulated after treatment and the expression of a nucleic acid molecule whose expression in plasma is down-regulated before treatment but is up-regulated after treatment.

In a fifth aspect, the present invention relates to a method for preventing or treating hepatocellular cancer, the method comprising: (a) identifying in plasma a nucleic acid expression signature by using a method, as defined herein; and (b) modifying in blood the expression of one or more nucleic acid molecules encoding a microRNA sequence that is/are comprised in the nucleic acid expression signature in such way that the expression of a nucleic acid molecule whose expression is up-regulated in plasma is down-regulated and the expression of a nucleic acid molecule whose expression is down-regulated in plasma is up-regulated.

In a sixth aspect, the present invention relates to a pharmaceutical composition for the prevention and/or treatment of hepatocellular cancer in blood, the composition comprising one or more nucleic acid molecules, each nucleic acid molecule encoding a sequence that is at least partially complementary to a microRNA sequence encoded by a nucleic acid molecule whose expression is up-regulated in plasma from hepatocellular cancer patients, as defined herein, and/or that corresponds to a microRNA sequence encoded by a nucleic acid molecule whose expression is down-regulated in plasma from hepatocellular cancer patients, as defined herein.

Finally, in a seventh aspect, the present invention relates to the use of said pharmaceutical composition for the manufacture of a medicament for the prevention and/or treatment of hepatocellular cancer.

Other embodiments of the present invention will become apparent from the detailed description hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a flow chart schematically illustrating the essential method steps for determining an expression signature according to the present invention for identifying one or more target plasma exhibiting hepatocellular cancer.

Figure 2 illustrates the human miRNAs comprised in particularly preferred expression signatures in the first aspect according to the present invention for identifying one or more target plasma exhibiting hepatocellular cancer. Also indicates the expression levels and accuracy of these miRNAs in the patients with hepatocellular cancer as compared to healthy controls (i.e. an up-regulation or a down-regulation). The top 5 combinations with 6 different miRNAs had 87%-94% accuracy as diagnostic biomarkers in differentiating hepatocellular cancer from healthy individuals.

Figure 3 depicts examples of ROC curve analysis for two tumor-related miRNAs (hsa-miR-122 and has-miR-139-3p) in plasma of hepatocellular cancer patients as compared to healthy controls (0: healthy individuals; 1: hepatocellular cancer). The results indicate high sensitivity and specificity of these miRNAs as diagnostic biomarkers. The data obtained on the microarrays were normalized against an internal stable control hsa-miR-1238.

Figure 4 illustrates the human miRNAs comprised in particularly preferred expression signatures in the second aspect according to the present invention for further discriminating hepatocellular cancer from healthy individuals, colorectal cancer and lung cancer. Also indicates the expression levels and accuracy of these miRNAs in the patients with hepatocellular cancer as compared to healthy control, colorectal cancer and lung cancer (i.e. an up-regulation or a down-regulation). The top 5 combinations showed 85% - 88% accuracy as diagnostic biomarkers for hepatocellular cancer.

Figure 5 depicts platform comparison of 4 combinations with 5 different miRNAs. The quantitative correlation (R) of the fold changes acquired on the arrays with that obtained from quantitative RT-PCR was 0.76. The results demonstrate that the miRNA

signatures discovered on Agilent miRNA microarrays are highly reliable.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the unexpected finding that hepatocellular cancer can be reliably identified based on particular miRNA expression signatures in plasma with high sensitivity and specificity, wherein the expression signatures as defined herein typically comprises both up- and down-regulated human miRNAs. More specifically, said miRNA expression signatures – by analyzing the overall miRNA expression pattern and/or the respective individual miRNA expression level(s) in plasma – allow the detection of hepatocellular cancer at an early disease state and discriminating healthy individuals, colorectal cancer and lung cancer.

The present invention illustratively described in the following may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein.

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. The drawings described are only schematic and are to be considered non-limiting.

Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. For the purposes of the present invention, the term "consisting of" is considered to be a preferred embodiment of the term "comprising of". If hereinafter a group is defined to comprise at least a certain number of embodiments, this is also to be understood to disclose a group, which preferably consists only of these embodiments.

Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated.

The term "about" in the context of the present invention denotes an interval of accuracy that the person skilled in the art will understand to still ensure the technical effect of the feature in question. The term typically indicates deviation from the

indicated numerical value of ± 10%, and preferably ± 5%.

Furthermore, the terms first, second, third, (a), (b), (c), and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

Further definitions of term will be given in the following in the context of which the terms are used.

The following terms or definitions are provided solely to aid in the understanding of the invention. These definitions should not be construed to have a scope less than understood by a person of ordinary skill in the art.

It is an objective of the present invention to provide novel approaches for diagnosing hepatocellular cancer, monitoring the cancer therapy and/or treating the cancer by determining a plurality of nucleic acid molecules in blood, each nucleic acid molecule encoding a microRNA (miRNA) sequence, wherein one or more of the plurality of nucleic acid molecules are differentially expressed in plasma of hepatocellular carcinoma, analyzed as compared to healthy controls, and/or as compared to healthy individuals, colorectal cancer and lung cancer, wherein the one or more differentially expressed nucleic acid molecules together represent a nucleic acid expression signature that is indicative for the presence of hepatocellular cancer, wherein the nucleic acid expression signatures include tumor-related signatures and plasma-specific signatures.

The term "cancer" (also referred to as "carcinoma"), as used herein, generally denotes any type of malignant neoplasm, that is, any morphological and/or physiological alterations (based on genetic re-programming) of target cells exhibiting or having a predisposition to develop characteristics of a carcinoma as compared to unaffected (healthy) wild-type control cells. Examples of such alterations may relate *inter alia* to cell size and shape (enlargement or reduction), cell proliferation (increase in cell number), cell differentiation (change in physiological state), apoptosis (programmed cell death) or cell survival.

The term "hepatocellular" (or "hepatic"), as used herein, relates to the liver. Hence, the term "hepatocellular cancer" refers to cancerous growths in the liver.

The most common type of hepatocellular (liver) cancer is hepatocellular carcinoma (also referred to as "hepatoma" and commonly abbreviated as "HCC"). The term "hepatocellular carcinoma", as used herein, denotes a primary malignancy of the liver. Most cases of HCC are secondary to either a viral hepatitide infection (hepatitis B or C) or cirrhosis (alcoholism being the most common cause of hepatic cirrhosis). In countries where hepatitis is not endemic, most malignant cancers in the liver are not primary HCC but metastasis (spread) of cancer from elsewhere in the body, e.g. the colon. Treatment options of HCC and prognosis are dependent on many factors but especially on tumor size and staging. Tumor grade is also important. High-grade tumors will have a poor prognosis, while low-grade tumors may go unnoticed for many years. The usual outcome is poor, because only 10% to 20% of hepatocellular carcinomas can be removed completely using surgery. If the cancer cannot be completely removed, the disease is usually deadly within 3 to 6 months.

Hepatocellular carcinoma, like any other cancer, develops when there is a mutation to the cellular machinery that causes the cell to replicate at a higher rate and/or results in the cell avoiding apoptosis. In particular, chronic viral infections of hepatitis B and/or C can aid the development of hepatocellular carcinoma by repeatedly causing the body's own immune system to attack the liver cells, some of which are infected by the virus, others merely bystanders. While this constant cycle of damage followed by repair can lead to mistakes during repair which in turn lead to carcinogenesis, this hypothesis is more applicable, at present, to hepatitis C. In hepatitis B, however, the integration of the viral genome into infected cells is the most consistently associated factor in malignancy. Alternatively, repeated consumption of large amounts of ethanol can have a similar effect.

Thus, within the scope of the present invention hepatitis B and/or C infections or hepatic cirrhosis are not merely to be considered as risk factors for tumor etiology but as early/intermediate stages in tumor progression (i.e. "pre-cancerous states") that are associated with hyper-proliferative tissue growth resulting in (often benign) non-invasive neoplasm which, in turn, may progress to malignant tumors such as HCC.

Such malignant tumors invade other tissues and often metastasize given enough time to do so. Malignant cells are often characterized by progressive and uncontrolled growth. Macroscopically, HCC appears as a nodular or infiltrative tumor. The nodular type may be solitary (having a large mass) or multiple (when developed as a complication of cirrhosis). Tumor nodules are round to oval, well circumscribed but not encapsulated. The diffuse type is poorly circumscribed and infiltrates the portal veins, rarely the hepatic veins.

The mammalian target plasma employed in the present invention may be of human or non-human origin. However, the invention is typically performed with human plasma. The term "one or more plasma", as used herein, is to be understood not only to include individual plasma. The term "target plasma", as used herein, refers to a plasma being at least supposed to exhibit hepatocellular cancer, whereas the term "control plasma" typically denotes a healthy individual not having characteristics of such a cancerous phenotype. However, in some applications, for example, when comparing plasma exhibiting different cancer types, the plasma not having characteristics of such a hepatocellular cancerous phenotype are typically considered the "control plasma".

The term “plasma”, as used herein, is the yellow liquid component of blood, in which the blood cells in whole blood would normally be suspended. It makes up about 55% of the total blood volume. It is mostly water (90% by volume) and contains dissolved proteins, glucose, clotting factors, mineral ions, hormones and carbon dioxide (plasma being the main medium for excretory product transportation). Blood plasma is prepared by spinning a tube of fresh blood in a centrifuge until the blood cells fall to the bottom of the tube. The blood plasma is then poured or drawn off. Blood plasma has a density of approximately 1025 kg/m^3, or 1.025 kg/l. Recent research showed that miRNA is stable in plasma. The term “plasma sample” refers to plasma taken from individuals being examined or from healthy control.

The term “patient”, as used herein, refers to a human being at least supposed to have hepatocellular cancer; where as “target plasma”, as used herein, refers to plasma collected from patients; The term “healthy individual” or “healthy control” typically denotes a healthy person not having characteristics of such a cancerous phenotype. And “control plasma”, as used herein, denotes plasma collected from healthy individuals.

However, in some applications, for example, when comparing different cancer types, the individual having the other cancer types and plasma collected from these individuals is typically considered the "control".

Typically, the plasma samples used are derived from biological specimens collected from the subjects to be diagnosed for the presence of hepatocellular cancer. Furthermore, in order to corroborate the data obtained "comparative samples" may also be collected from subjects having a given known disease state. The biological samples may include body tissues and fluids, such as tissue, serum, blood cell, sputum, and urine. Furthermore, the biological sample may be obtained from individual have hepatocellular cancerous characteristics or suspected to be cancerous. Furthermore, the sample may be purified from the obtained body tissues and fluids if necessary, and then used as the biological sample. According to the present invention, the expression level of the nucleic acid markers of the present invention is determined in the subject-derived biological sample(s).

The sample used for detection in the *in vitro* methods of the present invention should generally be collected in a clinically acceptable manner, preferably in a way that nucleic acids (in particular RNA) or proteins are preserved. The samples to be analyzed are typically from blood. Furthermore, liver tissue and other types of sample can be used as well. Samples, in particular after initial processing may be pooled. However, also non-pooled samples may be used.

The term "microRNA" (or "miRNA"), as used herein, is given its ordinary meaning in the art (Bartel, D.P. (2004) *Cell* 23, 281-292; He, L. and Hannon, G.J. (2004) *Nat Rev Genet* 5, 522-531). Accordingly, a "microRNA" denotes an RNA molecule derived from a genomic locus that is processed from transcripts that can form local RNA precursor miRNA structures. The mature miRNA is usually 20, 21, 22, 23, 24, or 25 nucleotides in length, although other numbers of nucleotides may be present as well, for example 18, 19, 26 or 27 nucleotides.

The miRNA encoding sequence has the potential to pair with flanking genomic sequences, placing the mature miRNA within an imperfect RNA duplex (herein also referred to as stem-loop or hairpin structure or as pre-miRNA), which serves as an intermediate for miRNA processing from a longer precursor transcript. This processing

typically occurs through the consecutive action of two specific endonucleases termed Drosha and Dicer, respectively. Drosha generates from the primary transcript (herein also denoted "pri-miRNA") a miRNA precursor (herein also denoted "pre-miRNA") that typically folds into a hairpin or stem-loop structure. From this miRNA precursor a miRNA duplex is excised by means of Dicer that comprises the mature miRNA at one arm of the hairpin or stem-loop structure and a similar-sized segment (commonly referred to miRNA*) at the other arm. The miRNA is then guided to its target mRNA to exert its function, whereas the miRNA* is degraded. In addition, miRNAs are typically derived from a segment of the genome that is distinct from predicted protein-coding regions.

The term "miRNA precursor" (or "precursor miRNA" or "pre-miRNA"), as used herein, refers to the portion of a miRNA primary transcript from which the mature miRNA is processed. Typically, the pre-miRNA folds into a stable hairpin (i.e. a duplex) or a stem-loop structure. The hairpin structures typically range from 50 to 80 nucleotides in length, preferably from 60 to 70 nucleotides (counting the miRNA residues, those pairing to the miRNA, and any intervening segment(s) but excluding more distal sequences).

The term "nucleic acid molecule encoding a microRNA sequence", as used herein, denotes any nucleic acid molecule coding for a microRNA (miRNA). Thus, the term does not only refer to mature miRNAs but also to the respective precursor miRNAs and primary miRNA transcripts as defined above. Furthermore, the present invention is not restricted to RNA molecules but also includes corresponding DNA molecules encoding a microRNA, e.g. DNA molecules generated by reverse transcribing a miRNA sequence. A nucleic acid molecule encoding a microRNA sequence according to the invention typically encodes a single miRNA sequence (i.e. an individual miRNA). However, it is also possible that such nucleic acid molecule encodes two or more miRNA sequences (i.e. two or more miRNAs), for example a transcriptional unit comprising two or more miRNA sequences under the control of common regulatory sequences such as a promoter or a transcriptional terminator.

The term "nucleic acid molecule encoding a microRNA sequence", as used herein, is also to be understood to include "sense nucleic acid molecules" (i.e.

molecules whose nucleic acid sequence (5'→ 3') matches or corresponds to the encoded miRNA (5'→ 3') sequence) and "anti-sense nucleic acid molecules" (i.e. molecules whose nucleic acid sequence is complementary to the encoded miRNA (5'→ 3') sequence or, in other words, matches the reverse complement (3'→ 5') of the encoded miRNA sequence). The term "complementary", as used herein, refers to the capability of an "anti-sense" nucleic acid molecule sequence of forming base pairs, preferably Watson-Crick base pairs, with the corresponding "sense" nucleic acid molecule sequence (having a sequence complementary to the anti-sense sequence).

Within the scope of the present invention, two nucleic acid molecules (i.e. the "sense" and the "anti-sense" molecule) may be perfectly complementary, that is, they do not contain any base mismatches and/or additional or missing nucleotides. Alternatively, the two molecules comprise one or more base mismatches or differ in their total numbers of nucleotides (due to additions or deletions). Preferably, the "complementary" nucleic acid molecule comprises at least ten contiguous nucleotides showing perfect complementarity with a sequence comprised in corresponding "sense" nucleic acid molecule.

Accordingly, the plurality of nucleic acid molecules encoding a miRNA sequence that are comprised in a diagnostic kit of the present invention may include one or more "sense nucleic acid molecules" and/or one or more "anti-sense nucleic acid molecules". In case, the diagnostic kit includes one or more "sense nucleic acid molecules" (i.e. the miRNA sequences as such), said molecules are to be considered to constitute the totality or at least a subset of differentially expressed miRNAs (i.e. molecular markers) being indicative for the presence of or the disposition to develop a particular condition, here hepatocellular cancer. On the other hand, in case a diagnostic kit includes one or more "anti-sense nucleic acid molecules" (i.e. sequences complementary to the miRNA sequences), said molecules may comprise *inter alia* probe molecules (for performing hybridization assays) and/or oligonucleotide primers (e.g., for reverse transcription or PCR applications) that are suitable for detecting and/or quantifying one or more particular (complementary) miRNA sequences in a given sample.

A plurality of nucleic acid molecules as defined within the present invention

may comprise at least two, at least ten, at least 50, at least 100, at least 200, at least 500, at least 1.000, at least 10.000 or at least 100.000 nucleic acid molecules, each molecule encoding a miRNA sequence.

The term "differentially expressed", as used herein, denotes an altered expression level of a particular miRNA in the disease plasma as compared to the healthy controls, or as compared to other types of disease samples, which may be an up-regulation (i.e. an increased miRNA concentration in the plasma) or a down-regulation (i.e. a reduced or abolished miRNA concentration in the plasma). In other words, the nucleic acid molecule is activated to a higher or lower level in the disease plasma samples than in the control plasma.

Within the scope of the present invention, a nucleic acid molecule is to considered differentially expressed if the respective expression levels of this nucleic acid molecule in disease plasma samples and control samples typically differ by at least 5% or at least 10%, preferably by at least 20% or at least 25%, and most preferably by at least 30% or at least 50%. Thus, the latter values correspond to an at least 1.3-fold or at least 1.5-fold up-regulation of the expression level of a given nucleic acid molecule in the disease plasma samples compared to the control plasma samples or *vice versa* an at least 0.7-fold or at least 0.5-fold down-regulation of the expression level in the disease plasma samples, respectively.

The term "expression level", as used herein, refers to extent to which a particular miRNA sequence is transcribed from its genomic locus, that is, the concentration of a miRNA in the plasma sample to be analyzed.

As outlined above, the term "control plasma" typically denotes a plasma sample collected from (healthy) individual not having characteristics of a colorectal cancer phenotype. However, in some applications, for example, when comparing other cancer types, the plasma collected from the patients with other cancer types is typically considered the "control plasma".

The determining of expression levels typically follows established standard procedures well known in the art (Sambrook, J. et al. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Ausubel, F.M. et al. (2001) *Current Protocols in Molecular Biology*.

Wiley & Sons, Hoboken, NJ). Determination may occur at the RNA level, for example by Northern blot analysis using miRNA-specific probes, or at the DNA level following reverse transcription (and cloning) of the RNA population, for example by quantitative PCR or real-time PCR techniques. The term "determining", as used herein, includes the analysis of any nucleic acid molecules encoding a microRNA sequence as described above. However, due to the short half-life of pri-miRNAs and pre-mRNAs typically the concentration of only the mature miRNA is measured.

In specific embodiments, the standard value of the expression levels obtained in several independent measurements of a given sample (for example, two, three, five or ten measurements) and/or several measurements within several samples or control samples are used for analysis. The standard value may be obtained by any method known in the art. For example, a range of mean ± 2 SD (standard deviation) or mean ± 3 SD may be used as standard value.

The difference between the expression levels obtained for disease and control plasma may be normalized to the expression level of further control nucleic acids, e.g. housekeeping genes whose expression levels are known not to differ depending on the disease states of the individual from whom the sample was collected. Exemplary housekeeping genes include *inter alia* β-actin, glycerinaldehyde 3-phosphate dehydrogenase, and ribosomal protein P1. In preferred embodiments, the control nucleic acid is another miRNA known to be stably expressed during the various non-cancerous and (pre-)cancerous states of the individual from whom the sample was collected.

However, instead of determining in any experiment the expression levels for plasma sample it may also be possible to define based on experimental evidence and/or prior art data on or more cut-off values for a particular disease phenotype (i.e. a disease state). In such scenario, the respective expression levels for the plasma sample can be determined by using a stably expressed control miRNA for normalization. If the "normalized" expression levels calculated are higher than the respective cutoff value defined, then this finding would be indicative for an up-regulation of gene expression. *Vice versa*, if the "normalized" expression levels calculated are lower than the

respective cutoff value defined, then this finding would be indicative for a down-regulation of gene expression.

In the context of the present invention, the term "identifying hepatocellular cancer and/or discriminating other cancer types" is intended to also encompass predictions and likelihood analysis (in the sense of "diagnosing"). The compositions and methods disclosed herein are intended to be used clinically in making decisions concerning treatment modalities, including therapeutic intervention, diagnostic criteria such as disease stages, and disease monitoring and surveillance for the disease. According to the present invention, an intermediate result for examining the condition of a subject may be provided. Such intermediate result may be combined with additional information to assist a doctor, nurse, or other practitioner to diagnose that a subject suffers from the disease. Alternatively, the invention may be used to detect cancerous changes through plasma sample, and provide a doctor with useful information for diagnosis. Furthermore, the invention may also be used to discriminate between hepatocellular cancer and other cancer types including colorectal cancer and lung cancer.

Within the present invention, one or more differentially expressed nucleic acid molecules identified together represent a nucleic acid expression signature that is indicative for hepatocellular cancer through plasma sample. The term "expression signature", as used herein, denotes a set of nucleic acid molecules (e.g., miRNAs), wherein the expression level of the individual nucleic acid molecules differs between the plasma collected from hepatocellular cancer patient and the healthy control. Herein, a nucleic acid expression signature is also referred to as a set of markers and represents a minimum number of (different) nucleic acid molecules, each encoding a miRNA sequence that is capable for identifying a phenotypic state of an individual.

In a first aspect, the present invention relates to a diagnostic kit of molecular markers in blood for identifying hepatocellular cancer, the kit comprising a plurality of nucleic acid molecules, each nucleic acid molecule encoding a microRNA sequence, wherein one or more of the plurality of nucleic acid molecules are differentially expressed in the target plasma as compared to healthy controls, and wherein the differentially expressed signatures are derived from tumor-related or plasma-specific

signatures, and wherein the one or more differentially expressed nucleic acid molecules together represent a nucleic acid expression signature that is indicative for the presence of hepatocellular cancer.

The nucleic acid expression signature, as defined herein, may comprise at least six nucleic acid molecules, preferably at least twelve nucleic acid molecules, and particularly preferably at least thirty-two nucleic acid molecules.

In preferred embodiment, the nucleic acid expression signature comprises at least one nucleic acid molecule encoding a microRNA sequence whose expression is up-regulated in the one or more target plasma compared to the one or more healthy controls and at least one nucleic acid molecule encoding a microRNA sequence whose expression is down-regulated in the one or more target plasma compared to the one or more healthy control plasma.

The term “plasma-specific”, as used herein, refers to signatures that are that differentially expressed in plasma from colorectal cancer patients and in control plasma are not found significantly differentially expressed in hepatocellular cancer tissues cells and non-cancer tissue cells.

Typically, the nucleic acid molecules comprised in the nucleic acid expression signature are human sequences (hereinafter designated "hsa" (Homo sapiens).

In preferred embodiments of the invention, the nucleic acid expression signature comprises any one or more of the nucleic acid molecules encoding tumor-related signatures hsa-miR-122 (SEQ ID NO:1), hsa-miR-199b-3p (SEQ ID NO:2), hsa-miR-192 (SEQ ID NO: 3), hsa-miR-21 (SEQ ID NO:4), hsa-miR-139-3p (SEQ ID NO:5), hsa-miR-10a (SEQ ID NO: 6), hsa-miR-103 (SEQ ID NO:7), hsa-miR-181d (SEQ ID NO:8), hsa-miR-125b-2* (SEQ ID NO:9), hsa-miR-125a-3p (SEQ ID NO:10); plasma-specific signatures hsa-miR-34a (SEQ ID NO:11), hsa-miR-136 (SEQ ID NO:12), hsa-miR-151-5p (SEQ ID NO:13), hsa-miR-193b* (SEQ ID NO:14), hsa-miR-124 (SEQ ID NO:15), hsa-miR-936 (SEQ ID NO:16), hsa-miR-198 (SEQ ID NO:17), hsa-miR-149* (SEQ ID NO:18), hsa-miR-138 (SEQ ID NO:19), hsa-miR-601 (SEQ ID NO:20), hsa-miR-769-3p (SEQ ID NO:21), hsa-miR-513c (SEQ ID NO:22), hsa-miR-525-5p (SEQ ID NO:23), hsa-miR-654-5p (SEQ ID NO:24), hsa-miR-518c* (SEQ ID NO:25), hsa-miR-500 (SEQ ID NO:26), hsa-miR-181c* (SEQ ID NO:27), hsa-miR-1226* (SEQ ID

NO:28), hsa-miR-202 (SEQ ID NO:29), hsa-miR-629* (SEQ ID NO:30) and internal stable controls hsa-miR-1238 (SEQ ID NO:36) and hsa-miR-1228 (SEQ ID NO:37).

The nucleic acid sequences of the above-referenced miRNAs are listed in Table 1.

TABLE 1

miRNA	Sequence (5' → 3')
Tumor-related miRNA	
hsa-miR-122	uggagugugacaaugguguuug
hsa-miR-199b-3p	acaguagucugcacauugguua
hsa-miR-192	cugaccuaugaauugacagcc
hsa-miR-21	uagcuuaucagacugauguuga
hsa-miR-139-3p	ggagacgcggcccuguuggagu
hsa-miR-10a	uacccuguagauccgaauuugug
hsa-miR-103	agcagcauuguacagggcuauga
hsa-miR-181d	aacauucauuguugucgguggguu
hsa-miR-125b-2*	ucacaagucaggcucuuugggac
hsa-miR-125a-3p	acaggugagguucuugggagcc
Plasma-specific miRNA	
hsa-miR-34a	uggcagugucuuagcugguugu
hsa-miR-136	acuccauuuguuuugaugaugga
hsa-miR-151-5p	ucgaggagcucacagucuagu
hsa-miR-193b*	cgggguuuugagggcgagauga
hsa-miR-124	uaaggcacgcggugaaugcc
hsa-miR-936	acaguagagggaggaaucgcag
hsa-miR-198	aacccguagauccgaacuugug
hsa-miR-149*	agggagggacgggggcugugc
hsa-miR-138	agcugguguugugaaucaggccg
hsa-miR-601	uggucuaggauuguuggaggag
hsa-miR-769-3p	cugggaucuccgggguguuggguu
hsa-miR-513c	uucucaaggaggugucguuuau
hsa-miR-525-5p	cuccagagggaugcacuuucu
hsa-miR-654-5p	ugguggggccgcagaacaugugc
hsa-miR-518c*	ucucuggagggaagcacuuucug
hsa-miR-500	uaauccuugcuaccugggugaga
hsa-miR-181c*	aaccaucgaccguugaguggac
hsa-miR-1226*	gugagggcaugcaggccuggaugggg
hsa-miR-202	agagguauagggcaugggaa
hsa-miR-629*	guucucccaacguaagcccagc

Internal stable control	
hsa-miR-1238	cuuccucgucugucugcccc
hsa-miR-1228	ucacaccugccucgcccccc

All miRNA sequences disclosed herein have been deposited in the miRBase database (http://microrna.sanger.ac.uk/; see also Griffiths-Jones S. et al. (2008) *Nucl. Acids Res.* 36, D154-D158).

Particularly preferably, the expression of any one or more of the nucleic acid molecules encoding hsa-miR-122, hsa-miR-199b-3p, hsa-miR-192, hsa-miR-21, hsa-miR-10a, hsa-miR-103, hsa-miR-34a, hsa-miR-136, hsa-miR-151-5p is up-regulated and the expression of any one or more of the nucleic acid molecules encoding hsa-miR-139-3p, hsa-miR-193b*, hsa-miR-124, hsa-miR-181d, hsa-miR-125b-2*, hsa-miR-125a-3p, hsa-miR-936, hsa-miR-198, hsa-miR-149*, hsa-miR-138, hsa-miR-601, hsa-miR-769-3p, hsa-miR-513c, hsa-miR-525-5p, hsa-miR-654-5p, hsa-miR-518c*, hsa-miR-500, hsa-miR-181c*, hsa-miR-1226*, hsa-miR-202, hsa-miR-629* is down-regulated; hsa-miR-1238 and hsa-miR-1228 is un-changed in the one or more target plasma compared to the one or more healthy controls.

The terms "any one or more of the plurality of nucleic acid molecules" or "any one or more of the plurality of nucleic acid molecules" as used herein, may relate to any subgroup of the plurality of nucleic acid molecules, e.g., any one, any two, any three, any four, any five, any six, any seven, any eight, any nine, any ten, and so forth nucleic acid molecules, each encoding a microRNA sequence that are comprised in the nucleic acid expression signature, as defined herein.

In particularly preferred embodiments, the nucleic acid expression signature comprises any one or more nucleic acid combinations encoding hsa-miR-34a/hsa-miR-193b*, hsa-miR-21/hsa-miR-936, hsa-miR-192/hsa-miR-124, hsa-miR-122/hsa-miR-193b*, hsa-miR-34a/hsa-miR-138, hsa-miR-34a/hsa-miR-198, hsa-miR-122/hsa-miR-124, hsa-miR-103/hsa-miR-139-3p, hsa-miR-192/hsa-miR-193b*, hsa-miR-122/hsa-miR-138, hsa-miR-34a/hsa-miR-139-3p, hsa-miR-122/hsa-miR-198, hsa-miR-122/hsa-miR-769-3p, hsa-miR-103/hsa-miR-193b*, hsa-miR-34a/hsa-miR-124, hsa-miR-192/hsa-miR-139-3p, hsa-miR-34a/hsa-miR-769-3p, hsa-miR-192/hsa-miR-936, hsa-miR-10a/hsa-miR-193b*, hsa-miR-122/hsa-miR-601, hsa-miR-21/hsa-miR-769-3p,

hsa-miR-199b-3p/hsa-miR-193b*, hsa-miR-103/hsa-miR-138, hsa-miR-103/hsa-miR-198, hsa-miR-199b-3p/hsa-miR-139-3p, hsa-miR-21/hsa-miR-139-3p and hsa-miR-21/hsa-miR-193b*.

The term "nucleic acid combinations", as used herein, refers to the usage of at least two nucleic acid expression levels as a whole. Preferably may use the relative changes or calculate results through a formulation as a whole.

In a second aspect, the present invention relates to a diagnostic kit of molecular markers for discriminating hepatocellular cancer from healthy individuals, colorectal cancer and lung cancer, the kit comprising a plurality of nucleic acid molecules, each nucleic acid molecule encoding a microRNA sequence, wherein one or more of the plurality of nucleic acid molecules are differentially expressed in the target plasma and in one or more healthy individuals, colorectal cancer and lung cancer, and wherein the one or more differentially expressed nucleic acid molecules together represent a nucleic acid expression signature that is indicative for the presence of hepatocellular cancer.

The nucleic acid expression signature, as defined herein, may comprise at least sixteen nucleic acid molecules, preferably at least six nucleic acid molecules.

In preferred embodiments, the nucleic acid expression signature comprises at least one nucleic acid molecule encoding a microRNA sequence whose expression is up-regulated in the one or more target plasma compared to the one or more healthy individuals, colorectal cancer and lung cancer, and at least one nucleic acid molecule encoding a microRNA sequence whose expression is down-regulated in the one or more target plasma compared to the one or more healthy individuals, colorectal cancer and lung cancer.

In more preferred embodiments, the nucleic acid expression signature comprises any one or more of the nucleic acid molecules encoding tumor-related signatures: hsa-miR-122 (SEQ ID NO:1), hsa-miR-192 (SEQ ID NO:4), hsa-miR-215 (SEQ ID NO:31), hsa-let-7c (SEQ ID NO:32), hsa-miR-103 (SEQ ID NO:5), hsa-miR-139-3p (SEQ ID NO: 7), hsa-miR-26b (SEQ ID NO:33); plasma-specific signatures: hsa-miR-936 (SEQ ID NO:16), hsa-miR-193b* (SEQ ID NO:14), hsa-miR-124 (SEQ ID NO:15), hsa-miR-34a (SEQ ID NO:11), hsa-miR-198 (SEQ ID NO:18), hsa-let-7g (SEQ ID NO:34), hsa-miR-363 (SEQ ID NO:35) and internal stable controls: has-miR-1228 (SEQ ID NO:36)

and hsa-miR-1238 (SEQ ID NO:37).

The nucleic acid sequences of the above-referenced miRNAs are listed in Table 1.

TABLE 2

miRNA	Sequence (5' → 3')
Tumor-related miRNA	
hsa-miR-122	uggagugugacaaugguguuug
hsa-miR-192	cugaccuaugaauugacagcc
hsa-miR-215	augaccuaugaauugacagac
hsa-let-7c	ugagguaguagguuguaugguu
hsa-miR-103	agcagcauuguacagggcuauga
hsa-miR-139-3p	ggagacgcggcccuguuggagu
hsa-miR-26b	uucaaguaauucaggauaggu
Plasma-specific miRNA	
hsa-miR-936	acaguagagggaggaaucgcag
hsa-miR-193b*	cgggguuuugagggcgagauga
hsa-miR-124	uaaggcacgcggugaaugcc
hsa-miR-34a	uggcagugucuuagcugguugu
hsa-miR-198	aacccguagauccgaacuugug
hsa-let-7g	ugagguaguaguuuguacaguu
hsa-miR-363	aauugcacgguauccaucugua
Internal stable control	
hsa-miR-1238	cuuccucgucugucugcccc
hsa-miR-1228	Ucacaccugccucgccccc

All miRNA sequences disclosed herein have been deposited in the miRBase database (http://microrna.sanger.ac.uk/; see also Griffiths-Jones S. et al. (2008) *Nucl. Acids Res.* 36, D154-D158).

Particularly preferably, the expression of any one or more of the nucleic acid molecules encoding hsa-miR-122, hsa-miR-192, hsa-miR-215, hsa-let-7c, hsa-miR-103, hsa-miR-26b, hsa-miR-34a, hsa-let-7g, hsa-miR-363 is up-regulated and the expression of any one or more of the nucleic acid molecules encoding hsa-miR-936, hsa-miR-193b*, hsa-miR-124, hsa-miR-139-3p, hsa-miR-198 is down-regulated; hsa-miR-1238 and hsa-miR-1228 is un-changed in the one or more target plasma compared to the one

or more healthy individuals, colorectal cancer and lung cancer.

In particularly preferred embodiments, the nucleic acid expression signature comprises any one or more nucleic acid combinations encoding hsa-miR-122/hsa-miR-936, hsa-miR-34a/hsa-miR-193b*, hsa-miR-34a/hsa-miR-198, hsa-miR-192/hsa-miR-936, hsa-miR-122/hsa-miR-193b*, hsa-miR-122/hsa-miR-198, hsa-miR-192/hsa-miR-124, hsa-miR-192/hsa-miR-193b*, hsa-miR-122/hsa-miR-124, hsa-miR-192/hsa-miR-198, hsa-miR-363/hsa-miR-936, hsa-miR-215/hsa-miR-193b*, hsa-miR-103/hsa-miR-936, hsa-miR-122/hsa-miR-139-3p, hsa-let-7c/hsa-miR-936, hsa-miR-215/hsa-miR-198, hsa-miR-192/hsa-miR-139-3p, hsa-miR-27b/hsa-miR-198, hsa-miR-26b/hsa-miR-936, hsa-let-7g/hsa-miR-936, hsa-miR-103/hsa-miR-198, hsa-let-7c/hsa-miR-193b*, hsa-miR-103/hsa-miR-193b*, hsa-miR-26b/hsa-miR-139-3p, hsa-let-7c/hsa-miR-198, hsa-miR-27b/hsa-miR-193b*, hsa-let-7g/hsa-miR-193b*, hsa-miR-363/hsa-miR-139-3p, hsa-let-7g/hsa-miR-198, hsa-miR-363/hsa-miR-198, hsa-miR-26b/hsa-miR-198, hsa-miR-103/hsa-miR-139-3p, hsa-miR-301a/hsa-miR-198, hsa-miR-26b/hsa-miR-193b*, hsa-let-7g/hsa-miR-139-3p, hsa-miR-363/hsa-miR-124, hsa-let-7c/hsa-miR-139-3p, hsa-miR-301a/hsa-miR-193b*, hsa-miR-301a/hsa-miR-139-3p, hsa-miR-26b/hsa-miR-124, hsa-let-7d/hsa-miR-198, hsa-let-7d/hsa-miR-139-3p, hsa-miR-103/hsa-miR-124, hsa-miR-363/hsa-miR-193b* and hsa-let-7d/hsa-miR-193b*.

In a third aspect, the present invention relates to a method for identifying one or more target plasma exhibiting hepatocellular cancer, the method comprising: (a) determining in the one or more target plasma the expression levels of a plurality of nucleic acid molecules, each nucleic acid molecule encoding a microRNA sequence; (b) determining the expression levels of the plurality of nucleic acid molecules in one or more healthy control plasma; and (c) identifying from the plurality of nucleic acid molecules one or more nucleic acid molecules that are differentially expressed in the target and control plasma by comparing the respective expression levels obtained in steps (a) and (b), wherein the one or more differentially expressed nucleic acid molecules together represent a nucleic acid expression signature, as defined herein, that is indicative for the presence of hepatocellular cancer.

In preferred embodiments of the invention, the method comprising: (a) determining in the one or more target plasma the expression levels of a combination of

nucleic acid molecules, each nucleic acid molecule encoding a microRNA sequence, and calculate with certain formula, then ; (b) determining the expression levels of the combination of nucleic acid molecules in healthy control plasma, and calculate with certain formula; and (c) identifying the difference of the combination in the one or more target and control plasma by comparing the respective calculation results obtained in steps (a) and (b), wherein the one or more differentially expressed combinations together represent a signature, as defined herein, that is indicative for the presence of hepatocellular cancer.

In more preferred embodiments of the invention, the method is for the further use of discriminating hepatocellular cancer from healthy individuals, colorectal cancer and lung cancer.

In a fourth aspect, the present invention relates to a method for monitoring treatment of hepatocellular cancer, the method comprising: (a) identifying in the one or more target plasma a nucleic acid expression signature by using a method, as defined herein; and (b) monitoring in blood the expression of one or more nucleic acid molecules encoding a microRNA sequence that is/are comprised in the nucleic acid expression signature in such way that the expression of a nucleic acid molecule whose expression in plasma is up-regulated before treatment but is down-regulated after treatment and the expression of a nucleic acid molecule whose expression in plasma is down-regulated before treatment but is up-regulated after treatment.

The term "modifying the expression of a nucleic acid molecule encoding a miRNA sequence", as used herein, denotes any manipulation of a particular nucleic acid molecule resulting in an altered expression level of said molecule, that is, the production of a different amount of corresponding miRNA as compared to the expression of the "wild-type" (i.e. the unmodified control). The term "different amount", as used herein, includes both a higher amount and a lower amount than determined in the unmodified control. In other words, a manipulation, as defined herein, may either up-regulate (i.e. activate) or down-regulate (i.e. inhibit) the expression (i.e. particularly transcription) of a nucleic acid molecule.

In a fifth aspect, the present invention relates to a method for preventing or treating hepatocellular cancer, the method comprising: (a) identifying in plasma a

nucleic acid expression signature by using a method, as defined herein; and (b) modifying in blood the expression of one or more nucleic acid molecules encoding a microRNA sequence that is/are comprised in the nucleic acid expression signature in such way that the expression of a nucleic acid molecule whose expression is up-regulated in plasma is down-regulated and the expression of a nucleic acid molecule whose expression is down-regulated in plasma is up-regulated.

Within the present invention, expression of one or more nucleic acid molecules encoding a microRNA sequence comprised in the nucleic acid expression signature is modified in such way that the expression of a nucleic acid molecule whose expression is up-regulated in plasma is down-regulated and the expression of a nucleic acid molecule whose expression is down-regulated in plasma is up-regulated. In other words, the modification of expression of a particular nucleic acid molecule encoding a miRNA sequence occurs in an anti-cyclical pattern to the regulation of said molecule in plasma of cancer patients in order to interfere with the "excess activity" of an up-regulated molecule and/or to restore the "deficient activity" of a down-regulated molecule in plasma.

In a preferred embodiment of the inventive method, down-regulating the expression of a nucleic acid molecule comprises introducing into the patient a nucleic acid molecule encoding a sequence that is complementary to the microRNA sequence encoded by nucleic acid molecule to be down-regulated.

The term "introducing into blood", as used herein, refers to any manipulation allowing the transfer of one or more nucleic acid molecules into blood. Examples of such techniques include injection, digestion or any other techniques may be involved.

The term "complementary sequence", as used herein, is to be understood that the "complementary" nucleic acid molecule (herein also referred to as an "anti-sense nucleic acid molecule") introduced into blood is capable of forming base pairs, preferably Watson-Crick base pairs, with the up-regulated endogenous "sense" nucleic acid molecule.

Two nucleic acid molecules (i.e. the "sense" and the "anti-sense" molecule) may be perfectly complementary, that is, they do not contain any base mismatches and/or additional or missing nucleotides. In other embodiments, the two molecules comprise

one or more base mismatches or differ in their total numbers of nucleotides (due to additions or deletions). In further embodiments, the "complementary" nucleic acid molecule comprises a stretch of at least ten contiguous nucleotides showing perfect complementarity with a sequence comprised in the up-regulated "sense" nucleic acid molecule.

The "complementary" nucleic acid molecule (i.e. the nucleic acid molecule encoding a nucleic acid sequence that is complementary to the microRNA sequence encoded by nucleic acid molecule to be down-regulated) may be a naturally occurring DNA- or RNA molecule or a synthetic nucleic acid molecule comprising in its sequence one or more modified nucleotides which may be of the same type or of one or more different types.

For example, it may be possible that such a nucleic acid molecule comprises at least one ribonucleotide backbone unit and at least one deoxyribonucleotide backbone unit. Furthermore, the nucleic acid molecule may contain one or more modifications of the RNA backbone into 2'-*O*-methyl group or 2'-*O*-methoxyethyl group (also referred to as "2'-*O*-methylation"), which prevented nuclease degradation in the culture media and, importantly, also prevented endonucleolytic cleavage by the RNA-induced silencing complex nuclease, leading to irreversible inhibition of the miRNA. Another possible modification – which is functionally equivalent to 2'-*O*-methylation – involves locked nucleic acids (LNAs) representing nucleic acid analogs containing one or more LNA nucleotide monomers with a bicyclic furanose unit locked in an RNA-mimicking sugar conformation (Orom, U.A. et al. (2006) *Gene* 372, 137-141).

Another class of silencers of miRNA expression was recently developed. These chemically engineered oligonucleotides, named "antagomirs", represent single-stranded 23-nucleotide RNA molecules conjugated to cholesterol (Krutzfeldt, J. et al. (2005) *Nature* 438, 685–689). As an alternative to such chemically modified oligonucleotides, microRNA inhibitors that can be expressed in cells, as RNAs produced from transgenes, were generated as well. Termed "microRNA sponges", these competitive inhibitors are transcripts expressed from strong promoters, containing multiple, tandem binding sites to a microRNA of interest (Ebert, M.S. et al. (2007) *Nat. Methods* 4, 721-726).

In particularly preferred embodiments of the inventive method, the one or more

nucleic acid molecules whose expression is to be down-regulated encode microRNA sequences selected from the group consisting of hsa-miR-139-3p, hsa-miR-181d, hsa-miR-125b-2*, hsa-miR-125a-3p, hsa-miR-193b*,hsa-miR-124, hsa-miR-936, hsa-miR-138, hsa-miR-198, hsa-miR-769-3p, hsa-miR-149*, hsa-miR-654-5p, hsa-miR-525-5p, hsa-miR-629*, hsa-miR-181c*, hsa-miR-202, hsa-miR-513c, hsa-miR-500, hsa-miR-518c*, hsa-miR-601 and hsa-miR-1226* with respect to the expression signature, presumably indicative for hepatocellular cancer as defined above.

In a further preferred embodiment of the inventive method, up-regulating the expression of a nucleic acid molecule comprises introducing into blood a nucleic acid molecule encoding the microRNA sequence encoded by nucleic acid molecule to be up-regulated. In other words, the up-regulation of the expression of a nucleic acid molecule encoding a miRNA sequence is accomplished by introducing into the one or more cells another copy of said miRNA sequence (i.e. an additional "sense" nucleic acid molecule). The "sense" nucleic acid molecule to be introduced into blood may comprise the same modification as the "anti-sense" nucleic acid molecules described above.

In a particularly preferred embodiment, the one or more nucleic acid molecules whose expression is to be up-regulated encode microRNA sequences selected from the group consisting of hsa-miR-122, hsa-miR-199b-3p, hsa-miR-192, hsa-miR-21, hsa-miR-10a, hsa-miR-103, hsa-miR-34a, hsa-miR-136, hsa-miR-151-5p, hsa-miR-215, hsa-let-7c, hsa-miR-26b, hsa-let-7g and hsa-miR-363 with respect to the expression signature, presumably indicative for hepatocellular cancer as defined above.

The "sense" and/or the "anti-sense" nucleic acid molecules to be introduced into blood in order to modify the expression of one or more nucleic acid molecules encoding a microRNA sequence that is/are comprised in the nucleic acid expression signature may be operably linked to a regulatory sequence in order to allow expression of the nucleotide sequence.

In order to unravel any potential implication of the miRNAs identified in the cancerous or pre-cancerous samples preliminary functional analyses may be performed with respect to the identification of mRNA target sequences to which the miRNAs may bind. Based on the finding that miRNAs may be involved in both tumor suppression and tumorigenesis (Esquela-Kerscher, A. and Slack, F.J (2006) *supra*; Calin, G.A. and

Croce, C.M. (2007) *supra*; Blenkiron, C. and Miska, E.A. (2007) *supra*) it is likely to speculate that mRNA target sites for such miRNAs include tumor suppressor genes as well as oncogenes.

A nucleic acid molecule is referred to as "capable of expressing a nucleic acid molecule" or capable "to allow expression of a nucleotide sequence" if it comprises sequence elements which contain information regarding to transcriptional and/or translational regulation, and such sequences are "operably linked" to the nucleotide sequence encoding the polypeptide. An operable linkage is a linkage in which the regulatory sequence elements and the sequence to be expressed (and/or the sequences to be expressed among each other) are connected in a way that enables gene expression.

The precise nature of the regulatory regions necessary for gene expression may vary among species, but in general these regions comprise a promoter which, in prokaryotes, contains both the promoter *per se*, i.e. DNA elements directing the initiation of transcription, as well as DNA elements which, when transcribed into RNA, will signal the initiation of translation. Such promoter regions normally include 5' non-coding sequences involved in initiation of transcription and translation, such as the -35/-10 boxes and the Shine-Dalgarno element in prokaryotes or the TATA box, CAAT sequences, and 5'-capping elements in eukaryotes. These regions can also include enhancer or repressor elements as well as translated signal and leader sequences for targeting the native polypeptide to a specific compartment of a host cell. In addition, the 3' non-coding sequences may contain regulatory elements involved in transcriptional termination, polyadenylation or the like. If, however, these termination sequences are not satisfactory functional in a particular host environment, then they may be substituted with signals functional in that environment.

Furthermore, the expression of the nucleic molecules, as defined herein, may also be influenced by the presence, e.g., of modified nucleotides (cf. the discussion above). For example, locked nucleic acid (LNA) monomers are thought to increase the functional half-life of miRNAs *in vivo* by enhancing the resistance to degradation and by stabilizing the miRNA-target duplex structure that is crucial for silencing activity (Naguibneva, I. et al. (2006) *Biomed Pharmacother* 60, 633–638).

Therefore, a nucleic acid molecule of the invention to be introduced into blood provided may include a regulatory sequence, preferably a promoter sequence, and optionally also a transcriptional termination sequence. The promoters may allow for either a constitutive or an inducible gene expression. Suitable promoters include *inter alia* the *E. coli lac*UV5 and *tet* (tetracycline-responsive) promoters, the T7 promoter as well as the SV40 promoter or the CMV promoter.

The nucleic acid molecules of the invention may also be comprised in a vector or other cloning vehicles, such as plasmids, phagemids, phages, cosmids or artificial chromosomes. In a preferred embodiment, the nucleic acid molecule is comprised in a vector, particularly in an expression vector. Such an expression vector can include, aside from the regulatory sequences described above and a nucleic acid sequence encoding a genetic construct as defined in the invention, replication and control sequences derived from a species compatible with the host that is used for expression as well as selection markers conferring a selectable phenotype on host. Large numbers of suitable vectors such as pSUPER and pSUPERIOR are known in the art, and are commercially available.

In a sixth aspect, the present invention relates to a pharmaceutical composition for the prevention and/or treatment of hepatocellular cancer in blood, the composition comprising one or more nucleic acid molecules, each nucleic acid molecule encoding a sequence that is at least partially complementary to a microRNA sequence encoded by a nucleic acid molecule whose expression is up-regulated in plasma from hepatocellular cancer patients, as defined herein, and/or that corresponds to a microRNA sequence encoded by a nucleic acid molecule whose expression is down-regulated in plasma from hepatocellular cancer patients, as defined herein.

Finally, in a seventh aspect, the present invention relates to the use of said pharmaceutical composition for the manufacture of a medicament for the prevention and/or treatment of hepatocellular cancer.

Within the scope of the present invention, suitable pharmaceutical compositions include *inter alia* those compositions suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), peritoneal and parenteral (including intramuscular, subcutaneous and intravenous) administration, or for administration by inhalation or

insufflation. Administration may be local or systemic. Preferably, administration is accomplished via the oral or intravenous routes. The formulations may also be packaged in discrete dosage units.

Pharmaceutical compositions according to the present invention include any pharmaceutical dosage forms established in the art, such as *inter alia* capsules, microcapsules, cachets, pills, tablets, powders, pellets, multi-particulate formulations (e.g., beads, granules or crystals), aerosols, sprays, foams, solutions, dispersions, tinctures, syrups, elixirs, suspensions, water-in-oil emulsions such as ointments, and oil-in water emulsions such as creams, lotions, and balms.

The ("sense" and "anti-sense") nucleic acid molecules described above can be formulated into pharmaceutical compositions using pharmacologically acceptable ingredients as well as established methods of preparation (Gennaro, A.L. and Gennaro, A.R. (2000) *Remington: The Science and Practice of Pharmacy*, 20th Ed., Lippincott Williams & Wilkins, Philadelphia, PA; Crowder, T.M. et al. (2003*) A Guide to Pharmaceutical Particulate Science.* Interpharm/CRC, Boca Raton, FL; Niazi, S.K. (2004) *Handbook of Pharmaceutical Manufacturing Formulations*, CRC Press, Boca Raton, FL).

In order to prepare the pharmaceutical compositions, pharmaceutically inert inorganic or organic excipients (i.e. carriers) can be used. To prepare e.g. pills, tablets, capsules or granules, for example, lactose, talc, stearic acid and its salts, fats, waxes, solid or liquid polyols, natural and hardened oils may be used. Suitable excipients for the production of solutions, suspensions, emulsions, aerosol mixtures or powders for reconstitution into solutions or aerosol mixtures prior to use include water, alcohols, glycerol, polyols, and suitable mixtures thereof as well as vegetable oils.

The pharmaceutical composition may also contain additives, such as, for example, fillers, binders, wetting agents, glidants, stabilizers, preservatives, emulsifiers, and furthermore solvents or solubilizers or agents for achieving a depot effect. The latter is to be understood that the nucleic acid molecules may be incorporated into slow or sustained release or targeted delivery systems, such as liposomes, nanoparticles, and microcapsules.

To target most tissues within the body, clinically feasible noninvasive strategies are required for directing such pharmaceutical compositions, as defined herein, into cells. In the past years, several approaches have achieved impressive therapeutic benefit following intravenous injection into mice and primates using reasonable doses of siRNAs without apparent limiting toxicities.

One approach involves covalently coupling the passenger strand (miRNA* strand) of the miRNA to cholesterol or derivatives/conjugates thereof to facilitate uptake through ubiquitously expressed cell-surface LDL receptors (Soutschek, J. et al. (2004) *Nature* 432, 173-178). Alternatively, unconjugated, PBS-formulated locked-nucleic-acid-modified oligonucleotides (LNA-antimiR) may be used for systemic delivery (Elmen, J. et al. (2008) *Nature* 452, 896-899). Another strategy for delivering miRNAs involves encapsulating the miRNAs into specialized liposomes formed using polyethylene glycol to reduce uptake by scavenger cells and enhance time spent in the circulation. These specialized nucleic acid particles (stable nucleic acid-lipid particles or SNALPs) delivered miRNAs effectively to the liver (and not to other organs (Zimmermann, T.S. et al. (2006) *Nature* 441, 111-114). Recently, a new class of lipid-like delivery molecules, termed lipidoids (synthesis scheme based upon the conjugate addition of alkylacrylates or alkyl-acrylamides to primary or secondary amines) has been described as delivery agents for RNAi therapeutics (Akinc, A. et al. (2008) *Nat Biotechnol* 26, 561-569).

A further targeting strategy involves the mixing of miRNAs with a fusion protein composed of a targeting antibody fragment linked to protamine, the basic protein that nucleates DNA in sperm and binds miRNAs by charge (Song, E. et al. (2005) *Nat. Biotechnol.* 23, 709-717). Multiple modifications or variations of the above basic delivery approaches have recently been developed. These techniques are known in the art and reviewed, e.g., in de Fougerolles, A. et al. (2007) *Nat. Rev. Drug Discov* 6, 443-453; Kim, D.H. and Rossi, J.J. (2007) *Nat Genet* 8, 173-184).

The invention is further described by the figures and the following examples, which are solely for the purpose of illustrating specific embodiments of this invention, and are not to be construed as limiting the scope of the invention in any way.

EXAMPLES

Example 1: Tissue sample collection and preparation

Sixty-three liver tissue specimens were taken during surgery. Surgical specimens were snap-frozen in liquid nitrogen at or immediately after collection. Samples were stored at -80°C. Baseline characteristics of the tumor specimens used in the invention are shown in Table 3.

Table 3

Baseline characteristics of liver tissue specimens

Tissue specimens	Number
Normal liver tissue	31
HCC tissue	32
Total number	63

Patient data (age, sex, imaging data, therapy, other medical conditions, family history, and the like) were derived from the hospital databases for matching the various samples collected. Pathologic follow-up (for example, histological analysis via hematoxylin and eosin (H&E) staining) was used for evidently determining the disease state (i.e. control, pre-cancerous stage (e.g., hepatic cirrhosis), primary malignancy (e.g., hepatocellular carcinoma) of a given sample as well as to ensure a consistent classification of the specimens.

Laser-capture micro-dissection was optionally performed for each cancerous sample in order to specifically isolate tumor cell populations (about 200.000 cells). In brief, a transparent transfer film is applied to the surface of a tissue section or specimen. Under a microscope, the thin tissue section is viewed through the glass slide on which it is mounted and clusters of cells are identified for isolation. When the cells of choice are in the center of the field of view, a near IR laser diode integral with the microscope optics is activated. The pulsed laser beam activates a spot on the transfer film, fusing the film with the underlying cells of choice. The transfer film with the bonded cells is then lifted off the thin tissue section (Emmert-Buck, M.R. et al. (1996) *Science* 274, 998-1001; Espina, V. et al. (2007) *Expert Rev. Mol. Diagn* 7, 647-657). The preparation of the cryostat sections and the capturing step using a laser capture microspope (Arcturus Veritas™ Laser Capture Microdissection Instrument (Molecular Devices, Inc.,

Sunnyvale, CA, USA) were performed essentially according to the instructions of the manufacturer.

Total RNA was extracted from the tissue sections by using mirVana miRNA isolation kit according to the instructions from the manufacturer (Ambion, Austin, TX). The concentration was quantified by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA). The quality control of RNA was performed by a 2100 Bioanalyzer using the RNA 6000 Pico LabChip kit (Agilent Technologies, Santa Clara, CA).

Example 2: Analysis of the miRNA expression profile in the tissue samples

A qualitative analysis of the miRNAs (differentially) expressed in a particular sample may optionally be performed using Agilent miRNA microarray platform (Agilent Technologies, Santa Clara, CA, USA). The microarray contains probes for 723 human miRNAs from the Sanger database v.10.1. Total RNA (100 ng) derived from each of 63 LCM-selected liver tissues were used as inputs for labeling via Cy3 incorporation. Microarray slides were scanned by XDR Scan (PMT100, PMT5). The labeling and hybridization were performed according to the protocols in the Agilent miRNA microarray system.

For the data analysis, the raw data obtained for single-color (CY3) hybridization were normalized by applying a Quantile method and using GeneSpring GX10 software (Agilent Technologies, Santa Clara, CA, USA) known in the art. Unpaired t-test (p value <0.01) after Fisher test (F-test) was used to identify differentially expressed miRNAs between normal liver tissues and HCC tissues.

Independent experiments on 63 tissue specimens were performed for each measurement and the miRNA expression level determined represents the mean value of the respective individual data obtained.

Example 3: Plasma sample collection and preparation

The principal method steps for identifying a patient in a blood sample exhibiting hepatocellular cancer are shown in Figure 1.

154 blood specimens from the cancer patients and healthy individuals were collected at Zhongshan and Huashan Hospitals in Shanghai between 2008 and 2009. Baseline

characteristics of the blood specimens used in the invention are shown in Table 4. All of the samples from the patients were procured before surgery. Patient data (age, sex, imaging data, therapy, other medical conditions, family history, and the like) were derived from the hospital databases. Tumor histopathology was classified according to the World Health Organization Classification of Tumor system by three pathologists independently.

Table 4

Baseline characteristics of blood specimens

Genome-wide miRNA analysis	Number
Controls	
Healthy individuals	23
Colorectal cancer	31
Lung cancer	36
Hepatocellular cancer	24
Quantitative RT-PCR analysis	
Healthy individuals	16
Hepatocellular cancer	24
Total number	154

Peripheral blood (2 ml) was drawn into EDTA tubes. Within two hours, the tubes were subjected to centrifuge at 820g for 10 min. Then, 1-ml aliquots of the plasma was transferred to 1.5-ml tubes and centrifuged at 16,000g for 10 min to pellet any remaining cellular debris. Subsequently, the supernatant was transferred to fresh tubes and stored them at –80 °C.

Total RNA was extracted from the plasma by using mirVana PARIS miRNA Isolation kit according to the instructions from the manufacturer (Ambion, Austin, TX). The concentration was quantified by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA). The quality control of RNA was performed by a 2100 Bioanalyzer using the RNA 6000 Pico LabChip kit (Agilent Technologies, Santa Clara, CA).

Example 4: Analysis of the miRNA expression profile in the plasma samples

A qualitative analysis of the miRNAs (differentially) expressed in a particular

plasma sample may optionally be performed using the Agilent miRNA microarray platform (Agilent Technologies, Santa Clara, CA, USA). The microarray contains probes for 723 human miRNAs from the Sanger database v.10.1. Total RNA (100 ng) derived from each of 114 plasma samples were used as inputs for labeling via Cy3 incorporation. Microarray slides were scanned by XDR Scan (PMT100, PMT5). The labeling and hybridization were performed according to the protocols in the Agilent miRNA microarray system.

For the data analysis, the raw data obtained for single-color (CY3) hybridization were normalized by applying an internal stable control (hsa-miR-1238). Unpaired t-test after Fisher test (F-test) was used to identify differentially expressed miRNAs between HCC vs. healthy individuals, and/or HCC vs. healthy individuals, colorectal cancer and lung cancer, respectively.

For the specificity and sensitivity of the individual miRNA as diagnostic biomarkers, MedCalc software was used to perform receiver operating characteristic (ROC) curve analysis of the individual miRNA in HCC vs. healthy individuals, and/or HCC vs. healthy individuals, colorectal cancer and lung cancer, respectively. 95% confidence interval was used to determine the significance.

For assessing whether a particular miRNA is differentially expressed in HCC as compared to healthy individuals, and/or healthy individuals, colorectal cancer and lung cancer the following criteria were used:

i) p-value (probability value) of < 0.05 with fold change >2

ii) AUC (accuracy as a diagnostic biomarker) AUC of > 0.700

In case, the two criteria were fulfilled, the miRNA was considered to be differentially expressed in HCC as compared to healthy individuals and/or healthy individuals, colorectal cancer and lung cancer, respectively.

Independent experiments on 114 plasma samples were performed for each measurement and the miRNA expression level determined represents the mean value of the respective individual data obtained.

The experimental data in the differential miRNA expression analysis between HCC vs. healthy controls are summarized in Tables 5-7 below. Table 5 lists the miRNAs exhibiting significantly differential expressions in both tissue and plasma of

HCC patients as compared to control tissues and healthy control plasma, respectively. Table 6 summarizes the miRNAs exhibiting a differential expression only in plasma of HCC patients as compared to healthy individuals, whereas Table 7 lists the best combinations of the miRNA signatures in plasma of HCC patients. The top 5 combinations with 6 different miRNAs had 87%-94% accuracy as diagnostic biomarkers in differentiating hepatocellular cancer from healthy individuals. In the column "t" denotes the HCC tissue, and "n" denotes normal liver tissue, whereas "p" denotes the HCC patients and "h" denotes healthy controls. Particularly preferred miRNAs (SEQ ID NO: 1 to SEQ ID NO: 7 in Table 5 and SEQ ID NO: 11 to SEQ ID NO: 17 in Table 6, respectively) are shown in bold.

TABLE 5
Tumor-related miRNA signatures in plasma of hepatocellular cancer

Name	Liver tissue		Plasma					
	t-test	fold	t-test	fold	ROC analysis			
	p-value	t/n	p-value	p/h	Sensitivity	Specificity	AUC	95% CI
hsa-miR-122	9.0E-11	0.3	3.6E-04	6.4	100	61	0.801	0.659 to 0.903
hsa-miR-199b-3p	1.9E-03	0.5	2.5E-03	7.9	67	78	0.723	0.573 to 0.843
hsa-miR-192	1.9E-06	0.7	1.6E-02	3.1	92	39	0.676	0.524 to 0.805
hsa-miR-21	6.0E-06	2.0	3.4E-02	2.3	92	39	0.670	0.518 to 0.800
hsa-miR-139-3p	4.5E-09	0.3	2.0E-04	0.1	63	91	0.817	0.677 to 0.915
hsa-miR-10a	7.7E-07	0.4	2.2E-03	4.7	79	65	0.716	0.565 to 0.837
hsa-miR-103	2.1E-05	1.4	7.0E-03	2.6	79	57	0.707	0.556 to 0.830
hsa-miR-181d	1.6E-02	1.6	1.4E-02	0.3	79	65	0.702	0.551 to 0.827
hsa-miR-125b-2*	2.2E-08	0.4	7.5E-03	0.2	75	74	0.710	0.560 to 0.833
hsa-miR-125a-3p	4.4E-05	0.4	2.1E-03	0.2	50	96	0.727	0.578 to 0.847

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TABLE 6
Plasma-specific miRNA signatures for hepatocellular cancer

Name	t-test	fold	ROC analysis			
	p-value	p/h	Sensitivity	Specificity	AUC	95% CI
hsa-miR-34a	6.8E-04	7.3	71	78	0.781	0.636 to 0.888
hsa-miR-136	4.1E-03	5.3	83	57	0.739	0.591 to 0.856
hsa-miR-151-5p	1.1E-02	5.7	75	65	0.716	0.565 to 0.837

hsa-miR-193b*	1.8E-04	0.1	88	65	0.807	0.666 to 0.907
hsa-miR-936	2.2E-03	0.2	71	78	0.759	0.612 to 0.872
hsa-miR-124	2.2E-03	0.1	75	74	0.758	0.611 to 0.871
hsa-miR-198	9.3E-04	0.1	54	91	0.757	0.610 to 0.870
hsa-miR-149*	1.9E-04	0.1	63	91	0.793	0.650 to 0.898
hsa-miR-138	8.0E-04	0.1	63	87	0.779	0.634 to 0.887
hsa-miR-601	2.2E-03	0.3	75	83	0.770	0.624 to 0.880
hsa-miR-769-3p	5.9E-03	0.2	88	61	0.759	0.612 to 0.872
hsa-miR-513c	4.1E-03	0.2	75	70	0.748	0.600 to 0.863
hsa-miR-525-5p	1.0E-03	0.1	63	87	0.741	0.592 to 0.858
hsa-miR-654-5p	1.4E-03	0.2	79	83	0.740	0.591 to 0.857
hsa-miR-518c*	1.3E-02	0.2	75	70	0.739	0.591 to 0.856
hsa-miR-500	1.1E-02	0.2	92	61	0.726	0.576 to 0.845
hsa-miR-181c*	1.0E-02	0.2	83	61	0.724	0.574 to 0.844
hsa-miR-1226*	1.4E-02	0.3	46	100	0.716	0.565 to 0.837
hsa-miR-202	1.4E-02	0.2	58	87	0.707	0.556 to 0.830
hsa-miR-629*	1.7E-03	0.2	75	78	0.704	0.553 to 0.828

TABLE 7
Combined miRNA signatures in plasma of hepatocellular cancer

Combination	t-test	fold	ROC analysis			
	p-value	p/h	Sensitivity	Specificity	AUC	95% CI
hsa-miR-34a/hsa-miR-193b*	9.3E-09	77.3	92	96	0.947	0.840 to 0.991
hsa-miR-21/hsa-miR-936	2.0E-06	14.5	96	78	0.909	0.789 to 0.973
hsa-miR-192/hsa-miR-124	4.3E-06	21.2	92	83	0.904	0.782 to 0.970
hsa-miR-122/hsa-miR-193b*	8.2E-08	67.8	88	87	0.901	0.779 to 0.969
hsa-miR-34a/hsa-miR-138	2.6E-07	51.9	75	91	0.899	0.776 to 0.968
hsa-miR-34a/hsa-miR-198	1.9E-06	67.4	92	87	0.896	0.772 to 0.966
hsa-miR-122/hsa-miR-124	2.7E-07	43.4	92	74	0.892	0.767 to 0.964
hsa-miR-103/hsa-miR-139-3p	6.6E-07	21.0	75	96	0.888	0.762 to 0.961
hsa-miR-192/hsa-miR-193b*	2.0E-06	33.0	88	83	0.884	0.757 to 0.959
hsa-miR-122/hsa-miR-138	3.9E-07	45.6	75	91	0.882	0.755 to 0.958
hsa-miR-34a/hsa-miR-139-3p	1.2E-06	58.8	79	83	0.88	0.753 to 0.957
hsa-miR-122/hsa-miR-198	9.3E-07	59.1	100	74	0.879	0.750 to 0.956

hsa-miR-21/hsa-miR-198	1.0E-05	21.0	88	83	0.875	0.746 to 0.953
hsa-miR-122/hsa-miR-769-3p	2.0E-06	28.2	92	74	0.875	0.746 to 0.953
hsa-miR-103/hsa-miR-193b*	3.9E-06	27.6	83	83	0.873	0.744 to 0.952
hsa-miR-34a/hsa-miR-124	3.9E-06	49.5	71	91	0.872	0.743 to 0.952
hsa-miR-192/hsa-miR-139-3p	7.0E-06	25.1	71	96	0.868	0.737 to 0.949
hsa-miR-34a/hsa-miR-769-3p	4.0E-06	32.2	88	83	0.866	0.735 to 0.948
hsa-miR-192/hsa-miR-936	7.7E-06	19.9	83	83	0.866	0.735 to 0.948
hsa-miR-10a/hsa-miR-193b*	2.9E-06	49.9	88	74	0.863	0.732 to 0.946
hsa-miR-122/hsa-miR-601	1.5E-06	22.4	96	78	0.862	0.730 to 0.945
hsa-miR-21/hsa-miR-769-3p	1.8E-05	10.0	79	83	0.862	0.730 to 0.945
hsa-miR-199b-3p/hsa-miR-193b*	3.2E-06	83.2	92	70	0.861	0.729 to 0.945
hsa-miR-103/hsa-miR-138	2.4E-06	18.5	79	83	0.859	0.726 to 0.943
hsa-miR-103/hsa-miR-198	6.1E-06	24.0	83	78	0.859	0.726 to 0.943
hsa-miR-199b-3p/hsa-miR-139-3p	1.1E-06	63.3	71	96	0.856	0.723 to 0.941
hsa-miR-21/hsa-miR-139-3p	1.0E-05	18.3	71	96	0.855	0.722 to 0.941
hsa-miR-21/hsa-miR-193b*	1.9E-05	24.1	88	74	0.85	0.715 to 0.937

The expression data on the preferred expression signatures in the second aspect for discriminating hepatocellular cancer from healthy individuals, colorectal cancer and lung cancer are summarized in Table 8-10 below. Table 8 lists tumor-related miRNA signatures for hepatocellular cancer; Table 9 shows plasma-specific miRNA signatures, whereas Table 10 displays the best combinations of the miRNA signatures for hepatocellular cancer. The top 5 combinations with 6 different miRNAs had 85%-88% accuracy as diagnostic biomarkers in differentiating hepatocellular cancer from healthy individuals, colorectal cancer and lung cancer. In the column "t" denotes the HCC tissue, and "n" denotes normal liver tissue, whereas column "HCC" denotes plasma from HCC patients, and "control" denotes plasma derived from healthy individuals, colorectal cancer and lung cancer patients. Particularly preferred miRNAs (SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 31 in Table 8; SEQ ID NO: 14 to SEQ ID NO:16 in Table 9, respectively) are shown in bold.

Table 8

Tumor-related miRNA signatures for hepatocellular cancer in discriminating healthy individuals, colorectal cancer and lung cancer

Name	Liver tissue		Plasma					
	t-test	fold	t-test	fold	ROC analysis			
	p-value	t/n	p-value	HCC/control	Sensitivity	Specificity	AUC	95% CI
hsa-miR-122	9.00E-11	0.3	2.10E-06	7.7	100	66	0.855	0.777 to 0.914
hsa-miR-192	1.90E-06	0.7	2.40E-06	5.1	92	57	0.794	0.708 to 0.864
hsa-miR-215	4.80E-06	0.7	8.90E-05	8	58	84	0.759	0.670 to 0.834
hsa-let-7c	2.90E-04	0.6	1.30E-05	4.5	92	52	0.715	0.623 to 0.796
hsa-miR-103	2.10E-05	1.4	1.00E-05	4.4	79	61	0.714	0.622 to 0.795
hsa-miR-139-3p	4.50E-09	0.3	1.20E-03	0.2	63	70	0.71	0.618 to 0.792
hsa-miR-26b	1.00E-05	0.7	5.80E-08	7.2	79	69	0.703	0.610 to 0.785

Table 9

Plasma-specific miRNA signatures for hepatocellular cancer in discriminating healthy individuals, colorectal cancer and lung cancer patients

Name	t-test	fold	ROC analysis			
	p-value	HCC/control	Sensitivity	Specificity	AUC	95% CI
hsa-miR-936	1.3E-03	0.2	71	82	0.769	0.681 to 0.843
hsa-miR-193b*	4.4E-05	0.1	98	62	0.762	0.673 to 0.837
hsa-miR-124	1.0E-04	0.2	70	69	0.744	0.654 to 0.821
hsa-miR-34a	2.8E-04	5.8	71	68	0.738	0.648 to 0.816
hsa-miR-198	9.8E-05	0.1	54	88	0.724	0.632 to 0.803
hsa-let-7g	1.1E-06	4.4	96	50	0.711	0.618 to 0.792
hsa-miR-363	1.7E-06	3.3	92	56	0.707	0.615 to 0.789

Table 10

Combinations of miRNA signatures for hepatocellular cancer in discriminating healthy individuals, colorectal cancer and lung cancer

Combination	t-test	Fold	ROC analysis			
	p-value	HCC/control	Sensitivity	Specificity	AUC	95% CI
hsa-miR-122/hsa-miR-936	1.4E-12	50.3	100	77	0.931	0.868 to 0.970
hsa-miR-34a/hsa-miR-193b*	8.9E-10	42.5	92	82	0.865	0.789 to 0.922
hsa-miR-34a/hsa-miR-198	9.6E-10	40.1	96	78	0.868	0.792 to 0.924

hsa-miR-192/hsa-miR-936	7.7E-11	33.3	88	84	0.907	0.838 to 0.953
hsa-miR-122/hsa-miR-193b*	6.4E-10	56.5	96	74	0.885	0.812 to 0.937
hsa-miR-122/hsa-miR-198	4.9E-10	53.2	100	68	0.887	0.815 to 0.939
hsa-miR-192/hsa-miR-124	4.3E-09	29.2	96	72	0.875	0.800 to 0.929
hsa-miR-192/hsa-miR-193b*	6.0E-09	37.4	96	72	0.865	0.788 to 0.922
hsa-miR-122/hsa-miR-124	5.7E-10	44.1	92	73	0.878	0.803 to 0.931
hsa-miR-192/hsa-miR-198	1.9E-08	35.2	96	69	0.866	0.789 to 0.922
hsa-miR-363/hsa-miR-936	9.7E-08	21.7	88	80	0.862	0.784 to 0.919
hsa-miR-215/hsa-miR-193b*	2.3E-08	58.5	92	79	0.862	0.785 to 0.919
hsa-miR-103/hsa-miR-936	8.0E-08	28.6	92	66	0.856	0.778 to 0.915
hsa-miR-122/hsa-miR-139-3p	6.7E-09	32.1	92	62	0.844	0.764 to 0.905
hsa-let-7c/hsa-miR-936	2.9E-07	29.0	96	62	0.844	0.764 to 0.905
hsa-miR-215/hsa-miR-198	3.6E-08	55.1	88	68	0.844	0.764 to 0.905
hsa-miR-192/hsa-miR-139-3p	3.8E-08	21.2	96	58	0.842	0.762 to 0.904
hsa-miR-27b/hsa-miR-198	2.8E-08	29.4	92	71	0.832	0.751 to 0.896
hsa-miR-26b/hsa-miR-936	1.1E-08	47.0	92	62	0.830	0.748 to 0.894
hsa-let-7g/hsa-miR-936	4.4E-07	28.7	96	63	0.830	0.748 to 0.894
hsa-miR-103/hsa-miR-198	7.5E-07	30.3	92	64	0.829	0.747 to 0.893
hsa-let-7c/hsa-miR-193b*	9.4E-09	32.6	92	66	0.827	0.745 to 0.892
hsa-miR-103/hsa-miR-193b*	1.3E-06	32.2	83	71	0.824	0.741 to 0.889
hsa-miR-26b/hsa-miR-139-3p	1.1E-08	30.0	92	61	0.823	0.740 to 0.888
hsa-let-7c/hsa-miR-198	9.9E-07	30.7	100	58	0.823	0.741 to 0.888
hsa-miR-27b/hsa-miR-193b*	1.4E-07	31.2	92	70	0.822	0.739 to 0.887
hsa-let-7g/hsa-miR-193b*	4.7E-08	32.2	92	61	0.822	0.739 to 0.887
hsa-miR-363/hsa-miR-139-3p	2.3E-06	13.8	92	62	0.820	0.737 to 0.886
hsa-let-7g/hsa-miR-198	1.5E-06	30.4	88	67	0.819	0.736 to 0.885
hsa-miR-363/hsa-miR-198	1.2E-06	22.9	88	69	0.818	0.735 to 0.884
hsa-miR-26b/hsa-miR-198	5.0E-08	49.8	92	62	0.818	0.735 to 0.884
hsa-miR-103/hsa-miR-139-3p	5.1E-07	18.3	75	83	0.817	0.733 to 0.883
hsa-miR-301a/hsa-miR-198	1.2E-06	24.4	88	68	0.816	0.733 to 0.882
hsa-miR-26b/hsa-miR-193b*	9.1E-09	52.8	75	77	0.816	0.733 to 0.883
hsa-let-7g/hsa-miR-139-3p	1.8E-07	18.3	83	67	0.813	0.730 to 0.880
hsa-miR-363/hsa-miR-124	1.8E-06	19.0	92	62	0.813	0.729 to 0.879
hsa-let-7c/hsa-miR-139-3p	3.2E-06	18.5	96	57	0.811	0.727 to 0.879
hsa-miR-301a/hsa-miR-193b*	2.5E-06	25.9	79	77	0.811	0.727 to 0.878
hsa-miR-301a/hsa-miR-139-3p	5.1E-06	14.7	75	74	0.809	0.725 to 0.877
hsa-miR-26b/hsa-miR-124	5.3E-08	41.3	92	62	0.808	0.723 to 0.875
hsa-let-7d/hsa-miR-198	3.2E-06	28.9	92	59	0.805	0.720 to 0.873
hsa-let-7d/hsa-miR-139-3p	2.7E-06	17.5	92	54	0.803	0.718 to 0.871

hsa-miR-103/hsa-miR-124	3.4E-06	25.1	79	72	0.802	0.717 to 0.870
hsa-miR-363/hsa-miR-193b*	2.5E-06	24.3	75	79	0.802	0.717 to 0.871
hsa-let-7d/hsa-miR-193b*	2.9E-07	30.7	92	60	0.800	0.714 to 0.869

Example 5: Verification of the microarray data

For verifying (and/or quantifying) the miRNA expression data acquired on microarrays, an established quantitative RT-PCR employing a TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions. Briefly, reverse transcription (RT) was performed with Taqman microRNA RT Kits according to the instruction from Applied Biosystem. 10ng total RNA was reverse-transcripted in 15ul RT solution mix that contains 1X Reverse Transcription Buffer, 1X RT primer, 1nM dNTP, 4U RNase Inhibitor and 50U MultiScribe Reverse Transcriptase. Then the RT solutions were performed by using the thermal program of 16°C, 30min; 42°C, 30min; 85°C, 5min on the PCR machine (Thermal cycler alpha engine, Bio-rad). Quantitative PCR was performed with TaqMan Universal PCR Master Mix kit and and Taqman microRNA assays kits according to the instruction from Applied Biosystem. 2ul RT products were PCR amplified in 1X TaqMan Universal PCR Master Mix, No AmpErase UNG, 1X TaqMan MicroRNA Assay mix. Each reaction was duplicated in triple. The real-time PCR was performed in Roch Light Cycling 480 machine with the program of 96°C, 5min initial heating; then 45 or 50 cycles of 95°C, 15s; 60°C, 60s. Cp value was calculated with 2nd derivative method in LC480 software. Then miRNAs were absolutely quantified with the standard samples CP values. For the CP value for each miRNA was normalized one internal stable control hsa-miR-1228.

The experimental data on platform comparison with 4 miRNA combinations with 5 miRNAs from plasma samples of 16 healthy individuals and HCC patients show in Figure 4. The quantitative correlation (R) of the fold changes acquired on the arrays with that obtained from quantitative RT-PCR was 0.76. The results demonstrate that the miRNA signatures discovered on Agilent miRNA microarrays are highly reliable.

The results obtained demonstrate a global highly specific regulation of miRNA expression in hepatocellular cancer. Thus, the respective subsets of miRNAs specified herein represent unique miRNA expression signatures for expression profiling of

hepatocellular cancer that do not only allow the identification of a cancerogenous state as such but also enables the discrimination of colorectal cancer and lung cancer

The identification of the miRNA expression signatures of the present invention provides a unique molecular marker that allows screening, detection, diagnosing hepatocellular cancer in blood. Furthermore, the expression signatures can be used to monitor the therapy response and guide the treatment decision in hepatocellular cancer patients. Additionally, the expression signatures may be also used for development of anti- hepatocellular cancer drugs.

The present invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by embodiments and optional features, modifications and variations of the inventions embodied therein may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

CLAIMS

1. Diagnostic kit of molecular markers in blood for identifying one or more target plasma exhibiting hepatocellular cancer, the kit comprising a plurality of nucleic acid molecules, each nucleic acid molecule encoding a microRNA sequence,

wherein one or more of the plurality of nucleic acid molecules are differentially expressed in the target plasma and in one or more control plasma, and

wherein the one or more differentially expressed nucleic acid molecules together represent a nucleic acid expression signature that is indicative for the presence of hepatocellular cancer.

2. The kit of claim 1, wherein the nucleic acid expression signature may comprise at least thirty-two nucleic acid molecules, preferably at least six nucleic acid molecules.

3. The kit of claim 1 or 2, wherein the nucleic acid expression signature comprises at least one nucleic acid molecule encoding a microRNA sequence whose expression is up-regulated in the one or more target plasma compared to the one or more control plasma and at least one nucleic acid molecule encoding a microRNA sequence whose expression is down-regulated in the one or more target plasma compared to the one or more control plasma.

4. The kit of any of claims 1 to 3, wherein the nucleic acid expression signature comprises any one or more of the nucleic acid molecules encoding tumor-related signatures hsa-miR-122, hsa-miR-199b-3p, hsa-miR-192, hsa-miR-21, hsa-miR-139-3p, hsa-miR-10a, hsa-miR-103, hsa-miR-181d, hsa-miR-125b-2*, hsa-miR-125a-3p; plasma-specific signatures hsa-miR-34a, hsa-miR-136, hsa-miR-151-5p, hsa-miR-193b*, hsa-miR-124, hsa-miR-936, hsa-miR-198, hsa-miR-149*, hsa-miR-138, hsa-miR-601, hsa-miR-769-3p, hsa-miR-513c, hsa-

miR-525-5p, hsa-miR-654-5p, hsa-miR-518c*, hsa-miR-500, hsa-miR-181c*, hsa-miR-1226*, hsa-miR-202, hsa-miR-629* and internal stable controls hsa-miR-1238 and hsa-miR-1228.

5. The kit of any of claims 4 , wherein the expression of any one or more of the nucleic acid molecules encoding hsa-miR-122, hsa-miR-199b-3p, hsa-miR-192, hsa-miR-21, hsa-miR-10a, hsa-miR-103, hsa-miR-34a, hsa-miR-136, hsa-miR-151-5p is up-regulated and the expression of any one or more of the nucleic acid molecules encoding hsa-miR-139-3p, hsa-miR-193b*, hsa-miR-124, hsa-miR-181d, hsa-miR-125b-2*, hsa-miR-125a-3p, hsa-miR-936, hsa-miR-198, hsa-miR-149*, hsa-miR-138, hsa-miR-601, hsa-miR-769-3p, hsa-miR-513c, hsa-miR-525-5p, hsa-miR-654-5p, hsa-miR-518c*, hsa-miR-500, hsa-miR-181c*, hsa-miR-1226*, hsa-miR-202, hsa-miR-629* is down-regulated; hsa-miR-1238 and hsa-miR-1228 is un-changed in the one or more target plasma compared to the one or more healthy controls.

6. The kit of any of claim 1 to 3, wherein the nucleic acid expression signature comprises any one or more nucleic acid combinations encoding hsa-miR-34a/hsa-miR-193b*, hsa-miR-21/hsa-miR-936, hsa-miR-192/hsa-miR-124, hsa-miR-122/hsa-miR-193b*, hsa-miR-34a/hsa-miR-138, hsa-miR-34a/hsa-miR-198, hsa-miR-122/hsa-miR-124, hsa-miR-103/hsa-miR-139-3p, hsa-miR-192/hsa-miR-193b*, hsa-miR-122/hsa-miR-138, hsa-miR-34a/hsa-miR-139-3p, hsa-miR-122/hsa-miR-198, hsa-miR-122/hsa-miR-769-3p, hsa-miR-103/hsa-miR-193b*, hsa-miR-34a/hsa-miR-124, hsa-miR-192/hsa-miR-139-3p, hsa-miR-34a/hsa-miR-769-3p, hsa-miR-192/hsa-miR-936, hsa-miR-10a/hsa-miR-193b*, hsa-miR-122/hsa-miR-601, hsa-miR-21/hsa-miR-769-3p, hsa-miR-199b-3p/hsa-miR-193b*, hsa-miR-103/hsa-miR-138, hsa-miR-103/hsa-miR-198, hsa-miR-199b-3p/hsa-miR-139-3p, hsa-miR-21/hsa-miR-139-3p and hsa-miR-21/hsa-miR-193b*.

7. The kit of any of claims 1 to 6, for the further use of discriminating hepatocellular cancer from healthy individuals, colorectal cancer and lung cancer.

8. The kit of claim 7, wherein the nucleic acid expression signature may comprise at least sixteen nucleic acid molecules, preferably at least six nucleic acid molecules.

9. The kit of any of claims 1 to 8, wherein the nucleic acid expression signature comprises any one or more of the nucleic acid molecules encoding tumor-related signatures hsa-miR-122, hsa-miR-192, hsa-miR-215, hsa-let-7c, hsa-miR-103, hsa-miR-139-3p, hsa-miR-26b; plasma-specific signatures hsa-miR-936, hsa-miR-193b*, hsa-miR-124, hsa-miR-34a, hsa-miR-198, hsa-let-7g and hsa-miR-363 and internal stable controls: has-miR-1228 and hsa-miR-1238.

10. The kit of any of claims 9, wherein the expression of any one or more of the nucleic acid molecules encoding molecules encoding hsa-miR-122, hsa-miR-192, hsa-miR-215, hsa-let-7c, hsa-miR-103, hsa-miR-26b, hsa-miR-34a, hsa-let-7g, hsa-miR-363 is up-regulated and the expression of any one or more of the nucleic acid molecules encoding hsa-miR-936, hsa-miR-193b*, hsa-miR-124, hsa-miR-139-3p, hsa-miR-198 is down-regulated; hsa-miR-1238 and hsa-miR-1228 is un-changed in the one or more target plasma compared to the one or more healthy individuals, colorectal cancer and lung cancer.

11. The kit of any of claim 7 to 10, wherein the nucleic acid expression signature comprises any one or more nucleic acid combinations encoding hsa-miR-122/hsa-miR-936, hsa-miR-34a/hsa-miR-193b*, hsa-miR-34a/hsa-miR-198, hsa-miR-192/hsa-miR-936, hsa-miR-122/hsa-miR-193b*, hsa-miR-122/hsa-miR-198, hsa-miR-192/hsa-miR-124, hsa-miR-192/hsa-miR-193b*, hsa-miR-122/hsa-miR-124, hsa-miR-192/hsa-miR-198, hsa-miR-363/hsa-miR-936, hsa-

miR-215/hsa-miR-193b*, hsa-miR-103/hsa-miR-936, hsa-miR-122/hsa-miR-139-3p, hsa-let-7c/hsa-miR-936, hsa-miR-215/hsa-miR-198, hsa-miR-192/hsa-miR-139-3p, hsa-miR-27b/hsa-miR-198, hsa-miR-26b/hsa-miR-936, hsa-let-7g/hsa-miR-936, hsa-miR-103/hsa-miR-198, hsa-let-7c/hsa-miR-193b*, hsa-miR-103/hsa-miR-193b*, hsa-miR-26b/hsa-miR-139-3p, hsa-let-7c/hsa-miR-198, hsa-miR-27b/hsa-miR-193b*, hsa-let-7g/hsa-miR-193b*, hsa-miR-363/hsa-miR-139-3p, hsa-let-7g/hsa-miR-198, hsa-miR-363/hsa-miR-198, hsa-miR-26b/hsa-miR-198, hsa-miR-103/hsa-miR-139-3p, hsa-miR-301a/hsa-miR-198, hsa-miR-26b/hsa-miR-193b*, hsa-let-7g/hsa-miR-139-3p, hsa-miR-363/hsa-miR-124, hsa-let-7c/hsa-miR-139-3p, hsa-miR-301a/hsa-miR-193b*, hsa-miR-301a/hsa-miR-139-3p, hsa-miR-26b/hsa-miR-124, hsa-let-7d/hsa-miR-198, hsa-let-7d/hsa-miR-139-3p, hsa-miR-103/hsa-miR-124, hsa-miR-363/hsa-miR-193b* and hsa-let-7d/hsa-miR-193b*.

12. Method for identifying one or more target plasma exhibiting hepatocellular cancer, the method comprising:

(a) determining in the one or more target plasma the expression levels of a plurality of nucleic acid molecules, each nucleic acid molecule encoding a microRNA sequence;

(b) determining the expression levels of the plurality of nucleic acid molecules in one or more healthy control plasma; and

(c) identifying from the plurality of nucleic acid molecules one or more nucleic acid molecules that are differentially expressed in the target and control plasma by comparing the respective expression levels obtained in steps (a) and (b), wherein the one or more differentially expressed nucleic acid molecules together represent a nucleic acid expression signature, as defined in any of 1 to 11, that is indicative for the presence of hepatocellular cancer.

13. The method of claim 12, for the further use of discriminating hepatocellular cancer from healthy individuals, colorectal cancer and lung cancer.

14. Method for monitoring treatment of hepatocellular cancer, the method comprising:

(a) identifying in the one or more target plasma a nucleic acid expression signature by using a method, as defined herein; and

(b) monitoring in blood the expression of one or more nucleic acid molecules encoding a microRNA sequence that is/are comprised in the nucleic acid expression signature in such way that the expression of a nucleic acid molecule whose expression in plasma is up-regulated before treatment but is down-regulated after treatment and the expression of a nucleic acid molecule whose expression in plasma is down-regulated before treatment but is up-regulated after treatment.

15. Method for preventing or treating hepatocellular cancer, the method comprising:

(a) identifying a nucleic acid expression signature in blood by using a method, as defined claim 12 or 13, and

(b) modifying in blood the expression of one or more nucleic acid molecules encoding a microRNA sequence that is/are comprised in the nucleic acid expression signature in such way that the expression of a nucleic acid molecule whose expression is up-regulated in blood is down-regulated and the expression of a nucleic acid molecule whose expression is down-regulated in blood is up-regulated.

16. Pharmaceutical composition for the prevention and/or treatment of hepatocellular cancer in blood, the composition comprising one or more nucleic acid molecules, each nucleic acid molecule encoding a sequence that is at least partially complementary to a microRNA sequence encoded by a nucleic acid

molecule whose expression is up-regulated in plasma from hepatocellular cancer patients, as defined herein, and/or that corresponds to a microRNA sequence encoded by a nucleic acid molecule whose expression is down-regulated in plasma from colorectal cancer patients, as defined in any of claim 1 to 13.

17. Use of the pharmaceutical composition of 16 for the manufacture of a medicament for the prevention and/or treatment of hepatocellular cancer.

FIGURE 1

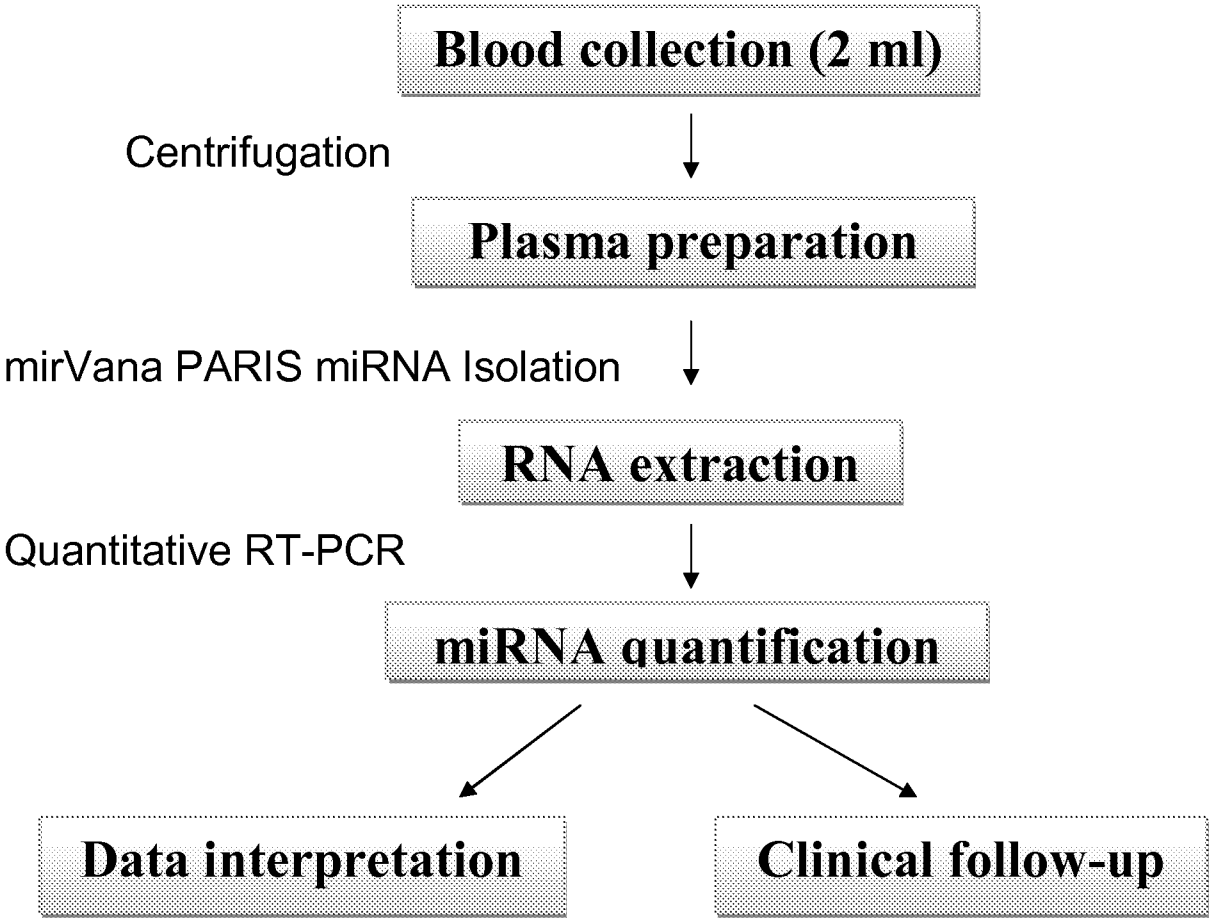

Blood collection (2 ml)
Centrifugation
Plasma preparation
mirVana PARIS miRNA Isolation
RNA extraction
Quantitative RT-PCR
miRNA quantification
Data interpretation
Clinical follow-up

FIGURE 2

Name	Expression	Accuracy (%)
Tumor-related miRNA		
hsa-miR-122	UP	80
hsa-miR-199b-3p	UP	73
hsa-miR-10a	UP	72
hsa-miR-192	UP	65
hsa-miR-103	UP	68
hsa-miR-21	UP	65
hsa-miR-139-3p	DOWN	77
Plasma-specific miRNA		
hsa-miR-34a	UP	75
hsa-miR-136	UP	70
hsa-miR-151-5p	UP	70
hsa-miR-193b*	DOWN	76
hsa-miR-124	DOWN	74
hsa-miR-936	DOWN	75
hsa-miR-198	DOWN	73
Combination		
hsa-miR-34a/hsa-miR-193b*	UP	94
hsa-miR-34a/hsa-miR-198	UP	89
hsa-miR-192/hsa-miR-124	UP	87
hsa-miR-122/hsa-miR-193b*	UP	87
hsa-miR-122/hsa-miR-198	UP	87

FIGURE 3

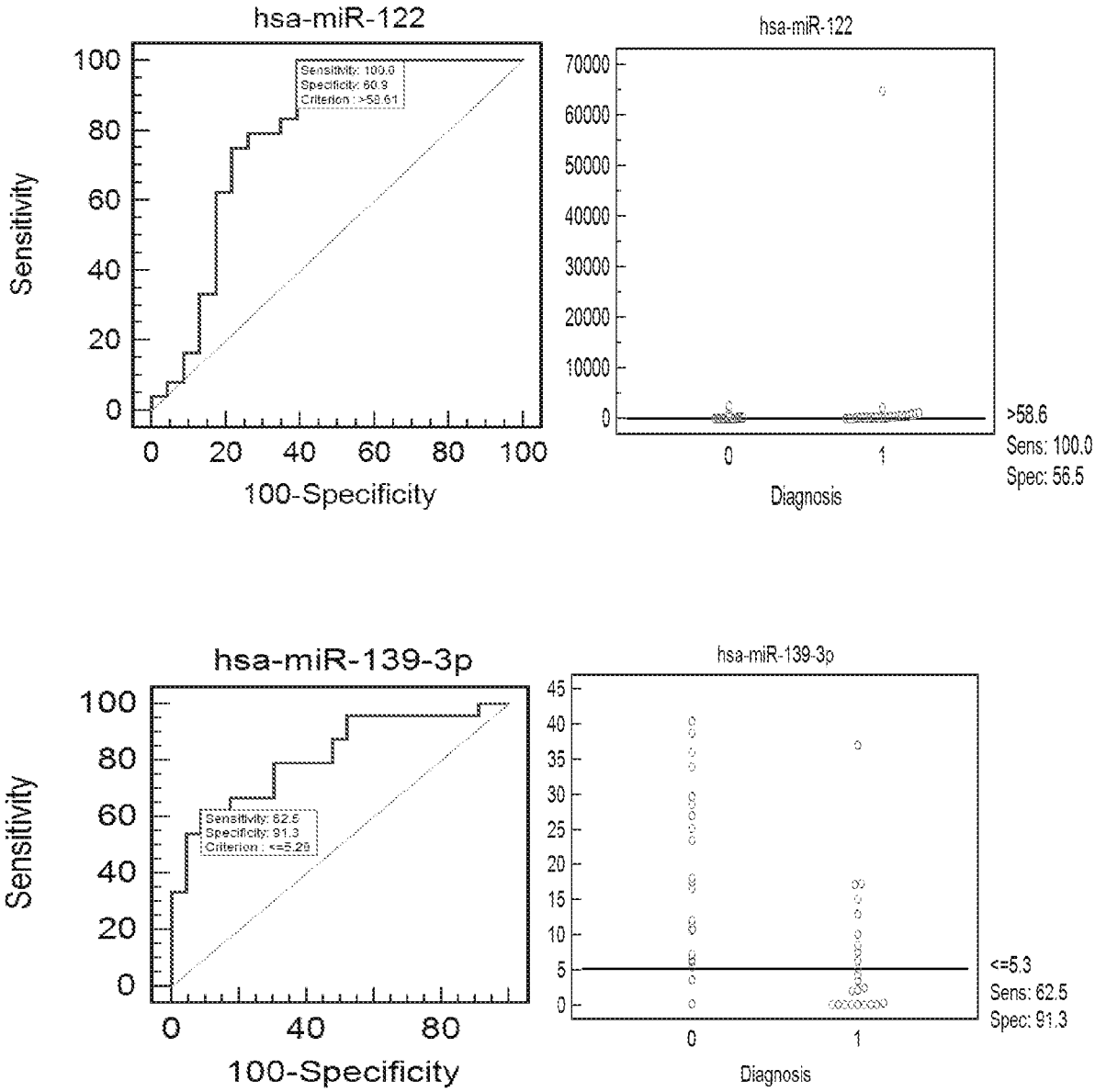
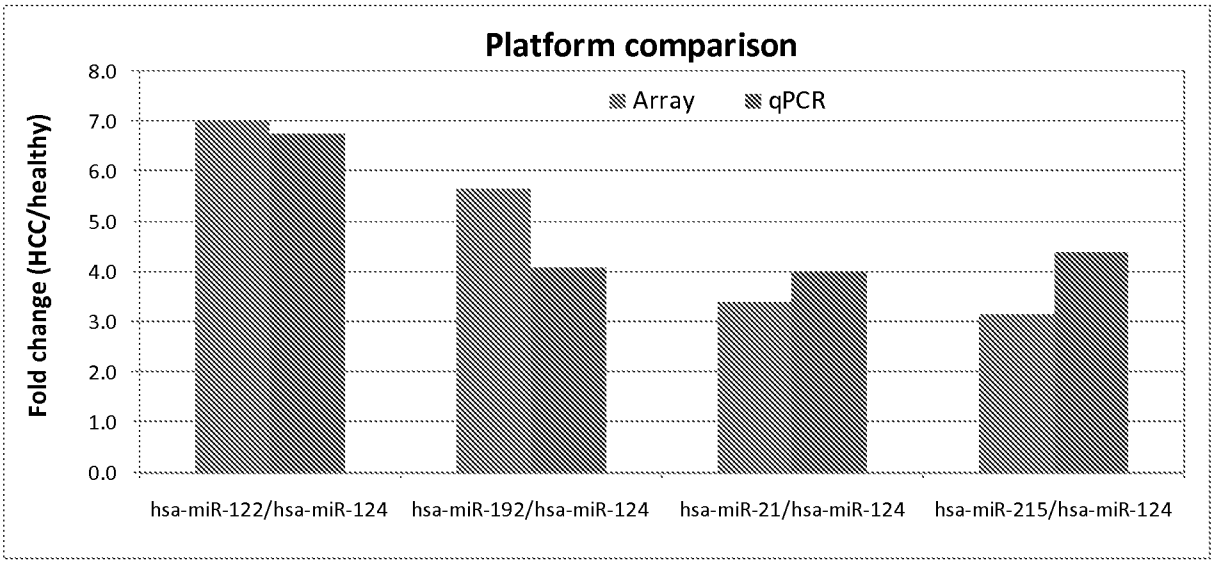

hsa-miR-122
Sensitivity: 100.0
Specificity: 60.9
Criterion : >58.61
Sensitivity
100-Specificity
hsa-miR-122
Diagnosis
>58.6
Sens: 100.0
Spec: 56.5
hsa-miR-139-3p
Sensitivity: 62.5
Specificity: 91.3
Criterion : <=5.28
Sensitivity
100-Specificity
hsa-miR-139-3p
Diagnosis
<=5.3
Sens: 62.5
Spec: 91.3

FIGURE 4

Name	Expression	Accuracy (%)
Tumor-related miRNA		
hsa-miR-122	UP	83
hsa-miR-192	UP	74
hsa-miR-215	UP	71
hsa-let-7c	UP	72
hsa-miR-103	UP	70
hsa-miR-26b	UP	74
hsa-miR-139-3p	DOWN	66
Plasma-specific miRNA		
hsa-miR-34a	UP	69
hsa-let-7g	UP	73
hsa-miR-363	UP	74
hsa-miR-193b*	DOWN	80
hsa-miR-124	DOWN	70
hsa-miR-198	DOWN	71
Combination		
hsa-miR-122/hsa-miR-936	UP	88
hsa-miR-34a/hsa-miR-193b*	UP	87
hsa-miR-34a/hsa-miR-198	UP	87
hsa-miR-192/hsa-miR-936	UP	86
hsa-miR-122/hsa-miR-193b*	UP	85

FIGURE 5


Platform comparison
Array
qPCR
Fold change (HCC/healthy)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
hsa-miR-122/hsa-miR-124
hsa-miR-192/hsa-miR-124
hsa-miR-21/hsa-miR-124
hsa-miR-215/hsa-miR-124

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2010/080230

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

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)

IPC: C12Q, A61K, A61P

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)

CNPAT, CNKI, WPI, EPODOC, ISI Web of Knowledge: microRNA, miRNA, hepatocellular cancer, miR-122, miR-199b-3p, miR-192, miR-21, miR-139-3p, miR-10a, miR-103, miR-181d, miR-125b-2, miR-125a-3p, miR-34a, miR-136, miR-151-5p, miR-193b, miR-124, miR-936, miR-198, miR-149, miR-138, miR-601, miR-769-3p, miR-513c, miR-525-5p, miR-654-5p, miR-518c, miR-500, miR-181c, miR-1226, miR-202, miR-629, miR-1238, miR-1228.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	EP2196543 A1(KONINK PHILIPS ELECTRONICS NV), 16 Jun. 2010(16.06.2010), See abstract, claims 1-15.	1-14, 16-17
X	WO2009036236 A1 (UNIV OHIO STATE RES FOUND), 19 Mar. 2009(19.03.2009), See claims 1-48, pages 12, 16-17, 30 in description.	1-14, 16-17
A	CN101386848 A(UNIV NANJING), 18 Mar. 2009(18.03.2009), See claims 1-8.	1-14, 16-17
A	WO2009111643 A3(ASURAGEN INC), 29 Oct. 2009(29.10.2009), See the whole document.	1-14, 16-17

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

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 Mar. 2011(21.03.2011)	**07 Apr. 2011 (07.04.2011)**
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451	Authorized officer WU,Li Telephone No. (86-10)62411046

Form PCT/ISA /210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2010/080230

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO2009058907 A2(ISIS PHARM INC), 7 May 2009(07.05.2009), See claims 1-169, pages 15-16 in description.	1-14, 16-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2010/080230

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.: 15
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 15 relates to treatment methods practiced on the live human or animal body (Rule 39.1 (iv) PCT).

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:

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 fee.

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.:

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.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2010/080230

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
WO2009036236A1	19.03.2009	EP2190992A1	02.06.2010
		JP2010538653W	16.12.2010
		CA2699646A1	19.03.2009
		CN101842484A	22.09.2010
		IN201001981P4	03.09.2010
		AU2008298744A1	19.03.2009
		US2010279292A1	04.11.2010
WO2009058907A2	07.05.2009	WO2009058907A3	17.12.2009
		CA2704043A1	07.05.2009
		US20100267814A1	21.10.2010
		AU2008318778A1	07.05.2009
		EP2217248A2	18.08.2010
WO2009111643A3	29.10.2009	US2009233297A1	17.09.2009
		EP2268832A2	05.01.2011
		WO2009111643A2	11.09.2009
EP2196543 A1	16.06.2010	None	
CN101386848 A	18.03.2009	None	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2010/080230

A. CLASSIFICATION OF SUBJECT MATTER

C12Q1/68 (2006.01) i

A61K31/7088 (2006.01) i

A61P 35/00 (2006.01) i