❷ ラベリングされたペレットを5 μLのNuclease Free Waterで溶かす.

⬇

❸ 同一のアレイにハイブリダイゼーションするAlexa Flour 546でラベルされた小分子RNAとAlexa Flour 647でラベルされた小分子RNAを混ぜ，10 μLとする.

⬇

❹ 10 μLの2×Hybridization Buffer（キット）を加えてピペッティングにてよく混ぜる．できるだけ泡立てないように注意する.

⬇

❺ 95℃で3分間インキュベートした後，氷冷して熱変性を行う.

⬇

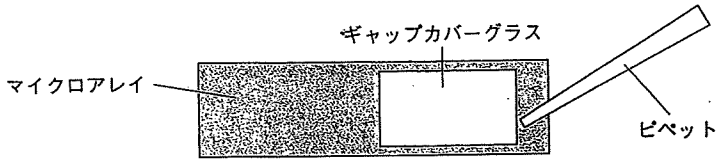
❻ 遠心機の最高回転数で2分間遠心し，スピンダウンするとともに生じた泡を消す.

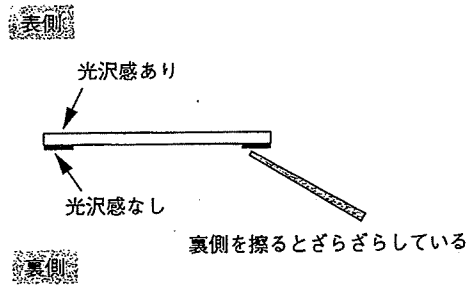
⬇

❼ アレイをハイブリチャンバーにセットする.

⬇

❽ アレイ上にギャップカバーグラスを置き（上下に気をつける），隙間から液を注入する（下図）この時気泡が入らないように注意する.





⬇

❾ ハイブリチャンバーを閉め，恒温水槽に沈めて遮光下で16時間ハイブリダイゼーションを行う.

⬇

❿ ハイブリダイゼーションが終了したら，室温の洗浄バッファー1（6×SSC，0.005％ Triton X-102）で10分間洗浄した後，4℃の洗浄バッファー2（0.1×SSC，0.005％ Triton X-102）で5分間洗浄する.

⬇

⓫ マイクロアレイスキャナーでスキャンする.

⬇

⓬ マイクロアレイ解析ソフトでデータ解析を行う.

【マイクロRNAの発現異常と肝発癌の関与】

The association between aberrant expression of miRNA and hepatocarcinogenesis

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Murakami　Yoshiki

Key words
miRNA, hepatocallular carcinoma, oncogene

要　約

肝細胞癌は慢性ウイルス性肝炎特にC型肝炎ウイルス(HCV)が年余の炎症を繰り返し，肝線維化が進行した結果発生する事が多い。マイクロRNAの発現は肝発癌，炎症の原因となるHCVの複製制御，慢性C型肝炎治療薬であるインターフェロンの応答，肝線維化等と関係し慢性肝疾患の進展に大きく影響していると考えられる。また近年ではマイクロRNAの情報基盤を元に診断，治療を目標とした臨床応用への試みも報告されている。本稿では肝細胞癌とマイクロRNAの発現についてその病態と，臨床応用の可能性についてこれまでの報告に自検例を加えて概説する。

はじめに

マイクロRNAはそれ自身タンパク質をコードしていない20塩基配列前後の小分子RNAである。マイクロRNAは塩基配列上相補的な標的部位をもつ様々な遺伝子（標的遺伝子）の発現を調節している。具体的にはマイクロRNAはRNA-induced silencing complex (RISC)と呼ばれるタンパク質との複合体を形成する。この複合体において，中心的な役割を果たすのが，マイクロRNAと直接結合するArgonaute (Ago)というタンパク質である。この複合体の中でマイクロRNAは（1）標的遺伝子と結合しその翻訳を阻害する，（2）または標的遺伝子のCap構造を不安定化し標的遺伝子のRNAのdegradationを起こす事により遺伝子発現の調節を行なう[1]。この遺伝子発現調節機能は発生のタイミングや形態形成，アポトーシス，細胞増殖や癌化など，生命現象を精密に制御していることがこれまでの研究から知られている。これまでに，動物，植物，ウイルス等において約9,000種のマイクロRNAが報告されており，ヒトのマイクロRNAは721種(miRbase ver. 14.0)が登録されている。マイクロRNAは塩基配列特異的に標的遺伝子を認識しその遺伝子を制御するが，標的遺伝子との塩基配列相補性は100%マッチを要求しないため，標的遺伝子は単一のマイクロRNAに対し数百あると考えられ，それを総和するとマイクロRNA全体で遺伝子全体の1/3以上を制御していると考えられている[2]。近年，マイクロRNAの働きを適切に制御することによって，疾患の治療として利用することやマイクロRNAの発現パターンを腫瘍マーカーなどのバイオマーカーとしての臨床応用への利用が急速に高まっている。

肝細胞癌は本邦の男性の中で3番目に多く，5年生存率は40-50%程度であるため発癌の抑制は患者のQOLの向上とともに医療費の削減にも有用である。発癌の主たる原因は慢性ウイルス感染であり，その70%をC型慢性肝炎が占める，このC型慢性肝炎は適切な治療を行わないと，年余を経て肝硬変に至り肝細胞癌に発生する。我々は慢性肝疾患（慢性肝炎，肝硬変，肝細胞癌）慢性肝疾患別にマイクロRNA発現プロファイルを作成した，本稿ではこの結果を元に（1）肝発癌に関係したマイクロRNA，（2）肝癌の悪性度に関係したマイクロRNA（3）バイオマーカーとしてのマイクロRNA利用について概説する。

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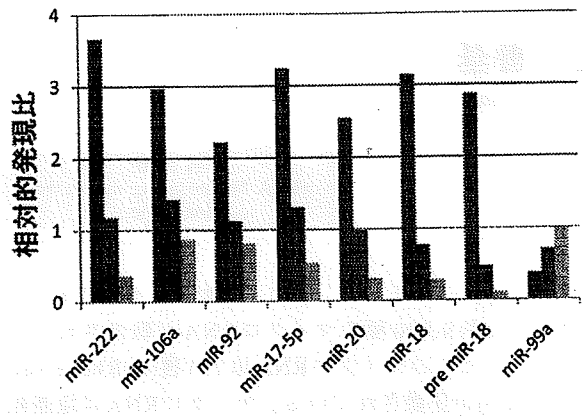

miRNA	p-value
miR-222	0.015
miR-106a	0.0451
miR-92	0.000821
miR-17-5p	0.0121
miR-20	0.00658
miR-18	0.00897
pre miR-18	0.00471
miR-99a	0.0105

図1　ここにキャプションが入ります）— 図表のキャプションをお願いします

1．肝発癌に関係しているマイクロRNA

肝臓癌組織と非癌部組織（外科的に一塊として採取した場合周辺部非癌部組織は大半が慢性肝炎または肝硬変となる）を用いてマイクロRNA発現の自検例を含めた比較解析結果を表1に示す。これらの中で同じクラスターに存在しているマイクロRNAや，他の癌種でも発現異常を示すものが認められる。miR-21は発癌に関与するマイクロRNAいわゆるoncogeneとして注目されており，肝細胞癌ではPTENを制御しmiR-21が過剰発現すると癌細胞の増殖を促進する事が示されている。他に卵巣癌，子宮頸癌，肺癌，乳癌，前立腺癌，膵癌，頭頸部腫瘍，甲状腺癌，胆管細胞癌，白血病，脳腫瘍（膠芽種）との関連が報告されている（表2）。発癌の際にmiR-21はどのようにその発現の調節を受けているのかと言う点に対し，骨髄腫細胞ではsignal transducer and activator of transcription 3 (STAT3)存在下にmiR-21の発現が亢進する[3]，activator protein 1 (AP-1) はmiR-21の転写活性を上昇させる[4]，transforming growth factor beta (TGF-b)がmiR-21の発現を亢進させる5)等の報告がある。miR-17-92 clusterもoncogeneとして多くの癌種との関連が報告されている，HBV陽性肝細胞癌とその実験動物モデルのwoodchuck hepatitis virusに感染したwoodchuck肝細胞癌において，miR-17-92 clusterの過剰発現が癌細胞の増殖に関与している報告がある。このクラスターの過剰発現は他にも造血器腫瘍，前立腺癌，肺癌，甲状腺癌，脳腫瘍（膠芽種，神経芽種）（表2）で観察されている。また発癌に関係するメカニズムとしてpro-apoptosis機能を持ち細胞死を制御するBCL2L11/BIMを標的としている，c-Myc oncogeneがTsp-1とCTGFに制御されているが，miR-17-92 clusterはTsp-1とCTGFの発現を制御しc-Mycの発現を亢進させるとの報告がある。肝癌では他にmiR-18, miR-30d, miR-143, miR-151, miR-181b, miR-224が細胞増殖を亢進する，apoptosisからの回避等の機能を持ち，これらのマイクロRNAも癌遺伝子として発癌に関与している（表1）。反対にマイクロRNAが癌抑制遺伝子として機能している例もmiR-23a, miR-23b, miR-24, miR-27a, miR-34a, miR-101, miR-122, miR-124, miR195, miR-203, miR-223, miR-375等があり，これらのマイクロRNAは癌組織中ではその発現が抑制されている。癌におけるマイクロRNAの発現異常については上述のようにマイクロRNAを制御する遺伝子が明らかになっている場合，ゲノム上でマイクロRNAをコードしている領域が変異，欠失を起こしている場合[6]，メチル化等で発現そのものを制御されているなどが報

miRNA	target gene	function	reference
let-7	RAS		Johnson SM et al. Cell 2005
let-7g	type 1 collagen alpha 2 (COL1A2)	inhibition of cell migration	Ji J et al. J Hepatol 2010
	c-myc/ $p16^{INK4A}$	inhibition of proliferation	Lan FF et al. Int J cancer 2010
miR-100	insulin like growth factor 1 receptor (IGF-1R)		Tovar V et al. J hepatol 2010
miR-101	v-fos	inhibition of migration and invasion	Li S et al. Hepatology 2009
	myeloid cell leukemia sequence 1 (Mcl-1)	proliferation of apoptosis	Su H et al. Cancer Res 2009
	myeloid cell leukemia sequence 1 (Mcl-1)	inhibition of proliferation	Su H et al. Cancer Res 2009
miR-122	HNF1A, HNF3A, HNF3B	loss of cell migration and invasion	Coulouarn C et al. Oncogene 2009
	bcl-w		Li CJF et al. Biochem Biophys Res Commun 2008
miR-124		tumor suppressor	Furuta M et al. Carcinogenesis 2009
miR-143	fibronectin type III domain containing 3B (FNDC3B)	promotion of metastasis	Zhang X et al. Hepatology 2009
miR-151	RhoGDIA	cell migration and cell spread	Ding J et al. Nat Cell Biol 2009
miR-17-92a		accelarated tumorgenesis	Connolly E et al. Am J Pathol 2008
miR-18	estrogen receptor alpha	proliferation of cancer cell	Liu WH et al. Gastroenterology 2009
miR-181	caudal type homeobox transcription factor 2 (CDX2)	proliferation of cancer cell	Ji J et al. Hepatology 2009
	GATA binding protein 6 (GATA6)	proliferation of cancer cell	Ji J et al. Hepatology 2009
	nemo like kinase (NLK)	proliferation of cancer cell	Ji J et al. Hepatology 2009
miR-181b	Tissue inhibitor of metalloprotease 3 (TIMP3),	promotion of hepatocarcinogenesis	Wang Bet al. Oncogene 2010
miR-203		tumor suppressor	Furuta M et al. Carcinogenesis 2009
miR-21		accelarated tumorgenesis	Connolly E et al. Am J Pathol 2008
	PTEN	proliferation of cancer cell	Meng F et al. Gastroenterology 2007
miR-221	CDKN1C/p57	cell proliferation	Fornari F et al. Oncogene 2008
	bmf (proapoptotic BH3-only protein)	oncogenecity/multifocality	Gramantieri L et al. Clin Cancer Res 2009
	CDKN1C/p57		Gramantieri L et al. Clin Cancer Res 2009
	CDKN1B/p27		Gramantieri L et al. Clin Cancer Res 2009
	DNA damege-inducible transcript 4 (DDIT4)	proliferation of cancer cell	Pascal P et al. Proc Natl Acad Sci U S A 2010
miR-222	PPP2R2A	promotion of cell migration	Wong QWL et al. Clin Cancer Res 2010
miR-223	stathmin 1	inhibition of proliferation	Wong QWL et al. Gastroenterology 2008
miR-224	Apoptosisi inhibitor 5 (API5)	anti-apoptosis	Wang Y et al. J Biol Chem 2008
miR-23a	TGFb	tumor suppressor	Huang S et al. Int Natll cancer 2008
miR-23b	urokinase, c-met	inhibition of proliferation	Salvi A et al. FEBS 2009
miR-24	TGFb	tumor suppressor	Huang S et al. Int Natll cancer 2008
miR-27a	TGFb	tumor suppressor	Huang S et al. Int Natl cancer 2008
miR-30d	Ganphai2 (GNAI2)	promotion of invasion and metastasis	Yao J et al. Hepatology 2010
miR-338		tumor progression	Huang et al. Hepatol Res 2009
miR-34a	CDK4, CDC2	nhibition of cell invasion and migratio	Cheng J et al. Proteomics 2010
	c-met	inhibition of migration and invasion	Li N et al. Cancer letter 2009
miR-375	Hippo-Signal effector YAP	inhibition of proliferation	Liu AM et al. Biochem Biophys Res Commun 2010
miR-9	E-cadherin		Hao-Xiang T et al. Med Oncol 2009

表1　肝細胞癌で発現異常を示すマイクロRNA

告されている[7]。

2．癌の悪性度と関与しているマイクロRNA

自検例で癌の組織学的悪性度と発現が相関しているマイクロRNAを表2に示す。臨床的に高分化型(well differentiated)肝臓癌は腫瘍が小さく悪性度が低い，中分化型(moderately differentiated)，未分化型(poorly differentiated)になるにつれ周辺組織への浸潤，遠隔転移（肝内転移を含む）腫瘍そのものの大きさも増大し悪性度が高くなるとされている。高分化型5例，中分化型15例，未分化型3例の肝臓癌組織よりマイクロアレイにてマイクロRNA発現解析を行なった結果を示す。miR-17-5p, miR-18, miR-20, miR-92, miR-106a, miR-222の発現は高分化から未分化への悪性度が高くなるにつれ亢進していた，またmiR-99aは逆に悪性度が高くなるにつれその発現が低下している事が分かった[8]。興味深いのは肝発癌に関与しているmiR-18だけではなくoncogeneとして知られているmiR-92-17 clusterの発現と癌の悪性度が正に相関している事である。自検例では肝細胞癌の組織学的分化度別の発現差としてとらえられていなかったmiR-181はcaudal type homeobox transcription factor 2 (CDX2), GATA binding protein 6 (GATA6), nemo like kinase (NLK)を介して肝癌の進展に関係している（表1）。これら悪性度と関係したマイクロRNA発現解析結果を用いる事により，癌の発育（浸潤，転移）等のメカニズム解析を行なう事が可能になり，さらには前骨髄性白血病(APL)等で行なわれているような癌の分化誘導療法への応用等の新たな遺伝子治療開

miRNA	other cancer	reference
let-7, let-7e	Lung cancer	Takamizawa J et al. Cancer Res 2004
	Colorectal cancer	Akao Y et al. Biol Pharm Bull 2006
	Ovarian cancer	Yang N et al. Cancer Res 2008
	Head & neck cancer	Childs G et al. Am J Pathol 2009
	Pancreatic cancer	Torrisani J et al. Hum Gene Ther 2009
	Glioblastoma	Kefas B et al. Cancer Res 2008
miR-101	Colorectal cancer	Strillacci A et al. Exp Cell Res 2009
	Prostatic cancer	Varambally S et al. Science 2008
	Bladder cancer	Friedman JM et al. Cancer Res 2009
miR-125a, miR-125b	Breast cancer	Mattie R et al. Chromosoma 2009
	Ovarian cancer	Corden Dahl KD et al. Neoplasia 2009
	Leukemia	Bousquet M et al. J Exp Med 2008
	Lung cancer	Izzotti A et al. FASEB J 2009
miR-143	Colorectal cancer	Akao Y et al. Oncol Rep 2006
	Gastric cancer	Takagi T et al. Oncology 2009
	Cervical cancer	Lui WO et al. Cancer Res 2007
	Prostatic cancer	Clape C et al. PLoS One 2009
	Bladder cancer	Lin T et al. J Urol 2009
miR-150	Gastric cancer	Wu Q et al Biochem Biophys Res Commun 2010
	Leukemia	Wang M et al. J Pathol 2008
miR-15, miR-15a	Leukemia	Simmino A et al. PNAS 2005
	Gastric cancer	Xia L et al. Int J Cancer 2008
	Prostatic cancer	Bonci D et al. Nat Med 2008
	Ovarian cancer	Bhattacharya R et al. Cancer Res 2009
miR-16, miR-16-1	Leukemia	Scaglione BJ et al. Br J Haematol 2007
	Gastric cancer	Xia L et al. Int J Cancer 2008
	Ovarian cancer	Bhattacharya R et al. Cancer Res 2009
	Prostatic cancer	Bonci D et al. Nat Med 2008
miR-17-5p, miR-17-92	Lung cancer	Hayashita Y et al. Cancer Res 2005
	Neuroblastoma	Shulte JH et al. Int J Cancer 2008
	Thyroid carcinoma	Takakura S et al. Cancer Sci 2008
	Lymphoma	Fontana L et al. Nat Cell Biol 2007
	Glioblastoma	Ernst A et al. Oncogene 2010
	Prostatic cancer	Zhang X et al. Clin Exp Metastasis 2009
miR-181	Leukemia	Debernardi S et al. Leukemia 2007
	Glioblastoma	Chen G et al. Oncol Rep 2010
	Colorectal cancer	Nakajima G et al. Cancer Genomics Proteomics 2006
	Gastric cancer	Zhu W et al. Int J Cancer 2010
miR-199a	Ovarian cancer	Yin G et al. Oncogene 2010
miR-199b	Leukemia	Altaro F et al. Br J Haematol 2009
	Lung cancer	Kelsohucer S et al. Carcinogenesis 2008
miR-200b	Ovarian cancer	Bendoraite A et al. Gynecol Oncol 2010
miR-200	Pancreatic cancer	Burk U et al. EMBO Rep 2008
	Head & neck cancer	Gibbons DL et al. Genes Dev 2009
	Prostatic cancer	Kong D et al. Stem Cell 2009
	Bladder cancer	Adam L et al. Clin Cancer Res 2009
miR-203	Head & neck cancer	Lena AM et al. Cell Death Differ 2008
miR-21	Cholangiocarcinoma	Selaru M et al. Hepatology 2009
	Head & neck cancer	Lu Z et al. Oncogene 2008
	Leukemia	Fulci V et al. Blood 2007
	Cervical cancer	Yao Q et al. Biochem Biophys Res Commun 2009
	Pancreatic cancer	Park JK et al. Pancreas 2010
	Ovarian cancer	Nam EJ et al. Clin Cancer Res 2008
	Breast cancer	Qian B et al. Breast Cancer Res Treat. 2008
	Thyroid carcinoma	Tolatta F et al. Oncogene 2009
	Glioblastoma	Chan JA et al. Cancer Res 2005
	Prostatic cancer	Li T et al. Biochem Biophys Res Commun 2009
	Lung cancer	Volinia S et al. Proc Natl Acad Sci U S A 2006
miR-214	Cervical cancer	Yang Z et al. IUBMB Life 2009
miR-221	Glioblastoma	Gillies JK et al. Cell Cycle 2007
	Thyroid carcinoma	Visone R et al. Endocr Relat Cancer 2007
	Pancreatic cancer	Park JK et al. Pancreas 2010
	Colorectal cancer	Wang P et al. Cancer Res 2009
	Prostatic cancer	Galardi S et al. J Biol Chem 2007
	Gastric cancer	Kim YK et al. Nucleic Acids Res 2009
	Leukemia	Felicetti F et al. Cancer Res 2008
	Bladder cancer	Lu Q et al. Urol Oncol 2009
miR-222	Thyroid carcinoma	Visone R et al. Endocr Relat Cancer 2007
	Prostatic cancer	Galardi S et al. J Biol Chem 2007
	Leukemia	Felicetti F et al. Cancer Res 2008
	Pancreatic cancer	Greither T et al. Int J Cancer 2010
	Glioblastoma	Gillies JK et al. Cell Cycle 2007
	Gastric cancer	Kim YK et al. Nucleic Acids Res 2009
miR-223	Leukemia	Fazi F et al. Cancer Cell 2007
	Ovarian cancer	Laios N et al. Mol Cancer 2008
miR-224	Prostatic cancer	Meza ST et al. AnnSurg Oncol 2009
	Thyroid carcinoma	Nikiforova MV et al. J Clin Endocrinol Metab 2008
miR-27a	Gastric cancer	Liu T et al. Cancer Lett 2009
miR-301	Pancreatic cancer	Lee EJ et al. Int J Cancer 2007
miR-34a	Pancreatic cancer	Ji Q et al. PLoS One 2009
	Cervical cancer	Wang X et al. RNA 2009
	Neuroblastoma	Welch C et al. Oncogene 2007
	Colorectal cancer	Yamakuchi M et al. Proc Natl Acad Sci U S A 2008
	Leukemia	Zenz T et al. Blood 2009
	Prostatic cancer	Chang TC et al. Mol Cell 2007
	Prostatic cancer	Shi XB et al. J Cell Mol Med 2008
	Gastric cancer	Ji Q et al. BMC Cancer 2008
miR-9	Neuroblastoma	Ma L et al. Nat Cell Biol 2010
	Ovarian cancer	Guo LM et al. FEBS J 2009
	Gastric cancer	Wan HY et al. Mol Cancer 2010

表2.肝細胞癌以外の癌でも共通して発現の変化しているマイクロRNA

発へのシーズになると考えられる。また臨床応用として各患者の臨床情報を照らし合わせる事によって治療効果，予後予測等に応用臨床的に利用する事が可能となる。

3．肝疾患におけるバイオマーカーとしてのマイクロRNAの利用

Ji Jらは455例の肝細胞癌検体を用いたマイクロRNA解析の結果を元にmiR-26の発現の低下は予後不良因子の一つとして報告している。またmiR-26の発現が低下し予後不良と考えられた症例に対するインターフェロンα投与はこの予後を改善するものとしている[9]。別項を記載しておられる黒田先生のグループと共同で末梢血のエクソソーム画分に含まれるマイクロRNAを肝細胞癌の治療前後でマイクロRNA発現を比較したところmiR-92/miR-638の比が治療後は治療前に比べて有意に上昇している事を見いだした[10]。この解析を元に現在用いられているAFPやPIVKA-II等の腫瘍マーカーと特異性，感度につき比較し新たな腫瘍マーカーとしての実用化を目指している。

おわりに

マイクロRNAは肝細胞癌をはじめとした種々の癌において共通した発現パターンを示し，マイクロRNAそのものが癌遺伝子または癌抑制遺伝子のように作用し，病態の進行と密接に関係していると考えられる。マイクロRNAの機能を明らかにする事は病態の解明につながり，将来的に臨床応用が期待できる。

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Original Article

Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development

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MicroRNAs (miRNAs) belong to a class of the endogenously expressed non-coding small RNAs which primarily function as gene regulators. Growing evidence suggests that microRNAs have a significant role in tumor development and may constitute robust biomarkers for cancer diagnosis and prognosis. The *miR-17-92* cluster especially is markedly overexpressed in several cancers, and is associated with the cancer development and progression. In this study, we have demonstrated that miR-92a is highly expressed in hepatocellular carcinoma (HCC). In addition, the proliferation of HCC-derived cell lines was enhanced by miR-92a and inhibited by the anti-miR-92a antagomir. On the other hand, we have found that the relative amount of miR-92a in the plasmas from HCC patients is decreased compared with that from the healthy donors. Interestingly, the amount of miR-92a was elevated after surgical treatment. Thus, although the physiological significance of the decrease of miR-92a in plasma is still unknown, deregulation of miR-92 expression in cells and plasma should be implicated in the development of HCC.

Key words: hepatocellular carcinoma, microRNA, miR-638, miR-92a, plasma

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MicroRNAs (miRNAs) are small endogenous non-coding RNAs that regulate gene expression and have a critical role in many biological and pathological processes.[1] Recent studies have shown that deregulation of microRNA expression contributes to the multistep processes of carcinogenesis, and have shown promise as tissue-based markers for cancer classification and prognostication.[2,3] However, biological roles of only a small fraction of known miRNAs have been elucidated to date.

The miR-17-92 cluster at 13q31.3 is consists of six miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1, and plays an important role for development of lung cancer,[4] B-cell lymphomas,[5] chronic myeloid leukemia,[6] medulloblastomas,[7] colon cancer[8] and hepatocellular carcinoma (HCC).[9] In addition, mice deficient in the miR-17-92 cluster died shortly after birth with lung hypoplasia, and B-cell development was impaired in the mice.[10] It has been reported, however, that miR-92a increases cell proliferation by negative regulation of an isoform of the cell-cycle regulator p63.[11] Furthermore, miR-92a regulates angiogenesis.[12] Thus, it is clear that the miR-92a has some oncogenic characteristics. However, the specific biological role of miR-92a in the processes of human cancer development has remained unclear.

Here, we have revealed that miR-92a is implicated in human HCC development. Furthermore, we have demonstrated that miR-92a in human blood has the potential to be a noninvasive molecular marker for diagnosis of human HCC.

MATERIALS AND METHODS

In situ hybridaization of miR-92a

Locked nucleic acid (LNA)-modified probes for miR-92a and negative control (miRCURY-LNA detection probe, Exiqon, Vedbaek, Denmark) were used. The probe sequences were as follows; *miR-92a*, 5′-ACAGGCCGGGACAAGTGCAATA-3′; and a scrambled oligonucleotides used for negative control, 5′-GTGTAACACGTCTATACGCCCA-3′. *In situ* hybridization was performed using the RiboMap *in situ* hybridization kit (Ventana Medical Systems, Tucson, AZ, USA) on the Ventana Discovery automated *in situ* hybridization instrument (Ventana Medical Systems). The *in situ* hybridization steps were performed as previously described.[13] Staining was evaluated by two investigators and graded as follows: negative (–), no or occasional (<5%) staining of tumor cells; positive (+), mild to strong (>5%) staining of tumor cells. Paraffin–embedded tissue samples of hepatocellular carcinoma (HCC) and adjacent non-tumorous liver cirrhosis (LC) were obtained from HCC patients at Ogaki Municipal Hospital (Ogaki, Japan). Details of the clinical data are provided in Table 1.

Plasma collection, RNA isolation and quantitative RT-PCR

Whole blood samples were collected from healthy donors and the patients with HCC at Ogaki Municipal Hospital. This study was approved by the institutional review board (IRB) of Tokyo Medical University, and all subjects provided written informed consent under the institutional review board. Details of clinical data are provided in Table 1. Diagnoses were confirmed using the post-operated tissues. Blood samples of the patients (Cases 1–10) were collected one day before the operation and then properly stored. One week after operation, blood samples of the patients were collected again. Whole blood was separated into plasma and cellular fractions by centrifugation at 1600 *g* for 15 min. Total RNA in the

Table 1 Summary of clinical details of hepatocellular carcinoma (HCC)used for in situ hybridaization and serum analysis

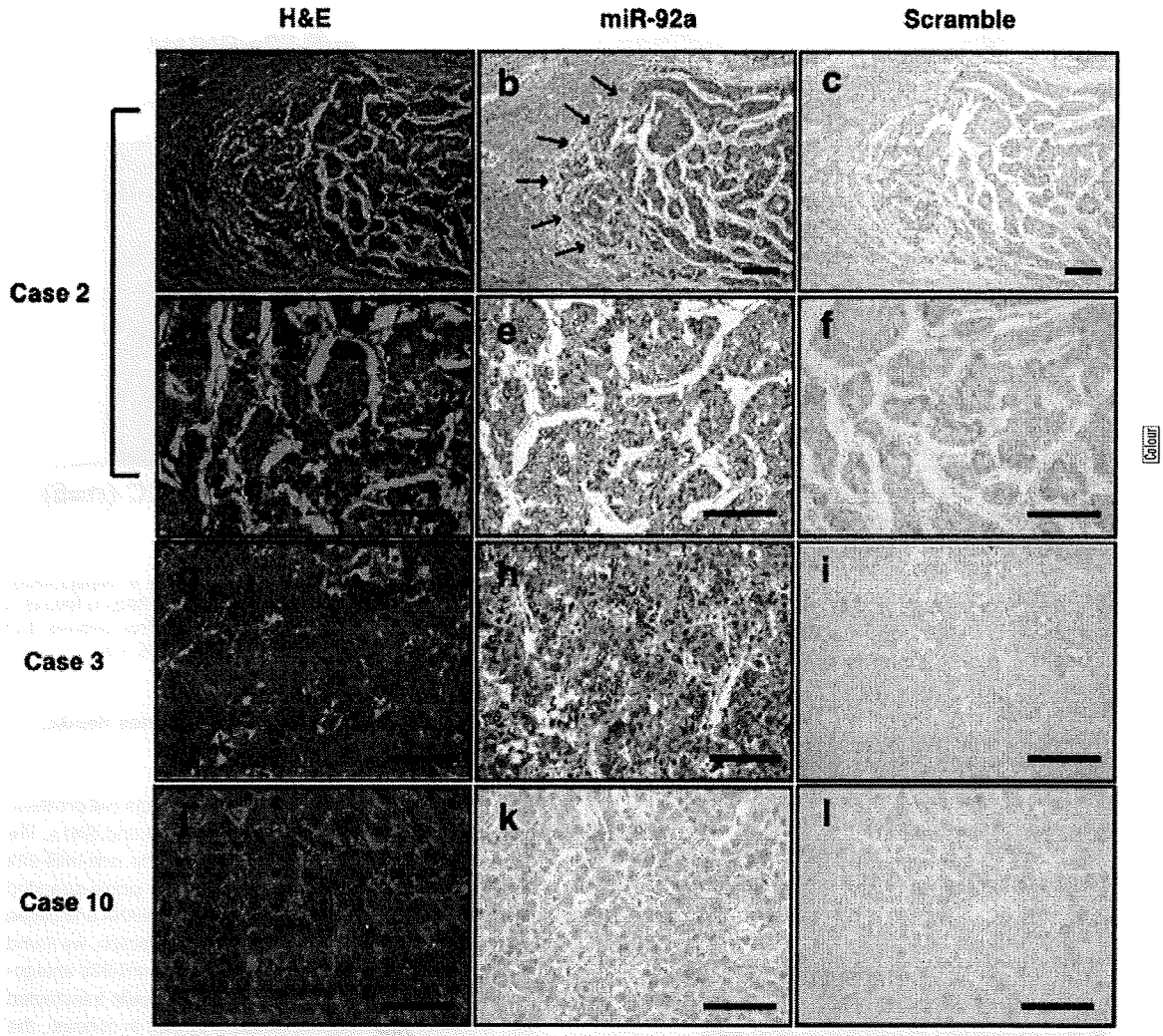
	Year	Sex	Virus type	Histologic type	Stage	Child-Pugh	miR-92a
Case 1	53	Male	HVB	Poorly	I	A	+
Case 2	59	Male	HVB	Moderate	II	A	+
Case 3	79	Male	NBNC	Moderate	III	A	+
Case 4	73	Male	HVC	Well	I	A	+
Case 5	76	Female	HVC	Moderate	IV-A	A	+
Case 6	59	Male	HVC	Moderate	II	A	+
Case 7	69	Female	HVC	Moderate	I	A	+
Case 8	71	Male	HVC	Moderate	I	A	+
Case 9	59	Female	HVB	Well	I	A	–
Case 10	69	Male	NBNC	Moderate	IV-A	A	–
Case 11	61	Female	HVB	Poorly	IV-A	B	+
Case 12	73	Male	NBNC	Moderate	II	A	+
Case 13	67	Male	NBNC	Moderate	IV-A	A	+
Case 14	61	Male	NBNC	Moderate	III	A	+
Case 15	45	Male	HVB	Moderate	I	A	+
Case 16	68	Female	HVC	Moderate	III	A	+
Case 17	70	Male	NBNC	Poorly	II	A	+
Case 18	59	Male	HVC	Moderate	III	A	+
Case 19	43	Male	HVB	Moderate	II	A	+
Case 20	69	Male	HVC	Moderate	II	A	–
Case 21	76	Male	HVC	Moderate	III	A	–
Case 22	53	Male	HVC	Moderate	II	A	–

HCV, hepatitis C virus; HVB, hepatitis B virus; NBNC, non-B non-C virus.

Table 2 Summary of clinical details of hepatocellular carcinoma (HCC) used for qPCR analysis

Code no.	Year	Sex	Virus type	Histologic type	Non-tumorous tissue	AFP	PIVKA-II
91	53	Male	HVC	Moderate	LC	5	0.06
160	59	Male	HVC	Moderate	LC	NI	NI
O89	68	Male	HVC	Moderate	LC	8	25
O90	70	Male	HVC	Moderate	LC	686	962
K89	51	Male	HVC	Moderate	LC	NI	NI

LC, liver cirrhosis; HCV, hepatitis C virus; NI, no information.



Colour

Figure 1 MicroRNA expression in hepatocellular carcinoma (HCC). *In situ* hybridization was performed using Locked nucleic acid (LNA)-modified probes for miR-92a and negative control. Case 2 and Case 3 were positive cases for miR-92a. Case 10 was a negative case for miR-92a. **(a–c)** Low power field of boundary of HCC and non-tumor lesion. Arrowheads indicated a border. Only HCC regions were positive for miR-92a. **(d–l)** High power field of HCC. Blue signals represent positive for miR-92a. Bars indicate 100 μm.

plasma was isolated using Isogen-LS (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. The RNA sample was suspended in 20 μL of nuclease free water. In general, we obtained 400 ng of RNA from 1 mL of plasma. MicroRNAs were quantified using TaqMan MicroRNA Assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) as previously described.[13]

For miR-92a quantification in tissue samples, five pairs of fresh HCC and non-tumorous LC samples were surgically resected from HCC patients (Table 2). All the patients or their guardians provided written informed consent, and the Ethics Committee of the Kyoto University Graduate School and Faculty of Medicine approved all aspects of this study. The amounts of miR-92a were normalized to RNU48 that is one of rRNAs (Applied Biosystems).

Cell culture and transfection

Hepatocellular carcinoma (HCC) cell lines HepG2, OR6 and SN1a were cultured in Dulbecco's modified Eagle's medium

(DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS). OR6 and SN1a are derived from the Huh7 HCC cell line and maintain hepatitis C virus (HCV) replicon.[14–16] The miR-92a oligonucleotide used in the transfection experiments is a synthetic double-strand 19 nucleotide RNA oligonucleotide (5′-UUGCACUUGUCCC GGCCUG-3′) purchased from B-Bridge International (Tokyo, Japan). The scrambled oligonucleotide represents a mix of two different frames of the miR-92 sequence (5′-UAUUGC ACUUGUCCCGGCCUGUCCCGGCC-3′ and 5′-AUUGCAC UUGUCCCGGCCUTT-3′). Locked nucleic acid (LNA) oligonucleotide miR-92 knockdown (antagomir) was obtained from Exiqon (Vedbaek, Denmark, http://www.exiqon.com). The oligonucleotides were individually transfected by Hiper-Fect (QIAGEN K. K., Tokyo, Japan) into the cells at a final concentration of 100 nM.

In vitro proliferation assays

The effects of miR-92a and the anti-miR-92a antagomir on the growth of HepG2, OR6 and SN1a were evaluated using the MMT metabolic growth assay kit (Cell Count Reagent SF, Nacalai tesque, Kyoto, Japan). The cells were transfected with miR-92a or the antagomir. The cell numbers were then assessed with MMT assay at 48 or 72 h after the transfection. The MMT assay was performed according to the manufacturer's recommendation. The reagents were added to each well and incubated at 37°C for 4 h. The MMT reduced by living cells into a formazan product was assayed with a multiwell scanning spectrophotometer at 450 nm.

RESULTS

Highly expression of miR-92a in HCC cells

We first examined whether or not miR-92a is expressed in hepatocellular carcinoma (HCC). We performed *in situ* hybridization using locked nucleic acid (LNA)-modified probes digoxigenin (DIG) labelled. We found that miR-92a was strongly expressed in cancer cells of 17 out of 22 HCC cases (Table 1 and Fig. 1). No significant differences were observed in age, sex, virus type, clinical stage and tumor differentiation of the clinical samples. In contrast, we did not detect miR-92a expression in non-cancerous hepatocytes around the HCCs.

Furthermore, we quantified miR-92a levels in HCC sections ($n = 5$) and their adjacent non-tumorous liver cirrhosis (LC) sections ($n = 5$) by *Taq*Man qRT-PCR (Table 2 and Fig. 2). The levels of miR-92a expression in HCC sections were higher than that in adjacent LC sections (Fig. 2).

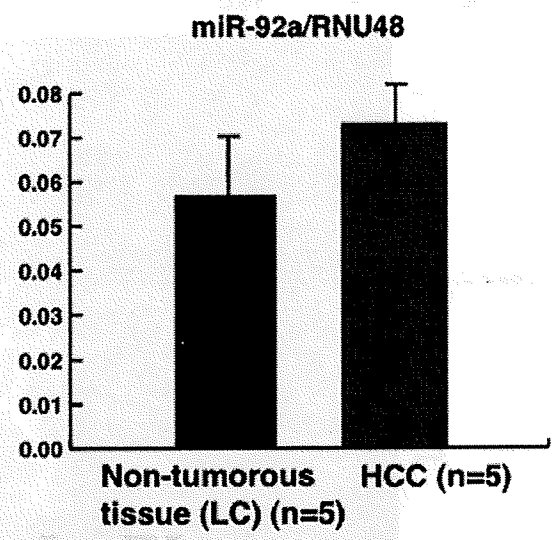


Figure 2 Quantification of miR-92a expression in hepatocellular carcinoma (HCC) tissue samples. The ratios of miR92a to RNU48 in HCC tissues and their adjacent non-tumorous liver cirrhosis (LC) tissues were analyzed by *Taq*Man qRT-PCR. *Bars*, s.d.

Effects of miR-92a on a Hepatoma cell lines HepG2, OR6 and SN1a

Next, we investigated whether miR-92a affects cell proliferation of human HCC cell lines, HepG2, OR6 and SN1a. We transiently transfected either miR-92a or the anti-miR-92a antagomir into the cells. Antagomirs are single-stranded RNAs that are complementary to a specific miRNA and cause the depletion of the miRNA.[17] After the transfection, we found that all of the cells transfected with the anti-miR-92a antagomir showed lower proliferation rate than the cells transfected with a control RNA oligonucleotide (Fig. 3a). In contrast, the cells except for HepG2 showed increased proliferation rate when miR-92a was transfected (Fig. 3a). We also confirmed the amounts of miR-92a in the cells by quantitative real time PCR (Fig. 3b).

The ratio of miR-92a to miR-638 serves as a biomarker for HCC

Finally, we sought to determine whether the expression level of miR-92a in blood sera could discriminate HCC patients from healthy individuals. Previously, we have revealed that miR-92a is dramatically reduced in the plasmas of acute leukemia patients although in leukemic cells it is strongly expressed.[13] We analyzed the miR-92a levels in the plasma samples from normal individuals ($n = 10$) and HCC patients

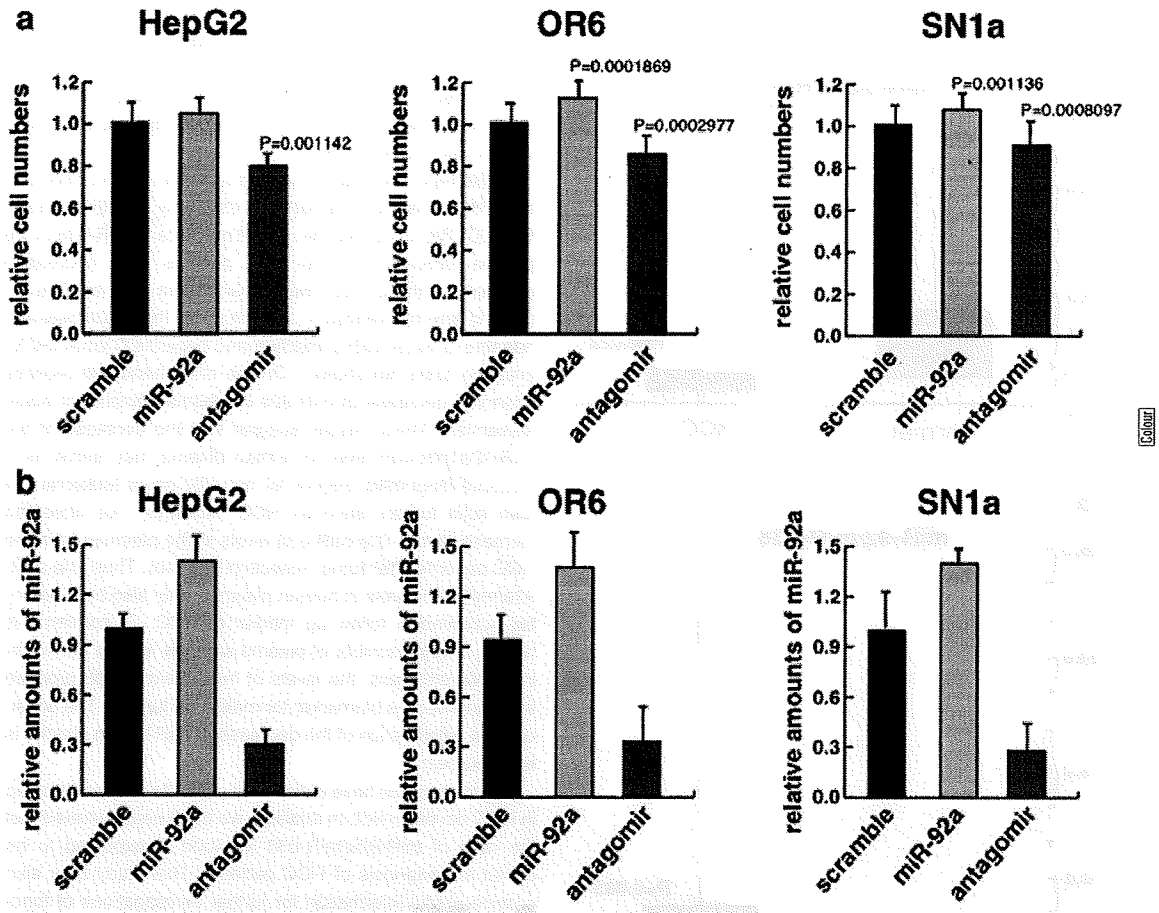


Figure 3 miR-92a modulates proliferation of HepG2, OR6 and SN1a cells. (**a**) Cell numbers of the HepG2, OR6 and SN1a cells transfected with synthetic miR-92a, anti-miR-92a antagomir, or scrambled control oligonucleotide were analyzed by MTT assays at 48 h for OR6 and SN1a and 72 h for HepG2 after transfection. *Bars*, s.d. (**b**) qRT-PCR analysis of miR-92a amounts in the cells transfected with miR-92a, anti-miR-92a antagomir or scrambled control at 48 h for OR6 and SN1a and 72 h for HepG2 after the transfection.

(n = 10) by *Taq*Man qRT-PCR. Because miR-638 is stably present in human plasmas,[13] we used miR-638 as the standard to improve the precision of the data. The ratio of miR-92a to miR-638 in the plasma samples from the HCC patients were decreased compared with that from the normal donors (Fig. 4a). Then, we further examined the ratio from the patients after surgical resection. Interestingly, the miR-92a/miR-638 levels were significantly higher than that in the plasmas from the patients before surgical resection (Fig. 4b).

DISCUSSION

In this study, we found that miR-92a was highly expressed in HCC (Figs 1,2). In addition, we demonstrated that the expression level of miR-92a affects the proliferation of hepatoma cell lines, HepG2, OR6 and SN1a (Fig. 3). These results suggest that miR-92a may play an important role in tumor progression of hepatocyte. We do not know why, but addition of miR-92a did not significantly increase the proliferation of HepG2 cells. It may be possible that HepG2 cells themselves already contain enough miR-92a to promote cancer cell proliferation. In addition, miR-92a is a part of the miR-17-92 cluster, which is actively involved in the development and progression of various cancers.[4–10] However, the molecular function of miR-92a is still unknown, and its mRNA targets have not been identified. Recently, it has been shown that one of the molecular mechanisms through which miR-92a increases cell proliferation is by negative regulation of an isoform of the cell-cycle regulator p63.[11] Thus, we examined

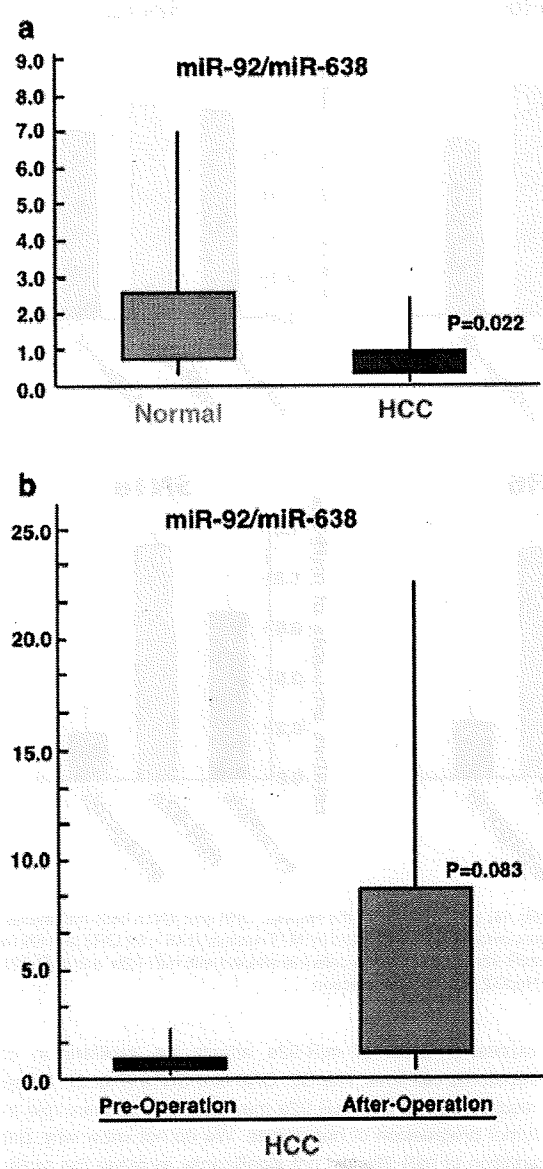


Figure 4 Comparison of miR-92a levels in the plasmas from normal individuals and hepatocellular carcinoma (HCC) patients. (**a**) The ratios of miR92a to miR-638 in the plasmas from normal donors and HCC patients were analyzed by *Taq*Man qRT-PCR. Student's *t*-test was used to determine statistical significance. (**b**) The ratios of miR92a to miR-638 in the plasmas from HCC patients before and after tumor resection were analyzed by *Taq*Man qRT-PCR.

the expression of p63 in HCC by immunohistochemistry. However, we could not find the positive nuclear staining both in HCC and normal hepatocyte (data not shown). On the other hand, the miRanda software found 300 different genes that have putative miR-92a binding sites conserved among *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus* at the 3′-UTR regions of their transcripts. Therefore, at least in HCC, there may be novel miR-92a targets that are involved in cancer cell proliferation.

In this report, we have revealed that the value of miR-92a/miR-638 in plasma has potential as a very sensitive marker for HCC. We found that the ratio of miR-92a to miR-638 in the plasma samples from the HCC patients were decreased compared with that from the normal donors (Fig. 4a). We did not find any differences in the values of the ratios between hepatitis B virus (HBV) infection and hepatitis C virus (HCV) infection (data not shown). On the other hand, we recently observed decrease of miR-92a in plasma samples of acute leukemia.[13] These results suggest that the decrease of the miR-92a/miR-638 level in human plasma may serve as a valuable diagnostic marker for not only acute leukemia but also solid tumors such as HCC. Moreover, we observed increase of miR-92a/miR-638 levels in the plasmas from the HCC patients after tumor resection (Fig. 4b). Thus, the miR-92a/miR-638 levels in human plasmas may also be a potential noninvasive follow up marker of HCC. To confirm this notion, a large number of plasma samples should be examined. Nevertheless, the levels of miR-92a/miR-638 promise to be an effective biomarker for malignant tumors. The physiological significance of the decrease of miR-92a in plasma is still unknown.

In summary, we have shown that miR-92a may be involved in HCC development. In addition, we have demonstrated that the ratio of miR-92a/miR-638 in blood is expected to be useful for diagnosis of HCC patients. This study may also provide useful information for further investigations of functional association between miRNAs and HCC.

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ORIGINAL ARTICLE

MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma

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Abstract

We identified that microRNA expression changed dynamically during liver development and found that miR-500 is an oncofetal miRNA in liver cancer. miR-500 was abundantly expressed in several human liver cancer cell lines and 45% of human hepatocellular carcinoma (HCC) tissue. Most importantly, an increased amount of miR-500 was found in the sera of the HCC patients. In fact, miR-500 levels in sera of the HCC patients returned to normal after the surgical treatment in three HCC patients. Our findings reveal that diverse changes of miRNAs occur during liver development and, one of these, miR-500 is an oncofetal miRNA relevant to the diagnosis of human HCC.

Keywords: *miRNA; miR-500; hepatocellular carcinoma, liver development, diagnosis*

Introduction

MicroRNAs (miRNAs) are small RNA molecules of 21–25 nt that have the potential to a play central role in physiological and pathological processes, including cell differentiation, apoptosis and oncogenesis (Ambros 2004, Esquela-Kerscher et al. 2006). The biogenesis of miRNAs involves nucleolytic processing of precursor transcripts, which are transcribed from different genomic locations as long primary transcripts (pri-miRNA) by RNA polymerase II in the nucleus (Lee et al. 2004). Pri-miRNAs are processed by the RNase-III family of an enzyme, Drosha, to a ~70 nt precursor called the pre-miRNA. The pre-miRNA is exported to the cytoplasm by Exportin-5 and then cleaved in the cytoplasm by Dicer to ~22 nt double-strand mature miRNA (Han et al. 2006, Lund et al. 2004, Ketting et al. 2001). A single strand of the mature miRNA is assembled into effecter complexes called miRNPs (miRNA-containing ribonucleoprotein particles), which share a considerable amount of similarity with an RNA-induced silencing complex (RISC) (Nelson et al. 2004). They induce gene suppression post-transcriptionally by inducing mRNA degradation or by regulating the translational efficiency of mRNA (Bartel 2004).

Several reports have shown the importance of miRNA functions in tissue development. More recent reports, in particular those regarding comprehensive microRNA profiling analysis, have shown that miRNAs are expressed in a tissue-specific manner and their expression altered

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in the process of development, such as cardiogenesis and haematopoiesis (Chen et al. 2006, 2004). For example, miR-1, which is expressed specifically in cardiac and skeletal muscle, is essential for cardiac morphogenesis and conduction (Zhao et al. 2007). Another study showed that miR-181a regulates intrinsic antigen sensitivity during T-cell development (Li et al. 2007). Another important aspect of miRNA study is the association of its gene targets and disease, which have been investigated by many researchers. Mir-17-92 polycistron has been designated as oncomiR-1 (He et al. 2005), and let-7 family miRNAs and miR-34 function as tumour suppressors (Johnson et al. 2005, Yu F et al. 2007, He et al. 2007); moreover, a number of studies have given evidence that several miRNAs are associated with carcinogenesis and regulate the expression of cancer-related genes.

Although emerging evidence suggests that several miRNAs are involved in the process of liver development (Esau et al. 2006, Fu et al. 2005, Gramantieri et al. 2007), the roles of miRNAs in hepatogenesis and their possible relation to hepatocarcinogenesis have not been thoroughly examined. In this study, to investigate liver development from the biological aspects of microRNA, we performed a mouse miRNA microarray carrying 340 miRNA probes. We report that some of these miRNAs are strongly expressed, and that dynamic changes in their expression profile are observed in the process of liver development. We also show that miR-500 is an oncofetal miRNA, which is highly expressed in fetal liver, more than in adult normal liver, and aberrantly expressed in hepatocellular carcinoma (HCC) tissue. Thus, dynamic miRNA regulation is an important feature as an oncofetal non-coding small RNA relevant to the diagnosis of human liver cancer.

Materials and methods

RNA extraction

C57BL/6J mice were used in this study. Total RNA from mouse liver tissues (embryo (E) 14, E16, E18, neonate and adult), *in vitro* fetal hepatocyte cultured samples (days 0, 1, 3, 5 and 7), and liver cancer cell lines (HepG2, Huh-7, JHH-7, Alexander, Li-7, and Hep3B) were extracted using the mirVana™ miRNA Isolation Kit (Ambion, Tokyo, Japan). Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute.

Locked nucleic acid (LNA)-based miRNA microarray

The miRCURY™ LNA array version 8.0, which contains capture probes targeting all human, mouse and rat miRNA listed in the miRBASE version 8.0, was applied to detect the expression of mouse miRNA (Exiqon, Vedbaek, Denmark). Total RNA samples were collected from fetal (E14, 16 and 18), neonate and adult (8-week-old) mice (n=7–10). Total RNA samples (2000 ng) from liver tissue and reference (Universal control, which is made from mouse tissue mixtures) were labelled with the Hy3™ and Hy5™ fluorescent stain, respectively, using the miRCURY™ LNA Array labelling kit according to the procedure described by the manufacturer (Exiqon). Hybridisation and normalisation were performed according to the miRCURY™ LNA array manual, and image analysis of the miRCURY™ LNA array microarray slides was acquired using an Agilent Technologies Microarray Scanner and Agilent Feature Extraction 9.1 (Agilent Technologies, Tokyo, Japan). A hierarchical cluster was produced from microarray data using a Euclidean distance calculation based on Ward's methods by GenMaths software (Applied Maths). All the miRNA microarray data are shown in Supplementary Table 1 (see the online version of this article).

Cell culture

Liver cancer cell lines (HepG2, Huh-7, JHH-7, Alexander, Li-7 and Hep3B) were cultured in liquid culture with Dulbecco's modified eagle medium (DMEM; GIBCO Laboratories, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA) and a 1% antibiotic antimycotic solution (Invitrogen, Tokyo, Japan). The cells were maintained *in vitro* at 37°C in a humidified atmosphere with 5% CO_2.

Patients and RNA specimens

Liver tissues were obtained surgically with informed consent from patients at the National Cancer Center Hospital (Tokyo, Japan). The study was approved by the Institutional Review Board of the National Cancer Center Research Institute. Liver tissue total RNAs were extracted from 40 HCC patients and their associated non-cancerous tissue. The clinical data and pathological diagnosis are summarized in Supplementary Table 2 (see the online version of this article).

Real-time polymerase chain reaction

Total RNAs of approximately 100 ng were reverse-transcribed using the Taqman miRNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). Real-time quantitative polymerase chain reaction (PCR) amplification of the cDNA template was done using Taqman Universal PCR Master Mix (Applied Biosystems) in

an ABI PRISM 7300 (Applied Biosystems). The PCR conditions were 50°C for 2 min and 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Taqman probes for human and mouse miRNA were used to assess the expression levels of miRNA (mmu-miR-101b, ID 4373159; mmu-122a, ID 4373151; mmu-miR-142-5p, ID 4373135; mmu-miR-223, ID 4373075; mmu-miR-451, ID 4373360; has-miR-346, ID 4373038; has-miR-500, ID 4373225; Applied Biosystems). The expression levels were normalised against U6 (RNU6B, ID 4373381; Applied Biosystems) or total RNA volume.

RNA isolation from human serum samples

Whole blood samples were obtained from patients with HCC at the Kyoto University (Kyoto, Japan). All of the donors or their guardians provided written consent and ethics permission was obtained for the use of all samples. Blood samples were taken before and after completion of surgery. Serum samples were stored at -80°C until analysis. For serum RNA isolation, total RNA was isolated using Isogen (Nippon Gene, Japan), according to the manufacturer's instructions.

Measurement of serum miRNA levels by using TaqMan qRT-PCR assays

A fixed volume of 5 µl of RNA solution (14 ng) was used as input into the reverse transcription reaction. Input RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied BioSystems) in a small-scale reverse transcription reaction (comprising 2 µl of H_2O, 1 µl of 10x reverse-transcription buffer, 0.2 µl of RNase inhibitor (20 units ml^{-1}), 0.1 µl of 100 mM dNTPs, 0.7 µl of Multiscribe reverse transcriptase and 5 µl of input RNA), using a Tetrad2 Peltier Thermal Cycler (BioRad, Tokyo, Japan) at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Teverse transcription product (4.75 µl) was combined with 5.25 µl of PCR assay reagents (comprising 5 µl of TaqMan 2x Universal PCR Master Mix, No AmpErase UNG and 0.25 µl of TaqMan miRNA assay) to generate a PCR of 10.0 µl of total volume. Real-time PCR was performed as described above. Serum levels of miR-16 were measured as internal normalisation control as they were not significantly different between controls and patients in prostate cancer and colorectal cancer (Mitchell et al. 2008).

Statistical analysis

The results are given as mean ± SD. The Student's *t*-test was performed for statistical evaluation; $p<0.05$ or $p<0.001$ was considered significant.

Table 1. MicroRNAs (miRNA) abundantly expressed in liver development.

Liver stage	MiRNA name[a]
E14	miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-346, miR-374-5p, miR-451, miR-486, miR-500
E16	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-295, miR-346, miR-367, miR-374-5p, miR-451, miR-464, miR-471, miR-486, miR-500, miR-547
E18	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-324-3p, miR-374-5p, miR-451, miR-486
Neonate	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-463
Adult	miR-21, miR-22, miR-29a, miR-29b, miR-29c, miR-101a, miR-101b, miR-122a, miR-126-5p, miR-192, miR-374-5p

[a]miRNAs are listed in ascending order. E, embryo.

Results

Analysis of the global expression levels of miRNA in the process of liver development by LNA-based miRNA microarray

To examine how the expression profile of miRNA changed in the process of mouse liver development, we performed an LNA-based miRNA microarray at different developmental stages. Total RNAs from E14, 16, 18, neonate and adult liver were isolated and labelled with Hy3, and total RNAs of universal control consisted of several tissue mixtures labelled with Hy5 as a common reference. After normalisation of the miRNA expression, the number of high- and low-expressed miRNAs at different time stages was counted. High-expressed miRNA represents twofold or more upregulated miRNA, and low-expressed miRNA represents twofold or more downregulated miRNA, when compared with an average expression level of all miRNAs (see Supplementary Figure 1 in the online version of this article). Throughout all developmental stages of the liver, most of the miRNA expression levels were classified as low-expressed miRNA; in contrast, the number of high-expressed miRNAs was quite limited and are listed in Table 1. These data indicated that expression levels of the general miRNAs were very low and that a limited number of miRNAs were highly expressed in mouse liver development.

Differential expression patterns of miRNAs in the process of mouse liver development

To determine differentially expressed miRNA and to quantify the expression changes in the process of liver

development, hierarchical unsupervised clustering analysis was performed using microarray data of E14, 16, 18, neonate and adult mouse liver. The case cluster analysis of the microarray data indicated a similarity of clusters from the viewpoint of the expression pattern between E14 and E16 fetal liver and between neonate and adult liver (Figure 1), indicating that the miRNA expressions changed depending on the developmental stage. These results indicated that expression of most of the miRNAs was regulated precisely in the process of liver development.

The expression pattern of miRNA selected from highly expressed miRNAs (Table 1) was verified by real-time PCR to show the accuracy of miRNA expression acquired from the microarray analysis. The left panels of Figure 2 present the results of microarray analysis for five miRNAs (miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451). Expressions of miR-101b and miR-122a were low at the early stage of liver development and were upregulated during maturation. In contrast, expressions of miR-142-5p, miR-223 and miR-451 were high at the early stage of liver development and were already known as miRNAs expressed in haematopoietic cells (Chen et al. 2004, Zhan et al 2007, Johnnidis et al 2008). The right panels of Figure 2 are the results of real-time PCR for the same set of miRNAs. In comparison to the microarray results and the real-time PCR results, these data obtained from two different methods showed approximately similar expression patterns of miRNAs, confirming the validity of our microarray analysis.

Interestingly, miRNAs (miR-142-5p, miR-451 and miR-223) expressed in haematopoietic cells were highly expressed at the early stages (E14 and E16) and then gradually downregulated in the process of liver development (Figure 2). Because whole fetal liver is a haematopoietic organ and a large number of haematopoietic cells are contained there, this also indicated the accuracy of expression profiling of miRNA in the process of liver development by LNA-based microarray.

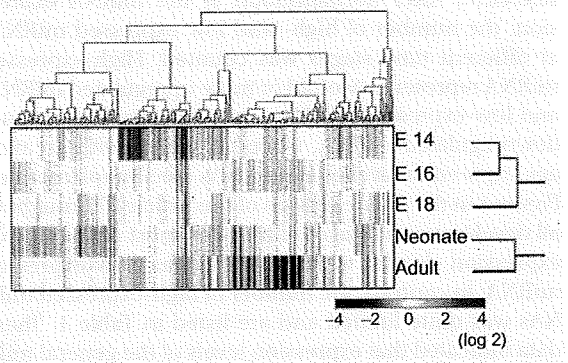

Figure 1. A global expression pattern of miRNA in the process of mouse liver development. The data were subjected to a hierarchical cluster analysis using a Euclidean distance calculation based on Ward's methods. The liver samples are aligned vertically: embryo (E) 14, E16, E18, neonate and adult. Samples were linked by the dendrogram shown on the right to highlight the similarity in their miRNA expression patterns. The expression profile of each miRNA is depicted in the respective row. The expressions of miRNA are linked by the dendrogram shown on the top to highlight the similarity in their expression patterns.

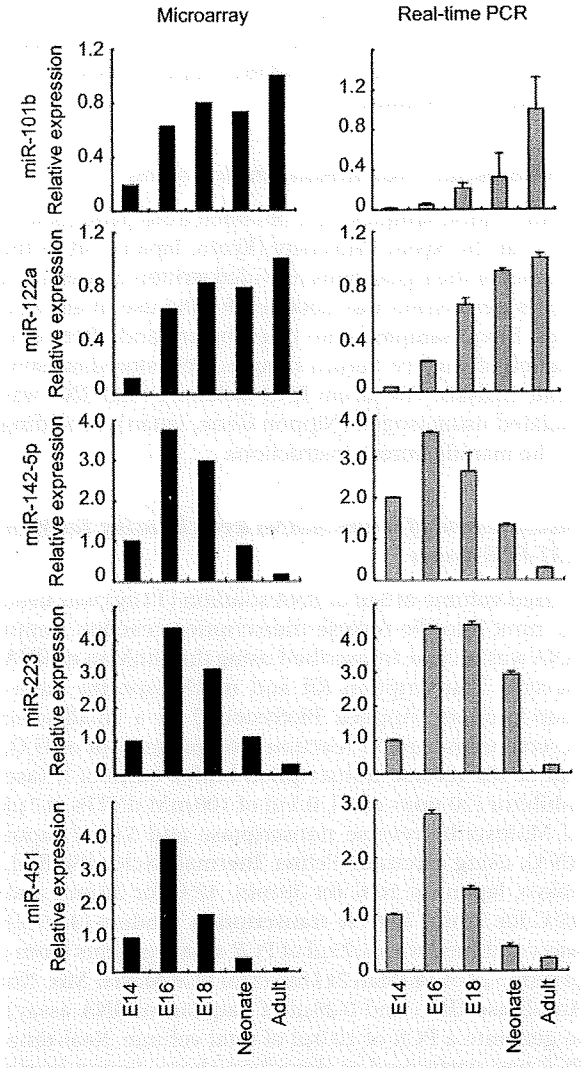

Figure 2. Differential expression of selected miRNA in mouse liver development by microarray and real-time polymerase chain reaction (PCR). miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451 were selected from highly expressed miRNAs to confirm the expression levels of microarray analysis by real-time PCR. The left panels represent the miRNA expression levels by microarray analysis. The right panels represent the miRNA expression levels by real-time PCR. The expression profile is compared for mouse fetal (embryo (E) 14, E16 and E18), neonate and adult liver. In the graphs of miR-142-5p, miR-223 and miR-451, the expression level of E14 fetal liver is set to 1.0. Real-time PCR analyses were performed in triplicate and expression values are normalized with total RNA volume. Data are shown as mean ± SD.

Differential expression patterns of cancer-related miRNAs in the process of mouse liver development

Interestingly, when analysing the expression patterns of the hierarchical clustering data in detail, we found that the expression of several let-7 miRNA family known as 'tumour suppressor miRNA' was upregulated, and, in contrast, the expression of miRNAs known as 'potential oncogenes' which are involved in cell proliferation, was downregulated in the process of liver development. Therefore, to reveal the expression pattern of cancer-related miRNAs in the process of mouse liver development, the expression profile of 21 selected miRNAs (11 miRNAs as oncogenes and 10 miRNAs as tumour suppressors) is summarized in Figure 3. Many oncogenic miRNA expressions, such as those of miR-17-5p, miR-20, and miR-92, tended to decrease in the process of mouse liver development (Figure 3A). In contrast, except for let-7d* and let-7e, the expression pattern of the let-7 miRNA family was elevated in the process of liver development (Figure 3B). This study provides evidence that the expression of oncogenic miRNA is downregulated and that the expression of tumour suppressor miRNA is upregulated in the process of liver development.

Expression of miR-500 is high in human fetal liver

As reported above, the expression levels of oncogenic miRNAs were downregulated in liver development. We tried to identify new miRNA candidates that act as an oncogenic miRNA in the liver from the microarray data. As a first step toward the elucidation of the role of miRNAs in liver carcinogenesis, we focused on downregulated miRNAs during liver maturation, which are possibly related to cell proliferation; high expressions of miR-140, miR-346, miR-411, miR-470 and miR-500 were detected at an early stage (E14) of liver development and downregulated at the late developmental stages (E16 and E18) (Figure 4A). Among these, miR-500 and miR-346 expressions were remarkably downregulated during development; thus, we concentrated on miR-500 and miR-346, which could be expected to be a potential target relevant to fetal liver development to control the time and spatial expression of sets of mRNA.

In the next step, the occurrence of miR-500 and miR-346 was assessed in human fetal and adult liver. Real-time PCR analysis revealed that the expression of miR-500 in human fetal liver, but not that of miR-346, was significantly higher than that in normal adult liver (Figure 4B and Supplementary Figure 2A (see online version of this article)). Taken together, as miR-500 expression was downregulated in human adult liver, our data suggest that miR-500 is developmentally associated with human fetal hepatocyte specification and functions. The

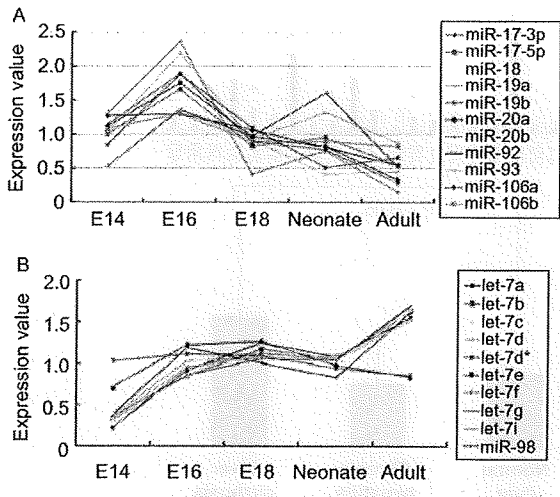

Figure 3. Expression patterns of cancer-related miRNAs in the process of mouse liver development. (A) Expression pattern by a microarray analysis (each sample: n=7–10) of miRNA that may act as an oncogene. (B) The expression pattern by the microarray analysis (each sample: n=7–10) of the let-7 family miRNAs functioned as a tumour suppressor. Expression levels are normalised by average expression value of each miRNA and shown in the graph.

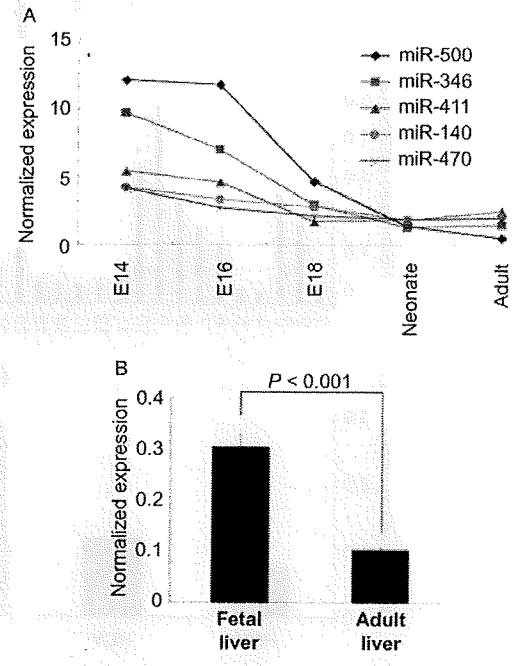

Figure 4. The expression of miR-500 is higher in the fetal stage than in the adult stage (A). The expression profile of miRNA decreased in the process of mouse liver development. Expression values are based on microarray data. (B) Expression of miR-500 in human fetal and adult liver. Real-time polymerase chain reaction analyses were performed in triplicate. Expression values are normalised with U6 snRNA value. The data represent the mean ± SD, p<0.001.

results of our ongoing knock-down analysis of miRNA in liver cancer cells will be presented in a future work.

Expression of miR-500 is high in human liver cancer

We next examined the expression level of miR-500 in six human liver cancer cell lines (JHH-7, Li-7, Huh-7, HepG2, Hep3B and Alexander) to assess whether miR-500 acts as an oncofetal miRNA and found that it increases 2.4- to 47.6-fold more in Alexander, JHH-7, HepG2, Huh-7 and Hep3B than in normal liver (Figure 5A); in contrast, no detectable amount of miR-500 was found in Li-7. On the other hand, the expression levels of miR-346 in the six liver cancer cell lines were not high (see Supplementary Figure 2B in the online version of this article). To evaluate the potential of miR-500 as an oncofetal miRNA, the expression levels of human miR-500 were analysed by real-time PCR in 40 pairs of malignant neoplasias of hepatocyte lineage (T) and adjacent non-tumorous tissue (NT). Differences in the miR-500 expression level were statistically significant ($p<0.001$) between T and NT (Figure 5B), but miR-346 expression was not significantly changed (see Supplementary Figure 2C in the online version of this article). Some of the samples exhibited remarkably high expression levels of miR-500, and 45% (18/40 patients) of the samples showed 1.2- to 8.6-fold higher upregulation in the cancerous samples than in each non-tumorous sample and

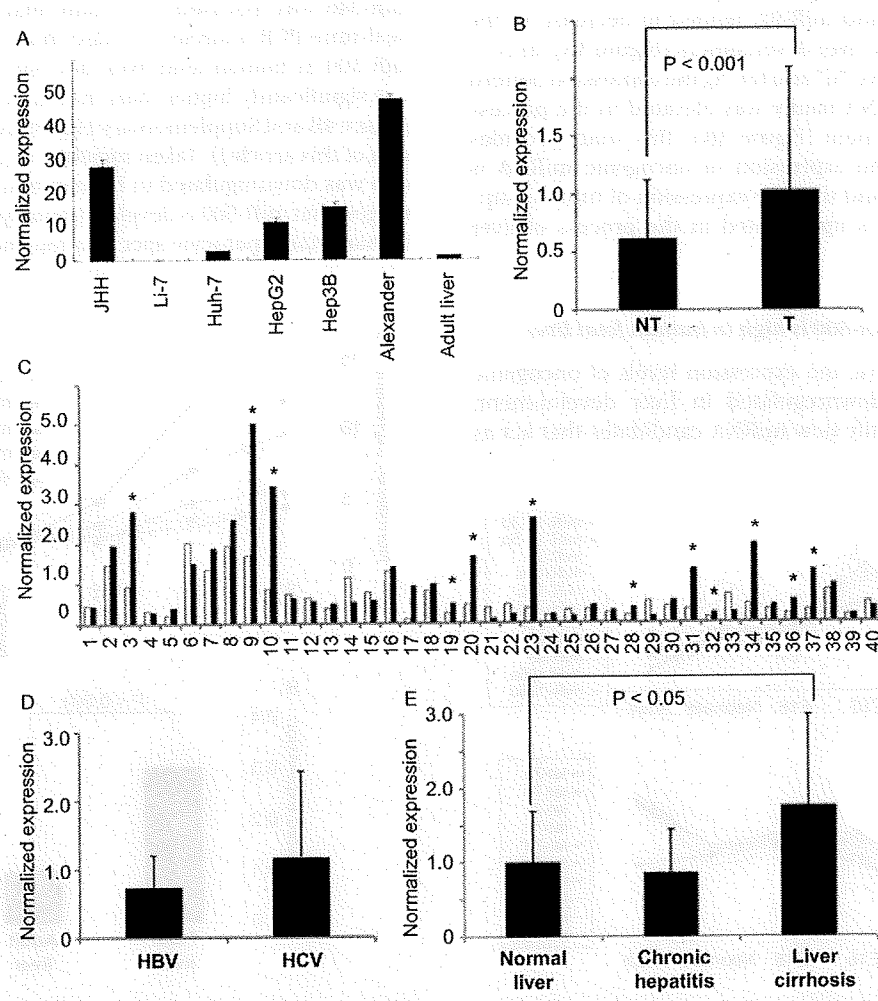

Figure 5. The expression of miR-500 is clearly upregulated in human liver cancer (A) miR-500 expression abundantly detected in liver cancer cell lines (JHH-7, Li-7, Huh-7, HepG2, Hep3B and Alexander). The expression level of normal liver is set to 1.0. The data represent the mean ± SD. (B) Forty pairs of hepatocellular carcinoma (HCC) patients (tumour (T) and non-tumour (NT)) were analysed by real-time polymerase chain reaction of human miR-500. The data represent the mean ± SD, $p<0.001$. (C) Expression levels of miR-500 in each patient (T and NT). Samples of 12 patients (*) showed twofold or more upregulation in HCC. (D) Expression levels of miR-500 in hepatitis B virus (HBV, $n=10$) and hepatitis C virus (HCV, $n=26$). (E) miR-500 expression was upregulated in liver cirrhosis ($n=17$) more than normal liver ($n=11$) and chronic hepatitis samples ($n=19$). The data represent the mean ± SD, $p<0.05$. Expression values are normalised with U6 snRNA value.

12 patients showed more than 2.0-fold higher expression (30%) (Figure 5C). Based on the clinical data and pathological diagnosis (see Supplementary Table 2 in the online version of this article), there is no significant difference in miR-500 expression between hepatitis virus B and C infection (Figure 5D). Importantly, significant difference in miR-500 expression was found between normal liver and liver cirrhosis samples, but not chronic hepatitis (Figure 5E), suggesting that miR-500 expression was upregulated during cirrhosis development. Thus, although only limited samples expressed miR-500 higher, miR-500 might be useful as a biomarker in the early stage of liver cancer.

Expression profiling of miR-500 in HCC patient serum

Recently, it has been reported that miRNAs are circulating in serum (Chim et al. 2008, Gilad et al. 2008) and tumour-derived miRNAs such as miR-155, miR-21, miR-15b, miR-16 and miR-24 are detected in the plasma and serum of tumour patients (Mitchell et al. 2008, Lawrie et al. 2008). In fact, an increased amount of miR-500 was found in the sera of three out of ten HCC patients, which means that liver cancer-specific miRNA such as miR-500 is circulating in the peripheral blood and can be a novel diagnostic marker. To determine whether or not serum levels of miR-500 truly reflect the presence of cancer in the HCC patients, the presence of miR-500 in the sera of three human HCC patients, post- and presurgical treatment, was also assessed. As can be seen in Figure 6, elevated serum levels of miR-500 in the three HCC patients were significantly reduced after surgery and returned to normal levels. These results expect that the miR-500s abundance profile in serum of the HCC patients might reflect physiological and/or pathological conditions.

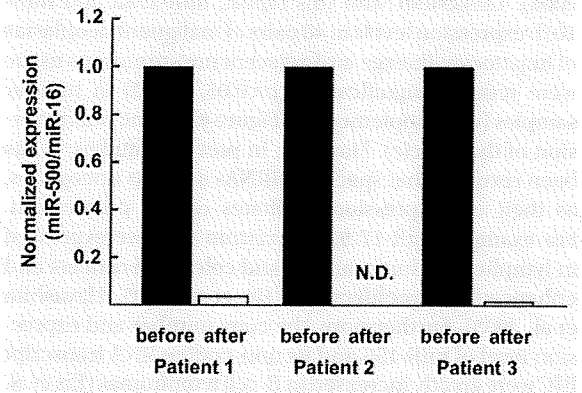

Figure 6. Serum levels of miR-500 in hepatocellular carcinoma (HCC) patients. Changes of serum levels of miR-500 in HCC patients ($n=3$) before (preoperation) and after (postoperation within 6 months) surgical removal of the tumour. Expression levels of the miR-500 are normalised to miR-16. N.D., not detected.

Discussion

Using a global miRNA expression profile in mouse liver development analysed by an LNA-based miRNA microarray, our data indicate that dynamic changes in miRNA expression occur in mouse liver development. However, the number of high-expressed miRNAs was quite limited at all developmental stages of the liver. This finding is also consistent with several reports that dominant miRNA expression is rigidly controlled in a developmental stage-specific and tissue- or cell-type-specific manner (Chen et al 2006, Shan et al. 2007). For example, it has been reported that the expressions of miR-1 and miR-133 are high and specific in adult cardiac and skeletal muscles and modulate skeletal muscle proliferation and differentiation by negatively regulating the histone deacetylase-4 or serum response factor (Chen et al. 2006). On the other hand, expression levels of the general miRNA are low at all stages of liver development. However, our data indicate that the expression pattern of some of the low-expressed miRNAs, including let-7 family, also dramatically change in the process of mouse liver development (Figure 4B). Using this platform, the overall regulation of individual miRNAs of sequential stages of liver development was determined, providing us with a useful baseline for understanding the developmental dynamics of liver miRNA expression.

In this study, we identified a novel cancer biomarker candidate, miR-500, which was designated as an oncofetal miRNA in the early stage of liver cancer, because miR-500 expression is highly expressed in a fetal liver and downregulated in the developmental process and then upregulated in the process of liver cirrhosis. When the expression profile of miR-500 in human tissues was examined, its expression was not specific in the liver and was broadly detected in all tissues (see Supplementary Figure 3 in the online version of this article). However, the expression level of miR-500 is high at the early stages of liver development in mice and humans. Furthermore, miR-500 was abundantly expressed in human liver cancer cell lines (JHH-7, Huh-7, HepG2, Hep3B and Alexander) and liver cancer tissues. Interestingly, six miRNAs (mir-532, 188, 362, 501, 660, 502) in addition to miR-500 make a cluster within a 10-kb distance from miR-500, and their expressions could be modulated by the same transcriptional regulatory unit. However, the levels were not remarkably changed during mouse liver development. Therefore, by analysing these miRNAs together, miR-500 might be a better biomarker in HCC.

We tried to test the effect of miR-500 using liver cancer cell lines. In a knock-down analysis of miR-500 with miR-500 LNA, significant changes in cell proliferation and colony formation were not observed in both Alexander and JHH-7 cells (see Supplementary Figure 5A and B in the online version of this article). Likewise, mature