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ウイルス性慢性肝疾患の病態に影響を与える miRNA多型の網羅的探索に関する研究

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総括研究報告書

ウイルス性慢性肝疾患の病態に影響を与えるmiRNA多型の網羅的探索に関する研究

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研究要旨：肝疾患の診療現場では、宿主のゲノム情報を活用した個別化医療が現実のものとなりつつあるが、依然として宿主の遺伝的要因が全て解明された訳ではないし、医療の個別化も満足のいくものではない。本研究は、臨床応用に近い研究シーズである miRNA とそれに関連した遺伝子多型に照準を絞り、臨床で有用な情報を導き出すことを目的としている。

これまでに GWAS で得られた関連シグナルやデータベースから得られる人種別頻度情報、miRNA 配列情報、発現組織特異性、過去の文献などから、網羅的にスクリーニング対象とする miRNA 多型を絞り込み、スクリーニングから追認試験を進めた。一定の意義があっても多型からのアプローチのみでは検出し難い miRNA も多数あることが想定されたため、並行して、miRNA アレイにより肝組織および血清中での発現量を網羅的に解析し、種々の phenotype と関連する miRNA の絞り込みを行った。一連の絞り込みにあたっては、*IL28B* 多型や HLA 型との関連、予測される標的が IFN シグナル経路であるか、といった免疫システムとの関連性に特に注目して優先候補とした。また、宿主の標的遺伝子のみならず、ウイルスゲノムとの相補性についても解析を進めてきた。miRNA とその多型を軸に、ウイルス性肝炎の病態解明に様々のアプローチを試行錯誤する中から、いくつかの興味深い知見を得ることができた。

血清試料によって、肝炎の dynamic な病勢を反映できる可能性のある miRNA を複数同定しており、臨床で有用なバイオマーカーとなるよう、他のマーカーも取り入れた予測モデルの構築を試みている。また、HBV/HCV 感染や HBV ウイルスマーカーと連動する複数の miRNA について、これらがどのようなメカニズムで肝炎の病態とリンクするのかを明らかにした上で、治療標的となり得るかどうかを見極めるために、現在、*in vitro*/*in vivo* の実験系で検討している。

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A．研究目的

ゲノムワイド関連解析（GWAS）によって、C 型慢性肝炎患者に対するインターフェロン治療の効果を強く規定する *IL28B* 遺伝子多型が発見され、ガイドラインにも盛り込まれるなど、すでに臨床応用がなされた。他にも、GWAS はＢ型慢性肝炎・C 型慢性肝炎の易罹患性、肝硬変・肝癌への進展などに関連する遺伝子多型あるいは一塩基多型（SNP）を明らかにしてきた。しかし、GWAS ではカバーしきれないゲノム領域の存在や、統計学的検出力の不足などの問題が依然として残っている。一方で、機能を有するノン・コーディング RNA、中でも21～25塩基ほどのmiRNAが肝疾患を含む多くの疾患で新規バイオマーカーとして期待され、これを核酸医薬の標的としたＣ型慢性肝炎治療薬の臨床試験が進行中であるなど、注目が集まっている。わが国における肝炎対策が急務であるという点では、臨床応用に比較的近い位置にある miRNA とそれに関連した多型を研究対象にすることは期待度が高い。また、医療の個別化を意識する点では、SNP 解析という実績のある手法を用いることは妥当である。発展目覚しい種々の大規模データベースを活用することで、効率的に、これまでの GWAS では明らかにできなかったウイルス性慢性肝疾患の病態・種々の表現型に関連する miRNA とそれに関連した多型の同定を目指す。

Ｂ．研究方法

網羅的アプローチとしてGWASデータを活用し、候補アプローチとして免疫関連遺伝子との関係、ウイルスゲノムとの相補性に注目し、種々のバイオデータベースを参照した上で、200個強の候補SNPを抽出した。最大でB型慢性肝炎2,000例、C型慢性肝炎5,000例、健常人1,000人超のゲノム試料を用いてSNP genotyping（Invader法）によるスクリーニングと追認試験を行った（1～2年目に予定通り遂行した）。

また、ここまでの解析過程から、一定の意義があっても多型からのアプローチのみでは検出し難いmiRNAも多数あることが想定されたため、並行してmiRNAアレイで肝組織・血清の網羅的発現解析を行った（2年目に追加した）。miRNAの発現量解析には、バイオマーカーとしての応用を念頭にヒト血清を用いるとともに、肝内の経時的な発現量変化を検討する目的で、ヒト肝細胞キメラマウスの実験系も用いている。

現在、miRNAやそれに関連した多型が表現型に影響するメカニズムにつき*in vitro/in vivo*の実験系での機能解析を進めている（2～3年目予定通り進行中）。

サンプルと付随する臨床情報は虎の門病院、札幌厚生病院、広島大学病院とその関連病院といった研究協力施設で収集されており、解析対象とする種々の表現型については、各研究協力者からのアドバイスを受けながら選別を行った。

（倫理面への配慮）

「ヒトゲノム・遺伝子解析研究に関する倫理指針」に基づいて、インフォームド・コンセントが得られた検体を用いて実施した。「動物実験等の実施に関する基本指針」を遵守して実施した。各所属研究機関の倫理委員会の承認を得て実施した。

C．研究結果

中国を中心にB型肝癌との関連が報告されている *miR-146a* 上の SNP と *miR-196a2* 上の SNP について、1,500 人以上の B 型慢性肝炎患者を解析した結果、わが国における B 型肝癌においても関連があることを明らかにし、学会発表した（Miki, *et al.* Two microRNA polymorphisms are associated with hepatitis B virus-related but not hepatitis C virus-related hepatocellular carcinoma in the Japanese population. *AASLD 2014*）。

当初、肝癌関連解析を行った GWAS のシグナルを頼りに上記のような既報のもの以外の miR-SNP の絞込みを試みたが、その方法では再現性をもって肝癌との関連を確認し得たものはなかった。そこで次に、miRNA およびその遺伝子多型の肝癌における意義を考察する上でも重要であろうと考えて、癌組織の全ゲノム解析で報告のあった高頻度変異遺伝子の発現量を baseline で規定している germline の SNP について、肝癌関連解析の GWAS データで検討した。C 型慢性肝炎 977 例（うち肝癌合併 212 例）の解析から、これら SNP と肝癌易罹患性とは関連が無いことを明らかにし、学会発表した（Miki, *et al.* Germline variants of highly point-mutated genes in hepatocellular carcinoma do not have strong effects on HCV-related hepatocarcinogenesis. *AASLD 2014*）。

miRNA の作用を解析するに先立ち、HCV 持続感染下での肝臓内遺伝子発現パターンと遺伝子多型との関連について予備的検討を行った。C 型慢性肝炎 133 例について解析した結果、インターフェロン誘導遺伝子群（ISGs）の一つである *OAS1* の HCV 持続感染肝内における発現量は、HCV 非感染時リンパ球とは全く異なるパターンで *IL28B* 遺伝子多型の影響を受けていることが明らかになった。マイクロアレイ等で遺伝子発現解析を行った結果を解釈する際、感染の有無といった外的要因や遺伝子発現の臓器特異性についても十分考慮する必要があることを示す重要な知見と考え、学会発表した（Miki, *et al.* The *IL28B* SNP has a stronger regulatory effect on the expression of *OAS1* than a nearby SNP located downstream of *OAS1* in chronic HCV patients. *AASLD 2014*）。

IL28B 遺伝子多型と *miR-122* 発現量の相関の報告がある。今回、*IFNL4-IL28B* の領域に存在する互いに連鎖の強い 3 つの SNP について、C 型慢性肝炎 4,630 症例と健常人 1,122 名を用いてハプロタイプを推定した上で、Peg-IFN+Rib 併用療法の SVR 率との関連を解析した結果、ss469415590、rs12979860 の 2 つが治療効果とより強く関連することが示され、論文発表した（Ochi, Miki, *et al. J Gen Virol 2014*）。

HCV 慢性化と *HLA-DQB1*03* および *IFNL4* あるいは *IL28B* 遺伝子多型との関連を確認するとともに、両者の相互作用を示唆する知見を得て、論文発表した（Miki, *et al. PLoS One 2013*）。さらに、

Peg-IFN+Rib 併用療法 1,140 例を解析し、HCV 持続感染の成立に防御的に働く *HLA-DQB1*03* は SVR 率とは関連しないことを明らかにした。同じく持続感染の成立に重要な *IFNL4* 遺伝子多型が治療効果にも強く関連するのとは対照的な結果であり、学会で発表した（Miki, *et al.* A single amino acid substitution in HLA-DQB1 as well as an *IFNL4* variant strongly affect susceptibility to chronic hepatitis C. *AASLD 2014*）。

ヒト肝細胞キメラマウスを用いて、肝細胞を miRNA アレイにより検討した結果、HBV 感染によって増加する miRNA を 5 つ認め、このうち 4 つは臨床材料でも再現された（*miR-486-3p*、*miR-1908*、*miR-675*、*miR-1231*）。最も増加が顕著かつ HBV ゲノムとの相補性の高い *miR-1231* に注目して機能解析を行った。HepG2 で HBV に *miR-1231* をあわせて発現させると、HBV 複製中間体、HBV-RNA、HBc 蛋白がいずれも抑制された。*miR-1231* と相補性の高いコア領域の発現プラスミドを *miR-1231* 発現プラスミドと共に HepG2 へ導入すると HBc 蛋白の発現量が低下した。*miR-1231* が HBV コア領域の mRNA に結合してコア蛋白の産生を阻害することで HBV 複製を抑制することが示唆されたため、論文で発表した(Kohno, *et al. J Viral Hepat 2014*)。

miRNA のバイオマーカーとしての利用を念頭に、B 型・C 型慢性肝炎患者（42 例・30 例）の血清中 miRNA のプロファイリングを行った。B 型よりも C 型肝炎群でより多くの miRNA の変動が認められた。*miR-122* はB・C 両群で最も強い発現亢進を認めた。HBe 抗原陽性群（e+群）と陰性群（e-群）は同様のプロファイルを示したものの、e-群に比して e+群で発現が有意に亢進している miRNA も複数認められた（*miR-122, 194, 125b, 99a, 100*）。多変量解析を行った結果、*miR-122* は HBV-DNA 量に、*miR-125b* は HBV-DNA と HBs 抗原と HBe 抗原の量に関連していることが明らかとなった。以上、血清中の miRNA 量が肝炎の etiology の違いや病期・病勢の違いを反映し得ることを論文で発表した（Akamatsu, *et al. J Infect 2015*）。

D．考察

これまでの網羅的解析で、miRNAの増減やその多型の効果は単独では決して大きなものではないことが分かってきた。多数のmiRNAが種々の程度で増減し、複数の標的遺伝子あるいはウイルスに対して様々な効果量で作用して病態を形成・修飾していることが、明らかになってきた。

これまでに得られた基礎研究の成果を臨床応用していくために、バイオマーカーとしての利用においては、周辺情報も統合しつつ総合的な変動を捉えるような多変量を含む予測モデルの構築が必要であるし、また、創薬へと進めるためには、そのメカニズムを一つ一つ機能的な解析で明らかにしていくことが必要である。

E．結論

ゲノム情報あるいは発現量情報を網羅的に解析することで、ウイルス性慢性肝疾患と関連する複数のmiRNAあるいはそれに関連した遺伝子多型を同定した。バイオマーカーや創薬といった臨床応用を目指し、*in vitro/in vivo*の実験系での機能解析など、さらなる検証が必要である。

F．健康危険情報

なし

G．研究発表

1.　論文発表

1) Akamatsu S, Hayes CN, Tsuge M, Miki D, Akiyama R, Ochi H, Hiraga N, Imamura M, Aikata H, Kawaoka T, Kawakami Y, Chayama K. Differences in serum microRNA profiles in hepatitis B and C virus infection. *J Infect.* 70(3):273-87, Mar, 2015.

2) Honda Y, Takahashi S, Zhang Y, Ono A, Murakami E, Shi N, Kawaoka T, Miki D, Tsuge M, Hiraga N, Abe H, Ochi H, Imamura M, Aikata H, Chayama K. The effects of bisphosphonate zoledronic acid in hepatocellular carcinoma, depending on mevalonate pathway. *J Gastroenterol Hepatol.* 30(3):619-27, Mar, 2015.

3) Akamatsu S, Hayes CN, Tsuge M, Murakami E, Hiraga N, Abe H, Miki D, Imamura M, Ochi H, Chayama K; Hiroshima Liver Study Group. Ribavirin dose reduction during telaprevir/ribavirin/peg-interferon therapy overcomes the effect of the ITPA gene polymorphism. *J Viral Hepat.* 22(2):166-74, Feb, 2015.

4) Kosaka K, Imamura M, Hayes CN, Abe H, Hiraga N, Yoshimi S, Murakami E, Kawaoka T, Tsuge M, Aikata H, Miki D, Ochi H, Matsui H, Kanai A, Inaba T, Chayama K. Emergence of resistant variants detected by ultra-deep sequencing after asunaprevir and daclatasvir combination therapy in patients infected with hepatitis C virus genotype 1. *J Viral Hepat.* 22(2):158-65, Feb, 2015.

5) Tanimine N, Tanaka Y, Kobayashi T, Tashiro H, Miki D, Imamura M, Aikata H, Tanaka J, Chayama K, Ohdan H. Quantitative Effect of Natural Killer-Cell Licensing on Hepatocellular Carcinoma Recurrence after Curative Hepatectomy. *Cancer Immunol Res.* 2(12):1142-7, Dec, 2014.

6) Fujino H, Imamura M, Nagaoki Y, Kawakami Y, Abe H, Hayes CN, Kan H, Fukuhara T, Kobayashi T, Masaki K, Ono A, Nakahara T, Honda Y, Naeshiro N, Urabe A, Yokoyama S, Miyaki D, Murakami E, Kawaoka T, Hiraga N, Tsuge M, Hiramatsu A, Hyogo H, Aikata H, Takahashi S, Miki D, Ochi H, Ohishi W, Chayama K; Hiroshima Liver Study Group. Predictive value of the IFNL4 polymorphism on outcome of telaprevir, peginterferon, and ribavirin therapy for older patients with genotype 1b chronic hepatitis C. *J Gastroenterol.* 49(12):1548-56, Dec, 2014.

7) Kawaoka T, Takahashi S, Tatsukawa Y, Hiramatsu A, Hiraga N, Miki D, Tsuge M, Imamura M, Kawakami Y, Aikata H, Ochi H, Ishiyama K, Ide K, Tashiro H, Ohdan H, Chayama K. Two patients treated with pegylated interferon/ribavirin/telaprevir triple therapy for recurrent hepatitis C after living donor liver transplantation. *Hepatol Res.* 44(12):1259-64, Nov, 2014.

8) Kohno T, Tsuge M, Murakami E, Hiraga N, Abe H, Miki D, Imamura M, Ochi H, Hayes CN, Chayama K. Human microRNA hsa-miR-1231 suppresses hepatitis B virus replication by

targeting core mRNA. *J Viral Hepat.* 21(9):e89-97, Sep, 2014.

9) Ochi H, Miki D, Hayes CN, Abe H, Hayashida Y, Kubo M, Chayama K. IFNL4/IL-28B haplotype structure and its impact on susceptibility to hepatitis C virus and treatment response in the Japanese population. *J Gen Virol.* 95(Pt 6):1297-306, Jun, 2014.

10) Murakami E, Imamura M, Hayes CN, Abe H, Hiraga N, Honda Y, Ono A, Kosaka K, Kawaoka T, Tsuge M, Aikata H, Takahashi S, Miki D, Ochi H, Matsui H, Kanai A, Inaba T, McPhee F, Chayama K. Ultradeep sequencing study of chronic hepatitis C virus genotype 1 infection in patients treated with daclatasvir, peginterferon, and ribavirin. *Antimicrob Agents Chemother.* 58(4):2105-12, Apr, 2014.

2. 学会発表

1) Miki D, Ochi H, Takahashi A, Hayes CN, Urabe Y, Abe H, Ono A, Akamatsu S, Nakahara T, Seki N, Murakami E, Zhang Y, Uchida T, Honda Y, Yoshimi S, Kobayashi T, Masaki K, Kan H, Kawaoka T, Tsuge M, Hiraga N, Imamura M, Kawakami Y, Aikata H, Takahashi S, Akuta N, Suzuki F, Ikeda K, Kumada H, Karino Y, Toyota J, Tsunoda T, Kubo M, Kamatani N, Nakamura Y, Chayama K. A single amino acid substitution in HLA-DQB1 as well as an IFNL4 variant strongly affect susceptibility to chronic hepatitis C. *The Liver Meeting 2014.* Boston, USA. 2014/11/11

2) Miki D, Abe H, Hayes CN, Ochi H, Kawaoka T, Akamatsu S, Ono A, Nakahara T, Seki N, Murakami E, Zhang Y, Uchida T, Honda Y, Masaki K, Kan H, Tsuge M, Hiraga N, Imamura M, Kawakami Y, Aikata H, Kubo M, Chayama K. The IL28B SNP has a stronger regulatory effect on the expression of OAS1 than a nearby SNP located downstream of OAS1 in chronic HCV patients. *The Liver Meeting 2014.* Boston, USA. 2014/11/11

3) Miki D, Ochi H, Hayes CN, Abe H, Kawaoka T, Ono A, Akamatsu S, Nakahara T, Seki N, Murakami E, Zhang Y, Uchida T, Honda Y, Masaki K, Kan H, Tsuge M, Hiraga N, Imamura M, Kawakami Y, Aikata H, Kubo M, Chayama K. Two microRNA polymorphisms are associated with hepatitis B virus-related but not hepatitis C virus-related hepatocellular carcinoma in the Japanese population. *The Liver Meeting 2014.* Boston, USA. 2014/11/9

4) Miki D, Ochi H, Hayes CN, Ono A, Akamatsu S, Y. Urabe, Masaki K, Abe H, Kawaoka T, Nakahara T, Seki N, Murakami E, Zhang Y, Uchida T, Honda Y, Kan H, Tsuge M, Hiraga N, Imamura M, Kawakami Y, Aikata H, Kubo M, Chayama K. Germline variants of highly point-mutated genes in hepatocellular carcinoma do not have strong effects on HCV-related hepatocarcinogenesis. *The Liver Meeting 2014.* Boston, USA. 2014/11/9

5) Ochi H, Miki D, Hayes CN, Abe H, Kubo M, Chayama K. IFNL4/IL-28B haplotype structure and predictability of their variations for spontaneous and treatment-induced clearance of HCV infection in the Japanese population.

The Liver Meeting 2014. Boston, USA. 2014/11/9

6) Tsuge M, Hiraga N, Murakami E, Imamura M, Abe H, Miki D, Ochi H, Hayes CN, Chayama K. Nucleotide analogue improves interferon responsiveness in HBV-infected human hepatocytes. *The Liver Meeting 2014.* Boston, USA. 2014/11/11

7) Abe H, Hayes CN, Hiraga N, Imamura M, Tsuge M, Miki D, Aikata H, Ochi H, Ishida Y, Tateno C, Yoshizato K, Chayama K. Pretreatment induction of ISGs in the presence of IFNL4 in hepatocytes but not in white blood cells is related with poor induction of ISGs following IFN-α therapy. *The Liver Meeting 2014.* Boston, USA. 2014/11/11

8) Hayes CN, Abe H, Akamatsu S, Hiraga N, Imamura M, Tsuge M, Miki D, Aikata H, Ochi H, Ishida Y, Tateno C, Chayama K. Hepatitis B virus infection efficiency and immune response decreases with cell density in primary cultured hepatocytes. *The Liver Meeting 2014.* Boston, USA. 2014/11/11

9) 三木大樹、相方浩、大野敦司、卜部祐司、柘植雅貴、川上由育、久保充明、中村祐輔、茶山一彰. わが国における miR-146a および miR-196a2 の遺伝子多型と肝炎ウイルス関連肝発癌リスク. *第 73 回日本癌学会学術総会.* 横浜. 2014/9/26

10) 三木大樹、越智秀典、ヘイズ・ネルソン、阿部弘美、大野敦司、中原隆志、村上英介、河岡友和、柘植雅貴、平賀伸彦、今村道雄、川上由育、相方浩、高橋祥一、茶山一彰. HCV 慢性化における HLA-DQB1 の重要性：ゲノムワイド関連解析. *第 50 回日本肝臓学会総会.* 東京. 2014/5/30

11) 阿部弘美、三木大樹、平賀伸彦、今村道雄、柘植雅貴、小林知樹、河岡友和、川上由育、相方浩、越智秀典、茶山一彰. HCV 感染における IFNL4 の多型の違いによる IFN-λs の発現量とインターフェロン投与前後のインターフェロン誘導遺伝子の発現量の関係. *第 50 回日本肝臓学会総会.* 東京. 2014/5/30

12) 狩野吉康、山口将功、木村睦海、荒川智宏、中島知明、桑田靖昭、小関至、佐藤隆啓、大村卓味、髭修平、豊田成司、三木大樹、越智秀典、茶山一彰. HCV 関連肝疾患における IFNL3(IL28B),IFNL4 遺伝子型乖離例の頻度と抗ウイルス療法の効果の検討. *第 50 回日本肝臓学会総会.* 東京. 2014/5/30

13) 村上英介、柘植雅貴、藤野初枝、菅宏美、福原崇之、小林知樹、本田洋士、中原隆志、苗代典昭、大野敦司、宮木大輔、三木大樹、河岡友和、平賀伸彦、平松憲、今村道雄、兵庫秀幸、川上由育、相方浩、茶山一彰. 当院における HBs 抗原陰性化例の解析. *第 50 回日本肝臓学会総会.* 東京. 2014/5/29

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版
Akamatsu S, Hayes CN, Tsuge M, Miki D, Akiyama R, Ochi H, Hiraga N, Imamura M, Aikata H, Kawaoka T, Kawakami Y, Chayama K.	Differences in serum microRNA profiles in hepatitis B and C virus infection.	J Infect	70(3)	273-87	2015
Honda Y, Takahashi S, Zhang Y, Ono A, Murakami E, Shi N, Kawaoka T, Miki D, Tsuge M, Hiraga N, Abe H, Ochi H, Imamura M, Aikata H, Chayama K.	The effects of bisphosphonate zoledronic acid in hepatocellular carcinoma, depending on mevalonate pathway.	J Gastroenterol Hepatol	30(3)	619-27	2015
Akamatsu S, Hayes CN, Tsuge M, Murakami E, Hiraga N, Abe H, Miki D, Imamura M, Ochi H, Chayama K; Hiroshima Liver Study Group.	Ribavirin dose reduction during telaprevir/ribavirin/peg-interferon therapy overcomes the effect of the ITPA gene polymorphism.	J Viral Hepat	22(2)	166-74	2015
Kosaka K, Imamura M, Hayes CN, Abe H, Hiraga N, Yoshimi S, Murakami E, Kawaoka T, Tsuge M, Aikata H, Miki D, Ochi H, Matsui H, Kanai A, Inaba T, Chayama K.	Emergence of resistant variants detected by ultra-deep sequencing after asunaprevir and daclatasvir combination therapy in patients infected with hepatitis C virus genotype 1.	J Viral Hepat	22(2)	158-65	2015

Tanimine N, Tanaka Y, Kobayashi T, Tashiro H, Miki D, Imamura M, Aikata H, Tanaka J, Chayama K, Ohdan H.	Quantitative Effect of Natural Killer-Cell Licensing on Hepatocellular Carcinoma Recurrence after Curative Hepatectomy.	Cancer Immunol Res	2(12)	1142-7	2014
Fujino H, Imamura M, Nagaoki Y, Kawakami Y, Abe H, Hayes CN, Kan H, Fukuhara T, Kobayashi T, Masaki K, Ono A, Nakahara T, Honda Y, Naeshiro N, Urabe A, Yokoyama S, Miyaki D, Murakami E, Kawaoka T, Hiraga N, Tsuge M, Hiramatsu A, Hyogo H, Aikata H, Takahashi S, Miki D, Ochi H, Ohishi W, Chayama K; Hiroshima Liver Study Group.	Predictive value of the IFNL4 polymorphism on outcome of telaprevir, peginterferon, and ribavirin therapy for older patients with genotype 1b chronic hepatitis C.	J Gastroenterol	49(12)	1548-56	2014
Kawaoka T, Takahashi S, Tatsukawa Y, Hiramatsu A, Hiraga N, Miki D, Tsuge M, Imamura M, Kawakami Y, Aikata H, Ochi H, Ishiyama K, Ide K, Tashiro H, Ohdan H, Chayama K.	Two patients treated with pegylated interferon/ribavirin /telaprevir triple therapy for recurrent hepatitis C after living donor liver transplantation.	Hepatol Res	44(12)	1259-64	2014

Kohno T, Tsuge M, Murakami E, Hiraga N, Abe H, Miki D, Imamura M, Ochi H, Hayes CN, Chayama K.	Human microRNA hsa-miR-1231 suppresses hepatitis B virus replication by targeting core mRNA.	J Viral Hepat	21(9)	e89-97	2014
Ochi H, Miki D, Hayes CN, Abe H, Hayashida Y, Kubo M, Chayama K.	IFNL4/IL-28B haplotype structure and its impact on susceptibility to hepatitis C virus and treatment response in the Japanese population.	J Gen Virol	95(Pt 6)	1297-306	2014
Murakami E, Imamura M, Hayes CN, Abe H, Hiraga N, Honda Y, Ono A, Kosaka K, Kawaoka T, Tsuge M, Aikata H, Takahashi S, Miki D, Ochi H, Matsui H, Kanai A, Inaba T, McPhee F, Chayama K.	Ultradeep sequencing study of chronic hepatitis C virus genotype 1 infection in patients treated with daclatasvir, peginterferon, and ribavirin.	Antimicrob Agents Chemother	58(4)	2105-12	2014

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Differences in serum microRNA profiles in hepatitis B and C virus infection

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Summary *Objectives:* Patients infected with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) are at greater risk of cirrhosis and hepatocellular carcinoma. The objective of this study was to identify virus-specific serum microRNA profiles associated with liver function and disease progression. Microarray analysis of serum microRNAs was performed using the Toray 3D array system in 22 healthy subjects, 42 HBV patients, and 30 HCV patients. Selected microRNAs were then validated by qRT-PCR in 186 HBV patients, 107 HCV patients, and 22 healthy subjects.

Results: Microarray analysis showed up-regulation of a number of microRNAs in serum of both HBV and HCV patients. In qRT-PCR analysis, miR-122, miR-99a, miR-125b, miR-720, miR-22, and miR-1275 were up-regulated both in HBV patients relative to healthy subjects, and all except

List of abbreviations: HBV, Hepatitis B virus; HCV, Hepatitis C virus; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; HBsAg, HBV surface antigen; HBeAg, HBe antigen; HBeAb, HBe antibody; HBcAg, HBV core antigen; γGTP, γ-glutamyl transpeptidase.

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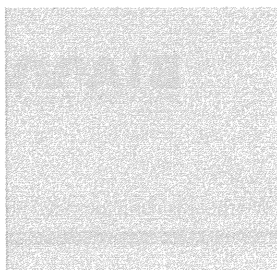

miR-1275 were up-regulated in HBeAg-positive patients relative to HBeAg-negative patients. Specific microRNAs were independently associated with different aspects of HBV infection. MiR-122 was independently associated with HBV DNA level, whereas miR-125b was independently associated with levels of HBV DNA, HBsAg, and HBeAg. MiR-22 and miR-1275 were independently associated with serum γ-glutamyl transpeptidase levels.
Conclusions: Serum microRNA levels reflect differences in the etiology and stage of viral hepatitis.
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Introduction

Chronic infection with hepatitis B virus (HBV), a partially double-stranded DNA virus, and hepatitis C virus (HCV), a single stranded RNA virus, increases the risk of cirrhosis and hepatocellular carcinoma (HCC). Despite improvements in antiviral therapy, many patients fail to respond to current therapies.[1–3] Therefore, non-invasive methods are needed for early detection of changes in liver function. One such approach is to measure changes in levels of small RNAs present in the serum of infected patients. In addition to messenger RNA, transfer RNA, and ribosomal RNA, there are many other classes of RNAs, many of which act to fine-tune gene expression and may play a role in disease pathogenesis. MicroRNAs are among the most important classes of non-coding RNA and consist of short linear RNA sequences that range in size from 19 to 24 nucleotides. MicroRNAs may influence gene expression by binding to a partially complementary region in the 3′ untranslated region of a targeted messenger RNA, thereby inhibiting translation or promoting degradation of the transcript. Because a single microRNA may regulate multiple genes, and a single gene may be regulated by multiple microRNAs, microRNAs may form complex regulatory networks.[4] Viral pathogenesis and inflammation may disrupt these intricate networks, resulting in changes in microRNA levels inside and outside of the cell. Given the liver's dual blood supply and central role in circulation, pathogenic changes in gene expression in the liver are likely to be reflected in changes in microRNA profiles in the serum.

Understanding the origin and function of serum microRNAs is important in the development of strategies to eradicate HCV and HBV and to monitor the degree of liver damage. Analysis of differential microRNA expression in liver tissues has revealed HCV- and HBV-specific microRNAs as well as microRNAs associated with the stage of liver disease.[5–9] MicroRNA levels in the liver have been found to be correlated with serum levels for a number of microRNAs,[10,11] suggesting that serum microRNAs might act as a surrogate measure of microRNA activity in the liver. While RNA typically has a short-half life and is quickly degraded by RNases, microRNAs tend to exist stably in serum when bound to argonaute proteins such as AGO2 as part of the RNA-induced silencing complex, the molecular scaffold that facilitates interaction of a microRNA with its target sequence.[12] Circulating microRNAs may exist in this form as vesicle-free ribonucleoprotein complexes, or they may be transported within HBV surface antigen (HBsAg) particles or contained within exosomes/microvesicles.[12–14] However, serum microRNAs are typically concentrated in exosomes.[15]

Exosomes are 30–150 nm endosome-derived microvesicles that are released from multiple cell types and are detectable in blood, urine, saliva, and other body fluids. Exosomes are involved in removal of cellular waste products as well as cell–cell communication and immune activation but may also be exploited by pathogens and contribute to tumor proliferation. Exosomes contain characteristic RNA transcripts, including microRNAs, transfer RNAs and other types of non-coding RNAs[16] and have been shown to affect gene expression in recipient cells. MiR-99a, miR128, miR-124, miR-22, and miR-99b account for 49% of identified exosome-associated microRNAs.[16] While exosomal RNA profiles vary by cell type, they do not completely mirror the RNA profile of the parent cell due to selective sorting and may change in response to cellular conditions.[16] Hepatocyte-derived exosomes are enriched for gene products involved in lipoprotein metabolism and xenobiotic processing and therefore have potential as a diagnostic tool by reflecting hepatic changes linked to disease.[17] Interferon-stimulated release of exosomes containing antiviral products and internalization by HBV-infected hepatocytes may also play a role in antiviral defense by bypassing viral interference in interferon signal transduction.[18] It is likely that analysis of serum microRNA profiles will provide insight into disease progression and antiviral activity in the liver, particularly in the case of HBV infection.

In order to investigate the relationship between serum microRNA profiles and viral hepatitis, we performed microarray and quantitative real-time polymerase chain reaction (qRT-PCR) analysis to identify host microRNAs that differ between healthy subjects and patients with chronic HBV or HCV infection as well as between HBeAg-positive and negative patients.

Methods

Study subjects

All patients had either chronic hepatitis B or C infection and were negative for HIV and HCC. No patients were co-infected with both HBV and HCV. All healthy subjects were negative for HBsAg and HCV antibody. Patient profiles are shown in Table 1. Histopathological diagnosis was determined as in Desmet et al.[19] The study was approved *a priori* by the ethical committee of Hiroshima University and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All patients provided written informed consent.

Microarray analysis of serum microRNA expression levels

Host microRNA expression in serum samples was measured using the Toray Industries microRNA analysis system, in which serum microRNA samples were hybridized to 3D-Gene human microRNA ver17.1 chips containing 1200 microRNAs (Toray Industries, Inc., Tokyo, Japan). Serum from 42 patients with chronic HBV infection and 30 patients with chronic HCV infection were compared with serum from 12 healthy males and 10 healthy females using a separate microarray for each sample.

Quantitative RT-PCR microRNA analysis

A subset of microRNAs was selected for validation using qRT-PCR based on preliminary microarray results and a search of the literature. Expression of 7 microRNAs was measured in serum from 186 HBV patients, 107 HCV patients, and 22 healthy subjects. Circulating microRNA was extracted from 300 μl of serum samples using the mirVana PARIS Kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. RNA was eluted in 80 μl of nuclease free water and reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life technologies Japan Ltd, Tokyo, Japan). Each sample was spiked with *Caenorhabditis elegans* miR-238 (cel-miR-238) as a control for extraction and amplification. The reaction mixture contained 5 μl of RNA solution, 2 μl of 10x reverse transcription buffer, 0.2 μl of 100 mM dNTP mixture, 4 μl of 5x RT primer, 0.25 μl of RNase inhibitor and 7.22 μl of nuclease free water in a total volume of 20 μl. The reaction was performed at 16 °C for 30 min followed by 42 °C for 30 min. The reaction was terminated by heating the solution at 85 °C for 5 min. MicroRNAs were amplified using primers and probes provided by Applied Biosystems Inc. using TaqMan MicroRNA assays according to the manufacturer's instructions. The reaction mixture contained 12.5 μl of 2x Universal PCR Master Mix, 1.25 μl of 20x TaqMan Assay solution, 1 μl of reverse transcription product and 10.25 μl of nuclease free water in a total volume of 25 μl. Amplification conditions were 95 °C for 10 min followed by 50 denaturing cycles for 15 s at 95 °C and annealing and extension for 60 s at 60 °C in an ABI7300 thermal cycler. For the cel-miR-238 assay, a dilution series using chemically synthesized microRNA was used to generate a standard curve that permitted absolute quantification of molecules. A separate internal normalization factor was not used.

Statistical analysis

MicroRNA microarray expression data was normalized using cyclic loess and analyzed using moderated *t*-tests using the limma package in the R statistical framework (http://www.r-project.org). *P*-values were adjusted for multiple testing using the false discovery rate (P_{FDR}). qRT-PCR expression levels were compared between healthy subjects and HBV or HCV using the non-parametric Mann–Whitney *U* test. Association between qRT-PCR microRNA levels and clinical parameters such as HBsAg, HBV DNA, HBeAg, HBeAb, AST, and ALT were evaluated using multiple linear regression. Factors that were significant at 0.05 in univariate analysis were included as candidates in the multivariate model, and forward-backward stepwise selection based on Akaike information criterion (AIC) was used to identify independently associated factors.

Pathway analysis

Target genes of differentially expressed microRNAs were predicted using the miRWalk database (http://www.umm.

Table 1 Clinical characteristics of healthy controls and patients with chronic viral HBV or HCV infection. Continuous variables are shown as median and range, and categorical variables are shown as counts.

Factor	Healthy(N = 22)	Hepatitis B virus (N = 186)	Hepatitis C virus (N = 107)
Age	33 (27–45)	48 (22–79)	64 (24–85)
Sex (male/female)	12/10	122/64	47/60
Alanine aminotransferase (IU/l)	18.5 (15–22)	73.5 (10–1867)	30.5 (18–145)
Aspartate aminotransferase (IU/l)	13.5 (6–44)	47.5 (15–982)	33.5 (11–141)
γ-glutamyl transpeptidase (IU/l)	20 (11–52)	41.5 (9–459)	22 (8–161)
rs8099917 genotype (TT/GT/GG/unknown)	5/0/0/17	89/76/3/18	–
Liver fibrosis (1/2/3/4/unknown)	–	65/76/28/3/14	39/35/11/4/18
Necroinflammatory activity (1/2/3/unknown)	–	58/80/34/14	32/48/9/18
Alpha-fetoprotein (ug/l)	–	6.1 (<5.0–2510.0)	5.0 (<5.0–104.8)
Promthrombin time (s)	–	95 (35–123)	98 (71–116)
Albumin (g/dl)	–	4.4 (2.8–4.9)	4.3 (3.5–5.0)
Platelets ($x10^4/mm^3$)	–	17.4 (5.0–35.7)	17.6 (5.3–29.8)
rs8099917 genotype (TT/GT/GG/unknown)	5/0/0/17	89/76/3/18	–
HBV DNA (IU/ml)	–	6.7 (<2.1–≥9.1)	–
HBsAg (IU/l)	–	3650 (1.2–239000)	–
HBeAg (−/+)	–	82/104	–
HBeAb (−/+)	–	88/98	–
HBV genotype (A/B/C/unknown)	–	3/14/129/40	–
HCV RNA (Log IU/ml)	–	–	6.5 (1.7–7.3)
HCV genotype (1a/1b/2a/2b/3a)	–	–	5/42/18/9/1/32

Table 2 Top up- or down-regulated serum microRNAs associated with chronic HBV or HCV infection. MicroRNAs that have been detected in exosomes are noted.

Contrast	Direction	miRNA	logFC	AveExpr	t	P	P_{FDR}	Exosome
HBV-Healthy	Up	hsa-miR-122	2.80	8.30	7.63	2.42E-11	3.23E-09	exosome
	Up	hsa-miR-3648	1.39	13.63	8.26	1.20E-12	2.14E-10	
	Up	hsa-miR-642b	1.07	9.64	9.16	1.63E-14	9.76E-12	
	Up	hsa-miR-22	1.04	8.16	5.12	1.70E-06	3.01E-05	exosome
	Up	hsa-miR-1246	1.02	10.75	5.29	8.59E-07	1.78E-05	
	Up	hsa-miR-486-3p	0.89	8.32	7.43	6.06E-11	5.66E-09	
	Up	hsa-miR-191	0.80	7.65	6.04	3.46E-08	1.30E-06	exosome
	Up	hsa-miR-1915*	0.63	7.64	4.85	5.22E-06	7.76E-05	
	Up	hsa-miR-3665	0.62	14.38	5.69	1.58E-07	4.54E-06	
	Up	hsa-miR-658	0.61	7.72	8.80	9.24E-14	3.70E-11	exosome
	Up	hsa-miR-550a	0.59	7.24	10.56	2.00E-17	2.40E-14	
	Up	hsa-miR-320b	0.57	7.22	7.13	2.43E-10	2.08E-08	
	Up	hsa-miR-320a	0.54	7.29	6.63	2.47E-09	1.41E-07	exosome
	Up	hsa-miR-320c	0.54	7.05	6.67	2.00E-09	1.24E-07	
	Up	hsa-miR-3663-3p	0.51	10.69	5.63	2.08E-07	5.67E-06	
	Up	hsa-miR-99a	0.51	6.56	5.30	8.38E-07	1.78E-05	exosome
	Down	hsa-miR-223	−0.89	7.69	−5.15	1.56E-06	2.79E-05	exosome
	Down	hsa-miR-4294	−0.86	10.91	−5.50	3.59E-07	8.98E-06	
	Down	hsa-miR-575	−0.75	7.63	−6.05	3.31E-08	1.28E-06	exosome
	Down	hsa-miR-1268	−0.57	11.77	−6.83	1.00E-09	6.66E-08	
	Down	hsa-miR-1202	−0.54	8.10	−5.40	5.51E-07	1.25E-05	
	Down	hsa-miR-1275	−0.52	8.92	−5.06	2.20E-06	3.71E-05	
HCV-Healthy	Up	hsa-miR-122	1.81	8.30	4.74	8.05E-06	7.37E-05	exosome
	Up	hsa-miR-3648	1.52	13.63	8.63	2.04E-13	2.23E-11	
	Up	hsa-miR-642b	1.42	9.64	11.67	1.12E-19	6.69E-17	
	Up	hsa-miR-24	1.11	8.80	6.58	3.06E-09	5.92E-08	exosome
	Up	hsa-miR-3925-5p	1.10	7.28	7.98	4.61E-12	2.49E-10	
	Up	hsa-miR-296-3p	1.10	7.76	7.30	1.10E-10	3.56E-09	
	Up	hsa-miR-3162-5p	1.08	8.42	8.30	9.94E-13	7.95E-11	
	Up	hsa-miR-3622b-5p	1.08	7.82	6.13	2.33E-08	3.77E-07	
	Up	hsa-miR-3665	1.06	14.38	9.27	9.51E-15	1.90E-12	
	Up	hsa-miR-3917	1.01	7.99	7.59	2.92E-11	1.11E-09	
	Up	hsa-miR-762	1.01	14.16	10.63	1.48E-17	5.93E-15	
	Up	hsa-miR-4258	0.96	8.57	7.00	4.39E-10	1.15E-08	
	Up	hsa-miR-4257	0.92	7.83	9.45	4.05E-15	9.73E-13	
	Up	hsa-miR-663	0.86	10.87	5.38	5.82E-07	7.27E-06	exosome
	Up	hsa-miR-4299	0.86	7.19	7.65	2.13E-11	9.33E-10	
	Up	hsa-miR-486-3p	0.83	8.32	6.65	2.20E-09	4.48E-08	
	Up	hsa-miR-149*	0.78	10.33	7.73	1.49E-11	6.88E-10	exosome
	Up	hsa-miR-4259	0.74	7.74	5.06	2.22E-06	2.32E-05	
	Up	hsa-miR-1469	0.74	10.93	5.28	8.83E-07	1.05E-05	
	Up	hsa-miR-3934	0.74	7.43	7.62	2.48E-11	1.03E-09	
	Up	hsa-miR-658	0.73	7.72	10.14	1.52E-16	4.57E-14	exosome
	Up	hsa-miR-3663-3p	0.73	10.69	7.65	2.18E-11	9.33E-10	
	Up	hsa-miR-671-5p	0.67	8.15	8.31	9.52E-13	7.95E-11	exosome
	Up	hsa-miR-187*	0.67	8.45	8.20	1.61E-12	1.02E-10	
	Up	hsa-miR-3131	0.66	7.71	8.40	6.21E-13	6.21E-11	
	Up	hsa-miR-3154	0.64	8.13	6.32	1.00E-08	1.77E-07	
	Up	hsa-miR-320a	0.59	7.29	6.94	5.85E-10	1.40E-08	exosome
	Up	hsa-miR-4300	0.55	6.89	6.43	6.06E-09	1.12E-07	
	Up	hsa-miR-3126-5p	0.53	6.85	7.43	6.11E-11	2.16E-09	
	Up	hsa-miR-3153	0.51	6.99	5.16	1.46E-06	1.56E-05	
	Up	hsa-miR-550a	0.51	7.24	8.70	1.50E-13	1.80E-11	
	Up	hsa-miR-3616-3p	0.50	6.87	8.18	1.78E-12	1.07E-10	
	Up	hsa-miR-371-5p	0.50	7.70	5.91	6.09E-08	9.14E-07	
	Up	hsa-miR-3147	0.50	7.60	6.20	1.68E-08	2.88E-07	

Table 2 (*continued*)

Contrast	Direction	miRNA	logFC	AveExpr	t	P	P_{FDR}	Exosome
	Down	hsa-miR-451	−2.00	10.87	−5.76	1.16E-07	1.68E-06	exosome
	Down	hsa-miR-223	−1.42	7.69	−7.91	6.28E-12	3.14E-10	exosome
	Down	hsa-miR-92a-2*	−1.30	10.11	−7.20	1.76E-10	5.03E-09	
	Down	hsa-miR-4294	−1.22	10.91	−7.42	6.33E-11	2.17E-09	
	Down	hsa-miR-575	−1.17	7.63	−9.06	2.67E-14	4.57E-12	exosome
	Down	hsa-miR-16	−1.13	7.77	−4.99	2.96E-06	2.96E-05	exosome
	Down	hsa-miR-1275	−0.75	8.92	−7.08	3.05E-10	8.52E-09	
	Down	hsa-miR-1915	−0.75	11.10	−12.24	7.86E-21	9.44E-18	
	Down	hsa-miR-1202	−0.69	8.10	−6.61	2.67E-09	5.34E-08	
	Down	hsa-miR-887	−0.68	8.13	−8.23	1.38E-12	9.30E-11	exosome
	Down	hsa-miR-1203	−0.64	8.50	−7.05	3.48E-10	9.49E-09	
	Down	hsa-miR-125a-3p	−0.62	6.90	−7.53	3.72E-11	1.35E-09	exosome
	Down	hsa-miR-17	−0.59	6.76	−5.00	2.79E-06	2.81E-05	exosome
	Down	hsa-miR-3141	−0.59	8.72	−7.02	4.11E-10	1.10E-08	
	Down	hsa-miR-20a	−0.59	6.60	−5.65	1.91E-07	2.57E-06	exosome
	Down	hsa-miR-1268	−0.58	11.77	−6.60	2.81E-09	5.52E-08	
	Down	hsa-miR-423-5p	−0.51	7.97	−7.75	1.38E-11	6.64E-10	
HCV-HBV	Up	hsa-miR-296-3p	0.80	7.76	6.07	3.06E-08	1.67E-06	
	Up	hsa-miR-3925-5p	0.74	7.28	6.09	2.79E-08	1.59E-06	
	Up	hsa-miR-4257	0.70	7.83	8.28	1.09E-12	4.34E-10	
	Up	hsa-miR-3162-5p	0.66	8.42	5.79	1.01E-07	4.67E-06	
	Up	hsa-miR-1469	0.65	10.93	5.28	8.82E-07	2.52E-05	
	Up	hsa-miR-149*	0.64	10.33	7.23	1.54E-10	2.65E-08	exosome
	Up	hsa-miR-3917	0.57	7.99	4.91	4.01E-06	8.74E-05	
	Up	hsa-miR-4299	0.53	7.19	5.36	6.43E-07	1.98E-05	
	Up	hsa-miR-762	0.52	14.16	6.27	1.25E-08	9.35E-07	

logFC: log2 fold-change; AveExpr: The average log2 expression level; t: moderated t-statistic; P: uncorrected *P*-value for *t*-test; P_{FDR}: *P*-value adjusted for multiple testing based on the false discovery rate.

uni-heidelberg.de/apps/zmf/mirwalk/ accessed on 14 September 2014)[20] based on maximum agreement among the following tools: DIANA-mT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR5, PITA, RNA22, and TargetScan. Gene set enrichment in canonical pathways was analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems, CA, USA).

Results

MicroRNA microarray results

MicroRNA microarray analysis was performed to identify differentially expressed microRNAs in serum of patients with chronic HBV or HCV compared to healthy individuals and between patients with chronic HBV compared to patients with chronic HCV. A larger number of microRNAs were significantly up- or down-regulated in serum of HCV patients compared to HBV patients (Table 2, Suppl. Table 1). MiR-122 was strongly up-regulated in both patients with HBV (logFC = 2.77) and HCV (logFC = 1.81), but the fold change was modest for other microRNAs. Several microRNAs were associated with HBV infection, including miR-22, miR-99a, miR-1246, miR-320a and miR-320b (Table 2; Fig. 1A). Serum microRNA profiles of HBeAg-positive and negative patients were compared with healthy subjects (Table 3, Fig. 1B, Suppl. Table 2). Results were similar for both HBeAg-positive and negative patients, but several microRNAs, including miR-122, miR-194, miR-125b, miR-99a, and miR-100, were up-regulated in HBeAg-positive patients compared to HBeAg-negative patients. MicroRNAs were annotated based on whether or not they have been reported to be detected within exosomes (www.exocarta.org accessed on 12 September 2014)[21,22] and/or within circulating HBsAg particles.[14] Nearly all of the significantly up-regulated microRNAs have been reported to be detected in exosomes, and miR-122, miR-30a, miR-30b, and miR-30c have been detected in HBsAg particles. However, further research is necessary to confirm in which compartments these microRNAs are present in these patients.

Quantitative RT-PCR analysis

qRT-PCR was used to validate expression of selected microRNAs (Table 4). MiR-122, miR-99a, miR-125b, miR-720, miR-22, and miR-1275 were significantly up-regulated in serum of HBV patients ($n = 185$) compared to healthy subjects ($n = 22$). MiR-122 and miR-720, but not miR-1246, were significantly up-regulated in serum of HCV patients ($n = 107$) relative to healthy subjects ($n = 10$). Microarray and qRT-PCR expression levels from the same individual were correlated ($P < 0.05$; data not shown). MiR-99a, miR-125b, miR-122, miR-720, and miR-22, but not miR-1275, were significantly elevated in HBeAg-positive versus HBeAg-negative individuals (Table 4; Fig. 2). In Fig.2, the points representing the highest

Figure 1 Heatmap of results of serum microRNA microarray analysis. Up-regulated microRNAs are shown in red, and down-regulated microRNAs are shown in green. Hierarchical clustering was performed in R using Euclidean distance and McQuitty clustering. A) Healthy (blue) versus HCV (purple) and HBV (yellow). B) HBe antigen-positive patients (yellow) versus HBe antigen-negative patients (purple). MicroRNAs that have been reported to be associated with exosomes are annotated based on a search of the Exocarta database.[21]

expression level for each of miR-122, miR-99a, and miR-125b corresponds to the same patient, who also had the highest HBsAg level (239000 IU/ml), but no other patients shared a similar rank pattern. The non-parametric Kruskal—Wallis test, based on the median instead of the mean, is robust to outliers and was used to prevent patients with high microRNA expression levels from having undo influence over the results. No internal normalization factors were selected *a priori*. However, because miR-1275 did not differ between HBeAg-positive and HbeAg-negative, it

Table 3 Top up- or down-regulated serum microRNAs associated with HBeAg-positive or negative chronic HBV infection. MicroRNAs that have been detected in exosomes or HBsAg particles are noted.

Contrast	Direction	miRNA	logFC	AveExpr	P	P_{FDR}	Exosome	HBsAg
HBeAg(+) vs Healthy	Up	hsa-miR-122	3.9	8.1	3.48E-14	4.18E-11	exosome	HBsAg
	Up	hsa-miR-22	1.3	8.1	3.52E-07	3.52E-05	exosome	
	Up	hsa-miR-3648	1.2	13.0	3.47E-06	2.08E-04		
	Up	hsa-miR-1246	1.0	10.5	7.43E-06	3.43E-04		
	Up	hsa-miR-642b	1.0	9.1	3.94E-08	5.91E-06		
	Up	hsa-miR-486-3p	0.9	8.0	3.79E-06	2.15E-04		
	Up	hsa-miR-191	0.8	7.5	7.67E-07	5.76E-05	exosome	
	Up	hsa-miR-4286	0.8	7.3	3.74E-04	6.31E-03		
	Up	hsa-miR-194	0.8	6.5	1.66E-05	5.88E-04	exosome	
	Up	hsa-miR-99a	0.7	6.6	3.99E-06	2.15E-04	exosome	
	Up	hsa-miR-125b	0.7	6.7	9.17E-06	3.84E-04	exosome	
	Up	hsa-miR-30d	0.7	7.4	5.54E-06	2.66E-04	exosome	
	Up	hsa-miR-3665	0.6	14.0	5.11E-04	8.07E-03		
	Up	hsa-miR-320b	0.6	7.1	6.74E-09	1.35E-06		
	Up	hsa-miR-100	0.6	6.5	1.70E-05	5.88E-04	exosome	
	Up	hsa-miR-1915*	0.6	7.5	9.81E-04	1.39E-02		
	Up	hsa-miR-320a	0.6	7.1	8.21E-09	1.41E-06		
	Up	hsa-miR-320d	0.6	6.8	4.01E-07	3.70E-05		
	Up	hsa-miR-550a	0.6	7.1	3.38E-11	2.03E-08		
	Up	hsa-miR-320c	0.5	6.9	2.17E-07	2.61E-05		
	Up	hsa-miR-658	0.5	7.4	3.73E-09	1.00E-06	exosome	
	Down	hsa-miR-4294	−1.0	11.3	1.08E-04	2.50E-03		
	Down	hsa-miR-575	−0.7	8.0	4.65E-04	7.54E-03	exosome	
	Down	hsa-miR-92a-2*	−0.7	10.6	1.29E-03	1.69E-02		
	Down	hsa-miR-3197	−0.6	10.8	1.28E-04	2.84E-03		
	Down	hsa-miR-1268	−0.5	12.0	2.96E-05	8.89E-04		
	Down	hsa-miR-1275	−0.5	9.2	4.72E-04	7.54E-03		
HBeAg(−) vs Healthy	Up	hsa-miR-122	2.1	7.6	1.68E-06	4.39E-05	exosome	HBsAg
	Up	hsa-miR-3648	1.5	13.3	2.78E-09	2.09E-07		
	Up	hsa-miR-642b	1.2	9.3	2.15E-11	6.45E-09		
	Up	hsa-miR-1246	1.0	10.6	3.12E-05	4.31E-04		
	Up	hsa-miR-486-3p	0.9	8.1	7.30E-11	1.75E-08		
	Up	hsa-miR-22	0.8	8.0	1.07E-03	7.36E-03	exosome	
	Up	hsa-miR-191	0.8	7.5	5.11E-06	1.04E-04	exosome	
	Up	hsa-miR-3622b-5p	0.7	7.6	1.49E-03	9.54E-03		
	Up	hsa-miR-658	0.7	7.6	4.34E-10	5.21E-08	exosome	
	Up	hsa-miR-4258	0.6	8.3	3.39E-05	4.58E-04		
	Up	hsa-miR-1915*	0.6	7.5	3.79E-06	8.93E-05		
	Up	hsa-miR-24	0.6	8.5	6.50E-04	4.97E-03	exosome	HBsAg
	Up	hsa-miR-3665	0.6	14.1	3.08E-05	4.30E-04		
	Up	hsa-miR-550a	0.6	7.1	7.37E-14	8.84E-11		
	Up	hsa-miR-663b	0.6	9.3	4.31E-05	5.56E-04		
	Up	hsa-miR-3663-3p	0.6	10.5	2.75E-09	2.09E-07		
	Up	hsa-miR-320b	0.5	7.1	5.71E-07	1.90E-05		
	Up	hsa-miR-762	0.5	13.9	1.21E-05	2.02E-04		
	Up	hsa-miR-320c	0.5	7.0	1.50E-06	4.10E-05		
	Up	hsa-miR-3917	0.5	7.7	7.78E-04	5.66E-03		
	Up	hsa-miR-135a*	0.5	8.4	2.13E-04	2.09E-03	exosome	HBsAg
	Up	hsa-miR-663	0.5	10.7	1.66E-03	1.04E-02	exosome	
	Up	hsa-miR-3934	0.5	7.3	3.00E-07	1.09E-05		
	Up	hsa-miR-320a	0.5	7.1	1.58E-06	4.21E-05		
	Down	hsa-miR-451	−1.5	11.3	9.61E-06	1.72E-04	exosome	
	Down	hsa-miR-223	−1.0	8.0	7.28E-05	8.56E-04	exosome	HBsAg
	Down	hsa-miR-16	−0.8	8.0	1.39E-03	9.03E-03	exosome	
	Down	hsa-miR-4294	−0.8	11.3	7.84E-07	2.30E-05		

(*continued on next page*)

Table 3 (*continued*)

Contrast	Direction	miRNA	logFC	AveExpr	P	P_{FDR}	Exosome	HBsAg
	Down	hsa-miR-575	−0.8	7.9	1.40E-06	3.89E-05	exosome	
	Down	hsa-miR-92a-2*	−0.8	10.5	9.47E-06	1.72E-04		
	Down	hsa-miR-1202	−0.6	8.3	2.12E-08	1.16E-06		
	Down	hsa-miR-1268	−0.6	11.9	1.99E-09	1.71E-07		
	Down	hsa-miR-1275	−0.5	9.1	4.35E-06	9.41E-05		
	Down	hsa-miR-17	−0.5	6.8	1.38E-05	2.24E-04	exosome	HBsAg
	Down	hsa-miR-20a	−0.5	6.7	2.58E-05	3.83E-04	exosome	
HBeAg(+) vs HBeAg(−)	Up	hsa-miR-122	2.8	8.3	1.57E-07	1.50E-04	exosome	HBsAg
	Up	hsa-miR-194	0.7	6.5	2.49E-07	1.50E-04	exosome	
	Up	hsa-miR-4286	0.6	7.3	3.97E-04	3.17E-02		
	Up	hsa-miR-30d	0.6	7.4	8.35E-06	2.01E-03	exosome	
	Up	hsa-miR-125b	0.5	6.7	1.07E-05	2.14E-03	exosome	
	Up	hsa-miR-99a	0.5	6.6	2.00E-04	1.85E-02	exosome	
	Up	hsa-miR-100	0.5	6.5	1.75E-04	1.75E-02	exosome	
	Up	hsa-miR-192	0.4	6.8	4.52E-05	6.23E-03	exosome	
	Up	hsa-miR-378	0.4	6.6	2.20E-06	6.61E-04	exosome	
	Up	hsa-miR-30a	0.3	6.5	8.66E-05	9.45E-03	exosome	HBsAg
	Up	hsa-miR-422a	0.3	6.5	1.50E-06	6.00E-04	exosome	
	Up	hsa-miR-30c	0.3	6.6	7.59E-05	9.11E-03	exosome	HBsAg
	Up	hsa-miR-378c	0.3	6.4	2.61E-04	2.23E-02		
	Up	hsa-miR-30b	0.2	6.5	4.67E-05	6.23E-03	exosome	HBsAg
	Up	hsa-miR-361-5p	0.2	6.4	3.11E-05	5.33E-03	exosome	

was used to renormalize miR-99a, miR-125b, miR-122, miR-720, and miR-22 qRT-PCR expression data. *P*-values using renormalized data decreased by approximately one order of magnitude but remained highly significant and did not affect any conclusions (data not shown).

Association between microRNA level and clinical factors in patients with chronic HBV

Multiple regression was used to identify associations among microRNA levels and clinical factors in HBV patients using

Table 4 Quantitative RT-PCR results of selected microRNAs in serum of chronic HBV or HCV patients and healthy controls and between HBeAg-positive and negative patients. Expression levels are shown as median and range and compared using the Mann–Whitney *U* test.

microRNA	Healthy (n = 22)	HBV (n = 185)	logFC	P	P_{FDR}
hsa-miR-122/cel-miR-238	0.021 (0.013–0.04)	0.204 (0.011–2.495)	3.31	1.54E-13	1.08E-12
hsa-miR-99a/cel-miR-238	0.014 (0.005–0.051)	0.132 (0.008–2.436)	3.24	3.64E-12	8.50E-12
hsa-miR-125b/cel-miR-238	0.023 (0.007–0.05)	0.146 (0.007–3.084)	2.70	3.36E-12	8.50E-12
hsa-miR-720/cel-miR-238	0.043 (0.024–0.123)	0.146 (0.035–3.732)	1.76	4.66E-11	8.15E-11
hsa-miR-22/cel-miR-238	0.226 (0.107–0.485)	0.335 (0.096–1.305)	0.57	4.69E-04	6.57E-04
hsa-miR-1275/cel-miR-238	0.405 (0.237–0.604)	0.517 (0.099–1.626)	0.35	4.90E-03	5.71E-03
microRNA	**Healthy (n = 10)**	**HCV (n = 107)**	**logFC**	**P**	**P_{FDR}**
hsa-miR-720/cel-miR-238	0.388 (0.232–0.749)	0.653 (0.198–1.731)	0.75	2.51E-03	7.53E-03
hsa-miR-122/cel-miR-238	0.671 (0.307–0.95)	1.096 (0.1–8.542)	0.71	1.78E-02	2.68E-02
hsa-miR-1246/cel-miR-238	2.893 (1.821–6.813)	4.360 (0.429–36.311)	0.59	7.28E-02	7.28E-02
microRNA	**HBeAg-negative (n = 82)**	**HBeAg-positive (n = 103)**	**logFC**	**P**	**P_{FDR}**
hsa-miR-99a/cel-miR-238	0.070 (0.009–0.585)	0.250 (0.008–2.436)	1.84	4.55E-11	1.59E-10
hsa-miR-125b/cel-miR-238	0.100 (0.007–0.507)	0.253 (0.012–3.084)	1.34	7.70E-10	1.80E-09
hsa-miR-122/cel-miR-238	0.143 (0.011–0.678)	0.337 (0.017–2.495)	1.24	8.60E-12	6.02E-11
hsa-miR-720/cel-miR-238	0.119 (0.035–0.517)	0.185 (0.040–3.732)	0.64	4.24E-06	7.42E-06
hsa-miR-22/cel-miR-238	0.302 (0.096–1.305)	0.391 (0.103–1.049)	0.37	2.36E-04	3.30E-04
hsa-miR-1275/cel-miR-238	0.494 (0.099–1.626)	0.541 (0.186–1.376)	0.13	1.07E-01	1.25E-01

logFC: log2 fold-change; P: uncorrected *P*-value for Mann-Whitney *U* test; P_{FDR}: *P*-value adjusted for multiple testing based on the false discovery rate.