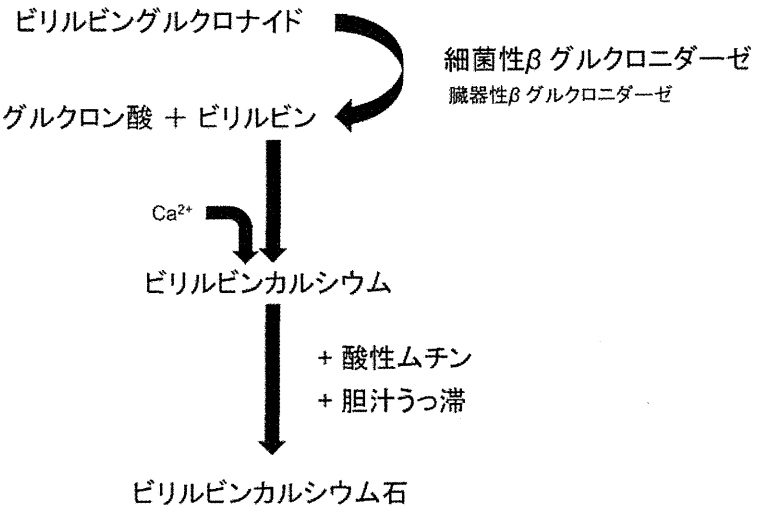

図5　ビリルビンカルシウム石の生成機序

養，迷走神経切離術後，脊髄損傷，ホルモン療法など）は，胆石形成のリスクファクターである．胆嚢収縮機能の減弱の原因に関しては，消化管ホルモンであるコレシストキニンに対する胆嚢平滑筋の収縮反応の低下が推測されている．

胆嚢収縮機能の低下は，循環胆汁酸プールの減少，腸肝循環サイクルの亢進による一次胆汁酸であるコール酸から二次胆汁酸であるデオキシコール酸の増加を引き起こし，肝から胆汁へのコレステロールの過剰分泌を誘発する．

(6) 腸管運動機能

胆石症患者では健常者に比較して，食物の小腸および大腸通過時間が遅延している．胆嚢収縮機能の低下に加えて食物の腸管通過時間の遅延は，腸内細菌叢の変化や小腸における胆汁酸吸収の低下に関連した胆汁酸組成の変化（二次胆汁酸であるデオキシコール酸の増加）を引き起こし，肝から胆汁へのコレステロールの過剰分泌を誘発する．また，循環胆汁酸プールの減少によると考えられる胆汁脂質組成（コレステロール飽和度の増加）の変化を引き起こすと考えられ，コレステロール胆石の形成のリスクとなる．

色素胆石

ビリルビンカルシウム石

ビリルビンカルシウム石の主成分は bilirubin と Ca が結合したビリルビンカルシウムである．胆汁中にビリルビンカルシウムが析出するためには非抱合型ビリルビンの carboxyl 基に Ca が結合し不溶性の色素となる必要がある．通常，胆汁中のビリルビンの多くはグルクロン酸抱合をうけビリルビングルクロナイドとして存在している．このビリルビングルクロナイドをビリルビンとグルクロン酸に加水分解する酵素として β-glucuronidase があり，細菌由来のものと臓器由来のものが存在する．

ビリルビンカルシウム石の胆汁中の β-glucuronidase 酵素活性を調査したところ，大腸菌由来の酵素とほぼ同一の特性を有していたことから胆汁中の β-glucuronidase は細菌由来と考えられている[9]．大腸菌のみならず多数の細菌が β-glucuronidase 活性を有していることから，胆道感染がビリルビンカルシウム石の主な原因であることには異論がない．一方，胆汁中の多核白血球も β-glucuronidase 活性を有することが報告されており[10]炎症との関連が示唆される．

このような過程で析出したビリルビンカルシウムは胆汁中に豊富に存在している酸性ムチンの架橋作用によって凝集し，強固な結石様凝塊が形成され，これが徐々に成長してビリルビンカルシウム石になると考えられている[11]（図5）．

黒色石

黒色石は感染を伴わない胆嚢に形成される胆石であり，欧米における胆嚢色素石の大部分は黒色石である．最近ではわが国においても，黒色石の症例数と割合が増加してきている[12][13]．黒色石の主成分である黒色色素の本態はビリルビン誘導体の重合体やビリルビン金属

表2　無症状胆石の転帰に関するエビデンス

報告者（年度）	対象	症例数	追跡期間（年）	急性胆嚢炎	重篤な合併症（%）			
					顕著な黄疸	胆管炎	膵炎	胆嚢癌
Comfort（1948）	無症状胆石	112	15	0	0	0	0	0
Lund（1960）	無症状胆石	95	13	?	?	1	0	0
Gracle（1982）	無症状胆石	123	11	2	0	0	1	0
McSherry（1987）	無症状胆石	135	5	3	0	0	0	0
Friedman（1989）	無症状胆石	123	7	4	2	2	0	0
Thistle（1984）	無症状胆石 有症状胆石	305	2	3	0	0	0	0

表3　胆石と胆嚢がん発生の関連に関するエビデンス

報告年	報告者	study design	対象と症例数	追跡期間	発癌の relative risk (95%CI)
1987	Maringhini	cohort study	胆嚢結石 2,583 例	20 年間	2.8（0.9 ～ 6.6） 男性 8.3（1.0 ～ 30.0） 女性 2.0（0.4 ～ 5.7）
1999	Chow	cohort study	胆嚢結石温存 17,715 例， 胆摘後 42,461 例（計 60,176 例）	4 ～ 16 年間	3.6（2.6 ～ 4.9）
2004	Yagyu	cohort study	113,394 例	11 年間	男性 1.2（0.3 ～ 4.7） 女性 1.1（0.4 ～ 2.9）
1985	Lowenfels	case-control study	胆嚢癌 131 例と非胆嚢癌 2,399 例		non-Indians 4.4（2.6 ～ 7.3） Indians 20.9（8.1 ～ 54.0）
1988	Nervi	case-control study	14,768 例の剖検例		7.0（5.9 ～ 8.3）
1989	WHO	case-control study	胆嚢癌 58 例と非胆嚢癌 355 例		2.3（1.2 ～ 4.4）
1989	Kato	case-control study	109 例の胆嚢癌，84 例の胆管癌， 386 例のコントロール		34.4（4.51 ～ 266.0）
1997	Zatonski	case-control study	胆嚢癌 196 例と非胆嚢癌 1,515 例		4.4（2.6 ～ 7.5）
1999	Okamoto	case-control study	194,767 例のコントロール， 胆嚢結石 7,985 例（4.1%）		10.8（4.1 ～ 28.4）
1999	Khan	case-control study	胆道癌 69 例，コントロール 138 例		26.6（7.0 ～ 101.4） 女性 28.6（4.7 ～ 173.0）

錯体であることは明らかにされているが，その成因はほとんど解明されていない．

黒色石の形成機序は，胆汁中の pH がアルカリ性を示す環境下において容易に非抱合型ビリルビンが析出する．最初にイオン化カルシウムの存在下でビリルビンカルシウムが形成されて，次いでビリルビンの重合が生じることで黒色色素が形成される．さらに，金属元素と複合体となり，黒色石が形成される．

溶血性貧血，肝硬変症，心臓弁置換術後などの患者では高頻度に胆嚢結石が認められるが，その殆どは黒色石である．これには，溶血によるビリルビンの過剰供給に伴い胆汁中非抱合型ビリルビンが増加するためと推測されている[14]．

胆石の自然史

日常臨床において無症候性胆石は経過観察で良いかどうか，方針に迷う場面は少なからず存在する．そこで，過去に行われたエビデンスレベルの高い臨床研究を参照しつつ，無症候性胆石の自然史（転帰）について検討した．無症候性胆石の転帰に関するエビデンスについて代表的な論文を表 2[15)~20)]に示した．表 2 のいずれの報告についても，無症候性胆石の経過観察の期間における重篤な合併症の頻度は数パーセントと非常に低く，また，胆嚢がんの発生を認めた症例は存在しなかったことが示されている．本邦における無症候性胆石からの胆嚢がんの発症は 2-3 例（0-0.5%）であったと報告されている[21)~24)]．このように国内外からの報告を合わせると，無症候性胆石保有者における胆嚢がんの

発生は稀であると解釈できる．

胆石と胆嚢がん

Randiらは胆嚢がんの危険因子に関するmeta-analysisの結果（表3）を報告し[25]，主要な危険因子として胆石を掲げている．胆嚢がんの発生に至る経路は広範囲にわたるが，主たる経路は胆石形成とその結果生じる胆嚢炎であるとしている．さらに本発がん経路は男性よりは女性において作用すると考えられている．本邦における胆嚢がんの疫学調査では，国立がんセンターが施行した日本人の大規模住民集団の前向き追跡研究である「多目的コホート研究（Japan Public Health Center-Based Prospective Study）」における報告が利用出来る[26]．胆石の既往のあるグループでは，既往のないグループに比較して，胆嚢がんのリスクが3.1倍，肝外胆管がんのリスクが2.1倍であった．これらの解析結果を考慮すると，胆石の保有と胆嚢がんの発生には関連性があることは考慮するべきである．一般人口に対して種々の因子（年齢，性別など）をmatchingさせた解析ではあるが，胆嚢がんの発生頻度が低いこと，また，解析された相対危険度も10倍以下の報告が殆どであることより，胆石の保有と胆嚢がんの発生には関連性はあるが，強い因果関係が存在するとの結論に至ることは出来ないと考える．

胆嚢がんの疫学，無症候性胆石の転帰に関するエビデンスレベルの高い臨床研究を参照しながら，「胆道癌診療ガイドライン」[27]と「胆石症診療ガイドライン」[8]の2つの診療ガイドラインでは，無症候性胆石における胆嚢がん発生の対応手順が示されている．いずれのガイドラインにおいても，無症候性胆石に対して胆嚢摘出術を勧める根拠は不十分であるとして，胆嚢がん発生に対応するための予防的胆嚢摘出術は推奨されない．無症候性胆は十分胆嚢を評価できる状況下では肝機能障害の発生，胆嚢がん合併の可能性を考慮した検査をしながら経過観察することが推奨されている．

おわりに

胆石の種類と成因について概説した．コレステロール胆石は，生活習慣病との関連性を有する結石症である．胆石形成の背景には，単なる胆嚢のイベントではなく，肝臓と腸管における脂質代謝が深く関与する．これまでに蓄積されてきたエビデンスに基づき，今後，予防を視野に入れた病態研究のさらなる発展が望まれる．また，日常臨床においては，診療ガイドラインの対応手順に従い，無症候性結石の自然史（転帰），胆石の保有と胆嚢がんの発生のリスクを考慮しながら，胆石症への診療が行われることを希望する．

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Classification and Pathogenesis of Gallstones

Junichi Shoda

A gallstone is defined as a concrement present in the biliary tract consisting of the gallbladder and bile duct. The etiological process of gallstones is different in terms of the location and composition. Gallstones are classified as cholesterol, pigment, and rare gallstones according to the main component (Japanese Gastroenterological Society, Committee for the Study of Cholelithiasis, 1986). The prevalence and category of gallstones in Japan are approaching those of the West; the proportion of cholesterol stones increased up to 70% of the gallbladder stones, and that of black pigment stones in the gallbladder also increased. While gallstone pathogenesis differs with respect to stone category, the process of stone formation consists of hypersecretion of main components in gallstones into bile, the crystallization and precipitation of the components in the bile, and then the rapid growth of the precipitated crystals. The knowledge of biliary physiology, e.g., formation, secretion and concentration of bile, is necessary for a better understanding of gallstone pathogenesis. This paper reviews the issue of classification and pathogenesis of gallstones in reference to the clinical practice guidelines for gallstone diseases published in 2009 (Japanese Gastroenterological Society).

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Key Words: gallstone, pathogenesis, pathophysiology, bile composition, clinical practice guidelines

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肝内結石症診療の現況

肝内結石症からの胆道発癌—分子生物学的立場から

正田 純一[1)]

要約：肝内胆管癌は肝内結石症の重要な予後規定因子である。難治性疾患克服事業「肝内結石症に関する調査研究班」における全国疫学調査では，本症の4.0～8.8%に肝内胆管癌が合併すると報告されている。疫学調査の結果より，肝内胆管癌のリスク因子として，「胆道再建の既往」，「肝萎縮」，「切石のみの治療」が抽出された。これらに関連して，多くの基礎および臨床研究より，本症における発癌メカニズムには，胆道上皮における慢性持続性炎症が関与することが以前より指摘されてきた。本稿では肝内胆管癌について，疫学よりみた発癌のリスクファクター，それらの発癌にかかわる分子メカニズムについて概説する。

Key words：肝内結石症，肝内胆管癌，リスクファクター，分子メカニズム

はじめに

肝内結石症は厚生労働省が指定する難治性疾患の一つである。難治性疾患克服事業調査研究班の全国疫学調査により，本症では肝内胆管癌の合併が予後因子として重要であることが判明している[1)]。調査の結果より本症の4.0～8.8%に肝内胆管癌が合併すると報告されている[2)]。肝内胆管癌はその発生と進展環境の複雑性，また，それに関連すると考えられる癌進展様式の多様性を示すことより，早期発見が難しく，早期癌の段階で外科切除できる例は極めてまれである。現行の化学療法や放射線療法に対して抵抗性を示すことが多く，完全治癒の期待できない難治性の癌と位置付けられている。肝内胆管癌の発症には，本症における慢性増殖性胆管炎[3)]，原発性硬化性胆管炎[4)]，炎症性腸疾患に付随する慢性胆管炎[5)]などの慢性胆道炎の病態が関与することが，多くの基礎および臨床研究より証明されている。本稿では，発癌のリスクファクターとそれらの発癌にかかわる分子メカニズムについて概説する。

Molecular Mechanism for Biliary Carcinogenesis in Hepatolithiasis
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Ⅰ．肝内結石症における肝内胆管癌のリスク

肝内結石症の全国疫学調査は現在までに計6回にわたり施行されている。最近行われた第6次調査では胆管癌の合併は5.5%（18/336例）に認められた[6)]。調査項目についてロジスティック回帰解析を施行したところ，有意な胆管癌合併因子として「胆道再建の既往（オッズ比3.8）」と「肝萎縮（オッズ比4.5）」の2項目が抽出された[7)]。これらの結果より胆道発癌の背景病態には，胆管-消化管吻合による反復性の胆管炎の存在，胆管炎による門脈循環障害の存在などが重要であると推測される。また，第5次調査においては，登録症例のコホート調査が行われ，コックス回帰分析の結果より，胆道癌の発症に影響を及ぼした因子として，「切石のみの治療（オッズ比2.5）」が抽出された[7)]。切石のみの治療では，慢性持続性の炎症や胆汁うっ滞の病態が改善されず，持続性胆道上皮の障害は，その後の再生，修復機転の異常と相まって，肝内胆管癌の発生に関与すると推測されている。

Ⅱ．肝内結石症における胆道発癌の分子メカニズム

肝内結石症の背景病態には慢性炎症や胆管閉塞に関連した胆汁うっ滞（胆汁酸などの胆汁成分が関与）に

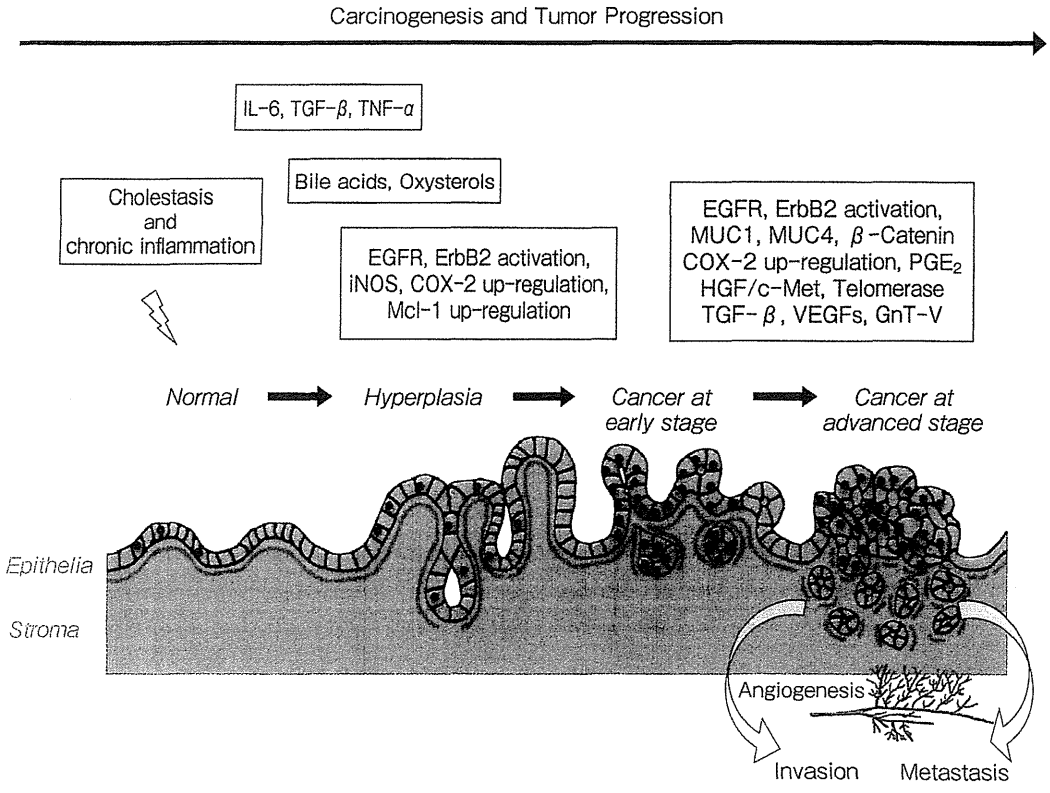

図 1 胆道上皮からの発癌メカニズムの概要（文献 10 より引用，一部改変）

よる慢性持続性に胆管上皮の障害（慢性増殖性胆管炎）が存在する[8]。本病態下では，炎症関連分子の発現異常に加えて，胆管上皮の再生，修復，増殖性変化が発生しており，胆管上皮の修復機転の異常，癌関連遺伝子の異常が生じる結果，胆管癌の発生に至ることが推測されている[9]。

胆道発癌に関与する形質発現の獲得には，主役である癌細胞のみではなく，脇役である宿主細胞の重要性が指摘されている。すなわち，炎症病態においては，宿主細胞や細胞外マトリックスよりなる微小環境において，増殖因子，サイトカイン，プロスタグランジンなどの生理活性物質が産生され，さらに胆汁中胆汁酸による慢性的な暴露により，これらの微小環境因子の変化が，癌遺伝子の活性化，癌抑制遺伝子の変異とそれによる細胞シグナル伝達の異常をきたし，アポトーシスからの回避を引き起こすことで癌細胞の発現を誘導すると考えられている（図 1）[10]。

胆道発癌に関与する腫瘍生物分子のうち重要な分子には，炎症性サイトカインである tumor necrosis factor-alpha（TNF-α），inducible nitric oxide synthetase（iNOS），cyclooxygenase-2（COX-2），epidermal growth factor（EGF）受容体，erbB2 受容体，myeloid cell leukemia protein 1（Mcl-1）などとそれら分子間の相互関係が重要であると報告されている[11~15]。炎症病態において発生した炎症性サイトカインは iNOS を発現誘導し，胆道系には過剰な NO が産生される結果，酸化ストレスによる胆管上皮の障害が生じる。DNA damage の増加や DNA repair の抑制により遺伝子変異が促進される[16]。また，誘導された iNOS や胆汁中の酸化ステロールである oxysterols は COX-2 を誘導し，その結果，アラキドン酸代謝の活性化によるプロスタグランジン E2（PGE2）の産生の増加が生じ，癌細胞の増殖が加速する。癌細胞の生存期間も延長する。われわれの解析[17]により，肝内結石症の胆管上皮および付属腺上皮には，正常胆管上皮に比較して強い COX-2 発現が観察され（図 2），また，プロスタグランジン受容体（EP2-EP4）も高密度に発現している（図 3）。PGE2 は EP2-EP4 を介して，細胞増殖を亢進することも判明している（図 4）。さらに，胆汁酸は EGF 受容体の transactivation を引き起こし，COX-2 の誘導や Mcl-1（抗アポトーシス蛋白）の分解を阻止することでアポトーシスを抑制する。erbB2 受容体は beta-catenin，mucin 1（MUC1），MUC4[18] の分子と会合することにより活性化する[19]。これらの細胞内イベントが蓄積していく結果，胆道発癌が促進するものと考えられている。

とくに胆道系の慢性炎症病態に関連して，炎症性サイトカインのうち IL-6 が胆管癌の発癌と進展機構に

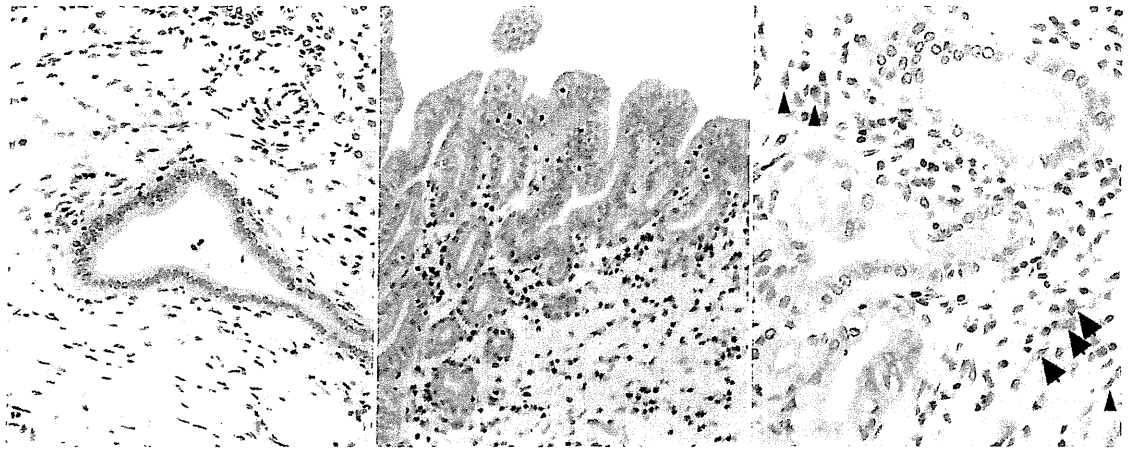

図 2 COX-2 の免疫染色（文献 17 より引用，一部改変）
a：正常胆管上皮（対照肝）
b：過形成胆管上皮（肝内結石症）
c：増殖する壁内胆管腺（肝内結石症），►は COX-2 を発現する線維芽細胞

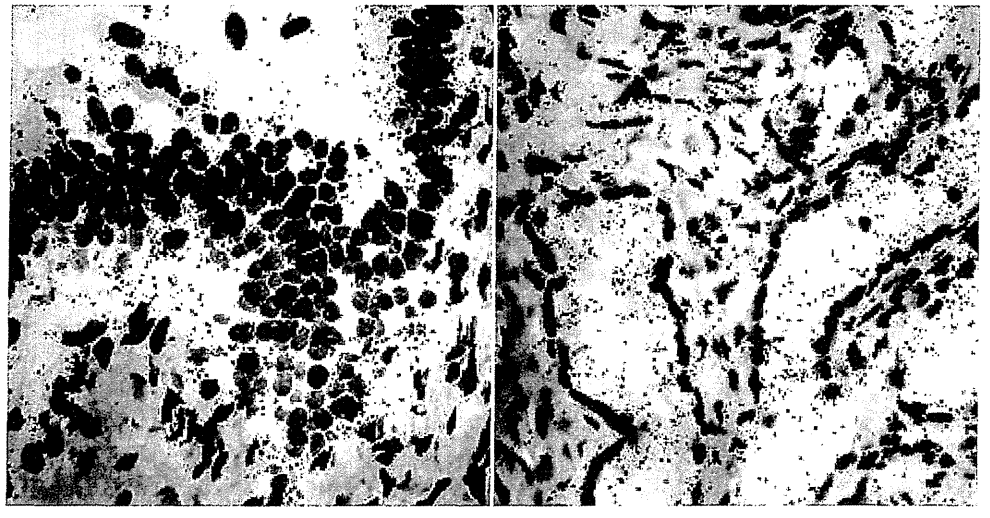

図 3 EP4 の in situ hybridization（文献 17 より引用，一部改変）
a：過形成胆管上皮（肝内結石症）
b：増殖する壁内胆管腺（肝内結石症）

深く関連することが報告されている[20,21]。IL-6 は癌周囲の間質細胞のみならず腫瘍細胞そのものからも産生される。すなわち，IL-6 は細胞膜表面の受容体に結合し，JAK/STAT，PI3K/Akt，MAPK のシグンル経路を活性化する（図 5）。JAK/STAT 経路では転写因子 STAT3 が核内移行し，抗アポトーシス分子である Mcl-1 を発現誘導する。また，同経路の活性化は増殖因子受容体である EGFR の発現レベルを増強する。PI3K/ Akt の活性化はアポトーシスを抑制し，細胞の成長を促進する。MAPK 経路の活性化は細胞周期関連の遺伝子発現を修飾し，細胞増殖を促進する[22]。また，IL-6 は DNA のメチルトランスフェラーゼの制御に関連し，発癌に関連する重要な遺伝子のメチル化に関与していることが報告されている[21]。

さらに，寄生虫関連肝内胆管癌はタイ東北部に蔓延する肝吸虫症がリスクファクターとして考えられている[23]。肝吸虫はニトロソアミンなどの発癌イニシエーターが加わった状態から，肝内胆管上皮に慢性炎症を引き起こすことにより胆管上皮の過形成が生じて発癌に至るとされている。川本ら[24]は，炎症性発癌に深く関与している COX-2 および COX-2/PGE2 pathway と link して活性化する EGFR，HER2，HER3（HER family）の発現が寄生虫関連肝内胆管癌の発生・進展にどのように関与しているかを検討した。COX-2 は正常胆管上皮，過形成上皮，癌の順に陽性率が増加した。HER family に関してはおのおの約 20～30％の陽性率を示したが，とくに EGFR の発現が有意に高かった。寄生虫関連肝内胆管癌においては，その発生・進

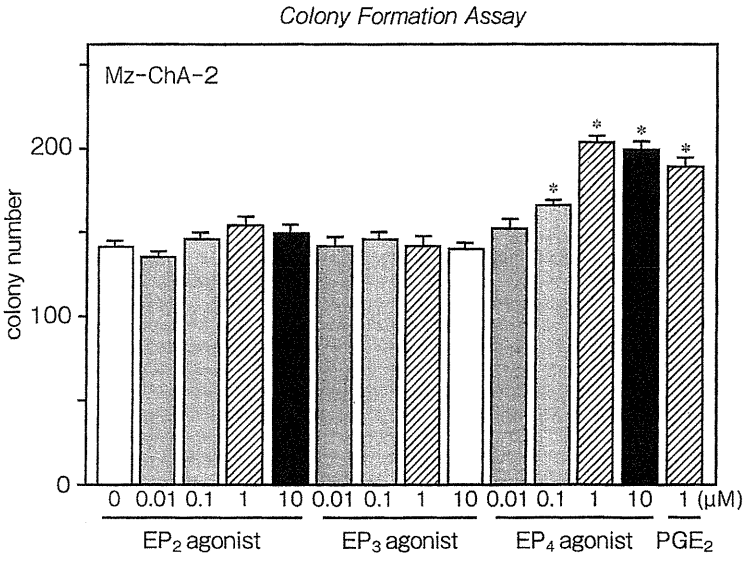

図 4　PGE2 および EP2, EP3, EP4 アゴニストの胆管上皮細胞に対する細胞増殖効果（文献 17 より引用，一部改変）

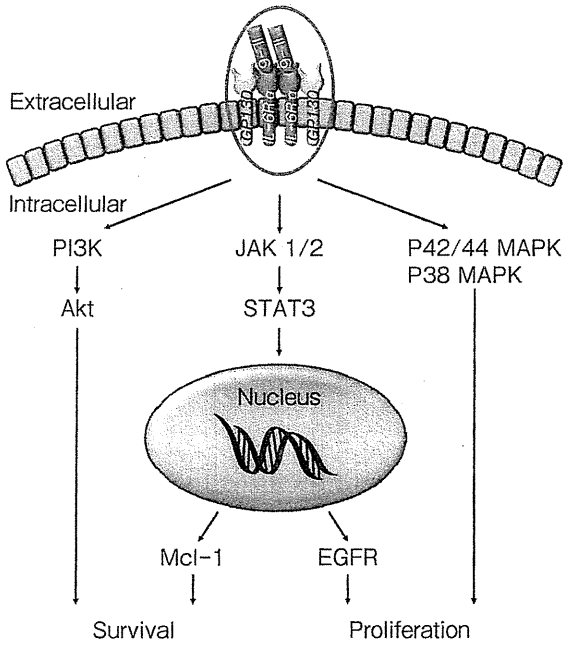

図 5　胆道癌における IL-6 とその細胞シグナル伝達経路（文献 22 より引用，一部改変）

展に COX-2-HER family pathway（図 6）が重要な役割を演じている可能性を強調している。すなわち，慢性炎症がトリガーとなって増加した COX-2 が arachidonic acid cascade を通じて PGE2 産生を亢進し，その PGE2 が EP1 受容体と結合することにより EGF や TGFα の産生，EGFR が誘導され，その結果，EGFR signal pathway が活性化する。EGFR signal pathway の下流にある PI3K/Akt pathway を介した癌細胞 apoptosis の抑制や癌細胞増殖の亢進などが誘導される。肝内結石症の慢性増殖性胆管炎からの発癌機序においても，前述した寄生虫関連肝内胆管癌と共通部分があると推測される。

肝内胆管癌の発症には，これらの腫瘍生物分子が過剰に発現し，それら分子間の相互関係が活発になることにより，胆道上皮の癌化が促進するものと考えられる。一方，これら分子に対する標的治療は，癌細胞の悪性挙動を抑制することにより癌の予防や進展の抑止に応用できる可能性がある。

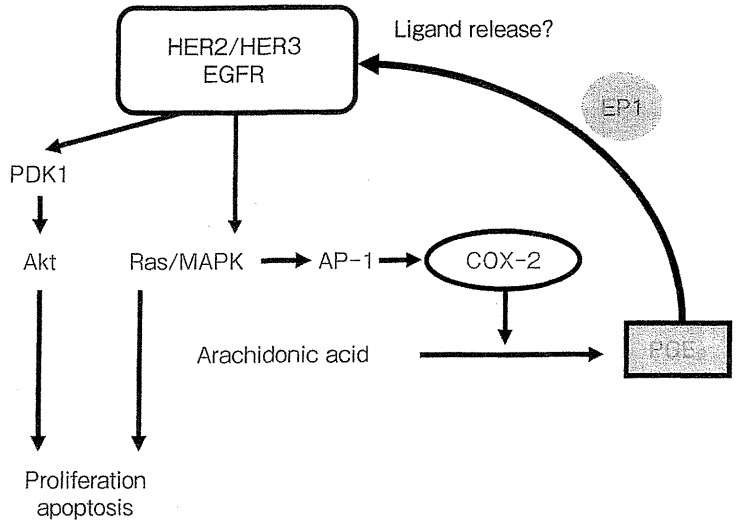

図 6　COX-2-HER2 family pathway とシグナル伝達系（文献 24 より引用，一部改変）

おわりに

肝内結石症に発生した肝内胆管癌は完全治癒の期待できない難治性の癌である。それらの治療成績の向上と生命予後の改善には，リスクファクターに関連する発癌の分子機構にかかわる腫瘍生物因子を同定し，それらの因子を用いた新しい有効な診断の開発，それらの因子を標的とする治療手段の開発が重要である。最近の研究成果では，それら胆道癌の細胞株，臨床標本，また，モデル動物における解析により，腫瘍生物学的因子の絞り込みがなされてきている。今後は，肝内胆管癌の腫瘍生物学的特色を捉えた新規診断法や治療法の登場が早急に望まれる。

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microRNA-122 Abundance in Hepatocellular Carcinoma and Non-Tumor Liver Tissue from Japanese Patients with Persistent HCV versus HBV Infection

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Abstract

Mechanisms of hepatic carcinogenesis in chronic hepatitis B and hepatitis C are incompletely defined but often assumed to be similar and related to immune-mediated inflammation. Despite this, several studies hint at differences in expression of miR-122, a liver-specific microRNA with tumor suppressor properties, in hepatocellular carcinoma (HCC) associated with hepatitis B virus (HBV) versus hepatitis C virus (HCV) infection. Differences in the expression of miR-122 in these cancers would be of interest, as miR-122 is an essential host factor for HCV but not HBV replication. To determine whether the abundance of miR-122 in cancer tissue is influenced by the nature of the underlying virus infection, we measured miR-122 by qRT-PCR in paired tumor and non-tumor tissues from cohorts of HBV- and HCV-infected Japanese patients. miR-122 abundance was significantly reduced from normal in HBV-associated HCC, but not in liver cancer associated with HCV infection. This difference was independent of the degree of differentiation of the liver cancer. Surprisingly, we also found significant differences in miR-122 expression in non-tumor tissue, with miR-122 abundance reduced from normal in HCV- but not HBV-infected liver. Similar differences were observed in HCV- vs. HBV-infected chimpanzees. Among HCV-infected Japanese subjects, reductions in miR-122 abundance in non-tumor tissue were associated with a single nucleotide polymorphism near the IL28B gene that predicts poor response to interferon-based therapy (TG vs. TT genotype at rs8099917), and correlated negatively with the abundance of multiple interferon-stimulated gene transcripts. Reduced levels of miR-122 in chronic hepatitis C thus appear to be associated with endogenous interferon responses to the virus, while differences in miR-122 expression in HCV- versus HBV-associated HCC likely reflect virus-specific mechanisms contributing to carcinogenesis. The continued expression of miR-122 in HCV-associated HCC may signify an important role for HCV replication late in the progression to malignancy.

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Introduction

Globally, liver cancer is the fifth and seventh most common malignancy in men and women, respectively, and the third most deadly [1]. Most (85-95%) of these cancers are hepatocellular carcinoma (HCC) [2], and many are associated with persistent intrahepatic infections with hepatitis C virus (HCV) or hepatitis B virus (HBV) [2,3]. Although the total cancer death rate decreased within the United States by over 1.5% between 2001-2007, deaths due to liver cancer increased by 50% among males and by 29% in females [4]. These changes in the incidence of HCC are largely due to increases in HCV-associated malignancy. Similarly, while HBV infection historically has been the major risk factor underlying development of HCC in Asia, in Japan it has been supplanted in recent decades by HCV infection [5].

The exact mechanisms underlying HCV- and HBV-associated malignancy are unknown [6,7]. Chronic infections with either virus may result in cirrhosis, which alone is a major risk factor for liver cancer [2]. However, there may also be

virus-specific mechanisms at work. While immune-mediated mechanisms are both necessary and sufficient for the development of HBV-related cancer in murine models, liver cancer arises in the absence of inflammation in HCV-transgenic mice [8,9]. Moreover, some HCV proteins may interact with host tumor suppressors and possibly impair cellular responses to DNA damage [10]. If virus-specific mechanisms of oncogenesis are important in the development of HCC, it is reasonable to anticipate that the pathways leading to HCV- and HBV-associated cancer might differ, possibly leaving distinguishing genetic or epigenetic marks in the tumors that arise. If so, understanding these differences would be important for biomarker discovery, and potentially design of preventative and therapeutic interventions.

Here, we describe a study that was aimed at determining whether the abundance of microRNA-122 (miR-122) is different in liver cancer arising in patients with chronic HCV infection compared to cancers arising in the context of chronic HBV infection. Mature microRNAs (miRNAs) are 20-23 nucleotides in length and encoded either by microRNA genes or from within conventional protein-coding genes. They act generally by binding to specific sites within the 3' untranslated region (3' UTR) of cellular mRNAs, to which they recruit RNA-induced silencing complexes (RISC) that repress translation and destabilize the mRNA [11–13]. miR-122 is a liver-specific miRNA that accounts for the majority of miRNAs in hepatocytes [14]. It regulates a large number of genes within the liver [15], and has several tumor suppressor-like properties [16,17]. Importantly, miR-122 is a crucial host factor for HCV replication, binding to the 5' untranslated RNA segment of the viral genome, physically stabilizing it, and promoting viral protein expression [18–20].

Because of its liver-specific nature and tumor suppressor-like qualities [16,17], it is of interest to know whether miR-122 expression is altered in liver cancers. Prior studies investigating miR-122 expression in liver cancers have produced conflicting results, particularly as related to the underlying viral causes of cancer. Two early studies suggest that miR-122 abundance is generally reduced in HCC [21,22]. However, Hou et al. [23] reported that miR-122 expression was maintained in both HBV- and HCV-associated cancer, while Varnholt et al. [24] reported that miR-122 levels were increased significantly in HCV-associated cancers when compared to non-cancerous tissue. Coulouarn et al. [25] reported higher miR-122 expression levels in HCV- versus HBV-associated cancers. To some extent, these conflicting results may reflect different patient populations, or possibly methodologic differences, not only in the measurement of miR-122 abundance but also in how miR-122 abundance was compared across tissue samples.

In an effort to resolve this controversy, we conducted a comprehensive analysis of miR-122 expression in liver cancers arising in a genetically homogenous group of Japanese patients. Using a highly accurate, miR-122-specific quantitative reverse-transcription, polymerase chain reaction (qRT-PCR) assay, and paying particular attention to how miR-122 measurements are compared between tissue samples, we show that miR-122 expression is significantly reduced in HBV-associated HCC but not in most HCV-associated cancers. We also demonstrate that miR-122 abundance is reduced in non-tumor HCV-infected liver in association with increased expression of interferon (IFN)-stimulated genes (ISGs).

Materials and Methods

Ethics statement

Liver tissue was obtained from Japanese patients undergoing surgical resection of liver cancer (primary or metastatic) at the Liver Center of Kanazawa University Hospital (Kanazawa, Japan). All subjects provided written informed consent for participation in the study, and tissue acquisition procedures were approved by the ethics committee of Kanazawa University under a protocol entitled "Gene expression analysis of peripheral blood cells and liver in patients with liver and gastrointestinal cancers". Archived liver tissue and serum samples were collected prior to December 15, 2011 from chimpanzees housed and cared for at the Southwest National Primate Research Center (SNPRC) of the Texas Biomedical Research Institute in accordance with the Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee. SNPRC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and operates in accordance with the NIH and U.S. Department of Agriculture guidelines and the Animal Welfare Act.

Human subjects and tissue samples

Paired samples of HCC and non-tumor liver tissue were obtained from Japanese patients undergoing surgical resection of HCC at the Liver Center of Kanazawa University Hospital (Kanazawa, Japan). Non-infected 'normal' liver tissue was similarly collected from patients undergoing resection of metastases of non-hepatic primary cancers. Patients were categorized as HCV-infected by the presence of HCV RNA (COBAS Ampli-Prep/COBAS TaqMan System) and absence of hepatitis B surface antigen (HBsAg) in serum or plasma at the time of surgery, while HBV infection was defined by the presence of HBsAg and absence of anti-HCV antibodies. HCC was categorized according to the degree of cellular differentiation, while fibrosis and inflammation in non-tumor tissue from HBV- and HCV-infected patients were compared after scoring each [26,27]. The IL28B genotype of study subjects with HCV infection was defined at the rs8099917 locus as described previously [28].

Chimpanzee care and sample collection

We studied archived liver tissue and serum samples collected prior to December 15, 2011 from chimpanzees housed and cared for at the Southwest National Primate Research Center (SNPRC) of the Texas Biomedical Research Institute. At the time samples were obtained, animals considered to be non-infected ('normal') were negative for HBV and HCV markers; HBV infection was defined as the presence of serum HBsAg, and HCV infection by the presence of HCV RNA detectable in sera by RT-PCR.

Small RNA quantitation in human samples

Human tissue samples were stored in liquid nitrogen until processed for RNA extraction. Approximately 1 mg of tissue was ground using a tissue homogenizer and total RNA isolated using the mirVana miRNA isolation kit (Ambion). Liver RNA samples were subsequently stored at -80°C or on dry ice during shipment. The quality of the isolated RNA (RIN score) was assessed using an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent Technologies) [29]. Quantification of miR-122, miR-191, Let-7a, miR-24, and the small nuclear RNA (snRNA) U6 was carried out by quantitative reverse-transcription, polymerase chain reaction (qRT-PCR) in a two-step process. RNA (12.5 ng) was reversed transcribed in a 10 µl reaction mix using reagents provided with the Universal cDNA Synthesis kit (Exiqon) and the manufacturer's recommended procedure. Quantitative PCR was carried out subsequently with the SYBR Green Master Mix Kit (Exiqon), mixed locked-nucleic acid primer sets specific for each miRNA or snRNA (Exiqon), and the CFX96 PCR System (Bio-Rad). Results are presented as relative copy number normalized to total RNA. Alternatively, absolute miR-122 copy numbers were estimated using serial dilutions of single-stranded synthetic miR-122 (Dharmacon) as a standard.

miR-122 and HCV RNA quantitation in chimpanzee samples

Total RNA was extracted from serum and liver using RNA Bee (Leedo Medical Labs, Houston, TX), chloroform extraction and isopropanol precipitation. Detection of miR-122 was performed using primers and probes for miR-122 included in the ABI TaqMan assay (Cat No. 4373151) and the ABI TaqMan microRNA Reverse Transcription Kit (Cat No. 4366596). The RT reaction was performed with 5 ng of total cell RNA, and the PCR amplification was performed with one-tenth of the resulting cDNA. The RT reaction was performed at 16°C for 30 min, followed by 42°C for 30 min, and 85°C for 5 min. The TaqMan Universal PCR Master Mix with no AmpErase UNG was used for PCR amplification with reaction conditions of 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. A standard curve was generated using a synthetic RNA equivalent to mature miR-122. HCV viral RNA levels in the serum and liver were determined using a real-time, quantitative RT-PCR (TaqMan) assay detecting sequences in the viral 5' noncoding RNA using an ABI 7500 sequence detector (PE Biosystems, Foster City, CA) as previously described [30]. Synthetic HCV RNA was used to generate a standard curve for determination of genome equivalents. The forward primer was from nucleotide 149 to 167 (5'-tgcggaaccggtgagtaca-3'), the reverse primer was from nucleotide 210 to 191 (5'-cgggtttatccaagaaagga-3') and the probe was from nucleotide 189 to 169 (5'-ccggtcgtcctggcaattccg-3') in the 5' NCR of HCV.

Affymetrix array analysis

Human RNA samples were subjected to high-density oligonucleotide microarray analysis as described previously [28]. In brief, cDNA amplified using the WT-Ovation Pico RNA Amplification System (NuGen, San Carlos, CA, USA) was used for fragmentation and biotin labeling with the FL-Ovation cDNA Biotin Module V2 (NuGen). Biotin-labeled cDNA suspended in hybridization cocktail (NuGen) was hybridized to Affymetrix U133 Plus 2.0 GeneChips, followed by labeling with streptavidin-phycoerythrin. Probe hybridization was determined using a GeneChip Scanner 3000 (Affymetrix) and analyzed using GeneChip Operating Software 1.4 (Affymetrix).

Statistical analysis

Statistical analyses were carried out using Prism V software (Graphpad Software, Inc). The paired t test was used for comparison of results arising from groups of paired tissue specimens (HCC versus non-tumor tissue), while the unpaired t test or Mann-Whitney test was used for comparisons between groups of unrelated tissues (e.g., HBV versus HCV infection). Nonparametric analysis of the correlation between miR-122 and ISG expression levels was done by the Spearman method. Other statistical tests were as described in the text.

Results

miR-122 abundance in HCV- versus HBV-associated liver cancer

We measured miR-122 abundance in paired tumor and non-tumor tissues collected from 26 patients undergoing surgical resection of HCC: 16 with concomitant chronic HCV infection, and 10 infected with HBV. The age, gender, histological classification of HCC, and fibrosis score of non-tumor tissues are shown in Figure 1 (see also Table 1). Subjects infected with HCV (predominantly genotype 1b) were approximately one decade older than those with HBV infection (66.6 ± 8.0 s.d. versus 54.3 ± 9.1 s.d. years, p=0.001, Figure 1A), consistent with previous studies indicating that HCC is generally diagnosed at an earlier age in HBV-infected Japanese patients [31]. There were no significant differences in the histological classification of HCC or scores for fibrosis or inflammatory activity in non-tumor tissues between the two groups (Figure 1B and C, and Table 1). There were more females among those with HCV infection (10 male and 6 female) than HBV (9 male and 1 female), but this difference did not achieve statistical significance (Chi square test with Yate's correction).

qRT-PCR revealed significant differences in the abundance of miR-122 in both tumor and non-tumor tissue samples when the HBV- and HCV-infected groups were compared (Figure 2). miR-122 abundance (miR-122 copy number per µg total RNA) was significantly lower in HCC tissue from HBV-infected versus HCV-infected subjects (p=0.009 by two-sided t test). In contrast, the miR-122 abundance in non-tumor tissue from HBV-infected patients was significantly greater than that in the HCV-infected patients (p=0.0005 by two-sided t test). The mean miR-122 abundance in HCC tissue was less than half that in non-tumor tissue in HBV-infected patients (p=0.003 by two-sided, paired t test). Strikingly, this relationship was reversed in the HCV-infected patients, in whom miR-122 abundance in HCC tissue was almost twice that in the non-tumor tissue (p=0.008 by two-sided paired t test). There was no significant difference in the abundance of miR-122 in the non-tumor tissue from HBV-infected patients and HCV-associated

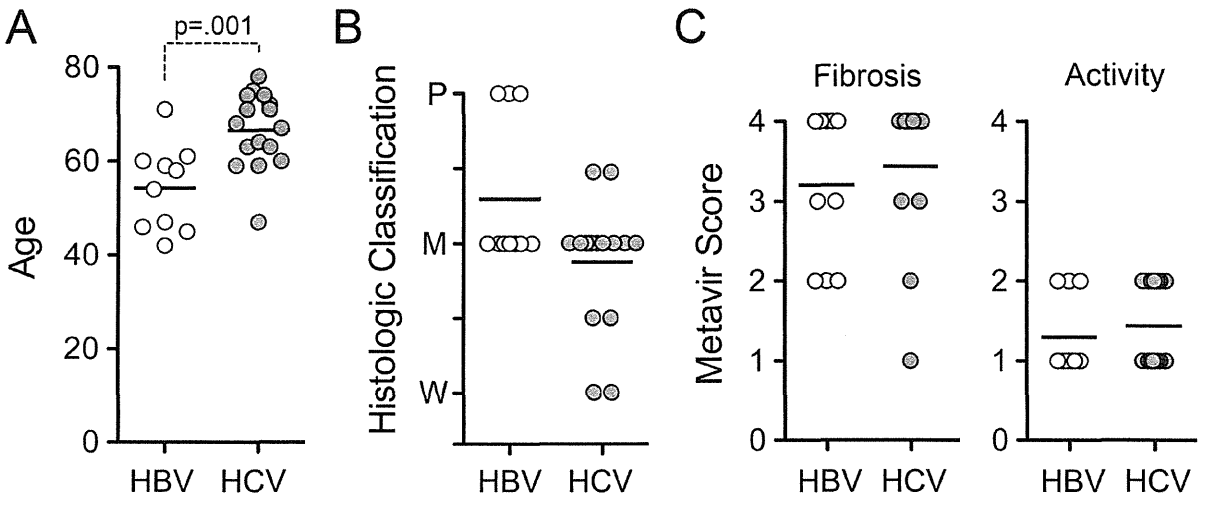

Figure 1. Age, histological classification of tumors, and scoring of non-tumor tissue for inflammation and fibrosis. (**A**) Age of subjects from whom HBV- and HCV-associated HCC and paired non-tumor samples were obtained. (**B**) Histological classification of tumors: W = well differentiated, M = moderately differentiated, P = poorly differentiated. (**C**) Individual scores for fibrosis and inflammatory activity in non-tumor tissue. Bars represent mean values. See also Table 1.

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HCC. miR-122 abundance varied quite widely in liver tissue collected from non-infected individuals undergoing resection of metastatic tumors. Despite this, miR-122 abundance was significantly less in HBV-associated cancer tissue and non-tumor HCV-infected tissue than in the non-infected tissues (p=0.016 and 0.013, respectively).

To account for potential differences in degradation of the RNA or efficiency of reverse transcription between tissue samples, we assessed the abundance of several other small RNAs against which we could normalize the abundance of miR-122. U6, a noncoding snRNA component of the spliceosome, is commonly used to normalize miRNA abundance. However, we observed substantial differences in U6 abundance in these tissues, suggesting that U6 would be a poor normalizer (Figure 3A). Substantially less variation was observed in the abundance of the miRNAs, miR-24 or Let-7a (Figure 3B and C), for which the standard deviation of the critical threshold [25] in the PCR assay was 0.79 and 1.27, respectively, compared to 1.34 for U6. Notably, we observed no difference in the abundance of Let-7a in HBV-associated cancer and non-tumor tissues (p=0.52 by two-sided, paired t test), despite a prior report suggesting that Let-7a expression is regulated by the HBx protein and increased in abundance in HBV-associated HCC [32]. In addition, although miR-24 negatively regulates the expression of hepatocyte nuclear factor 4-alpha (HNF4-alpha) and thus might be up-regulated in some liver cancers [33], we did not observe this. A strong positive correlation was evident between the abundance of miR-24 and Let-7a (Figure 3E, Spearman r_s=0.7959, p<0.001 by two-tailed t test), suggesting that these miRNAs might belong to a common regulatory network and that either could be used to normalize miR-122 abundance. In contrast, there was no correlation between miR-24 and either U6 or miR-122 abundance (Figure 3D and F), which indicates that U6 and miR-122 are regulated independently of miR-24. Importantly, when the miR-122 abundance was normalized to miR-24 levels, miR-122 expression remained significantly depressed in HBV-associated HCC when compared either with paired non-tumor tissue, or HCC tissue from HCV-infected subjects (p<0.001 and p=0.002, respectively, Figure 2B). In replicate assays, the abundance of miR-122 in non-tumor HCV-infected tissue also remained significantly lower than either non-infected or HBV-infected liver tissues (Figure 2B). Similar associations were found when miR-122 abundance was normalized to Let-7a (data not shown).

To assess further the possibility of bias in these results due to differences in the quality of the RNA samples, we compared the RNA integrity number (RIN) [29] of each sample with the abundance of each of the small RNAs detected. Interestingly, while the quantity of U6 snRNA detected correlated positively with the RIN score (Spearman r_s = 0.5216, two-tailed p = 0.0001) (Figure S1A in Supporting Information), this was not the case with miR-24 or Let-7a (r_s = -0.124 and -0.045, respectively). RIN scores also did not vary significantly between tumor and non-tumor tissue-derived RNA samples, or RNA from HBV- vs. HCV-infected tissue. Thus, although the quality of the RNA samples was generally high (mean RIN = 8.0 ± 0.17 s.e.m.), it was an important factor in determining the abundance of U6 but not either of these miRNAs. These data suggest that U6 is less stable than the miRNAs and provide additional support for the use of miR-24 (or Let-7a) as a standard against which to normalize miR-122 abundance (see Discussion). Nonetheless, when miR-122 results were normalized to U6 abundance, the correlations described above

Table 1. Characteristics of Study Subjects.

	HCV (n = 16)	HBV (n=10)	Non-infected (n=9)
Mean Age (years)	66.6 ± 8.0 s.d.	54.3 ± 9.1	60.1 ± 14.3
Gender (M/F)	10M/6F	9M / 1F	5M / 4F
HCV Genotype			
1a	0	n/a	n/a
1b	14		
2	2		
3	0		
Fibrosis Stage	n (%)	n (%)	n (%)
0	0 (0)	0 (0)	9 (100)
1	1 (6)	0 (0)	0 (0)
2	1 (6)	3 (30)	0 (0)
3	4 (25)	2 (20)	0 (0)
4	10 (63)	5 (50)	0 (0)
Inflammation	n (%)	n (%)	n (%)
0	0 (0)	0 (0)	9 (100)
1	9 (56)	7 (70)	0 (0)
2	7 (44)	3 (30)	0 (0)
3	0 (0)	0 (0)	0 (0)
4	0 (0)	0 (0)	0 (0)
HCC Histologic Differentiation	n (%)	n (%)	
Well	2 (13)	0 (0)	n/a
Moderate-Well	2 (13)	0 (0)	
Moderate	10 (63)	7 (30)	
Poor-Moderate	2 (13)	0	
Poor	0 (0)	3 (30)	
IL28B genotype (rs8099917)	n (%)		
TT	9 (56)	n.d.	n.d.
TG	7 (44)		
GG	0 (0)		

n/a = "not applicable"; n.d. = "not done"

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between miR-122 abundance, in both tumor and non-tumor tissues, and the type of virus infection remained strongly statistically significant (Figure S1B in Supporting Information). The mean miR-122 abundance was substantially lower in HBV-associated HCC tissue than in HBV-infected non-tumor tissue ($p = 0.003$ by paired t-test), while this relationship was reversed in HCV-infected liver ($p = 0.001$). miR-122 abundance was also significantly lower in non-tumor tissue from HCV-infected subjects than HBV-infected subjects ($p < 0.001$).

To exclude the possibility of bias due to the trend toward a less differentiated histologic classification among HBV-associated cancers (Figure 1B), we limited the comparison of miR-122 abundance to those HCC tissues that were scored as moderately differentiated and their corresponding paired non-tumor samples. While this reduced the number of subjects available for analysis, miR-122 abundance remained significantly lower in HBV- versus HCV-associated cancer tissue: $p=0.007$ when compared on the basis of miR-122 copy number/mg RNA (Figure 2C) vs. $p=0.033$ when normalized to miR-24 (Figure 2D). Thus differences in miR-122 abundance in HCC associated with HBV vs. HCV infection are independent of the degree of histologic differentiation of the cancer.

Collectively, these results provide strong evidence that miR-122 expression is reduced in HCC associated with HBV infection, but not in most HCV-associated liver cancers.

Reduced miR-122 abundance is associated with interferon responses in HCV-infected liver

The data shown in Figure 2 indicate that miR-122 is frequently reduced in abundance in non-tumor, HCV-infected liver tissue, but not in liver infected with HBV. To determine whether similar HCV-induced suppression of miR-122 expression occurs in chimpanzees (*Pan troglodytes*), the only animal species other than humans that is permissive for HCV infection, we measured miR-122 abundance in liver tissues

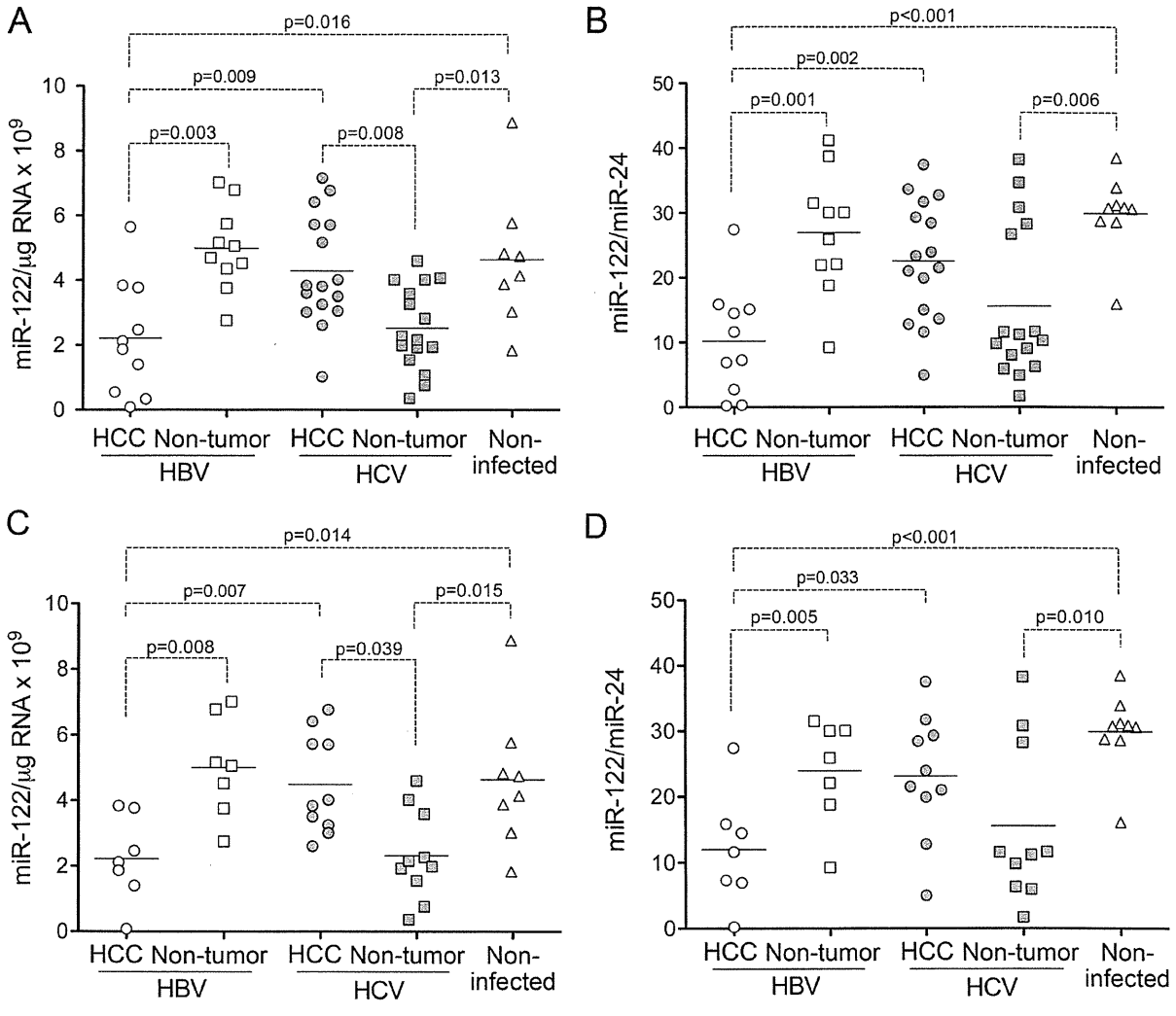

Figure 2. miR-122 expression in paired HCC and non-tumor liver tissue from patients with chronic HBV and HCV infection and control, non-infected liver tissue. (**A**) miR-122 abundance quantified by qRT-PCR in paired tumor and non-tumor tissues and non-infected ('normal') liver from patients undergoing resection of metastatic tumors, normalized to total RNA. (**B**) Relative miR-122 abundance normalized to miR-24 abundance in the same tissues. (**C**) miR-122 abundance in HCC classified histologically as "moderately differentiated", paired non-tumor tissue from the same patients, and non-infected ('normal') liver. (**D**) miR-122 abundance in the subset of tissues shown in panel C, normalized to miR-24 abundance. The statistical significance of differences between paired observations was estimated using the paired t test, while differences between non-paired observations were analyzed by the Mann-Whitney test.

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collected previously from 45 HCV-infected chimpanzees, and compared this to that present in 10 HBV-infected animals, and 6 that were not infected with either virus. These results showed that miR-122 expression was significantly reduced in HCV-infected liver compared to both HBV-infected ($p<0.0001$) and normal, non-infected ($p=0.007$) chimpanzee liver (Figure 4A). A strong, negative correlation (Spearman $r_s= -0.63$, $p<0.0001$) existed between hepatic miR-122 expression levels and HCV RNA copy numbers in serum (Figure 4B). The mean miR-122 abundance was lower in HBV-infected liver than in uninfected chimpanzee liver (Figure 4A), but the difference did not achieve statistical significance ($p=0.059$ by two-tailed t test). Thus, intra-hepatic miR-122 abundance is reduced in HCV-infected chimpanzees as well as humans. This is consistent with earlier studies that have found reduced intrahepatic expression of miR-122 in patients with advanced chronic hepatitis C [34–36].

Sarasin-Filipowicz et al. [36] reported previously that miR-122 levels were reduced in liver from HCV-infected

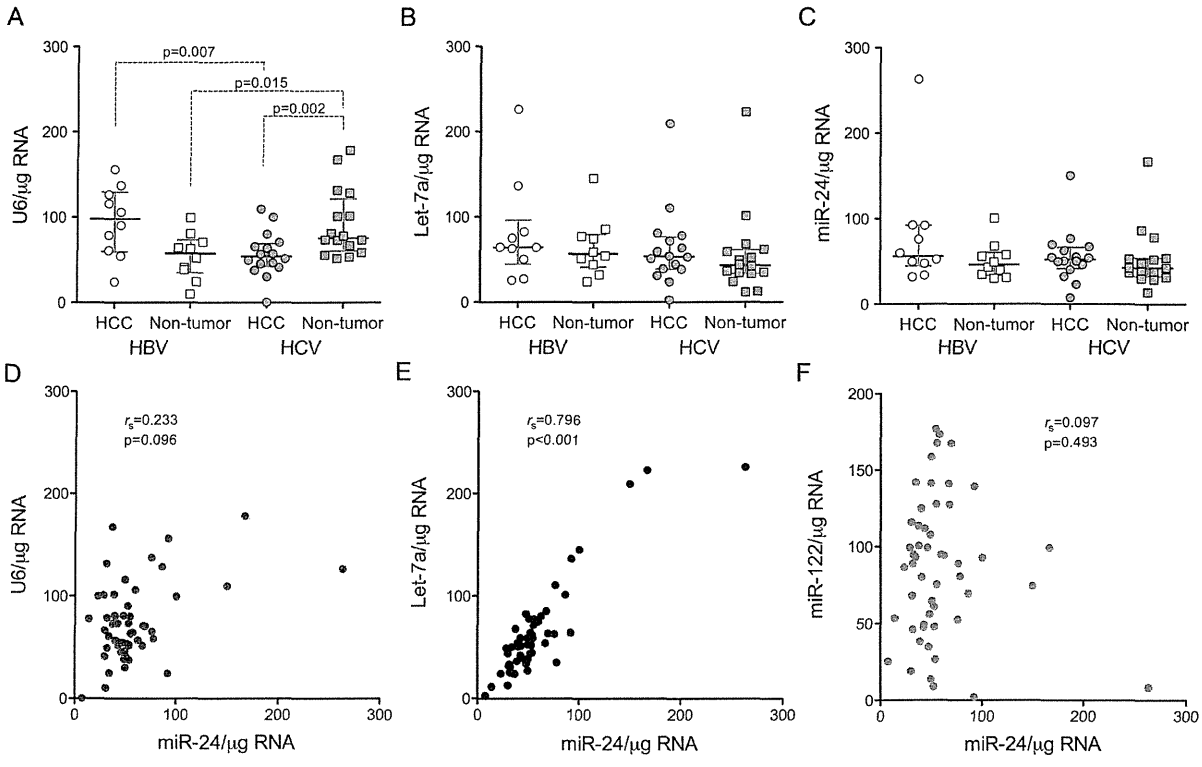

Figure 3. Comparison of small RNAs as normalizers for assessing miR-122 abundance. Shown in the panels at the top are the relative abundance of (**A**) U6 snRNA, (**B**) Let-7a, and (**C**) miR-24 miRNAs in paired tumor and non-tumor tissues from subjects with HBV or HCV infection, normalized to total RNA. Bars represent median and quartiles for each group. Statistical comparisons between groups were made with paired or unpaired t tests, and are shown only if p<.05. In the lower set of panels, (**D**) U6, (**E**) Let-7a, and (**F**) miR-122 abundance are plotted as a function of miR-24 abundance. r_s = Spearman rank-order correlation coefficient.

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patients who responded poorly to treatment with pegylated IFN-α and ribavirin (Peg-IFN/RBV). Consistent with this, we observed a negative correlation between miR-122 abundance in non-tumor tissue from HCV-infected human subjects and the GT versus TT genotype at the rs8099917 locus in the IL28B gene (p=0.011, Figure 5A) that is predictive of a poor response to Peg-IFN/RBV therapy [37]. HCV-infected patients with the TT genotype are prone to a greater inflammatory response than those with TG or GG [38]. Thus, differences in IL28B genotype may have contributed to a correlation we observed between miR-122 abundance and A1 versus A2 Metavir activity scores (Figure 5B, 6 of 7 subjects with an A2 Metavir score had the TT genotype). Importantly, the association between IL28B genotype and miR-122 abundance was observed only in non-tumor liver from HCV-infected patients, and not in paired HCC tissue (Figure 5A).

Patients who are non-responsive to Peg-IFN/RBV, or who have IL28B genotypes predictive of a poor response to Peg-IFN/RBV therapy, are likely to have increased pre-treatment intra-hepatic ISG transcript levels compared to those who respond well to treatment [39–41]. We thus asked whether a correlation existed between miR-122 abundance and levels of selected ISG transcripts in HCV-infected non-tumor tissue determined by Affymetrix 133U Plus 2.0 GeneChip assay. For this analysis, we selected ISGs that were shown previously to be correlated with treatment response [39] (Figure 5C). We also included Mx1 and OAS1, both well-characterized ISGs. Overall, the Affymetrix signals for these genes showed a strong trend toward negative correlations with miR-122 abundance. Fourteen of 24 ISGs demonstrated a Spearman rank-order coefficient, r_s, ≤ -0.300; this negative correlation was significant (p<0.05) for 7 of the ISGs by one-tailed t test (Figure 3C). These data are consistent with the notion that reduced miR-122 abundance is associated with strong intrahepatic IFN-mediated responses to the virus.

miR-191 abundance is increased in HBV-associated HCC

Since Elyakim et al. [42] reported recently that miR-191 was increased in HCC arising in a study population comprised mostly of HBV-infected subjects, we also quantified miR-191 expression levels in the human tissue samples. We confirmed

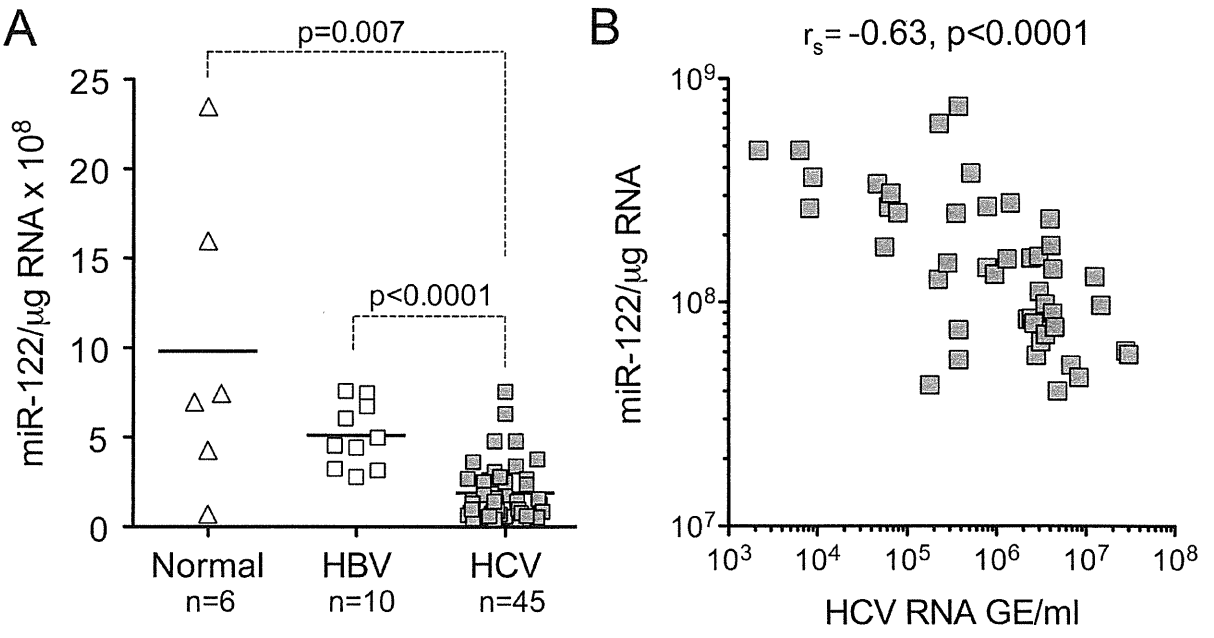

Figure 4. miR-122 expression in chimpanzee liver tissue. (**A**) Hepatic miR-122 abundance in liver biopsies from chimpanzees infected with HBV or HCV, or not infected with either virus ('normal'). Statistical significance was assessed by non-paired two-sided t test. Bars represent mean values. (**B**) Liver miR-122 expression plotted against serum HCV RNA abundance from acutely HCV-infected chimpanzees. r_s = Spearman rank-order correlation coefficient.

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miR-191 levels were modestly increased in HBV-associated HCC compared to non-tumor HBV-infected tissue when normalized to total RNA (p=0.049 by two-sided, paired t test, Figure 6). This trend remained significant only by one-sided t test when the miR-191 abundance was normalized to miR-24 abundance (p=0.045), and was absent when miR-191 levels were normalized to U6 snRNA. miR-191 abundance in non-tumor, HBV-infected tissue was similar to that in both tumor and non-tumor liver from HCV-infected subjects (Figure 6).

Discussion/Conclusions

miR-122 is a critical regulator of hepatic gene expression and an essential host factor for HCV replication [15,18,43]. It also has important tumor suppressor properties [16,44], and recent reports indicate that loss of its expression promotes carcinogenesis in knockout mice [45,46]. While its abundance is often reduced in HCC [21,22], two previous studies suggest that miR-122 expression may be preserved in liver cancer arising in patients with HCV infection [24,25]. We confirm this, showing in a genetically and geographically homogeneous population of patients, and normalizing results either to total RNA or to levels of miR-24, that miR-122 abundance is significantly reduced from normal in HBV-associated HCC but not in liver cancer associated with HCV infection (Figure 2A and B). This difference in miR-122 expression is independent of the histologic classification of the tumors (Figure 2C and D), as well as the degree of fibrosis or inflammation in paired non-tumor tissue from the same patients. Conversely, we show that miR-191 tends to be increased in abundance in HBV-associated cancer, but not HCV-associated HCC (Figure 6). These observations have important implications for the pathogenetic mechanisms involved in viral carcinogenesis within the liver. While HCC may arise as a result of factors common to both HBV and HCV infection (such as chronic inflammation, oxidative stress, and progressive fibrosis leading to cirrhosis), distinctive molecular signatures associated with HBV- versus HCV-associated cancer suggest there are fundamental differences in the ways these two viruses cause cancer.

Our study highlights the challenges inherent in comparing miRNA abundance in different clinical samples. In addition to potential differences in the proportion of cells present within a biopsy that are of hepatocellular origin vs. derived from other cell lineages, a constant concern is the quality of the RNA. While our initial analysis, like many studies, compared miR-122 copy numbers based on the quantity of total RNA subjected to RT-PCR, this approach can be biased by differences in the quality of the RNA and degree of RNA degradation. Although our RNA samples were of generally high quality (see Figure S1A in Supporting Information), we determined miR-24, Let-7a, and U6 snRNA copy numbers and evaluated each as a standard against which miR-122 abundance could be normalized to account for potential differences in RNA integrity (Figure 3). Median miR-24 and Let-7a copy numbers did not

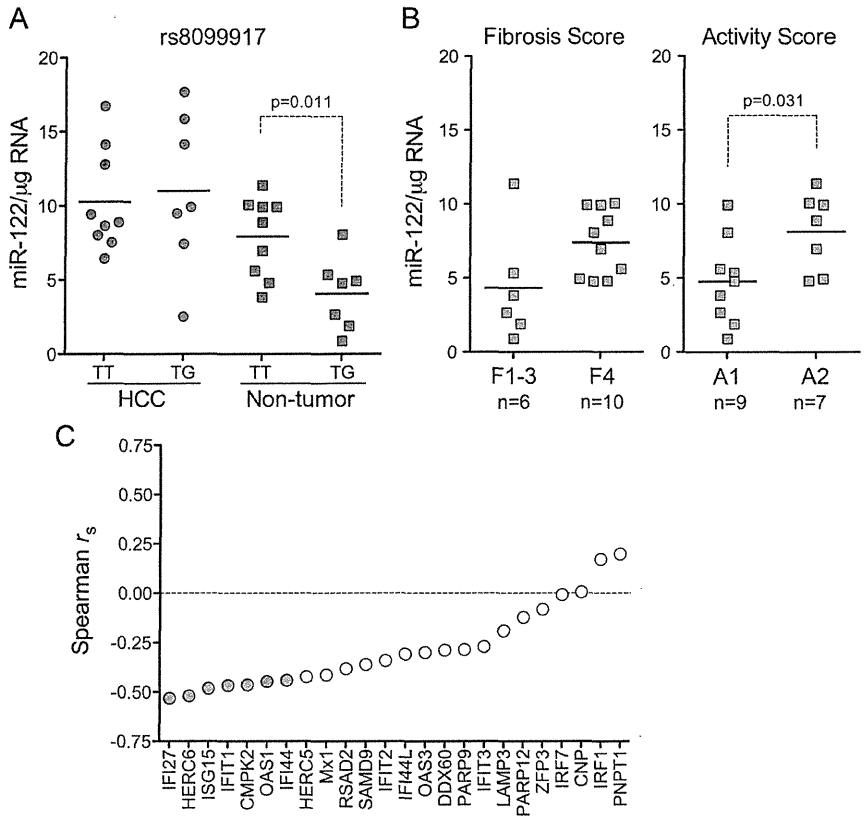

Figure 5. miR-122 expression, IL28B genotype, Metavir scores and ISG transcript levels in HCV-infected human liver. (**A**) miR-122 expression in HCC and paired non-tumor samples from subjects with HCV infection, grouped according to rs8099917 genotype (TT or GT). (**B**) miR-122 expression levels in non-tumor tissue from HCV-infected subjects categorized according to Metavir score for (left) fibrosis and (right) inflammatory activity. (**C**) Correlation between miR-122 abundance and expression levels of selected ISGs determined by Affymetrix U133 Plus 2.0 Array analysis. With the exception of OAS1 and Mx1, intrahepatic transcript levels of these ISGs have been shown previously to be predictive of Peg-IFN/RBV treatment outcome [31]. "r_s" = Spearman rank-order correlation coefficient. Filled symbols indicate a statistically significant negative correlation ($p<0.05$ by one-sided t test).

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vary significantly between tumor and non-tumor tissue samples from HBV- and HCV-infected subjects (one-way ANOVA), suggesting that the expression of these miRNAs is relatively constant in liver and that either could serve as a standard for normalizing miR-122 abundance. In contrast, median U6 copy numbers varied significantly between these tissue groups ($p = 0.004$ by one-way ANOVA with Kruskal-Wallis test) and, more importantly, were strongly correlated negatively with the RIN score, a measure of RNA integrity [29] (Figure S1A in Supporting Information). There was no correlation between the RIN score and miR-24 or Let-7a abundance, suggesting that U6 snRNA may be less stable and more prone to degradation than the miRNAs. This may be due to the greater length of U6 (106 nts vs. ~20-23 nts for miRNAs), or the absence of terminal modifications that may influence the stability of miRNAs [47]. Consistent with this, Let-7a was found to have greater biological stability and to be superior to U6 for normalization of miRNA abundance in previous studies of rat hepatocyte RNA [48]. Nonetheless, even though these data argue against the use of U6 as a standard for normalizing miR-122 copy numbers, we found the abundance of miR-122 was significantly reduced in HCC associated with HBV but not HCV infection, and that miR-122 abundance was significantly depressed in non-tumor tissue infected with HCV but not HBV, using any of these small RNAs, including U6, to normalize the miR-122 results.

While it remains unclear exactly how miR-122 contributes to the HCV lifecycle, it is known to promote viral replication independently of its regulation of hepatic genes [49]. It binds to two sites near the 5' end of the viral genome [18], recruiting argonaute 2 (EIF2C2) and physically stabilizing the RNA by protecting it from 5' exonucleolytic Xrn1-mediated decay [19,20]. However, miR-122 has other, positive effects on HCV replication beyond its ability to physically stabilize the viral

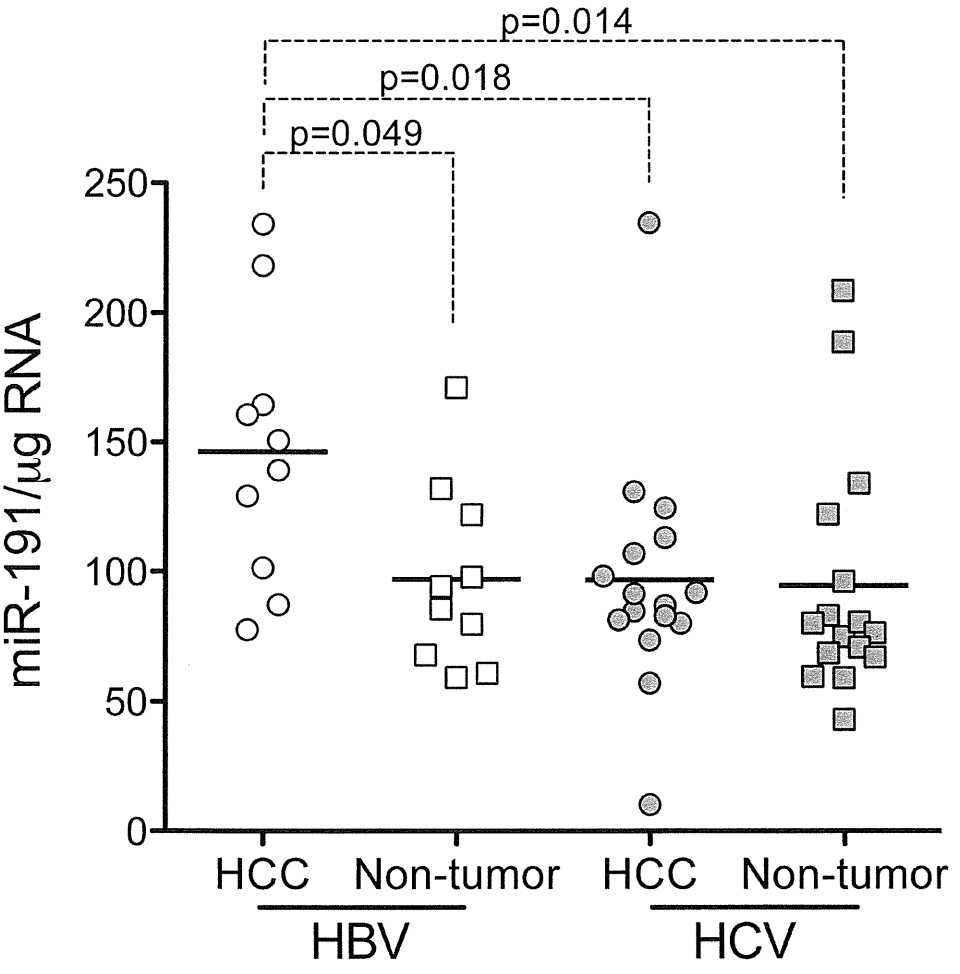

Figure 6. Relative abundance of miR-191 in paired HCC and non-tumor tissue from subjects with HBV or HCV infection. Relative miR-191 abundance in paired tumor and non-tumor samples from HBV- and HCV-infected subjects normalized to total RNA. Statistical significance was assessed in two-sided paired t tests for comparisons between tumor and non-tumor tissue, or two-sided unpaired t test for comparison between infection groups.

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genome [20,50]. It is essential for HCV replication, and its therapeutic silencing with an antisense oligonucleotide has potent antiviral effects [15,51]. No other RNA virus is known to rely so completely on a cellular miRNA for its replication cycle. Thus, the continued expression of miR-122 in HCV-associated HCC could reflect close linkage between carcinogenesis and HCV replication and viral protein expression. We speculate that miR-122 expression is preserved in HCV-associated HCC (in contrast to HBV-associated cancer) because HCV-encoded proteins help to drive a multi-stage process of carcinogenesis within infected cells. This may result from the ability of the virus to directly disable DNA damage responses or other cellular tumor suppressor functions, and to contribute directly to malignant conversion of hepatocytes as reviewed elsewhere [10,52]. Early loss of miR-122 during the progression to cancer would eliminate virus replication, protecting the cell from further effects of viral protein expression. In contrast, in HBV-infected cells, a loss of miR-122 expression could both accelerate tumorigenesis and enhance replication, as miR-122 appears to restrict, rather than promote, HBV replication [53–56]. Although speculative, this hypothesis raises the interesting possibility that HCV-associated cancers arise within the small minority of hepatocytes infected with the virus, and not the much larger number of uninfected bystander cells [52,57].

Epigenetic mechanisms are likely to contribute to the differential expression of miR-122 and miR-191 in HCC. The miR-122 promoter is hyper-methylated in the HBV-associated HCC-derived cell line, Hep3B [58]. It remains to be seen whether differences exist in methylation of the promoter in vivo in HBV- versus HCV-associated cancers, but bacterial artificial