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症例報告

インターフェロン・リバビリン併用療法中に脾摘後劇症型感染症を発症し，救命し得た生体肝移植後患者の1例

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要旨：症例は60代女性．C型肝硬変に対して生体肝移植，脾臓摘出術を施行．C型肝炎再発に対するインターフェロン療法中に発熱，下痢，嘔気が出現，ショックとなり脾摘後劇症型感染症と診断されたが，集中治療により救命した．脾摘後劇症型感染症は生命予後が不良であり，救命率の改善には予防の徹底，症状が軽度の段階から劇症化する可能性があることを念頭において，きわめて迅速に治療を開始することが必要である．

索引用語：OPSI，生体肝移植，脾摘，C型肝炎，肺炎球菌ワクチン

はじめに

脾臓摘出後，主に肺炎球菌などの有莢膜細菌の感染が原因で劇症型感染症を引きおこす場合があり，overwhelming postsplenectomy infection（OPSI）として報告されている[1)~4)]．急激な経過と高い死亡率を特徴とし，感染予防の徹底や軽微な症状からもOPSIを念頭において治療を開始することが重要である．一方，C型肝硬変に対する生体肝移植術の際，C型肝炎再発を考慮した脾臓摘出術が施行されるが，移植後には免疫抑制下となるため，さらなる易感染状態となる可能性もある．今回，生体肝移植，脾摘後のC型肝炎再発に対するインターフェロン（interferon；IFN）療法中にOPSIを発症したが，集中治療により救命し得た1例を経験したので，文献的考察を加えて報告する．

I 症　例

症例：60代 女性．

主訴：なし．

既往歴：糖尿病，甲状腺腫，乳癌，特発性血小板減少性紫斑病．

手術歴：虫垂切除術（20歳），帝王切開術（27歳），左乳癌切除術（59歳）．

家族歴：父 肝硬変，脳梗塞，母 解離性大動脈瘤，夫 C型慢性肝炎．

生活歴：専業主婦，飲酒 なし，喫煙 なし，輸血 なし．

現病歴：1998年にHCV抗体陽性を指摘，2000年の検診にて肝機能異常を指摘され近医を受診し，腹部CTにて典型的な肝硬変の所見を認めた．その後のフォローアップ中，2008年3月に肝S6，10mm大の初発肝細胞癌（hepatocellular carcinoma；HCC）に対し経皮的ラジオ波焼灼療

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法が施行された．2009年1月から腹水貯留が出現し，非代償性肝硬変と診断された．同年2月に局所再発HCCに対して肝動脈化学塞栓療法が施行され，8月にも局所再発（S6，17mm）と新規病変(S8, 8mm)を認めたが肝予備能はChild-Pugh 10点，Cであり，非代償性肝硬変，HCC，血小板減少に対して次男をドナーとした生体肝移植と，脾臓摘出術を施行した．脾摘前後で肺炎球菌ワクチンの投与は施行しなかった．免疫抑制剤はプレドニゾロンとタクロリムスを用い，術後経過は良好で第31病日に転院した．術前28000/μlだった血小板数は転院時には正常値220000/μlまで上昇した．術後3カ月までプレドニゾロンとタクロリムス，その後はタクロリムス単剤での外来フォローアップをしていたが，2010年4月に肝機能異常を認めた．肝生検ではA1，F0で門脈域の炎症は軽度だったが肝炎主体の像であり，また急性拒絶の所見は認めなかった．HCV-RNA定量は7.1logIU/ml，genotypeは1bであり，C型肝炎再発と診断し，IFNβ（600万IU/日）+リバビリン（400mg/日）を開始した．糖尿病については食事療法，ミチグリニド食直前内服，インスリンアスパルトを各食前4U使用し，コントロールは良好であった．PEG-IFNα2b（70μg/週）+リバビリン（400mg/日）に変薬し同年8月にはHCV-RNA陰性となったが，その後陽転化したためIFNβ再導入となり，免疫抑制剤変更を目的に12月当院入院となった．

入院翌日にタクロリムスからシクロスポリンに変更した．入院時HCV-RNA定量は1.2logIU/mlであり，入院後2日目にIFNβ（600万IU/日）+リバビリン（400mg/日）を開始したが，4日目朝から発熱，嘔気，下痢などの消化器症状が出現し，その後血圧低下と酸素化の悪化を認めショックとなった．プロカルシトニンの上昇をともなうことから敗血症性ショックと考え，集中治療を開始した．

発症時現症：身長145cm，体重43kg，意識レベルE4V5M6/GCS，体温38.6℃，脈拍110/分，血圧68/31mmHg，SpO_2 85～90%（room air）．

発症時検査所見：WBC 500/μl，Plt 36000/μlと低下，PCT >100ng/mlの上昇を認め重症感染症が示唆された．IFNの副作用のためと考えられるHb 7.7g/dlと貧血を認めた．PT 43%，APTT 66secと凝固異常を認め，感染症による影響が考えられた．Cre 1.28mg/dlと腎機能障害も認めた．AST 1002IU/l，ALT 306IU/lと肝機能障害も認めた．

経過：ICU入室後よりメロペネム（MEPM）3g/日とバンコマイシン（VCM）投与を開始したが全身状態はさらに悪化し，人工呼吸管理を開始した．また腎機能障害も進行し乏尿となったため，持続血液透析濾過法（CHDF）を導入した．免疫抑制療法中で易感染状態でありホスカルネットナトリウム（FN）+シプロフロキサシン（CPFX）600mg/日+アムホテリシンB（AMPH-B）150mg/日を加え，CHDFを施行しながら十分量を投与した．さらにポリミキシン吸着療法（PMX）を施行，免疫グロブリン製剤（γ-globulin）を投与した．血液培養結果から*Streptococcus pneumoniae*が検出され，脾臓摘出後の劇症型感染症の原因と考えられた．VCMをリネゾリド（LZD）1200mg/日に変更後，呼吸，循環動態は安定し炎症反応も改善した．FN，CPFX，AMPH-B，LZDは血液培養の陰転化を確認して14日間投与，MEPMのみ21日間投与し終了した．腎機能障害についても改善したため発症20日目にCHDFを離脱，25日目に一般病棟へ退室した（Figure 1，Table 1）．退室後は長期臥床によるADL低下や帯状疱疹などを認めたが全身状態およびADLは改善し，2011年3月に退院した．

II 考 察

脾摘後劇症型感染症（OPSI）とは脾摘後に発症しうる劇症型感染症で，時間単位での急激な経過をとり，集中治療にもかかわらず50～70%と死亡率は高い[2)5)~8)]．脾摘後からOPSI発症までの期間は脾摘後1週間から20年以上とさまざまな報告があり，一生涯発症する危険性がある[9)]．また発症頻度は，小児では0.13～8.1%，成人では0.28～4.3%程度といわれており小児例と比較して成人例は少なく，発症年齢は2歳以下の成長期の小児に多い[2)~4)]．

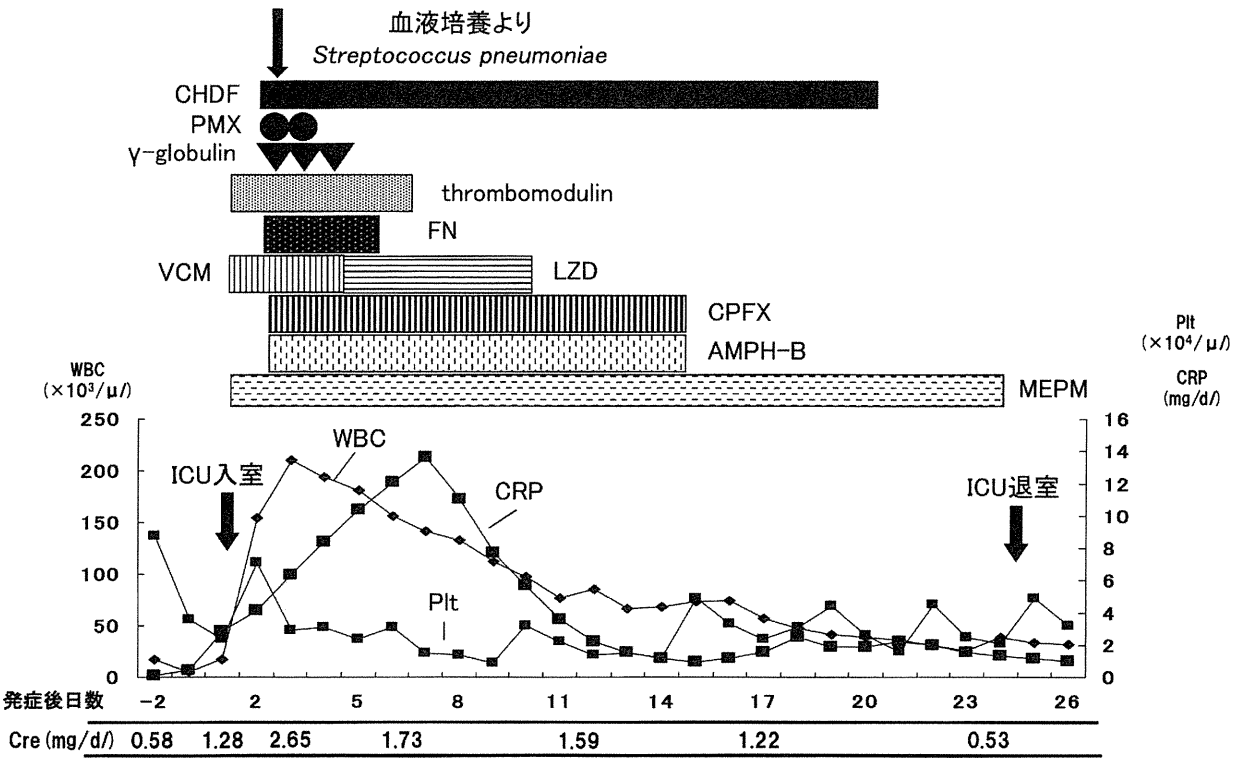

Figure 1. OPSI発症後の治療経過．CHDF（持続血液透析濾過法），PMX（ポリミキシン吸着療法），FN（ホスカルネットナトリウム），VCM（バンコマイシン），LZD（リネゾリド），CPFX（シプロフロキサシン），AMPH-B（アムホテリシンB），MEPM（メロペネム）．

Table 1. 血液検査結果の推移

	入院時	発症時	発症3日目	発症7日目	発症14日目	発症25日目
WBC (μl)	1800	500	21000	14100	6800	3400
Hb (g/dl)	8.3	7.7	8.8	8.7	8.9	8.8
Plt (μl)	108000	36000	29000	15000	12000	49000
PT (%)	89	43	39	66	59	59
APTT (sec)	29.3	66	86.7	64.5	41.1	37.4
BUN (mg/dl)	15	28	34	40	88	47
Cre (mg/dl)	0.54	1.28	2.65	1.73	1.72	1.62
TP (g/dl)	7.7	6.7	5.8	5.8	5.6	6.7
Alb (g/dl)	4	3.2	3.7	3.3	3.2	3.4
T-bil (mg/dl)	0.4	2.3	4.9	11	6.2	2.3
AST (IU/l)	28	1002	680	100	40	24
ALT (IU/l)	18	306	198	47	8	19
LDH (IU/l)	243	846	1284	399	305	244
CRP (mg/dl)	0.01	0.43	6.33	13.61	1.17	1.16
BS (mg/dl)	93	179	95	233	157	145
HbA1c (%)	5.5					
HCV-RNA 定量 (logIU/ml)	<1.2				6.8	

原因生物は細菌，ウイルス，真菌，原生動物などあらゆる生物が挙げられるが，有莢膜細菌が中心で，特に肺炎球菌（50～90%），髄膜炎菌，インフルエンザ菌，溶連菌（25%）が多く[8)10)～12)]，肺炎球菌に関して，脾摘後の患者はそれ以外と比べて12～25倍の肺炎球菌感染のリスクがある[13)14)]．

脾臓の感染防御機能に関して，脾臓は毎分全血液の4%を浄化しており貪食能は肝臓の10倍あり，抗体産生能は脾臓がない場合，抗体価が約10分の1に低下するといわれている．脾摘により細菌貪食能，抗原提示とそれに続く抗体産生，オプソニン産生などといった機能の欠如や有莢膜細菌に対する特異的抗体産生の低下が，劇症型感染症を引きおこすと考えられている[1)2)15)]．

OPSIの初発症状は，発熱，頭痛，倦怠感から，腹痛，嘔吐，下痢，便秘といった消化器症状など非特異的で軽微なものが多く[4)6)]，本症例でも発熱や嘔吐，下痢といった消化器症状を初発としている．

本症例は生体肝移植における同時脾摘症例に発症したOPSIである．今回脾摘を併施した理由は術後のIFN導入のためであり，一般的にHCV陽性症例では移植後1年以内に70～90%の症例で組織学的な肝炎再発が確認され，適切なコントロールがされても5～10年の間に8～44%の症例で肝硬変となる[16)～19)]．またHCV陽性と陰性症例において移植後5年のグラフト生着率と患者生存率を比較した際，生着率は69.9% vs 80.6%，生存率は74.6% vs 83.5%との報告があり[20)]，このような成績をふまえたとき，移植後のC型肝炎再発に十分な対策を考慮する必要がある．肝炎再発時のIFN・リバビリン併用療法において，脾摘により移植後血小板数を高いレベルで維持できるため，血小板低下を理由とするIFNの中断を防ぎ，患者のコンプライアンスを高めることが期待できる．

本症例では，脾摘は生体肝移植時に行われたもので，脾摘後およそ1年でOPSIを発症した．同様に生体肝移植，脾摘後にOPSIを発症し救命し得た報告例は1例のみで，40代男性，HBVによる劇症肝炎に対して施行された血液型不適合生体肝移植症例であり，術後80日で退院したが術後136日目に湿性咳嗽を主訴に外来受診した．*Streptococcus pneumoniae*, *Pneumocystis carinii*による重症肺炎，OPSIと診断され，挿管，抗菌薬，抗真菌薬，γ-globulinでの集中治療により，治療開始後18日で抜管，41日目に退院している[21)]．

50～70%と高い致死率にもかかわらず本症例を救命できた理由に，まず入院中に発症したことが挙げられる．先述の通りOPSIの初発症状は消化器症状をはじめとした非特異的なものが多く，時間単位で容態が変化する本症を外来で診ることも想定される．本症例では症状やバイタルを時間を追って観察でき，血液検査や画像検査も容易に行えたため治療開始の時期を逸することなく，また人工呼吸器管理や透析といった集中治療を行えた．次に，敗血症性ショックの際に陥りやすい尿量低下，急性腎障害に対して，CHDFを施行しながら最大投与量での抗菌薬加療を行った点が挙げられる．

IFN・リバビリン併用療法とOPSI発症について，C型肝硬変症例の外傷性脾損傷に対する脾摘術後，IFN・リバビリン併用療法中にOPSIを発症した報告例があるが[22)]，IFNとOPSI発症との関連については明らかではなく，今後の検討が必要である．本症例は生体肝移植後の患者であり，通常のC型肝炎脾摘施行後のOPSIと異なり，免疫抑制下にあることが経過に関与した可能性もある．

OPSIの予防についてであるが，脾摘により免疫抑制状態にある患者にとって，このOPSIをいかに予防できるかが重要な課題である．一般的な脾摘術が予定されている場合，少なくとも2週間前までに肺炎球菌ワクチンを投与し，6年ごと，もしくは5～10年ごとの追加投与が推奨されている[9)]が，本症例のように臓器移植周術期の投与に関して，免疫抑制状態での効果・安全性が不確実であり，また投与時期や回数についても明確なコンセンサスは得られていないのが現状である．当科では原則として移植の14日以上前，もしくは移植後に全身状態が安定した後に投与する方針と

しているが，今後，多施設共同での投与プロトコールの作成が望ましい．

一般的に，肺炎球菌の病原因子pneumolysinに対する抗体価は感染後回復期に上昇するが[23)24)]，HIV感染者などの免疫不全患者では侵襲性肺炎球菌感染症（invasive pneumococcal disease；IPD）に罹患した後でもpneumolysin抗体価の上昇を認めないことも報告されている[25)]．また，本症例のような無脾患者における肺炎球菌ワクチン接種者でも，ワクチンと異なる血清型の肺炎球菌感染により致命的となった報告がある[26)27)]．ワクチン未接種患者では，IPD罹患後に生存できたとしても2.3%が再発し，その罹患率は通常IPD感染率の50倍にも及ぶ[28)]．以上から，ワクチン未接種患者では罹患後でもワクチン接種が推奨され，本症例においてもワクチン接種予定である．

今後，医療従事者や患者にOPSIを周知させることも重要である．脾摘後の患者のうち11～50%は患者自身が低免疫状態であり，劇症型感染症のリスクにさらされていることを知らない，といった報告もある[9)29)30)]．

結　語

生体肝移植，脾臓摘出後のC型肝炎再発に対するIFN療法中に発症し，救命し得たOPSIの1例を経験した．脾摘患者においては非特異的で軽微な訴えでもOPSIを念頭においた厳重なフォローと治療が必要であり，患者教育の徹底や臓器移植周術期における肺炎球菌ワクチン投与プロトコールの確立を進める必要がある．また肺炎球菌ワクチン未接種での罹患患者においても，再発予防のためワクチン接種が望ましい．

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本論文内容に関連する著者の利益相反
：なし

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Overwhelming postsplenectomy infection during combination therapy with interferon ribavirin after living donor liver transplantation for hepatitis C : a case report

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A 60-year old woman was admitted for reintroduction of interferon/ribavirin combination therapy to prevent the recurrence of hepatitis C following living donor liver transplantation (LDLT). She had also undergone splenectomy during LDLT to avoid postoperative pancytopenia due to hypersplenism. However, a few days after reintroduction of the therapy, she developed severe diarrhea and fever that progressed to circulatory and respiratory shock. Blood culture was positive for *Streptococcus pneumoniae*, leading to a diagnosis of overwhelming postsplenectomy infection (OPSI). Although the patient developed multi-organ failure, she ultimately recovered after intensive care including mechanical ventilation and hemodialysis. Once OPSI is suspected, intensive care should be commenced immediately given the disease's fulminant clinical course and high mortality. Postoperative prophylaxis with the pneumococcal vaccine needs to be tested in a multicenter study.

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LETTER FROM THE FRONTLINE

Preoperative Simulation With a 3-Dimensional Printed Solid Model for One-Step Reconstruction of Multiple Hepatic Veins During Living Donor Liver Transplantation

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TO THE EDITORS:

A 53-year-old male patient underwent living donor liver transplantation (LDLT) for hepatitis C virus–infected liver cirrhosis complicated with hepatocellular carcinoma. Preoperative 3-dimensional (3D) images were obtained with a 3D image analysis system (Synapse Vincent; Fujifilm Medical, Tokyo, Japan) so that we could evaluate the graft volume and possible congested volume after implantation in LDLT. This revealed that a large middle hepatic vein (MHV) drained a vast area in the right lobe (Fig. 1A,B). The estimated volume of the donor's whole liver was 1048 mL. The extended left graft was considered to be small for the size of the recipient and corresponded to 30% of the recipient's standard liver volume; also, it had an estimated congested area of 407 mL, which was equivalent to 39% of the donor's liver volume in the remnant right lobe (Fig. 1C). As a result, if a left lobe graft could be procured, the functional remnant liver volume was estimated to become 20% of the donor's liver volume. Hence, the left lobe was considered inappropriate not only because of the small-for-size graft for the recipient but also because of safety concerns for the donor.

After ensuring sufficient drainage of the left lobe by medial segmental vein (V4) and the left hepatic vein, we decided to use a right lobe graft with the MHV because the volume was considered sufficient and was equivalent to 47.5% of the standard liver volume of the recipient. A preoperative contrast-enhanced computed tomography scan revealed a distance of 2 cm between the donor's right hepatic vein (RHV) and MHV at the estimated Cantlie line. Because of the location and alignment, we planned to use an autologous portal vein (PV) Y-graft interposition for the hepatic vein anastomosis. The image of the Y-graft from the recipient's PV was also made with the Synapse Vincent program (Fig. 2).

An extended right lobe graft was transplanted from the patient's wife, and the actual graft weight was 493 g, which corresponded to 42.3% of the recipient's standard liver volume. A hilar PV was harvested from the recipient, and ex vivo hepatic vein reconstruction was performed through the connection of a right portal branch to the MHV and a left portal branch to the RHV with continuous 5-0 polypropylene monofilament sutures. An end-to-end anastomosis was performed between the explanted main PV graft and the inferior vena cava with continuous 5-0 polypropylene monofilament sutures. In addition, the right inferior hepatic vein (RIHV) was directly anastomosed to the inferior vena cava. After reperfusion, intraoperative Doppler ultrasound showed a stable and sufficient hepatic inflow and outflow from the 3 anastomosed hepatic veins. The length of the recipient operation was 13 hours 15 minutes, and the blood loss was 11,700 g. At the time of this writing, 8 months after LDLT, the patient was doing well with good liver function. The fact that both the MHV and the RHV were patent in the early postoperative period is considered to have contributed to the patient's recovery. The donor favorably recovered without any complications. The study protocol received a priori approval by the appropriate institutional review board committee.

DISCUSSION

An extended right lobe graft is uncommon in Japan because of concerns about donor safety. On the other hand, approximately 58% of individuals have an MHV that is larger than or the same size as the RHV, and in 13% of right liver lobes, the MHV partially or totally drains a vast area, including segment 6,[1] as with our patient. A preoperative 3D image simulation depicting

Potential conflict of interest: Nothing to report.

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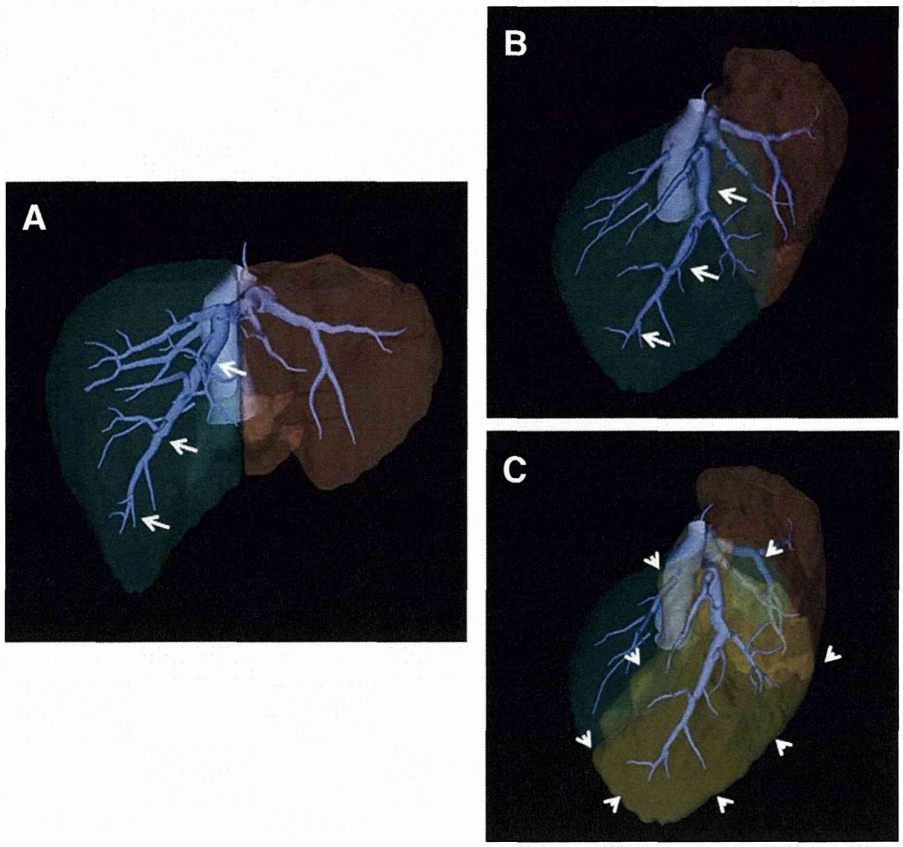

Figure. 1. (A,B) A preoperative 3D image evaluation of the donor's graft revealed that the large MHV drained a vast area in the right lobe (arrows). (C) The estimated volume of the congested area (the yellow area with arrowheads) was 407 mL, which was equivalent to 39% of the donor's liver volume in the remnant right lobe.

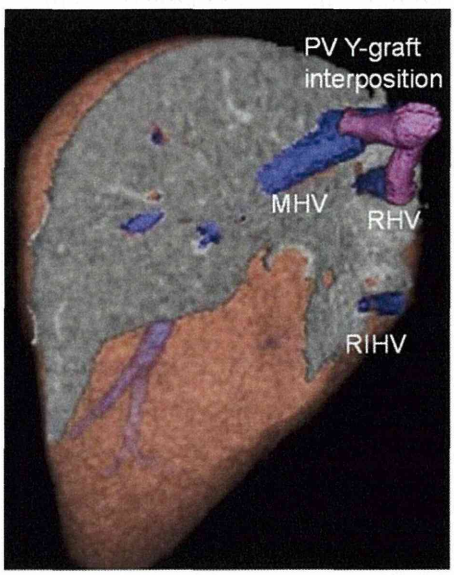

Figure. 2. A preoperative 3D simulation of the autologous PV Y-graft interposition for the hepatic vein anastomosis connecting a right portal branch to the MHV and a left portal branch to the RHV was performed with the Synapse Vincent program.

the anatomy in detail and providing an accurate estimate of the possible congested volume prompted us to make the decision to use an extended right lobe graft in this case.

The usefulness of explanted autologous PV grafts for hepatic vein tributaries in LDLT has recently been reported.[1,2] Although previous reports have described the use of a PV graft for MHV tributaries, including segment 5 and 8 veins, in our case, both main drainage veins, including the MHV and the RHV, were reconstructed with an interposition Y-shaped PV graft. Subsequently, the main outflow of the liver graft depended on this reconstruction. The reconstruction of the main drainage veins of the right liver graft with an explanted hilar PV graft is considered to be an effective management strategy for cases with a large distance between the hepatic veins in LDLT.

The useful application of a 3D volume analyzer in the field of liver surgery has recently been reported.[3,4] Meticulous preoperative volumetry of the partial liver graft is essential in terms of both postoperative graft function and donor safety. One of the strongest merits of using a 3D volume analyzer is that it is possible to

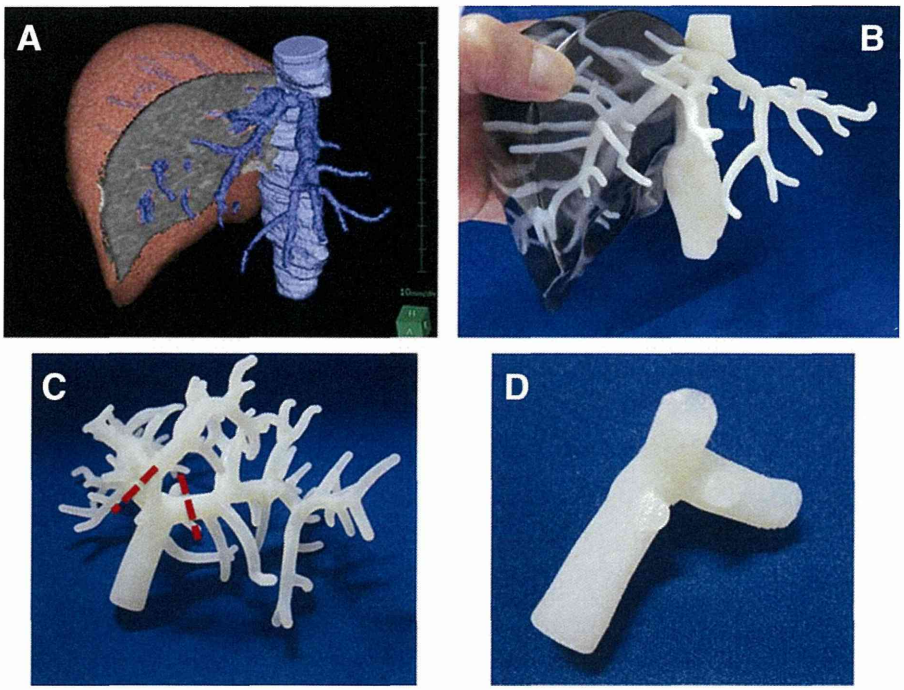

Figure. 3. (A,B) A 3D solid model of the graft obtained from a 3D printer was constructed from the data obtained with the Synapse Vincent program. A gumlike material was used to make (C) the recipient's PV tree with (D) a PV Y-graft, and these models were effective for allowing the reconstructed shape of the anastomosis to be understood with appropriate spatial perception.

estimate each vessel's circulating and draining areas. On the basis of this estimate, we are able to evaluate whether to reconstruct the MHV tributaries or anomalous hepatic veins in LDLT.

With the recent development of 3D printers, solid 3D models can be made from images obtained with a 3D volume analyzer. In this case, we made a 3D model from the data generated by the Synapse Vincent program (Fig. 3A,B). The solid 3D model was effective as a preoperative simulation, and the plastic liver and gum-like material made to represent the vessels made it easy to imagine the reconstructed shape and angle of the anastomosis with appropriate spatial perception (Fig. 3C,D), which helped us to choose an appropriate surgical strategy. It should also be noted that the use of a 3D printed model during surgical planning was helpful not only for the operating surgeons, but also for young surgeons as training, so that they could understand the detailed representation of the future complex anastomosis in LDLT. Although making a 3D solid model for every case is currently expensive, it is considered to be worthwhile for select cases such as ours because of its efficacy in facilitating operation planning. We highly recommend using a 3D printed model in cases with complex vascular or biliary anatomy, which require multiple or complicated anastomoses with or without the use of graft interposition.

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Hepatology Research

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Original Article

Clinical significance of intragraft miR-122 and -155 expression after liver transplantation

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Aim: Recurrent hepatitis C (RHC) and acute cellular rejection (AR) remain critical problems following liver transplantation (LT) in hepatitis C virus (HCV) positive recipients because of the similar clinical features. Discrimination between these conditions can be problematic, and adjunctive biomarkers would be useful to discriminate these processes. The aim of our study was to investigate the possibility of the intragraft miR-122 and -155 expression as new biomarkers after LT.

Methods: A total of 29 HCV positive recipients were enrolled in this study. Intragraft expressions of miR-122 and -155 were studied between RHC predominant (n = 17) and AR predominant cases (n = 12) using quantitative reverse transcription polymerase chain reaction. Furthermore, we investigated the correlations between these expression levels and clinical serum parameters.

Results: Intragraft miR-122 expression had a good correlation with serum alkaline phosphatase (P = 0.02), but it was not correlated with the serum HCV viral load. The expression levels of miR-122 in the AR group were significantly higher than those in the RHC group (P = 0.0006) and, inversely, the expression levels of miR-155 in the AR group were significantly lower than those in the RHC group (P = 0.01).

Conclusion: Our study emphasizes a useful pattern of miR-122 and -155 as ancillary markers to discriminate AR predominant cases from RHC in HCV positive patients after LT.

Key words: acute cellular rejection, hepatitis C, liver transplantation, miRNA, molecular biomarker

INTRODUCTION

LIVER TRANSPLANTATION (LT) has been a life-saving and well-established treatment for acute liver failure and various end-stage liver diseases. However, both acute cellular rejection (AR) and recurrent hepatitis C (RHC) as complications after LT have life-threatening implications and they are the leading causes of graft loss and retransplantation.[1] In particular, in hepatitis C virus (HCV) patients, one of the most serious problems is the fact that sometimes they can reveal overlapping features in both entities. Although histology remains the gold standard for differentiating AR from RHC, it is often challenging to distinguish which process is more predominant in those cases.[2] This has significant clinical implications because the treatment of AR can adversely potentiate HCV recurrence. In this regard, the findings of new biomarkers and a deeper understanding of the regulation of AR and RHC will lead to better control of liver allograft viability.[3]

miRNA are small non-coding RNA molecules that function at the post-transcriptional level. miRNA consist of approximately 22 nucleotide RNA that suppress the expression of messenger RNA bearing partially complementary sequences by accelerating their degradation and inhibiting their translation into protein.[4] They have been implicated in various molecular mechanisms such as viral infection and immune responses.[5,6]

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Author contribution: Tadafumi Asaoka performed research and participated in the study design, writing the paper and data analysis. Dayami Hernandez participated in the technical support for the experiments. Panagiotis Tryphonopoulos and Jennifer Garcia participated in obtaining clinical data. Akin Tekin, Thiago Beduschi, Seigo Nishida, Ji Fan and Rodrigo Vianna participated in the study design and suggestions. Phillip Ruiz participated in the study design, revising the paper, pathological diagnosis and data analysis.

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miR-122 is a liver-specific member and it accounts for approximately 70% of the total miRNA content of liver.[7] miR-122 binds to two highly conserved sites in HCV RNA, and protects it from degradation rather than causing it to be degraded. Actually, liver-specific miR-122 was recently reported to be crucial for efficient replication of HCV RNA and has been shown to facilitate the replication of HCV *in vitro*.[8–11] It also modulates the expression of genes involved in hepatic lipid and cholesterol metabolism.[12,13] A change in levels of miR-122 in the liver and blood has been confirmed as an indicator for liver disease[14,15] and the use of miR-122 as biomarker for hepatitis C has been proposed.[16]

The miRNA have a critical role in regulating the expression of genes relevant to allograft rejection and the induction of immune tolerance. miR-155 has been shown to dramatically impact both innate and adaptive immune processes, including inflammation, antigen presentation, T-cell differentiation, cytokine production and regulatory T-cell (Treg) functions.[17–19]

As described above, miR-122 and -155 have been reported as key molecules for HCV replication and $CD4^+$ T-cell differentiation, respectively. However, the clinical significance of these miRNA in recipients after LT remains unclear. So, we focused on miR-122 and -155 as HCV and host related factors, respectively.

We hypothesized that intragraft miRNA are dysregulated in liver allografts with RHC and AR after LT. In order to reveal the clinical significance of miR-122 and -155 expressions after LT, we developed an assay to measure their levels in formalin-fixed tissue from HCV-infected human liver allografts and correlated them with clinical parameters. The aim of our study is to investigate the intragraft expression levels of miR-122 and -155 in liver transplants and evaluate the clinical relevance and discriminatory capacity in conjunction with histology.

METHODS

Study sites and internal review board approval

ALL PATIENTS GAVE their written informed consent. This study was performed at the University of Miami, Miami Transplant Institute. The study protocol was approved by the internal review board of the University of Miami.

Patients and controls

We evaluated a total of 29 liver allograft biopsy specimens from 29 recipients after LT available for this study from March 2008 to September 2010. All of these cases were HCV positive patients, and the biopsy samples were obtained when the patients had liver dysfunction, with all changes in immunosuppressive or antiviral therapy being recorded. We defined liver dysfunction as elevated total bilirubin (>2.0 mg/dL), aspartate aminotransferase (>40 IU/L) and alanine aminotransferase (>40 IU/L) levels. Additional liver tissues from 20 normal livers were used as a pooled control mixture. A mixture of RNA from the normal parts of liver specimens of 20 patients with liver metastases from colon cancer was used as a reference for analysis.

Histopathological examination of liver biopsy samples and sample classifications

Hematoxylin–eosin-stained sections of the 29 samples were examined by experienced pathologists at the University of Miami. AR-labeled specimens were graded according to the Banff classification and the inflammatory grade and fibrosis stage for RHC were scored with the METAVIR scoring system. After the evaluation of these biopsy samples, prior to the assay, the investigators followed the patients and confirmed that those pathological diagnoses matched the clinical course of the patients. On the basis of the clinicopathological assessment, patients were assigned to two groups:

1 The RHC group: no evidence of AR or mild AR on the basis of the Banff criteria and an inflammatory grade of hepatitis greater than G1. These patients were pathologically diagnosed as RHC predominant with possible superimposed AR.
2 The AR group: mild (rejection activity index, 4–5) or moderate (rejection activity index, 6–7) AR on the basis of the Banff criteria and an inflammatory grade of hepatitis greater than G1. These patients were pathologically diagnosed as AR predominant with possible superimposed RHC.

Blood sampling

A peripheral blood sample was collected from each individual just before the liver biopsy. Standard parameters of liver allograft function were measured at the Immune Monitoring Laboratory, University of Miami.

Total RNA isolation

Total RNA was extracted from formalin-fixed paraffin-embedded (FFPE) biopsy samples using the RecoverAll

Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA, USA) for FFPE tissues according to the manufacturer's instructions. The concentration and quality of total RNA were measured by a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA) and checked by the ultraviolet absorbance at 260 nm and 280 nm (A260/280). A 260/280 absorbance ratio range of 1.9–2.1 was considered acceptable for miRNA assay.

Quantification of miR-122 and -155

Real-time polymerase chain reaction (PCR) analysis of miR-122 and -155 expression were performed in 29 FFPE tissue samples (AR/RHC; 12/17) using TaqMan miRNA assays with individual miRNA-specific primers (Applied Biosystems). The real-time PCR was run on the Applied Biosystems 7900HT Fast Real-Time PCR System. The expression of each miRNA was normalized using the small RNU44 as internal control. Relative quantification was done based on pooled normal livers using a comparative Ct method. Ct values of more than 35 were eliminated from analysis. Reverse transcription (RT)–PCR was performed in triplicate and the average data of each sample was used for further analysis. The data of each group was compared by the Mann–Whitney *U*-test.

Real-time PCR for miRNA targets

In order to discover the functional interactions between miRNA and mRNA genes in liver allografts, we selected a couple of genes (signal transducers and activators of transcription 1, STAT1; bile acid coenzyme A, BAAT) related to miR-155 and -122. STAT1 is well known as one of the key molecules in the Janus kinase (JAK)/STAT signaling pathway which is involved in miR-155 expression.[20] BAAT was selected by mimiRNA (http://mimirna.centenary.org.au), a resource designed to graphically represent human miRNA expression profiles and discover miRNA targets. We analyzed STAT1 and BAAT expression by quantitative real-time RT–PCR using the original pooled samples. The real-time PCR is run on the Applied Biosystems 7900HT Fast Real-Time PCR System with individual mRNA-specific primers. The expression values of genes were normalized relative to glyceraldehyde 3-phosphate dehydrogenase of the same samples.

Statistical analysis

Data were expressed as median and range values. Differences were tested by the exact χ^2-test or Mann–Whitney *U*-test, and correlation between two variables was analyzed using the Pearson correlation coefficient. All differences were considered statistically significant at $P < 0.05$. SPSS for Windows version 8.0J software (SPSS, Chicago, IL, USA) was used for all analyses.

RESULTS

Clinicopathological features

ON THE BASIS of the classification described in the Methods section, the set of 29 samples used in this study consisted of 17 samples belonging to the RHC group and 12 samples belonging to the AR group. All patients underwent liver transplantation for HCV-related cirrhosis and received a graft from a heart beating donor. Most patients were treated with a combination of calcineurin inhibitor, corticosteroids and mycophenolate mofetil (7/29 were on mycophenolate mofetil and 14/29 received maintenance corticosteroids).

There were no significant differences in age, sex, primary liver disease, serum total bilirubin, serum alanine aminotransferase or serum HCV RNA levels between the RHC and AR groups (Table 1).

Intragraft miR-122 and -155 expression levels

Intragraft (i.e. from paraffin block) expression of miR-122 was decreased in all recipients relative to normal liver. Particularly, intragraft miR-122 expression was significantly decreased in the RHC group (median, 0.07) rather than the AR group (median, 0.34) ($P = 0.0006$). On the other hand, miR-155 expression was significantly increased in the RHC group (median, 1.92) as compared with the AR group (median, 0.90) ($P = 0.01$) (Fig. 1).

Clinical relevance between intragraft miR-122 and -155 expression and standard laboratory parameters in HCV positive recipients

We correlated miR-122 and -155 levels in liver allografts with standard laboratory parameters by Spearman's rank correlation coefficient analysis. Intragraft miR-122 expression demonstrated a positive correlation with serum ALP levels, but it was not correlated with serum AST, ALT and HCV viral load. miR-155 expression had no correlation with any laboratory parameters. Values of correlation coefficient (r^2) and *P*-values are shown in Figure 2.

Table 1 Clinicopathological features

Category	RHC ($n = 17$)	AR ($n = 12$)	P
Age	57 (15–72)	57 (49–67)	0.94
Sex (M/F)	9/8	8/4	0.46
Days after transplantation	146 (74–4327)	31 (7–525)	0.003†
Primary disease			
HCV/LC	12	7	0.49
HCV/HCC	5	5	
Immune suppression			
FK based	15	9	
CyA based	2	3	
Steroid addition	6	8	
MMF addition	2	5	
Rapamycin addition	4	1	
Rejection grade			
No evidence of rejection	4	0	
Indeterminate	10	0	
Mild	3	5	
Moderate	0	6	
Severe	0	1	
Chronic hepatitis C			
Grade			
0	0	10	
1	1	0	
2	4	2	
3	11	0	
4	1	0	
Stage			
0	1	10	
1	5	2	
2	5	0	
3	5	0	
4	1	0	
Laboratory data			
T-Bil (mg/dL)	1.8 (0.1–23.5)	2.9 (0.5–14)	0.53
AST (IU/L)	203 (36–566)	110 (27–665)	0.23
ALT (IU/L)	192 (26–1306)	375 (58–935)	0.27
ALP (IU/L)	264 (90–650)	244 (103–1177)	0.67
HCV RNA (PCR, $\times 10^4$ IU/mL)	1185×10^4 ($4790–416 \times 10^6$)	1780×10^4 ($10^4 \times 10^4–301 \times 10^6$)	0.50

†The level of statistical significance was set at $P < 0.05$.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AR, acute cellular rejection; AST, aspartate aminotransferase; CyA, cyclosporin; FK, tacrolimus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LT, liver transplantation; MMF, mycophenolate mofetil; PCR, polymerase chain reaction; RHC, recurrent hepatitis C; T-Bil, total bilirubin; Treg, T regulatory cell.

Correlation assay between miRNA and target gene expression in liver allografts

We selected BAAT and STAT1 as the target genes for miR-122 and -155, respectively. Intragraft miR-122 expressions had a positive correlation with BAAT ($P = 0.0034$), but there was no correlation between miR-155 and STAT1 (Fig. 3).

Distribution chart of total 29 samples based on intragraft miR-122 and -155 expression levels

The distribution map revealed that the intragraft expression pattern of high miR-122/low miR-155 was characteristic of the AR group, while the pattern of high miR-155/low miR-122 was a distinctive trend in the

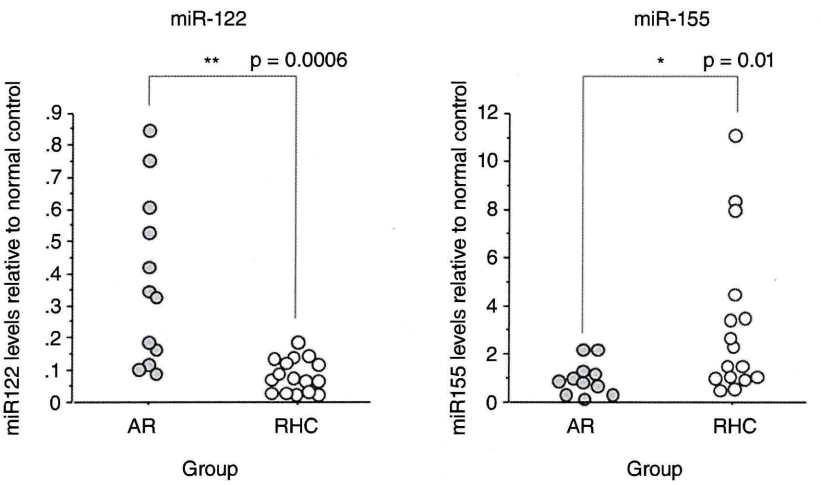

Figure 1 Intragraft expression level of miR-122 and -155. Expression data was represented based on comparative Ct method and normalized using RNU44 as the endogenous control. The relative expression of each sample was calculated using normal liver mixture as a reference. ●, acute cellular rejection (AR) ($n = 12$); ○, recurrent hepatitis C (RHC) ($n = 17$). **, *$P < 0.001$; *, **$P < 0.05$.

RHC group. Interestingly, miR-122 expression levels were inversely correlated with miR-155 expression levels ($P = 0.047$) (Fig. 4).

DISCUSSION

IN THIS STUDY, we investigated two miRNA that are dysregulated in HCV positive recipients after LT and evaluated the clinical relevance of intragraft miR-122 and -155 expressions for RHC and AR.

The liver miRNA expression profile is dominated by a single sequence, miR-122. It accounts for approximately 70% of all miRNA in the liver and appears critically related to the functional state of hepatocytes. miR-122 regulates many genes in the liver that control cell cycle, differentiation, proliferation and apoptosis.[21–23] Several reports have suggested that hepatic miR-122 expression in HCV seronegative patients is higher than in HCV seropositive patients and, furthermore, the level of hepatic miR-122 expression may be inversely correlated with the severity of liver damage.[14,24] On the other hand, serum miR-122 has been reported to be higher in patients with chronic hepatitis C than that in healthy controls.[16] They also suggested that serum miR-122 levels correlated well with ALT and histological activity index score.[16] These previous reports supported the hypothesis that miR-122 may leak out of injured hepatocytes into circulating blood, and the decline of miR-122 levels in the liver allograft may correspond to the destruction of HCV-infected hepatocytes.

We found that intragraft miR-122 expression in liver allograft was significantly downregulated in recipients with RHC and AR compared with healthy controls. In

	Correlation with (P value)	
	miR-122	miR-155
T-Bil	*0.58*	*0.74*
AST	*0.22*	*0.17*
ALT	*0.50*	*0.11*
ALP	*0.02**	*0.99*
HCV-RNA	*0.30*	*0.40*

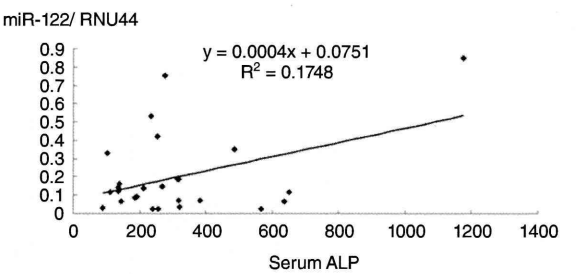

Figure 2 Correlation assay between intragraft miR-122 and -155 expression and standard laboratory parameters in hepatitis C virus (HCV) positive recipients. We correlated miR-122 and -155 levels in liver allografts with standard laboratory parameters by Spearman's rank correlation coefficient analysis. Intragraft miR-122 expression has a positive correlation with serum alkaline phosphatase (ALP) levels, but it was not correlated with peripheral HCV viral load. Values of correlation coefficient (r^2) and *P*-values are shown. ALT, alanine aminotransferase; AST, aspartate aminotransferase; T-Bil, total bilirubin.

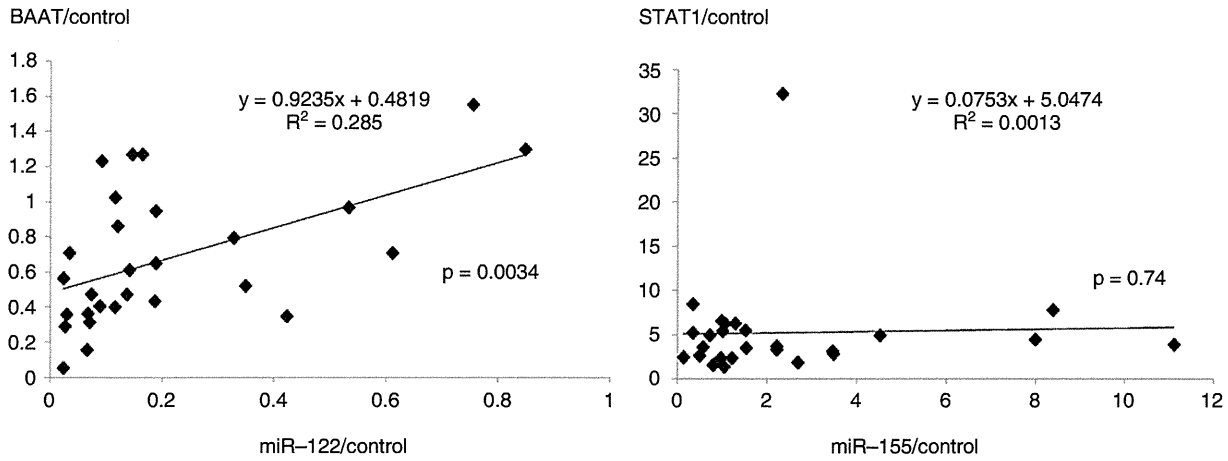

Figure 3 Correlation assay between intragraft miRNA and related gene expression in hepatitis C virus (HCV) positive recipients. Levels of intragraft miR-122 correlated significantly with levels of intragraft bile acid coenzyme A (BAAT) in the same samples ($P = 0.0034$). But, intragraft miR-155 expression did not have the significant correlation with intragraft signal transducer and activator of transcription (STAT)1 expression ($P = 0.74$).

particular, HCV positive recipients with active viral hepatitis showed that intragraft miR-122 expression significantly decreased in comparison with AR after LT. From our results, it is not possible to determine if miR-122 downregulation is a cause or consequence of active hepatitis but this finding was consistent with previous findings. Our data demonstrated the first clinical findings of differential miR-122 expression in liver allograft between AR and RHC.

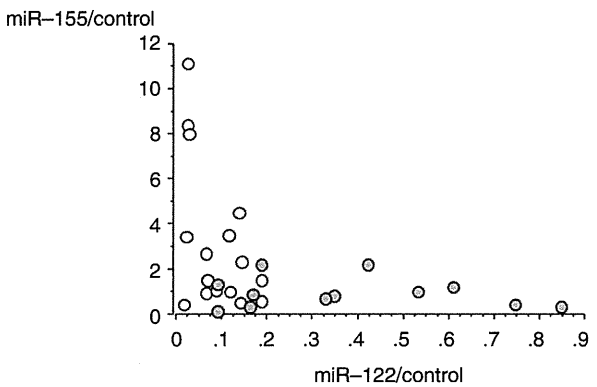

Figure 4 Distribution chart of total 29 samples based on intragraft miR-122 and -155 expression levels. The distribution map revealed that the intragraft expression pattern of high miR-122/low miR-155 was characteristic of the acute cellular rejection (AR) group, while the pattern of high miR-155/low miR-122 was a distinctive trend in the recurrent hepatitis C (RHC) group. Interestingly, miR-122 expression levels were inversely correlated with miR-155 expression levels ($P = 0.047$). ●, AR ($n = 12$); ○, RHC ($n = 17$).

miR-122 has been reported to have another critical role in regulating lipid homeostasis by controlling cholesterol synthesis and lipoprotein secretion in the liver.[25] A recent study demonstrated that mice lacking the gene encoding miR-122 develop extensive lipid accumulation and reduced glycogen storage in the livers.[12,13,26] They suggested that long-term suppression of miR-122 causes persistent steatohepatitis, fibrosis and spontaneous hepatocellular carcinoma. Actually, some studies showed that hepatic steatosis could be present in liver allografts with RHC after LT and that ballooning degeneration and cholestasis correlate with more rapid development of allograft cirrhosis.[2,27,28] In our study, the decreased miR-122 expression in liver allograft may be associable with these clinical and pathological features.

We selected BAAT as the target gene for miR-122 based on the mimiRNA database (http://mimirna.centenary.org.au), discovering functional interactions between miRNA and mRNA genes. We found that intragraft miR-122 expression has a strong positive correlation with BAAT ($P = 0.0034$) (Fig. 3) thus confirming that indeed the miRNA and the target gene are similarly influenced during RHC in the graft. It has been reported that a defect of BAAT can cause intrahepatic cholestasis;[29] thus, suppression of miR-122 and BAAT and their interactions as our studies indicate, could be associated with altered

lipid metabolism and steatosis in the liver allograft in HCV positive recipients.

miR-155 has been shown to regulate STAT1 expression and, conversely, STAT1 also partially regulates the JAK/STAT) signaling pathway. One recent study reported the presence of a positive feedback cycle of miR-155 and STAT1 in response to inflammatory signals or infection.[30] In our study, we could not detect a significant correlation between miR-155 and STAT1, but there are several possible explanations for our results. For example, miR-155 could be regulated by additional other target molecules yet to be described, because the field of miR-mRNA interactions is still very young.

It has been reported that miR-155 is highly expressed in Treg, as a direct target of Foxp3,[31,32] and Treg development required miR-155.[33] In this study, we found a repression of miR-155 in liver allografts undergoing AR. Our results raise the possibility that reduction of miR-155 may reflect insufficient Treg activity in the rejecting liver allograft.

It is known that miR-155 is not limited to immune cells (dendritic cells, Kupffer cells, monocytes, natural killer cells, T cells), and is also prevalent in non-immune cells such as hepatocytes and endothelial cells. These studies have suggested that there is a positive correlation between serum miR-155 and miR-122 increase in HCV positive patients.[19] Interestingly, in our study, miR-122 expression levels were inversely correlated with miR-155 expression in liver allograft ($P = 0.047$). The expression balance of miR-122 and -155 in the RHC group was remarkably different from that in the AR group (Fig. 4). We analyzed cut-off values of miR-122 and -155 expression using a receiver–operator curve. Our analysis showed that AR can be diagnosed using the levels of miR-122 and -155 in the liver graft (miR-122: cut-off, 0.12; area under the curve [AUC], 0.864; sensitivity, 81.8%; specificity, 62.5%; miR-155: cut-off, 0.99; AUC, 0.79; sensitivity, 81.3%; specificity, 63.6%) (Supplemental Fig. S1). Our novel finding implies that the intragraft expression of miR-122 and -155 may serve as potential molecular biomarkers to distinguish between AR and RHC after LT.

One of the limitations of this study is the heterogeneous sample populations which were biopsied at different time intervals from LT. However, there were no significant expression differences between early (≤6 months after LT) and late (>6 months after LT) biopsied samples after LT (Supplemental Fig. S2).

The other limitation of this study is the small sample size. An external validation cohort should be evaluated to make sure of reproducibility. Furthermore, various additional patient samples, such as patients transplanted with alcoholic liver disease, are needed to confirm our preliminary findings.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

Figure S1 Receiver–operator curve (ROC). Cut-off values for diagnosis were ascertained using the ROC, and the sensitivity and specificity were calculated for each cut-off value.

Figure S2 Intragraft miR-122 and -155 expression levels according to time interval after liver transplantation (LT). There were no significant differences of miR-122 and -155 expressions between early (≤6 months after LT) and late (>6 months later after LT) biopsied samples after LT. Expression data was represented based on comparative Ct method and normalized using RNU44 as the endogenous control. The relative expression of each sample was calculated using normal liver mixture as a reference.

Minimum graft size calculated from pre-operative recipient status in living donor liver transplantation

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Running title

Minimum graft size for LDLT

Abbreviations

EGL, early graft loss

GW, graft weight

LDLT, living donor liver transplantation

MELD, model of end-stage liver disease

MHV, middle hepatic vein

SAGL, small-for-size associated graft loss

SFSS, small-for-size syndrome

SLV, standard liver volume

Keywords

Graft selection, donor hepatectomy, small for size