Protective effect of taurine on APAP-induced liver damage

APAP overdose can cause acute liver injury in both humans and animals. At normal and therapeutic doses, APAP is principally and rapidly metabolized in liver by glucuronidation and sulfation; however, an overdose of APAP that exceeds the capacities of these processes results in additional oxidation by CYP2E1 that generates a large amount of a highly reactive and cytotoxic intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI). This metabolite can be detoxified by conjugation with GSH, but may also bind covalently to hepatic parenchymal cell proteins and DNA, with resultant liver injury (Vermeulen et al. 1992; Fig. 3). Thus, hepatic damage in CYP2E1-knockout mice was mitigated, because these mice were less sensitive to APAP than wild-type mice (Lee et al. 1996). Furthermore, APAP overdose caused upregulation of CYP2E1 and direct activation of JNK-dependent cell death pathway (Das et al. 2010a). APAP-induced hepatic damage is typically found in the PC region. In a rat model with single APAP administration, Waters et al. (2001) showed that taurine administration (200 mg/kg intraperitoneal injection) 12 h before, simultaneous with, and 1–2 h after APAP treatment significantly inhibited histological damage including hepatic necrosis and inflammation in the PC region, DNA fragmentation, and hepatic lipid peroxidation. Acharya and Lau-Cam (2010) also showed the protective action of taurine as well as *N*-acetylcysteine and hypotaurine, which are the immediate metabolic precursor of taurine and the analog of cysteine, respectively, against hepatic injury induced by APAP overdose in rat via suppression of oxidative stress and alterations in GSH redox cycling, utilization, and transfer. Furthermore, taurine treatment has been recently reported to reduce APAP-induced hepatic damages and nephrotoxicity through suppression of CYP2E1 upregulation and oxidative stress and enhancement of urinary excretion of APAP (Das et al. 2010a, b).

Protective effect of taurine on TAA-induced liver damage

TAA is also widely used to induce hepatic damage experimentally. TAA-induced hepatotoxicity arises through a two-step bioactivation to thioacetamide sulfoxide (TASO) (step I) and then to thioacetamide-*S,S*-dioxide ($TASO_2$) (step II) (Fig. 2) by CYP2E1, with consequent generation of oxidative stress (Chilakapati et al. 2005; Dogru-Abbasoglu et al. 2001; Fig. 3). In TAA-treated rats, Uysal's group showed that taurine was protective against both acute and chronic hepatic damages (Balkan et al. 2001; Dogru-Abbasoglu et al. 2001). Taurine ameliorated histopathological and biochemical abnormalities and reduced lipid peroxidation in the liver in models of acute and chronic hepatic damages in the PC region induced by TAA administered in three IP injections at 24-h intervals and for 3 months in drinking water, respectively. In zebrafish, taurine treatment has been shown to improve hepatic steatosis and damage due to oxidative stress induced by TAA through significant improvement of adipocytokine-related effects via altered expression of tumor necrosis factor α (TNF-α) and adiponectin receptor 2 (Hammes et al. 2012).

Protective effect of taurine on EtOH-induced liver damage

With irregular and lower quantity intake, alcohol is metabolized in the liver through a two-step pathway of metabolism by alcohol dehydrogenase to acetaldehyde, which is toxic for the liver, and degradation of acetaldehyde to acetate by the mitochondrial enzyme acetaldehyde dehydrogenase (Fig. 3). With chronic and excessive intake that exceeds the capacity of alcohol dehydrogenase, both alcohol and acetaldehyde are also metabolized by CYP2E1, which is upregulated by EtOH (Lu and Cederbaum 2008). Since oxidative stress is generated in the CYP2E1-mediated EtOH catabolism pathway, CYP2E1 activity plays an important role in the pathogenesis of EtOH-induced liver damage and lipid peroxidation. Many previous studies have shown a protective effect of taurine on EtOH-induced liver injury through an action against oxidative stress (Bleich and Degner 2000; Erman et al. 2004; Kerai et al. 1999; Ogasawara et al. 1993; Pushpakiran et al. 2004; Watanabe et al. 1985). The efficacy of taurine for alcoholic steatohepatitis (ASH) has also been examined in EtOH intake animal models (Balkan et al. 2002; Kerai et al. 1998, 1999; Wu et al. 2009). Taurine administration at a level of 1–2 % in drinking water significantly decreased fatty acid degeneration and inflammation in histological observation, with inhibition of serum aminotransferases, inflammatory-related cytokines (interleukin-2, -6, TNF-α), and oxidative stress in a hepatic steatosis rat model induced by co-administration of EtOH and pyrazole combined with a high-fat diet (Wu et al. 2009). In the EtOH-intake rat, the hepatic taurine level is inhibited due to suppression of cysteine metabolism, including synthesis and catabolism of cysteine and GSH synthesis (Kim et al. 2003), and the influence of EtOH consumption on the taurine transport is not cleared. In addition to the anti-oxidative stress effect, Kerai et al. (1998) showed in rat that taurine inhibited the hepatic CYP2E1 activity induced by alcohol consumption. This was initially proposed to be due to a possible increase in taurine-conjugated bile acid (taurocholic acid), a potent

inhibitor of microsomal enzymes including CYP2E1, after taurine administration, but in a subsequent study the same authors rejected the effect of a taurine-enhanced increase in the taurocholic acid level on inactivation of CYP2E1 (Kerai et al. 1999). Furthermore, Chen et al. (2009) also showed that taurine supplementation reduced EtOH-induced hepatic steatosis in rat associated with the attenuations of oxidative stress and TNF-α expression in the liver. This study emphasized the relevance of taurine to the serum adiponectin that is a adipokine primarily secreted from adipose tissue and stimulates fatty acid oxidation and decreases triglyceride accumulation in the liver, because taurine prevented the decrease of serum adiponectin concentration through the inhibitions of oxidative stress, inflammatory cytokine (interleukin-6; IL-6), and transcription factors of adiponectin expression (early growth response-1; Egr-1, CCAAT/enhancer binding protein α; C/EBPα, peroxisome proliferator-activated receptor α; PPARα) in the subcutaneous adipose tissue. The authors suggested that taurine might play roles as anti-oxidative stress and as chemical chaperone/osmolyte in the subcutaneous adipose tissues.

Potential effect of taurine on non-alcoholic steatohepatitis (NASH)

Similarly to ASH, taurine may be a potent therapeutic agent for NASH. In many advanced countries, the number of patients with NASH has increased due to intake of high-fat and high-calorie diets. NASH is a necroinflammatory form of non-alcohol fatty liver disease that can promote hepatic fibrogenesis and lead to cirrhosis, liver failure, and hepatocellular carcinoma. The pathology of NASH has similar histological features to those of ASH, including fatty changes, ballooning degeneration, apoptosis and necrosis of hepatocytes, appearance of Mallory bodies, and fibrosis (Ludwig et al. 1980). These histological abnormalities in the PC region are particularly characteristic of NASH. In the development of NASH, the "two-hit theory" has been widely accepted: the first hit producing steatosis (fatty liver and/or diabetes mellitus) and the second as a source of oxidative stress capable of initiating significant lipid oxidation (Day and James 1998). Importantly, the expression and activity of CYP2E1 are also upregulated in patients and animals with NASH (Weltman et al. 1996, 1998). Therefore, there is a close relationship in NASH between the heterogeneity of expression of CYP2E1 and the predominant occurrence of steatosis and inflammation in the PC region (Buhler et al. 1992). In NASH model rats induced by a chronic high-fat diet (10 % lard + 2 % cholesterol for 12 weeks), taurine administration at 250 mg/kg/day significantly improved the distinctive morphological and histological features of NASH, including inflammation fibrosis in the PC region and hepatic lipid and glucose metabolism (Chen et al. 2006). In the fatty liver of children with simple obesity, oral taurine administration improved fatty liver and serum ALT level, along with improved body weight control (Obinata et al. 1996). The effectiveness of taurine on liver injury has also been reported in streptozotocin-induced diabetic rats (El-Batch et al. 2011). In this model, increased hepatic activity and expression of CYP2E1 were associated with the elevation of plasma ketone bodies (β-hydroxybutyrate), which might be an inducer of CYP2E1. CYP2E1 expression was significantly decreased by taurine administration, together with reduced hepatic damage and oxidative stress markers, compared to untreated controls and to animals treated with melatonin, which is also a potent scavenger of hydroxy and peroxyl radicals. Because some previous studies have shown that taurine could downregulate CYP2E1 activated by APAP (Das et al. 2010a, b) and EtOH (Kerai et al. 1998) in the liver and kidney, these findings suggest that the beneficial effects of taurine on the pathology of NASH may relate to the expression or activity of CYP2E1 in the NASH models, in addition to its role as anti-oxidative stress. Therefore, evidences for a direct relationship between CYP2E1 and taurine in NASH are needed to be obtained in future study.

Furthermore, a recent study also proposed the preventative and therapeutic potentials of dietary taurine supplementation against non-alcoholic fatty liver disease, because the inhibitive effects of taurine on nutrient- and chemical-induced hepatic steatosis, ER stress, inflammation, and injury were observed in four experimental models including palmitate-exposed primary rat hepatocytes and rat hepatoma cell line (H4IIE), long-term high sucrose fed rat, and acute ER stress (tunicamycin)-injected mice (Gentile et al. 2011).

Conclusion

The liver is the central organ of vital metabolism and functions including protein synthesis, nutrient metabolism, and detoxification. In the liver, taurine is abundantly maintained by endogenous biosynthesis and exogenous transport systems. It is recognized to be an essential nutrient due to its many physiological and biochemical roles. Decreased taurine content in the liver has been found in cases of experimental interruption of taurine transport and in liver diseases and, consequently, unexpected symptoms in the whole body would be observed due to reduced protection against oxidative stress and toxins, absence of conjugation with bile acids, and a reduced metabolic role in β-oxidation. Since previous studies have

reported that the liver damages in the PC region were induced in the TAUT KO mice and taurine has a protective action against many hepatotoxins that cause damage in the PC region, the protective effect of taurine on liver damage is suggested to be associated with the heterogeneous distribution of taurine in the hepatic lobule. Furthermore, these hepatotoxins are catabolized by CYP2E1, which is also expressed in the PC region. In addition to the inhibitive effect of taurine on the oxidative stress caused by the catabolic process of hepatotoxins through CYP2E1, some studies have shown that taurine also suppressed the activated CYP2E1. This suggests that taurine might be a useful agent for CYP2E1-related liver diseases including NASH. The therapeutic efficacy of taurine for NASH requires investigation in a future clinical study.

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Conflict of interest The authors declare that they have no conflict of interest.

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グリチルリチン製剤

商品名 強力ネオミノファーゲンシー，ミノフィット，ヒシファーゲン，グリチロン，ネオファーゲンC

松﨑 靖司

静注用グリチルリチン製剤

同種薬剤

強力ネオミノファーゲンシー®，ミノフィット®*，ヒシファーゲン®*(*はナショナルセンター採用の後発品)

作用機序

抗アレルギー作用(抗アレルギー作用，アラキドン酸代謝系酵素の阻害作用)，免疫調節作用，肝細胞傷害抑制作用，肝細胞増殖促進作用，ウイルス増殖抑制・不活化作用などが実験的に確認されている．

代表的な病態に応じた使い方

強力ネオミノファーゲンシー®(SNMC)は，国内36施設における慢性肝炎133症例を対象に，1日40 m*l*，1カ月間連日投与の二重盲検比較試験が行われた．その結果，本剤投与群67例中の有効率は68.7%，プラセボ群27.3%に比し，明らかな有意の差をもって有効であること，肝機能検査項目別では，AST，ALTおよびγ-GTP値の改善が有意の差をもって認められた．これにより，1979年「慢性肝疾患における肝機能異常の改善」が追加承認された．

SNMC100 m*l*を8週間投与して治療前後の肝組織を採取した．肝生検標本は投与前後をブラインド化．その判定の結果は，投与後の肝組織は明らかに改善していた．これによって，トランスアミナーゼ値の改善された症例は，組織像も良くなっていることが証明された．

さらに1日投与量40 m*l*では効果の不十分な症例もあることから，国内11施設における慢性肝炎，肝硬変178症例を対象に，1日40 m*l*，3週間連日静注投与を行い，2週目のALT値が正常値上限値の1.5倍以上に改善しなかった症例93例を対象に，40 m*l*継続投与群と100 m*l*増量投与群との用量別比較試験が行われた．その結果，100 m*l*増量投与群が40 m*l*継続投与群に比し，有意にALT値を改善することが認められ，40 m*l*でALT値改善不十分な症例に100 m*l*増量投与は有用であることが認められた．これにより，1994年，慢性肝疾患の用法・用量に関する承認事項の一部変更が承認され，1日100 m*l*を限度とした，大量投与が可能となった．

①薬剤名：強力ネオミノファーゲンシー®，ミノフィット®，ヒシファーゲン®

②用法・用量：通常，成人には1日1回5～20 m*l*を静脈内に注射する．なお，年齢，症状により適宜増減する．慢性肝疾患に対しては1日1回40～60 m*l*を静脈内に注射または 点滴静注する．年齢，症状により適宜増減する．な

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Column

肝庇護療法は肝細胞癌の発癌予防に有効か？

Ikedaらは表1に示す如く，342名の慢性C型肝炎患者のうち，SNMC投与を行った群での肝発癌率は5年で13.3%，10年で21.5%，非投与群では5年で26%，10年で35.5%であったと報告した．比例ハザードモデルを用いた解析では，SNMC注射の有無が有意に発癌率を低下させる要因として抽出された．

SNMCの肝発癌予防については，本邦における後ろ向き研究であるが，投与群が非投与群に比べ有意に肝発癌率が低下したとする報告がある．SNMC投与継続群84例，非投与群109例をレトロスペクティブに15年間追跡した．その結果，SNMC継続投与によりほぼ半数に発癌率の低下がみられ，ALT値が正常値の2倍以下に下降した症例では明らかな発癌抑制がみられた．この報告からC型慢性肝炎後の発癌予防には，ウイルスの排除が第一であるが，炎症を抑制してトランスアミナーゼ値を落ち着かせておくことも重要との認識が得られた．ただし，この報告では長期予後の遅いについては言及されていない．この報告からC型慢性肝炎後の発癌予防には，炎症を抑制してトランスアミナーゼ値を落ち着かせておくことも重要との認識が得られた．

お，増量する場合は1日100 m*l*を限度とする．

③効果：慢性肝疾患における肝機能異常の改善

④使用上の注意点：高齢者の投与：低カリウム血症などの副作用の発現率が高い傾向が認められるので，患者の状態を観察しながら慎重に投与する．さらに，ショックなどの発現を予測するため，十分な問診を行う，ショック発現時に救急処置のとれる準備をしておく，投与後，患者を安静な状態に保たせ，十分な観察を行うなどの基本的注意が必要である．また，甘草を含有する製剤との併用は，本剤に含まれるグリチルリチン酸が重複し，偽アルドステロン症が現れやすくなるので注意する．妊婦への投与に関する安全性は確立していないので，これらの患者には治療上の有益性が危険性を上回ると判断される場合にのみ投与する．

注射速度：静脈内投与は，患者の状態を観察しながらできるだけ投与速度を緩徐にする必要がある．グリチルリチン酸または甘草を含有する製剤の経口投与により，横紋筋融解症が現れたとの報告がある．

【表1】強力ミノファーゲン注は肝発癌のリスクを低下させる(文献3より抜粋)

Factors	Category	Risk Ratio(95% CI)	*p*
線維化レベル	F1	1	
	F2～3	2.94(1.20～7.21)	.018
	F4	9.21(3.73～22.8)	<.001
性別	1：Female	1	
	2：Male	2.80(1.35～5.81)	.006
SNMC注の有無	1：No	1	
	2：Yes	0.49(0.27～0.86)	.014

注意すべき副作用・相互作用

ループ利尿薬などを併用した場合，カリウム排泄が増強し血清カリウム低下が現れやすくなる．重大な副作用として，ショック，アナフィラキシーショック，アナフィラキシー様症状，偽アルドステロン症(頻度不明)：低カリウム血症，血圧上昇，ナトリウム・体液の貯留，浮腫，尿量減少，体重増加などの偽アルドステロン症が現れることがある．また低カリウム血症のため，脱力感，筋力低下，などの症状が現れることがある．

適応外使用

中等度以上の肝細胞障害型薬物性肝障害例(ALT 300 IU/m*l*以上)においては，SNMCの静注を行いALT改善に努める．SNMCは1回，20～100 m*l*の静注をする．

経口グリチルリチン製剤

同種薬剤

グリチロン®錠

作用機序

前記SNMCと同様に抗アレルギー作用，免疫調節作用，肝細胞傷害抑制作用，肝細胞増殖促進作用，などが実験的に確認されている．

代表的な病態に応じた使い方

慢性肝炎についての二重盲検比較試験が1980年代に施行された．その結果，国内19施設における慢性肝炎224例に対して本剤1日9錠，連日12週間経口投与を行われ，本剤投与群はプラセボ群に比し有意に肝機能の改善が認められた．

①薬剤名：グリチロン®，ネオファーゲンC®錠

②用法・用量：通常，成人には1回～3錠，小児には1錠を1日3回食後経口投与する．なお，年齢，症状により適宜増減する．

③効果：慢性肝疾患における肝機能異常の改善

④使用上の注意点：高齢者の投与には，低カリウム血症などの副作用の発現率が高い傾向が認められるので，患者の状態を観察しながら慎重に投与する．そのほか，前記SNMCの内容と同様である．

注意すべき副作用・相互作用

ループ利尿薬などを併用した場合，カリウム排泄が増強し血清カリウム低下が現れやすくなる．重大な副作用として，偽アルドステロン症（頻度不明）：低カリウム血症，血圧上昇，ナトリウム・体液の貯留，浮腫，尿量減少，体重増加などの偽アルドステロン症が現れることがある．また，脱力感，筋力低下，筋肉痛，四肢痙攣・麻痺などの横紋筋融解症の症状が現れることがあるので注意を要する．

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▷回　答
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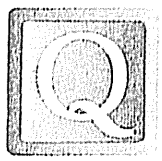

IFN治療不応・高齢者C型慢性肝炎難治例の肝庇護療法

70歳以上高齢者のC型慢性肝炎で，PEG-IFN＋リバビリン療法で治療が完結せず，SNMC，UDCA併用療法でもALT値が十分低下しない難治例について，最も効果が期待される肝庇護療法（組み合わせ，投与法）のデータがあれば併せて．
（長野県　H）

UDCA，SNMCなどの肝庇護剤は安全性も高いが，患者個人の反応によってテーラーメイド化して施行すべきであり，きめ細やかな対応ができるかかりつけ医に向いた治療法であると言える

SNMC＋UDCAは我が国においては肝庇護療法のスタンダードであり，多くの医師がこれを使用している．しかし，それらのALT値改善効果は限定されており，これらのみではALT値を正常化することはなかなか難しい．UDCAの投与量は，慢性C型肝炎には1日600mgの投与が推奨されており[1]，この効果は濃度依存性であり，保険診療上は1日900mgまでは認められている．下痢などの副作用が問題ない用量までこれを増量することが可能である．

SNMCの使用量，使用間隔については，通常は1回40mL・週3回投与が一般的であるが，1回量100mLまで増量可能であり，また，回数も連日まで増やすことが可能である．血圧上昇や低カリウム血症（hypokalemia）に注意を払う必要があること，来院が頻繁になることなどから患者の利便性が低いことなどの問題があるが，検査データを見ながら患者それぞれに合わせて行う．

これらの組み合わせでALT値がまだ高い場合，IFNに対する副作用などで治療が中止になった例以外で，本人の理解が得られる場合は，IFN少量長期投与も検討対象となる．以前のIFN治療で，SVRには至らなかったもののALTが正常化した症例がよい適応となる．PEG-IFNα-2a（ペガシス®）90μgを1～2週に1回の割合，あるいは自己注射が可能なIFNα（スミフェロン®DS）を1回量300万IU，週3回投与する．いずれの場合も効果・副作用により適宜増減し，治療は可及的に長期に行う．通常のIFN治療と同様，血小板数の減少や開始当初のインフルエンザ症状などの副作用はあるが，投与量が少なく，また治療間隔も空くため，比較的耐容性がよいことが多いが，一般の診療所で行

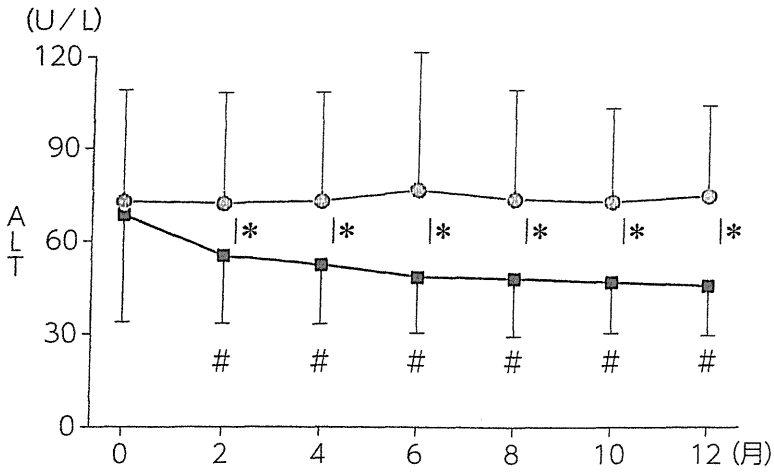

図1 SNMC+少量瀉血療法 (*n*=36) 群 (■) とSNMC療法 (*n*=36) 群 (◎) でのALTレベルの変化 (平均値±標準偏差)

＊*P*<0.05 同時期のSNMC群に比べて有意差あり.
＃*P*<0.05 ベースラインのALT値に比べて有意差あり.
(文献[4] より改変)

う場合は，導入期は肝臓専門医の意見を聞きながら施行したほうが安全である．ウイルス量の減少が得られなくても，ALT値が正常化することが多いが，投与開始後6カ月でALT値あるいはAFP値が改善しない場合は中止を検討するべきである．

IFNが使用できない症例に対して考えるべき肝庇護療法としては，瀉血療法がある．瀉血は一般的には2～4週ごとに200～400mL行い，血清フェリチン値10ng/mLあるいはHb値10～11g/dLを目標とする[2]．70歳以上の高齢者では，Hbもあまり高くないことが多く，貧血に伴う合併症（心不全や脳梗塞など）も懸念されるため，慎重に行うべきである．瀉血はUDCAまたはSNMCとの併用で相加的な効果が認められ，UDCAと瀉血療法の併用ではUDCA単独療法よりもALT値の低下が見られること[3]，またSNMCとの併用では，SNMCを投与する際に少量の瀉血を併用することによってALT値がさらに改善することが報告されている（図1）[4]．これはSNMCの注射前に週60mL程度の血液を瀉血する方法で，高齢者や代償性肝硬変（compensated cirrhosis）症例にも安全に施行できたという．例えば，週3回注射に来た場合は，1回20mLの瀉血を行う．

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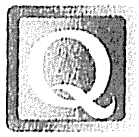
内科



γ-GTPとLDHのみ高値の理由

65歳，男性．臨床検査で20年ほど前からγ-GTP 130 IU/L前後（基準値75 IU/L），LDH 280 IU/L前後（基準値115～245 IU/L）と高値が続いている．AST 29 IU/L，ALT 23 IU/Lと常に正常．全身状態は正常．飲酒はほとんどしない．心，肝，腎その他疾患なし．中肉中背，肥満なし．γ-GTP，LDHが常に高い理由について．

（埼玉県　S）

まずAST，ALTが完全に正常値である間隔，異常値の継続期間を明確にした上でγ-GTP，LDHともに疾患を鑑別する．本例のような場合，病的に問題となるケースは少ないことが多い

γ-GTP（γ-glutamyl transpeptidase）は，ペプチドのN末端のグルタミン酸を他のペプチドまたはアミノ酸に転移する酵素である．解毒に関与している物質であるグルタチオンを作るのに重要な役割を果たしている．グルタチオンは肝ミクロソームにおける薬物代謝などに重要な役割を持つため，γ-GTPは肝細胞に多量に含まれる．ある種の薬物（ジアゼパム，フェニトイン，フェノバルビタールなどの向精神薬）などにより，γ-GTPはミクロソーム酵素としての誘導を受け血中濃度は上昇する．

さらにγ-GTPは胆道から分泌される酵素でもある．また，生体内には腎臓に最も多く含まれ，次いで膵臓，肝臓，脾臓，精巣，小腸などに多く含まれるが，血液中に存在するγ-GTPのほとんどが肝臓由来のため，血液中のγ-GTPは主に肝臓・胆道系の異常を反映する．つまり，γ-GTPは肝臓などの組織が障害を受けることによって，組織内のγ-GTPが血液中に流れ出てしまう場合（逸脱）と，アルコールや薬物などによって組織の酵素量が増える（酵素誘導）ことによって異常値を示す．またγ-GTP値には性差があり，女性ホルモンがγ-GTPの生成を抑制していると考えられ，女性に比べて男性のほうが高値を示しやすいとされる．また，年齢とともに軽度の上昇傾向があるとされている．

γ-GTPの存在は毛細胆管側や胆管上皮細胞などに多く見られるので，高値になる場合は，胆汁うっ滞を呈する薬物性，ウイルス性肝炎，原発性胆汁性肝硬変（primary biliary cirrhosis；PBC），原発性硬化性胆管炎（primary sclerosing cholangitis；PSC）などの胆管病変を呈するもの，胆道閉塞などや，アルコール性肝障害，脂肪肝といった肝細胞障害でも認められる．胆石，肝臓癌などでも上昇する．γ-GTPのみ高値を示すケースでは，その原因の多くはアルコール摂取によるものである．最近，アルコールとは無関係に，栄養過剰や肥満がもとでγ-GTPやALTが上昇する，非アルコール性脂肪性肝疾患（non-alcoholic fatty liver disease；NAFLD），非アルコール性脂肪性肝炎（non-alcoholic steatohepatitis；NASH）と言われる病気が増えてきている．これらの場合，γ-GTPのみが高い場合もある．

以上のような異常値を示す機序を勘案し，病態を考えることになろう．乳酸脱水素酵素（lactate dehydrogenase；LDH）は，細胞内

で糖をエネルギーに変える際に必要な酵素で，あらゆる臓器に含まれているが，特に心臓，肺，腎臓，脳，肝臓，骨格筋，血球（赤血球，白血球）に多く含まれる．よって，それらの細胞が障害（破壊）を受けると細胞外に出てくるため，血液中のLDHは高値を示す．高値の場合，肝疾患，心疾患，筋疾患，その他（悪性貧血，白血病，溶血性貧血など）が考えられる．

食事による変動はないが，運動によって上昇することがある．ジョギングなどの日常の軽い運動でも高値となり，時には1週間近く続くこともある．検査数日前はなるべく運動を控えたほうがよい．基準値は検査方法や測定方法，測定機器，用いる試薬，単位などにより値が異なる．また，個人個人の生理的変動によってかなり左右されることもあるため，少し値が基準値から外れていたからといって神経質にならないことである．

これらの機序を考えると，本例の場合，まず両検査値とも20年前から同じ施設で同じ測定法でチェックされているか，また数値はどのくらいの値で，どのくらいの間隔で検査を行い，どのくらいの期間異常値を指摘されていたかなどを，まず明確にしなくてはならないことが前提である．またLDHに関しては，同施設で連続的に異常値を示しているかは重要な点である．採血の時に溶血してもLDHは高値となるため，よく吟味することが必要である．

γ-GTPに関しては，上記のようにAST，ALTが完全に正常値であることがどのくらいの間隔で，どのくらいの期間，同施設で異常値が継続しているか，明確にすることが肝要である．γ-GTP，LDHともに前記のような疾患を鑑別することが第一であろう．次に画像検査などでも異常もなく，諸検査によりすべての疾患が否定された場合，どのように考えればよいかが問題となる．

明確な文献，EBMはないのであるが，筆者らの経験を述べる．LDHが異常値である点に関しては，上記の鑑別診断を施行することで比較的容易に判断できるものと考える．γ-GTPの異常値に関しての考察が重要であろう．現在，比較的多く見られる疾患は，飲酒が完全に否定されている場合，栄養過剰や肥満が原因で起こる単純な脂肪肝，特にNAFLDが多い．ただ，肝生検を施行しないと完全には診断できるものではない．

筆者らの経験例では，同様の事例に肝生検を施行してもほとんど異常を認めないことがあり，診断が明確につかない場合があった．肝生検は肝臓すべての場所をチェックできるものではなく，小さな組織片であるので，サンプリング・エラーもあり一概にすべて正常かは明確にできない．また，薬物，健康食品，食品添加物などで異常を来す場合もあり，原因の特定には大変苦労する．また，過去に薬物性肝障害を起こし，正常化せずγ-GTPの異常値だけが長期にくすぶる場合もある．

以上の通りであり，本例のような場合で病的に問題となるケースは少ないことが多いことを最後に記す．

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HEPATOBILIARY MALIGNANCIES

FGF3/FGF4 Amplification and Multiple Lung Metastases in Responders to Sorafenib in Hepatocellular Carcinoma

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The response rate to sorafenib in hepatocellular carcinoma (HCC) is relatively low (0.7%-3%), however, rapid and drastic tumor regression is occasionally observed. The molecular backgrounds and clinico-pathological features of these responders remain largely unclear. We analyzed the clinical and molecular backgrounds of 13 responders to sorafenib with significant tumor shrinkage in a retrospective study. A comparative genomic hybridization analysis using one frozen HCC sample from a responder demonstrated that the 11q13 region, a rare amplicon in HCC including the loci for *FGF3* and *FGF4*, was highly amplified. A real-time polymerase chain reaction–based copy number assay revealed that *FGF3/FGF4* amplification was observed in three of the 10 HCC samples from responders in which DNA was evaluable, whereas amplification was not observed in 38 patients with stable or progressive disease ($P = 0.006$). Fluorescence *in situ* hybridization analysis confirmed *FGF3* amplification. In addition, the clinico-pathological features showed that multiple lung metastases (5/13, $P = 0.006$) and a poorly differentiated histological type (5/13, $P = 0.13$) were frequently observed in responders. A growth inhibitory assay showed that only one *FGF3/FGF4*-amplified and three *FGFR2*-amplified cancer cell lines exhibited hypersensitivity to sorafenib *in vitro*. Finally, an *in vivo* study revealed that treatment with a low dose of sorafenib was partially effective for stably and exogenously expressed *FGF4* tumors, while being less effective in tumors expressing *EGFP* or *FGF3*. *Conclusion:* *FGF3/FGF4* amplification was observed in around 2% of HCCs. Although the sample size was relatively small, *FGF3/FGF4* amplification, a poorly differentiated histological type, and multiple lung metastases were frequently observed in responders to sorafenib. Our findings may provide a novel insight into the molecular background of HCC and sorafenib responders, warranting further prospective biomarker studies. (HEPATOLOGY 2013;57:1407-1415)

Abbreviations: 5FU, 5-fluorouracil; CGH, comparative genomic hybridization; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ *hybridization; HCC, hepatocellular carcinoma; IC_{50}, 50% inhibitory concentration; mRNA, messenger RNA; PCR, polymerase chain reaction; PIVKA-II, protein induced by vitamin K absence or antagonist-II; RPMI-1640, Roswell Park Memorial Institute 1640; RT-PCR, reverse-transcription PCR.*

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**These authors contributed equally to this work.*

treatment. The present study was approved by the institutional review boards of all the centers involved in the study, and informed consent was obtained from the patients.

Isolation of Genomic DNA. Genomic DNA samples were extracted from deparaffinized tissue sections preserved as FFPE tissue using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA samples were extracted from surgical frozen sections using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. The DNA concentration was determined using the NanoDrop2000 (Thermo Scientific, Waltham, MA).

Comparative Genomic Hybridization Analysis. The Genome-wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA) was used to perform array comparative genomic hybridization (CGH) on genomic DNA from HCC and paired liver samples according to the manufacturer's instructions. A total of 250 ng of genomic DNA was digested with both Nsp I and Sty I in independent parallel reactions, subjected to restriction enzymes, ligated to the adaptor, and amplified using polymerase chain reaction (PCR) with a universal primer and TITANIUM Taq DNA Polymerase (Clontech, Palo Alt, CA). The PCR products were quantified, fragmented, end-labeled, and hybridized onto a Genome-wide Human SNP6.0 Array. After washing and staining in Fluidics Station 450 (Affymetrix), the arrays were scanned to generate CEL files using the GeneChip Scanner 3000 and GeneChip Operating Software version 1.4. In the array CGH analysis, sample-specific copy number changes were analyzed using Partek Genomic Suite 6.4 software (Partek Inc., St. Louis, MO).

Copy Number Assay. The copy numbers for *FGF3* and *FGF4* were determined using commercially available and predesigned TaqMan Copy Number Assays according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) as described.[10] The primer IDs used for the *FGFs* were as follows: *FGF3*, Hs06336027_cn; *FGF4*, HS01235235_cn. The *TERT* locus was used for the internal reference copy number. Human Genomic DNA (Clontech) and DNA from noncancerous FFPE tissue were used as a normal control.

Real-Time Reverse-Transcription PCR. Real-time reverse-transcription PCR (RT-PCR) was performed as described.[11] In brief, complementary DNA was prepared from the total RNA obtained from each surgical frozen section using a GeneAmp RNA-PCR kit (Applied Biosystems). Real-time RT-PCR amplification was performed using a Thermal Cycler Dice (TaKaRa, Otsu, Japan) in accordance with the manufacturer's instructions under the following conditions: 95°C for 5 minutes, followed by 50 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The primers used for the real-time RT-PCR were as follows: *FGF3*, 5′-TTT GGA GAT AAC GGC AGT GGA-3′ (forward) and 5′-CGT ATT ATA GCC CAG CTC GTG GA-3′ (reverse); *FGF4*, 5′-GAG CAG CAA GGG CAA GCT CTA-3′ (forward) and 5′-ACC TTC ATG GTG GGC GAC A-3′ (reverse); *GAPD*, 5′-GCA CCG TCA AGG CTG AGA AC-3′ (forward) and 5′-ATG GTG GTG AAG ACG CCA GT-3′ (reverse). *GAPD* was used to normalize expression levels in the subsequent quantitative analyses.

Fluorescence* In Situ *Hybridization Analysis. Fluorescence *in situ* hybridization (FISH) was performed as described.[10] Probes designed to detect the *FGF3* gene and *CEN11p* on chromosome 11 were labeled with fluorescein isothiocyanate or Texas red and were designed to hybridize to the adjacent genomic sequence spanning approximately 0.32 Mb and 0.63 Mb, respectively. The probes were generated from appropriate clones from a library of human genomic clones (GSP Laboratory, Kawasaki, Japan).

Immunoblotting. Western blot analysis was performed as described.[11] The following antibodies were used: monoclonal FGF3 (R&D Systems, Minneapolis, MN), FGF4 and FGFR2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and phosphorylated FGFR and horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA). NIH-3T3 cells were exposed to the indicated concentrations of sorafenib for 2 hours and were then stimulated with FGF4-conditioned medium for 20 minutes.

Cell Growth Inhibitory Assay. To evaluate growth inhibition in the presence of various concentrations of sorafenib, we used an MTT assay as described.[12]

Plasmid Construction, Viral Production, and Stable Transfectants. The methods used in this section have been described.[12] The complementary DNA fragment encoding human full-length *FGF3* or *FGF4* was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa, Otsu, Japan) with following primers: *FGF3*, 5′-GG GAA TTC GCC GCC ATG GGC CTA ATC TGG CTG CTA-3′ (forward) and 5′-CC CTC GAG GCC CAG CTA GTG CGC ACT GGC CTC-3′ (reverse); *FGF4*, 5′-GG GAA TTC GCC GCC ATG TCG GGG CCC GGG ACG GCC GCG GTA GCG C-3′ (forward) and 5′-CC CTC GAG

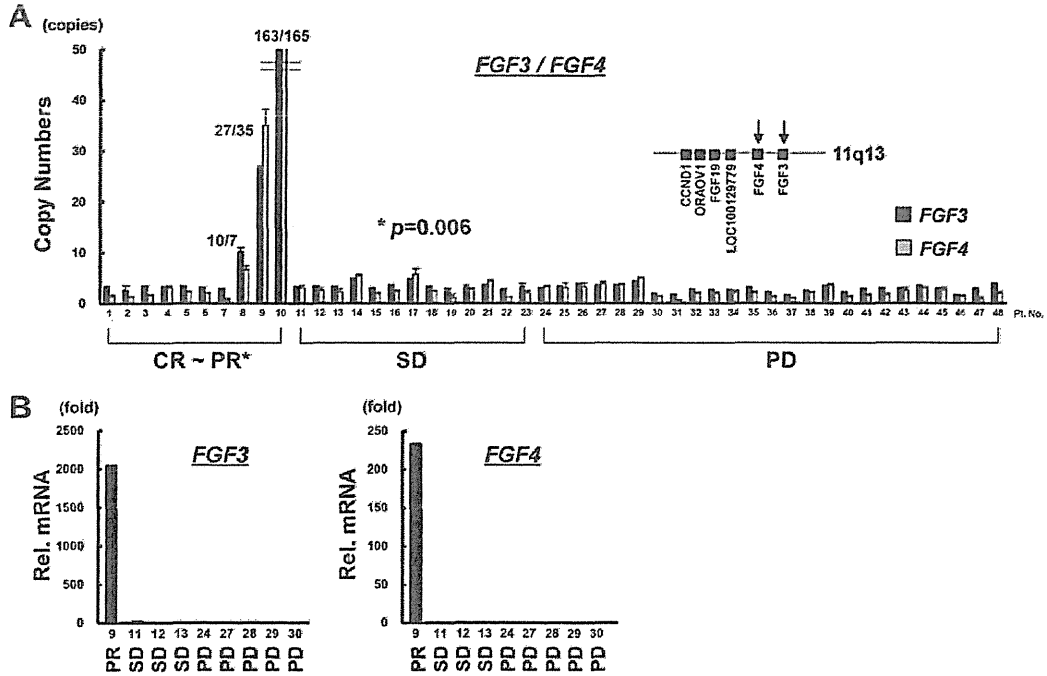

Fig. 2. *FGF3/FGF4* gene amplification is frequently observed in responders to sorafenib in HCC. (A) *FGF3/FGF4* gene amplification was determined using the TaqMan copy number assay in DNA samples obtained from 48 HCC samples that had been treated with sorafenib. *FGF3* amplification of >5 copies was observed in three of the sorafenib responders. *Complete response + partial response versus stable disease + progressive disease. (B) *FGF3/FGF4* gene amplification mediates the overexpression of *FGF3/FGF4* mRNA. The mRNA expression levels of *FGF3* and *FGF4* were examined in nine HCC samples that were available as frozen samples among 48 HCC samples that were treated with sorafenib. Rel. mRNA, *target gene/GAPD* × 10^6.

The 11q13 locus is known to be a frequently amplified region in several human cancers except HCC.[13] Thus, we hypothesized that the amplification of 11q13 may be involved in a marked response to sorafenib.

FGF3/FGF4 ***Gene Amplification Is Frequently Observed in Responders to Sorafenib.*** To address the question of whether *FGF3/FGF4* gene amplification is also found in the HCC of other responders to sorafenib, we examined HCC specimens collected from 11 other medical centers in Japan. Because most of the HCC samples were collected as FFPE samples, we used a TaqMan Copy number assay.[10] A copy number assay revealed that *FGF3/FGF4* amplification was observed in three of the 10 (30%) HCC samples that responded to sorafenib, whereas no amplification was observed in the 38 specimens from patients with stable or progressive disease ($P = 0.006$, Fig. 2A). The copy numbers for *FGF3/FGF4* were 10.2 ± 0.8/6.7 ± 0.8, 26.7 ± 0.4/35.1 ± 3.1, and 162.5 ± 9.0/165.0 ± 12.5 copies in the amplified samples, whereas the copy numbers of *FGF3* for all the other samples were below 5 copies. The correlation between the *FGF3* locus and the *FGF4* locus copy numbers was very high (R = 0.998), indicating that the DNA copy number assay for *FGF3/FGF4* was a sensitive and reproducible method.

FGF3/FGF4 ***Gene Amplification Mediates the Overexpression of*** **FGF3/FGF4** ***Messenger RNA.*** We examined the messenger RNA (mRNA) expression levels of *FGF3/FGF4* in nine HCC samples that were available as frozen samples among the 48 sorafenib-treated samples, as shown in Fig. 2A. One amplified sample expressed extremely high mRNA levels of *FGF3/FGF4* compared with nonamplified samples (Fig. 2B). The results demonstrated that *FGF3/FGF4* gene amplification mediates the overexpression of *FGF3/FGF4* mRNAs and proteins (Figs. 2B and 1D).

FISH Analysis Confirmed **FGF3/FGF4** ***Gene Amplification.*** We used FISH analysis to examine *FGF3/FGF4* amplification and to verify the results of the above-described PCR-based DNA copy number assay. All *FGF3/FGF4*-amplified clinical samples were confirmed as exhibiting high-level *FGF3* amplification using FISH analysis (Fig. 3). One patient showed multiple scattered signals, whereas two patients showed large clustered signals. Nonamplified HCC yielded a negative result for gene amplification. These results clearly demonstrate the presence of *FGF3/FGF4*-

Table 2. Clinicopathological Characteristics and *FGF3/FGF4* Gene Amplification in Responders and Nonresponders to Sorafenib

Characteristic	Responders (n = 13)	Nonresponders (n = 42)	*P* Value*
Age, years (range)	63 (47-84)	66 (22-89)	0.98
Sex, M/F	10/3	30/12	0.97
Viral status, no.			0.69
HBV	5	10	
HCV	6	16	
B+C	0	1	
Non-B, non-C	2	15	
AFP, ng/mL (range)	378 (8-404,100)	56 (2-114,248)	0.33
PIVKA-II, mAU/mL (range)	728 (14-847,000)	81 (11-147,000)	0.78
Clinical stage, no.			0.73
II	0	1	
III	3	13	
IV	10	28	
Primary tumor, cm (range)	5 (0-14)	3 (0-15)	0.20
Lung metastasis, no.			0.13
(−)	6	31	
(+)	7	11	
Multiple lung metastases, no.			0.006
<5	8	40	
≥5	5	2	
Other metastases, no.			0.24
(−)	11	26	
(+)	2	16	
Histological type, no.			0.13
Well	1	7	
Moderate	6	26	
Poor	5	6	
Combination†	1	3	
Response, no.			ND
Complete response	6	–	
Partial response	7	–	
Stable disease	–	16	
Progressive disease	–	24	
Not evaluable	–	2	

Abbreviations: AFP, alpha-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus; ND, not done.

**P* values of viral status and histological type were calculated between HBV versus HCV and poorly differentiated versus nonpoorly differentiated.

†HCC with cholangiocarcinoma component.

more common among responders to sorafenib (responders, 5/13 [38%]; nonresponders, 6/42 [14%]; $P = 0.13$). These results suggest that multiple lung metastases and a poorly differentiated histology may be clinical biomarkers for sorafenib treatment in patients with HCC.

Sorafenib Potently Inhibits Cellular Growth in* FGF3/FGF4-*Amplified and* FGFR2-*Amplified Cell Lines. We examined the growth inhibitory effect of sorafenib in various cancer cell lines to evaluate whether activated FGFR signaling is involved in the response to sorafenib. Among 26 cell lines, KYSE220 was the only *FGF3/FGF4*-amplified cell line (data not shown), and HSC-43, HSC-39, and KATOIII were the only *FGFR2*-amplified cell lines.[14] Sorafenib potently inhibited cellular growth in these four cell lines at a sub-μM 50% inhibitory concentration (IC_{50}) (Fig. 5A). The IC_{50} values were as follows: HSC43, 0.8 μM; HSC39, 0.6 μM; KATOIII, 0.4 μM; and KYSE220, 0.18 μM. These results suggest that activated FGFR signaling may be involved in the response to sorafenib.

***Sorafenib Inhibits Tumor Growth in* FGF4-*Introducing Cell Lines* In Vivo.** Finally, we established cancer cell lines stably overexpressing *EGFP*, *FGF3*, or *FGF4* to examine the relationship between the gene function of *FGF3* or *FGF4* and drug sensitivity to sorafenib *in vivo*. Western blotting confirmed that exogenously expressed FGF3 and FGF4 were secreted into the culture medium (Fig. 5B). Sorafenib inhibited the FGF4-conditioned, medium-mediated expression levels of phosphorylated FGFR (Figure 5C). A similar result was obtained using recombinant FGF4 (data not shown). Mice inoculated with these cell lines were treated with a low dose of oral sorafenib (15 mg/kg/day) or without sorafenib (vehicle control). *FGF3* overexpression did not increase the tumor volume compared with EGFP tumors; however, *FGF4* overexpression aggressively increased tumor volume and clearly enhanced the malignant phenotype (Fig. 5D). Notably, the low-dose sorafenib treatment significantly inhibited the growth of the A549/FGF4 tumors, whereas it was not effective against A549/EGFP and A549/FGF3 tumors (Fig. 5D). These results suggest that overexpression of *FGF4* is partially involved in the response to sorafenib.

Discussion

The *FGF3* gene was first identified and characterized based on its similarity to the mouse *fgf3/int-2* gene, which is a proto-oncogene activated in virally induced mammary tumors in mice.[15] Meanwhile, the *FGF4* gene was first identified in gastric cancer as an oncogene *HST*, which has the ability to induce the neoplastic transformation of NIH-3T3 cells upon transfection.[16] These genes were initially regarded as proto-oncogenes. *FGF3* and *FGF4* genes are located side-by-side and are also closely located to the *FGF19* and *CCND1* genes (within 0.2 Mb of the 11q13 region).[13] The 11q13 region is known as a gene-dense region, and gene amplification of this region is frequently observed in various solid cancers (including breast cancer, squamous cell carcinoma of the head and neck, esophageal cancer, and melanoma) at frequencies of 13%-60%.[13] On the other hand, the frequency of *FGF3/FGF4* amplification in HCC remains

sorafenib is necessary. We are presently undertaking a prospective molecular translational study (2010-2012) in a cohort of Japanese patients with sorafenib-treated HCC.

Multiple lung metastases were frequently observed among responders to sorafenib (38%) but were less common among nonresponders (5%). Based on a Japanese follow-up survey of patients with primary HCC, lung metastasis was observed in 7% (169/2355) of the patients at the time of autopsy.[20] Another study demonstrated that 15% of patients were found to have extrahepatic metastases, and lung metastasis was detected in 6% of 995 consecutive HCC patients.[21] When compared with these data from large-scale studies, the frequency of lung metastasis among responders to sorafenib seems quite high. In addition, a poorly differentiated histological type tended to be more common among responders, although the correlation was not significant.

In conclusion, we found that *FGF3/FGF4* gene amplification, multiple lung metastases, and a poorly differentiated histological type may be involved in the response to sorafenib.

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Baseline Factors and Early Viral Response (Week 4) to Antiviral Therapy With Peginterferon and Ribavirin for Predicting Sustained Virologic Response in Patients Infected With Hepatitis C Virus Genotype 1: A Multicenter Study

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Both baseline predictive factors and viral response at week 4 of therapy are reported to have high predictive ability for sustained virologic response to peginterferon and ribavirin combination therapy in patients with hepatitis C virus (HCV) genotype 1. However, it is not clear how these baseline variables and week 4 response should be combined to predict sustained virologic response. In this multicenter study, the authors investigated the impact of baseline predictive factors on the predictive value of week 4 viral response. Receiver-operating characteristic curve analyses were performed to evaluate the ability of week 4 reduction in HCV RNA levels to predict sustained virologic response in 293 Japanese patients infected with HCV genotype 1b. Analyses were performed in all patients and in patient subgroups stratified according to baseline variables. Overall, week 4 viral reduction demonstrates a high predictive ability for sustained virologic response. The sensitivity, specificity, positive predictive value (PPV), negative predictive value, and accuracy were higher than those of viral reduction at week 12. However, the best cut-off levels differ depending on the baseline factors and they were lower in patients with unfavorable baseline predictors. When patients had the TG/GG rs8099917 genotype, the best cut-off was markedly low with low PPV. Week 4 viral response can be a predictor of sustained virologic response in patients with HCV genotype 1 and is better than week 12 viral response. However, the cut-off levels should be modified based on the baseline predictive variables. ***J. Med. Virol. 85:65–70, 2013.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: chronic hepatitis C; peginterferon and ribavirin; week 4 viral response; baseline predictive factors, genetic polymorphism near the *IL28B* gene

INTRODUCTION

Although the combination antiviral therapy with peginterferon (PEG-IFN) and ribavirin has increased markedly the rate of patients with a sustained virologic response, that is, the eradication of hepatitis C virus (HCV), only 50% of patients infected with HCV genotype 1 had achieved a sustained virologic response, approximately. Several studies reported that early HCV viral dynamics during therapy have a high

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predictive value for a sustained virologic response in HCV genotype 1-infected patients. Previous studies reported that the response of HCV during combination therapy, that is, the changes in serum HCV RNA levels after starting therapy, has been shown to be an important predictor of the treatment outcome [Zeuzem et al., 2001; Buti et al., 2002; Berg et al., 2006]. Several recent reports have emphasized the importance of evaluating the viral dynamics at 4 weeks after starting therapy to predict a sustained virologic response. A rapid virologic response, in which serum HCV RNA is undetectable at 4 weeks after starting therapy, has been a strong predictive factor of a sustained virologic response reportedly [Martinez-Bauer et al., 2006; Poordad et al., 2008; Martinot-Peignoux et al., 2009; de Segadas-Soares et al., 2009]. In addition to a rapid virologic response, reduced serum HCV RNA levels at 4 weeks after starting therapy has also been reported to have a strong predictive value for the likelihood of achieving sustained virologic response to PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1 [Yu et al., 2007; Huang et al., 2010; Toyoda et al., 2011; Marcellin et al., 2012]. These studies suggested that a reduction in HCV RNA levels at week 4 is closely associated with the probability of achieving sustained virologic response.

Aside from early viral response to therapy, several baseline host and viral factors are associated with treatment outcome. Genetic polymorphism near the *IL28B* gene (rs12979860 or rs8099917) is the strongest baseline factor associated with treatment outcome in patients with HCV genotype 1 reportedly [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. In addition, studies from Japan have reported that amino acid substitutions at residue 70 of the HCV core region and amino acids 2,209–2,248 of the NS5A region of HCV (i.e., interferon sensitivity-determining region, ISDR) are viral factors associated with treatment outcome in patients with HCV genotype 1b [Enomoto et al., 1996; Akuta et al., 2005, 2007a; Donlin et al., 2007; Maekawa and Enomoto, 2009; Hayes et al., 2011]. Given these various predictors for a sustained virologic response, that is, week 4 viral response and baseline variables, how should they be combined to predict treatment outcome more precisely? In the present study, the authors investigated how to incorporate week 4 viral response to PEG-IFN and ribavirin combination therapy with baseline predictive factors to predict a sustained virologic response.

MATERIALS AND METHODS

Patients and Analyses

In this multicenter study, 682 patients who underwent PEG-IFN alpha-2b and ribavirin combination therapy in a standard treatment regimen at one of the participating institutions, (Musashino Red Cross Hospital, Kurume University Hospital, Shin-Matsudo Central General Hospital, Kagawa Prefectural Central Hospital, and Ogaki Municipal Hospital) between December 2004 and January 2010 were initially included into the retrospective analyses. All patients were infected with HCV genotype 1b; patients with HCV genotype 1a are usually not found in the Japanese general population. Pretreatment HCV RNA levels were $\geq$5.0 $\log_{10}$ IU/ml, based on a quantitative real-time PCR-based method (COBAS AmpliPrep/COBAS TaqMan HCV Test; Roche Molecular Systems, Pleasanton, CA; lower limit of quantification, 1.7 $\log_{10}$ IU/ml: lower limit of detection, 1.0 $\log_{10}$ IU/ml) [Colucci et al., 2007; Pittaluga et al., 2008], because the use of ribavirin along with PEG-IFN is not approved by Japanese National Medical Insurance System for patients with pretreatment HCV RNA levels <5.0 $\log_{10}$ IU/ml. No patients had co-infection with hepatitis B virus or human immunodeficiency virus. All patients had 100% medication adherence for both PEG-IFN and ribavirin during the initial 4 weeks of therapy and 80% or more throughout the treatment period. Among these 682 patients, three baseline factors, genetic polymorphism near the *IL28B* gene, amino acid substitution at residue 70 of the HCV core region, and ISDR sequence had been measured prior to treatment in 405 patients. We excluded 112 of these 405 patients with extended treatment duration up to 72 weeks because the extension of treatment duration might influence outcomes, leaving 293 patients who underwent 48-week standard regimen included in the final sample (Fig. 1).

Receiver-operating characteristic (ROC) analyses were performed to evaluate the value of week 4 reduction in HCV RNA levels in predicting sustained virologic response and an area under the ROC curve (AUROC) was generated. Best cut-off levels were determined based on the sensitivity and specificity. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were also calculated using these cut-off levels. Analyses were performed for all patients and subgroups according to baseline variables. The same analyses were performed on the reduction in HCV RNA levels at week 12 after starting therapy.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of each participating institution.

Measurements of Serum HCV RNA Levels, Amino Acid Substitution at Residue 70 in the HCV Core, Amino Acid Sequence of ISDR, and Genetic Polymorphism Near the *IL28B* Gene

After each patient gave informed consent, serum samples were obtained during the patient's regular hospital visits just prior to beginning treatment, every 4 weeks during the treatment period, and during the 24-week follow-up period after treatment. Serum samples were stored at −80°C until they were analyzed. HCV RNA levels were measured using a quantitative

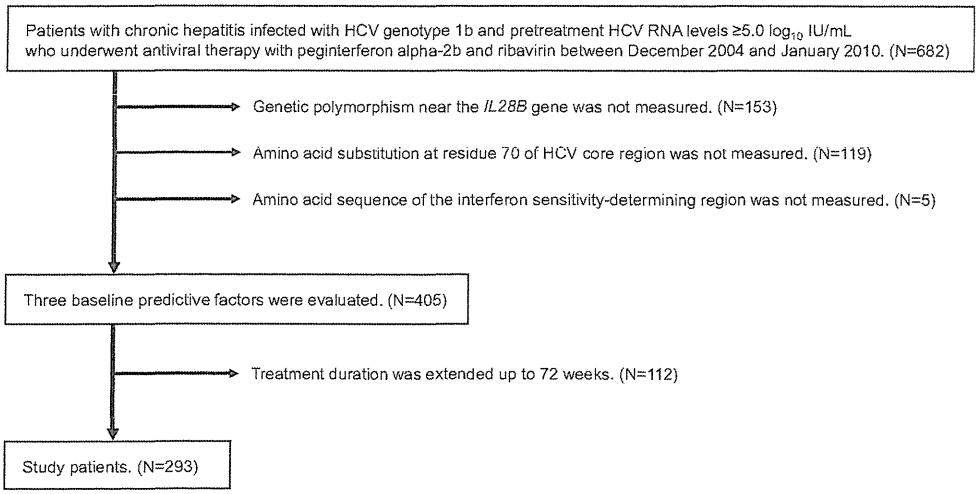

Fig. 1. Schematic representation of the study patients.

real-time PCR-based method (COBAS AmpliPrep/COBAS TaqMan HCV Test). The reductions in HCV RNA 4 and 12 weeks after starting therapy were calculated based on the pretreatment HCV RNA levels. When calculating the reduction in HCV RNA levels, HCV RNA concentration was defined as zero when HCV RNA was undetectable (i.e., rapid virologic response at week 4 and complete early virologic response at week 12).

Amino acid 70 of the HCV core region and the amino acid sequence of the ISDR were analyzed by direct nucleotide sequencing of each region as described previously [Enomoto et al., 1996; Akuta et al., 2007b]. The following PCR primer pairs were used for direct sequencing of the HCV core region:

5′-GCCATAGTGGTCTGCGGAAC-3′ (outer, sense primer), 5′-GGAGCAGTCCTTCGTGACATG-3′ (outer, antisense primer), 5′-GCTAGCCGAGTAGTGTT-3′ (inner, sense primer), and 5′-GGAGCAGTCCTTCGTGACATG-3′ (inner, antisense primer). The following PCR primers were used for direct sequencing of ISDR: 5′-TTCCACTACGTGACGGGCAT-3′ (outer, sense primer), 5′-CCCGTCCATGTGTAGGACAT-3′ (outer, antisense primer), 5′-GGGTCACAGCTCCCTGTGAGCC-3′ (inner, sense primer), and 5′-GAGGGTTGTAATCCGGGCGTGC-3′ (inner, antisense primer). When evaluating the ISDR, HCV was defined as wild-type when there were zero or one amino acid substitutions in residues 2,209–2,248 as compared with the HCV-J strain [Kato et al., 1990], and as non-wild-type when there was more than one substitution.

Genotyping of rs8099917 polymorphisms near the *IL28B* gene was performed using the TaqMan SNP assay (Applied Biosystems, Carlsbad, CA) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C_11710096_10, Applied Biosystems). Genetic polymorphism of rs8099917 reportedly corresponds to rs12979860 in more than 99% of individuals of Japanese ethnicity [Tanaka et al., 2010]. The TT genotype of rs8099917 corresponds to the CC genotype of rs12979860, the GG genotype of rs8099917 corresponds to the TT genotype of rs12979860, and the TG heterozygous genotype of rs8099917 corresponds to the CT of rs12979860.

RESULTS

Patients Characteristics and Baseline Variables

Table I summarizes patient characteristics. The polymorphism of rs8099917 was TT genotype in 204 patients (69.6%). Amino acid substitution at residue 70 was arginine in 200 patients (68.3%). HCV-ISDR was non-wild-type in 78 patients (26.6%). All these variables (TT genotype of rs8099917, arginine at residue 70, and non-wild-type ISDR) were reportedly associated with favorable response to therapy.

As a final outcome, 113 patients (38.6%) achieved sustained virologic response. Sensitivity, specificity, PPV, NPV, and accuracy were 97%, 48%, 54%, 97%, and 67%, respectively, according to genotypes of rs8099917 near the *IL28B* gene. They were 85%, 42%, 48%, 82%, and 59%, respectively, according to amino acid substitutions at residue 70 in the HCV core region, and 43%, 84%, 63%, 70%, and 78%, respectively, according to ISDR of HCV NS5A region.

Association Between Week 4 Viral Reduction and Treatment Outcome Based on Baseline Predictive Factors

Table II shows the predictive value of a reduction in serum HCV RNA levels at week 4 of therapy in all patients and based on each baseline predictive variable. Week 4 viral reduction demonstrates a high predictive ability for a sustained virologic response with