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分担研究報告書

異なる2種類のReal-time PCR法を用いたペグインターフェロン・リバビリン併用療法の治療効果予測

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研究要旨：C型慢性肝炎に対するPEG-IFNとRBV併用療法のHCV RNAのモニタリングには、従来アンプリコア定量法・定性法が用いられていたが、より高感度で広い測定範囲を有するリアルタイムPCR法が用いられるようになった。現在2種類のリアルタイムPCR法を用いることができ臨床的な有用性についてPEG-IFNとRBV併用療法中の検体を用い検討した。同一検体でのHCV RNA陰性化率はリアルタイムPCR法で低く、感度が高いことを反映していた。リアルタイムPCR法を用いることによりアンプリコア定性法と比べ特に治療開始12週でのSVRの予測能が向上した。2つのリアルタイムPCR法の臨床的な有用性は同等と考えられた。リアルタイムPCR法を用いることにより、従来よりも治療期間を適切に設定することができ治療効果の向上に寄与すると考えられた。

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A. 研究目的

難治性慢性C型肝炎の標準的治療は，Pegylated interferon (Peg-IFN)とRibavirin (RBV) の併用療法となっており、難治例であるgenotype 1b、高ウィルス量例でのsustained virological response (SVR)率は40～50%で、年齢、性別、ウィルス陰性化時期などいくつかの因子により治療効果が予測されることが報告されている。とりわけ、HCV RNAの測定は治療前においては治療内容の決定、治療中のモニタリングに必須であり，投与開始4，8，12，24週でのHCV RNAの陰性化が治療効果予測のための重要な因子である。ウイルスが12週で検出され、24週で陰性化するlate virological response (LVR)症例においては、治療期間を延長することにより治療効果の向上が得られることが報告され治療期間の決定のためにも必須の検査である。

従来は、HCV RNA 量の測定は，PCR 法を用いたアンプリコア定量法、定性法が広く用いられていたが、より高感度でかつ広い測定範囲を有するリアルタイムPCR法を用いたHCV RNA定量法が開発された．2007年11月にリアルタイムPCR法を用いたコバスTaqMan HCV®オート（Roche Diagnostics）が発売され、その後，アキュジーンm-HCV（Abbott Molecular Inc.）も使用可能となった．今回、この2種類のリアルタイムPCR法を用いた治療効果予測について保存血清を用い、従来のアンプリコア定性法と比較検討した。

B. 研究方法

PEG-IFNα2b(1.5μg/kg)とRBV併用療法を48週間行ったゲノタイプ1b、高ウィルス量のC型慢性肝炎患者60例（男：女＝37：23、平均年齢53歳）について、凍結保存血清を用い，治療開始後4，8，12，16，20，24，48週，終了後24週の HCV RNA を，アキュジーン m-HCV，TaqMan® HCV オートとアンプリコア定性法で測定し以下の検討を行った。

1) 各ポイントにおける それぞれの測定法によるHCV RNA の陰性化率。
2)それぞれの測定法を用いてHCV RNAの検出の有無について乖離がみられた検体の検討。
3) 各測定法でHCVRNA検出感度未満となった時期別の治療効果予測について比較検討した．
4) 治療終了時のHCV RNAの測定．再燃例でRNAが検出される例があるか否かについて検討した。

なお、それぞれの測定法の感度は、開発時のデータによると、アンプリコア定性法50IU/mL、アキュジーン m-HCV12IU/mL，TaqMan® HCV オート15IU/mLである。

(倫理面への配慮)

臨床試験の目的・方法、治療の副作用、患者に関する個人情報の守秘義務、患者の権利保護等について十分な説明を行い、患者が熟考するに十分な時間と理解の後に書面による同意を得たうえで臨床試験を遂行した（新 GCP に遵守）。既に医療保険が認められている治療法においても上記に準じて書面の同意書を得ている。

C. 研究結果

治療成績はSVR26例、治療終了後再燃16例、無効15例、中止3例であった。

1) 各ポイントにおける それぞれの測定法によるHCV RNA の陰性化率（図1）

各週における HCV RNA 検出感度未満の割合を比較すると、アンプリコア定性法と比較して、アキュジーンm-HCV、TaqMan® HCV オートともに検出感度未満となる割合が低く、感度が高いことを反映していた。アキュジーンm-HCVとTaqMan® HCV オートを比較すると、有意差はないが、若干TaqMan® HCV オートのほうが，同時期において検出感度未満となる割合が低い傾向がみられた。特に治療開始12週での各検査での HCV RNA 検出感度未満の割合の乖離が大きく、アンプリコア定性法58.3%、アキュジーンm-HCV41.7%、TaqMan®

HCV オート35.0%であった(アンプリコア定性法 vs. TaqMan® HCV オート；$p<0.05$)。

2) それぞれの測定法を用いて HCV RNA の検出の有無について乖離がみられた検体の検討

2つのリアルタイム PCR 法で HCV RNA が検出感度未満となった検体において、アンプリコア定性法で陽性であった検体はなかった。アンプリコア定性法で陰性であった検体251例中、アキュジーン m-HCV で検出された検体は36例、TaqMan® HCV オートで検出された検体は50例、両者で検出された検体は25例みとめた。一方、2種類のリアルタイム PCR 法の間で乖離がみとめられた検体については、アキュジーン m-HCV で検出されず、TaqMan® HCV オートで検出された検体25例、TaqMan® HCV オートで検出されずアキュジーン m-HCV で検出された検体11例であった。

3) 各測定法で HCVRNA 検出感度未満となった時期別の治療効果予測について

治療開始12週での HCV RNA 検出感度未満の著効に対する positive predictive value(PPV)は、アンプリコア定性法 72.7%、アキュジーン m-HCV 83.3%、TaqMan® HCV オート 92.3%、negative predictive value(NPV)は、アンプリコア定性法100%、アキュジーン m-HCV 95.5%、TaqMan® HCV オート 85.2%であった。

また HCV RNA がはじめて検出感度未満となった時期別の SVR 率についての検討を示す（図2）。いずれの測定法を用いても8週までに RNA が検出感度未満となった例では、ほぼ全例で SVR が得られていた。9−12週で検出感度未満となった例の SVR 率はアンプリコア定性法 55%、アキュジーン m-HCV 75%、TaqMan® HCV オート87%であった。また13−24週で検出感度未満となった例の SVR 率はアンプリコア定性法 0%、アキュジーン m-HCV 26%、TaqMan® HCV オート40%であった。なお、いずれの測定法においても、25週以降に検出感度未満となった例での SVR は認めなかった。

12週でのアンプリコア定性法とリアルタイム PCR 法での乖離例についての検討では、アンプリコア陰性でアキュジーン m-HCV で検出された10例中6例、アンプリコア陰性で TaqMan® HCV オートで検出された14例中8例で高確率に再燃していた。

4) 治療終了時の HCV RNA の測定

再燃例12例の治療終了時の HCV RNA を測定し、アンプリコア定性法、アキュジーン m-HCV ではすべての検体で陰性、検出感度未満であったが、TaqMan® HCV オートでは2例において HCV RNA が検出された。一方、著効例においては、アキュジーン m-HCV の1例と別の症例で TaqMan® HCV オートで測定した2例において HCV RNA が検出された。

D. 考察

今回検討した2種類のリアルタイム PCR 法は従来のアンプリコア定性法と比較し感度、測定範囲について精度が向上している。今回の PEG-IFN と RBV 併用療法を施行中の検体で検討しても、同一時期の HCV RNA 陰性化率はリアルタイム PCR 法で低く、感度の向上を反映する結果であった。従来のアンプリコア定性法を用いた検討では、治療開始12週までに HCV RNA が陰性化した症例においての SVR 率はおおむね70%と報告されており、本検討においても 72.7%であったが、アキュジーン m-HCV 83.3%、TaqMan® HCV オート 92.3%と感度が高くなったことにより12週での治療効果予測が向上した。また HCV RNA がはじめて検出感度未満となった時期別の SVR 率についての検討においてはいずれの測定法を用いても8週までに RNA が検出感度未満となった例では、ほぼ全例で SVR が得られていたが、9−12週で検出感度未満となった例の SVR 率はアンプリコア定性法 55%、アキュジーン m-HCV 75%、TaqMan® HCV オート 87%であった。12週で HCV RNA が検出され、24週で検出感度未満となる LVR 症例では治療期間を72週に延長することが望ましいとされており、リアルタイム PCR 法を用いることにより、12週時点で、より適切に治療延長が望ましい症例を判別することができ、治療効果の向上が見込めると考えられる。実際に今回の検討において、12週でアンプリコア陰性であったがアキュジーン m-HCV で検出された10例中6例、アンプリコア陰性で TaqMan® HCV オートで検出された14例中8例で48週の治療期間では高確率に再燃しており、このような症例の SVR 率の向上につながるものと考えられる。

また、アキュジーン m-HCV と TaqMan® HCV オートの比較については、有意差はないが、TaqMan® HCV オートのほうが、12週での PPV が高い傾向にあった。臨床的な有用性は同等と考えられるが、現在、感度については同一検体を用い検討中である。

治療終了時の HCV RNA 測定においては、12例の再燃例のうち TaqMan® HCV オートで測定した2例で微量の RNA が検出されており、治療後再燃症例の予測ができる可能性が示唆された。しかしながら、著効例においてもアキュジーン m-HCV1例、TaqMan® HCV オート2例で検出された。原因として、コンタミネーション、測定系の疑陽性の可能性も否定はできない。また、今まで測定することのできなかった極めて微量のウィルス動態については、今後の前向き検体での検討が必要である。

E. 結論

リアルタイム PCR 法を用いて HCVRNA を測定することによって PEG-IFN と RBV 併用による治療効果を正確にモニターできるようになり、治療効果予測能が向上した。また、従来よりも治療期間を適切に設定することができ治療効果の向上に寄与すると考えられた。2つのリアルタイム PCR 法の有用性は同等と考えられた。

F. 健康危険情報

特記すべきことなし。

G. 研究発表

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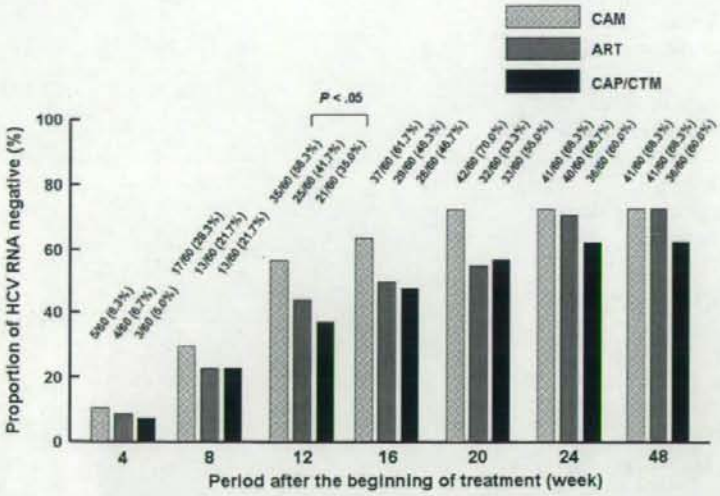
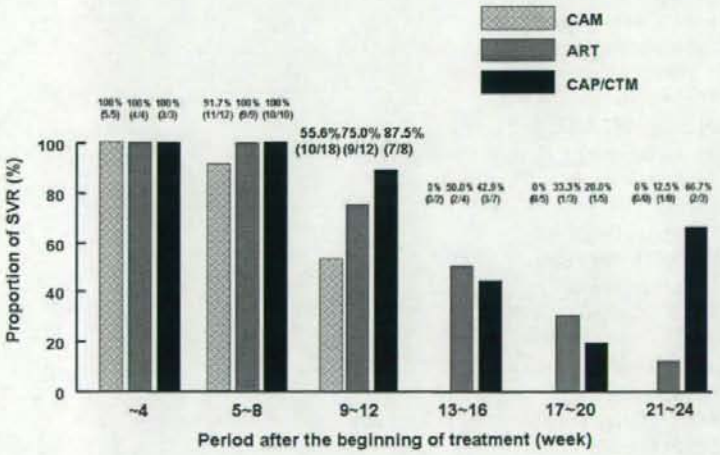


（図 1）
各週における HCV RNA 検出感度未満の割合（n=60）

CAM（アンプリコア定性法）
ART（アキュジーン m-HCV）
CAP/CTM（TaqMan HCV オート）



（図 2）
HCV RNA が 検出感度未満となった時期別の SVR 率

CAM（アンプリコア定性法）
ART（アキュジーン m-HCV）
CAP/CTM（TaqMan HCV オート）

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Original Article

Suppressive effect of oral administration of branched-chain amino acid granules on oxidative stress and inflammation in HCV-positive patients with liver cirrhosis

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Aim: In chronic hepatitis C virus (HCV) infection, it is thought that both chronic persistent inflammation and oxidative stress contribute to the development of hepatocellular carcinoma (HCC), and it has been reported that long-term oral supplementation with branched-chain amino acid (BCAA) granules could inhibit liver carcinogenesis. However, the extent of the involvement of these factors remains obscure.

Methods: To clarify the involvement of inflammation and oxidative stress in the inhibition of liver carcinogenesis, we evaluated the effect of oral administration of BCAA granules on oxidative stress and inflammation in HCV-positive patients with liver cirrhosis.

Results: Twenty-seven patients were enrolled in the study: 18 of the patients were treated with BCAA granules (administered group) and nine were observed without BCAA granules (non-administered group). In the non-administered group, the production of oxidative stress, as indicated by urine 8-hydroxydeoxyguanosine (8-OHdG) and 15-F2t-isoprostane (8-IsoPs), significantly increased with time, while in the administered group the levels of ferritin and 8-OHdG decreased significantly. Comparison of the two groups demonstrated that highly sensitive CRP, ferritin, 8-OHdG and 8-IsoPs were significantly reduced by taking BCAA granules. The time-course analysis showed that ferritin and highly sensitive CRP seemed to decrease first, followed by a decrease of 8-OHdG and 8-IsoPs.

Conclusion: These findings indicated that the administration of BCAA granules influenced microinflammation and the metabolism of iron in HCV-positive patients with liver cirrhosis, and subsequently seemed to reduce the production of oxidative stress, possibly leading to a decrease in the occurrence of HCC.

Key words: branched-chain amino acid, hepatitis C virus, liver cirrhosis, oxidative stress

INTRODUCTION

THERE ARE APPROXIMATELY 170 million people infected with hepatitis C virus (HCV) worldwide.[1] HCV is a major causative agent of liver disease, including chronic hepatitis, liver cirrhosis (LC) and hepatocellular carcinoma (HCC). It is estimated that 70–80% of HCV infections evolve into chronic infection. Once HCV develops into cirrhosis, HCC develops at an annual rate of 5–7%.[2] Nevertheless, the precise mechanism underlying HCV-associated HCC is not completely understood. HCV may contribute to the development of HCC by facilitating the accumulation of genetic damage as a result of continuous cell death followed by regeneration in the course of chronic hepatitis. If this is the case, HCV would only be associated indirectly with hepatocarcinogenesis. Another possibility is the direct involvement of HCV in hepatocarcinogenesis, whereby some products of the virus may be oncogenic and involved in cell transformation. Recently, the core protein of HCV has been shown to induce HCC in a transgenic mouse model and has been suggested to play a central role in the development of HCC in chronic

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hepatitis C.[3] However, it still remains unclear how the core protein influences the development of HCC. An additional possibility is the involvement of oxidative stress. Recently, numerous studies have shown that HCV infection itself causes oxidative stress to a greater degree than other inflammatory liver disease.[4,5]

Oxidative stress is defined as a disturbance of the normal balance between oxidants and antioxidants in the body. Under oxidative stress, reactive oxygen species (ROS) can modify macromolecules such as lipids, proteins and nucleic acids (DNA). If ROS-mediated DNA damage is not repaired, it leads to the production of genetic mutations as well as gross chromosomal alterations and contributes to cancer development in multistep carcinogenesis.[6]

Clinically, when long-term supplementation with branched-chain amino acids (BCAA) is given to patients with compensated or decompensated cirrhosis, their nutritional status, as indicated by hypoalbuminemia, ammonia metabolism and glucose metabolism, is improved.[7] Additionally, a recent study has shown that long-term oral supplementation with BCAA can inhibit liver carcinogenesis, especially in overweight or obese patients.[8]

Against this background, we hypothesized that when BCAA granules are administered orally to patients with LC, the improvement of hypoalbuminemia, glucose and ammonia metabolism, as well as the inhibitory effect on hepatocarcinogenesis, might be due to a reduction of oxidative stress by the BCAA supplementation. In the present study, we investigated the effects on oxidative stress of administering BCAA granules orally to patients with LC.

METHODS

Subjects, design and protocol

THE SUBJECTS INCLUDED in the present study were outpatients with compensated cirrhosis who had hypoalbuminemia despite adequate food intake.

A total of 27 subjects, 18 of whom were given a 4 g BCAA preparation (LIVACT Granules; Ajinomoto, Tokyo, Japan) administered orally three times daily after meals (administered group), and nine of whom were followed up without the BCAA granules as a control (non-administered group), were enrolled in the present study. All of subjects were confirmed to be HCV-positive and HBV-negative patients with liver cirrhosis and without other liver diseases, including HCC as shown by image techniques, and not to be have received any medication with BCAA granules, intravenous albumin administration, or enteral nutrition for hepatic insufficiency within 8 weeks before enrollment. Patients were followed up for at least 6 months and blood and urine samples were collected at treatment initiation (0 M) and at 1 month (1 M), 2 M, 3 M, 4 M and 6 M for as long as possible. Routine laboratory parameters as shown in Table 1, highly sensitive CRP, ferritin and oxidative stress markers (urine 8-hydroxydeoxyguanosine [8-OHdG] and urine 15-F2t-Isoprostane [8-IsoPs]) were evaluated.[9] Routine laboratory parameters were measured on every visit to hospital. Blood and urine samples were stored at −70°C until highly sensitive CRP, ferritin, urine 8-OHdG and urine 8-IsoPs were centrally measured at SRL MediSearch (Tokyo, Japan). The raw values (nanogram: ng/mL) of urine 8-OHdG and urine 8-IsoPs were conventionally corrected by urinary creatinine (mg/dL) to make it possible to compare them with each other.[9]

It was confirmed that the patients' medications had not been changed within 8 weeks before enrollment or

Table 1 Characteristics of HCV patients with LC

	P	C
Number of patients	18	9
Age	56–80	52–79
Median	70.2	66.8
Sex (male/female)	9/9	4/5
Routine parameters		
Uric acid (mg/dL)	5.44	5.8
NH3 (μg/dL)	64.44	68.56
T-protein (g/dL)	7.59	7.53
Alb (g/dL)	3.84	3.68
AST (IU/L)	68.39	67.44
ALT (IU/L)	49.94	46
LDH (IU/L)	228.29	230.44
ALP (IU/L)	338.12	325.67
T-chol (mg/dL)	153.94	168.56
FBS (mg/dL)	138.56	119.44
PT-INR	1.1	1.13
AFP (ng/mL)	33.39	16.06
Centrally measured parameters		
Ferritin (ng/mL)	65.93	45.23
Highly sensitive CRP (ng/mL)	1043.56	428.11
8-OHdG (ng/mL)	11.07	9.35
8-IsoPs (ng/mL)	373.96	338.57
Hepatocellular carcinoma	0	0

ALT, alanine transaminase; C, control patients; HCV, hepatitis C virus; LC, liver cirrhosis; P, patients who were orally administered branched-chain amino acid (BCAA) preparation thrice a day.

during the administration of BCAA. The eligibility and exclusion criteria were made according to the manufacturer's instructions.

This study was a post-marketing clinical trial and was performed with the approval of the review board of Social Insurance Chukyo Hospital. Written informed consent to participate in this trial was obtained from all subjects.

Statistical analysis

The primary end point was the amount of change in oxidative stress markers. The *t* statistic was used to evaluate whether the study drug was useful for reducing oxidative stress. Data were further analyzed by the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.

RESULTS

THE CHARACTERISTICS OF the two groups of patients were comparable in terms of age, sex and underlying liver disease severity, as shown in Table 1.

To assess how the evaluated variables altered during the observation, the data from before (0 M) and at 6 months (6 M) were compared in each group (Table 2). The results showed that total cholesterol (T-chol) was significantly decreased and PT-INR was significantly prolonged, and also that urine 8-OHdG and 8-IsoPs were significantly increased at 6 M in the non-administered group. The decrease in T-chol and the prolongation of PT-INR might mean that the liver diseases were becoming more severe and that oxidative stress was increasing in the patients over time. In contrast, in administered group, urine 8-OHdG and serum ferritin at 6 M significantly decreased, suggesting that administering BCAA granules regularly might reduce oxidative stress. Considering that highly sensitive CRP was slightly, although not significantly, increased in the non-administered group and slightly decreased in the administered group during the observation period, it was considered necessary to compare the alterations between the non-administered group and the administered group. Therefore, the time-dependent alterations (Δp or $\Delta c = value_{6M} - value_{0M}$) of all parameters were compared between the two groups using the Mann–Whitney *U*-test. The results showed that ferritin, highly sensitive CRP, urine 8-OHdG and urine 8-IsoPs were significantly decreased by BCAA administration. Considering that ferritin reflects the body's iron storage[10] and that highly sensitive CRP can detect changes in microinflammation, our data suggested that taking

Table 2 Results of statistic analysis

	Uric acid	NH3	T-protein	Alb	AST	ALT	LDH	T-chol	FBS	PT-INR	AFP	Ferritin	H-CRP	8-OHdG	8-IsoPs
P (0 M)	5.44	64.44	7.59	3.84	68.39	49.94	228.29	154.94	138.56	1.10	33.29	65.03	1043.56	11.07	373.96
P (6 M)	5.70	75.22	7.63	3.87	63.44	48.56	213.92	160.33	122.89	1.11	34.88	53.15	351.28	0.03	337.89
P-value*	0.284	0.334	0.877	0.62	0.602	0.619	0.238	0.664	0.236	0.454	0.758	0.024	0.095	0.0003	0.518
C (0 M)	5.80	68.56	7.53	3.68	67.44	46.00	230.44	168.56	119.44	1.13	16.06	45.23	428.11	9.35	338.57
C (6 M)	5.61	80.89	7.29	3.59	59.33	41.11	211.89	158.00	120.56	1.16	26.21	44.87	591.44	10.64	512.41
P-value*	0.433	0.439	0.086	0.303	0.181	0.359	0.055	0.021	0.865	0.004	0.445	0.881	0.311	0.042	0.047
Mann–Whitney**	0.245	>0.10	0.291	0.194	0.857	>0.10	0.483	$P > 0.010$	0.799	$P > 0.05$	$P > 0.10$	0.037	0.041	0.00038	0.025

ALT, alanine transaminase; AST, aspartate aminotransferase; C, control patients; H-CRP, highly sensitive CRP; P, patients who were orally administered branched-chain amino acid (BCAA) preparation thrice a day.
*$P < 0.05$ versus data of 0 M for data of 6 M (*T*-test). **$P < 0.05$ versus data of ΔP ($P_{6M} - P_{0M}$) for ΔC ($C_{6M} - C_{0M}$) (Mann–Whitney *U*-test).
Data depict mean values.

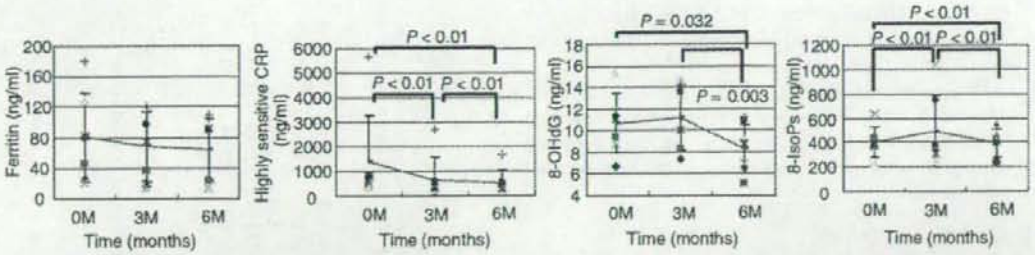

Figure 1 Time-course analysis of ferritin, highly sensitive CRP, 8-OHdG and 8-IsoPs. In seven patients of the administered group, markers of oxidative stress (8-OHdG and 8-IsoPs), ferritin and highly sensitive CRP were measured at 0 M, 3 M and 6 M. Each point represents the mean ± SD. Ferritin decreased slightly, although not significantly, and highly sensitive CRP significantly decreased over time. The values of 8-OHdG and 8-IsoPs did not decrease, but rather increased at 3 M and then significantly decreased at 6 M.

BCAA granules regularly reduced iron storage, microinflammation and oxidative stress.

Next, we evaluated how these parameters that were reduced by BCAA administration altered over time in seven patients of the administered group. As shown in Figure 1, ferritin decreased slightly although not significantly, while highly sensitive CRP significantly decreased with time. In contrast, urine 8-OHdG and 8-IsoPs were increased at 3 M and then significantly decreased at 6 M, compared to their levels before BCAA administration. Considering that it has been reported that hepatic iron accumulation was present in patients with chronic hepatitis C, the decrease of ferritin might indicate the improvement of not only hepatic iron accumulation, but also iron metabolism.[10] Therefore, these data may indicate that the reduction of oxidative stress was followed by an improvement of the iron metabolism and microinflammation.

DISCUSSION

THE AIM OF this study was to analyze the effects on oxidative stress of the oral administration of BCAA granules to HCV-positive patients with LC. In terms of the usefulness of BCAA granules, in the present study the level of albumin did not change significantly, which was probably due to the number of enrolled patients, although some papers have described BCAA granules as improving nutritional disorders associated with abnormal protein and amino acid metabolism in patients with LC.[4,11] Additionally, one *in vitro* study has shown that an increase in the molar ratio of BCAA to aromatic amino acids reduced the growth of HepG2 cells;[12] and recently Muto *et al.* reported that long-term supplementation with BCAA decreased the risk for HCC in patients with LC. These authors pointed out that hyperinsulinemia and peripheral insulin resistance are important mechanisms, although these mechanisms are not yet fully understood.[8] Under these circumstances, if BCAA granules did suppress the production of oxidative stress in HCV-positive patients with LC, it would be another potential mechanism by which the incidence of HCC in HCV-LC patients could be reduced. Therefore, in this study, which we believe is the first report on this topic, we focused on the effect of BCAA on oxidative stress. However, the present study was not a control study but a preliminary study; in the future a larger scale study will be required.

The risk of developing HCC is significantly increased in patients with chronic hepatitis. Hepatocarcinogenesis is considered a multi-step process, in a similar way to the carcinogenesis of other cancers, and is thought to involve at least two factors. One is HCV viral protein, especially the core protein that has been shown to alter the oxidant–antioxidant status of the liver,[4] and another is chronic inflammation. Regarding the former, several studies have investigated the possible role of oxidative injury in the pathogenesis of hepatitis C.[13,14] In particular, oxidative damage is evident in both HCV-infected patients and in HCV-transgenic mice in the absence of necroinflammatory changes or transaminoferase release.[4,15] Moreover, a direct relationship between HCV core protein and HCC has been reported recently.[3,16] Certainly, it is easily to imagine that when DNA damage caused by oxidative stress is not repaired, the accumulation of genetic damage leads to the development of hepatic neoplasm. It has actually been demonstrated that antioxidant treatment improved the liver injury of chronic hepatitis C patients in a prospective study.[17] Clinically, with oral administration of BCAA, we expe-

rienced not only that the nutritional status of such patients improved, but also that the incidence of HCC was reduced.[8] These findings prompted us to evaluate the effects of BCAA granules on the production of oxidative stress. The results, as expected, showed that urine 8-OHdG and 8-IsoPs were significantly reduced compared with those patients followed up without BCAA administration.

In addition to the substantial role of oxidative stress in HCV-associated hepatocarcinogenesis, chronic inflammation is also important. In asymptomatic patients who show normal alanine transaminase (ALT) levels despite having HCV-RNA, the possibility of progression of the disease to LC and HCC has been reported, but HCV-positive HCC is rarely observed in healthy carriers, and is mainly observed in patients with LC.[18] This means that chronic inflammation, creating a cycle of repeated hepatocyte destruction and regeneration, is very important to the development of HCC. On the other hand, chronic hepatitis in the absence of hepatitis virus infection, such as autoimmune hepatitis, carries a low risk for the development of HCC.[19] Based on these findings, it is thought that both HCV proteins and chronic inflammation contribute to the development of HCC additively or synergistically. In the present study, the level of ALT and aspartate aminotransferase (AST) was not significantly changed, but microinflammation, as indicated by highly sensitive CRP, not usual CRP, was found to be first reduced, followed by the reduction of oxidative stress by oral administration of BCAA granules.

However, oxidative stress is reported to be influenced by various things: alcohol consumption, supplementations such as vitamin A tablets, other inflammation and malignancy complications. In considering the mechanism by which the production of oxidative stress was reduced by the oral administration of BCAA granules, we experienced one interesting patient. This patient took iron supplementation for anemia from before entry into this study until 6 M after the enrollment. Despite administrating BCAA granules, the production of oxidative stress was not decreased in this patient, but rather gradually increased. When the patient stopped taking the iron supplements, the production of oxidative stress gradually decreased (data not shown). In the present study ferritin, which is considered to reflect the body's iron store, was shown to be significantly decreased, along with oxidative stress, in the administered group. There are some papers in the literature reporting that iron stimulates the production of ROS and that hepatic iron overload induces HCC.[20] These findings may suggest that BCAA is able to reduce the production of ROS by involving with the iron metabolism.

BCAA granules certainly seemed to reduce the production of oxidative stress and microinflammation, which could possibly lead to a decrease in the occurrence of HCC with long-term supplementation, but as the number of enrolled patients in this study was limited, in the future large-scale studies in which patients with LC, including patients without HCV infection, are administered BCAA granules, will be necessary.

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Abbott RealTime HCV and Roche COBAS AmpliPrep/COBAS TaqMan HCV assay in predicting of sustained virological response to pegylated interferon and ribavirin in chronic hepatitis C patients

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Abbreviations: HCV, hepatitis C virus; ALT, alanine aminotransferase; PCR, polymerase chain reaction; PEG-IFN, pegylated interferon; RBV, ribavirin

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ABSTRACT

Two commercial real-time PCR assays are currently available for sensitive hepatitis C virus (HCV) RNA quantification; Abbott RealTime HCV (ART) and Roche COBAS AmpliPrep/COBAS TaqMan HCV assay (CAP/CTM). We assessed whether the two real-time PCR assays were more effective than Roche COBAS Amplicor HCV Monitor test, v.2.0 (CAM) in prediction of the sustained virological response (SVR) to pegylated interferon (PEG-IFN) plus ribavirin (RBV) in chronic hepatitis C. Sixty patients chronically infected with HCV genotype 1b (M:F=37:23, 53±12 years) were treated with PEG-IFNα2b plus RBV for 48 weeks. Stored specimens at 9 points for each patient (at baseline, on-treatment and 24 weeks after treatment) were tested by the two real-time PCR assays and CAM. Twenty-six (43.3%) patients reached SVR. Positive predictive value (PPV) for SVR of undetectable HCV RNA at week 12 by CAM, ART and CAP/CTM were 74.3%, 88.0% and 95.2%, respectively. Undetectable HCV RNA by CAM, ART and CAP/CTM correctly predicted SVR at week 4 in 100%, 100% and 100%, at week 5-8 in 91.7%, 100% and 100%, at week 9-12 in 55.6%, 75% and 87.5%, and at week 13-24 in 0%, 26.7% and 40%, respectively. Of 16 patients relapsed after treatment, in two patients HCV RNA were detectable at end of treatment by CAP/CTM, but undetectable by ART and CAM. HCV RNA test using ART and CAP/CTM are considered to be more effective in predicting SVR than CAM, and PPV for SVR was slightly higher in CAP/CTM than ART.

INTRODUCTION

The quantification of hepatitis C virus (HCV) RNA is essential for the management of chronic hepatitis C therapy based on the combination of pegylated interferon (PEG-IFN) and ribavirin (RBV). Based on pivotal trials in large multicenter studies, positive and negative predictions of sustained virological response (SVR) using viral load kinetics have been established and are now used for recommendations on antiviral therapy management by the American and European international consensus conference (1, 7, 10). Initially, the effectiveness of antiviral therapy had been controlled by HCV RNA tests using end-point PCR assays sensitive to a level of 50-100 IU/ml, Roche COBAS Amplicor HCV Monitor test, v.2.0 (CAM; Roche Molecular Systems Inc., Pleasanton, CA, US). Recently, the two novel commercial real-time PCR assays became available for highly sensitive HCV RNA quantification; Abbott RealTime HCV (ART; Abbott Molecular Inc., Des Plaines, IL, US) and Roche COBAS AmpliPrep/COBAS TaqMan HCV assay (CAP/CTM; Roche Molecular Systems Inc., Pleasanton, CA, US). Reported sensitivity of these real-time PCR assays is higher than that of CAM, they are reportedly not prone to carryover contamination, and have a consistently wider dynamic range of quantification, which makes them particularly useful for quantifying the full range of viral genome levels observed in treated and untreated patients. Real-time PCR is rapidly replacing other technologies for the routine quantification of HCV RNA.

In present study using stored serum samples collected from patients chronically infected with HCV receiving PEG-IFNα2b and RBV, ART and CAP/CTM were clinically evaluated, with a particular regard to predicting effectiveness of the treatment. The serum samples were obtained at various time points during the therapy and the viral load in each of the specimens was evaluated using CAM, ART and CAP/CTM.

MATERIAL AND METHODS

Patients. A total of 60 patients were enrolled in this study from those received

PEG-IFNα2b plus RBV therapy for chronic HCV infection at three hospitals (Naogoya City University Hospital, Social Insurance Chukyo Hospital and Nagoya City Johoku Hospital). The mean age of the population was 53 years, and 37 (61.7%) were male. All patients had genotype 1b. The mean alanine transaminase (ALT) level was 63.4 ± 39.1 IU/L; mean platelet count was 16.1 ± 6.0 × 10^4/mm^3; mean HCV RNA level was 2311 ± 1600 KIU/mL by CAM. Other causes of chronic hepatitis and HIV infection were excluded by appropriate serological testing and/or liver histology. All patients were treated with PEG-IFNα2b (1.5 mg/kg subcutaneous injection once a week) plus RBV (600-1000 mg daily according to body weight), orally, for 48 weeks. The PEG-IFNα2b plus RBV doses were individually reduced during the treatment whenever needed to lessen side effects, and these dose reductions were done according to the labeling.

Written informed consent was obtained from each patient, and the study was approved by the local Ethics committee in accordance with the 1975 declaration of Helsinki.

Definitions of Response. All patients had HCV RNA testing at weeks 4, 8, 12, 16, 20, 24, and 48 of the combination therapy. Follow-up testing was performed at week 72. On-treatment virological response, SVR, and relapse were defined on the basis of CAM results in accordance with standard definitions. On-treatment response was defined as undetectable serum HCV RNA determined during the antiviral therapy. SVR was defined as serum HCV RNA tested undetectable 6 months after the end of therapy.

Detection of HCV RNA. A total of 486 serum samples were obtained from the 60 patients. Each of the specimens was frozen to -80℃ within 2 hours of collection (11). HCV RNA quantitation in the sera was performed by CAM, ART and CAP/CTM according to the corresponding manufacturer's instruction. ART, which is provided with an automated sample preparation, was carried on the m2000sp and the m2000rt instruments, and CAP/CTM, which is also provided with an automated sample preparation, was carried on the COBAS AmpliPrep and COBAS TaqMan instruments.

The lower limit of detection was 12 IU/ml as reported for ART (15), 15 IU/ml for CAP/CTM (8) and 50 IU/ml for CAM (16). Positive results (signals) below the quantitative HCV RNA concentrations are referred to as "low-positive" when registered by ART and CAP/CTM.

In general, the HCV RNA levels were measured for each patient in 9 serum specimens obtained at baseline, at week 4, 8, 12, 16, 20, 24 and 48 of the treatment and finally after 6 months following the discontinuation of therapy.

Statistical Analysis. Descriptive statistics are shown as the mean ± SD or the median and interquartile range as appropriate. Categorial variables were compared between groups by the $\chi 2$-test or Fisher's exact test, and non-categorial variables by the Mann–Whitney's U-test. A P value less than 0.05 were considered significant.

RESULTS

Virological Response. Among the 60 patients, 26 (43.3%) had SVR, 16 (26.7%) relapsed, 15 (25%) did not respond, 3 (5%) discontinued the treatment due to side effects.

Comparison of CAM, ART and CAP/CTM Testing. The proportion of cases with undetectable HCV RNA revealed by each of the three assays among the patients tested at different time points during the therapy, is depicted in Figure 1. At week 12, the difference between CAM and CAP/CTM was significant ($P<.05$), but at the other time-points the difference was not significant.

Of the 243 specimens with HCV RNA undetectable by CAM, ART and CAP/CTM revealed presence of the viral genome copies in 36 and 50 specimens, respectively. In addition, CAP/CTM allowed detection of HCV RNA in 25 specimens that were negative by ART. On the other hand, there were some 11 specimens with HCV RNA undetectable by CAP/CTM but positive by ART (Table 1).

Prediction of SVR Based on each assay. Positive predictive value (PPV) and negative predictive value (NPV) for SVR evaluated on basis of undetectable HCV RNA as revealed by each of the three assays at weeks 4, 8, 12, 16, 20 and 24 of the treatment start (Table 2). At week12, PPV by CAM, ART and CAP/CTM were 74.3%, 88.0% and 95.2%, respectively. When we combined results of these 2 assays at week 12, PPV and NPV of undetectable HCV RNA by both ART and CAP/CTM were 94.4% and 78.6%, respectively, and those by ART and/or CAP/CTM were 89.3% and 96.9%, respectively. Of note, all cases undetectable HCV RNA by ART or CAP/CTM at 8 week reached SVR. As shown in Fig. 2, firstly undetectable HCV RNA by CAM, ART and CAP/CTM could predicted SVR at week 9-12 in 55.6%, 75% and 87.5%, and at week 13-24 in 0%, 26.7% and 40%, respectively.

Prediction of Relapse by CAP/CTM Testing at the End of Treatment. Of 16 patients relapsed after treatment, in two patients very low levels of HCV RNA (positive results below the limit of quantification) were detectable at end of treatment by CAP/CTM, but undetectable by ART or CAM. On the other hand, of 26 patients reached SVR, very low levels of HCV RNA at the end of treatment were detectable in two patients by CAP/CTM and in one by ART but undetectable by CAM (Fig. 3). In these three cases, HCV RNA was undetectable afterward.

DISCUSSION

The two commercial real-time PCR assays currently available for highly sensitive HCV RNA quantification were comparatively evaluated in this study. These assays are reportedly more sensitive, have a wider dynamic range of quantification than CAM, are "specific", "accurate", "precise", and "reproducible" (4, 8, 15, 17, 23, 24).

The proportion of HCV RNA-negative specimens as determined by CAP/CTM or ART was lower than that by CAM during the whole treatment periods, especially at week 12 (CAP/CTM vs. CAM; $P < .05$); implying higher sensitivity of these new

real-time PCR assays, which makes them superior in their clinical value than CAM.

According to pivotal trials in large multicenter studies, positive and negative predictions of SVR estimated on basis of viral load kinetics are clinically reliable and are now used in antiviral therapy management by recommendation of the American and European international conferences consensus (1, 7, 10). The ability to predict either a positive or negative therapeutic response is of obvious benefit to clinicians and patients (13, 21). Positive predictive evidence early in the course of treatment could be used to reinforce the importance of compliance in ensuring a successful outcome. Conversely, negative predictive capability would allow clinicians to discontinue therapy early during the course of treatment, which would save health care resources and, more important, could prevent drug-related adverse events. A dynamic prediction model based on the early virological response showed that the outcome of combination therapy with PEG-IFN and RBV is dependent on the rapidity of the viral response (7, 19, 20). Therefore, early virologic response monitoring is now the standard of care for tailoring treatment to the individual patient's response (3, 5, 6, 12). The data for the proposal algorithm in the management of antiviral therapy in patients chronically infected with HCV were mainly based on measurement of viral load by CAM. We sought to determine whether testing by the two real-time PCR assays during therapy could further improve accuracy of predicting SVR to PEG-IFN and RBV therapy. Almost all patients whose HCV RNA went undetectable until week 8 as determined by ART, CAP/CTM or CAM had reached SVR. The rates of SVR among patients who had firstly undetectable HCV RNA during week 9 and 12 by CAM, ART or CAP/CTM were 55.6%, 75.0% or 87.5%, respectively (Fig. 2), suggesting that the ART or CAP/CTM could be more specific than CAM in predicting the SVR during the first 12 weeks of the therapy. Compared ART to CAP/CTM, PPV for SVR of undetectable HCV RNA during the first 12 weeks were slightly higher in CAP/CTM than ART, but the difference did not reach significance. Recently, it has been reported that the sensitivity of CAP/CTM was

slightly higher than ART on the basis of 'limit of detection (LOD)' using international HCV WHO standard specimens (genotype 1a) (14). Further 'LOD' studies using serum samples with other genotypes are needed to conclude the sensitivity between ART and CAP/CTM.

Recently, it has been reported that extension of treatment with PEG-IFN plus RBV from 48 to 72 weeks increases the rate of SVR in patients with late virological response defined as HCV RNA positive at week 12 but negative at week 24 (2). In this study, some of the patients, who at week 12 had HCV RNA undetectable by CAM, were found to be positive by ART or CAP/CTM, and 60% (6/10, ART) or 57% (8/14, CAP/CTM) of these patients failed to achieve SVR. In such cases, the extended treatment up to 72 weeks might provide higher rate of SVR.

The rate of successful HCV eradication in chronically infected patients has significantly increased following the introduction of PEG-IFN plus RBV combination therapy. Nevertheless, still from 15 to 25% of the patients who become HCV RNA negative by CAM during and at the end of therapy had viral relapse after treatment withdrawal. There are no identified on-treatment markers able to predict whether the patient will develop SVR or will relapse. Recently, it has been reported that minimal residual viremia detected by transcription-mediated amplification assay (the lower limit of detection is 5-10 IU/ml) (22) at the end of therapy in CAM negative cases could reliably predict relapse after therapy withdrawal (9). In this study, of 16 patients had relapsed after treatment; in two of the patients HCV RNA was detectable by CAP/CTM, but undetectable by ART or CAM at the end of treatment. Minimal residual viremia detected by CAP/CTM might be predictive of post-treatment relapse. Whether relapse could have been prevented by continuing treatment for a longer duration remains to be confirmed in prospective controlled trials.

On the other hand, of 26 patients reached SVR, 3 patients had HCV RNA detectable by ART or CAP/CTM at the end of treatment (Fig. 3). Similarly, it has been

reported that minimal residual viremia were detectable by transcription-mediated amplification assay (the lower limit of detection is 5-10 IU/ml) at the end of treatment in the patients reached SVR (18). One possibility is that some patients with SVR became undetectable for HCV RNA even after discontinuation of therapy. However, it might be difficult to exclude the contamination, falsely positive or the detection of low levels of noninfectious fragments of the virus genome of a little clinical significance. These explanations remain hypotheses, and then it is necessary to examine minimal residual viremia by highly sensitive assays on or after treatment with PEG-IFN and RBV in large-scale prospective cohorts.

In conclusions, the results show that real-time PCR using any of the two newly available commercial assays instead of CAM can provide higher clinical value for the management of therapeutic responses to chronic hepatitis C. The ability to detect extremely low levels of serum HCV RNA may allow clinicians to predict outcome of treatment with higher confidence and develop more individualized protocols for treatment in terms of optimal duration.

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