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HEPATOLOGY

8-Hydroxy-2′-deoxy-guanosine is a risk factor for development of hepatocellular carcinoma in patients with chronic hepatitis C virus infection

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Key words
8-hydroxy-2′-deoxy-guanosine (8-OHdG), hepatitis C Virus, hepatocellular carcinoma.

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Abstract

Background and Aim: Increased production of reactive oxygen species, which cause oxidative DNA damage, is considered to be related to hepatocarcinogenesis. 8-Hydroxy-2′-deoxy-guanosine (8-OHdG) is a useful marker of DNA damage induced by oxidative stress. The aim of this study was to determine whether expression of 8-OHdG is a risk factor for the development of hepatocellular carcinoma (HCC) in patients with hepatitis C virus (HCV) infection.

Methods: The expression of 8-OHdG in liver biopsy specimens was assessed immunohistochemically. In total, 104 patients with chronic HCV infection who were diagnosed on liver biopsy between January 1987 and December 2002 were studied retrospectively. Univariate and multivariate analyses using age, gender, habitual drinking, tobacco exposure, diabetes mellitus, serum alanine aminotransferase level, HCV genotype, hepatic fibrosis, inflammation, steatosis, and 8-OHdG expression in liver biopsy specimens were conducted to identify factors related to the development of HCC.

Results: On multivariate analysis, 8-OHdG and fibrosis were independent and significant risk factors for HCC development (relative risk, 2.48; $P = 0.023$; relative risk, 5.35; $P = 0.001$, respectively). Furthermore, the cumulative incidence rate of HCC in 39 patients with high 8-OHdG expression levels was significantly greater than that in 65 patients with low 8-OHdG expression levels ($P = 0.043$). In addition, liver 8-OHdG expression was correlated with hepatic inflammation.

Conclusions: 8-OHdG is a risk factor for the development of HCC in patients with chronic HCV infection. Patients with chronic HCV who express 8-OHdG should be monitored carefully for the development of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and one of the leading causes of cancer death in Japan.[1] During the past several decades, the incidence of HCC has increased substantially in Japan. The increase in the incidence of HCC has been attributed to the increased number of patients with hepatitis C virus (HCV)–associated liver cirrhosis.[2] Several risk factors for the development of HCV-associated HCC have been identified, including age,[3] gender,[4] total alcohol intake,[5,6] tobacco exposure,[6] diabetes mellitus,[7] cirrhosis,[5,8] irregular regeneration of hepatocytes,[9] HCV genotype,[10] elevated serum alanine aminotransferase (ALT),[11] and interferon (IFN) treatment.[12] Failure to achieve a sustained virological response (SVR) to IFN-based therapy, as well as preexisting advanced hepatic fibrosis and/or cirrhosis, are the major predictors for the development of HCC and mortality.[12] The identification of additional variables associated with the risk of developing HCC is important as this would help in designing preventive medical programs.

8-Hydroxy-2′-deoxy-guanosine (8-OHdG), a DNA base-modified product generated by reactive oxygen species, is a mutation prone to induce G-C to T-A transversion at DNA replication.[13] Previous studies have shown that 8-OHdG is a good marker of oxidative DNA damage,[14] and is implicated in carcinogenesis[14,15] and hepatocarcinogenesis.[16] 8-OHdG is also produced in chronic liver diseases, including chronic hepatitis C.[17] The objectives of this study were to investigate the relationship between 8-OHdG expression and histological findings (inflammation, fibrosis) in the liver and, using univariate and multivariate analyses, to determine whether 8-OHdG is an independent risk factor for the development of HCC in patients with chronic HCV infection.

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Methods

Patients

Liver biopsies were obtained from 548 patients with no detectable HCC at Hokkaido University Hospital, Sapporo, Japan, from January 1987 to September 2002. Patients with bleeding tendencies or clinically evident cirrhosis associated with ascites or hepatic encephalopathy were excluded from the biopsy procedure. In this retrospective study, of the 548 patients who were assessed initially, 104 fulfilled the following criteria and were enrolled: (i) liver biopsy that showed histopathological features of chronic hepatitis or cirrhosis; (ii) HCV-RNA-positive based on the amplicor monitor assay[18] (Roche Diagnostic Systems, Tokyo, Japan); (iii) negative for hepatitis B surface antigen (HBsAg), antinuclear antibody (ANA), and antimitochondrial antibody (AMA) in the serum, based on radioimmunoassay and spot hybridization techniques; (iv) no sustained virological response to IFN therapy; (v) more than 60 months of follow-up at our hospital; and (vi) no occurrence of HCC within 6 months after liver biopsy. At the time of the liver biopsy, the physicians asked about alcohol drinking habits. Habitual heavy drinking was defined as an average daily consumption of an amount equivalent to 65 g of pure ethanol over a period of 5 years. Tobacco exposure was defined as ≥ 20 pack-years, because 1 pack (20 cigarettes)/day has been shown to be a risk for HCC over a 20-year period.[6] Diabetes mellitus was diagnosed on the basis of the American Diabetes Association guidelines.[19]

Serologic tests

Frozen-stored sera obtained from patients at the time of liver biopsy were tested. The serum HCV-RNA level was determined using an Amplicor GT HCV monitor (Roche Diagnostic Systems). HCV genotypes 1 and 2 were determined using a serologic genotyping assay.[20] In this assay, genotypes 1 and 2 correspond to genotype 1 (1a, 1b) and genotype 2 (2a, 2b) as proposed by Simmonds *et al.*[21]

Follow-up of patients

During follow-up, clinical evaluations and biochemical tests were performed every 1–3 months. Patients underwent liver ultrasonography or computed tomography at least every 6 months. HCC was diagnosed based on the American Association for the Study of Liver Disease guidelines.[22] The endpoint used in this study was the appearance of HCC; 30 September 2007 was used as the reference date. Ninety-five patients were followed until the endpoint was reached. The duration of follow-up was 61 to 204 months (mean observation time, 122 months). The patients' background characteristics at study entry are summarized in Table 1.

Histopathological examination of the liver

Liver histology was evaluated according to the degree of fibrosis and the degree of inflammatory activity. The degree of fibrosis (staging) and the degree of necroinflammatory activity (grading) were classified according to Desmet *et al.*[23] as follows: mild fibrosis included F0 (none), F1 (periportal expansion), and F2 (portoportal septa), and severe fibrosis included F3 (portocentral linkage or bridging fibrosis) and F4 (cirrhosis). Mild necroinflammatory activity included grade A1, while severe activity included grades A2 and A3. Hepatic steatosis was graded according to Brunt *et al.*[24] In short, steatosis observed in up to 33%, 33%–66%, and more than 66% of the liver histology was classified as grade 1, 2, and 3, respectively. If no hepatic steatosis was observed, grade 0 was assigned.

Table 1 Clinical and laboratory characteristics of 104 patients

Characteristic	No of patients
Gender (male/female)	44/60
Median age (years, mean ± SD)	50.5 ± 11.5
Habitual drinking (yes/no)	28/76
Tobacco exposure (yes/no)	18/86
Diabetes mellitus (yes/no)	19/85
ALT (IU/L, mean ± SD)	79.8 ± 50.9
HCV genotype (1/2/undetermined)	79/18/7
IFN (yes/no)	75/29

ALT, alanine aminotranferase; HCV, hepatitis C virus; IFN, interferon.

Immunohistochemistry

Immunohistochemical staining was done on formalin-fixed, paraffin-embedded tissue sections using an immunoperoxidase method, as described previously.[25] Briefly, each section was deparaffinized, rehydrated, and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature and then washed in phosphate-buffered saline. Normal swine serum (Dako, Glostrup, Denmark) was applied for 30 min and then removed. The sections were incubated with mouse monoclonal antibodies for anti-8-OHdG antibody (Japan Institute for the Control of Aging, Shizuoka, Japan) at a dilution of 1 : 50 overnight at 4°C. The specimens were subsequently washed three times in phosphate-buffered saline, and then incubated with a secondary antibody for 30 min at room temperature. A Vectastatin Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) was used for antibody detection. The degree of 8-OHdG immunostaining was expressed as the labeling index (LI), based on the percentage of positive cells identified by dark brown staining of the nucleus per 1000 cells; the relationship between the LI and the development of HCC was examined.

Statistical analysis

The cumulative HCC incidence rates were calculated and plotted using the Kaplan–Meier method. Univariate and multivariate analyses of the risk ratios for the occurrence of HCC were done using Cox's proportional hazards regression analysis. The risk factors examined included age, gender, habitual heavy drinking, diabetes mellitus, mean follow-up serum ALT (≥ 90 IU/L or < 90 IU/L), staging (mild fibrosis or severe fibrosis), and grading (mild activity or severe activity). Differences between the two groups were analyzed using the log-rank test. All P values were two-tailed, and P-values < 0.05 were considered significant. Statistical analysis was performed using Stat View software (version 5.0; SAS Institute, Cary, NC, USA).

Results

Histopathological findings

Examples of the histopathological findings are shown in Table 2. Based on the histopathological staging, five patients (4.8%) had F0 fibrosis, 45 patients (43.3%) had F1 fibrosis, 33 patients (31.7%) had F2 fibrosis, 15 patients (14.4%) had F3 fibrosis, and six patients (5.7%) had F4 fibrosis. Based on the histopathological grading, 45 patients (43.3%) had A1 necroinflammatory activity, 54 patients (51.9%) had A2 activity, and five patients (4.8%) had A3 activity. Hepatic steatosis was present in 25 (24%) of the 104 patients. Based on the histopathological grading, 79 patients (76%) had grade 0 steatosis, 22 patients (21.6%) had grade 1, 3 patients (2.9%) had grade 2, and no patients had grade 3 steatosis.

Hepatocyte expression of 8-OHdG

8-OHdG expression was detected in 62 (59.6%) of the 104 patients with chronic hepatitis C. However, 8-OHdG expression was very weak in 23 of the 62 cases; the 8-OHdG LI was less than 30% in these 23 cases. Thus, to objectively evaluate the relationship between quantitative expression of 8-OHdG and HCC development, 30% was defined as the 8-OHdG LI cut-off point. Nuclear expression of 8-OHdG in hepatocytes was especially abundant in periportal areas with piecemeal necrosis and prominent cell infiltration (Fig. 1).

Relationship between liver 8-OHdG LI and grading and staging

The 8-OHdG LI was correlated with the pathologic grading of necroinflammatory activity: the mean 8-OHdG LIs were 25.1% for A1, 34.8% for A2, and 48% for A3 (Fig. 2a). However, the 8-OHdG LI was not strongly correlated with pathologic staging of liver fibrosis: the mean 8-OHdG LI increased gradually as the stage increased from F0 (15%) to F1 (24.8%) and then F2 (39.1%), but the mean 8-OHdG LI values were similar in the F2 (39.1%), F3 (37.3%), and F4 (36.7%) stages (Fig. 2b).

Table 2 Histologic characteristics of 104 patients

Characteristic	No. of patients (%)
Distribution of stage of fibrosis (F)	
0	5 (4.8)
1	45 (43.3)
2	33 (31.7)
3	15 (14.4)
4	6 (5.7)
Distribution of grade of inflammation (A)	
1	45 (43.3)
2	54 (51.9)
3	5 (4.8)
Distribution of grade of steatosis	
0	79 (76.0)
1	122 (21.6)
2	23 (2.9)
3	30 (0)

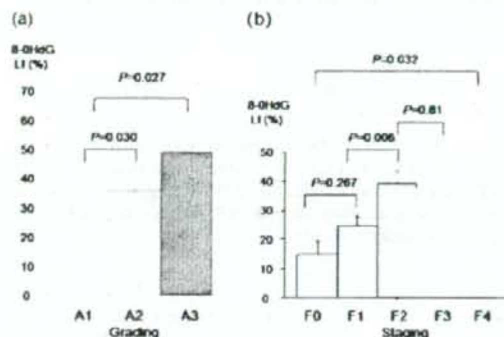

Figure 2 Differences in the 8-hydroxy-2′-deoxy-guanosine (8-OHdG) labeling index by liver biopsy grade (a) and stage (b).

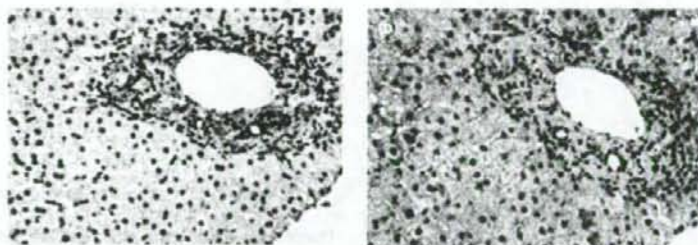

Figure 1 Immunohistochemical localization of 8-hydroxy-2′-deoxy-guanosine (8-OHdG) in patients with chronic hepatitis C (CH-C). Representative histology of CH-C (b) (hematoxylin–eosin stain, original magnification × 20), and 8-OHdG immunostaining of a serial section (a). Many 8-OHdG-positive hepatocytes are present in the portal area of tissue obtained from a patient with CH-C. Cells with a brown-stained nucleus were considered positive. In this patient, the positive rate was 70%.

Analysis of the clinicopathological factors affecting HCC development

During follow-up, 35 of the 104 patients (33.7%) developed HCC; 22 patients were diagnosed as having HCC based on the results of ultrasonography-guided biopsy, and 13 patients were diagnosed as having HCC based on the presence of the typical vascular pattern on dynamic imaging. The cumulative incidence rates of HCC were 14.7% at 7 years, 29.1% at 10 years, and 50.6% at 15 years after the liver biopsy. To determine the factors that may influence the development of HCC, a Cox's proportional hazards regression analysis was performed. On univariate analysis, the following three factors significantly affected the incidence of HCC: patient age at the time of liver biopsy (relative risk, 2.02; 95% confidence interval [CI], 1.03–3.97; $P = 0.042$), histopathological stage (fibrosis: relative risk, 4.10; 95% CI, 2.00–8.39; $P = 0.001$), and 8-OHdG expression (relative risk, 2.09; 95% CI, 1.07–4.11; $P = 0.031$) (Table 3). Gender, alcohol intake, tobacco exposure, diabetes mellitus, HCV genotype, serum ALT, and pathological grade were not correlated with HCC development. On multivariate analysis of the determinants of HCC using a Cox regression model, fibrosis (relative risk, 5.35; 95% CI, 1.96–14.6; $P = 0.001$) and 8-OHdG expression (relative risk, 2.48; 95% CI, 1.13–5.41; $P = 0.023$) were identified as statistically independent risk factors (Table 4).

Relationship between the liver 8-OHdG LI and HCC development

Overall, 39 (37.5%) of the 104 patients had a high 8-OHdG LI (30%). As shown in Figure 3, the cumulative incidence rate of HCC in these 39 patients with high 8-OHdG expression levels was significantly greater than that in the 65 patients with low 8-OHdG expression levels ($P = 0.043$).

Table 3 Univariate analysis of risk ratios for hepatocellular carcinoma

Characteristic	Risk ratio	95% CI	*P*-value
Gender (compared with female)	1.37	0.70–2.75	0.37
Age (compared with < 55)	2.02	1.03–3.97	0.042[†]
Habitual drinking (compared with no habitual drinking)	1.38	0.64–2.99	0.41
Tobacco exposure (compared with no tobacco exposure)	1.02	0.52–2.00	0.95
Diabetes mellitus (compared with absent)	1.30	0.61–3.00	0.49
HCV genotype (compared with genotype 2)	1.15	0.48–2.80	0.75
Mean ALT (compared with < 90)	1.23	0.62–2.44	0.56
Inflammation (compared with mild)	1.45	0.72–2.95	0.30
Fibrosis (compared with mild)	4.10	2.00–8.39	0.001[†]
Steatosis (compared with grade 0)	1.62	0.67–3.93	0.29
8-OHdG expression (compared with < 30%)	2.09	1.07–4.11	0.031[†]

[†]Statistically significant.
95% CI, 95% confidence interval; ALT, alanine aminotranferase; HCV, hepatitis C virus; IFN, interferon; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

Discussion

The current study evaluated the incidence of HCC and analyzed the factors that affect the incidence of HCC in patients with HCV-related chronic liver disease diagnosed on liver biopsy. Patients who were sustained virological responders to IFN therapy were not enrolled because it is well known that the development of HCC in such patients is significantly suppressed.[11] On univariate and multivariate analyses, it was found that the 8-OHdG expression level and fibrosis were significant independent risk factors for

Table 4 Multivariate analysis of risk ratios for hepatocellular carcinoma

Characteristic	Risk ratio	95% CI	*P*-value
Gender (compared with female)	1.33	0.58–3.03	0.50
Age (compared with < 55)	2.00	0.88–4.44	0.10
Habitual drinking (compared with no habitual drinking)	1.48	0.60–3.65	0.40
Tobacco exposure (compared with no tobacco exposure)	1.17	0.31–4.40	0.82
Diabetes mellitus (compared with absent)	1.30	0.35–4.40	0.73
HCV genotype (compared with genotype 2)	0.92	0.29–3.00	0.89
Mean ALT (compared with < 90)	1.13	0.30–4.29	0.86
Inflammation (compared with mild)	1.51	0.67–3.36	0.31
Fibrosis (compared with mild)	5.35	1.96–14.6	0.001[†]
Steatosis (compared with grade 0)	1.50	0.66–3.40	0.33
8-OHdG expression (compared with < 30%)	2.48	1.13–5.41	0.023[†]

[†]Statistically significant.
95% CI, 95% confidence interval; ALT, alanine aminotranferase; HCV, hepatitis C virus; IFN, interferon; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

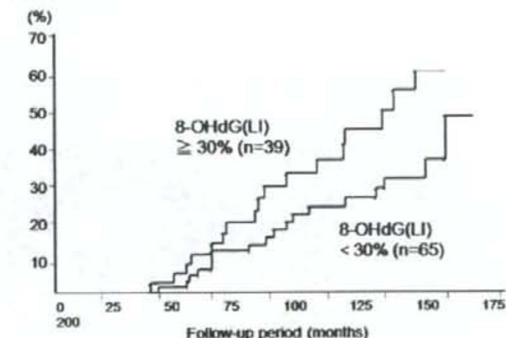

Figure 3 Cumulative incidence rates of hepatocellular carcinoma (HCC) based on the prevalence of 8-hydroxy-2′-deoxy-guanosine (8-OHdG) expression in patients with chronic hepatitis C. The cumulative incidence rate of HCC in 39 patients with high 8-OHdG expression levels was significantly higher than that in 65 patients with low 8-OHdG expression levels ($P = 0.043$).

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HCC development. Age, gender, total alcohol intake, tobacco exposure, diabetes mellitus, HCV genotype, and elevated serum ALT were not correlated with HCC development. A relationship between the 8-OHdG expression level and HCC development was also demonstrated.

It is not clear why the rate of HCC occurrence was high in patients with high liver 8-OHdG expression. 8-OHdG is one of the main markers of oxidative DNA injury. During hepatocarcinogenesis, the increased production of reactive oxygen species is thought to cause oxidative DNA damage. Studies of hepatocarcinogenesis related to the presence of chemical carcinogens, such as peroxisome proliferators and N-nitrosodiethylamine, have also shown that 8-OHdG is induced during liver DNA damage.[16] 8-OHdG is considered to be a useful indicator for investigating the involvement of active oxygen in hepatocarcinogenesis.[26] The expression of 8-OHdG has been reported in the liver of patients with chronic hepatitis C,[27,28] cirrhosis,[29] and HCC.[30] These reports noted that there is a relationship between the 8-OHdG expression level in the noncancerous region and postoperaive recurrence of HCC in the remnant liver.[31] It was also found that the number of 8-OHdG-positive hepatocytes was greater in HCC than in chronic hepatitis.[30] Based on our results and these reports, it is possible that 8-OHdG-positive hepatocytes have a high-grade malignancy potential in HCC and that 8-OHdG could thus be a useful marker of HCC development. In the present study, the 8-OHdG LI was shown to be correlated with the pathologic inflammation, but the univariate and multivariate analyses showed that pathologic inflammation did not affect the incidence of HCC. The reason for this discrepancy is not clear. In several reports in which it was reported that 8-OHdG caused by oxidative DNA damage was involved in hepatocarcinogenesis, Sekoguchi et al. showed that 8-OHdG-positive hepatocytes had shorter telomeres than 8-OHdG-negative hepatocytes,[32] Kinoshita et al. reported formation of 8-OHdG and cell-cycle arrest in the rat liver via generation of oxidative stress in association with p21 and cyclin D1,[33] and Shen et al. reported an association between 8-OHdG and cell proliferation.[34] On the basis of these reports, 8-OHdG formation may be involved in alteration of cellular proliferation and apoptosis in the liver; these phenomena do not always accompany necroinflammation of the liver. Thus, the presence of 8-OHdG is considered to not only reflect the severity of liver cell injury (inflammation, fibrosis), but also to induce gene mutations, which implies that the presence of 8-OHdG is closely involved with hepatocarcinogenesis. Therefore, it is likely that 8-OHdG plays a role in hepatocarcinogenesis via a mechanism other than inflammation. Further analyses are required to identify the role of 8-OHdG in hepatocarcinogenesis.

In this study, the relationship between serum and liver tissue levels of 8-OHdG was investigated. Although there was a tendency for serum and tissue 8-OHdG levels to be correlated, it was not significant (data not shown).

The results of the present study indicate that the presence of 8-OHdG expression at the time of liver biopsy could be a useful marker of the development of HCC. Thus, these results emphasize the need for careful monitoring for the development of HCC in patients with chronic HCV and 8-OHdG expression. Given the small number of patients included in the present study, the current findings need to be confirmed in a larger population of patients with chronic HCV infection.

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血液中で測定されるHCV-RNA定量値の意味

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はじめに

C型肝炎患者の臨床的検討を行う際にC型肝炎ウイルス(HCV)関連マーカーの測定は重要である．なかでも，血液中のウイルス核酸(HCV-RNA)量の測定は，抗ウイルス治療の効果予測，治療開始後の反応性の評価，などに大きな意義を有する．しかし，HCVは肝臓内で増幅し，その一部が血液中に放出されていることを考慮すると，通常，われわれが測定しているHCV-RNA量は，体内・肝内のHCVに関する間接情報である．さらに，HCVの体内動態は非常にダイナミックであることも明らかになってきた．本稿では，臨床検査として測定可能な「HCV-RNA量」値の意味について，われわれのデータを示して考察する．

Ⅰ．HCV-RNA量の変動

C型肝炎患者の経過中のHCV-RNA量の経時的変化について，当科における検査結果を示す．C型慢性肝疾患患者のなかで，1年以上，月1回のHCV-RNA量定量測定を行った138例についてHCV-RNA量の測定結果を示す．

対象症例は慢性肝炎121例，肝硬変症17例，平均年齢は62.8歳，男女比は60：78であった．HCVグループ別には，1型107例，2型27例，判定不能4例であった．肝庇護療法(強力ネオミノファーゲンCあるいはウルソデオキシコール酸)施行例では，観察開始時から内容に変更のない例を選択した．

HCV-RNA量は，基本的には分岐プローブ法(b-DNA法)で測定し，0.24Meq/mlの測定感度未満となる例ではPCR法(Amplicor法)で測定した．その結果，b-DNA測定可能例は111例(1型98例，2型9例，判定不能4例)，Amplicor法測定例は27例(1型19例，2型8例)で，b-DNA法では測定感度以下になる比較的低値例は2型に有意に高率であった．測定したHCV-RNA量実測値は対数変換し，観察期間中のHCV-RNA量の変動を，平均値(M)，標準偏差(SD)，最高値と最低値の差(D)により評価した．

Key words：HCV-RNA量，HCV体内動態，肝組織中HCV，FSSA法，DFPP

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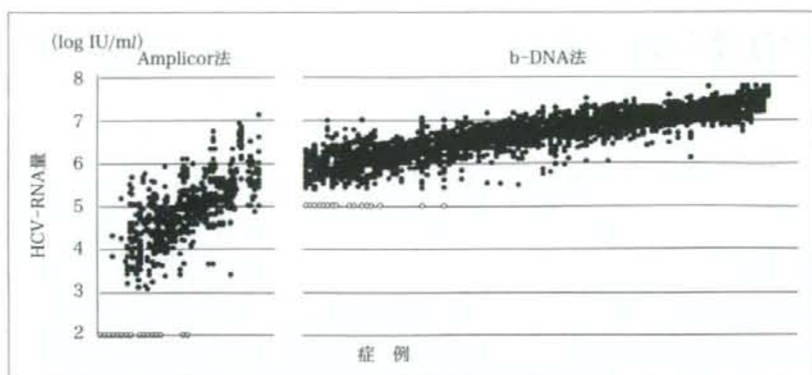

図1 C型慢性肝疾患患者のHCV-RNA量の変動
観察期間中の平均HCV-RNA値の低い順に症例を横軸に並べ，各症例の期間中の実測値を縦軸にプロットした(○は測定感度以下).

実際のHCV-RNA量測定結果を**図1**に示す．1〜2年の観察期間内で，HCV-RNA量の変動は数倍以内の例が多く，5〜10倍以上の比較的大きな変動を示した例は少数であった．HCV-RNA量の平均値(M)分布では，b-DNA量測定例では6(log IU/ml)台が52例(51.0%)，Amplicor測定例では4(log IU/ml)台が11例(44.0%)と最多であった．標準偏差(SD)の分布では，0.1が48例(41.7%)，0.2が29例(25.2%)，0.3が13例(11.3%)で，SD値0.3以下の症例が約80%を占めた．一方，SD値が0.7以上を示す変動の多い症例は11例で全体の1割程度であった．M値とSD値に有意な逆相関($R^2=0.67$, $p<0.05$)を認め，HCV-RNA量が低値な例ほど経時的変動が大きい傾向を示した．D値(観察期間中の最大値と最小値の差)が1.0(log IU/ml)以上を示した例も，b-DNA法27例(29.0%)，Amplicor法18例(81.8%)と，後者のウイルス量低値例で変動が大きいことを示した．

この結果は，治療内容を含めて変化のない，多くのC型肝炎症例では，経過中2〜3倍以上のHCV-RNA量変動はよく観察されることを示している．副腎皮質ステロイドや免疫抑制剤などがHCV-RNA量に影響するか議論になることもあるが，その場合も，本稿に示されるようなC型肝炎患者のHCV-RNA量のベースラインの変動状態を理解したうえで評価することが望まれる．また，これらの症例の多くはウイルス量の変動と血清ALT値の変化に関連が認められず，経過中の評価は別個に行う必要があることにも留意すべきである．

Amplicor法で測定したHCV-RNA量低値例では経過中の変動幅が比較的大きい傾向を示したが，このような症例にはウイルス量低値の時期があることを示しており，抗ウイルス治療開始のタイミングをはかる際の参考になる．HCV-RNA量低値群にはHCV-RNA量とALT値の変動が連動している症例があり，ウイルス量が低値でRNA量とALT値の連動を認めた例は11例(8.0%)であった．このような例でALT値からRNAレベルが推測可能な場合がある．

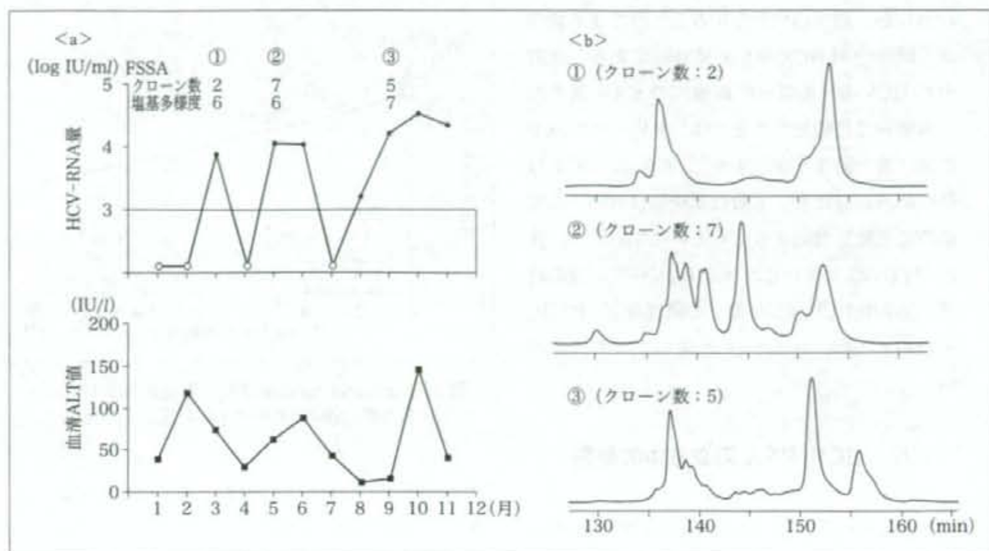

図2 ウイルス量の変動幅の大きい症例

a：HCV-RNA 量と ALT 値変動の関連：①〜③は，FSSA 法による HCV 変異の測定点を示す．

b：SSCP 法による電気移動度の違いによる HCV クローン数の測定．

Ⅱ．HCV-RNA 量の変動と quasispecies の変化

ウイルス量変動の大きい症例の，HCV 超可変領域（hypervariable region；HVR-1）における経時的変化を FSSA 法〔fluorescence single strand conformation polymorphism（SSCP）and sequence analysis〕を用いて検討した．本法は，SSCP 法により 1 本鎖 DNA 断片を電気泳動の移動度の違いにより分離して測定領域から検出されたクローン数を算出し，さらに direct sequence 法で測定領域中の変異率を塩基多様度として示すことで，HCV 変異の状態を解析するものである．HCV-RNA/ALT 連動例について，肝炎再燃時ごとの HCV を FSSA 法で解析した結果，再燃時ごとに HCV population は変化をしていることがウイルス学的に示された．1 例を図 2 に示す．

【症　例】

26 歳，女性．グループ 2 型の C 型慢性肝炎例である．経過中に HCV-RNA 量は 10 倍以上の変動を繰り返し，ALT もウイルス量に連動していた．それぞれのウイルス量増加時の血清から FSSA 法による HCV 変異の解析をしたところ，各肝炎再燃時のウイルスの population は異なっていた．したがって，このような例の HCV-RNA 量変動は，単にウイルス量の増減ではなく，quasispecies の変化と関連する経時的変化である可能性がある．

Ⅲ．血液中と組織内の HCV-RNA

HCV は体内では，おもに肝細胞内で増幅して血液中に放出されることが知られている．わ

れわれが，通常の検査で知ることのできる情報は，流血中のHCV-RNA量のみである．血液中のHCV-RNA量と肝組織内のRNA量を同一容積内で比較した場合には，肝内には血液中に比べ数十倍多く存在する[1]．さらに，HCVは肝細胞内以外にも，末梢血単核球(PBMC)に能動的に感染し増幅すると考えられているが，体内のPBMCプールにおけるHCV-RNA量の評価，血液中HCV-RNA量との関連など，PBMCを含めたHCV dynamicsに関しては報告がない．

Ⅳ．HCV-RNAの血液中の動態

Neumannら[2]は，interferon(IFN)α2bを投与したC型慢性肝炎患者のHCV dynamicsをmathematical modelを用いて解析した．HCV-RNA量をVとし，血液中HCV-RNA量は肝細胞内で増幅し放出されるウイルス量と血液中から排除されるウイルス量の差で規定される．

すなわち経時的変化(dV/dt)は，

$dV/dt=pI-cV$と示される．

上式においては，p：1日当りの感染肝細胞内HCV産生率，I：感染肝細胞数，c：ウイルス消失率(定数)である．

また，β：感染肝細胞への感染率(定数)，δ：感染肝細胞の消失率(定数)，T：肝細胞数，s：肝細胞増殖率(定数)，d：肝細胞消失率(定数)と表した場合，

$$dT/dt=s-dT-(1-\eta)\beta VT$$

$$dI/dt=(1-\eta)\beta VT-\delta I$$

$$dV/dt=(1-\varepsilon)pI-cV$$

と示される．なお，上式の$(1-\varepsilon)$はウイルス粒子産生率を示し，$(1-\eta)$はHCVの*de novo*感染率を表している．

Neumannらは，上記の解析をIFNα2b投与後の慢性肝炎患者で行い，IFNのおもな抗ウイルス効果が，ウイルスの複製や放出の抑制であることを明らかにした．

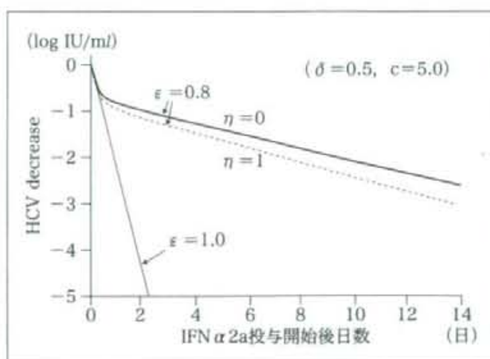

図3 mathematical modelから推定されるIFNα2b投与開始後のHCV-RNAの減少

上式のパラメータを変えた場合のIFNα2b投与後HCV減少パターンにつき**図3**に示す．ウイルス複製がまったく行われないと仮定した場合($\varepsilon=1$)，IFN投与後にHCV-RNAは直線的に減少する．しかし，ウイルス複製が行われている体内の状態($\varepsilon\neq1$)では，HCV-RNA量の減少は図3のような二相性を示す．さらに，HCVの再感染の有無(η：0〜1)もHCV-RNA減少に影響することが示されている．

血液中のHCV-RNA量は，肝細胞内でのウイルスの産生と細胞外への放出，血液中でのウイルスの消失，肝細胞へのウイルスの再感染，感染肝細胞の破壊・排除などによるウイルス動態のバランスの結果として規定されるものと考えられる．Neumannらの解析では，HCVの産生および消失は1日に10^{12}個程度と算出され，無治療状態では体内のダイナミックな動きが示された．一方で，産生・消失のバランスは動的には比較的安定し，総体としては定常状態を示しているものと考えられる．

V．二重濾過血漿交換（DFPP）における HCV-RNA 量の解析

DFPP とは，患者血液を一次膜で血球と血漿に分離し，二次膜で血漿からウイルスなど不要な物質を除去する血漿浄化法である．わが国では，多発性骨髄腫，原発性マクログロブリン血症などに対し保険適応を有し，臨床応用されている．DFPP の二次膜の孔は最大 40 nm であり，径が 55～65 nm である HCV 粒子は二次膜の孔を通過できずに排除されると考えられる．Yamashita ら[3]は，C 型慢性肝炎患者の IFNα2b＋リバビリン治療に DFPP を併用し，安全性と治療初期の有効性を報告し，Fujiwara ら[4]は C 型慢性肝炎に対する IFNα2b 治療に DFPP を併用し，治療初期の HCV-RNA 量減量効果や substained viral response（SVR）において有用であることを報告した．

DFPP は HCV を物理的に排除する治療法で，二次膜通過後の濾液中 HCV-RNA は，ほとんどの例で測定感度以下となる．本法は肝細胞における HCV の複製や放出を抑制する IFN とは作用機序が異なり，前述した HCV の体内動態を解析する際に，新たな視点を導入できる可能性がある．多くの症例では DFPP 単独治療前後に HCV-RNA 量は低下するが，一部に開始前よりも上昇する症例が認められた．この違いは，ウイルスの増殖力の差や，肝臓と循環血液中の間の turn over の差などによる可能性が考えられ，DFPP が個々の症例における HCV 体内動態の解析手段になりうることが示された．

まとめ

HCV はおもに肝細胞内で増幅され血液中に放出されている．HCV は体内ではダイナミックな動態を示しているが，血液中 HCV-RNA 量はそのなかで定常状態を維持していることが示された．血液中 HCV-RNA 量は，このような体内動態の結果が示されていることを理解したうえで評価すべきである．

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ウイルス肝炎（壊死炎症の繰り返し）の線維進展

髭　修平*

索引用語：肝線維化，ウイルス肝炎，壊死炎症反応，アポトーシス，staging

1 はじめに

肝臓は，肝細胞および胆管細胞の上皮組織と支持組織からなりたっており，本来の構成要素として種々の程度に線維結合織が含まれている．結合組織は，肝臓の支持組織のひとつであるが，基質(matrix)とその中に埋まっている細胞および線維からなる．この線維には，膠原線維，細網線維，弾性線維がある．

"肝線維化"とは，肝内で線維が異常に増加した状態であるが，ウイルス性肝炎の病因や病態の解明が進み，線維化の機序や評価も明らかにされてきた．本稿では臨床的な意義を含めて概説する．

2 線維化の病態

肝の線維化は，その形成機序により病態を分けてみることができる．まず，刺激に反応した線維増生の有無からみて，受動的線維化と能動的線維化とがある．前者では，脱落した肝細胞の枠としてあった線維が集合し，結果的に線維化が認められるようになる．後者では，線維産生細胞が積極的にプロコラーゲンを産生し細胞外に線維が沈着するものである．実際には両者が複合して線維化が生じていることが多い．

また，先行する組織障害が明らかでないまま，膠原線維産生細胞が直接刺激を受けて積極的に線維が増殖する1次的線維化と，先行する組織障害に対して反応性にコラーゲンが増殖する2次的線維化とに分類することもできる．

3 ウイルス性肝炎における肝の線維化

ウイルス性肝炎に伴う肝臓の線維化は，肝内の慢性的な炎症による組織障害に伴って2次的に起こる．肝障害が一過性の場合，肝細胞の再生が十分であれば，細胞外マトリックスは吸収されて障害組織は痕を残さない．しかし，慢性的に壊死炎症反応が繰り返されるウイルス性肝炎においては，肝再生よりも結合織の増生が勝り，その結果，細胞外マトリックスは吸収されずに沈着し，線維化が進

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行していくことになる[1]．そして，コラーゲン沈着量の増加は肝臓の組織構築を改変させていく．

ウイルス性肝炎おける肝細胞死は，壊死(necrosis)だけではなく，アポトーシスも大きく関与していることが明らかとなってきた．肝細胞のアポトーシスから肝線維化への進展に関しては，以下の機序が考えられている[2]．アポトーシスを起こし分解された肝細胞からはアポトーシス小体が形成されるが，この小体を取り込んだ周囲のクッパー細胞や肝星細胞から，強力な線維化促進因子であるTGF-βやFas ligandなどの分泌が亢進する．これらの細胞が持続的に活性化されると，IL-8やMIP-2（macrophage inflammatory protein-2）などのCXCケモカインが誘導され，肝炎は増強しアポトーシスがさらに進行する．このような"feed-forward-loop"が形成された状態で，肝星細胞はさらに活性化を受ける．活性化した星細胞は筋線維芽細胞に形質転換するが，この細胞からTGF-βの他にも，抗アポトーシス作用を持つTIMP-1（tissue inhibitor of metalloproteinase-1）などが分泌される．その結果，活性化星細胞の分解が抑制され，細胞外マトリックスの蓄積が進行する．

4 B型肝炎とC型肝炎―組織像の違い

肝の線維化は持続する炎症の結果であり，ウイルス性慢性肝炎の組織所見は基本的には同じである．しかし，起因ウイルスによる経過・病態の違いを反映し，B型肝炎とC型肝炎の組織像にも違いが認められる．

B型肝炎では，C型肝炎に比べて活動期に壊死炎症反応が比較的強く，線維化が目立たない段階からinterface hepatitisの所見を認める頻度も高い．また，肝細胞の再生傾向が強く，結節形成も目立つ．広範な壊死炎症反応のために短期間で肝硬変まで進展する例も観察される．C型肝炎では，壊死炎症反応は比較的弱く，再生活動・結節形成傾向も乏しい．長期間にわたり炎症が持続することで徐々に病期が進展し，肝硬変への進展過程で膠原線維の沈着がより強くなる[3]．また，Disse腔や類洞に線維化の目立つ症例もあり，アルコール性肝障害に類似することもある．

同様に，線維化，再生結節形成が進んだ肝硬変においても，起因ウイルスによる差を認める．B型肝硬変においては，比較的大きめの再生結節を認め，線維性隔壁は狭い例が多い．一方，C型肝硬変では小さな結節を呈するものが多く，さらに一旦形成された再生結節をさらに細分割するような線維化がみられる．C型では門脈域および門脈域周囲の炎症細胞浸潤が比較的強く，肝硬変の進行に伴い線維化はさらに強くなる．その結果，線維性隔壁は不規則に厚くなる傾向を認める[4]．

5 慢性肝炎の組織診断基準の変遷と線維化の評価

慢性肝炎における肝臓の線維化が，より客観的に評価可能となったのは，肝組織診断基準の改訂によるところが大きい．従来は記述的であった肝組織の病理学的所見がスコア化された結果，肝の炎症や線維化の程度をより客観的に表現することが可能となった．

慢性肝炎の組織分類について，1990年頃までは1970年前後に提唱された分類（わが国では犬山分類，欧米ではヨーロッパ分類）が基本として利用されてきた．1981年にはKnodellら[5]により，肝組織病変の程度をスコア化して表現するHAI（histological activity index）スコアの手法がはじめて提唱され，

表1　慢性肝炎の線維化分類

score	新犬山分類	modified HAI staging	HAI (fibrosis)	Ishak et al.	METAVIR system
0	線維化なし	No fibrosis	No fibrosis	No fibrosis	No fibrosis
1	門脈域の線維性拡大	Fibrous expansion of some portal areas, with or without short fibrous septa	Fibrous portal expansion	Mild fibrosis	Periportal fibrosis expansion
2	bridging fibrosis (線維性架橋形成)	Fibrous expansion of most portal areas, with or without short fibrous septa	-	Moderate fibrosis	P-P septae (>1 septum)
3	小葉のひずみを伴う bridging fibrosis	Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging	Bridging fibrosis (portal-portal or portal-central linkage)	Severe fibrosis	P-C septae
4	肝硬変	Fibrous expansion of portal areas with marked bridging [portal to portal (P-P) as well as portal to central (P-C)]	Cirrhosis	Cirrhosis	Cirrhosis
5		Marked bridging (P-P and / or P-C) with occasional nodules (incomplete cirrhosis)			
6		Cirrhosis, probable or definite			

肝炎の客観的評価に利用されてきた(表1).

1990年代に入り，C型肝炎の診断が容易になると，C型肝炎自体の診断，病理学的評価が可能となり，さらにアルコール性肝障害や脂肪肝との鑑別も明確となった．その結果，慢性肝炎の組織診断基準の見直しが行われるようになり，HAIスコアリング以外にさまざまな組織評価法の提唱がなされた[6]．1991年にScheuerら[7]が炎症の活動性と線維化を分けた分類を示し，1994年にはDesmetら[8]により新ヨーロッパ分類がまとめられた．同じ年，フランスの研究グループからもC型肝炎組織の評価法(METAVIRシステム)が提唱された[9]．1995年にはIshakらによって新しいHAIスコアの分類法が報告され，炎症はgradingで，線維化はstagingとして表現される方法が一般化した．この時点で，piecemeal necrosis（削り取り壊死）と呼ばれていた門脈周囲の肝細胞死の主体がアポトーシスであることが明らかになってきたため，interface hepatitisと表現されるようになっている．わが国でも，同様の時期に犬山分類の検討が行われ，1994年に新犬山分類として提案され，1996年に現在の基準が確定した[10]．

6　B型肝炎とC型肝炎—線維化の進展予測

B型慢性肝炎は，活動性の強い時期には炎症が強く，線維化も急速に進行することがあるが，一方で，HBe抗原のseroconversionを起こしたあとでは炎症は消失し，その後の線維化進展がほとんどみられない症例も多い．このようにB型肝炎では病勢の変動が大きいため，線維化は一定の速度では進展せず，長期的な予後を推測することは容易ではない．

C型慢性肝炎の場合にも，線維化進展には

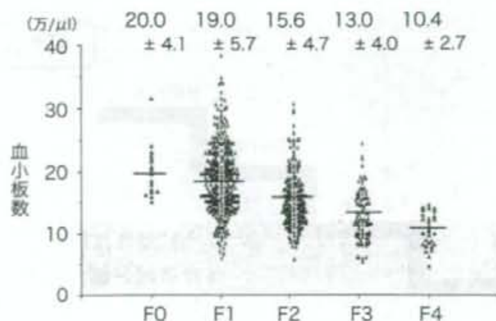

図1　Fスコアと血小板数

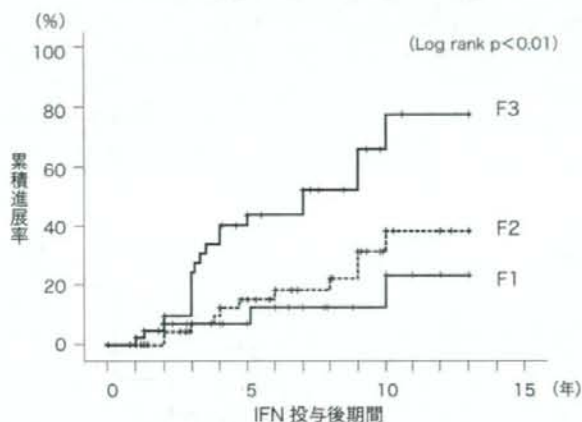

図2　肝線維化と肝硬変への進展

個人差があるが，C型肝炎症例全体としては，経過とともに進展する例が多い．Poynardら[11]は，感染時期が特定できる患者の肝生検結果から，線維化スコアの進展速度を年率0.133と報告した．また，Shiratoriら[12]は，同一患者への経時的な2回の肝生検から線維化スコアの進展速度を年率0.10と算出している．これらの結果からは，肝硬変までの進展が約30年であることを表しており，臨床的な成績ともほぼ合致する．なおC型肝炎例では，ALT持続正常例の線維化進展は緩徐で，一方，男性，アルコール多飲，高齢時感染などでは線維化進展は速い傾向にあると考えられている．

7　C型肝炎の線維化と血小板数

C型慢性肝炎では，線維化の進行と血小板数の減少が相関することが知られている．肝の線維化を非侵襲的に評価する方法としては，線維化マーカーやFibroscanによるものなどがあるが，血小板数は検査が簡単で，かつ臨床的な有用性が高く評価されている．

図1は，当科のC型慢性肝疾患患者の肝生検によるFスコア別の血小板数を示してい

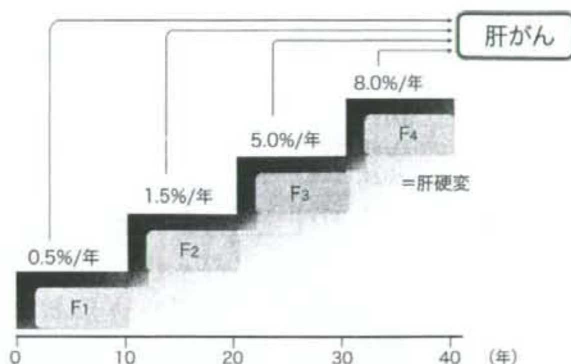

図3　C型慢性肝炎における肝線維化の進行

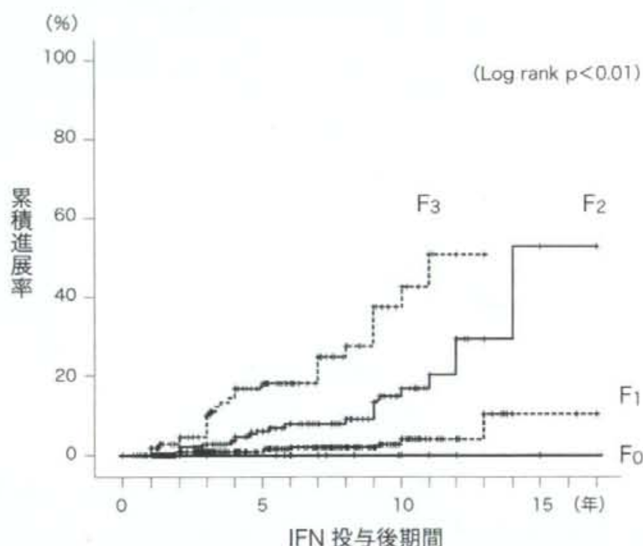

図4　IFN治療時の線維化の程度と肝硬変への進展

る．各スコアの平均血小板数(/μL)は，F0で20.0万，F1で19.0万，F2で15.6万，F3で13.0万，F4で10.4万であった．Fスコアが高値になるほど血小板数の平均値は減少傾向を示している．ただし，同一スコア内でも血小板数のばらつきが大きいことには注意が必要である．個々の症例では，線維化に関連する他の検査結果や，経時的な血小板数の変動も含めて，臨床的に判断することが望ましい．

8　C型肝炎の線維化進展

C型慢性肝炎の自然経過において線維化は経時的に進展し，年率で約3%の頻度で慢性肝炎から肝硬変へ進行する．無治療・自然経

表2 IFN治療効果別の肝硬変への進展例

	著効	再燃	無効	計
F_0	0/13 (0%)	0/8 (0%)	–	0/21 (0%)
F_1	0/191 (0%)	3/187 (0.2%)	3/35 (1.5%)	6/413 (0.2%)
F_2	0/87 (0%)	11/87 (1.8%)	11/59 (3.4%)	22/233 (1.6%)
F_3	0/38 (0%)	6/44 (2.5%)	20/47 (9.3%)	26/129 (4.0%)
計	0/329 (0%)	20/326 (0.9%)	34/141 (4.7%)	54/796 (1.2%)

(カッコ内は年率)

過観察例を対象とした解析では，病期進展例の頻度が実際より低率になる可能性が高いため，ここではインターフェロン治療例の内，無反応例を対象として肝硬変進展率を検討した．これらの対象では，治療を要すると判断される症例の無治療・自然経過に類似するものと考えられる．

対象は1990年代に，肝生検施行後，従来型のインターフェロン治療(6カ月以内)を施行したC型慢性肝疾患症例796例である．治療効果は，著効329例(41.3%)，再燃326例(41.0%)，無効141例(17.7%)であった．この無効例における，治療開始時のFスコアとそののちの肝硬変進展率をKaplan-Meier法で示す(図2)．インターフェロン治療開始後の5年後および10年後の肝硬変進展率は，F1例では6.9%，23.3%，F2例では14.9%，38.1%，F3例では43.5%，77.3%と，Fスコアが高い症例ほど，早期かつ高率に肝硬変への進展を示した．

同様にFスコアが高いほど，肝癌発生の可能性も高くなる．特に，F4の肝硬変と診断されたのちでは，肝癌発生のリスクも非常に高くなる(図3)．

9 C型肝炎に対するインターフェロン治療と肝線維化

肝の線維化は，インターフェロン治療効果にも関連し，線維化が進行するほど，難治傾向を示す．前述のインターフェロン治療症例全体を対象として，インターフェロン治療を行ったのちの経過観察における肝硬変への進展度をFスコア別に表す(図4)．この解析は著効・再燃・無効のすべての例が対象であり，図2と比較すると，同じFスコアの症例でも，インターフェロン治療がそののちの予後を改善し，肝硬変への進展を抑制していることが示されている．この対象症例を，Fスコア別・治療効果別に分けて，治療後の肝硬変進展率を示す(表2)．Fスコア別の肝硬変進展に関する年率は，F0では0%，F1が0.2%，F3が1.6%，F3で4.0%であった．ウイルス学的著効例からの肝硬変進展は認めなかったが，再燃例あるいは無効例のそれぞれの対象に限定した場合も，線維化の程度と肝硬変進展率に相関がみられる．

また，Shiratoriら[12]の検討で，インターフェロン治療によりC型肝炎ウイルスが完全に排除された症例では，Fスコアが年率0.28の速度で低下することが明らかとなり，治療前の線維化進展速度よりも速く，線維化の改善が得られることが報告されている．このように肝の病態，あるいは治療効果評価をFスコアを用いて解析することにより，長期経過のなかでの肝疾患の予後の評価も客観化が可能となった．

10 おわりに

ウイルス性肝炎に伴う肝臓の線維化は，肝内の慢性的な炎症の繰り返しの結果，沈着が蓄積していく．したがって，肝線維化はウイルス性肝炎の「慢性度」を表し，スコア化して評価することは臨床的に有効である．ウイルス性肝炎に対する治療効果も，短期的なウイルス学的・生化学的有効性から評価するだけでなく，病期の進行あるいは発癌など，長期予後の観点からの評価の必要性が認識されるようになり，肝線維化はさらに重要な臨床的指標となってきた．

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Human T-cell leukemia virus type 1 Tax modulates interferon-α signal transduction through competitive usage of the coactivator CBP/p300

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ABSTRACT

We describe here Tax protein of human T-cell leukemia virus type 1 (HTLV-1) as an interferon (IFN)-α antagonist counteracting the transactivation function of IFN-stimulated gene factor 3 (ISGF3). Co-expression of Tax, but not the Tax mutant unable to bind to CBP, significantly inhibited the reporter gene expression directed by IFN-stimulated regulatory elements, despite that the formation of DNA-binding ISGF3 complex was unaffected. Gene activation induced by STAT2 transcription domain was also inhibited by expression of Tax. Furthermore, Tax-mediated transcriptional inhibition was reversed by overexpression of p300. These observations indicate that Tax interferes with IFN-α-induced JAK-STAT pathway by competition with STAT2 for CBP/p300 binding. Consistently, GST pull-down assay showed that Tax dose-dependently inhibited binding of STAT2 to p300. This study suggests that Tax may prevent IFN-α from exerting its antiviral, antiproliferative and proapoptotic effects, thereby contributing to persistent viral infection and HTLV-1-associated oncogenesis.

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Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is an oncogenic retrovirus responsible for development of adult T-cell leukemia/lymphoma (ATLL) (Yoshida et al., 1984) and HTLV-1-associated neurodegenerative diseases such as myelopathy/tropical spastic paraparesis (HAM-TSP) (Gessain et al., 1985; Osame et al., 1986). ATLL is highly chemoresistant and its prognosis is usually poor. After conventional chemotherapy failure, interferon (IFN)-α combined with antiretroviral reagents such as zidovudine produces clinical remission followed by progression and death (Gill et al., 1995; Hermine et al., 1995). HTLV-1-associated oncogenesis has been demonstrated to be largely attributable to the expression of the viral regulatory protein Tax (Grassmann et al., 1989; Grassmann et al., 1992). In addition to its role in activating proviral transcription, Tax has been shown to activate transcription through protein–protein interactions with multiple transcription factors including the cyclic AMP-responsive element-binding factor (CREB), NF-κB, and the serum-responsive element-binding factor (SRF). Tax stimulates viral transcription through interacting with three conserved 21-bp repeat DNA elements known as viral CREs located within the HTLV-1 promoter. Tax binds to the CRE sequences in complex with the transcriptional factor CREB, which subsequently facilitates the transcription by recruitment of the cellular coactivators CBP (CREB-binding protein) and p300 (Giebler et al., 1997; Kwok et al., 1996).

CBP and p300 were originally identified as targets of CREB and adenovirus E1A, respectively (Chrivia et al., 1993; Eckner et al., 1994). They are large nuclear phosphoproteins that act as global transcriptional coactivators by binding numerous transcription factors (Janknecht and Hunter, 1996). CBP/p300 functions as coactivator or adapter by bridging transcription factors to general transcription machinery such as TFIIB, TBP and RNA polymerase II. In addition, CBP/p300 might contribute to transcriptional regulation by acetylation of chromatin via its intrinsic histone acetyltransferase (HAT) activity or its association with another HAT, p/CAF (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996). The simultaneous interaction of multiple transcription factors with CBP/p300 has been proposed to contribute to transcriptional synergy. Conversely, competition for limiting amounts of CBP/p300 has been suggested as a potential mechanism for transcriptional repression.

IFNs are a family of immunomodulatory cytokines that are produced in response to virus infection and possess both antiviral and antiproliferative functions. Secreted IFNs act in an autocrine or paracrine fashion to activate JAK-STAT pathway by binding to IFN receptors (IFN-α/β to IFNAR1/IFNAR2 and IFN-γ to IFNGR1/IFNGR2).

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The activated (phosphorylated) STAT proteins form homodimers, or heterodimers, with other STAT proteins, which subsequently translocate to nucleus and bind specific DNA sequences within the promoter region of IFN-stimulated genes (ISGs). In the case of signaling via IFN-α/β, heterodimer of phosphorylated STAT1 and STAT2 interacts with IFN regulatory factor 9 (IRF9; p48) to form the trimer transcription complex ISGF3 (IFN-stimulated gene factor 3). By recruiting the transcriptional coactivator CBP/p300, ISGF3 triggers the expression of a variety of genes driven by the promoter containing IFN stimulated response elements (ISRE). Viruses have evolved different strategies to subvert IFN response, mainly by interfering IFN induction, perturbing IFN signaling and inhibiting IFN-induced effectors. Viral proteins acting as IFN antagonists have been identified in a numbers of viruses belonging to different families. Additionally, employing its antiproliferative and proapoptotic properties, IFN has been used as a therapeutic agent for multiple hematological and non-hematological malignancies. The anti-tumor efficacy, however, is limited by emergence of IFN resistance, a phenomenon attributable to the functional defects involved in IFN signaling pathway (Sun et al., 1998).

Here we present evidence demonstrating that HTLV-1 Tax interferes with the JAK-STAT signal transduction pathway in response to IFN-α, and the mechanism underlying the antagonistic function involves interaction of Tax with coactivator CBP/p300 in competition with STAT2, thereby inhibiting the transcription activation of STAT2-containing ISGF3 complex.

Results

Inhibition of IFN-α-induced ISRE signaling by Tax

By analyzing the influence of Tax protein on replication of hepatitis C virus (HCV) replicon, we previously showed an attenuated IFN-α-induced antiviral response in Tax-expressing Huh-7 cells (Zhang et al., 2007). If Tax inhibits IFN-α response, interference could occur via global interruption of the JAK-STAT signal transduction pathway or via inhibition of specific ISG products such as protein kinase R or 2′,5′-oligoadenylate synthetase. To define a possible role of Tax in modulating IFN-α signaling, Tax- or Tax mutant-expressing plasmid was transfected into Huh-7 cells together with the reporter vector pISRE-luc to monitor ISRE-directed gene expression. Cells were either left untreated or treated with 100 IU/ml of human IFN-α 24 h later, and the luciferase activity was measured following a further incubation for 24 h. As shown in Fig. 1A, both the wild type and the Tax mutant m148, previously demonstrated to be functional in activating CREB pathway while failing to activate NF-κB pathway, significantly inhibited IFN-α induced ISRE-directed luciferase expression, while the Tax mutant m319, unable to activate CREB pathway although retaining the ability to activate NF-κB pathway (Yamaoka et al., 1996), had no significant effect on ISRE-driven gene expression, indicating that Tax inhibits ISRE-directed gene expression and its competence for signaling through CREB is important for the observed inhibition. Western blot analysis showed a comparable expression of Tax and Tax mutants (Fig. 1B). Similar results were also observed with Jurkat T cells (Fig. 1C), suggesting that Tax-mediated inhibition of JAK-STAT signaling in response to IFN-α was not cell-type specific. These results suggest that Tax interferes with IFN-α response by a global transcriptional repression of downstream effector gene expression.

Tax does not affect the formation of DNA-binding ISGF3 complex

As forementioned above, IFN-α signaling through the JAK-STAT pathway involves a series of events including tyrosine phosphorylation of STAT1 and STAT2, formation and nuclear translocation of ISGF3 followed by its binding to ISREs. Impairment at any of these steps might consequently lead to blockade of IFN-α-induced signaling. To understand the mechanism employed by Tax to counteract IFN's

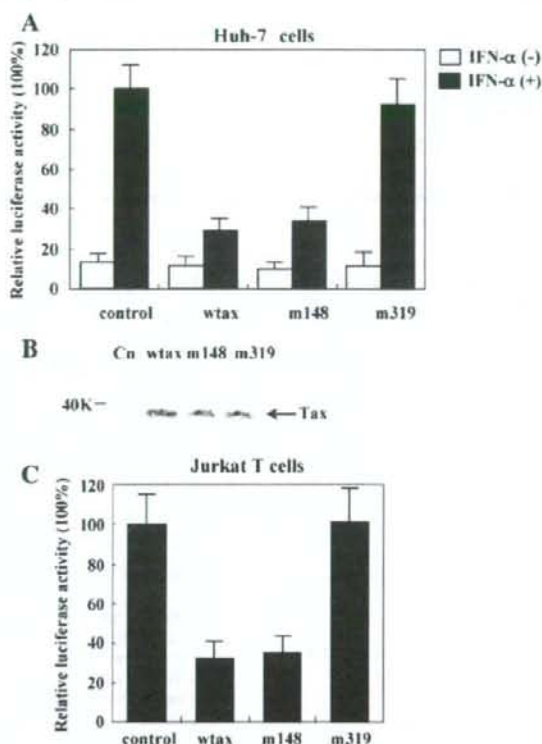

Fig. 1. Effect of Tax or its mutants on IFN-α-induced ISRE signaling. Huh-7 (A) or Jurkat T cells (C) were transfected with ISRE-luc together with pCn, pCnwtax, pCnm148, or pCnm319. Cells were either left untreated or treated with 100 IU/ml of human IFN-α 24 h later, and the luciferase activity was measured following a further incubation for 24 h. Renilla luciferase activities from cotransfected pRL-TK were used to normalize the transfection efficiency. Normalized luciferase activity from an otherwise identical control transfection with pCn backbone vector was set as 100%, and those in other transfectants are expressed as relative percentage. Results are presented as the means and standard deviations of four independent triplicate transfections. (B) Expression of Tax protein in each transfectant was confirmed by Western blot analysis. Solid arrow indicates the signals of Tax protein.

actions, we next investigated the formation of ISRE-binding ISGF3 in Huhwtax. Huhwtax is a cell line stably expressing Tax at a level comparable to HTLV-1-infected MT-2 cells (Fig. 2B), which was established by transfection with pCnwtax followed by G418 selection and limiting dilution (Zhang et al., 2007). Nuclear extracts were prepared and subjected to electrophoretic mobility shift assay (EMSA) using the labeled ISRE as an oligonucleotide probe. As seen in Fig. 2A, in IFN-α-treated HuhCn cells, which were stably transfected with the empty vector pCn and served as a negative control, ISRE–protein complexes of delayed electrophoretic mobility was detected (lane 2), which was diminished by addition of antibody against STAT1 or STAT2 (lanes 7–8) but not by irrelevant antibody to β-actin (lane 9), confirming the identity of the ISRE-binding factor as ISGF3. The formation of ISRE–protein complex was significantly inhibited by addition an excess of unlabeled ISRE as a competitor (lane 3), revealing the specificity of the observed ISRE–protein binding. Unexpectedly, IFN-α-induced ISGF3 formation in Huhwtax was comparable to that detected in HuhCn (lanes 4–6). While expression of Tax conferred an attenuated IFN-α-induced antiviral response in Huhwtax cells (Zhang et al., 2007), the formation of ISRE-binding ISGF3 complex in their nuclear extract was not affected, which suggests that Tax inhibits IFN-