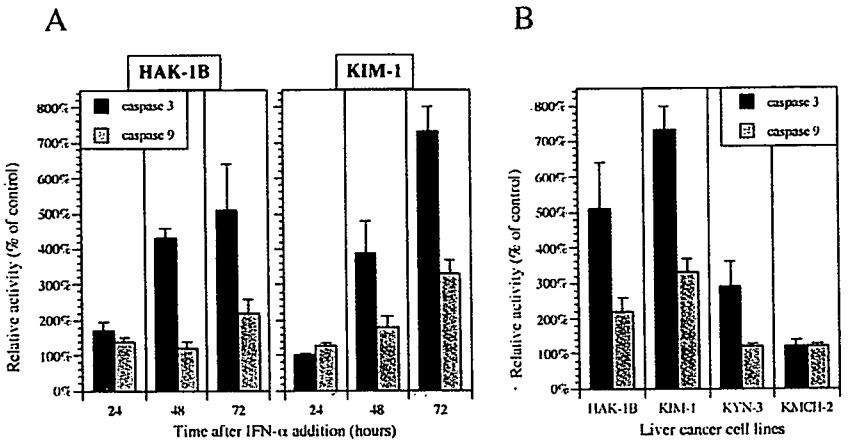


Figure 2. Effects of 1,000 IU/ml IFN-α on activation of caspase-3 and -9, analyzed by fluorometric protease assay. (A), Time-course change of relative activity (in comparison to the activity in the no-treatment group) of caspase-3 and -9 in the 2 IFN-α-mediated apoptosis-sensitive cell lines cultured with 1,000 IU/ml IFN-α. (B), Relative activity of caspase-3 and -9, 72 h after the addition of 1,000 IU/ml IFN-α to the cultures of the 2 sensitive cell lines (KIM-1 and HAK-1B) and 2 resistant cell lines (KYN-3 and KMCH-2). The experiment was repeated at least twice to confirm the reproducibility of the test results.

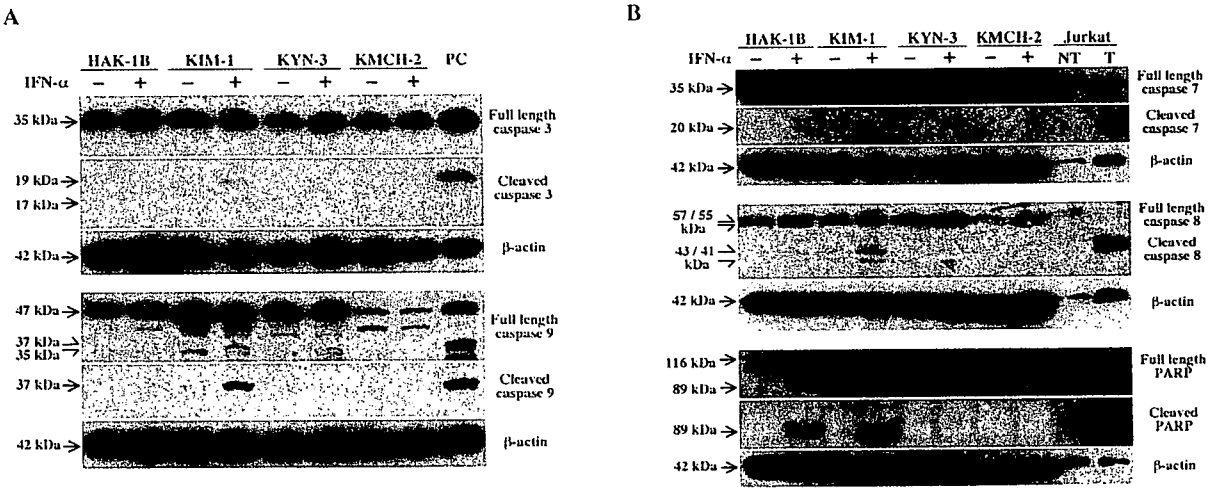


Figure 3. Effects of IFN-α on full-length expression and cleavage of caspase-3, -9 (A), -7 and -8, and PARP (B) in the 4 liver cancer cell lines, analyzed by Western blotting. Two IFN-α-mediated apoptosis-sensitive cell lines (KIM-1 and HAK-1B) and 2 resistant cell lines (KYN-3 and KMCH-2) were incubated with medium alone (-) or 1,000 IU/ml IFN-α for 72 h, and then used in analysis. Seventy-five μg proteins from each cell line were used for the detection of caspases-3, -7, and -9, and PARP. For caspase-8 detection, samples were prepared from $1.0x10^5$ KYN-3 cells, $1.5x10^5$ KIM-1 and HAK-1B cells, and $2.0x10^5$ KMCH-2 cells. In the analysis of caspase-7, caspase-8 and PARP, and for positive control of caspase and PARP cleavage, protein extracts from Jurkat cells untreated with the chemotherapeutic agent etoposide were used. The ß-actin level was used as the control for equal loading.

(HAK-1B and KIM-1) that are sensitive to IFN-α-mediated apoptosis. However, the activity levels increased with the contact hours, and the levels reached the highest at 72 h after IFN-α addition (Fig. 2A), when the activities were also measured in the other 2 cell lines (KYN-3 and KMCH-2) that are resistant to IFN-α-mediated apoptosis. As a result, the activity levels were 5.1 times higher in HAK-1B, 7.3 times in KIM-1, 2.9 times in KYN-3, and 1.2 times in KMCH-2, for caspase-3, and 2.2, 3.3, 1.2, and 1.2 times higher, respectively, for caspase-9 (Fig. 2B).

In the Western blot analysis, KIM-1 (sensitive cells) cultured with IFN-α had weak bands of cleaved caspase-3 and -7, and clear bands of cleaved caspase-8 and -9, while HAK-1B cells cultured with IFN-α had weak bands of cleaved caspase-3, -8 and -9 (Fig. 3). Both IFN-α-treated KIM-1 and HAK-1B had cleaved product of PARP (Fig. 3B). In the resistant cell lines (KYN-3 and KMCH-2), these activation bands were not found.

*Activation of mitochondrial proteins and Bcl-2 family proteins by IFN-α.* The expression of 2 mitochondrial proteins, i.e. cytochrome c and Smac/DIABLO, at 24, 48 and 72 h with or without IFN-α treatment was examined in IFN-α-mediated apoptosis-sensitive KIM-1 cells. As a result, the cytochrome c and Smac/DIABLO levels in the mitochondrial fraction decreased at 72 h after treatment, and cytochrome c level in

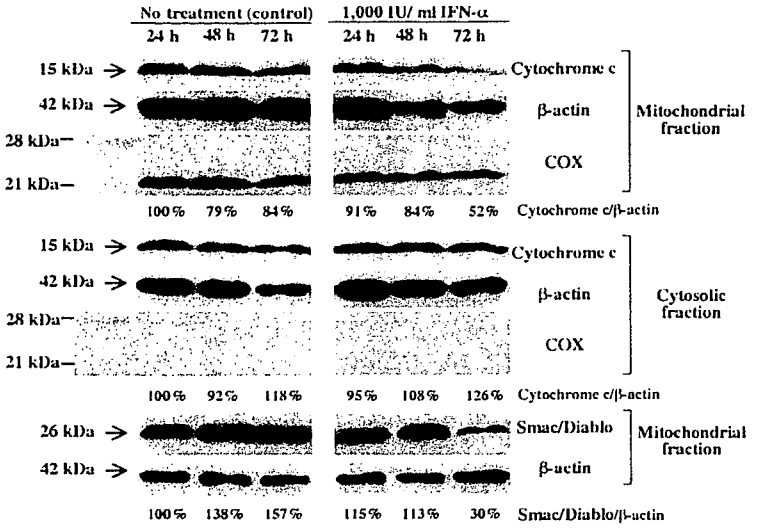


Figure 4. Effects of 1,000 IU/ml IFN-α on the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol in KIM-1 cells, analyzed by Western blotting. Equal amounts (50 μg) of protein were subjected to electrophoresis. Cytochrome oxidase subunit II (COX) was expressed in mitochondrial fraction, but not in cytosolic fraction. This shows that proteins in the cytosolic fraction were not intermixed with those in the mitochondrial fraction. The intensities of the bands were quantified densitometrically using the NIH Image 1.61 software program. Cytochrome c and Smac/DIABLO levels were normalized against ß-actin levels that were used as an internal control. The normalized levels of cytochrome c and Smac/DIABLO were comparatively analyzed.

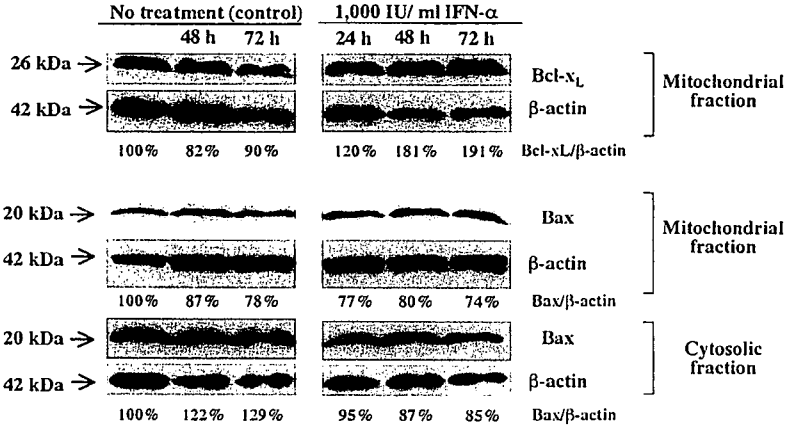


Figure 5. Effects of 1,000 IU/ml IFN-α on Bcl-$x_L$ and Bax expression in the mitochondrial and cytosolic fractions, analyzed by Western blotting. The intensities of the bands were quantified densitometrically using the NIH Image 1.61 software program. Cytochrome c and Smac/DIABLO levels were normalized against the ß-actin level used as an internal control. The normalized levels of cytochrome c and Smac/DIABLO were comparatively analyzed.

the cytosolic fraction increased (Fig. 4). This indicates the release of these proteins from mitochondria to cytosole. The band of cytochrome oxidase subunit II (COX) was expressed in the mitochondrial fraction but not in the cytosolic fraction, which shows that the proteins in the mitochondrial fraction were not intermixed with the proteins in the cytosolic fraction.

In the mitochondrial fraction of KIM-1 cells, the expression of Bcl-$x_L$ increased over time after the IFN-α treatment, whereas the expression of Bax slightly decreased in comparison to the no-treatment group at 24 h (77% vs. 100%) but then the expressions in the 2 groups were maintained at similar levels at the 48 and 72 h (Fig. 5). Bax in the cytosolic fraction of the no-treatment group slightly increased over time, while

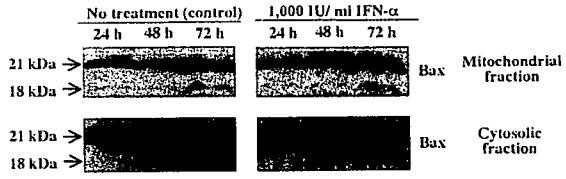


Figure 6. Effects of 1,000 IU/ml IFN-α on the expression of p18 Bax, which is the cleaved form of p21 Bax in the mitochondrial and cytosolic fractions, analyzed by Western blotting.

that of the treated group slightly decreased. Regarding Bax, expression of p18 Bax, which is the cleaved form of p21

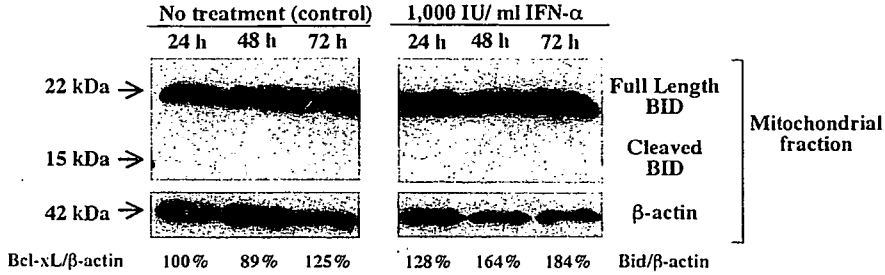


Figure 7. Effects of 1,000 IU/ml IFN-α on the expression of full-length and cleaved forms of Bid in the mitochondrial fraction, analyzed by Western blotting. Bid level was normalized against the level of ß-actin, which was used as an internal control. The normalized Bid level was comparatively analyzed.

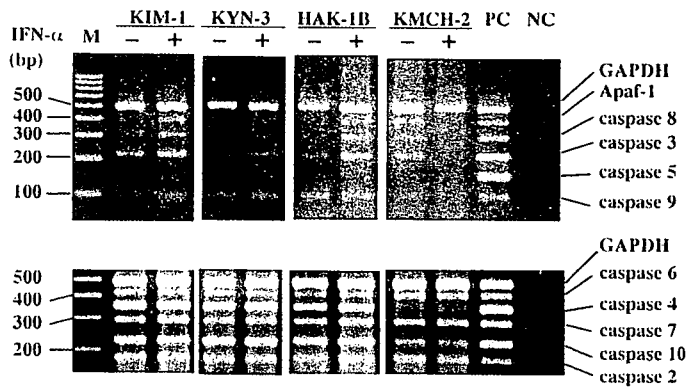


Figure 8. RT-PCR analysis on the mRNA expression of the apoptosis-related molecules in the 4 liver cancer cell lines. PCR products were electrophoresed in 4% NuSieve agarose gel and stained with ethidium bromide. A positive control (PC) for each product was provided by the manufacturer. No DNA bands were produced with identical volumes of the PCR reaction mixtures without the addition of cDNA, and they served as negative controls (NC). Lane M shows DNA molecular-weight markers.

Bax, was examined by using another antibody, but there were no differences between the no-treatment and treated groups (Fig. 6). The presence of the full-length Bid in the mitochondrial fraction was confirmed but there was no cleaved form (15 kDa band, Fig. 7). The expression of the full-length Bid in the treated group increased over time more than in the no-treatment group.

*Apoptosis-related molecule mRNA expression induced by IFN-α.* Expression of mRNA of 10 apoptosis-related molecules was examined in the no-treatment and treated groups at the 72 h in the 4 cell lines. In 2 IFN-α-mediated apoptosis-sensitive cells (KIM-1 and HAK-1B), the bands of caspase-3, -8 and -9, and Apaf-1 were expressed or their intensities increased. On the other hand, in the 2 resistant cell lines, KYN-3 had slight increases of band intensities for caspase-3, -8 and -9, but no other increases were observed in either cell line (Fig. 8).

## Discussion

In the experiment using the broad spectrum caspase inhibitor Z-VAD-fmk, IFN-α-mediated apoptosis was completely suppressible in HAK-1B. This indicates that IFN-α-mediated apoptosis in this cell line depends on caspase. On the other hand, IFN-α-mediated apoptosis in KIM-1 was unsuppressible when the cells were treated according to the same procedures as for HAK-1B. In KIM-1, however, IFN-α-mediated apoptosis was suppressed more apparently, but incompletely by adding an inhibitor at a higher concentration before the culture and at the 24 h. This suggests 2 possibilities: a) caspase activity in KIM-1 is higher than in HAK-1B, and a higher concentration of the inhibitor is necessary to suppress their apoptosis, and b) instability of the inhibitor is present in the culture of KIM-1 cells.

Regarding the IFN-α-mediated apoptosis of KIM-1, we obtained several findings that suggest the involvement of the mitochondrial apoptotic pathway. In the protein level, there were releases of mitochondrial proteins (cytochrome c and Smac/DIABLO); activation of initiator caspase-8 and -9, and effector caspase-3, and -7; and cleavage of cellular substrate PARP. In the mRNA level, involvement of caspase-3, -8 and -9, and Apaf-1 is suggested.

Previous studies suggest that upon activation caspase-8 directly activates several caspases including caspase-3, -6, -7 and -10 as an initiator in different forms of apoptosis (11,26). In addition, caspase-8 induces Bid cleavage and the translocation of truncated cleavage product (tBit) to mitochondria where it triggers cytochrome c release (27,28). In our current study, mild increase in the expression of full-length Bid was observed over time in IFN-α-treated KIM-1 cells, but there was no clear cleavage of Bid. Therefore, release of cytochrome c through tBid was not likely to occur. However,

Desagher *et al* (29) found that, during certain types of apoptosis, full-length Bid translocates to mitochondria and binds to Bax, leading to a change in conformation of Bax and to cytochrome c release from mitochondria. Therefore, further studies are necessary to clarify whether the increase of full-length Bid expression, not the cleavage of Bid, is related to the release of cytochrome c.

In the release mechanism of cytochrome c, multiple stimuli act on mitochondria and induce the opening of a large conductance channel known as the mitochondrial permeability transition (PT) pore, and this causes $\Delta\Psi_m$ disruption and release of caspase-activating proteins such as cytochrome c (14,30). Bcl-2 and Bcl-$x_L$ are reported to prevent PT whereas Bax is reported to induce PT and the release of cytochrome c (30,31). Expression level of Bcl-$x_L$ in mitochondrial fraction increases with IFN-α treatment, as seen with whole cell lysate in our previous study (10). We consider that an anti-apoptotic protein Bcl-$x_L$ level is not likely to contribute to the susceptibility of IFN-α-mediated apoptosis. Regarding Bax, a pro-apoptotic protein, various apoptotic stimuli are reported to induce its translocation from the cytosol to mitochondria (32). Juang *et al* (17) showed translocation of Bax from cytosol to mitochondria in IFN-ß-mediated apoptosis, however, Sangfelt *et al* (33), Yanase *et al* (18) and our own studies did not find such translocation in IFN-α-mediated apoptosis. Yanase *et al* (18) showed the expression of 18-kDa fragment (p18 Bax), which is a cleavage fragment of endogenous p21 Bax (21 kDa), in mitochondrial fraction, and state that modulation of endogenous p21 Bax is implicated in IFN-α-induced apoptosis. In the present study, p18 Bax expression in both the mitochondrial and cytosolic fractions was similar in the KIM-1 cells with or without IFN-α treatment. This suggests that at least in the KIM-1 cell line, p18 and p21 Bax are not essential factors for the induction of IFN-α-mediated apoptosis. A recent study of Panaretakis *et al* (20) that used a multiple myeloma cell line (U266), showed that IFN-α-induced apoptosis involves Bak and Bax activation with conformational changes via distinct mechanisms involving an unknown protease. It is necessary to examine whether similar changes occur in HCC cell lines. Smac/DIABLO is a protein that is released with cytochrome c from mitochondria during apoptosis, and this protein functions to promote caspase activation by associating with the Apaf-1 apoptosome and inhibiting apoptosis inhibiting proteins such as XIAP (34-36). Release of Smac/DIABLO from mitochondria was identified in IFN-α-mediated apoptosis of KIM-1 cells, and this would promote IFN-α-mediated apoptosis.

Although KYM-3 and KMCH-2 are resistant to IFN-α-induced apoptosis, IFN-α induces blockage of the cell-cycle at the S-phase in KYN-3, and the $G_1$ phase in KMCH-2 (10). Therefore, activation of Jak/signal transducer and activator of the transcription signaling pathway and certain IFN-inducible genes could be induced by IFN-α in these cells, but the cells may have abnormality in the caspase(s) and/or apoptosis-related molecules in mitochondria. Full-length caspase-9 and -8 expression levels in KMCH-2 were constitutively lower than in the other cell lines, and this would relate to the resistance. Recent studies indicate that caspase-8 expression acts as a key determinant of sensitivity for apoptosis induced by death ligands or cytotoxic drugs (37). We expect that the mechanisms of IFN-α-mediated apoptosis will become clearer by examining the expression patterns of multiple apoptosis-related genes using cDNA microarray analysis in the 4 cell lines with or without IFN-α treatment.

In conclusion, our current findings indicate the involvement of the mitochondrial apoptotic pathway with the activation of various caspases in the IFN-α-mediated apoptosis mechanism in liver cancer cells.

## Acknowledgments

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第4章 急性肝炎，劇症肝炎の診断と治療

# 急性肝炎の診断と治療（各論）：(2) B型急性肝炎とB型慢性肝炎急性増悪


## 要旨

B型急性肝炎やB型慢性肝炎急性増悪は，その重症度によって，適切な治療方針を立てなければならない．抗原抗体系やウイルス量などの血清学的検査を中心に診断を行う．急性感染は多くが一過性で軽快する．急性増悪ではインターフェロンやラミブジンによる治療を行う．重症化や劇症化時には肝移植を含めての治療を考慮する．劇症化の有無を的確に判断し，早期に適当な治療を行うことが重要である．



● キーワード

B型肝炎ウイルス
B型急性肝炎
B型慢性肝炎急性増悪


## はじめに

我が国におけるB型肝炎ウイルス（HBV）の感染者は人口の約1％，100万人強と考えられている．しかし，医療機関を受診しない無症候性キャリアも多いため，その正確な感染率はいまだに明らかでない．HBVによる感染は，日本ではほとんどが，母児による垂直感染である．それも，HBワクチンや抗HBs免疫グロブリン（HBIg）の開発により，予防可能となっている．しかし，不幸にも感染が成立した場合，そのほとんどはHBキャリアとなり，終生感染が持続し，自然治癒することはまれである．年齢を経るにつれて，肝機能異常（AST，ALTの上昇）が出現し，やがて慢性肝炎や肝硬変，中には肝癌を発症する．一方，成人になってからの感染は水平感染が主で，急性肝炎として一過性感染を起し，持続感染することはまれである．しかし，時に重症化や劇症化を引き起す．B型肝炎の診断については，以前から知られる抗原抗体系の血清マーカーに加え，HBV－DNAやprecore変異株やcore promoter変異株およびgenotypeの測定などが可能となり，病態の詳細な把握が可能となった．治療に関しては，近年新しい抗ウイルス薬の登場により，選択肢が広がった．そこで本稿では，前半でB型急性肝炎の診断と治療について，後半でB型慢性

肝炎急性増悪について述べたい．

## B型急性肝炎の診断

ここでは，母児感染を除く，成人期以降の水平感染による初感染について述べる．今日では医療従事者の針刺し事故や血液汚染を除けば，性行為を介して感染するいわゆる性行為感染症（STD）が大部分である[1]．輸血による感染はスクリーニング検査の確立された現在はほぼ皆無だが，一部で occult HBV による感染血が検査をすり抜けて感染を起す例が報告されている．慢性化率は2～10％と言われ，高齢者や免疫状態の悪い患者に多い．潜伏期間は1～6ヵ月と幅がある．

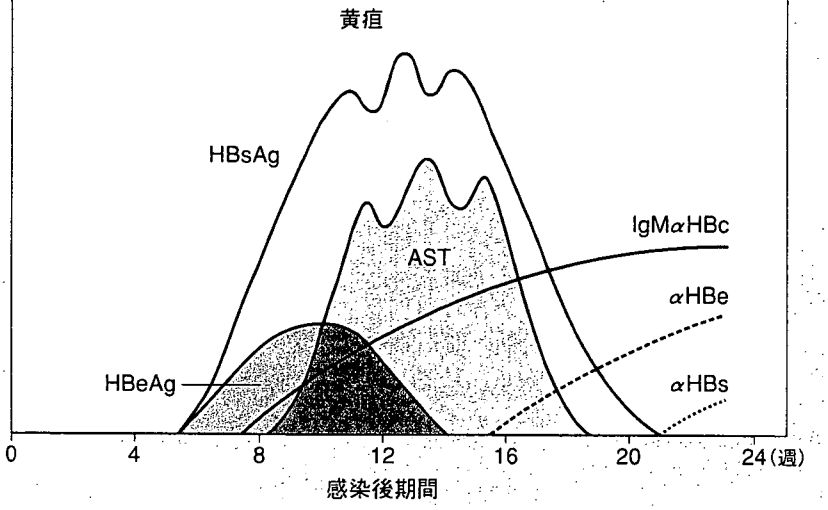
診断には血清学的検査が最も有効である．HBV の抗原抗体系にはB型肝炎ウイルス表面（HBs）抗原，HBs 抗体，B型肝炎ウイルスコア（HBc）抗原，HBc 抗体，B型肝炎ウイルスe（HBe）抗原，HBe 抗体，がある（表1）[2]．

HBs 抗原は血清トランスアミナーゼ値の上昇に先立ち，感染後約6週で血中に現れ，発病後，3ヵ月までには通常消失する（図1）．6ヵ月以上持続する場合は慢性化を考える．HBs 抗体は回復期と中和抗体を意味する．HBc 抗原は抹消血中には認められない．HBc 抗体には IgM 型と IgG 型がある．高力価 IgM 型 HBc 抗体の存在は感染初期を意味し，B型急性肝炎の診断となる．IgG 型 HBc 抗体はB型慢性肝炎もしくは既往感染を意味する．HBe 抗原の存在はウイルス産生が盛んで感染力が高いことを意味する．急性肝炎時も HBs 抗原とほぼ同時期に一過性に増加し早期に消失する．HBe 抗体は急性肝炎の回復期もしくは持続感染を意味する．急性肝炎で HBe 抗体が出現すれば，完全に回復する可能性が高い．B型急性肝炎の診断には HBs 抗原陽性，IgM 型 HBc 抗体強陽性が有用である．なお，IgM 型 HBc 抗体はB型慢性肝

表1　B型肝炎ウイルスマーカーの血清学的意義

| マーカー | 意義 |
|---|---|
| HBs 抗原 | 急性あるいは慢性B型肝炎 |
| IgM 型 HBc 抗体 | 急性B型肝炎（高力価）<br>慢性B型肝炎（低力価） |
| IgG 型 HBc 抗体 | B型肝炎既往感染（HBs 抗原陰性）<br>慢性B型肝炎（HBs 抗原陽性） |
| HBs 抗体 | B型肝炎に対する中和抗体 |
| HBe 抗原 | 急性B型肝炎，続けば持続感染状態 |
| HBe 抗体 | 回復期または持続感染状態 |
| HBV-DNA | 持続感染状態 |

図1　急性B型肝炎の経過




HBsAg：B型肝炎表面抗原，HBeAg：B型肝炎e抗原，
IgMαHBc：B型肝炎コア抗原に対するIgM抗体，
AST：アスパラギン酸アミノトランスフェラーゼ，αHBe：B型肝炎e抗原に対する抗体，
αHBs：B型肝炎表面抗原に対する抗体

炎の急性増悪時にも弱陽性を示すことがあるが，その場合は IgG 型 HBc 抗体が 200 倍希釈でも高力価であり，両者の鑑別が可能である．

血中 HBV－DNA の検出はウイルス増殖の最も鋭敏な指標であり，血清トランスアミナーゼ値ともよく相関する．血清トランスアミナーゼ値は現在の肝炎の程度を知る最も有用なマーカーとなる．多峰性に変動を繰り返す場合は慢性化に注意が必要である．

B型急性肝炎では感染の鎮静化とともに陰性化する．しかし，肝細胞内には，一過性感染後も HBV－DNA が検出され，肝炎鎮静後も肝内からは完全にウイルスが消失していないことを当院では報告している[3]．

劇症化率はB型急性肝炎全体で約2％と推定される．しかし，プロトロンビン時間が 40％ 未満のいわゆる重症型の急性肝炎では約 30％ が，肝性脳症Ⅱ度以上を合併し劇症肝炎に進展する[4]．劇症化の予測には，厚生労働省の研究班による予知式[5]と，与芝らの予知式[6]がよく用いられる（表2）．双方の式で算出される劇症化確率はしばしば異なるため，複数の式を用いて予後を判断するべきである．

表2 急性肝炎重症型の劇症化予知式

与芝の式

$\lambda = \text{logit}\ (P) = -0.89 + 1.74 \times$ 成因 $+ 0.056 \times$ T. Bil (mg / dl) $- 0.014 \times$ ChE (IU / L)

成因：HAV または HBV 初感染　1，その他　2

研究班の式

$\lambda = \text{logit}\ (P) = -2.7469 + 0.0914 \times$（年齢）$+ 0.1255 \times$ T. Bil (mg / dl) $- 0.1534 \times$ PT (%)

劇症化確率 $(p) = 1 / (1 + e^{-\lambda})$

## B型急性肝炎の治療

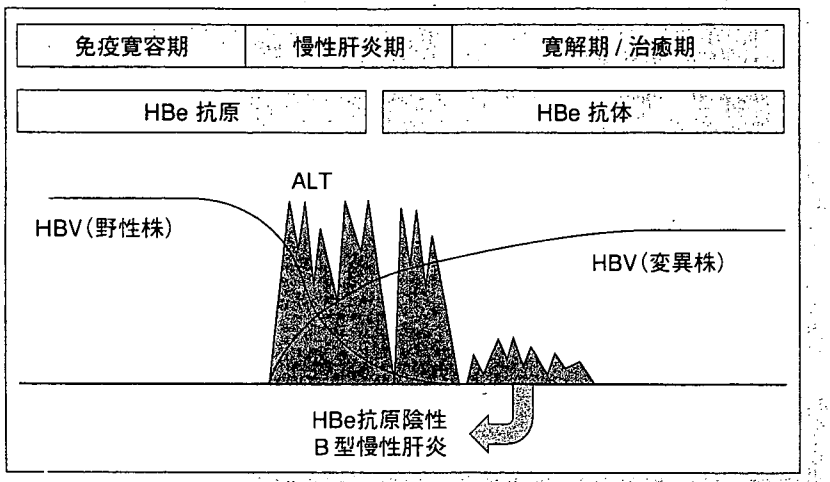
多くの場合3ヵ月以内に肝炎は鎮静化するので，入院による安静と食事療法の対処療法のみで根治可能である．食事は高タンパク，高カロリーが基本であるが，栄養過多による脂肪肝に注意が必要である．食欲低下を認める場合はビタミン剤を添加した補液が行われる．当院では全身倦怠感や食欲不振などの自覚症状の消失，血清トランスアミナーゼ値 100 IU / L 未満，血清総ビリルビン値 2.0 mg / dl 未満を退院の目処としている．

B型急性肝炎の中でも，重症化，劇症化を来す症例を数％で認める．血清総ビリルビン値やプロトロンビン時間などの肝予備能に注意しながら，劇症化の予知式[5)6)]などによる予測を行い，早めの対策が救命につながる．抗ウイルス薬のラミブジンをB型急性肝炎に使用し有効との報告[7)]もあるが，急性感染では血中のウイルスは早期に消失するため，ラミブジンがこの消失動態に影響するかは，まだ明らかなエビデンスはない．重症化，劇症化時の詳しい治療は劇症肝炎の治療の項に譲る．

## B型慢性肝炎急性増悪の診断

B型慢性肝炎や血清トランスアミナーゼ正常の HB キャリアの患者が，急激な血清トランスアミナーゼ値の上昇と，時に肝機能の低下や全身倦怠感を引き起す．これを急性増悪と呼ぶ．その際に HBe 抗原陽性から HBe 抗体陽性へのセロコンバージョンを起すことがある（図2）[2)]．自然経過でのセロコンバージョンは年率 10～15％と言われ，インターフェロン（IFN）などの抗ウイルス療法後にみられるこ

図2 HBe 抗原から HBe 抗体へのセロコンバージョン（SC）




SC は HBV（野性株）が pre-core 遺伝子あるいは core promoter 遺伝子に変異を来した HBV（変異株）となるために生じる．SC 後も HBV 増殖が持続し慢性肝炎（HBe 抗原陰性）が持続することがまれにある．

ともある．しかし，中には重症化，劇症化を来し，肝不全となる症例を認める．近年，HBe 抗原陰性でも HBV－DNA 陽性のB型慢性肝炎患者の存在が確認され，これらは HBe 抗原が産生されない pre-core 変異株や core promoter 変異株によることが報告されている[8]．HB キャリアやB型慢性肝炎からの急性増悪症例で肝不全となり死亡した症例の中には，HBV－DNA が 7.6 log copies / ml を超える高ウイルス症例のみならず，今述べた HBe 抗原陰性 HBe 抗体陽性の低ウイルス症例も認められる．したがって，抗原抗体系の血清マーカーのみにとらわれず，血清総ビリルビン値やプロトロンビン時間などの肝予備能を見ながら，重症化や劇症化を防ぐ必要がある．

## B型慢性肝炎急性増悪の治療

急性増悪時の治療目標は ALT の正常化と重症化の防止である．基本的にはB型慢性肝炎の治療すなわち，IFN もしくはラミブジンなどの抗ウイルス薬の使用が中心となる．HB キャリアからの急性増悪例では予後の悪い亜急性型の劇症肝炎に進展する可能性が高く，肝移植も考慮しながらの診療が必要である．HBe 抗体陽性 HB キャリアに，他疾患でステロイドや免疫抑制剤を使用すると，肝炎が増悪する

表3 B型肝炎治療の新しいステージ分類

| HB stage | 0 | I | II | III | IV | V |
|---|---|---|---|---|---|---|
| HBsAg | + | + | + | + | + | −** |
| HBeAg | + | + | + | − | − | − |
| HBV-DNA (copies/ml) | 不問 | $10^{7.6}$≦ | $10^{7.6}$> | $10^{5}$≦ | $10^{5}$> | 不問 |
| ALT | 持続正常 | 持続正常以外 | 持続正常以外 | 不問 | 不問 | 不問 |
| 年齢 | 不問 | 若年 / 高年* (Ia / Ib) | 若年 / 高年* (IIa / IIb) | 不問 | 不問 | 不問 |
| 発癌リスク | 極めて小 | 小 / 大 | 小 / 極めて大 | 極めて大 | 極めて小 | 極めて小 |
| 治療 | 不要 | $F_2$ 以上 IFN / IFN+ ラミブジン | IFN / ラミブジン | ラミブジン | 不要 | 不要 |

*：若年　男性 30 歳未満，女性 35 歳未満　　高年　男性 30 歳以上，女性 35 歳以上
**：HBsAg (+) の時期が確認されていること

略語：巻末の略語集参照

ことがあり，注意が必要である．一般にウイルス量の増加が高度な症例では，肝炎も重症化する例が多いので，早期より積極的にラミブジンを使用するべきである．いずれにしても，急性感染同様に，劇症化の予知式[5][6]などを用いて，早めの治療が必要である．

最も重要なのは，治療開始のタイミングである．特にラミブジンの開始時期にはさまざまな見解がある．プロトロンビン時間が 40 % をきるような重症例での早期使用は当然であるが，HB キャリアからの急性増悪と慢性肝炎からの急性増悪では異なった基準を設けるべきとの意見もある[9]．すなわち，ラミブジンの有効性の検討結果から，HB キャリアからの急性増悪例ではプロトロンビン時間が 60 % 未満，慢性肝炎からの急性増悪例では総ビリルビン値が 5 mg / dl 以上で速やかにラミブジンを投与すべきと考えられる．

ラミブジン長期投与例においてみられる耐性株出現による breakthrough hepatitis が原因の急性増悪例では他の抗ウイルス薬の併用が試みられる．我が国では 2004 年 12 月より，アデホビルが保険適用となり，ラミブジン投与中に HBV の持続的な再増殖を伴う肝機能異常が確認された症例で，ラミブジンとの併用治療が可能となった．また，entecavir など，他の抗ウイルス薬の開発も進んでおり，今後

の臨床への応用が期待される．

当院の加藤ら[10]は年齢やウイルス量によって，B型肝炎治療のステージ分類（表3）を作成し，治療法の選択に使用している．HBs抗原，HBe抗原，HBV－DNA，ALT，年齢によってステージングを行い，発がんリスクを考慮して，ラミブジン，IFNなどの治療の適否を示している．しかし，これらは新たな抗ウイルス薬が使用可能になるまでの暫定的な治療選択であり，今後，選択肢が広がる可能性が十分考えられる．

## おわりに

B型急性肝炎では，その多くが一過性感染で，無治療にて軽快する．まれに重症化や劇症化を引き起す．B型慢性肝炎急性増悪では，セロコンバージョンなど，一過性の悪化で改善する場合もあるが，一部に重症化や劇症化を起す症例を認める．どちらも治療の最大の目的は重症化や劇症化を未然に防ぎ，肝不全を引き起さないことである．そして，不幸にして，肝不全となったときには，肝移植を含めた集学的治療を行うことが重要である．今後，新たな抗ウイルス薬の開発により，B型肝炎に対する治療法は大きく変化する可能性が考えられる．

伊与田賢也　結城暢一　山本佳司
加藤道夫

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# Polymorphisms of interleukin-1β in Japanese patients with hepatitis B virus infection

Kiyoshi Migita[1,*], Yumi Maeda[1], Seigo Abiru[1], Minoru Nakamura[1], Atsumasa Komori[1], Seiji Miyazoe[2], Kazuhiko Nakao[2], Hiroshi Yatsuhashi[1], Katsumi Eguchi[2], Hiromi Ishibashi[1]

[1]*Clinical Research Center, National Nagasaki Medical Center, Kubara 2-1001-1, Omura 856-8562, Japan*
[2]*First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki 852-8501, Japan*

***Background/Aims*: Hepatitis B virus (HBV) induces liver cirrhosis (LC) and hepatocellular carcinoma (HCC) mainly by causing chronic necro-inflammatory hepatic disease. Our aim was to investigate the relationships between the polymorphisms of the interleukin-1B (*IL-1B*) promoter region and the interleukin-1 receptor antagonist gene (*IL-1RN*) and disease progression in an HBV-infected Japanese population.**

***Methods*: Genomic DNA was extracted from the peripheral blood of 237 HBV carriers. Polymorphisms in *IL-1B* and *IL-1RN* were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and PCR with confronting two-pair primers (PCR-CTPP) methods. These polymorphic sites include the promoter regions of *IL-1B* at positions −511 and −31, and *IL-1RN* variable tandem repeats.**

***Results*: The *IL-1B* −31 and −511 loci were in complete linkage disequilibrium, and the frequency of the *IL-1B* −31 T carrier (*IL-1B* −31 T/T or T/C) was significantly higher in HBV carriers with LC compared to those without LC (LC; 86.1% vs non-LC; 72.1%, $P = 0.009$). There was no difference in the genotype distribution of the *IL-1RN* polymorphism.**

***Conclusions*: This is the first report describing the association between *IL-1B* polymorphism and HBV-related hepatic fibrosis, and our data suggest that *IL-1B* polymorphisms may be related to disease progression of HBV-related hepatitis in Japan.**



***Keywords*: Cytokines; Hepatitis B virus; Interleukin-1β; Liver cirrhosis; Polymorphism; Hepatic fibrosis**

## 1. Background

Recent studies have emphasized that hepatic satellite cells (HSC) play an important role in the pathogenesis of hepatic fibrosis [1]. Following liver injury, HSC produce matrix metalloproteinase (MMPs) resulting in the development of hepatic fibrosis [2]. IL-1 has been implicated in the regulation of MMPs production by HSC [3]. IL-1β is known to be a proinflammatory cytokine and mediate several immune responses [4]. It is encoded by the *IL-1B* gene with several promoter elements, including a TATA box, a typical motif of includible genes [5,6]. The *IL-1B* gene has diallelic polymorphisms at −511, −31 base pairs (bp) from the transcriptional start site [7]. IL-1-receptor antagonist (*IL-1RN*) is an anti-inflammatory molecule that competes for receptor binding with IL-1β [8,9]. The *IL-1RN* gene contains an 86-bp variable number tandem repeat (VNTR) polymorphism in intron 2 [10]. These polymorphisms are, therefore, of

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* Corresponding author. Tel.: +81 957 52 3121; fax: +81 957 54 0292.

*E-mail address:* migita@nmc.hosp.go.jp (K. Migita).

*Abbreviations:* HBV, hepatitis B virus; LC, liver cirrhosis; HCC, hepatocellular carcinoma; IL-1B, interleukin-1B; IL-1RN, interleukin-1 receptor antagonist gene.

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**Table 1**
**Baseline characteristics of HBV carrier**

| | ASC ($n = 65$) | CH ($n = 58$) | LC ($n = 65$) | HCC ($n = 49$) (CH:6, LC:43) |
|---|---|---|---|---|
| Mean age (yr) | 55.7 ± 17.6 | 42.6 ± 14.4 | 58.2 ± 9.3 | 62.9 ± 9.4 |
| Sex (M/F) | 34/31 | 37/21 | 50/15 | 40/9 |
| Mean ALT (U/L) | 21.2 ± 8.1 | 134.7 ± 159.1 | 71.4 ± 89.3 | 59.2 ± 62.8 |
| Mean PLT ($10^3/\mu l$) | 197.8 ± 52.2 | 199.5 ± 51.1 | 125.9 ± 55.2 | 121.6 ± 84.0 |
| Mean albumin (g/dl) | 4.6 ± 0.3 | 4.5 ± 0.4 | 4.2 ± 0.6 | 3.8 ± 1.0 |

Abbreviations: ASC, asymptomatic carrier; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; PLT, platelet.

potential functional importance in their modulation of IL-1β protein production and its biological activity.

Increasing evidences indicate that genetic factors influence the natural history of chronic liver diseases [11]. Recent studies have proposed that a number of gene polymorphisms influence the progression of fibrosis in patients with hepatitis C virus (HCV) infections, autoimmune chronic cholestasis, and alcohol-induced liver disease [12–14]. The aim of the present study was to elucidate the association of *IL-1B* and *IL-1RN* loci polymorphism as host genetic factors with an increased risk of developing HBV-related liver diseases in a Japanese population.

## 2. Patients and methods

### 2.1. Patients

A total of 237 patients who were positive for hepatitis B surface antigen (HBsAg) visited the clinics for liver diseases at the Nagasaki University Hospital or Nagasaki Medical Center between August, 1999, and June, 2004. As controls, 63 healthy Japanese volunteers (33 men and 30 women, 22–66 years old, with a mean age of 36.6 ± 7.7 years) without any history liver disease were enrolled in the study after obtaining informed consent. The patients were regularly followed, with measurements of serum ALT and HBV markers such as HBsAg, HBeAg, anti-HBe using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan), and HBV DNA. Tumor markers such as alpha fetoprotein and/or des-γ-carboxy-prothrombin were also measured every month, and with ultrasonography or computed tomography of the liver every 3 months to detect HCC in a early stage. The diagnosis of HCC was made by several imaging modalities in all patients and confirmed histologically by sonography-guided fine-needle biopsy specimens, if needed in all patients. All patients did not have any other types of liver diseases such as chronic hepatitis C, alcoholic liver diseases, autoimmune liver diseases, or metabolic liver diseases. The study protocol was approved by the Ethics Committees of both Nagasaki University Hospital and National Nagasaki Medical Center, and informed consent was obtained from each individual.

Of the 237 HBV carriers, 65 patients were considered to be asymptomatic carriers (ASC) based on sustained normalization of the serum ALT levels together with seropositivity for anti-HBe throughout the study (Table 1). On the other hand, 172 of the 237 HBV carriers were considered to have chronic progressive liver disease (CPLD) such as chronic hepatitis (58), cirrhosis (65), or hepatocellular carcinoma (49) manifested by elevated ALT levels and by clinical or histological findings on examination of liver tissue during the follow-up period (Table 1). Of the 49 patients with HCC, 6 (12%) were found to have chronic hepatitis; 43 (88%) had cirrhosis. The clinical data, including bilirubin, albumin, prothrombin time and the presence of ascites or hepatic encephalopathy, were collected and Child–Pugh score was calculated in LC patients. Of 237 HBV carriers, 79 had undergone liver biopsy during the study period to assess the degree of liver fibrosis using the METAVIR system [15]. Liver biopsy was not performed in parts of patients who had apparent biochemical, endoscopic and ultrasound features of LC.

### 2.2. DNA extraction

Genomic DNA was isolated from whole blood using the QIAamp DNA blood protocol according to the manufacturer's instruction (Qiagen Ltd., UK).

### 2.3. Genotyping

The polymerase chain reaction (PCR) amplification was conducted using the primers listed in Table 2.

#### 2.3.1. IL-1RN

The *IL-1RN* intron 2 contains a VNTR of an 86-bp length of DNA. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Allele 1 (four repeats) was 410 bp, allele 2 (two repeats) was 240 bp, allele 3 (three repeats) was 325 bp, allele 4 (five repeats) was 500 bp, and allele 5 (six repeats) was 595 bp [16].

**Table 2**
**PCR condition for IL-1B and IL-1RN**

| Polymorphism | Primers | PCR conditions |
|---|---|---|
| IL-1B, T to C at −31 | F1 5′-AATGTGGACATCAACTGCA-3′<br>R1 5′-CTCCCTCGCTGTTTTTATA-3′<br>F2 5′-ACTTCTGCTTTTGAAAGCC-3′<br>R2 5′-TCAGCTGTTAGATAAGCAG-3′ | PCR-CTPP (confronting two-pair primers)<br>10 min at 95 °C, 30 cycles of 1 min at 95 °C, 48 °C and 72 °C and 5 min at 72 °C |
| IL-1B, C to T at −511 | 5′-GCCTGAACCCTGCATACCGT-3′<br>5′-GCCAATAGCCCTCCCTGTCT-3′ | PCR-RFLP (*Ava I*)<br>10 min at 94 °C, 5 cycles of 30 sec at 94 °C, 65 °C and 72 °C and 30 cycles of 30 sec at 94 °C, 60 °C and 72 °C and 5 cycles of 30 sec at 94 °C, 55 °C and 72 °C and 7 min 72 °C |
| IL-1RN 86 bp VNTR at intron 2 | F 5′-CTCAGCAACACTCCTAT-3′<br>R 5′-TCCTGGTCTGCAGGTAA-3′ | 10 min at 95 °C, 35 cycles of 1 min at 95 °C, 55 °C and 72 °C and 5 min at 72 °C |

Table 3
IL-1RN genotype frequencies in HBV carriers

| Variables | Patients with HBV | | | | | Healthy volunteers ($n = 63$) (%) |
|---|---|---|---|---|---|---|
| | Total ($n = 237$) (%) | ASC ($n = 65$) (%) | CH ($n = 58$) (%) | LC ($n = 65$) (%) | HCC ($n = 49$) (%) | |
| *IL-1RN* | | | | | | |
| 1/1 | 216 (91.1) | 59 (90.8) | 53 (91.4) | 58 (89.2) | 46 (93.9) | 53 (84.1) |
| 1/2 | 13 (5.5) | 5 (7.7) | 1 (1.7) | 6 (9.2) | 1 (2.0) | 7 (11.1) |
| 1/3 | 1 (0.4) | 0 | 1 (1.7) | 0 | 0 | 0 |
| 1/4 | 4 (1.7) | 0 | 2 (3.4) | 1 (1.5) | 1 (2.0) | 2 (3.2) |
| 2/2 | 1 (0.4) | 0 | 0 | 0 | 1 (2.0) | 0 |
| 2/4 | 2 (0.8) | 1 (1.5) | 1 (1.7) | 0 | 0 | 1 (1.6) |

*Note.* The genotype are shown as frequency (percentage).
Abbreviations: HBV, hepatitis B virus; ASC, asymptomatic carrier; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; IL-1RN, IL-1 receptor antagonist.

#### 2.3.2. *IL-1B −511*

A fragment containing the *Ava*I polymorphic site at position −511 of the *IL-1B* gene was amplified by PCR. Fragments were separated by electrophoresis on 3% agarose with ethidium bromide staining using appropriate commercially available size markers for comparison. The C allele was designated if two bands of 92 and 63 bp were obtained, and the T allele was designated if a signal band of the undigested 155 bp was obtained. Genotypes were designated as follows: C/C, two bands of 92 and 63 bp: C/T, three bands of 155, 92, and 63 bp; and T/T, a single band of 155 bp [17].

#### 2.3.3. *IL-1B −31*

For the polymorphisms at −31 of *IL-1B*, a new method named PCR-CTPP (PCR with confronting two-pair primers) was applied, which does not require a step to digest DNA products for single nucleotide polymorphism genotyping [18]. All PCR products were visualized on a 2% agarose gel with ethidium bromide staining.

### 2.4. Statistical analysis

Results are expressed as means ± SD. Comparisons were made by Fisher's extract probability test and the $\chi^2$ test. All p values were two-tailed, and $P$ values <0.05 were considered to indicate statistical significance.

## 3. Results

### 3.1. *IL-1RN* gene polymorphisms

Six *IL-1RN* genotypes (1/1, 1/2, 1/3, 1/4, 2/2, and 2/4) were included in our study. Genotype 1/1(4/4 repeats) was the most common genotype in the HBV carrier patients (91.1%). In contrast to Caucasian populations, the homozygote allele 2* (2/2 repeat) was found in only one patient with HCC. The heterozygote allele 2* (1/2, 2/2, and 2/4) was found in 9.2% of ASC, 3.4% CH, 9.2% LC, and 4.0% of the HCC group (Table 3). The present study found no significant difference in *IL-1RN* genotype frequencies among various liver diseases of HBV carriers.

### 3.2. *IL-1B* gene polymorphisms

Table 4 shows the genotype frequencies of *IL-1B* gene polymorphism. Since *IL-1B* −511C/T was in complete linkage equilibrium with *IL-1B* −31T/C, only *IL-1B* −31T/C was described in this haplotype analysis. Although no statistical difference was found in allelic frequencies between liver cirrhosis patients and healthy subjects, *IL-1B* −31C/C homozygotes were less frequently seen in HBV carriers with LC or HCC. The proportion of the C/C genotype of *IL-1B* −31 in patients with HCC (14.3%) was not different from that in patients with LC (15.4%). However, when cases were subdivided according to the presence of LC (Table 5), the frequency of the C/C genotype in patients with LC (13.9%) was significantly lower than that in patients without LC (27.9%), while inversely the frequency of

Table 4
IL-1B genotype frequencies in HBV carriers

| Variables | Patients with HBV | | | | | Healthy volunteers ($n = 63$) (%) |
|---|---|---|---|---|---|---|
| | Total ($n = 237$) (%) | ASC ($n = 65$) (%) | CH ($n = 58$) (%) | LC ($n = 65$) (%) | HCC ($n = 49$) (%) | |
| *IL-1B −511* | | | | | | |
| C/C | 69 (29.1) | 17 (26.2) | 18 (31.0) | 17 (26.2) | 17 (34.7) | 20 (31.8) |
| C/T | 117 (49.4) | 27 (41.5) | 27 (46.6) | 38 (58.5) | 25 (51.0) | 29 (46.0) |
| T/T | 51 (21.5) | 21 (32.3) | 13 (22.4) | 10 (15.4) | 7 (14.3) | 14 (22.2) |
| *IL-1B −31* | | | | | | |
| C/C | 51 (21.5) | 21 (32.3) | 13 (22.4) | 10 (15.4) | 7 (14.3) | 14 (22.2) |
| C/T | 117 (49.4) | 27 (41.5) | 27 (46.6) | 38 (58.5) | 25 (51.0) | 29 (46.0) |
| T/T | 69 (29.1) | 17 (26.2) | 18 (31.0) | 17 (26.2) | 17 (34.7) | 20 (31.8) |

*Note.* The genotype are shown as frequency (percentage).
Abbreviations: HBV, hepatitis B virus; ASC, asymptomatic carrier; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; IL-1B, interleukin-1B.

**Table 5**
**Differential distribution of IL-1B genotypes in HBV carriers**

| Locus | Genotype | Non-LC ($n = 129$) | LC ($n = 108$) | OR (95% CI) | $P$ |
|---|---|---|---|---|---|
| IL-1B −31 | T/C, T/T | 93 (72.1%) | 93 (86.1%) | 2.40 (1.23–4.68) | 0.0089 |
| | C/C | 36 (27.9%) | 15 (13.9%) | | |

*IL-1B* −31 T carrier (*IL-1B* −31 T/T or T/C) was significantly higher in HBV carriers with LC compared to those without LC (LC; 86.1% vs non-LC; 72.1%, $P = 0.009$). When the LC patients were divided using Child–Pugh classification, there was no significant difference in *IL-1B* gene genotype frequencies between LC patients with Child–Pugh classification A and Child–Pugh classification B or C (Table 6A). In *IL-1RN* genotypes, *IL-1RN* 1/1 genotype was the most common genotype and 1/2 genotype was increased in LC patients with Child–Pugh classification A. However, there was no significant difference in genotype of *IL-1RN* among these LC patient groups (Table 6B). Furthermore, we examined the relationship between *IL-1B* gene genotype and the stage of hepatic fibrosis (Table 7). The frequency of the C/C genotype was reduced in patients with fibrosis stage F3–F4 (11.8%) compared to those with F0–F2 (28.6%), however, the number of patients who had undergone liver biopsy was limited and statistically significant difference was observed.

## 4. Discussion

Liver fibrogenesis is initiated by hepatocyte damage and the subsequent recruitment and activation of inflammatory cells [19]. These inflammatory cells produce fibrogenic cytokines and growth factors that activate hepatic satellite cells (HSC) [20]. The role of cytokine gene polymorphism in the progression of liver fibrosis or development of cirrhosis in patients with chronic liver diseases has been investigated extensively. Yee et al. indicated that TNF2 allele (−238A) and TNF3 allele (−308A) are more frequently found in patients with cirrhosis in chronic HCV infection [21]. Polymorphisms of TGF-β gene are thought to be one of the determinants of fibrosis progression in viral hepatitis [11]. Therefore, cytokine polymorphism could be involved in fibrosis progression in HBV infection.

**Table 6A**
**Distribution of IL-1B genotype and Child–Pugh classification in LC patients**

| Locus | Genotype | Child–Pugh classification | | |
|---|---|---|---|---|
| | | A ($n = 79$) (%) | B ($n = 20$) (%) | C ($n = 9$) (%) |
| IL-1B −31 | C/C | 10 (12.7) | 4 (20.0) | 1 (11.1) |
| | C/T | 44 (55.7) | 12 (60.0) | 5 (55.6) |
| | T/T | 25 (31.6) | 4 (20.0) | 3 (33.3) |

Recent studies have indicated that HSC play an important role in hepatic fibrogenesis and that IL-1 is a potent cytokine that induces the myofibroblastic activation of HSC [3]. Our data indicate that the frequency *IL-1B* −31 T carrier (*IL-1B* −31 T/T or *IL1B* −31 T/C) was significantly higher in HBV carriers with LC compared to in those without LC. These results suggest that the *IL-1B* genotype may influence fibrotic progression in HBV-related hepatitis. On the other hand, the frequency of the *IL-1RN* (intron 2, VNTR)* A2 allele was extremely uncommon in the study subjects and was not significantly different between HBV carriers with or without liver cirrhosis.

In our study, *IL-1B* −511 was in a complete linkage disequilibrium (LD) with IL-1B −31 in the Japanese population. Therefore, the effect of the *IL-1B* −511 C/T may be due to LD with *IL-1B* −31 T/C. There are some confusions regarding the 1*L*-1*B* (−31) alleles in earlier literatures. El-Omar et al. reported that −31C alleles increased the risk of gastric cancer [5,6]. They considered the *IL-1B* −31C allele but not the T allele as a pro-inflammatory gene. The *IL-1B* −31 T/C polymorphism is situated on a TATA box in the promoter region. However, the mutation of T to C in the TATA box in the *IL-1B* gene promoter (−31) will result in down regulation of the *IL-1B* gene in electrophoretic mobility-shift assay [5,6]. Xuan et al. found that *IL-1B* polymorphisms (*IL-1B* −511 C/C and −31T/T) enhanced IL-1β production in the gastric body of Japanese patients [22]. Similarly, the *IL-1B* −31 T/T genotype has been shown to be associated with an increased risk for HCC in Japanese patients with HCV infection [23]. Therefore, it is possible that the *IL-1B* −31T/T allele could be implicated in inflammatory processes. Our finding that HBV carriers harboring an *IL-1B* −31C/C genotype were less frequent in LC patients

**Table 6B**
**Distribution of IL-1RN genotype and Child–Pugh classification in LC patients**

| Variables | Child–Pugh classification | | |
|---|---|---|---|
| | A ($n = 79$) (%) | B ($n = 20$) (%) | C ($n = 9$) (%) |
| *IL-1RN* | | | |
| 1/1 | 72 (91.1) | 19 (95.0) | 8 (88.9) |
| 1/2 | 6 (7.6) | 0 | 0 |
| 1/3 | 0 | 0 | 0 |
| 1/4 | 1 (1.3) | 1 (5.0) | 0 |
| 2/2 | 0 | 0 | 1 (11.1) |
| 2/4 | 0 | 0 | 0 |

Table 7
Distribution of IL-1B genotypes and METAVIR score in HBV carriers

| Locus | Genotype | Stage of fibrosis | | OR (95% CI) | P |
|---|---|---|---|---|---|
| | | F0–F2 ($n = 28$) (%) | F3–F4 ($n = 51$) (%) | | |
| IL-1B −31 | T/C, T/T | 20 (71.4) | 45 (88.2) | 3.0 (0.92–9.8) | 0.061 |
| | C/C | 8 (28.6) | 6 (11.8) | | |

than non-LC patients is in accord with these findings. IL-1 is a proinflammatory cytokine which is involved in the fibrotic response. IL-1 causes tissue injury, which induces the fibrotic response, by producing chemotactic molecules, such as chemokines [24]. IL-1 is also implicated in the proliferation of HSC [25] and the regulation of the expression of various matrix metalloproteinases, which play a key role in the turnover and the deposition of extracellular matrix (ECM) [3]. Therefore, it is possible that genetic polymorphism of *IL-1B* gene may influence the progression of hepatic fibrosis by affecting the hepatic expression of IL-1 during the process of liver injury. Since this is the first report of the association between *IL-1B* polymorphism and hepatic fibrosis in HBV carriers, further investigation is required to confirm and extend our findings.

Several studies reported that *IL-1B* −31T is a risk haplotype for the development of cancer. Hirankarn et al. reported that IL-1B- 511C (−31T) allele is a genetic marker for the development of HCC in chronic hepatitis B patients in Thai population [26]. More recently, Chen et al. reported that in the presence of the *IL-1RN**2 allele, a ~5-fold increased risk of HCC was found for HBV carriers harboring the *IL-1B* −31 T/T or IL-1B −511 C/C genotype compared with those harboring the IL-1B −31 C/C or *IL-1B* −511 T/T genotype [27]. The frequencies of the *IL-1B* −31 genotype between HCC and non-HCC patients were insignificant in our study. However, our study included only 49 HCC patients and could not assess the interaction between polymorphisms of the *IL-1RN* and *IL-1B* genes due to the infrequency of the *IL-1RA**2 allele among Japanese subjects. Further investigations using large-scale case-control studies are needed to elucidate the relationship between the HCC risk and the *IL-1B* gene polymorphism.

Takamatsu et al. reported that the presence of the *IL-1B* −31C/C genotype was found at a significantly higher frequency in patients with liver cirrhosis than in those without cirrhotic alcoholic liver disease [28]. The reason for this discrepancy between our data and those of this previous report is unclear, although one possible explanation is a difference of the pathogenesis of liver cirrhosis.

In summary, the findings of the present study suggest that polymorphism in the promoter region of the *IL-1B* gene (−31) is implicated in the regulation of liver fibrosis in patients with HBV infection. The interactions between HBV viral factors and host factors including cytokine polymorphisms may contribute to disease progression in HVB carriers. The interaction between *IL-1B* polymorphisms and liver fibrogenesis deserves further study.

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# Spatial and chronological differences in hepatitis B virus genotypes from patients with acute hepatitis B in Japan

Fuminaka Sugauchi[a,b], Etsuro Orito[a,b], Tomoyoshi Ohno[a,b], Yasuhito Tanaka[a], Atsushi Ozasa[a,b], Jong-Hon Kang[c], Joji Toyoda[d], Tomoyuki Kuramitsu[e], Kazuyuki Suzuki[f], Eiji Tanaka[g], Yoshihiro Akahane[h], Takafumi Ichida[i], Namiki Izumi[j], Kazuaki Inoue[k], Hiromi Hoshino[l], Shiro Iino[m], Hiroshi Yotsuyanagi[n], Shinichi Kakumu[o], Eiichi Tomita[p], Takeshi Okanoue[q], Shuhei Nishiguchi[r], Yoshikazu Murawaki[s], Keisuke Hino[t], Morikazu Onji[u], Hiroshi Yatsuhashi[v], Michio Sata[w], Yuzo Miyakawa[x], Ryuzo Ueda[b], Masashi Mizokami[a,*]

[a] *Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan*
[b] *Department of Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan*
[c] *Teinekeijinkai Hospital, Sappolo, Japan*
[d] *Sapporo Kosei General Hospital, Sappro, Japan*
[e] *Akita City Hospital, Akita, Japan*
[f] *Iwate Medical University, Morioka, Japan*
[g] *Shinshu University Graduate School of Medicine, Matsumoto, Japan*
[h] *University of Yamanashi, Yamanashi, Japan*
[i] *Department of Gastroenterology, Juntendo University School of Medicine, Shizuoka Hospital, Shizuoka, Japan*
[j] *Musashino Red Cross Hospital, Tokyo, Japan*
[k] *Showa University Fujigaoka Hospital, Yokohama, Japan*
[l] *Hepaptitis Research Institute, Tokorozawa, Japan*
[m] *Kiyokawa Hospital, Tokyo, Japan*
[n] *St. Marianna University School of Medicine, Kawasaki, Japan*
[o] *Aichi Medical University School of Medicine, Aichi, Japan*
[p] *Gifu Municipal Hospital, Gifu, Japan*
[q] *Kyoto Prefectural University of Medicine, Kyoto, Japan*
[r] *Osaka City University, Osaka, Japan*
[s] *Tottori University, Tottori, Japan*
[t] *Yamaguchi University School of Medicine, Yamaguchi, Japan*
[u] *Ehime University School of Medicine, Matsuyama, Japan*
[v] *National Hospital Organization Nagasaki Medical Center, Nagasaki, Japan*
[w] *Kurume University School of Medicine, Kurume, Japan*
[x] *Miyakawa Memorial Reserch Foundation, Tokyo, Japan*

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**Abstract**

Genotypes of hepatitis B virus (HBV) were determined in 485 patients with acute hepatitis B from all over Japan. They were A in 92 (19%), Ba in 26 (5%), Bj in 32 (7%), C in 330 (68%) and D in 5 (1%). Sexual contacts were the main route of transmission in them. Overall,

* Corresponding author. Tel.: +81 52 853 8292; fax: +81 52 842 0021.
*E-mail address:* mizokami@med.nagoya-cu.ac.jp (M. Mizokami).

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