Suzuki Y, Akuta N, et al. J Clin Virol 2010; 47 (1): 76—78 3) Gane EJ, Roberts SK, Stedman CA, et al. Lancet 2010; 376 (9751): 1467—1475 4) Moucari R, Forestier N, Larrey D, et al. Gut 2010; 59 (12): 1694—1698

英文要旨

Effect of early antiviral agent therapy (NS3 and NS5A inhibitors) in chronic hepatitis C null responders

Yoshiyuki Suzuki[1)*], Hitomi Sezaki[1)], Norio Akuta[1)], Fumitaka Suzuki[1)], Hiroya Seko[1)], Norihiro Imai[1)], Miharu Hirakawa[1)], Yuusuke Kawamura[1)], Tetsuya Hosaka[1)], Masahiro Kobayashi[1)], Satoshi Saitoh[1)], Yasuji Arase[1)], Kenji Ikeda[1)], Mariko Kobayashi[2)], Hiromitsu Kumada[1)]

To further improve therapeutic effect on chronic hepatitis C, we have administered NS3 inhibitor and NS5A inhibitor together, and examined effects of early antiviral agent therapy. The subjects were five cases where interferon is ineffective (null responders). The NS5A and NS3 inhibitors are oral drugs and were daily administered for 24 weeks. Figure 1 shows time-dependent change of the number of viruses after the therapy started, and rapid decrease of viruses is recognized. Within 12 hours, HCV-RNA decreased by more than 2 log IU/m*l* in every patient. Two patients became negative for the virus by the 15th day after the therapy started. Furthermore, 80% of cases by the 28th day and all the cases by the 56th day became negative. The new therapy has manifested excellent early antiviral effect.

Key words: hepatitis C virus, NS5A inhibitor, protease inhibitor

Kanzo 2011: 52: 147—149

1) Department of Hepatology, Toranomon Hospital
2) Liver Research Laboratory, Toranomon Hospital
*Corresponding author: suzunari@interlink.or.jp

肝臓　52巻4号　255-257（2011）

＜速　報＞

B型慢性肝疾患に対する核酸アナログ療法によるHBs抗原消失とその関連因子の検討

保坂　哲也[1)*]　鈴木　文孝[1)]　小林　正宏[1)]　瀬古　裕也[1)]　今井　則博[1)]
平川　美晴[1)]　川村　祐介[1)]　瀬崎ひとみ[1)]　芥田　憲夫[1)]　鈴木　義之[1)]
斎藤　聡[1)]　荒瀬　康司[1)]　池田　健次[1)]　小林万利子[2)]　熊田　博光[1)]

緒言：B型肝疾患に対する核酸アナログ療法の有効性は広く知られており，経過観察期間が長くなるにつれ，B型肝炎治療の最終目標であるHBs抗原（HBsAg）消失を得られる症例も散見されている．本邦及び海外からいくつかの報告もあるが[1)~4)]，いまだ長期に渡る核酸アナログ使用例での報告はない．今回我々は長期間の核酸アナログ治療によるHBsAg消失とその関連因子について検討した．

対象と方法：1995年～2006年までに当院でB型慢性肝疾患に対して，ラミブジン単独投与を開始した769例を対象とした．これら全ての症例で6カ月以上のHBV持続感染を確認した．核酸アナログ投与内容の内訳はラミブジン単独投与継続306例，ラミブジン投与開始後耐性ウイルス出現に対してラミブジン＋アデフォビル併用を行った症例297例，ラミブジン→エンテカビルへの切り替え症例166例であった．これらの症例のうち，何らかの理由で投与中止した症例は46例存在し，それ以外の症例はすべて継続投与を行った．HBsAg測定はCLIA法（ARCHITECT® HBsAg QT）を用いた．

Table　Factors associated with HBsAg clearance by univariate and multivariate analysis.

	Univariate		Multivariate	
factors	Hazard Ratio (95%CI)	P	Hazard Ratio (95%CI)	P
Age (≥50yr)	0.94 (0.48-1.89)	0.865		
Gender (F)	0.59 (0.21-1.68)	0.323		
Family history of HBV infection	**0.43 (0.22-0.84)**	**0.014**		
Presence of cirrhosis	0.79 (0.56-1.12)	0.192		
Previous IFN therapy	**2.70 (1.31-5.59)**	**0.007**	**2.96 (1.34-6.54)**	**0.008**
HBV genotype (A)	**3.39 (2.27-5.08)**	**<0.0001**	**3.64 (2.40-5.52)**	**<0.0001**
HBeAg (positive)	1.23 (0.61-2.48)	0.563		
HBV DNA (≥6.0 logcopies/mL)	1.20 (0.52-2.78)	0.674		
HBsAg (<2000 IU/mL)	1.40 (0.70-2.80)	0.346		
ALT (≥300 IU/L)	**1.47 (1.02-2.11)**	**0.040**		
Platelets count ($<1.2\times10^5$/mm^3)	0.91 (0.34-2.43)	0.123		
Treatment response at 6 months				
HBeAg positive → clearance	**3.15 (1.49-6.66)**	**0.003**	**2.22 (1.01-4.88)**	**0.046**
HBV DNA (<2.6 logcopies/mL)	**3.56 (1.22-10.4)**	**0.021**	**4.07 (1.36-12.2)**	**0.012**

The bolded numbers: statically significant.
Abbreviation: HBsAg, Hepatitis B surface antigen; IFN, interferon; HBeAg: Hepatitis B envelope antigen

1）虎の門病院肝臓センター
2）虎の門病院肝臓研究室
*Corresponding author: hosa-p@toranomon.gr.jp

＜受付日2011年2月1日＞＜採択日2011年3月2日＞

ラミブジン開始後のHBsAg消失に寄与する因子についてCox比例ハザードモデルを用いて，単変量及び多変量解析を行い検討した．

結果：ラミブジン投与開始からの観察期間の中央値は6.3年（0.7–13.5年）であった．ラミブジン投与前にIFN治療歴を有する症例が297例（39％）存在した（投与期間の中央値は27週（2–575週））．HBV感染の家族歴を有する症例が538例（70％）存在した．ラミブジン投与開始後のHBsAg消失は33例で認められた（内訳は投与中消失31例，投与終了後消失2例）．全体での累積HBsAg消失率は5年：1.8％，10年：7.3％であった．HBsAg消失に寄与する因子について単変量解析を行ったところ，抽出された因子は，家族歴あり（48％ vs. 74％），IFN治療歴あり（64％ vs. 37％），genotype A（25％ vs. 2.6％），開始時ALT高値（300 IU/L以上）（33％ vs. 20％），治療開始6カ月以内のHBe抗原消失（30％ vs. 12％：HBeAg持続陽性例や持続陰性例に比して），治療開始後6カ月時点でのHBVDNA陰性化（<2.6 log copies/ml）（85％ vs. 67％）の6因子が抽出された（Table）．また治療法別で検討すると，ラミブジン単独またはエンテカビル切り替え症例では，ラミブジン＋アデフォビル併用療法症例に比してHBsAg消失率が高率であった（P＝0.014）．

上記の因子を用いて，HBsAg消失に寄与する因子について多変量解析を行ったところ，独立因子としてgenotype A，IFN治療歴，治療開始6カ月時点でHBeAg陽性→陰性化，治療開始後6カ月時点でのHBVDNA陰性化の4因子が抽出された（Table）．

考察：今回の検討では核酸アナログ投与後のHBsAg消失にはHBV genotypeが強く関わっている事が分かった．これまでテルビブジンやPegIFNでの報告のように[4][5]，genotype AではHBsAg量の低下が，他のgenotypeより起こりやすいため，HBsAg消失が起こりやすいと考えられる．またIFN治療歴や核酸アナログ治療早期の反応性などがHBsAg消失に寄与し，治療開始時ALTの上昇が強い症例でもHBsAgが消失しやすい傾向にあったことから，核酸アナログ治療によりHBsAgを消失させるためには，核酸アナログ自体の抗ウイルス作用だけでなく，宿主の免疫反応が必要と推察される．今後HBsAg消失を目指した，核酸アナログ治療法の工夫が望まれる．この研究はラミブジン投与症例での検討であるが，今後は現在の標準治療であり，薬剤耐性出現が極めて低率のエンテカビル投与症例での検討も必要と思われる．

索引用語：HBsAg，核酸アナログ，IFN

文献：1）Kobayashi M, Suzuki F, Akuta N, et al. J Med Virol 2007; 79: 1472―1477　2）Manesis EK, Hadziyannis ES, Angelopoulou OP, et al. Antiviral Ther 2007; 12: 73―82　3）Gish RG, Chang TT, Lai CL, et al. J Viral Hepat 2010; 17: 16―22　4）Wursthorn K, Jung M, Riva A, et al. Hepatology 2010；52：1612―1620　5）Moucari R, Martinot-Peignoux M, Mackiewicz V, et al. Antivir Ther 2009; 14: 1183―1188

英文要旨

Clearance of hepatitis B surface antigen during long-term nucleot(s)ide analogues treatment in chronic hepatitis B

Tetsuya Hosaka[1]*, Fumitaka Suzuki[1], Masahiro Kobayashi[1], Yuya Seko[1], Norihiro Imai[1], Miharu Hirakawa[1], Yusuke Kawamura[1], Hitomi Sezaki[1], Norio Akuta[1], Yoshiyuki Suzuki[1], Satoshi Saitoh[1], Yasuji Arase[1], Kenji Ikeda[1], Mariko Kobayashi[2], Hiromitsu Kumada[1]

Clearance of HBsAg is considered the ultimate goal in the treatment for chronic hepatitis B. We analyzed clinical factors associated with HBsAg clearance during long-term nucleot(s)ide analogue treatment. By univariate analysis, HBV genotype, family history of HBV infection, previous IFN therapy, HBeAg clearance at 6 months, and undetectable HBV DNA at 6 months were significant predictive factors. By multivariate analysis, HBV genotype, previous IFN therapy, HBeAg clearance at 6 months, and undetectable HBV DNA at 6 months were independent and significant predictive factors of HBsAg clearance. We conclude that patients with genotype A have high probability of HBsAg clearance, and it seems that not only the antiviral potential of nucleot(s)ide analogue but host immune response is needed to achieve HBsAg clearance.

Key words: hepatitis B surface antigen, nucleot(s)ide analogues, interferon

Kanzo 2011; 52: 255—257

1) Department of Hepatology, Toranomon Hospital, Tokyo
2) Department of Research Institute for Hepatology, Toranomon Branch Hospital, Kawasaki
*Corresponding author: hosa-p@toranomon.gr.jp

肝臓　52巻11号　756-757（2011）

＜短　報＞

コバスTaqMan HBV「オート」v2.0における同一時の血清検体と血漿検体のHBV DNA検出率の検討

小林万利子[1)*]　鈴木　文孝[2)]　鈴木　義之[2)]　芥田　憲夫[2)]　瀬崎ひとみ[2)]
川村　祐介[2)]　瀬古　裕也[2)]　保坂　哲也[2)]　小林　正宏[2)]　斉藤　聡[2)]
荒瀬　康司[2)]　池田　健次[2)]　熊田　博光[2)]

緒言：HBV DNAの測定は，1996年に分岐HBV DNAプローブ法が臨床応用されてから，検査技術の進歩に伴いTMA（transcription-mediated amplification）法やPCR法などの高感度な測定法の開発が進んできた．現在，日常の臨床で使用されているreal-time PCR法は，HBV DNA量が1.5～2.0 Log copies/mL程度まで検出可能となった．今回我々は，TaqMan HBV v2.0法（コバスTaqMan HBV「オート」v2.0[1)]：ロシュ・ダイアグノスティックス，東京）を用い，血清と血漿の同時採血を行い，各検体の有用性について検討を行ったので報告する．

対象と方法：対象は，B型慢性肝炎および肝硬変の成人でEntecavir投与1年以上経過しALT（alanine aminotransferase）値が30 IU/l以下を持続している52症例（104検体）とした．内訳は，男性29例（55.8%），年齢52歳：中央値（27～81歳）であった．HBV genotypeはgenotype A：2例，genotype B：5例，genotype C：44例，typing不能：1例であった．52症例に対し治療効果の均一化を計るため同一検体で2回の採血を実施しHBV DNAを測定した．2回目のポイントの採血は，1回目の採血後，8週±2週の間に実施した．血清用採血管で全血5 mLと血漿用採血管（EDTA-2K）で全血8 mLを採血，速やかに遠心分離後，TaqMan HBV v2.0法（最小検出感度は，血清検体：2.0 Log copies/mL，血漿検体：1.7 Log copies/mL）にて測定を行った．統計解析は，統計解析ソフトウェアSTAT Flex ver. 5.0を用い，$P<0.05$で有意とした．本試験は，当院の倫理審査委員会の承認を受け，実施についてのインフォームド・コンセントを行った．

結果：血清・血漿ペア検体104例のうち，血清と血漿の両者でHBV DNAを検出したのは，25例（24.0%），両者ともに検出不能は，41例（39.4%）であったが，血清で検出したが血漿では検出不能であったのは，6例（5.8%）であり，血漿で検出したが血清では検出不能であったのは，32例（30.8%）で，血漿での検出率は，血清より有意（$P<0.001$［McNemar検定］）に高率であった（Table 1）．

考察：核酸アナログ製剤を長期に投与することによりその耐性株の出現および肝炎の悪化が認められることから，特に若年者においては核酸アナログ製剤を中止することも考え，HBV DNA量をはじめ，HBs抗原，HBコア関連抗原などの種々のHBVマーカーについて検討が行われている[2)]．Drug freeが可能な症例選定の必要条件の一つはHBV DNAの持続陰性化であり[3)]，投与中止後ALT値の再上昇による重症化・劇症化が懸念されることより，高感度にHBV DNAを検出することが重要である可能性がある．

そこで今回，我々は臨床検体を用いTaqMan HBV

Table 1　Detail correlation between plasma specimen (EDTA-2K) and serum specimen

		Serum	
		detected	not detected
plasma (EDTA-2K)	detected	25 (24.0%)	32* (30.8%)
	not detected	6* (5.8%)	41 (39.4%)

*: $P<0.001$［McNemar検定］

1）虎の門病院肝臓研究室
2）虎の門病院肝臓センター
*Corresponding author: vj7m-kbys@asahi-net.or.jp
＜受付日2011年8月19日＞＜採択日2011年9月2日＞

v2.0 の血清検体と血漿検体の有用性の検討を行った．対象の 104 検体のうち血清または血漿のいずれかで HBV DNA を検出したのは，血清は 5.8％に対し血漿では 30.8％と血漿での HBV DNA の検出率は統計学的有意差（P＜0.001）をもって高率であった．一方，血清で HBV DNA を検出したが血漿では検出不能であった検体も 5.7％存在したが，年齢，性別，genotype などに一定の偏りは無く，この現象は，最小検出感度未満の極めて低濃度の検体で発生するバラツキに起因する確率論的な現象と考えられた．

以上から，血漿検体を用いることにより血清検体より高感度に HBV DNA を測定することが可能となった．今後より高感度な測定が必要な分野での臨床応用が期待される．

索引用語：B 型肝炎ウイルス，
TaqMan PCR 法，高感度

文献：1）Goedel S, Rullkoetter M, Weisshaar S, et al. Journal of Clinical Virology 2009; 45: 232—236　2）Matsumoto A, Tanaka E, Minami N, et al. Hepatology Research 2007; 37: 661—666　3）田中榮司．厚生科学研究費補助金（肝炎等克服緊急対策研究事業）総括研究報告書，2010，H21-肝炎一般-001

英文要旨

The evaluation of the sensitivity between serum and plasma specimen for COBAS TaqMan HBV v2.0

Mariko Kobayashi[1)*], Fumitaka Suzuki[2)], Yoshiyuki Suzuki[2)], Norio Akuta[2)], Hitomi Sezaki[2)], Yusuke Kawamura[2)], Yuya Seko[2)], Tetsuya Hosaka[2)], Masahiro Kobayashi[2)], Satoshi Saitoh[2)], Yasuji Arase[2)], Kenji Ikeda[2)], Hiromitsu Kumada[2)]

The sensitivity in serum and plasma for HBV DNA was evaluated by using 104 clinical specimens from 52 patients who were treated with entecavir for ≥1 year and continued ALT levels ≤30 IU/l. The measurement employed the COBAS TaqMan HBV v2.0. Twenty-five specimens (24.0%) were detected from both serum and plasma, and 41 specimens (39.4%) were not detected from both. On the other hand, there were 32 specimens (30.8%) with detectable from plasma but undetectable from serum, and only 6 specimens (5.8%) with detectable from serum but undetectable from plasma. This result suggested the sensitivity of HBV DNA using plasma specimen is more sensitive than that of serum specimen with statistical significance (p＜0.001).

Key words: hepatitis B virus, TaqMan, high sensitivity

Kanzo 2011; 52: 756—757

1）Research Institute for Hepatology, Toranomon Hospital
2）Department of Hepatology, Toranomon Hospital
*Corresponding author: vj7m-kbys@asahi-net.or.jp

Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Reduced NKG2D ligand expression in hepatocellular carcinoma correlates with early recurrence

Hiroteru Kamimura[1], Satoshi Yamagiwa[1,*], Atsunori Tsuchiya[1], Masaaki Takamura[1], Yasunobu Matsuda[2], Shogo Ohkoshi[1], Makoto Inoue[3], Toshifumi Wakai[3], Yoshio Shirai[3], Minoru Nomoto[1], Yutaka Aoyagi[1]

[1]*Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan;*
[2]*Department of Medical Technology, School of Health Sciences, Faculty of Medicine, Niigata University, Niigata, Japan;*
[3]*Division of Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*

See Editorial, pages 308–310

Background & Aims: The activating receptor natural killer group 2, member D (NKG2D) and its ligands play a crucial role in immune response to tumors. NKG2D ligand expression in tumors has been shown to be associated with tumor eradication and superior patient survival, but the involvement of NKG2D ligands in the immune response against hepatocellular carcinoma (HCC) still remains to be elucidated.

Methods: We investigated the expression of NKG2D ligands in HCC tissues collected from 54 patients and HCC cell lines. We also examined the proteasome expression and the effect of inhibition of proteasome activity on NKG2D ligand expression in HCC tissues and cell lines.

Results: In dysplastic nodules (DN), well-differentiated (well-HCC), and moderately-differentiated HCCs (mod-HCC), UL16-binding protein (ULBP) 1 was expressed predominantly in tumor cells, but not in poorly-differentiated HCCs (poor-HCC). Remarkably, recurrence-free survival of patients with ULBP1-negative HCC was significantly shorter than that of patients with ULBP1-positive HCC ($p = 0.006$). Cox regression analysis revealed that loss of ULBP1 expression was an independent predictor of early recurrence ($p = 0.008$). We confirmed that ULBP1 was expressed in the well- and mod-HCC cell lines, but not in the poor-HCC cell line KYN-2. However, inhibition of proteasome activity resulted in significant up-regulation of ULBP1 expression in KYN-2. Moreover, we found that 20S proteasome expression was more abundant in KYN-2 than that in the well- and mod-HCC cell lines.

Conclusions: ULBP1 is prevalently expressed in DN to mod-HCC, but loss of its expression correlates with tumor progression and early recurrence.

Keywords: ULBP; MICA; NK cell; Immune surveillance; Proteasome inhibitor.
☆ DOI of original article: 10.1016/j.jhep.2011.07.008.
* Corresponding author. Address: Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachi-dori 1, Chuo-ku, Niigata 951-8510, Japan. Tel.: +81 25 227 2207; fax: +81 25 227 0776.
E-mail address: syamagi@med.niigata-u.ac.jp (S. Yamagiwa).
Abbreviations: HCC, hepatocellular carcinoma; well-HCC, well differentiated hepatocellular carcinoma; mod-HCC, moderately differentiated hepatocellular carcinoma; poor-HCC, poorly differentiated hepatocellular carcinoma; MHC, major histocompatibility complex; NKG2D, natural-killer group 2, member D; MIC, MHC class I-related chain; ULBP, UL16-binding protein; DN, dysplastic nodule; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PCR, polymerase chain reaction.

Introduction

Hepatocellular carcinoma (HCC) is a highly prevalent and lethal malignancy, being one of the leading causes of cancer death worldwide [1,2]. Despite recent advances in therapeutic modalities, a significant number of HCCs still show frequent recurrence and progression to an advanced stage [3]. It is well known that there are two distinct types of HCC recurrence: tumor growth from dissemination of the primary tumor (early recurrence) and *de novo* tumors arising from the "field cancerization" in diseased liver (late recurrence) [1,3]. Malignant phenotypes of the tumor, such as a larger size and the presence of vascular invasion and additional tumor sites besides the primary lesion (satellite lesions), are known to be predictive of early recurrence, which is usually observed within 2 years following surgery [4,5]. Several studies have shown that the specific gene expression patterns in cancerous tissues of HCC can accurately predict early intrahepatic recurrence [3,6]. However, it is possible that HCC metastatic propensity may be determined and/or influenced by the local tissue microenvironment of the host. In fact, Budhu *et al.* demonstrated that a Th2-dominant cytokine gene expression profile in non-cancerous liver tissues was associated with a greater risk of intrahepatic metastasis of HCC [7]. Besides the malignant potential of HCC itself, their study clearly indicated that immune responses against cancer cells are also crucial for the prevention of HCC recurrence.

One important mechanism that prevents cancer metastasis is immune surveillance against cancer cells, in which natural killer (NK) cells play a crucial role [8]. NK cell function is strictly regulated by a balance between positive and negative signals provided

by a diverse array of cell surface receptors. The activating receptor natural-killer group 2, member D (NKG2D) and its ligands play an important role in not only NK cell- but also $\gamma\delta^+$ and $CD8^+$ T cell-mediated immune response to tumors [9,10]. A distinctive feature of the NKG2D system is that it has a variety of ligands, all of which are distantly related homologues of major histocompatibility complex (MHC) class I proteins. Human ligands consist of the MHC class I-related chains A and B (MICA/B) and the unique long 16 (UL16)-binding protein (ULBP) family [10,11]. In healthy humans, MICA/B are detected only on gastrointestinal epithelial cells, possibly due to the close contact of these cells with intestinal microbes [12]. However, NKG2D ligand expression has been shown to be a consequence of genetic stress and the resulting DNA damage response that has been reported to occur in precancerous lesions and many tumors in humans [8,10,13]. The up-regulation of NKG2D ligands on transformed cells suggests that cells have sensing mechanisms that recognize changes associated with transformation, thereby activating pathways that up-regulate the cell surface expression of NKG2D ligands [8,10,14].

It is now widely accepted that tumors have developed ways to evade anticancer immunity through a process termed immunoediting. A number of mechanisms by which cancers could evade NKG2D-mediated immune responses have been proposed. One of these mechanisms is release of the MICA molecule from tumor cells in a soluble form [15,16]. Soluble MICA (sMICA) has been implicated in the systemic down-regulation of NKG2D on NK cells in cancer patients [15]. Moreover, NKG2D ligand shedding by tumor cells leads to a reduction in the density of cell surface NKG2D ligands, and reduced susceptibility to NKG2D-mediated cytotoxicity [15–17]. In HCC, Jinushi *et al.* have reported that sMICA is present in sera of patients with advanced HCC [18,19]. Moreover, Kohga *et al.* have recently demonstrated that a disintegrin and metalloproteinase 9 (ADAM9) was overexpressed in human HCC tissues, and that ADAM9 knockdown resulted in increased expression of membrane-bound MICA, decreased production of sMICA, and up-regulation of NK sensitivity of human HCC cells [20]. However, the involvement of NKG2D ligands, especially the ULBP family, in the immune response against HCC has not yet been fully elucidated.

In the present study, we investigated the expression of NKG2D ligands in HCC using human HCC tissue samples and cell lines, and the association between NKG2D ligand expression and HCC recurrence after hepatic resection. Moreover, we focused on the regulation of NKG2D ligand expression by proteasomes, and examined the proteasome expression and the effect of inhibition of proteasome activity on NKG2D ligand expression in human HCC tissues and cell lines.

Patients and methods

Patient samples

After obtaining appropriate informed consent in writing under Institutional Review Board-approved protocols (approved No. 983), blood and surgically removed liver tissues, or liver biopsy specimens were collected from 54 patients (10 female, 44 male) with dysplastic nodule (DN) and HCC, and 8 normal donors for living donor liver transplantation. Information regarding patient profiles and tumor stage of HCC patients is shown in Table 1.

Cell culture

The HCC cell lines used in the present study were Hep3B, PLC/PRF/5 (from the American Type Culture Collection, Rockville, MD), and KYN-2 (kindly provided by Prof. H. Yano at Kurume University, Kurume, Japan). The cervical carcinoma cell line HeLa was also purchased from ATCC. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY) in a humidified incubator at 5% CO_2 and 37 °C.

Reagents

Monoclonal antibodies (mAb) used for immunofluorescence assay were anti-MICA/B (159207), anti-ULBP1 (170818), anti-ULBP2 (165903), anti-ULBP3 (166514) (R&D Systems Inc., Minneapolis, MN), anti-HLA-A, B, C (G46-2.6), and

Table 1. Patient characteristics.

Factors	Total	Hepatocellular carcinoma		
		Well	Moderately	Poorly
Number of patients	54	13	24	10
Age (Mean ± SD, years)	65.4 ± 10.8	69.0 ± 9.2	63.9 ± 10.2	65.9 ± 8.8
Gender (male/female)	44/10	8/5	23/1	9/1
Etiology (HBV/HCV/NBNC/Alcohol)	15/23/6/10	4/6/0/3	7/10/3/4	2/5/1/2
Background (non-cirrhosis/cirrhosis)	17/36	4/9	11/13	2/8
Child-Pugh classification (A/B/C)	42/11/1	9/4/0	21/2/1	6/4/0
Histological grade				
Dysplastic nodule/well/moderately/poorly	7/13/24/10	-	-	-
Tumor factors				
Tumor size (≤30/>30, mm)	37/17	8/5	16/8	4/6
Number of tumors (single/double/multiple)	34/12/8	9/3/1	15/4/5	9/1/0
CLIP score (mean ± SD)	0.8 ± 0.9	0.6 ± 0.8	0.8 ± 0.9	0.9 ± 1.1
AFP (<400/≥400, ng/ml)	47/7	13/0	20/4	7/3
Operation (partial/segmentectomy/lobectomy)	17/20/17	8/1/4	2/14/8	2/3/5

HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-HBV non-HCV; Well, well-differentiated; Moderately, moderately-differentiated; Poorly, poorly-differentiated; CLIP, Cancer of the Liver Italian Program.

anti-CD107a (H4A3) (BD Biosciences, San Diego, CA). Monoclonal antibodies used for immunohistochemical staining were anti-MICA/B (H-300), anti-ULBP1 (3F1), anti-ULBP2 (6F6), anti-ULBP3 (2F9), and anti-20S proteasome α7 (GH6) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Control murine IgG1 and IgG2a were also purchased from Santa Cruz. Proteasome inhibitor, MG132 (474790), was purchased from CARBIOCHEM (Darmstadt, Germany).

Immunohistochemistry

Tissue sections were first deparaffinized with xylene and then rehydrated through graded alcohol. To retrieve antigenicity, sections were immersed in pH 6.0 citrate buffer. After autoclaving, sections were then immersed in phosphate-buffered saline (PBS) containing 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Sections were then incubated with a series of antibodies at a 1:50 or 1:100 dilution in PBS supplemented with 3% BSA at 4 °C overnight. Sections were followed sequentially by the biotinylated secondary antibody and the avidin–biotin–peroxidase complex method using the Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA). Sections were finally developed with diaminobenzidine (DAB) substrate (Muto Pure Chemicals, Tokyo, Japan). Specimens were counterstained with Methyl Green solution and mounted. Instead of DAB, the fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate-conjugated mouse and rabbit IgG antibody (Santa Cruz Biotechnology) were also used.

Immunofluorescence assay

Cells (10^5) were labeled with appropriate FITC- or phycoerythrin (PE)-conjugated mAbs at 4 °C for 30 min in darkness for the surface antigens, and then washed three times and acquired by FACScan (Becton Dickinson, San Jose, CA) and analyzed with FlowJo software (Ver. 7, Tree Star Inc., Ashland, OR). For the intracellular antigen assay, after the surface antigens became stained, cells were fixed and permeabilized using 100 µl each of cytofix and cytoperm solution (BD Biosciences), and then were stained with PE-anti-ULBP1 mAb.

CD107a degranulation assay

Degranulation assays were based on a previously described method [21]. Briefly, PBMCs (10^6 cells/ml) derived from healthy donors were stimulated with HCC cell lines and MHC-devoted targets, K562 cell line (ATCC), at an E:T ratio of 5:1. PE-anti-CD107a Ab (H4A3) was added directly to the culture plates at 20 µl/ml. Following 1 h of incubation at 37 °C in 5% CO_2, breferdin A and monensin (BD Biosciences) were added at a final dilution of 1:1000 and at a final concentration of 6 µg/ml, respectively, and incubated for an additional 5 h at 37 °C in 5% CO_2. Anti-ULBP1 Ab (170818) and isotype matched control Ab were added at 10 µg/ml during the culture. Samples were then surface-stained for 30 min for flow cytometric analysis.

Western blot analysis

Cell lysates were prepared by incubation in lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 1% Nonider P-40 and the protease inhibitors cocktail 1 tablet, for 20 min at 4 °C. Lysates, supernatants, and exosome preparations were run on 12% SDS–PAGE gels and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Detection of ULBP and 20S proteasome was performed by incubation with biotinylated anti-ULBP1 antibody and anti-20S proteasome antibody, followed by horseradish peroxidase-conjugated streptavidin. Blots were developed using an enhanced chemiluminescence detection kit (GE Healthcare, Piscataway, NJ). For protein loading analyses, a monoclonal beta-actin antibody (1:5000; clone AC-15, Sigma) was used.

Real-time polymerase chain reaction

Total cellular RNA was extracted from the surgically removed tissues, biopsy liver tissues, and HCC cell lines using RNeasy Mini Kit (Qiagen, Tokyo, Japan). Reverse transcription of the RNA samples to cDNA was performed with Transcriptor First Strand cDNA Synthesis Kit (Roche, Penzberg, Germany) with anchored-oligo(dT)18 primer. Real-time PCR was performed on a lightcycler (Roche) using the primers as described previously [22]. Expression levels of mRNA were normalized to *NADPH* mRNA levels.

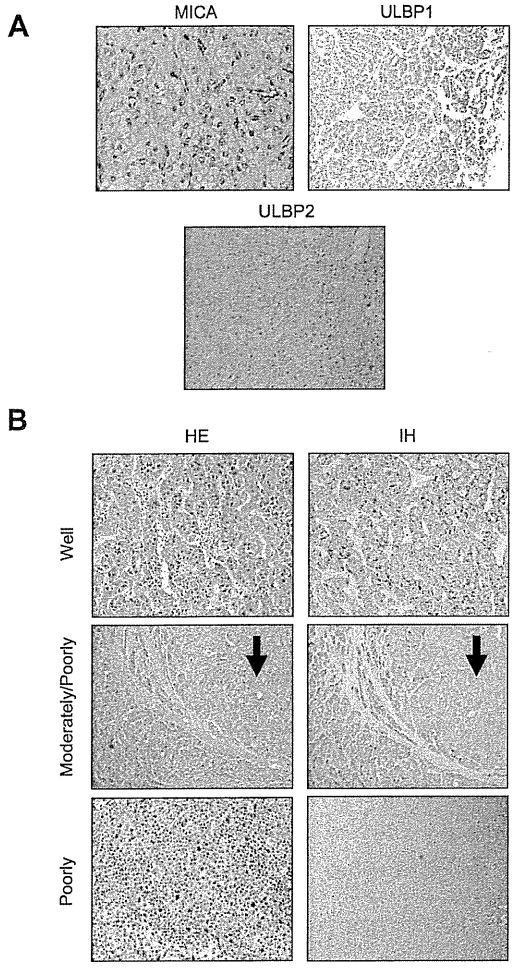

Fig. 1. Immunohistochemical staining of NKG2D ligands in human HCC tissues. (A) Representative staining for MICA, ULBP1, and ULBP2 in well-differentiated HCC. MICA immunoreactivity was seen mainly in the vascular endothelial cells, whereas ULBP1 was expressed in tumor cells. ULBP2 was negative. (B) Representative staining for ULBP1 in well- to poorly-differentiated HCC. ULBP1 was expressed in well- to moderately-differentiated HCCs, but not in poorly-differentiated HCCs. The loss of ULBP1 expression in poorly-differentiated HCC was clearly shown on the nodule-in-nodule growth of poorly-differentiated HCC (arrow in middle panels) in moderately-differentiated HCC. MICA, major histocompatibility complex class I-related chain A; ULBP, UL16-binding protein.

Statistical analysis

The significance of differences was analyzed statistically by the compared *t* test with Welch's correction, or Mann–Whitney *U* test, using SPSS software (Ver. 18, SPSS Inc., Chicago, IL). Kaplan–Meier curves were used to assess factors that influenced survival. The significance of differences in survival between groups with differing forms of expression was estimated using the log-rank test. The Cox proportional-hazards model was used for multivariate analysis to determine the relative risk and independent significance of individual factors. In all cases, the level of significance was set at $p < 0.05$.

Results

Expression of NKG2D ligands in HCC

We first investigated the expression of individual NKG2D ligands in human HCC tissues. MICA immunoreactivity was seen mainly in vascular endothelial cells of well- (well-HCC) or moderately-differentiated (mod-HCC) HCCs (Fig. 1A). ULBP1 was expressed in tumor cells of well- or mod-HCCs (Fig. 1A), and not in surrounding non-tumor tissues (data not shown). On the other hand, no positive expression of ULBP2, ULBP3, or ULBP4 was evident in HCCs (data for ULBP3 and ULBP4 not shown) (Fig. 1A).

Loss of ULBP1 expression in poorly-differentiated HCC

After the initial experiments, we focused on ULBP1, which was expressed in well- to mod-HCCs. Representative results are shown in Fig. 1B. When we examined the expression of ULBP1 in well- to poorly-differentiated (poor-HCC) HCCs, we found that ULBP1 was expressed predominantly in well- to mod-HCCs, but not in poor-HCCs. Notably, loss of ULBP1 expression in poor-HCC was clearly shown in nodule-in-nodule growth of poor-HCC (Fig. 1B) in mod-HCC. Table 2 summarizes the expression of ULBP1 in the normal liver, dysplastic nodules (DN), and HCCs. Although the normal liver did not express ULBP1, ULBP1 was up-regulated and predominantly expressed in DN, and in well- and mod-HCCs. These observations indicate that ULBP1 is induced in the early stage of hepatocyte transformation, and expressed widely in HCC. However, the expression of ULBP1 was significantly decreased in poor-HCCs relative to DN, and well- and mod-HCCs (all p values <0.01) (Table 2). These data suggest that the loss of predominant expression of ULBP1 in HCC may be associated with progression to invasive tumor or increasingly higher grades. Despite the loss of ULBP1 expression in poor-HCCs, we did not find any significant differences in the expression of *ULBP1* mRNA among the HCCs (Fig. 2). These data suggest that loss of ULBP1 expression may result from post-transcriptional events rather than from a decrease of mRNA expression.

Table 2. Expression of ULBP1 by immunohistochemical staining.

Normal	Dysplastic nodule	Hepatocellular carcinoma		
		Well	Moderately	Poorly
0/8	7/7*	10/13*	15/24*	0/10*,‡,‡‡
(0%)	(100%)	(76.9%)	(62.5%)	(0%)

Well, well differentiated; Moderately, moderately differentiated; Poorly, poorly differentiated. *p <0.01 (compared to control), **p <0.001 (compared to dysplastic nodule), ‡p = 0.003 (compared to Well), ‡‡p = 0.007 (compared to Moderately).

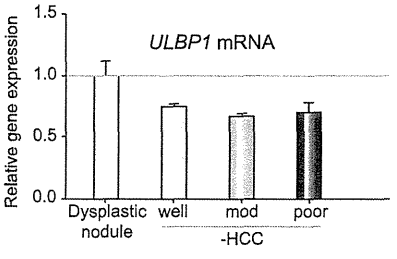

Fig. 2. Expression of *ULBP1* mRNA in human HCC tissues. Total cellular RNA was extracted from the surgically removed tissues or biopsy samples of patients with dysplastic nodule (DN) and well- to poorly-differentiated HCC. Following reverse transcription of the RNA samples to cDNA, expression levels of *ULBP1* mRNA were quantified by real-time PCR. Steady state levels of mRNA were normalized relative to *NADPH* mRNA levels. Then the relative expression levels were calculated with the levels in DN as baseline. The values are presented as means + SD. No significant changes were seen in *ULBP1* mRNA levels among DN to poorly-differentiated HCCs.

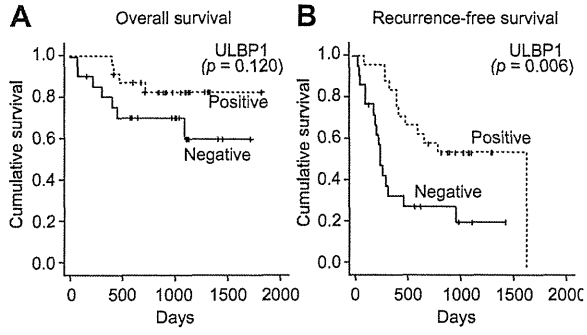

Fig. 3. Kaplan–Meier plots showing disease-specific overall survival and recurrence-free survival. (A) Disease-specific overall survival did not differ significantly between patients with positive, and those with negative expression of ULBP1. (B) Recurrence-free survival was significantly shorter in patients with negative expression of ULBP1 than in those with positive expression.

Table 3. Multivariate analysis using Cox regression model predicts independent risk factor of early recurrence within 1 year.

Factors	95% CI for Exp (B)		Cox regression p value
Age	0.994	1.111	0.080
Gender	0.293	4.618	0.830
Tumor factors			
Size	0.969	1.021	0.680
im (+)	0.036	0.455	0.001
Vp (+)	0.041	0.828	0.027
ULBP1 expression			
Negative	1.537	16.261	0.008

im, intrahepatic metastasis; Vp, tumor invasion in portal veins.

Loss of ULBP1 expression is associated with early recurrence of HCC

When we analyzed the factors associated with the overall survival of the enrolled patients with HCC after hepatic resection, we did not find any significant association with the loss of ULBP1 expression (p = 0.120) (Fig. 3A). This may have been partly attributable to the rather short observation period (mean ± SD; 2.2 ± 1.3 years). However, the loss of ULBP1 expression was significantly associated with shorter recurrence-free survival (p = 0.006) (Fig. 3B). Patients with ULBP1-negative HCC showed a significantly higher recurrence rate within 1 year than patients with ULBP1-positive HCC. Multivariable analysis showed that independent predictors of such early recurrence were the presence of intrahepatic metastasis (im) (p = 0.001), tumor invasion into the portal veins (Vp) (p = 0.027), and lack of expression of

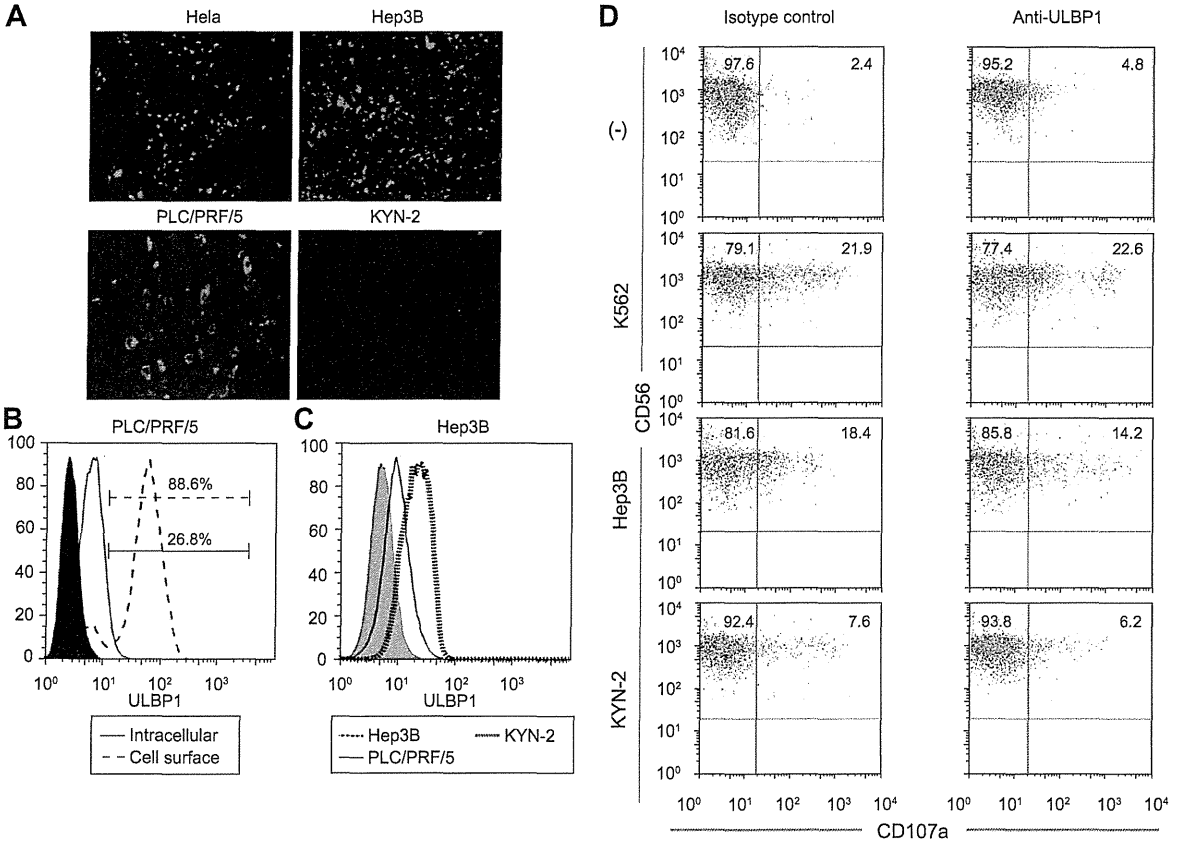

Fig. 4. Expression of ULBP1 in HCC cell lines. (A) Immunofluorescence representing ULBP1 expression is evident in the well-HCC cell line Hep3B and the mod-HCC cell line PLC/PRF/5, but not in the poor-HCC cell line KYN-2. (B) Representative results of flow cytometric analysis of ULBP1 expression in HCC cell lines. Expression of ULBP1 was detected on both the cell surface (solid line) and within the cell (dotted line) in the mod-HCC cell line PLC/PRF/5. Black profile represents staining with control mouse IgG. (C) Cell surface expression levels of ULBP1 differed among the HCC cell lines. ULBP1 was more highly expressed in the well-HCC cell line Hep3B (dotted line) than in the mod-HCC cell line PLC/PRF/5 (black line) and in the poor-HCC cell line KYN-2 (gray profile). (D) Representative results of CD107a degranulation assay of $CD3^-CD56^+$ NK cells derived from a healthy individual following co-culture with K562, Hep3B, and KYN-2 in the presence of anti-ULBP1 antibody or isotype-matched antibody. Five independent experiments were conducted.

ULBP1 ($p = 0.008$), as shown in Table 3. No other factors were found to have independent prognostic value (data not shown).

ULBP1 expression in HCC cell lines

We then investigated the expression of ULBP1 in HCC cell lines. As shown by immunofluorescence, ULBP1 was expressed abundantly in the well-HCC cell line Hep3B [23] and the mod-HCC cell line PLC/PRF/5 [24], but absent from the poor-HCC cell line KYN-2 [25] (Fig. 4A). Representative results of flow cytometric analysis of ULBP1 are also shown in Fig. 4B and C. ULBP1 showed a higher intensity of intracellular staining than of cell surface staining (Fig. 4B). When we compared the cell surface expression of ULBP1 among these HCC cell lines, KYN-2 showed relatively lower expression than Hep3B and PLC/PRF/5 (Fig. 4C). NK cell degranulation assays revealed that the HCC cell lines Hep3B and KYN-2 were less susceptible to NK lysis than the MHC-devoted cell line K562. Moreover, the poor-HCC cell line KYN-2 was less susceptible than the well-HCC cell line Hep3B, although blocking of ULBP1 only partially decreased the susceptibility of Hep3B to NK lysis (Fig. 4D). Such reduced susceptibility of KYN-2 to NK lysis may result from the loss of ULBP1 expression. The significantly lower cell surface and intracellular expression of ULBP1 in KYN-2 compared to Hep3B and PLC/PRF/5 ($p = 0.004$ and 0.0096, respectively) is shown in Fig. 5.

Increase of ULBP1 expression induced by proteasome inhibitor

As shown in experiments using human HCC tissues, we found no decrease in the expression of *ULBP1* mRNA, although the expression of ULBP1 itself was significantly decreased in poor-HCCs. Therefore, we focused on proteasome regulation of ULBP1, which has been demonstrated previously in head and neck squamous cell carcinoma cells [26]. Initially, we used a reversible proteasome inhibitor, MG132, which affects primarily the chymotrypsin-like activity of the proteasome, but also inhibits other cellular proteases such as cathepsins and calpains [26]. After treatment with MG132 at 2.5 μmol/L, an increase in the surface expression of ULBP1 was observed after 12 h, and maximum induction was observed after 24 h (data not shown). At this concentration of MG132, the majority of the cells were viable, and analysis of the viable cells showed that the cell lines

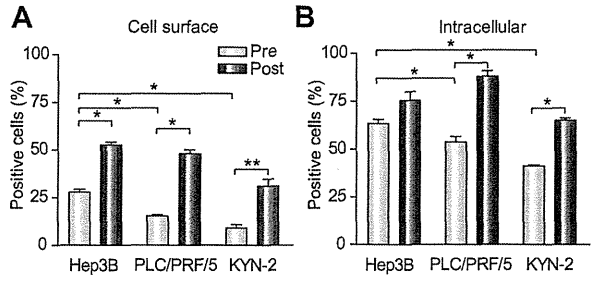

Fig. 5. The effect of a proteasome inhibitor on the expression of ULBP1. Expression levels of ULBP1 were analyzed by flow cytometry before and after treatment with the proteasome inhibitor MG132 for 24 h. Before the treatment with MG132, both cell surface (A) and intracellular (B) expression of ULBP1 were significantly lower in KYN-2 than in Hep3B or PLC/PRF/5. Treatment with MG132 led to significant up-regulation of the surface expression of ULBP1 in the HCC cell lines. Pre, before treatment with the proteasome inhibitor MG132; Post, after treatment with MG132. The values are presented as means + SD of five independent experiments. $^{*}p$ <0.01, $^{**}p$ <0.05.

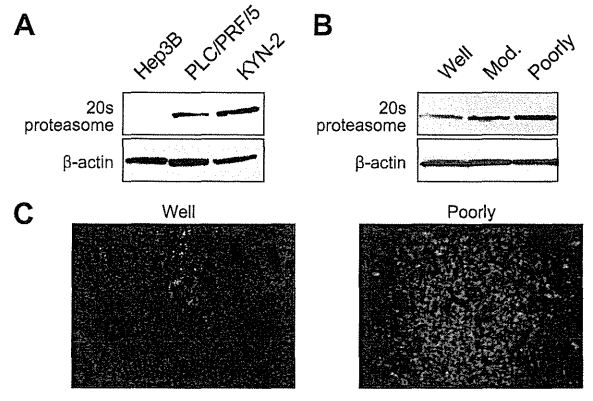

Fig. 6. Proteasome expression in human HCC tissues and HCC cell lines. Expression of 20S proteasome was examined by Western blotting and immunofluorescence. (A) The 20S proteasome was more abundantly expressed in the poor-HCC cell line KYN-2 than in Hep3B and PLC/PRF/5. (B and C) Expression of the 20S proteasome was more abundant in clinical samples of poor-HCC than in well- and mod-HCCs. Representative results are shown.

Hep3B, PLC/PRF/5, and KYN-2 had significantly increased cell surface expression of UPBP1 ($p = 0.0013$, 0.0039, and 0.036, respectively) (Fig. 5A). Moreover, intracellular expression of ULBP1 was also significantly increased in all of the cell lines treated with MG132 (Fig. 5B). These data suggest that loss of the predominant expression of ULBP1 in HCC cell lines may be associated with progression to higher grades, and that proteasome inhibition in HCC cell lines may reverse the loss of ULBP1expression.

20S proteasome in HCC cell lines and human HCC tissues

We then examined the proteasome expression of HCC cell lines by Western blotting. Using a 20S proteasome-specific antibody, we found that the poor-HCC cell line KYN-2 expressed more 20S proteasome than the well- to mod-HCC cell lines Hep3B and PLC/PRF/5 (Fig. 6A). Moreover, after extraction of proteins from well- to poor-HCC tissues, we found that the expression of 20S proteasome was increased to a greater extent in poor-HCC tissues than in well- and mod-HCC tissues (Fig. 6B). The results of immunohistochemistry also demonstrated increased expression of 20S proteasome in poor-HCC tissue (Fig. 6C). Thus, our results indicated that proteasome expression was increased to a greater degree in both the poor-HCC cell line and tissues than in well- and mod-HCCs.

Discussion

In the present study, we found that one of the ligands for NKG2D, ULBP1, was abundantly expressed in HCC, but that progression to a higher grade resulted in loss of ULBP1 expression. Although no association of ULBP1 expression with overall survival was demonstrated, the loss of ULBP1 expression was significantly associated with early recurrence of HCC after hepatic resection. Moreover, expression of 20S proteasome was more abundant in both the poor-HCC tissues and cell line, and proteasome inhibition reversed the loss of ULBP1 expression in the poor-HCC cell line. These results suggest that proteasome up-regulation is involved in loss of ULBP1 expression, which may result in defective NKG2D-mediated tumor surveillance.

NKG2D ligands can be expressed independently of each other in human cell lines and primary tumors [10]. It is also known that different ligands can be expressed in response to different cancer-specific pathways, and that it is rare for any cancer to express all NKG2D ligands [10,26]. Therefore, it is important to examine the NKG2D ligand expression pattern in different types of human tumors. Among various cancer cell lines, MICA expression is usually positive, ULBP2–4 expressions are variable, but ULBP1 expression is rare [26,27]. In contrast, T-cell leukemias express MICA less often, and express ULBP1 more often [28]. Our present results demonstrated a common expression pattern of NKG2D ligands in human HCC tissues and cell lines; MICA and ULBP1 proteins were expressed in the absence of ULBP2–4. Although MICA/B expression in human HCC has already been reported in several studies [18,29,30], to our knowledge the present study is the first to demonstrate that ULBP1 is also expressed abundantly in human HCC.

In both experimental animals and human cancer patients, tumor NKG2D ligand expression is associated with tumor eradication and superior patient survival [10,31–33]. NKG2D recognizes ligands that are often expressed at higher levels on tumor cells than in surrounding tissues and that can be induced further by cancer treatments [10]. In colorectal cancer, a high level of NKG2D expression in tumors is associated with good prognosis, and MICA is a strong independent predictor of long survival [31]. In prostate cancer, MICA/B is induced at the early stage of prostate luminal epithelial cell transformation and expressed widely in prostate carcinoma. Moreover, loss of predominant surface localization of MICA/B is associated with progression to invasive tumor or to progressively higher grades [32]. On the other hand, a high level of NKG2D ligand expression is inversely correlated with survival in patients with ovarian cancer [33]. These reports suggest a fundamental difference in the involvement of NKG2D-mediated immunity among various types of cancer. In the present study, we found that ULBP1 was expressed predominantly at early stage of hepatocyte transformation (DN) to mod-HCC, and that loss of ULBP1 expression was significantly associ-

Cancer

ated with early recurrence after hepatic resection. Although no association of ULBP1 expression with overall survival was demonstrated, this may have been attributable to the rather short observation period (mean ± SD; 2.2 ± 1.3 years). Notably, we demonstrated that loss of ULBP1 expression was an independent predictor of early recurrence after hepatic resection. Our results seem to be similar to those obtained in previous studies of prostate cancer.

Besides the NKG2D system, other NK receptors and ligands also play important roles in the recognition of tumor cells. NK cells express a repertoire of inhibitory receptors that regulate their activation. These receptors include the inhibitory killer cell immunoglobulin-like receptors (KIRs) that bind to HLA-A, -B, and -C, and the inhibitory CD94-natural killer group 2A (NKG2A) heterodimeric C-type lectin-like receptors that bind to HLA-E [10,14]. The NK activating receptor family, including NKp44, NKp46, and NKp30, also plays an important role in activating NK cells. NKp30 and NKp46 are expressed on resting and activated NK cells, whereas NKp44 is expressed only on activated NK cells [14]. Recently, several novel NK cell receptors have been reported to mediate NK cell adhesion and activation through interaction with ligands for the nectin and nectin-like (Necl) family of adhesion molecules [34]. In particular, DNAX accessory molecule-1 (DNAM-1) is reportedly involved in human HCC lysis by Vγ9Vδ2 T cells [35]. In the present study, although we demonstrated that HCC cell lines were less susceptibility to NK lysis than MHC-devoted cell line K562, we found that blocking of ULBP1 only partially decreased the susceptibility of Hep3B cell line. Therefore, it should be noted that involvement of NK cell receptors other than ULBP1 remains to be further investigated.

Although up-regulation of NKG2D ligands on transformed cells has been shown to be an important mechanism for sensing changes associated with transformation, a number of mechanisms by which cancers could evade NKG2D-mediated immune responses have been proposed. Although MICA/B are expressed on a subset of human HCCs and have been shown to play an important role in NK lysis against HCC cell lines [18,32,33], sMICA has been shown to be present in sera of patients with advanced HCC and might serve as a factor enabling tumors to evade immune surveillance [19]. Moreover, Kohga *et al.* have reported that therapeutic intervention for HCC can reduce the levels of sMICA and thereby up-regulate the expression of NKG2D [36]. Recently, Kohga *et al.* have also demonstrated that ADAM9 is involved in MICA shedding in HCC cells, and that a unique multitargeting kinase molecule, sorafenib, can modulate ADAM9 expression [20]. Thus, cancer therapy may have a beneficial effect on the NKG2D-mediated anti-tumor immune response. Although MICA shedding is thought to be one of the principal mechanisms by which tumor cells can escape NKG2D-mediated immunosurveillance, it has recently been shown that ULBP1 is shed at only relatively low levels [37]. Taken together, the data suggest that loss of ULBP1 expression not caused by shedding is also an important mechanism of immune-evasion by tumors.

There has been a growing interest in the role of proteasomes in malignant diseases, and proteasome inhibitors now represent a promising new class of anticancer agent for different types of tumors, including HCC [38,39]. The ubiquitin–proteasome system (UPS) plays a key role in many processes important for cellular homeostasis, such as regulation of the cell cycle, apoptosis, receptor signaling, and endocytosis [38]. Among the multiple roles for the UPS in the pathogenesis of HCC, proteasome regulation of NKG2D ligand expression has already been suggested. Armeane *et al.* have reported that the proteasome inhibitors MG132 and Bortezomib increased MICA/B expression in HCC cell lines, but that ULBP1–3 remained undetectable [29,30]. Contrary to their report, we found that MG132 was also able to upregulate the expression of ULBP1 in HCC cell lines, even in a poor-HCC cell line whose ULBP1 expression was significantly decreased. The reason for this inconsistency still remains unclear, as Armeane *et al.* did not present any data for the expression of ULBP1–3 protein [29,30]. However, those results suggest that, as well as inhibiting tumor cell proliferation, proteasome inhibitors may also enhance NKG2D-mediated antitumor immunity against HCC. Interestingly, Neo *et al.* have demonstrated that the 20S proteasome genes showed the most remarkable increase of expression in the UPS pathway in HCC tumors, and it can be speculated that overexpression of the proteasome subunit could be a crucial step in the malignant transformation of hepatocytes [39]. Consistent with these results, we demonstrated overexpression of the 20S proteasome in HCCs, and showed that this was markedly more pronounced in poor-HCC than in well- and mod-HCCs. Taken together with the results obtained using the proteasome inhibitor, we conclude that loss of ULBP1 expression in poor-HCCs is induced by overexpression of proteasomes, although the mechanism involved remains to be further investigated.

In conclusion, we have demonstrated for the first time that ULBP1 is predominantly expressed at the early stage of hepatocyte transformation (DN) to mod-HCCs, and that loss of ULBP1 expression is significantly associated with early recurrence after hepatic resection. These results suggest that loss of ULBP1 expression may result in a deficiency of NKG2D immune surveillance, thus contributing to the early recurrence of HCC. Our results obtained using a proteasome inhibitor to enhance NKG2D ligand expression *in vitro* have provided an alternative mechanism of proteasome inhibitor for HCC.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Financial support

This work was supported by Grant-in-Aid for Scientific Research (C) (21590834) from Japan Society for the Promotion of Science (JSPS).

Acknowledgements

The authors thank N. Honda for excellent technical assistance; and Prof. H. Yano at Kurume University, Kurume, Japan, for providing KYN-2 cell line.

References

[1] Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907–1917.
[2] Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and trends. Gastroenterology 2004;127:S35–S50.
[3] Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology 2005;42:1208–1236.

[4] Imamura H, Matsuyama Y, Tanaka E, Ohkubo T, Hasegawa K, Miyagawa S, et al. Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. J Hepatol 2003;38:200–207.
[5] Utsunomiya T, Shimada M, Imura S, Morine Y, Ikemoto T, Mori M. Molecular signatures of noncancerous liver tissue can predict the risk for late recurrence of hepatocellular carcinoma. J Gastroenterol 2010;45:146–152.
[6] Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, et al. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Eng J Med 2008;359:1995–2004.
[7] Budhu A, Forgues M, Ye QH, Jia HL, He P, Zanetti KA, et al. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. Cancer Cell 2006;10:99–111.
[8] Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene 2008;27:5932–5943.
[9] Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999;285:727–729.
[10] Moretta L, Bottino C, Pende D, Castriconi R, Mingari MC, Moretta A. Surface NK receptors and their ligands on tumor cells. Semin Immunol 2006;18:151–158.
[11] Cosman D, Müllberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 2001;14:123–133.
[12] Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci USA 1996;96:6879–6884.
[13] Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat Immunol 2001;2:255–260.
[14] Yamagiwa S, Kamimura H, Ichda T. Natural killer cell receptors and their ligands in liver diseases. Med Mol Morphol 2009;42:1–8.
[15] Groh V, Wu J, Yee C, Spie T. Tumor-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 2001;419:734–738.
[16] Salih HR, Rammensee HG, Steinle A. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. J Immunol 2002;169:4098–4102.
[17] Salih HR, Holdenrieder S, Steinle A. Soluble NKG2D ligands: prevalence, release, and functional impact. Front Biosci 2008;13:3448–3456.
[18] Jinushi M, Takehara T, Tatsumi T, Kanto T, Groh V, Spies T, et al. Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid. Int J Cancer 2003;104:354–361.
[19] Jinushi M, Takehara T, Tatsumi T, Hiramatsu N, Sakamori R, Yamaguchi S, et al. Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class-I-related chain A in advanced human hepatocellular carcinomas. J Hepatol 2005;43:1013–1020.
[20] Kohga K, Takehara T, Tatsumi T, Ishida H, Miyagi T, Hosui A, et al. Sorafenib inhibits the shedding of major histocompatibility complex class I-related chain A on hepatocellular carcinoma cells by down-regulating a disintegrin and metalloproteinase 9. Hepatology 2010;51:1264–1273.
[21] Alter G, Malenfant JM, Delabre RM, Burgett NC, Yu XG, Lichterfeld M, et al. Increased natural killer cell activity in viremic HIV-1 infection. J Immunol 2004;173:5305–5311.
[22] Apps R, Gardner L, Traherne J, Male V, Moffett A. Natural-killer cell ligands at the maternal–fetal interface. UL-16 binding proteins, MHC class-I chain related molecules, HLA-F and CD48. Hum Reprod 2008;23:2535–2548.
[23] Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 1979;282:615–616.
[24] Alexander JJ, Bey EM, Geddes EW, Lecatsas G. Establishment of a continuously growing cell line from primary carcinoma of the liver. S Afr Med J 1976;50:2124–2128.
[25] Yano H, Maruiwa M, Murakami T, Fukuda K, Ito Y, Sugihara S, et al. A new human pleomorphic hepatocellular carcinoma cell line, KYN-2. Acta Pathol Jpn 1998;38:953–966.
[26] Buttler JE, Moore MB, Presnell SR, Chan HW, Chalupny NJ, Lutz CT. Proteasome regulation of ULBP1 transcription. J Immunol 2009;182:6600–6609.
[27] Pende D, Rivera P, Marcenaro S, Chang CC, Biassoni R, Conte R, et al. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. Cancer Res 2002;62:6178–6186.
[28] Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, Rammensee HG, et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood 2003;102:1389–1396.
[29] Armeane S, Bitzer M, Lauer UM, Venturelli S, Pathil A, Krusch M, et al. Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. Cancer Res 2005;65:6321–6328.
[30] Armeanu S, Krusch M, Baltz KM, Weiss TS, Smirnow I, Steinle A, et al. Direct and natural killer cell-mediated antitumor effects of low-dose Bortezomib in hepatocellular carcinoma. Clin Cancer Res 2008;14:3520–3528.
[31] McGilvray RW, Eagle RA, Watson NFS, Al-Attar A, Ball G, Jafferji I, et al. NKG2D ligand expression in human colorectal cancer reveals associations with prognosis and evidence for immunoediting. Clin Cancer Res 2009;25:6993–7002.
[32] Wu JD, Higgins LM, Steinle A, Cosman D, Haugk K, Plymate SR. Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer. J Clin Invest 2004;114:560–568.
[33] McGilvray RW, Eagle RA, Rolland P, Jafferji I, Trowsdale J, Durrant LG. ULBP2 and RAET1E NKG2D ligands are independent predictors of poor prognosis in ovarian cancer patients. Int J Cancer 2010;127:1412–1420.
[34] Fuchs A, Colonna M. The role of NK cell recognition of nectin and nectin-like proteins in tumor immunosurveillance. Semin Cancer Biol 2006;16:359–366.
[35] Toutirais O, Cabillic F, Le Friec G, Salot S, Loyer P, Le Gallo, et al. DNAX accessory molecule-1 (CD226) promotes human hepatocellular carcinoma cell lysis by Vγ9Vδ2 T cells. Eur J Immunol 2009;99:1361–1368.
[36] Kohga K, Takehara T, Tatsumi T, Ohkawa K, Miyagi T, Hiramatsu N, et al. Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver diseases and changes during transcatheter arterial embolization for hepatocellular carcinoma. Cancer Sci 2008;99:1643–1649.
[37] Fernández-Messina L, Ashiru O, Boutet P, Agüera-González S, Skepper JN, Reyburn HT, et al. Differential mechanisms of shedding of the glycosylphosphatidylinositol (GPI)-anchored NKG2D ligands. J Biol Chem 2010;285:8543–8551.
[38] Dawson SP. Hepatocellular carcinoma and the ubiquitin-proteasome system. Biochim Biophys Acta 2008;1782:775–784.
[39] Neo SY, Leow CK, Vega VB, Long PM, Islam AF, Lai PB, et al. Identification of discriminators of hepatoma by gene expression profiling using a minimal dataset approach. Hepatology 2004;39:944–953.

Hepatology Research

Hepatology Research 2012; **42**: 213–218 doi: 10.1111/j.1872-034X.2011.00913.x

Case Report

Japanese case of Budd-Chiari syndrome due to hepatic vein thrombosis successfully treated with liver transplantation

Tomohiro Iwasaki,[1] Hirokazu Kawai,[4] Koushi Oseki,[1] Tadayuki Togashi,[1] Kazuhiko Shioji,[1] Satoshi Yamamoto,[2] Yoshinobu Sato,[2] Kenji Suzuki,[1] Ken Toba,[3] Minoru Nomoto,[1] Katsuyoshi Hatakeyama[2] and Yutaka Aoyagi[1]

[1]Division of Gastroenterology and Hepatology, [2]Division of Digestive and General Surgery, [3]Division of Hematology, Niigata University Graduate School of Medical and Dental Sciences, and [4]Department of Clinical Laboratory, Niigata University Medical and Dental Hospital, Niigata, Japan

A 22-year-old Japanese woman was found to have severe esophageal varices and then suffered from hepatic encephalopathy. She was diagnosed with Budd-Chiari syndrome (BCS) due to hepatic vein (HV) thrombosis accompanied by portal vein thrombosis without inferior vena cava (IVC) obstruction. Latent myeloproliferative neoplasm (MPN) lacking the JAK2-V617F mutation was considered to be the underlying disease. Liver transplantation was strikingly effective for treating the clinical symptoms attributable to portal hypertension. Although thrombosis of the internal jugular vein occurred due to thrombocythemia, which manifested after transplantation despite anticoagulation therapy with warfarin, the thrombus immediately disappeared with the addition of aspirin. Neither thrombosis nor BCS has recurred in more than 4 years since the amelioration of the last thrombotic event, and post-transplant immunosuppression with tacrolimus has not accelerated the progression of MPN. In Japan, IVC obstruction, which was a predominant type of BCS, is suggested to have decreased in incidence with recent improvements in hygiene. The precise diagnosis of BCS and causative underlying diseases should be made with attention to the current trend of the disease spectrum, which fluctuates with environmental sanitation levels. Because the stepwise strategy, including liver transplantation, has been proven effective for patients with pure HV obstruction in Western countries, this strategy should also be validated for utilization in Japan and in developing countries where HV obstruction potentially predominates.

Key words: Budd-Chiari syndrome, hepatic vein thrombosis, liver transplantation, myeloproliferative neoplasm, tacrolimus

INTRODUCTION

BUDD-CHIARI SYNDROME (BCS) is a heterogeneous disorder consisting of various underlying diseases with a common feature of hepatic venous outflow obstruction. This obstruction involves hepatic veins (HVs), inferior vena cava (IVC) and/or the right atrium in the outflow tract. The vulnerable site in the outflow tract is closely related to the etiology, with geographical variations.[1,2] IVC obstruction, particularly so-called membranous obstruction of IVC (MOVC), with undefined causes has been found to be the most frequent type and etiology of BCS in Japan and developing countries, such as Nepal, South Africa, India and China.[3–9] Although MOVC was previously thought to be a congenital vascular malformation, it has been proven to be a sequela of IVC thrombosis.[6] In contrast, HV thrombosis caused by prothrombotic states due to certain diseases (mostly hematological disorders) is common in Western countries.[2]

Herein, we report a Japanese case of BCS due to HV thrombosis with possible latent myeloproliferative neoplasm (MPN), which has been regarded as a rare pattern in Japan, which was successfully treated with liver transplantation.

CASE REPORT

THE PATIENT WAS a 22-year-old Japanese woman with no personal or family history of liver diseases or hematological abnormalities. In March 2006, the

Correspondence: Dr Hirokazu Kawai, Department of Clinical Laboratory, Niigata University Medical and Dental Hospital, 1-757 Asahimachi-dori Chuo-ku, Niigata 951-8510, Japan. Email: kawaih@med.niigata-u.ac.jp
Received 26 May 2011; revision 3 September 2011; accepted 6 September 2011.

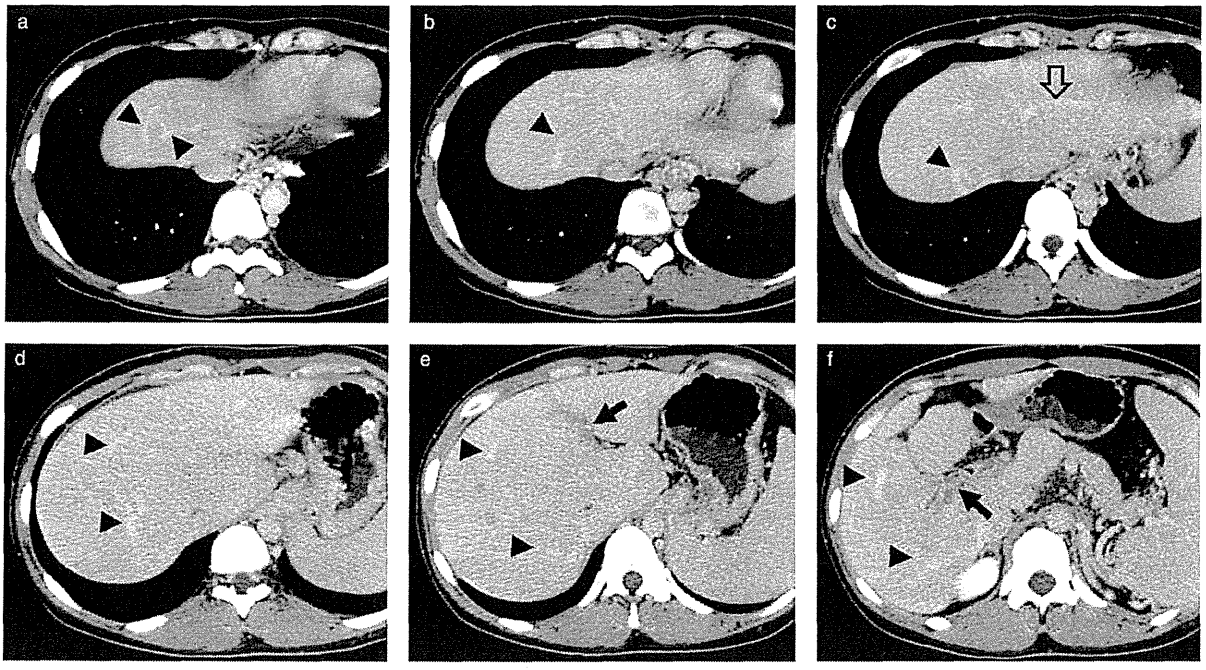

Figure 1 Contrast-enhanced abdominal computed tomography before liver transplantation. (a–f) A liver deformity with enlargement of the caudate lobe accompanied by huge splenomegaly and esophageal varices is shown. Extensive collateral veins (arrow heads) within the liver flowed into inferior vena cava, primarily through the right inferior hepatic vein. (f) The distal left hepatic vein (open arrow) was confluent with the collaterals, and the original middle and right hepatic veins were not revealed. (e, f) Thrombosed portal vein (closed arrows) is shown.

patient visited a gynecology clinic with a complaint of amenorrhea continuing for 6 months without pregnancy, and she received oral medications containing estrogen and progesterone for 10 days. However, the symptom did not improve, and she was referred to another hospital in April 2006. Contrast-enhanced abdominal computed tomography (CT) depicted a liver deformity with enlargement of the caudate lobe accompanied by huge splenomegaly (Fig. 1a–f). The distal left hepatic vein was confluent with collateral veins within the liver, and the original middle and right hepatic veins were not revealed (Fig. 1a–f). Portal vein (PV) was occluded with thrombus (Fig. 1e,f). An upper gastrointestinal endoscopy showed severe esophageal varices (Fig. 2a). One month later, she was referred and admitted to our hospital for further workup and treatment.

At admission to our hospital, hepatic encephalopathy was absent. Hepatomegaly and marked splenomegaly with mild tenderness were observed. Ascites, edema of the lower extremities, palmar erythema and vascular spiders were absent. A summary of the laboratory tests is shown in Table 1. The peripheral blood cell counts were within the normal range. Liver function tests showed a slight elevation of liver enzymes, although all of the serological markers for viral hepatitis type B and C, autoimmune liver diseases and metabolic liver diseases were negative. The prothrombin time-international normalized ratio was high, and the protein S antigen level was decreased; however, indicators for the activation of coagulation and fibrinolysis, such as fibrin degradation products, were not increased. An abdominal ultrasonography revealed HVs appearing with a hyperechogenic cord-like structure, which implied obstruction with thrombus (Fig. 2b). An abdominal angiography demonstrated extensively developed collateral veins that flowed into IVC through the right inferior hepatic vein or extrahepatic veins instead of innate HVs (Fig. 2c,d). Meanwhile, IVC was only compressed by the caudate lobe, without membranous obstruction or thrombus (Fig. 2e). PV occlusion with a cavernous transformation and the dilated left gastric vein was apparent (Fig. 2f). The series of imaging studies indicated BCS due to HV thrombosis without IVC obstruction, which was super-

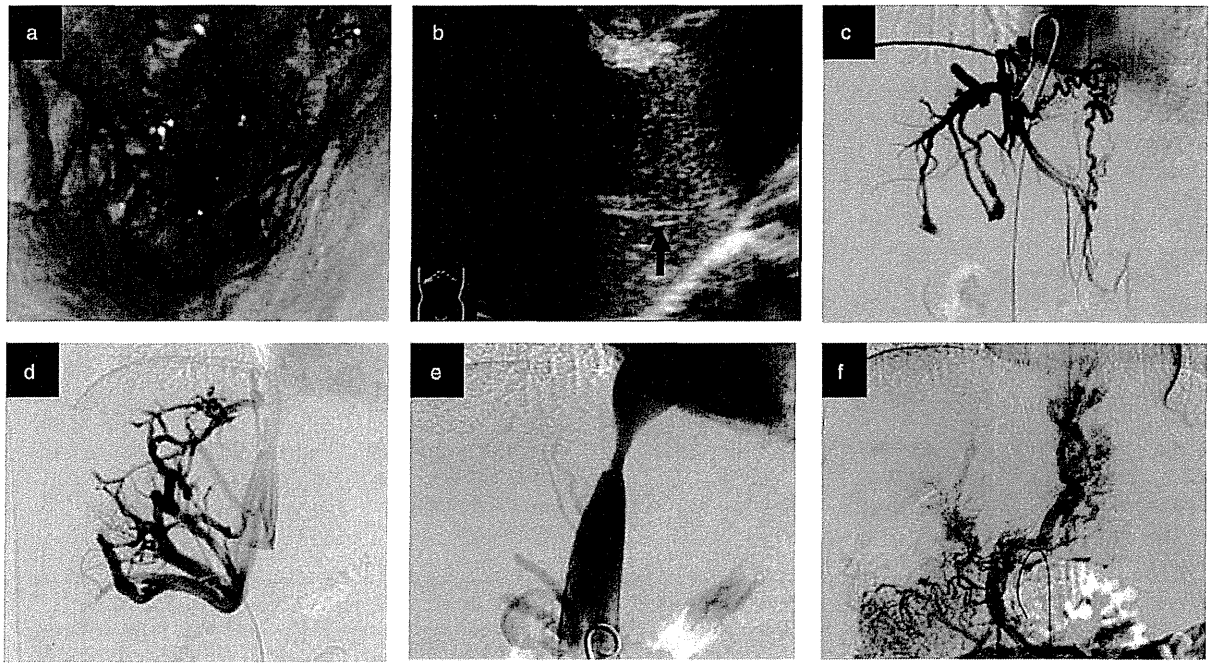

Figure 2 Imaging studies before liver transplantation. (a) Upper gastrointestinal endoscopy showed severe esophageal varices. (b) Abdominal ultrasonography revealed hepatic veins appearing with a hyperechogenic cord-like structure (arrows). Abdominal angiography demonstrated (c) extrahepatic collateral veins, such as the inferior phrenic vein and the ascending lumber vein, instead of innate hepatic veins (d) extensively developed collateral veins flowing through the right inferior hepatic vein in the liver (e) inferior vena cava compressed by the caudate lobe without membranous obstruction or thrombus and (f) occluded portal vein with cavernous transformation and the dilated left gastric vein.

imposed by PV thrombosis. We then suspected MPN as the underlying etiology because cytopenia was absent in the peripheral blood, despite huge splenomegaly. Although the JAK2-V617F mutation was not detected, a bone marrow biopsy showed compatible findings with an early stage of MPN, which comprised moderate cellularity and an increased number of megakaryocytes without fibrosis. A definite diagnosis of MPN, however, could not be made because the results did not fulfill the WHO diagnostic criteria.[10]

After admission, hepatic encephalopathy developed with an increase in blood ammonia to more than 200 μg/dL, and oral intake of kanamycin and lactulose was started. Because both the severe esophageal varices and hepatic encephalopathy were caused by prominent portal hypertension due to HV obstruction complicated by PV occlusion, the symptoms were likely to be refractory to medications or endoscopic therapies. A radical treatment for the basal pathophysiology was needed to improve the symptoms; therefore, a living donor liver transplantation and splenectomy were performed in October 2006. The pathological examination of the explanted liver revealed HV thrombosis with well-developed collateral veins and sinusoidal dilatation (Fig. 3a,b), which suggested BCS. The extramedullary hematopoiesis (Fig. 3c), which was compatible with MPN, was shown within the liver. In addition, a dysplastic nodule was detected (Fig. 3d), although it had not been recognized on the CT prior to the operation.

After transplantation, the esophageal varices and hepatic encephalopathy disappeared, and the ammonia level was normalized. The patient received immunosuppression with tacrolimus throughout the post-transplantation course. The immunosuppression has not developed rejection nor accelerated the progression of MPN. Furthermore, warfarin therapy was initiated to prevent thrombosis due to thrombocythemia of more than $100 \times 10^4/\mu L$, which occurred and persisted after the operation. Although thrombosis of the left internal jugular vein occurred 3 months after the operation despite anticoagulation, the thrombus immediately disappeared with the additional use of aspirin. Neither

Table 1 Laboratory data on admission

White blood cell	7280	/μL	Total protein	7.5	g/dL
Neutrophil	62.0	%	Albumin	5.2	g/dL
Basophil	2.0	%	BUN	10	mg/dL
Eosinophil	6.0	%	Creatinine	0.6	mg/dL
Lymphocyte	25.5	%	Total-Bilirubin	2.3	mg/dL
Monocyte	4.5	%	Direct-Bilirubin	0.4	mg/dL
Red blood cell	558×10^4	/μL	AST	41	IU/L
Hemoglobin	14.5	g/dL	ALT	38	IU/L
Hematocrit	44.8	%	LDH	348	mg/dL
Platelet	31.4×10^4	/μL	ALP	460	IU/L
PT-INR	1.46		γ-GTP	77	IU/L
APTT	40.1	sec	Ammonia	49	μg/dL
FDP	1.0	μg/mL	Fe	63	μg/dL
D-dimer	0.5	μg/mL	Ferritin	9	ng/mL
Protein C antigen	78	%	Antinuclear antibody	(−)	
Protein S antigen	63	%	Lupus anticoagulant	(−)	
HBs-antigen	(−)		Anti-DNA antibody	(−)	
Anti-HBc	(−)		JAK2-V617F	(−)	
Anti-HCV	(−)				

γ-GTP, γ-glutamyltranspeptidase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BUN, blood urea nitrogen; FDP, fibrin and fibrinogen degradation product; HBc, hepatitis B core; HBs, hepatitis B surface; HCV, hepatitis C virus; LDH, lactate dehydrogenase; PT-INR, prothrombin time-international normalized ratio.

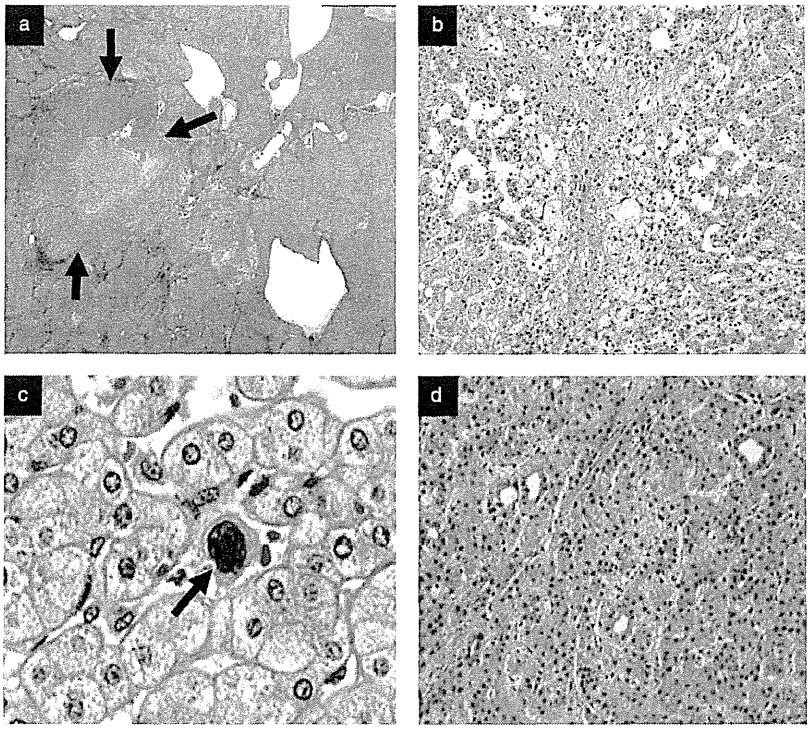

Figure 3 A pathological examination of the explanted liver (hematoxylin and eosin staining). (a) Hepatic veins were occluded by thrombosis (arrows) with developed collateral veins (original magnification ×20). (b) Sinusoids were dilated with thrombosis of hepatic veins (original magnification ×200). (c) Precursor cells, including megakaryocytes (arrow), erythroblasts and myeloblasts, were present, which indicated extramedullary hematopoiesis (original magnification ×400). (d) A dysplastic nodule was observed (original magnification ×200).

thrombosis nor BCS has recurred in more than 4 years since the amelioration of the last thrombotic event, even with thrombocythemia remaining at a consistently high level.

DISCUSSION

IN JAPAN AND developing countries, IVC obstruction was previously the major type of BCS.[1,2,4,5] The frequencies of IVC obstruction and HV obstruction without IVC obstruction were 93.0% and 5.7%, respectively, in 1989, according to a nation-wide survey in Japan.[1] However, a decrease in IVC obstruction in India was recently reported by Amarapurkar *et al.*; the frequencies of IVC obstruction, MOVC and HV obstruction were 16%, 4% and 59%, respectively.[11] This change in the BCS spectrum in India might be related to recent improvements in hygiene, because unsanitary environments are considered to be responsible for IVC obstruction.[12] A similar trend has possibly appeared in Japan, as has been suggested in a few reports,[6,13] and the affected site that was confined to HV in the present case might reflect this current trend. It is crucial to understand the latest characteristics of BCS for an accurate diagnosis because an antiquated disease profile might lead to neglecting BCS with intact IVC in differential diagnoses for Japanese patients.

A precise diagnosis of MPN as an underlying disease of BCS is important for preventing additional thrombotic events. Unfortunately, the diagnosis of MPN coexisting with BCS is difficult because peripheral blood cell counts do not increase due to hypersplenism, hemodilution or iron deficiency in most patients.[14,15] As shown in recent reports, the JAK2-V617F mutation, which is thought to play a causative role in the pathogenesis of MPN, is useful for diagnosing classical Philadelphia-negative MPN, even in BCS patients.[14–18] However, JAK2-V617F is not an absolute determinant for MPN diagnosis because the mutation is absent in approximately half of the patients with either essential thrombocythemia or primary myelofibrosis.[16,17] Indeed, despite lacking JAK2-V617F, MPN was strongly suggested through other hematological examinations and clinical manifestations in the present case. Thus, the diagnosis of MPN in BCS patients should be made not only by a JAK2-V617F mutation test but also by an overall hematological assessment, including clinical symptoms and a bone marrow biopsy.

European expert panels have elaborated a stepwise strategy for BCS, outlined as follows: (i) anticoagulation, treatment of the underlying condition and symptomatic treatment for complications of portal hypertension; (ii) angioplasty/stenting; (iii) transjugular intrahepatic portosystemic shunt; and (iv) liver transplantation.[19,20] This graded approach enabled us to rationally select the appropriate treatment method. Before the transplantation, we skipped the early steps of the BCS treatment strategy because of the potential risk of variceal hemorrhage with anticoagulation therapy and no accessible veins deserving recanalization or shunting with interventional radiology. We eventually performed a successful liver transplantation without any critical complications. Because the stepwise strategy has been proven effective in patients with pure HV obstruction in Western countries,[20] the strategy should be also validated for use in Japan and developing countries where HV obstruction potentially predominates.

Excellent outcomes of liver transplantation for BCS were demonstrated in a national registry analysis in the US (the 3-year survival of 84.9% in the model for the end-stage liver disease era)[21] and in a European registry survey (the 5-year survival of 71.4%).[22] The European survey did not clarify the impact of underlying diseases on the outcome, whereas the results indicated the poor outcome of patients with recurrence of venous thrombosis at various sites (mortality of 40.7%).[22] Thus, preventing recurrent thrombosis is critical for a positive outcome, regardless of the underlying diseases. A favorable effect of hydroxyurea with anticoagulation for thrombosis after liver transplantation has been suggested in patients with MPN.[23] In the present case, the addition of aspirin on anticoagulation with warfarin improved post-transplantation thrombosis. If thrombotic events recur or thrombocythemia deteriorates, we must consider further treatment with hydroxyurea, regardless of whether overt MPN develops or not.

Potential liver transplant candidates with pre-existing malignancies pose a risk for recurrence or tumor progression with the long-term immunosuppression that accompanies transplantation. Saigal *et al.* assessed the outcome of six MPN cases presenting as BCS who underwent liver transplantation and received subsequent immunosuppression with calcineurin inhibitors during a median follow-up of 71 months.[24] Of these cases, one patient with polycythemia vera developed acute leukemia 72 months after transplantation that resulted in death. The authors regarded this leukemic transformation as the natural history of the disease rather than an effect of immunosuppression because of a 14-year history after the primary diagnosis of MPN. Furthermore, Bahr *et al.* found no progression to acute leukemia in 21 transplanted patients with MPN during a

mean follow-up of 8.5 years.[25] Post-transplant immunosuppression with tacrolimus did not accelerate MPN progression also in our case. These data suggest no substantial impact of immunosuppression on the course of MPN in patients transplanted for BCS. However, only limited data are available, and a larger scale investigation is needed to be conclusive.

In conclusion, we report on a Japanese case of BCS due to HV thrombosis with possible latent MPN that was successfully treated with liver transplantation. It is necessary to recognize the current disease spectrum of BCS and to establish treatment strategies corresponding to the types or severity of the venous obstruction for optimal management of BCS.

REFERENCES

1 Okuda H, Yamagata H, Obata H *et al.* Epidemiological and clinical features of Budd-Chiari syndrome in Japan. *J Hepatol* 1995; **22**: 1–9.
2 Valla DC. Hepatic venous outflow tract obstruction etiopathogenesis: Asia versus the West. *J Gastroenterol Hepatol* 2004; **19**: S204–S211.
3 Nakamura T, Nakamura S, Karoji N *et al.* Obstruction of the inferior vena cava in the hepatic portion and the hepatic veins. Report of eight cases and review of the Japanese literature. *Angiology* 1968; **19**: 479–98.
4 Hirooka M, Kimura C. Membranous obstruction of the hepatic portion of the inferior vena cava. Surgical correction and etiologic study. *Arch Surg* 1970; **100**: 656–63.
5 Kage M, Arakawa M, Kojiro M *et al.* Histopathology of membranous obstruction of the inferior vena cava in the Budd-Chiari syndrome. *Gastroenterology* 1992; **102**: 2081–90.
6 Okuda K. Obliterative hepatocavopathy-Inferior vena cava thrombosis at its hepatic portion. *Hepatobiliary Pancreat Dis Int* 2002; **1**: 499–509.
7 Simson IM. Membranous obstruction of the inferior vena cava and hepatocellular carcinoma in South Africa. *Gastroenterology* 1982; **82**: 171–8.
8 Shrestha S, Okuda K, Uchida T *et al.* Endemicity and clinical pictures of liver disease due to obstruction of the hepatic portion of the inferior vena cava in Nepal. *J Gastroenterol Hepatol* 1996; **11**: 179–179.
9 Dilawari JB, Bambery P, Chawla Y *et al.* Hepatic outflow obstruction (Budd-Chiari syndrome). Experience with 177 patients and a review of the literature. *Medicine* 1994; **73**: 21–36.
10 Tefferi A, Thiele J, Vardiman JW. The 2008 World Health Organization classification system for myeloproliferative neoplasms: order out of chaos. *Cancer* 2009; **115**: 3842–7.
11 Amarapurkar DN, Punamiya SJ, Patel ND. Changing spectrum of Budd-Chiari syndrome in India with special reference to non-surgical treatment. *World J Gastroenterol* 2008; **14**: 278–85.
12 Eapen CE, Mammen T, Moses V, Shyamkumar NK. Changing profile of Budd Chiari syndrome in India. *Indian J Gastroenterol* 2007; **26** (2): 77–81.
13 Okuda K, Kage M, Sherestha SM. Proposal of a New Nomenclature for Budd-Chiari syndrome: hepatic vein thrombosis versus thrombosis of the inferior vena cava at its hepatic portion. *Hepatology* 1998; **28**: 1191–8.
14 Valla DC. Primary Budd-Chiari syndrome. *J Hepatol* 2009; **50**: 195–203.
15 Patel RK, Lea NC, Mufti GJ *et al.* Prevalence of the activating JAK2 tyrosine kinase mutation V617F in the Budd-Chiari syndrome. *Gastroenterology* 2006; **130**: 2031–8.
16 Baxter EJ, Scott LM, Campbell PJ *et al.* Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative diseases. *Lancet* 2005; **365**: 1054–61.
17 Kralovics R, Passamonti F, Buser AS *et al.* A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med* 2005; **352**: 1779–90.
18 Goulding C, B.Uttenthal CM, Foroni L. The JAK2-V617F tyrosine kinase mutation identifies clinically latent myeloproliferative disorders in patients presenting with hepatic or portal vein thrombosis. *Int Lab Hematol* 2008; **30**: 415–9.
19 Janssen HL, Garcia-Pagan JC, Elias E *et al.* Budd-Chiari syndrome: a review by an expert panel. *J Hepatol* 2003; **38**: 364–71.
20 Plessier A, Valla DC. Budd-Chiari syndrome. *Semin Liver Dis* 2008; **28**: 259–69.
21 Segev DL, Nguyen GC, Locke JE *et al.* Twenty years of liver transplantation for Budd-Chiari syndrome: a national registry analysis. *Liver Transpl* 2007; **13**: 1285–94.
22 Mentha G, Giostra E, Majno PE *et al.* Liver transplantation for Budd-Chiari syndrome: a European study on 248 patients from 51 centres. *J Hepatol* 2006; **44**: 520–8.
23 Melear JM, Goldstein RM, Levy MF *et al.* Hematologic aspects of liver transplantation for Budd-Chiari syndrome with special reference to myeloproliferative disorders. *Transplantation* 2002; **74**: 1090–5.
24 Saigal S, Norris S, Srinivasan P *et al.* Successful outcome of orthotopic liver transplantation in patients with preexisting malignant states. *Liver Transpl* 2001; **7** (1): 11–15.
25 Bahr MJ, Rosenau J, Tietge UJ *et al.* Immunosuppression and the prognosis of patients suffering from myeloproliferative disorders after liver transplantation. *Transplant Proc* 2002; **34**: 1493–4.