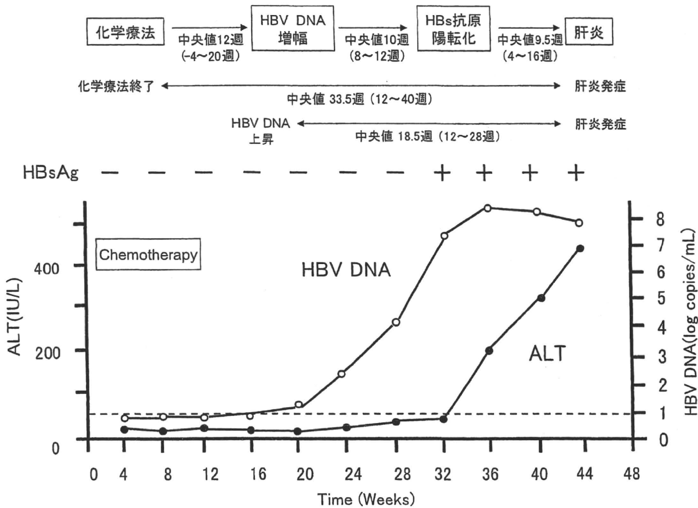

Figure 2. *De novo* B 型肝炎の臨床パターン（文献 22 より引用改変）.

29 例）を占めている[24]. HBs 抗原陽性キャリアからの再活性化では，基礎疾患として悪性リンパ腫が最も多いが，乳癌などの固形癌や慢性関節リウマチなどの膠原病などもみられる．前治療としては，ステロイドが最も多い．一方，*de novo* B 型肝炎はほとんどが，悪性リンパ腫に対するリツキシマブとステロイドの併用療法が原因である．臨床背景では *de novo* B 型肝炎は高齢者が多く，病型は劇症肝炎亜急性型を呈し，全例が核酸アナログで治療されていたが救命例はなかった（Table 1）. *de novo* B 型肝炎は発症時のトランスアミナーゼはあまり高くなく，治療薬による肝障害との鑑別も困難であり，診断が遅れる一因になっていると思われる．また，肝炎発症後の核酸アナログの治療効果も不良であり，その病態は不明である．

IV　HBV 再活性化対策

近年の HBV 再活性化肝炎の増加をうけて，厚生労働省の「難治性の肝・胆道疾患に関する調査研究」班劇症肝炎分科会と「肝硬変を含めたウイルス性肝疾患の治療の標準化に関する研究」班が合同で，HBV 再活性化に対するガイドラインを作成した[25]. 現在，本ガイドラインを検証する前向きコホート研究が実施されている．免疫抑制・化学療法前のスクリーニングで重要な点は，従来の HBs 抗原検査だけではなく HBc 抗体および HBs 抗体検査をできるだけ感度のよい方法で実施することである．以下にガイドラインの骨子を述べる．

1. HBV 再活性化対策ガイドライン

1）HBV キャリア

HBs 抗原陽性例に対しては，さらに HBe 抗原，HBe 抗体，HBV DNA 定量検査を実施し，感染状態を詳しく把握する必要がある．HBV DNA 量が高値であるほど再活性化のリスクは高い．HBs 抗原陽性例では HBe 抗原，HBe 抗体，HBV DNA 定量検査の結果にかかわらず治療開始前に核酸アナログ製剤の予防投与を行う．核酸アナログは最も耐性株出現率の低いエンテカビル投与が推奨される．核酸アナログにより血中 HBV DNA 量が低下するまでには日数を要するため，HBV DNA 量が多い症例などでは，できるだけ

Table 1. HBV 再活性化による劇症肝炎（2004～2008年）

	HBV キャリア (n = 15)	*de novo* B 型肝炎 (n = 14)
年齢（Median（range）歳）	59（29～80）	66（48～76）
性別（男/女）	9/6	8/6
病型（急性/亜急性/LOHF）	3/4/8	0/14/0
転帰（生存/死亡/肝移植）	2/12/1	0/14/0
基礎疾患		
悪性リンパ腫	7（46.7%）	11（78.6%）
その他の血液悪性腫瘍	1	2
血液以外の悪性腫瘍	2	1
膠原病（リウマチなど）	4	
その他	1	
前治療		
リツキシマブ	5（33.3%）	11（78.6%）
ステロイド	11（73.3%）	11（78.6%）
末梢血幹細胞移植		1
肝炎の治療		
核酸アナログ	15（100%）	14（100%）

早期に投与を開始するのが望ましい．

2）HBV 既往感染者

HBs 抗原陰性で HBc 抗体ないし HBs 抗体陽性の場合は更に HBV DNA 定量検査を実施する．HBV DNA 定量検査が陽性であれば核酸アナログの予防投与を開始する．HBV DNA が検出感度以下で陰性の場合は，治療中および治療終了後に HBV DNA を月1回モニタリングする．HBV DNA の測定には現在最も検出感度の高い，リアルタイム PCR 法での測定がのぞましい．経過観察中に HBV DNA が陽性化した時点で直ちに核酸アナログの投与を開始する．HBV DNA のモニタリングは治療中だけでなく治療終了後も12カ月間は継続する．HBs 抗原陰性で HBc 抗体，HBs 抗体いずれも陰性の場合は通常の対応とするが，患者が既に免疫抑制あるいは化学療法後で免疫抑制状態にある場合には抗体が検出されないことがあり，HBV DNA 定量検査まで測定することが望ましい．

核酸アナログ投与終了に関する明確な基準はないが，HBs 抗原陽性例では使用する各核酸アナログの投与終了基準に準ずる．HBs 抗原陰性，HBc 抗体ないし HBs 抗体陽性例では治療終了後も12カ月間は投与を継続し，この継続期間中に ALT と HBV DNA が持続陰性化している場合は投与終了の検討も可能である．ただし，核酸アナログ予防投与終了6～8カ月後にウイルス血症の再出現および重症肝炎の発症も報告されており[26]，投与終了後も更に12カ月間は厳重な経過観察が必要である．

2. HBV 再活性化に関連する薬剤

HBV 再活性化は血液悪性疾患以外の膠原病やアレルギー性疾患に対する免疫抑制療法でもみられるが，その頻度は低い[27]．少量のステロイドやアザチオプリンでは一般的にはみられないが，メソトレキセートの長期投与ではまれに報告例がある[28]．またステロイドと抗 TNF-α 抗体（インフリキシマブ）併用による HBV 再活性化の報告がみられる[6]．Crohn 病，慢性関節リウマチ，強直性脊椎炎に対するインフリキシマブ使用により重篤な HBV 再活性化や，少数ながら *de novo* B 型肝炎の報告もみられる[29)～31]．現在，わが国では HBV 感染者に対するインフリキシマブ，エタネルセプト，アダリムマブの使用に際しては HBV

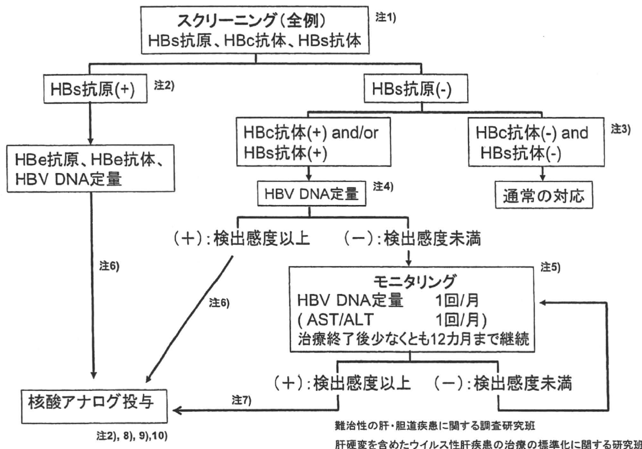

Figure 3. 免疫抑制・化学療法により発症するB型肝炎対策ガイドライン*.
*血液悪性疾患に対する強力な免疫抑制・化学療法中あるいは終了後に，HBs抗原陽性あるいはHBs抗原陰性例の一部に，HBV再活性化によりB型肝炎が発症し，その中には劇症化する症例があり，注意が必要である．その他の疾患においても治療によるHBV再活性化のリスクを考慮して対応する必要がある．また，ここで推奨する核酸アナログ予防投与のエビデンスはなく，劇症化予防効果を完全に保証するものではない．
注1）CLIA法で測定することが望ましい． 注2）HBs抗原陽性例は肝臓専門医にコンサルトすること．すべての症例で核酸アナログ投与にあたっては肝臓専門医にコンサルトするのが望ましい． 注3）初回治療時にHBc抗体，HBs抗体未測定の再治療例では抗体価が低下している場合があり，HBV DNA定量検査などによる精査が望ましい． 注4）PCR法およびリアルタイムPCR法により実施する．より検出感度の高いリアルタイムPCR法が望ましい． 注5）リツキシマブ・ステロイド使用例，造血細胞移植例はHBV再活性化の高リスクであり，注意が必要である．フルダラビンは強力な免疫抑制作用を有するが，HBV再活性化のリスクは不明であり，今後注意が必要である． 注6）免疫抑制・化学療法を開始する前，できるだけ早期に投与を開始するのが望ましい． 注7）免疫抑制・化学療法中はHBV DNA定量検査が検出感度以上になった時点で直ちに投与を開始する． 注8）核酸アナログはエンテカビルの使用を推奨する． 注9）下記の条件を満たす場合には核酸アナログ投与の終了を検討して良い．スクリーニング時にHBs抗原（+）例ではB型慢性肝炎における核酸アナログ投与終了基準を満たす場合．スクリーニング時にHBc抗体（+）and/or HBs抗体（+）例では，(1) 免疫抑制・化学療法終了後，少なくとも12カ月間は投与を継続すること．(2) この継続期間中にALT（GPT）が正常化していること．(ただしHBV以外にALT異常の原因がある場合は除く)(3)この継続期間中にHBV DNAが持続陰性化していること． 注10）核酸アナログ投与終了後12カ月間は厳重に経過観察する．経過観察方法は各核酸アナログの使用上の注意に基づく．経過観察中にHBV DNA定量検査が検出感度以上になった時点で直ちに投与を再開する．

再活性化に注意することが喚起されている．これまでにHBV再活性化が報告されている薬剤はTable 2のとおりである[32)33)]．今後も生物学的製剤，分子標的治療薬など新規の免疫抑制剤や抗癌剤の登場が予想され，HBV再活性化には十分注意が必要である．

おわりに

HBV再活性化は発症すれば危険な病態だが，

Table 2. B型肝炎再活性化に関連する薬剤

コルチコステロイド	プレドニゾロン，デキサメタゾン，メチルプレドニゾロン
抗腫瘍抗生物質	ドキソルビシン，エピルビシン，ダウノルビシン，ブレオマイシン（BLM），マイトマイシン-C（MMC），アクチノマイシン-D（ACT-D）
植物アルカロイド	ビンクリスチン（VCR），ビンブラスチン（VLB）
アルキル化薬	シクロホスファミド（CPA），イホスファミド（IFM），クロラムブシル，カルボプラチン（CBDCA），シスプラチン（CDDP），プロカルバジン（PCZ）
代謝拮抗薬	シタラビン（SPAC），アザウリジン，フルオロウラシル（5-FU），ゲムシタビン，メルカプトプリン（6-MP），メソトレキセート（MTX），チオグアニン
その他	L-アスパラギナーゼ（L-ASP），コラスパーゼ，フォリニックアシッド，プロカルバジン，ドセタキセル，エトポシド（VP-16），フルダラビン，インターフェロン（IFN）
分子標的治療薬	リツキシマブ（anti-CD20），アレムツズマブ（anti-CD52），インフリキシマブ（anti-TNF），メシル酸イマチニブ

文献32）33）より引用改変.

発症前に核酸アナログを投与できればその予防が可能である．個々の症例においてHBV再活性化およびその劇症化のリスクを正確に予測することは困難だが，スクリーニングによりHBV再活性化高リスク群を治療前に把握し，適切な時期に核酸アナログを使用することが，HBV再活性化対策において最も重要である．

文　献

1) Lok AS, Lai CL, Wu PC, et al: Spontaneous hepatitis B e antigen to antibody seroconversion and reversion in Chinese patients with chronic hepatitis B virus infection. Gastroenterology 92; 1839–1843: 1987
2) Yeo W, Chan PK, Zhong S, et al: Frequency of hepatitis B virus reactivation in cancer patients undergoing cytotoxic chemotherapy: a prospective study of 626 patients with identification of risk factors. J Med Virol 62; 299–307: 2000
3) Wands JR, Chura CM, Roll FJ, et al: Serial studies of hepatitis-associated antigen and antibody in patients receiving antitumor chemotherapy for myeloproliferative and lymphoproliferative disorders. Gastroenterology 68; 105–112: 1975
4) Dervite I, Hober D, Morel P: Acute hepatitis B in a patient with antibodies to hepatitis B surface antigen who was receiving rituximab. N Engl J Med 344; 68–69: 2001
5) Rostoker G, Rosenbaum J, Ben Maadi A, et al: Reactivation of hepatitis B virus by corticosteroids in a case of idiopathic nephrotic syndrome. Nephron 56; 224: 1990
6) Esteve M, Saro C, González-Huix F, et al: Chronic hepatitis B reactivation following infliximab therapy in Crohn's disease patients: need for primary prophylaxis. Gut 53; 1363–1365: 2004
7) Waite J, Gilson RJ, Weller IV, et al: Hepatitis B virus reactivation or reinfection associated with HIV-1 infection. AIDS 2; 443–448: 1988
8) Dickson RC, Everhart JE, Lake JR, et al: Transmission of hepatitis B by transplantation of livers from donors positive for antibody to hepatitis B core antigen. The National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation Database. Gastroenterology 113; 1668–1674: 1997
9) Uemoto S, Sugiyama K, Marusawa H, et al: Transmission of hepatitis B virus from hepatitis B core antibody-positive donors in living related liver transplants. Transplantation 65; 494–499: 1998
10) Knöll A, Boehm S, Hahn J, et al: Reactivation of resolved hepatitis B virus infection after allogeneic haematopoietic stem cell transplantation. Bone Marrow Transplant 33; 925–929: 2004
11) Onozawa M, Hashino S, Izumiyama K, et al: Progressive disappearance of anti-hepatitis B surface antigen antibody and reverse seroconversion after allogeneic hematopoietic stem cell transplantation in patients with previous hepatitis B virus infection. Transplantation 79; 616–619: 2005
12) Lok AS, Liang RH, Chiu EK, et al: Reactivation of hepatitis B virus replication in patients receiving

cytotoxic chemotherapy. Report of a prospective study. Gastroenterology 100 ; 182–188 : 1991
13) Zhong S, Yeo W, Schroder C, et al : High hepatitis B virus (HBV) DNA viral load is an important risk factor for HBV reactivation in breast cancer patients undergoing cytotoxic chemotherapy. J Viral Hepat 11 ; 55–59 : 2004
14) Chou CK, Wang LH, Lin HM, et al : Glucocorticoid stimulates hepatitis B viral gene expression in cultured human hepatoma cells. Hepatology 16 ; 13–18 : 1992
15) Omata M, Ehata T, Yokosuka O, et al : Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 324 ; 1699–1704 : 1991
16) Dai MS, Lu JJ, Chen YC, et al : Reactivation of precore mutant hepatitis B virus in chemotherapy-treated patients. Cancer 92 ; 2927–2932 : 2001
17) Lau GK, Yiu HH, Fong DY, et al : Early is superior to deferred preemptive lamivudine therapy for hepatitis B patients undergoing chemotherapy. Gastroenterology 125 ; 1742–1749 : 2003
18) Fong TL, Di Bisceglie AM, Gerber MA, et al : Persistence of hepatitis B virus DNA in the liver after loss of HBsAg in chronic hepatitis B. Hepatology 18 ; 1313–1318 : 1993
19) Michalak TI, Pasquinelli C, Guilhot S, et al : Hepatitis B virus persistence after recovery from acute viral hepatitis. J Clin Invest 93 ; 230–239 : 1994
20) Raimondo G, Pollicino T, Cacciola I, et al : Occult hepatitis B virus infection. J Hepatol 46 ; 160–170 : 2007
21) Kawatani T, Suou T, Tajima F, et al : Incidence of hepatitis virus infection and severe liver dysfunction in patients receiving chemotherapy for hematologic malignancies. Eur J Haematol 67 ; 45–50 : 2001
22) Hui CK, Cheung WW, Zhang HY, et al : Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. Gastroenterology 131 ; 59–68 : 2006
23) Umemura T, Tanaka E, Kiyosawa K, et al : Mortality secondary to fulminant hepatic failure in patients with prior resolution of hepatitis B virus infection in Japan. Clin Infect Dis 47 ; e52–e56 : 2008
24) 坪内博仁，桶谷　真，井戸章雄，他：①劇症肝炎及び遅発性肝不全の全国集計（2008 年）．② de novo　B 型肝炎における HBV 遺伝子解析および病態解明．厚生労働省難治性疾患克服研究事業「難治性の肝・胆道疾患に関する調査研究」班，平成 21 年度研究報告書，95–110 : 2010
25) 坪内博仁，熊田博光，清澤研道，他：免疫抑制・化学療法により発症する B 型肝炎対策—厚生労働省「難治性の肝・胆道疾患に関する調査研究」班劇症肝炎分科会および「肝硬変を含めたウイルス性肝疾患の治療の標準化に関する研究」班合同報告—．肝臓　50 ; 38–42 : 2009
26) Dai MS, Chao TY, Shyu RY, et al : Delayed hepatitis B virus reactivation after cessation of preemptive lamivudine in lymphoma patients treated with rituximab plus CHOP. Ann Hematol 83 ; 769–774 : 2004
27) Lok AS, McMahon BJ : Chronic hepatitis B. Hepatology 45 ; 507–539 : 2007
28) Ito S, Nakazono K, Murasawa A, et al : Development of fulminant hepatitis B (precore variant mutant type) after the discontinuation of low-dose methotrexate therapy in a rheumatoid arthritis patient. Arthritis Rheum 44 ; 339–342 : 2001
29) Ostuni P, Botsios C, Punzi L, et al : Hepatitis B reactivation in a chronic hepatitis B surface antigen carrier with rheumatoid arthritis treated with infliximab and low dose methotrexate. Ann Rheum Dis 62 ; 686–687 : 2003
30) Calabrese LH, Zein NN, Vassilopoulos D : Hepatitis B virus (HBV) reactivation with immunosuppressive therapy in rheumatic diseases : assessment and preventive strategies. Ann Rheum Dis 65 ; 983–989 : 2006
31) Zingarelli S, Frassi M, Bazzani C, et al : Use of tumor necrosis factor-alpha-blocking agents in hepatitis B virus-positive patients : reports of 3 cases and review of the literature. J Rheumatol 36 ; 1188–1194 : 2009
32) Yeo W, Johnson PJ : Diagnosis, prevention and management of hepatitis B virus reactivation during anticancer therapy. Hepatology 43 ; 209–220 : 2006
33) Lalazar G, Rund D, Shouval D : Screening, prevention and treatment of viral hepatitis B reactivation in patients with haematological malignancies. Br J Haematol 136 ; 699–712 : 2007

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The complement component C3a fragment is a potential biomarker for hepatitis C virus-related hepatocellular carcinoma

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Abstract

Background Hepatocellular carcinoma (HCC) has a high mortality rate, and early detection of HCC improves patient survival. However, the molecular diagnostic markers for early HCC have not been fully elucidated. The aim of this study was to identify novel diagnostic markers for HCC.
Methods Serum protein profiles of 45 hepatitis C virus infection (HCV)-related HCC patients (HCV-HCC) were compared to 42 HCV-related chronic liver disease patients without HCC (HCV-CLD) and 21 healthy volunteers using the ProteinChip SELDI system. One of the identified proteins was evaluated as a diagnostic marker for HCC in patients with HCV.
Results Five protein peaks (4067, 4470, 7564, 7929, and 8130 m/z) had *p*-values less than 1×10^{-7} and were significantly increased in the sera of HCV-HCC patients compared to HCV-CLD patients and healthy volunteers. Among these proteins, an 8130 m/z peak was the most differentially expressed and identified as the complement component 3a (C3a) fragment. For HCV-HCC and HCV-CLD, the relative intensity of this C3a fragment had the best area under the ROC curve [0.70], followed by des-γ-carboxy prothrombin (DCP) [0.68], lectin-bound alpha fetoprotein (AFP-L3) [0.58] and AFP [0.53] for HCC. A combined analysis of the C3a fragment, AFP and DCP led to a 98% positive identification rate. In addition, the measurable C3a fragment in some HCC patients was not only significantly higher in the year of HCC onset compared to the pre-onset year, but also decreased after treatment.
Conclusions The 8130 m/z C3a fragment is a potential marker for the early detection of HCV-related HCC.

Keywords Hepatocellular carcinoma · Complement component C3a · Serum proteomics · Serum biomarkers · Proteinchip SELDI system · Hepatitis C virus

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Introduction

Hepatocellular carcinoma (HCC) is reportedly the third most frequent cause of global cancer-related deaths, and the incidence of HCC is increasing worldwide [1, 2]. The clearly established risk factor for HCC is chronic hepatitis C virus (HCV) infection [3].

To date, both ultrasonography and serum tumor markers such as the alpha fetoprotein (AFP), and des-γ-carboxy prothrombin (DCP) assay are the principle methods for screening and detecting HCC. Routine screening is the best method to detect early HCC and improve patient survival; however, elevated serum AFP and DCP levels have insufficient sensitivity and specificity, respectively. The sensitivity and specificity of serum elevated AFP levels were reported to range from 39–64% and 76–91%, while those of the serum elevated DCP levels were 41–77% and 72–98%, respectively [4–9]. In addition, it was recently reported that only a small percentage of small HCC tumors were diagnosed based on AFP and DCP [6, 10]. The lens culinaris agglutinin-reactive fraction of AFP (lectin-bound AFP or AFP-L3) has been reported to be elevated in the serum of HCC patients. Although AFP-L3 has a high range of specificity for detecting HCC, the sensitivity is low [11, 12]. The ability to detect early HCC, prior to the onset of clinical symptoms, leads to curative treatment and significantly improves the disease prognosis. Thus, additional biochemical markers are necessary for the specific detection of early HCC.

Serum profiling using a proteomic approach is thought to be a useful technique to detect or predict early HCC in chronic liver disease patients. Studies using the ProteinChip SELDI system, which is a powerful tool to discover new biomarkers, have shown that this method may be successfully used to diagnose HCC. Zinkin et al. [13], Schwegler et al. [14] and our research group [15] previously detected early HCC using the profile of several protein peaks that were identified by the ProteinChip SELDI system. Paradis et al. [16] reported the highest discriminating peak (8900 Da), which was identified as the V10 fragment of vitronectin. Furthermore, Lee et al. [17] described complement 3a, which had a molecular weight of approximately 8900 Da, as a novel marker of HCC. Therefore, using this proteomic approach to identify specific proteins may not only help establish simple methods to detect HCC, but also further our understanding of the molecular mechanisms of hepatocarcinogenesis and facilitate the development of novel cancer therapies. Therefore, this study assessed and compared the protein expression profiles in the sera of HCC patients in order to identify a more useful biomarker of HCC-associated HCV infection using proteomic approach.

Table 1 Patient characteristics

	HCC[a]	CLD[b]	p value
Patients (male/female)	45 (40/5)	42 (40/2)	–
Age	73.6 [63–85]	61.8 [41–83]	<0.0001
PLT[c] ($\times 10^4$/ul)	12.5 ± 5.8	8.4 ± 4.6	0.001
Albumin (g/dl)	3.8 ± 0.8	4.2 ± 1.6	0.8
ALT[d] (IU/l)	57.7 ± 28.3	52.8 ± 37.5	0.7
AFP[e] (ng/ml)	311 ± 1144	51.6 ± 36.1 (38)	0.008
DCP[f] (mAU/ml)	235 ± 605 (44)	37.1 ± 59.8 (39)	<0.0001
HA[g] (ng/ml)	388 ± 446 (40)	280 ± 272 (27)	0.6
Diameter of HCC (mm)	23.2 [10–40]	–	–
TNM stage[h] (I/II/III/IV)	24/18/3/0	–	–

Data are shown as the means ± SD or means [range] (numbers)

[a] Hepatocellular carcinoma

[b] Chronic liver disease

[c] Platelet counts

[d] Alanine aminotransferase

[e] Alpha fetoprotein

[f] Des-γ-carboxy prothrombin

[g] Hyaluronic acid

[h] TNM; primary tumor/lymph node/distant metastasis

Materials and methods

Samples

Eighty-seven patients [45 HCC patients and 42 patients with chronic liver diseases without HCC (CLD)] with HCV infection were selected to participate in this study (Table 1). These patients provided informed consent. Serum samples were collected by the Faculty of Medicine, University of Miyazaki (Miyazaki, Japan), and some patients were in a hyperendemic HCV area with a cohort study in Miyazaki [18]. The sera of all patients with and without HCC, which was confirmed by abdominal ultrasonography or computed tomography, were obtained prior to treatment. All of the sera samples from HCV-infected patients were analyzed in a previous study [15]. In addition, sera from 10 HCV-HCC patients who were diagnosed with HCC within 1 or 2 years and sera from five patients who had received radiofrequency ablation (RFA), percutaneous ethanol injection therapy (PEIT) and/or transarterial chemoembolization (TACE) for HCC were collected through a cohort study in Miyazaki. We also analyzed the sera of 21 healthy volunteers without HCC as controls. After freezing and thawing once, all samples were separated into 50–100 μl aliquots and refrozen at −80°C. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Miyazaki, Kagoshima University Graduate School of Medical and Dental Sciences, and Harvard School of Public Health and Boston University School of Public Health.

SELDI-TOF/MS analysis of sera

Expression difference mapping analysis profiles of the samples were obtained using weak cation-exchange (CM10) ProteinChip Arrays (Bio-Rad Laboratories). Arrays were analyzed by ProteinChip reader as previously reported [15]. In addition, the laser intensity ranged from 220 to 245, with a detector sensitivity of 8, and spectra ranging from 1300 to 150000 m/z were selected for analysis in this study.

Separation of candidate biomarker (8.1 k m/z)

The purification strategy was determined by the ProteinChip Arrays. Two hundred microliters of sera from HCV-HCC patients were diluted 5-fold into 50 mM Na-phosphate buffer, pH 7.0, and loaded onto a CM-Ceramic HyperD F spin column (Bio-Rad Laboratories). After equilibrating with the same buffer, the samples were eluted with a stepwise sodium chloride gradient from 0, 200, 300, and 1000 mM. The elution was desalinated and concentrated using a centrifugal concentrator (VIVASPIN, Vivascience, Hannover, Germany), and the purification progress was monitored using NP20 arrays. The flow-through fraction was dialyzed and then separated by 16.5% tricine one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The SDS–PAGE samples were run in tricine sodium dodecyl sulfate buffer according to the manufacturer's instructions and then stained with Coomassie brilliant blue (CBB).

Identification of the candidate biomarker (8.1 k m/z)

Gel pieces containing the target 8.1 k m/z protein were excised. The excised bands were reduced and alkylated for 30 min at room temperature, and then digested with trypsin (Modified Sequence Grade, Roche Diagnostics, Basel, Switzerland) in Tris–HCl, pH 8.0, for 20 h at 35°. The reaction solution was applied to NP20 arrays and allowed to air dry. To identify the protein, the digested peptides were purified by high-performance liquid chromatography (HPLC; MAGIC 2002; Michrom Bioresources Inc., Auburn, CA) and analyzed by Q-Tof2 (Micromass; Waters Ltd., Hertsfordshire, UK). The HPLC solvent consisted of solvent A (2% acetonitlile/0.1% formic acid) and B (90% acetonitlile/0.1% formic acid). The digested peptides were separated with a linear gradient from 10 to 50% solvent B with a flow rate of 400 nl/min using HPLC [19]. Mass spectral data were searched with Mascot (http://www.matrixscience.com) to identify proteins based on the peptide mass [20, 21].

Immunodepletion assay

For immunodepletion, serum samples were prepared as follows. Sera (250 μl) from HCC patients were diluted 5-fold in 50 mM Tris–HCl buffer, pH 8.0, and loaded onto a CM-Sepharose Fast Flow spin column (GE Healthcare Bio-Sciences Corp., NJ). After equilibration with the same buffer, the samples were eluted with a stepwise sodium chloride gradient from 0, 500, and 1000 mM. The elution from each NaCl concentration was monitored using NP20 arrays. To prepare the antibodies for immunodepletion, 6 μl anti-human C3 antibody, which detected C3 and C3a expression, or anti-C4a antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 20 μl Interaction Discovery Mapping (IDM) affinity beads (Bio-Rad Laboratories) and Protein A (Sigma Chemical Co, St. Louis, MO) over night at 4° with shaking. These beads were centrifuged, and the supernatant was discarded. The beads were washed with 50 mM phosphate buffer (pH 7.0), and 3 μl of the prepared serum sample was incubated with 15 μl IDM affinity beads with shaking for 2 h at 4°. As a negative control, 3 μl sample was incubated with IDM affinity beads and Protein A with an anti-C4a antibody or without antibody. After the incubation, the samples were cleared by centrifugation, and 5 μl of each supernatant was analyzed on NP20 ProteinChip arrays in a PBS II reader.

Cell culture and SELDI-TOF/MS analysis of culture supernatants

The human hepatocarcinoma cell line HuH-7 and human hepatoblastoma cell line HepG2 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin G, and 100 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA). Before starting the experiments, the cells were cultured on 96-well microplates in medium without FBS for 24 h. After washing with FBS-free media, the cells were cultured for 24 h with FBS-free media with or without 500 μg/ml of C3a (Calbiochem, San Diego, CA). The supernatants were collected by centrifugation and analyzed for the expression of 8.1 k m/z using the ProteinChip system.

Statistical analysis

Values are shown as the means ± SD. Statistical differences, including laboratory data and individual peaks in SELDI TOF/MS, were determined using the Mann–Whitney U test. Values of $p < 0.05$ were considered statistically significant. The discriminatory power for each putative marker was described via receiver operating characteristics

(ROC) area under the curve (AUC). These statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA), SPSS software (SPSS Inc., Chicago, IL), JMP software, or Ciphergen ProteinChip Software, version 3.0.2.

Results

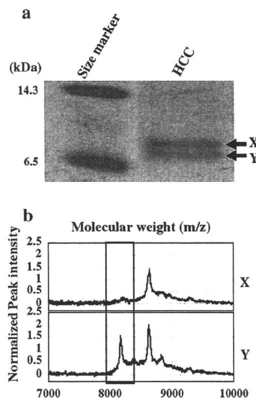
Profiling sera from HCC patients and healthy controls

We analyzed the sera of all patients with HCV-HCC or HCV-CLD and healthy controls without HCC using the CM10 ProteinChip array to identity the most differential protein peak. Peaks were automatically detected using the Ciphergen ProteinChip Software 3.0.2. following baseline subtraction as described previously [15, 22]. This analysis identified 178 protein peak clusters, as seen in the spectrum representations from the three groups (HCV-HCC, HCV-CLD, and healthy control) in the 3000- to 15000-m/z range. Peak expressions were increased for 18 proteins and decreased for 14 proteins in sera from HCV-HCC patients compared to HCV-CLD patients. Compared to healthy subjects, 68 protein peaks were increased, and 16 protein peak intensities were decreased in the sera of HCV-HCC patients. Five protein peaks (4067, 4470, 7564, 7929, and 8130 m/z) had a p-value less than 1×10^{-7} and were significantly increased in the sera of HCC patients compared to the sera of HCV-CLD patients and healthy volunteers. In particular, an 8130 m/z peak was the most significantly different peak and had the most differential expression profile between patients with HCV-HCC and with HCV-CLD.

Purification and identification of the 8.1 k m/z peak

We optimized the adsorption and desorption conditions on the arrays using an HCV-HCC patient serum sample and healthy volunteer serum sample in order to determine a procedure to purify the target 8.1 k m/z protein. The optimal pH for retention of the 8.1 k m/z protein was a *pI* value of approximately 7.0 on the CM10 arrays, which indicates that weak cation-exchange sorbents and buffer pH should be fixed for further experiments. The target protein was eluted by increasing the sodium chloride concentrations in a Na-phosphate buffer and was eluted in the 1000 mM sodium chloride fraction. The concentrated serum protein that was eluted with 1000 mM sodium chloride was applied to SDS–PAGE for further separation. The 8.1 k m/z protein was identified and excised by in-gel trypsin digestion for identification. The peptide sequences were analyzed using liquid chromatography (LC)-MS/MS and then examined by a database search with Mascot. The digested peptides matched human complement C3a (Fig. 1).

After reacting the HCC sera with anti-complement C3a or anti-C4 antibodies or without antibody, the supernatants were analyzed by the SELDI ProteinChip system for immunodepletion. Analysis of the supernatant showed that only the 8.1 k m/z peak corresponding to complement C3a



c **Complement component 3:**
Total score: 70, Peptides matched: 2

FISLGEACK , Start – End Sequence: 713 - 721

FISLGEACKK , Start – End Sequence: 713 - 722

Fig. 1 **a** Partially purified proteins were separated by SDS–PAGE using serum samples from HCV-HCC patients. The Coomassie-stained SDS-PAGE gel shows two clear bands at approximately 8 kDa (*X* and *Y*). **b** After each band (*X* and *Y*) was excised from the gel, the proteins were extracted and analyzed using the ProteinChip system. The target protein in the excised band was detected, and the 8.1 k m/z peak corresponded only to the "*Y*" band contained in gel. **c** The excised "*Y*" band was alkylated and digested using trypsin. The peptides were collected and subjected to LC–MS/MS analysis. The proteins, which were derived from complement C3a, were identified using a database search

was reduced. On the other hand, immunodepletion with a control anti-C4 antibody or without antibody did not reduce the 8.1 k m/z peak (Fig. 2).

Profiling the C3a of sera from patients with HCC and without HCC

The 8.1 k m/z peak was confirmed as the complement C3a fragment using an immunodepletion assay. However, C3a was stabilized as C3adesArg with a molecular weight of approximately 8.9 k m/z. Figure 3a, b compares the expression of the 8.1 k m/z peak in the sera of HCV-HCC or HCV-CLD patients and healthy controls. The intensities

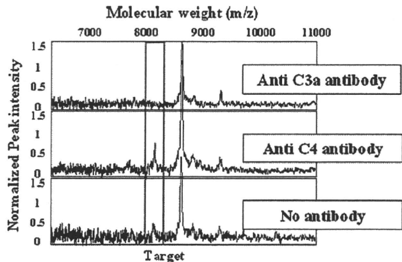


Fig. 2 Immunodepletion assay of the C3a fragment. Analysis of supernatant that had been immunodepleted with an anti-C3a antibody showed that only the 8.1 k m/z peak corresponding to complement C3a was reduced. Supernatants that had been immunodepleted with either a control anti-C4 antibody or without antibody did not have reduced 8.1 k m/z peaks by the ProteinChip system

in HCC patient sera were significantly higher than those in the HCV-CLD patients or healthy controls. The expression of the 8.9 k m/z peak in HCV-HCC patients was also higher than that in HCV-CLD patients or healthy controls (Fig. 3c, d). Although the 8.9 k m/z peak was not identified as C3adesArg, it is possible that both the 8.1 and 8.9 k m/z peaks were specific tumor markers for HCC. Furthermore, we analyzed sera from 10 HCV-HCC patients who were diagnosed with HCC within 1 or 2 years and sera from five patients who had received curative treatments using RFA, PEIT, and TACE for HCC. The 8.1 k m/z C3a fragment in the HCV-HCC patients was significantly increased in the year of disease onset compared to the pre-onset year. After treatment, expression of the C3a fragment significantly decreased in all five of the patients who had measurable samples after treatment (Fig. 4a). In contrast, the 8.9 k m/z peak did not change regardless of the occurrence of HCC over time (Fig. 4b). Thus, the 8.1 k m/z C3a fragment appears to be the most discriminatory tumor marker for HCV-HCC.

Relationship between the C3a fragment and other tumor markers

AFP and DCP levels were measured in sera from 83 of 87 patients with HCV-associated liver disease. The recommended cutoff levels for these tumor markers, AFP and DCP, are 20 ng/ml and 40 mAU/ml, respectively. AFP-L3 in 26 patients with HCV-associated liver disease was also investigated among measurable samples in which AFP in a total 35 patients was higher than 20 ng/ml. The cutoff level of AFP-L3 was set at 10%. When samples from patients

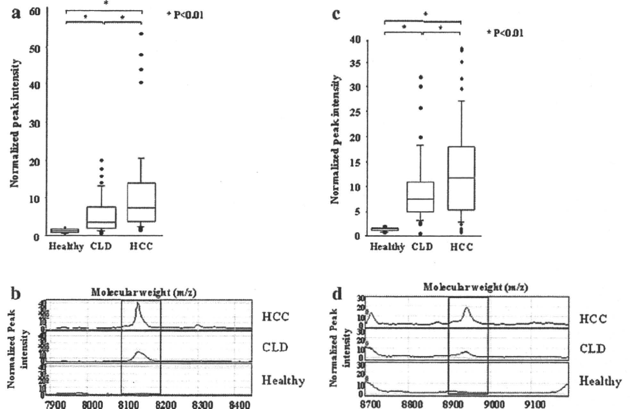


Fig. 3 **a** and **c** Comparisons of the expression profiles of the 8.1 and 8.9 k m/z peaks in HCV-HCC, HCV-CLD, and healthy sera. *Boxes* indicate the median ± 25th percentile. The *lower* and *upper bars* represent the 10th and 90th percentiles, respectively. **b** and **d** Representative spectra of the 8.1 and 8.9 k m/z peaks from patients in each group. The *horizontal axis* indicates the protein molecular weight, while the *vertical axis* designates the relative intensity

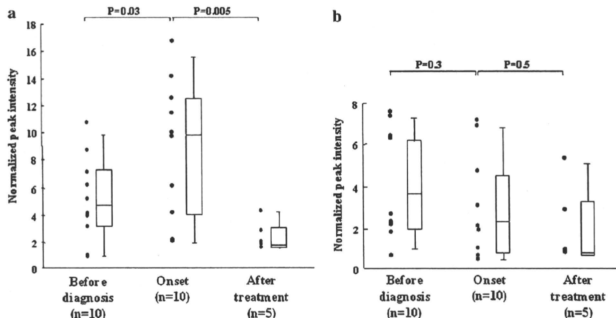


Fig. 4 Comparisons of the expression profiles of the 8.1 k m/z (**a**) and 8.9 k m/z (**b**) peaks in sera from HCV-HCC patients before diagnosis, during disease onset, and after treatment. The samples in the before diagnosis group included sera collected 1 or 2 years before the onset of HCC. *Boxes* indicate the median ± 25th percentile, the *lower bar* indicates the 10th percentile and the *upper bar* indicates the 90th percentile

Table 2 Diagnostic rates for hepatocellular carcinoma in the HCV infected patients

Markers	Sensitivity (%)	Specificity (%)	ROC AUC
AFP[a] (>20 ng/ml)	38 (17/45)	47 (18/38)	0.53
DCP[b] (>40 mAU/ml)	45 (20/44)	74 (29/39)	0.68
AFP-L3[c] (>10%)	58 (8/14)	50 (6/12)	0.58
C3a fragment (>3.5)	78 (37/45)	52 (22/42)	0.70
C3a fragment + AFP	91 (41/45)	26 (10/38)	0.72
C3a fragment + DCP	93 (41/44)	33 (13/39)	0.77
AFP + DCP	64 (28/44)	34 (12/35)	0.70
C3a fragment + AFP + DCP	98 (43/44)	20 (7/35)	0.80

[a] Alpha fetoprotein

[b] Des-γ-carboxy prothrombin

[c] Alpha fetoprotein, lectin lens culinaris agglutin-bound fraction

with HCV-HCC and HCV-CLD without HCC were compared, the sensitivity and specificity of AFP were 38 and 47%, whereas those of DCP were 45 and 74% and those of AFP-L3 were 58 and 50%, respectively. When the cutoff level for the relative intensity of the C3a fragment was set at 3.5, the sensitivity and specificity were 78 and 52%, respectively; the C3a fragment had the most sensitivity for the diagnosis of HCC. Furthermore, the ROC AUC of the C3a fragment, AFP, DCP, and AFP-L3 was 0.70, 0.53, 0.68, and 0.58, respectively (Table 2). There was no relationship between the C3a fragment and several other tumor and inflammation markers [AFP, DCP, AFP-L3, alanine aminotransferase (ALT), and high-sensitivity C-reactive protein (hs-CRP)], and each of these markers was independent of the diameter and number of tumors. The ROC AUC using AFP and DCP was highly similar to the ROC AUC with the C3a fragment alone. In addition, we investigated a combination assay that included the C3a fragment, AFP and DCP. This combination test, in which at least AFP, DCP, or the C3a fragment was positive, had a positive identification rate of 98%, although the specificity of this assay was too low at 20%. The ROC AUC of the combination test using AFP, DCP, and the C3a fragment was higher than those of any other markers. This result indicates that this combination assay using three markers is more useful than the combination assay using AFP ± DCP, which are measured worldwide to detect HCC (Table 2).

Profiling C3a expression in culture medium

C3a reacted with HCC cell lines, and the C3a peak in the culture medium was monitored by the ProteinChip system. The C3a fragment (approximately 8.1 k m/z) was not detected in the supernatants of HuH-7 and HepG2 cell cultures. However, the 8.9 k m/z peak was detected in the culture medium. This 8.9 k m/z peak was considered to be a stabilized form of C3a. This result indicated that the stabilized form of C3a (8.9 k m/z) was not undergoing proteasome-mediated degradation to yield the C3a fragment (8.1 k m/z) in these HCC cell lines.

Discussion

Because the HCC disease-associated mortality rate remains high, it is highly important to develop early diagnostic tools and treatments for HCC. Our study indicates that an 8.1 k m/z peak, which was identified as the C3a fragment by both peptide sequencing and an immunoassay, is upregulated in the serum of HCC patients, 93% (42/45) of whom were TNM stage I or II. The C3a fragment in some HCC cases was also significantly higher in the year of HCC onset compared to the pre-onset year and decreased after curative treatment. Therefore, the C3a fragment appears to

be a promising simple tumor marker for the diagnosis of early HCC. In addition, a combination serum HCC diagnostic test that included AFP, DCP, and the C3a fragment had higher sensitivity than each individual marker. These results suggest that this combination test may be a useful HCC screening method, although the low specificity may pose challenges. Further examinations are needed to determine whether the C3a fragment or a combination test can be used to detect early HCC.

The results of our study demonstrated that the C3a fragment (8.1 k m/z) is a highly expressed novel tumor marker that is abundant in the sera of early HCC patients but not in the sera of healthy volunteers or HCV-CLD patients. A similar study by Lee et al. [17] used the ProteinChip SELDI system to show that C3a is a potential candidate biomarker for HCV-HCC. However, Lee et al. found that the molecular weight of C3a was represented by an approximately 8.9 k m/z peak. C3a has a very short half-life and is immediately cleaved into the more stable C3adesArg (8.9 k m/z), which is the anaphylatoxin C3a that lacks the C-terminal arginine and is stable state in the serum [23]. In our study, the 8.9 k m/z peak was also significantly different among HCV-HCC patients, HCV-CLD patients, and healthy volunteers (Fig. 3c, d). However, the discriminatory power of the 8.9 k m/z peak (ROC AUC was 0.60) was lower than the 8.1 k m/z peak (ROC AUC was 0.70) to distinguish between HCV-HCC and HCV-CLD. In addition, unlike the 8.1 k m/z peak, the levels of the 8.9 k m/z peak did not significantly increase with time as HCC progressed in 10 HCV-HCC cases (Fig. 4b). In contrast, Li et al. identified two proteins (8926 m/z and 8116 m/z) as complement component C3adesArg and a C-terminal truncated form of C3adesArg; the latter was a C-terminal truncation of C3adesArg that lacked the C-terminal sequence RASHLGLA (referred to as C3adesArgΔ8) in breast cancer patients [24]. However, these two biomarkers cannot be used to discriminate between breast cancers and benign tumors, and there were minimal differences in the peak intensities between breast cancer patients and healthy controls. Therefore, the C3a fragment with a molecular weight of 8.1 k m/z appears to be a potential diagnostic marker for HCC, although we cannot explain why the 8.1 k m/z fragment of C3a is overexpressed in HCC patients and did not confirm whether our C3a fragment (8.1 k m/z) is C3adesArgΔ8.

C3a, including C3adesArg, was also previously identified as a tumor marker for lymphoid malignancies, breast and colorectal cancers using the ProteinChip SELDI system [24–26]. Complement activation and subsequent deposition of complement components on tumor tissues has been demonstrated in cancer patients [27]. Malignant ovarian cells isolated from ascitic fluid samples had C3 activation products deposited on their cell surface [28]. Complement components are important mediators of inflammation and help regulate the immune response. C3a is biologically active and binds to mast cells and basophils, triggering the release of their vasoactive contents [29]. We investigated C3a expression by immunochemical examination of HCC tissues and Western blot analysis of proteins extracted from human HCC cell lines, including HepG2 and HuH-7. However, specific C3a expression, including the C3a fragment (8.1 k m/z), was not detected.

The complement system can be activated after exposure to tumor antigens [30]. It is speculated that small tumors can trigger a systematic reaction. Therefore, elevated C3a (8.9 k m/z) levels in the serum of HCV-HCC patients may reflect both a systematic immune response to HCV infection and non-specific tumor antigens rather than a specific immune response to HCC [24–26, 31]. In contrast, it is possible that overexpression of the C3a fragment (8.1 k m/z) is specific for HCC in addition to non-specific C3 activation.

In contrast to our results, Steel et al. [32] searched for HCC biomarkers using HCC-associated HBV-infected patient sera and found that the C-terminal fragment of complement C3 was down-regulated. Kawakami et al. [33] searched for characteristic alterations in the sera of HBV- and HCV-HCC-infected patients who had undergone curative radiofrequency ablation treatment and showed that C3 was up-regulated after treatment. In these studies, C3 was separated and identified using 2-DE of a mixture of proteins from a small number of patient sera samples, and this process identified various molecular weights for C3. In addition, we analyzed the sera of 25 patients with HCC-associated HBV infections, and the profile of several proteins was different between HCV- and HBV-infected patients. Although 35 protein peaks, including the C3a fragment, were overexpressed in the sera of both HCV-HCC and HBV-HCC patients compared to sera from healthy volunteers, the C3a fragment (8.1 k m/z) was particularly overexpressed in the sera of HCV-HCC patients and was not significantly different between HBV-HCC patients and HCV-CLD patients without HCC (data not shown). The biologic and pathogenic activities of HCV and HBV are different, and the molecular mechanisms underlying the development of hepatitis and hepatocarcinogenesis may differ between HBV and HCV infections [34–36]. Although the number of samples, cause of liver disease, and method of protein identification may affect these results, we speculate that the C3a fragment with a molecular weight of 8.1 k m/z is a candidate tumor marker for HCV-HCC but not HBV-HCC.

AFP, which is a commonly used HCC tumor marker, is elevated not only during HCC, but also during hepatocyte regeneration following liver damage. Previous reports revealed that AFP was abnormally elevated in the sera of patients with acute hepatitis, chronic hepatitis, and liver

cirrhosis. This lack of specificity for HCC means that AFP has a comparatively high false-positive rate [37]. The C3a fragment may also be elevated during hepatocyte regeneration following liver damage [38], and early diagnosis of small HCC tumors may be difficult with one marker alone. Therefore, the false-positive rates for HCC must be carefully considered [39–41]. Also, a combination of markers, including AFP, DCP, and the C3a fragment, in the serum should be verified to improve the diagnostic rate.

The ProteinChip SELDI system can separate and partially characterize multiple proteins in tissue and serum samples. Our previous report used a panel of proteins to diagnose early HCC with the ProteinChip SELDI system [15]. This panel diagnosis of seven protein peaks included a discriminant peak of 4060 m/z. This 4060 m/z peak may be a double-charged 8130 m/z peak, although the C3a fragment (8130 m/z) was not used to develop this diagnostic method. These results suggest that the C3a fragment is a useful HCC biomarker, regardless of whether this fragment carries a single or double charge. In addition, the panel diagnosis method is more useful than measuring the C3a fragment alone to diagnose and predict the occurrence of HCC. However, this method must be performed using the ProteinChip SELDI system, which is expensive and does not detect putative interactions between various proteins. Identifying a specific HCC protein such as the C3a fragment will also further our understanding of the molecular mechanisms of hepatocarcinogenesis. Therefore, the C3a fragment should not only be considered a simple HCC tumor marker, but should also be evaluated for its contribution to HCC carcinogenesis.

In conclusions, serum profiling with the ProteinChip SELDI system may be used to distinguish HCC from chronic liver disease without HCC and to detect early HCC in HCV-infected patients. Because we identified the C3a fragment (8.1 k m/z) in serum samples from HCC patients, the C3a fragment is a promising marker that can be used to screen for HCV-HCC and to develop new therapeutic targets.

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References

1. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med. 1999;340:745–50.
2. Robert GG. Hepatocellular carcinoma: overcoming challenges in disease management. Clin Gastroenterol Hepatol. 2006;4:252–61.
3. Okuda K. Hepatocellular carcinoma. J Hepatol. 2000;32:225–37.
4. Oka H, Tamori A, Kuroki T, Kobayashi K, Yamamoto S. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. Hepatology. 1994;19:61–6.
5. Ishii M, Gama H, Chida N, Ueno Y, Shinzawa H, Takagi T, et al. Simultaneous measurements of serum alpha-fetoprotein and protein induced by vitamin K absence for detecting hepatocellular carcinoma. South Tohoku District Study Group. Am J Gastroenterol. 2000;95:1036–40.
6. Okuda H, Nakanishi T, Takatsu K, Saito A, Hayashi N, Takasaki K, et al. Serum levels of des-gamma-carboxy prothrombin measured using the revised enzyme immunoassay kit with increased sensitivity in relation to clinicopathologic features of solitary hepatocellular carcinoma. Cancer. 2000;88:544–9.
7. Grazi GL, Mazziotti A, Legnani C, Jovine E, Miniero R, Gallucci A, et al. The role of tumor markers in the diagnosis of hepatocellular carcinoma, with special reference to the des-gamma-carboxy prothrombin. Liver Transpl Surg. 1995;1:249–55.
8. Wang CS, Lin CL, Lee HC, Chen KY, Chiang MF, Chen HS, et al. Usefulness of serum des-gamma-carboxy prothrombin in detection of hepatocellular carcinoma. World J Gastroenterol. 2005;11:6115–9.
9. Marrero JA, Su GL, Wei W, Emick D, Conjeevaram HS, Fontana RJ, et al. Des-gamma carboxyprothrombin can differentiate hepatocellular carcinoma from nonmalignant chronic liver disease in American patients. Hepatology. 2003;37:1114–21.
10. Mita Y, Aoyagi Y, Yanagi M, Suda T, Suzuki Y, Asakura H. The usefulness of determining des-gamma-carboxy prothrombin by sensitive enzyme immunoassay in the early diagnosis of patients with hepatocellular carcinoma. Cancer. 1998;82:1643–8.
11. Taketa K, Okada S, Win N, Hlaing NK, Wind KM. Evaluation of tumor markers for the detection of hepatocellular carcinoma in Yangon General Hospital, Myanmar. Acta Med Okayama. 2002;56:317–20.
12. Khien VV, Mao HV, Chinh TT, Ha PT, Bang MH, Lac BV, et al. Clinical evaluation of lentil lectin-reactive alpha-fetoprotein-L3 in histology-proven hepatocellular carcinoma. Int J Biol Markers. 2001;16:105–11.
13. Zinkin NT, Grall F, Bhaskar K, Otu HH, Spentzos D, Kalmowitz B, et al. Serum proteomics and biomarkers in hepatocellular carcinoma and chronic liver disease. Clin Cancer Res. 2008;14:470–7.
14. Schwegler EE, Cazares L, Steel LF, Adam BL, Johnson DA, Semmes OJ, et al. SELDI-TOF MS profiling of serum for detection of the progression of chronic hepatitis C to hepatocellular carcinoma. Hepatology. 2005;41:634–42.
15. Kanmura S, Uto H, Kusumoto K, Ishida Y, Hasuike S, Nagata K, et al. Early diagnostic potential for hepatocellular carcinoma using the SELDI ProteinChip system. Hepatology. 2007;45:948–56.
16. Paradis V, Degos F, Dargère D, Pham N, Belghiti J, Degott C, et al. Identification of a new marker of hepatocellular carcinoma by serum protein profiling of patients with chronic liver diseases. Hepatology. 2005;41:40–7.
17. Lee IN, Chen CH, Sheu JC, Lee HS, Huang GT, Chen DS, et al. Identification of complement C3a as a candidate biomarker in human chronic hepatitis C and HCV-related hepatocellular carcinoma using a proteomics approach. Proteomics. 2006;6:2865–73.
18. Uto H, Hayashi K, Kusumoto K, Hasuike S, Nagata K, Kodama M, et al. Spontaneous elimination of hepatitis C virus RNA in individuals with persistent infection in a hyperendemic area of Japan. Hepatol Res. 2006;34:28–34.
19. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem. 1996;68:850–8.
20. Prahalad AK, Hickey RJ, Huang J, Hoelz DJ, Dobrolecki L, Murthy S, et al. Serum proteome profiles identifies parathyroid hormone physiologic response. Proteomics. 2006;6:3482–93.

21. Shiwa M, Nishimura Y, Wakatabe R, Fukawa A, Arikuni H, Ota H, et al. Rapid discovery and identification of a tissue-specific tumor biomarker from 39 human cancer cell lines using the SELDI ProteinChip platform. Biochem Biophys Res Commun. 2003;309:18–25.
22. Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, et al. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. Cancer Res. 2002;62: 3609–14.
23. Sahu A, Lambris JD. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. Immunol Rev. 2001;180:35–48.
24. Miguet L, Bogumil R, Decloquement P, Herbrecht R, Potier N, Mauvieux L, et al. Discovery and identification of potential biomarkers in a prospective study of chronic lymphoid malignancies using SELDI-TOF-MS. J Proteome Res. 2006;5:2258–69.
25. Ward DG, Suggett N, Cheng Y, Wei W, Johnson H, Billingham LJ, et al. Identification of serum biomarkers for colon cancer by proteomic analysis. Br J Cancer. 2006;94:1898–905.
26. Li J, Orlandi R, White CN, Rosenzweig J, Zhao J, Seregni E, et al. Independent validation of candidate breast cancer serum biomarkers identified by mass spectrometry. Clin Chem. 2005;51:2229–35.
27. Jurianz K, Ziegler S, Garcia-Schüler H, Kraus S, Bohana-Kashtan O, Fishelson Z, et al. Complement resistance of tumor cells: basal and induced mechanisms. Mol Immunol. 1999;36:929–39.
28. Bjørge L, Hakulinen J, Vintermyr OK, Jarva H, Jensen TS, Iversen OE, et al. Ascitic complement system in ovarian cancer. Br J Cancer. 2005;92:895–905.
29. Mollnes TE, Garred P, Bergseth G. Effect of time, temperature and anticoagulants on in vitro complement activation: consequences for collection and preservation of samples to be examined for complement activation. Clin Exp Immunol. 1988;73: 484–8.
30. Verhaegen H, De Cock W, De Cree J, Verbruggen F. Increase of serum complement levels in cancer patients with progressing tumors. Cancer. 1976;38:1608–13.
31. Habermann JK, Roblick UJ, Luke BT, Prieto DA, Finlay WJ, Podust VN, et al. Increased serum levels of complement C3a anaphylatoxin indicate the presence of colorectal tumors. Gastroenterology. 2006;131:1020–9.
32. Steel LF, Shumpert D, Trotter M, Seeholzer SH, Evans AA, London WT, et al. A strategy for the comparative analysis of serum proteomes for the discovery of biomarkers for hepatocellular carcinoma. Proteomics. 2003;3:601–9.
33. Kawakami T, Hoshida Y, Kanai F, Tanaka Y, Tateishi K, Ikenoue T, et al. Proteomic analysis of sera from hepatocellular carcinoma patients after radiofrequency ablation treatment. Proteomics. 2005;5:4287–95.
34. Honda M, Kaneko S, Kawai H, Shirota Y, Kobayashi K. Differential gene expression between chronic hepatitis B and C hepatic lesion. Gastroenterology. 2001;120:955–66.
35. Kim W, Oe Lim S, Kim JS, Ryu YH, Byeon JY, Kim HJ, et al. Comparison of proteome between hepatitis B virus- and hepatitis C virus-associated hepatocellular carcinoma. Clin Cancer Res. 2003;9:5493–500.
36. Koike K. Steatosis, liver injury, and hepatocarcinogenesis in hepatitis C viral infection. J Gastroenterol. 2009;44:82–8.
37. Lok AS, Lai CL. Alpha-fetoprotein monitoring in Chinese patients with chronic hepatitis B virus infection: role in the early detection of hepatocellular carcinoma. Hepatology. 1989;9:110–5.
38. Markiewski MM, Mastellos D, Tudoran R, DeAngelis RA, Strey CW, Franchini S, et al. C3a and C3b activation products of the third component of complement (C3) are critical for normal liver recovery after toxic injury. J Immunol. 2004;173:747–54.
39. Oka H, Kurioka N, Kim K, Kanno T, Kuroki T, Mizoguchi Y, et al. Prospective study of early detection of hepatocellular carcinoma in patients with cirrhosis. Hepatology. 1990;12:680–7.
40. Tanaka N, Horiuchi A, Yamaura T, Komatsu M, Tanaka E, Kiyosawa K. Efficacy and safety of 6-month iron reduction therapy in patients with hepatitis C virus-related cirrhosis: a pilot study. J Gastroenterol. 2007;42:49–55.
41. Tsamandas AC, Antonacopoulou A, Kalogeropoulou C, Tsota I, Zabakis P, Giannopoulou E, et al. Oval cell proliferation in cirrhosis in rats. An experimental study. Hepatol Res. 2007;37:755–64.

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NOTE

Presence of multiple copies of capsulation loci in invasive *Haemophilus influenzae* type b (Hib) strains in Japan before introduction of the Hib conjugate vaccine

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ABSTRACT

Despite the effectiveness of the Hib vaccine, multiple amplification of the *cap*b locus contributes to vaccine failure. However, there has been no report on the effect of Hib locus amplification in Japan. We examined 24 Hib strains from Japanese children with invasive diseases due to Hib. Although all strains showed the same *cap*b sequence, Southern blot analysis showed that four strains (16.7%) harbored multiple copies (more than two) of the *cap*b locus. Careful analysis of the locus in circulating Hib strains is necessary now that the Hib vaccine has been introduced into Japan.

Key words capsular polysaccharide, *Haemophilus influenzae* type b, Hib conjugate vaccine.

Hib occasionally causes invasive bacterial diseases such as meningitis, epiglottitis and sepsis, especially among young children. Hib conjugate vaccines, which consist of capsule polysaccharide conjugated with carrier protein, are very effective and safe. Since the Hib conjugate vaccine was introduced in Europe and America in the 1990s, the incidence of invasive Hib disease has decreased dramatically in many countries (1). However, despite the efficacy of the Hib vaccine, an increased number of cases of the rare invasive Hib diseases (i.e. cases of true vaccine failure) have now been reported in Europe in fully vaccinated children (2–5). Although possibly contributory host factors such as lower avidity of the anti-Hib antibody are known to occur (6, 7), amplification of the capsulation locus may also have contributed to vaccine failure (8, 9).

Type b polysaccharide capsules, polymers of PRP, are cell-surface components that serve as major virulence factors against host defense mechanisms. The genes involved in Hib capsule expression are found within the *cap*b locus, an 18-kb DNA segment of the chromosome (10). Most invasive Hib strains contain a partial duplication of the *cap*b locus which consists of one intact copy of the locus, and a second copy with a 1.2-kb deletion region containing the *bex*A gene and an IS1016 insertion element that flanks the locus (10). Polysaccharide capsule production relates to the number of copies of the locus (11). Recently, Cerquetti *et al.* reported that amplification of the *cap*b locus to as many as three to five copies is associated with vaccine failure (8, 9). In addition, Schouls *et al.* found two variants of the capsular gene cluster, designated type I and type II, which were assessed by considerable sequence divergence in the *hcs*A and *hcs*B genes of the *capb* locus. They found that type I strains carry approximately twice as much capsular polysaccharide on the cell surface as type II strains (12).

In Japan, the Hib conjugate vaccine was licensed in January 2007, and introduced in December 2008; however, the vaccination plan has not yet been fully implemented. Although 55% of bacterial meningitis cases in children in Japan were caused by Hib (13), there has been no national

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List of Abbreviations: capsulation b, *capb*; CSF, cerebrospinal fluid; DIG, digoxigenin; Hib, *Haemophilus influenzae* type b; PFGE, pulsed-field gel electrophoresis; PRP, polymers of ribose ribitol phosphate.

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Table 1. Sequence type and number of copies of the *capb* locus of the 21 *Haemophilus influenzae* strains examined in this study

No. of cases	No. of strains	Detected date (Year/month)	Age (months)	specimen	disease	Ampicillin susceptibility	PFGE pattern	the *capb* locus		
								Sequence type	Size of band	No. of copies
1	C1650	2004/11	14	blood	bacteremia	R†	H	I	45 kb	2
2	K4646	2005/7	9	blood	meningitis	R	G	I	81 kb	4
3	K5003	2005/11	53	blood	meningitis	S‡	A1	I	45 kb	2
4	K5154	2006/1	17	CSF	meningitis	S	D	I	45 kb	2
5	K5221	2006/1	5	CSF	meningitis	S	B	I	45 kb	2
6	K5331	2006/2	24	CSF	meningitis	S	E	I	45 kb	2
7	K5545	2006/4	12	blood	cellulitis	-	A1	I	45 kb	2
8	K5625	2006/5	31	CSF	meningitis	R	F	I	45 kb	2
9	K5905	2006/9	19	CSF	meningitis	S	A1	I	45 kb	2
10	K6066	2006/11	7	CSF	meningitis	S	B	I	63 kb	3
11	K6168	2006/12	56	CSF	meningitis	R	B	I	45 kb	2
12	K6519	2007/8	20	CSF	meningitis	S	A1	I	45 kb	2
13	K6803	2007/10	29	blood	epiglottitis	S	A1	I	45 kb	2
14	K6886	2007/12	21	CSF	meningitis	S	A1	I	45 kb	2
15	K6892	2007/12	9	CSF	meningitis	R	A1	I	45 kb	2
16	K6930	2008/1	63	blood	bacteremia	R	A1	I	45 kb	2
17	K6934	2008/1	2	CSF	meningitis	R	A1	I	45 kb	2
18	K7112	2008/3	15	blood	meningitis	S	A1	I	45 kb	2
19	K7448	2008/7	8	CSF	meningitis	S	C	I	45 kb	2
20	K7450	2008/7	7	CSF	meningitis	S	A1	I	45 kb	2
21	K7522	2008/9	14	CSF	meningitis	S	A1	I	45 kb	2
22	K7639	2009/4	4	blood	meningitis	S	A2	I	81 kb	4
23	K7641	2009/4	12	CSF	meningitis	S	A1	I	45 kb	2
24	K7721	2009/5	4	blood	bacteremia	S	I	I	63 kb	3

†resistant, ‡susceptible.

survey of strains isolated from patients with invasive Hib diseases including meningitis. Furthermore, there are no reports on the amplification or sequence divergence of the *cap*b locus. The principle aim of this study was to analyze the number of *cap*b copies, and to assess sequence divergence in the *hcs*A and *hcs*B genes of Hib strains isolated from children with Hib diseases in our district before the introduction of the Hib conjugate vaccine.

A total of 24 Hib strains isolated between November 2004 and May 2009 from 24 children with invasive Hib diseases who had not received Hib conjugate vaccine in Kagoshima Prefecture, Japan, were collected and examined. Of these strains, 15 were isolated from CSF and 9 from blood. The strains were epidemiologically unrelated and individually stored at −80°C. All isolates were identified as serotype b by PCR capsular genotyping (14). PFGE was performed using a CHEF-DR 3 apparatus (Nippon Bio-Rad Laboratories, Tokyo, Japan) according to previously reported methodology (15). Briefly, DNA was digested by *Sma*I and separated on 1% agarose gels by PFGE under the following conditions: current range, 100 to 130 mA at 14°C for 16 hr; initial switch time, 5.3 s, linearly increasing to a final switch time of 49.9 s; angle, 120°; field strength, 6 volts/cm. The gels were stained with ethidium bromide and photographed. A lambda with a size range of 48.5 kb to 1 Mb (BME, Rockland, ME, USA) was used as a size marker. For interpretation of banding patterns separated by PFGE, we referred to the criteria of Tenover *et al.* (16).

Two variants of the *cap*b locus DNA sequence, type I and type II, were determined by PCR using two primer sets targeting the *hcs*A gene which could discriminate between the two capsular genotypes as described in a previous report (12). The DNA sequences of the PCR products were determined by an ABI Prism 310 sequencer (Applied Biosystems Japan, Tokyo, Japan).

The number of *cap*b locus copies was detected by Southern blotting analysis according to previously reported methods (8). Because *Kpn*I and *Sma*I restriction sites flank the *cap*b locus, extracted DNA in an agarose plug was digested with these enzymes, separated by PFGE, and transferred to a nylon membrane. A Hib capsule-specific 480-bp probe was constructed by PCR (14) and labeled with DIG using a DIG high prime DNA labeling kit (Roche Diagnostics, Mannheim, Germany). The membrane was hybridized with the probe and visualized by

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chemiluminescent detection using a DIG detection kit (Roche Diagnostics). The *Kpn* I/*Sma* I fragment of a two copy strain was expected to be 45-kb, because it includes two repeats of the locus (18 + 17 kb) plus additional segments (~10 kb) upstream and downstream of the *cap* region (17). Three-, four-, and five-copy fragments showed increased size in 18-kb increments for each additional copy (63, 81, and 99-kb, respectively) (8).

A summary of results is shown in Table 1. The type I-associated *hcs*A gene was found in all of the strains examined. The DNA sequences of all the PCR products were completely identical. PFGE analysis showed nine distinctive restriction patterns (A to I) among the 24 isolates. Fourteen strains with the A pattern were divided into A1 subtype (13 strains) and the closely-related A2 subtype (one strain). Southern blotting analysis demonstrated that 20 strains showed a two-copy arrangement of the *cap*b locus (45-kb), two strains showed three copies (63-kb), and the other two showed four copies (81-kb) (Fig. 1). The incidence of multiple-copy strains (>two copies) among examined strains was 16.7% (4/24). All of the strains with the dominant PFGE pattern (A1) possessed two copies, while one with the closely-related A2 subtype harbored four copies. The other three strains with multiple copies showed minor PFGE patterns (B, G or I). All the patients infected by strains with multiple copies were treated successfully without neurological or physical sequelae.

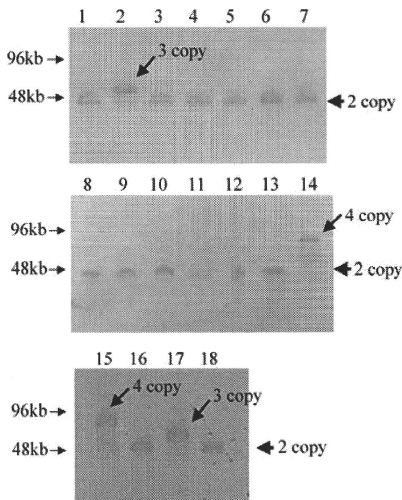

Fig. 1. Examples of Southern blot analysis of DNA from *Haemophilus influenzae* type b strains digested with KpnI/SmaI, separated by PFGE, and hybridized with the 480-bp DIG-labeled *cap*b probe. Strain K6066 in lane 2 and strain K7721 in lane 17 showed three-copy arrangement of the *cap*b locus (ca. 63-kb). Strain K4646 in lane 14 and K7639 in lane 15 had four-locus copies (ca. 81-kb). Other strains had two copies (ca. 45-kb).

Amplified *cap*b sequences were detected more frequently among strains from children with true vaccine failure than among those from unvaccinated children (24% vs. 10%) in the United Kingdom (8). Furthermore, the proportion of strains with multiple copies of the *cap*b locus increased over time in Italy (9). Amplification of the *cap*b locus is associated with decreased susceptibility to complement-mediated lysis and decreased complement-mediated opsonization (11). Thus, amplification of the *cap*b locus may result in the overcoming of host defenses and contribute to vaccine failure. We have found that Hib strains with multiple (three or four) copies of the *cap*b locus were present in Japan before the introduction of the Hib conjugate vaccine. The incidence of 16.7% (4/24) of multiple-copy strains found in our study is slightly higher than that found in the UK between 1991 and 1992 before routine immunization was introduced (10.1%, 9/89) (8). In our study, most of the multiple-copy strains showed rare PFGE patterns. Thus these strains might be selected and involved in vaccine failure after the introduction of Hib conjugate vaccination in Japan.

Sequence typing of the *capb* locus is based on the considerable sequence divergence in the *hcs*A and *hcs*B genes, which are involved in the transport of capsular polysaccharides across the outer membrane (18). Schouls *et al.* have reported that type II strains display less expression of capsular polysaccharide than do type I, and were isolated only during the pre-vaccination era in the Netherlands (12). The greater polysaccharide expression may have provided a selective advantage for type I strains, resulting in the rapid elimination of type II. In addition, there have been remarkable differences in the geographic distribution of type I and type II; with a higher incidence in the United States (73%) than the Netherlands (5%) of type II among Hib strains isolated from patients (12). While we did not find type II strains in this study, more Hib strains should be evaluated to clarify the exact incidence.

To our knowledge, this is the first study to investigate *cap*b locus copy number in invasive Hib strains isolated in Japan. We found that multiple-copy strains were in existence in Japan before the introduction of Hib conjugate vaccine. Molecular epidemiological surveillance of invasive Hib strains after the introduction of vaccines will allow prompt detection of any changes in bacterial properties. In addition, because higher antibody concentrations may be required to protect against Hib disease caused by strains with multiple copies of the *cap*b locus, we strongly

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recommend the complete implementation of Hib vaccination in young children in Japan.

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REFERENCES

1. Morris S.K., Moss W.J., Halsey N. (2008) *Haemophilus influenzae* type b conjugate vaccine use and effectiveness. *Lancet Infect Dis* **8:** 435–43.
2. Booy R., Heath P.T., Slack M.P., Begg N., Moxon E.R. (1997) Vaccine failures after primary immunisation with *Haemophilus influ*enzae type-b conjugate vaccine without booster. *Lancet* **349:** 1197–202.
3. Schouls L.M., Van Der Ende A., van de Pol I., Schot C., Spanjaard L., Vauterin P., Wilderbeek D., Witteveen S. (2005) Increase in genetic diversity of *Haemophilus influenzae* serotype b (Hib) strains after introduction of Hib vaccination in The Netherlands. *J Clin Microbiol* **43:** 2741–9.
4. Aracil B., Slack M., Perez-Vazquez M., Roman F., Ramsay M., Campos J. (2006) Molecular epidemiology of *Haemophilus influenzae* type b causing vaccine failures in the United Kingdom. *J Clin Microbiol* **44:** 1645–9.
5. Ramsay M.E., McVernon J., Andrews N.J., Heath P.T., Slack M.P. (2003) Estimating *Haemophilus influenzae* type b vaccine effectiveness in England and Wales by use of the screening method. *J Infect Dis* **188:** 481–5.
6. Breukels M.A., Jol-van der Zijde E., van Tol M.J., Rijkers G.T. (2002) Concentration and avidity of anti-*Haemophilus influ*enzae type b (Hib) antibodies in serum samples obtained from patients for whom Hib vaccination failed. *Clin Infect Dis* **34:** 191–7.
7. Lee Y.C., Kelly D.F., Yu L.M., Slack M.P., Booy R., Heath P.T., Siegrist C.A., Moxon R.E., Pollard A.J. (2008) *Haemophilus influenzae* type b vaccine failure in children is associated with inadequate production of high-quality antibody. *Clin Infect Dis* **46:** 186–92.
8. Cerquetti M., Cardines R., Ciofi Degli Atti M.L., Giufre M., Bella A., Sofia T., Mastrantonio P., Slack M. (2005) Presence of multiple copies of the capsulation b locus in invasive *Haemophilus influenzae* type b (Hib) strains isolated from children with Hib conjugate vaccine failure. *J Infect Dis* **192:** 819–23.
9. Cerquetti M., Cardines R., Giufre M., Sofia T., D'Ambrosio F., Mastrantonio P., Ciofi degli Atti M.L. (2006) Genetic diversity of invasive strains of *Haemophilus influenzae* type b before and after introduction of the conjugate vaccine in Italy. *Clin Infect Dis* **43:** 317–9.
10. Kroll J.S., Loynds B.M., Moxon E.R. (1991) The *Haemophilus influenzae* capsulation gene cluster: a compound transposon. *Mol Microbiol* **5:** 1549–60.
11. Noel G.J., Brittingham A., Granato A.A., Mosser D.M. (1996) Effect of amplification of the Cap b locus on complement-mediated bacteriolysis and opsonization of type b *Haemophilus influenzae. Infect Immun* **64:** 4769–75.
12. Schouls L., Van Der Heide H., Witteveen S., Zomer B., Van Der Ende A., Burger M., Schot C. (2008) Two variants among *Haemophilus influenzae* serotype b strains with distinct bcs4, hcsA and hcsB genes display differences in expression of the polysaccharide capsule. *BMC Microbiol* **8:** 35.
13. Sunakawa K., Ubukata K., Chiba N., Hasegawa K., Nonoyama M., Iwata S., Akita H., Sato Y. (2008) Childhood bacterial meningitis trends in Japan from 2005 to 2006. *Kansenshogaku Zasshi* **82:** 187–97.
14. Falla T.J., Crook D.W., Brophy L.N., Maskell D., Kroll J.S., Moxon E.R. (1994) PCR for capsular typing of *Haemophilus influenzae. J Clin Microbiol* **32:** 2382–6.
15. Saito M., Umeda A., Yoshida S. (1999) Subtyping of *Haemophilus influenzae* strains by pulsed-field gel electrophoresis. *J Clin Microbiol* **37:** 2142–7.
16. Tenover F.C., Arbeit R.D., Goering R.V., Mickelsen P.A., Murray B.E., Persing D.H., Swaminathan B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33:** 2233–9.
17. Corn P.G., Anders J., Takala A.K., Kayhty H., Hoiseth S.K. (1993) Genes involved in *Haemophilus influenzae* type b capsule expression are frequently amplified. *J Infect Dis* **167:** 356–64.
18. Sukupolvi-Petty S., Grass S., St Geme J.W. 3rd (2006) The *Haemophilus influenzae* Type b hcsA and hcsB gene products facilitate transport of capsular polysaccharide across the outer membrane and are essential for virulence. *J Bacteriol* **188:** 3870–7.

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