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# Investigation of simplified international diagnostic criteria for autoimmune hepatitis

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The simplified international diagnostic criteria for autoimmune hepatitis (AIH), re-revised by the International AIH Group in 2008, were investigated in 114 patients with AIH from 15 centers in Japan. While applying of the criteria, we had to pay attention to anti-nuclear antibody measurement methods, and liver histology scoring. Definite and probable AIH were diagnosed in 83 and 22 patients, respectively. The criteria were found to be useful for the diagnosis of AIH in Japan. However, 9 patients who did not meet the diagnostic criteria showed normal immunoglobulin G levels or were negative for autoantibodies. As the criteria were unreliable for diagnosing such atypical cases in the present series, we speculated that we should not rely solely on these, criteria and take a more holistic approach to diagnosis in such cases.

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# 検査値の読み方

連載

## 肺炎球菌による脾摘後重症感染症(OPSI)の1例

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### 緒言

C型慢性肝疾患において，血小板数が少ない患者では，インターフェロン(IFN)治療中にIFNの減量が必要になることが多く，ウイルス学的著効が得られにくい．近年，血小板数を増加させ，IFN治療効果を高める目的で脾臓摘出術が行われている．脾摘後は肺炎球菌敗血症のリスクが高まるため，ワクチン接種など適切な予防対策をとることが必要であるが，一般的にはあまり行われていない．肺炎球菌による脾摘後重症感染症(overwhelming postsplenectomy infection；OPSI)を生じた症例を経験したので，報告する．

### I．症例提示

**患　者**：59歳，女性

**既往歴**：35歳；子宮頸癌にて子宮・右卵巣摘出，その際，輸血．

**現病歴**：C型慢性肝炎治療目的のために2005年12月よりペグインターフェロン・リバビリン併用療法を開始．治療前血小板数が69,000/μlであり，治療後さらに低下し，50,000/μlを切るようになったため32週で中止となりウイルス学的著効には至らなかった．2006年9月，IFN再治療の際，血小板数を増加させるため腹腔鏡下脾臓摘出術施行．血小板数が157,000/μlと上昇したため2006年11月から2008年3月までペグインターフェロン・リバビリン併用療法を72週実施，ウイルス学的著効となった．その後，消化器科にて外来通院経過観察となっていた．

2009年3月X日より38℃台の発熱があり，翌日に嘔吐・下痢もみられ，ほとんど食事ができない状態となった．3日後，見当識障害と尿失禁が出現し立位困難となったため当院に救急搬送された．

**身体所見**：血圧138/79 mmHg，脈拍125/min，体温39.4℃，$SpO_2$ 99％(room air)，Glasgow Come Scale E4V3M6，眼球結膜異常なし，瞳孔および眼球運動異常なし，呼吸数14回/分，心雑音なし，腹壁軟，腸音減弱，病的皮疹

Key words：脾摘後重症感染症，肺炎球菌，C型慢性肝疾患，肺炎球菌ワクチン，インターフェロン治療

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表1　検査所見

| 血液 | | 尿 以外 | |
|---|---|---|---|
| WBC | 15,400/μl（好中球 94.0％） | Na | 126 mEq/l |
| Hb | 14.6 g/dl | K | 3.3 mEq/l |
| Plt | 55,000/μl | CRP | 25.83 mg/dl |
| AST | 124 IU/l | 尿 | |
| ALT | 62 IU/l | pH | 6.0 |
| LDH | 551 IU/l | RBC | 1～4/HPF |
| Alb | 3.5 g/dl | WBC | 0～1/HPF |
| BUN | 34 mg/dl | 硝子円柱（＋） | |
| Cre | 0.97 mg/dl | 顆粒円柱（2＋） | |
| Glu | 151 mg/dl | 蛋白（2＋） | |
| | | 糖（－） | |

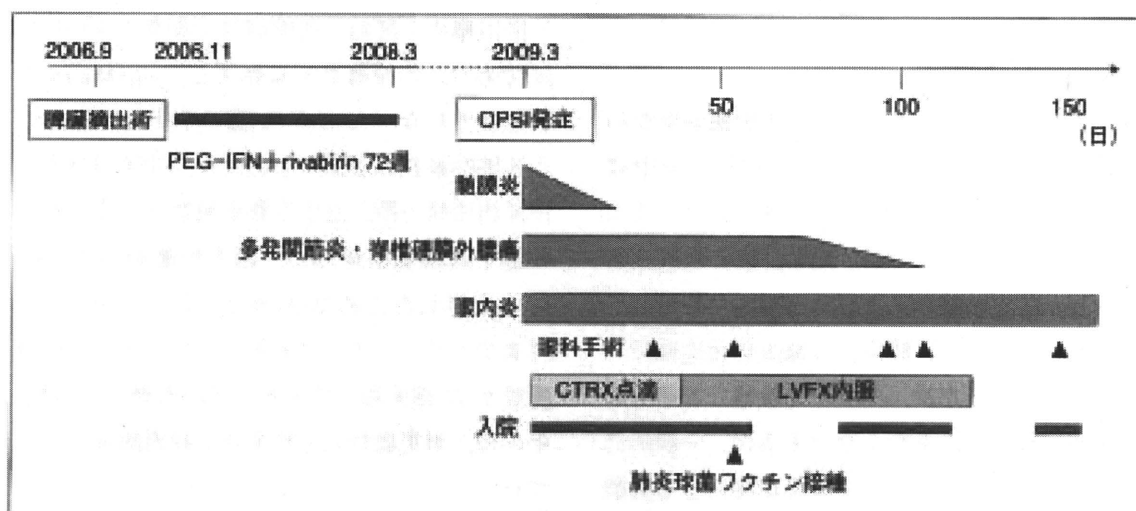


図1　経過表
CTRX：セフトリアキソン，LVFX：レボフロキサシン

なし，右股関節軽度圧痛あり，右腰背部圧痛あり．

質問に答えるときと答えないときがある．自分の名前と場所が答えられない．項部硬直なし，構音障害なし，明らかな四肢の運動の左右差や失調様の運動はみられなかった．

**検査所見**：表1

**胸腹部X線**：明らかな異常なし．

**腹部超音波**：肝表面に腹水あり．肝内胆管・総胆管の拡張なし，胆石なし．

**頭部CT**：明らかな異常なし．

**インフルエンザ迅速検査**：A，Bともに陰性．

**入院後経過（図1）**：入院時身体所見，検査所見から，鑑別診断として，急性腸炎，髄膜炎，感染性心内膜炎などが挙げられた．入院当日から各種培養を提出し，スルバクタム/セフォペラゾン（スルペラゾン®）による治療を開始した．入院翌日，血液培養からグラム陽性双球菌が検出され，肺炎球菌感染症と診断した．髄液は初圧21 $cmH_2O$で外見はやや白濁，細胞数6,920/3 μl（多核球95％），グルコース13 mg/dl，蛋白415 mg/dlであり，髄液塗抹は陰性で

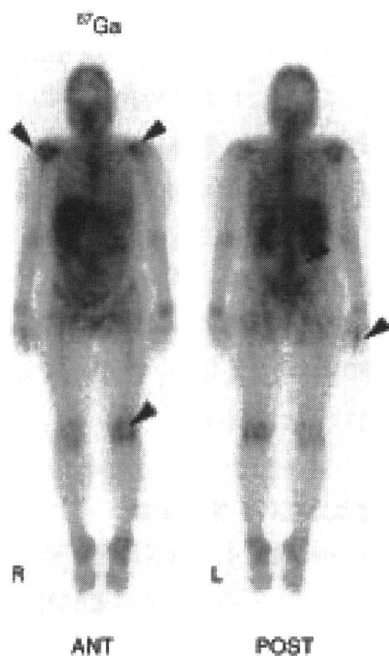


図2　Gaシンチ
両肩関節，左膝関節，右第三指，腰椎に集積がみられる．

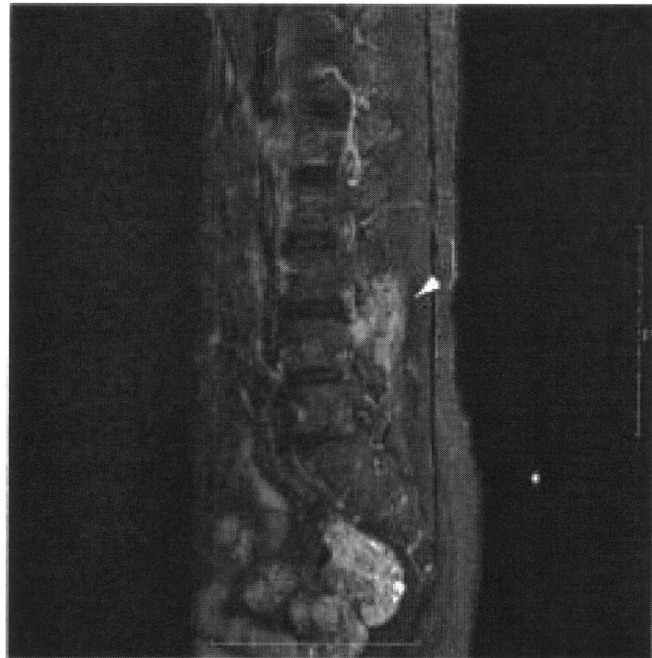

図3　腰椎造影MRI
L3/4椎間関節炎を認め，造影により増強されている．

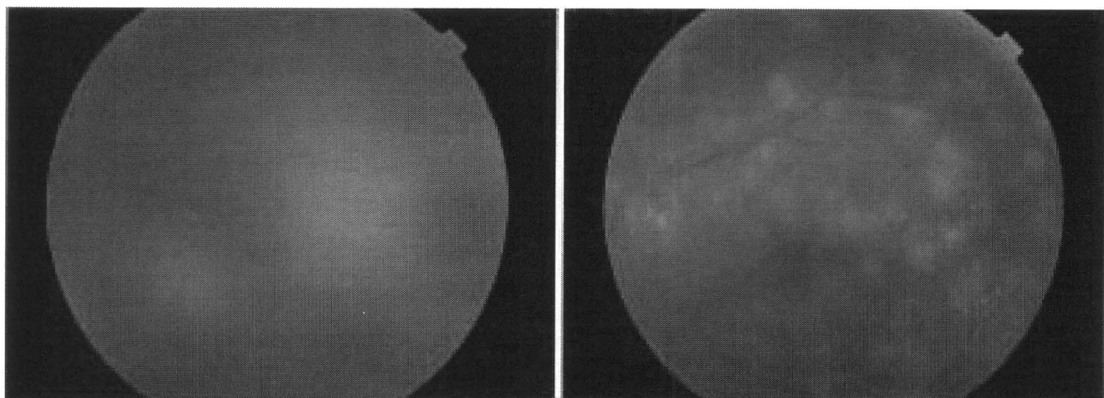

図4　眼底　　4a|4b
a：右眼底所見．硝子体混濁が高度で，眼底を観察できない．
b：左眼底所見．網絡膜に無数のfocusを認める．

あったが，経過と血液培養の結果から肺炎球菌髄膜炎と診断．抗菌薬をバンコマイシンとセフトリアキソン(ロセフィン®)に変更した．入院3日目には，ペニシリン感受性肺炎球菌と確定したため，バンコマイシンを中止した．

同日には見当識も回復し，症状を訴えられるようになった．両肩，右第3指，腰部，左膝の疼痛の訴えあり，Gaシンチを撮像したところ，

疼痛の訴えのあった部位に集積を認め(図2)，多発関節炎と診断した．また腰椎MRIではL3/4右椎間関節の関節炎(図3)と，L4/5硬膜外膿瘍も認めた．さらに視力が低下しているとの訴えがあり眼科受診したところ，右眼の視力は光覚弁でほとんど何も見えない状態，左も0.6と低下しており，右硝子体の高度混濁と左眼底脈絡膜に多数の病巣を認め(図4)，細菌性眼内炎と診断された．

入院14日目には髄膜炎は治癒していると考えられたが，多発関節炎，脊椎硬膜外膿瘍，眼内炎が持続していたために抗菌薬点滴は継続した．入院1カ月後には眼内炎が進行したため右眼硝子体切除＋眼内レンズ設置術を実施し，2カ月後には網膜裂孔を生じたため緊急硝子体切除・ガスタンポナーデ術を施行した．関節炎症状は次第に改善したため，抗菌薬はレボフロキサシン(クラビット®)内服に変更し，肺炎球菌ワクチンを接種し，入院70日目に退院とした．その後，右眼はさらに2回の手術を行ったが結局視力はほとんど回復せず，眼球癆に近い状態となった．

最終診断としては肺炎球菌による脾摘後重症感染症(敗血症，髄膜炎，多発関節炎，脊椎硬膜外膿瘍，眼内炎)であった．

## II．考　　察

### 1．脾臓摘出術とOPSI

C型慢性肝疾患に対するペグインターフェロン＋リバビリン併用療法が認可されて以降，その適応の拡大に伴い，血小板減少など血球減少の軽減を目的に脾臓摘出術や部分的脾動脈塞栓術(partial splenic arterial embolization；PSE)を施行される患者が近年増加傾向にある．こうした患者では肺炎球菌，髄膜炎菌，インフルエンザ桿菌といった細菌による脾摘後重症感染症(overwhelming postsplenectomy infection；OPSI)のリスクが高まる．理由としては，これらの菌は莢膜をもっておりオプソニン抗体によって修飾されることで白血球の貪食を受けて血液中から除去されるが，脾臓はオプソニン抗体産生の場としても細菌濾過の場としても重要なためである．門脈圧亢進症が原因の脾摘後患者における敗血症の発生率は6.7/100人年であり，特発性血小板減少性紫斑病(2.0/100人年)や外傷(2.1/100人年)などほかの理由による脾摘後に比べ高いとされている[1]．

### 2．肺炎球菌敗血症の診断と治療

脾摘後敗血症の半数以上は肺炎球菌が原因である．肺炎や中耳炎などの初期感染巣が明らかな場合もあるが，当症例のように発熱と非特異的な全身症状のみで感染巣がはっきりしない場合もあるため，血液培養検査はいかなる場合も必須である．髄膜炎が疑われる場合は髄液検査も行う．肺炎球菌尿中抗原検査も参考になる．数時間の経過で急速に全身状態が悪化するため，敗血症が疑われれば即座に抗菌薬治療を開始する．初期治療としては，セフトリアキソン(ロセフィン)2g，24時間ごと(髄膜炎が疑われる場合は12時間ごと)に加えてペニシリン耐性肺炎球菌の可能性を考慮しバンコマイシン1g 12時間ごとの点滴が推奨される．

当症例でみられた髄膜炎，関節炎，眼内炎，硬膜外膿瘍のほか，心内膜炎，脳膿瘍など全身に種々の播種性病変を形成し，後遺症を残すこともある．致死率は抗菌薬治療を行っても15～20％といわれ，発生した場合の被害は甚大であるため，医療従事者は脾摘後患者に発熱がみられた場合，常にOPSIの可能性を念頭において診療を進める必要がある．

表2　肺炎球菌によるOPSIの予防

| | |
|---|---|
| 肺炎球菌ワクチン（ニューモバックス®） | 脾臓摘出術の2週間前までに接種をすませる<br>5年後に再接種 |
| 患者指導 | 発熱時は早期に病院を受診するように説明しておく |
| 抗菌薬処方 | 発熱時，受診前に内服できるようにあらかじめ処方しておくことを考慮<br>アモキシシリン・クラブラン酸（オーグメンチン®），レボフロキサシン（クラビット®）などキノロン系薬剤が選択肢となる |

### 3．肺炎球菌によるOPSIの予防（表2）

このように重大な結果をもたらす脾摘後敗血症を減らすため，肺炎球菌ワクチンの接種が勧められている．成人は23価の莢膜多糖抗原を含むワクチン（ニューモバックス®）の適応があり，接種により敗血症や髄膜炎などの発症を減らすことが示されている[2]．PSEにおいても脾機能は低下していると考えられるため接種を考慮する．CDC（米国疾病予防管理センター）ではインフルエンザ菌b型（Hib），髄膜炎菌もワクチン接種を推奨している[3]．日本ではHibワクチンの適応は原則小児のみであるが接種は可能である．髄膜炎菌ワクチンは現在のところ発売されていない．

当症例で脾摘から2年半後に敗血症を起こしているように，脾摘後敗血症の発症時期は術後数年以内が多く，約1/3が1年以内，約半数が2年以内とされている[4]．そのため脾臓摘出の際にワクチン接種を済ませるのがよく，手術の14日前までに接種を完了しておくことが抗体の反応を高めるために望ましいので，手術を行う外科医と接種日について確認しておいたほうがよい．術後に接種する場合は，2週間経過してからのほうが高い効果を得られるという報告もある[5]．また，脾臓摘出による敗血症のリスクは生涯続くため，以前に脾摘した患者であってもワクチンを接種していなければ，接種を勧めるべきである．さらに，過去に肺炎球菌感染症に罹患した患者でも，ほかの血清型の肺炎球菌感染症に罹患する可能性があるため，接種が望ましい．当症例でも，退院前にワクチン接種を行った．

莢膜多糖抗原ワクチンは接種後徐々に効果が減弱する．そのためCDCでは5年後の再接種を勧めている[3]．日本では注射部位の疼痛，紅斑，硬結などの増強を理由に再接種が認められていなかったが，2009年10月に添付文書が改訂され『再接種の必要性を慎重に考慮したうえで，前回接種から十分な間隔を確保して』いれば再接種が可能となった．

### 4．脾摘患者への指導

脾摘患者に対しては，急速に進行し重篤な結果をもたらす敗血症を起こすリスクがあることを説明しワクチン接種を勧める．当症例では発熱がみられてから3日後の受診となったが，術後の生活において発熱をきたした場合は，早期に病院を受診する必要があることを指導する．抗菌薬をあらかじめ処方して，受診前に内服できるようにおくことも対応策の一つである．通常はアモキシシリン・クラブラン酸（オーグメンチン®）で十分だが，ペニシリン耐性肺炎球菌が問題となる場合にはレボフロキサシン（クラビット®）などキノロン系薬剤が選択肢となる[1]．

## 結　語

OPSIは脾摘後数年以内の発症率が高く，後遺症を残したり生命を脅かすこともある疾患である．脾摘患者には肺炎球菌ワクチン接種による予防を行うこと，発熱時はすぐに受診するよう指導することが肝要である．

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ORIGINAL ARTICLE

# Evaluation of long-term entecavir treatment in stable chronic hepatitis B patients switched from lamivudine therapy

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**Abstract**
*Purpose* Current Japanese guidelines recommend that patients should be switched from lamivudine to entecavir when they meet certain criteria. This analysis examines the efficacy and safety of long-term entecavir therapy in patients who were switched to entecavir after 24 weeks' lamivudine therapy in Japanese studies ETV-047 and ETV-060.
*Methods* The Phase II Japanese study ETV-047 assessed the efficacy of different entecavir doses when compared with lamivudine. A total of 33 Japanese patients who received lamivudine 100 mg daily in ETV-047 entered the open-label rollover study ETV-060 and subsequently received treatment with entecavir 0.5 mg daily. Hepatitis B virus (HBV) DNA suppression, alanine aminotransferase (ALT) normalization, hepatitis B e antigen (HBeAg) seroconversion, and resistance were evaluated among patients with available samples for up to 96 weeks. Safety was assessed throughout the treatment period.
*Results* After 96 weeks of entecavir therapy in ETV-060, 90% of patients achieved HBV DNA <400 copies/mL as compared to 21% of patients who completed 24 weeks of lamivudine therapy in ETV-047. Increasing proportions of patients achieved ALT normalization and HBeAg seroconversion following long-term entecavir treatment. No patients experienced virologic breakthrough, and substitutions associated with entecavir resistance were not

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observed in patients with detectable HBV DNA. Entecavir was well tolerated during long-term treatment.

*Conclusions* Switching lamivudine-treated patients with chronic hepatitis B to entecavir results in increased virologic suppression with no evidence of resistance through 2 years of entecavir therapy. These findings support recommendations in the current Japanese treatment guidelines that stable lamivudine patients should be switched to entecavir.

**Keywords** Japanese · Chronic hepatitis B · Entecavir · Lamivudine · Switch

## Introduction

Chronic hepatitis B virus (HBV) infection affects more than 350 million people worldwide, and is a leading cause of liver-related mortality [1]. Although Japan has one of the lowest prevalence rates for chronic hepatitis B (CHB) (0.8%) among Asian countries, it is still estimated that over 1 million people are chronically infected with HBV [2]. These individuals are at an increased risk of developing cirrhosis, liver failure or hepatocellular carcinoma (HCC) [3].

Lamivudine was the first nucleoside analog introduced for the treatment of CHB. In clinical trials, it demonstrated superior efficacy to placebo for HBV DNA suppression, alanine aminotransferase (ALT) normalization and hepatitis B e antigen (HBeAg) seroconversion [4, 5]. However, a major limitation of lamivudine therapy is the development of resistance, which occurs in up to 70% of patients through 4 years of therapy [6]. Entecavir is a potent inhibitor of HBV replication [7]. In global Phase III studies, entecavir demonstrated superior histologic, virologic and biochemical responses when compared with lamivudine in nucleoside-naïve patients and lamivudine-refractory patients at 48 weeks [8–10]. In the Japanese Phase II study ETV-047, treatment with entecavir resulted in a superior reduction in HBV DNA as compared to lamivudine [11]. In contrast to lamivudine, entecavir has been shown to have a high genetic barrier to resistance; the cumulative probability of resistance through 5 years of treatment has been reported to be 1.2% [12]. The genetic barrier is lower in patients who are infected with lamivudine-resistant HBV and consequently higher resistance rates are observed in this population with long-term treatment [12].

Current Japanese treatment guidelines recommend that all treatment-naïve CHB patients with ALT levels ≥31 IU/L should be treated, dependent on their viral load. The thresholds for treatment are HBV DNA ≥5 $\log_{10}$ copies/mL in HBeAg-positive patients, ≥4 $\log_{10}$ copies/mL in HBeAg-negative patients, and ≥3 $\log_{10}$ copies/mL in cirrhotic patients [13]. Lamivudine, adefovir, and entecavir are currently approved for the treatment of CHB in Japan. Entecavir 0.5 mg once daily is the first choice therapy for treatment-naïve HBeAg-positive and negative patients aged 35 years or older. In treatment-naïve patients <35 years, the guidelines recommend treating first with interferon for HBeAg-positive patients, and treating HBeAg-negative patients with HBV DNA ≥7 $\log_{10}$ copies/mL with entecavir until undetectable HBV DNA is achieved, followed by a combination of entecavir and interferon for 4 weeks, and finally interferon monotherapy for 20 weeks. HBeAg-negative patients with HBV DNA <7 $\log_{10}$ copies/mL should be monitored or can receive interferon therapy. For patients who are lamivudine experienced, but not necessarily resistant, the guidelines also recommend that patients can be switched to entecavir 0.5 mg daily if they have received lamivudine therapy, and have HBV DNA <2.1 $\log_{10}$ copies/mL. Patients with HBV DNA ≥2.1 $\log_{10}$ copies/mL can also be switched to entecavir 0.5 mg once daily if they do not have viral breakthrough. Limited data on the efficacy of entecavir in this patient population are available; however, the design of the Japanese study ETV-047 and the rollover study ETV-060 presents an opportunity to assess the efficacy of this treatment option. This report examines the long-term efficacy, safety and resistance of entecavir 0.5 mg daily among patients who were directly switched from lamivudine following 24 weeks' treatment in ETV-047.

## Materials and methods

### Study population

Study ETV-047 was a Phase II, randomized, double-blind study conducted to evaluate the dose–response relationship of entecavir and compare the antiviral activity and safety of entecavir to lamivudine in Japanese patients with CHB. In ETV-047, 137 patients were randomized to receive one of three entecavir doses [0.01 mg ($n = 35$), 0.1 mg ($n = 34$) or 0.5 mg ($n = 34$), once daily] or lamivudine [100 mg ($n = 34$), once daily] for 24 weeks. The study design and complete inclusion criteria have been described previously [11]. Briefly, eligible patients had HBeAg-positive or -negative CHB with compensated liver disease, HBV DNA ≥7.6 $\log_{10}$ copies/mL by PCR assay, <12 weeks' prior therapy with anti-HBV nucleoside analogs and ALT levels 1.25–10 × upper limit of normal (ULN). After completion of treatment in ETV-047, all patients were eligible to enroll immediately in the rollover study ETV-060, with no gap in dosing.

The rollover study ETV-060 was designed to provide open-label entecavir for patients who had completed therapy in the Japanese Phase II program. Patients who completed 24 weeks of treatment in ETV-047 enrolled in ETV-060 and received 0.5 mg entecavir once daily. After 96 weeks of treatment in study ETV-060, patients could complete the study and were eligible to receive commercially available entecavir, which was approved by Japanese health authorities while study ETV-060 was ongoing.

The current analysis describes results for a subset of 33 patients who received lamivudine for 24 weeks in ETV-047 and entecavir 0.5 mg once daily for up to 96 weeks in ETV-060.

## Efficacy analyses

Efficacy assessments evaluated the proportions of patients who had available samples (non-completer = missing) every 24 weeks through 120 weeks' treatment. Efficacy end points assessed included HBV DNA <400 copies/mL by PCR assay, ALT normalization ($\leq 1.0 \times$ ULN), HBeAg seroconversion among patients who were HBeAg-positive at baseline, and hepatitis B surface antigen (HBsAg) loss. Serum HBV DNA was determined by Roche Amplicor® PCR assay (Roche Diagnostics K.K., Tokyo, Japan; limit of quantification = 400 copies/mL) in a central laboratory. Clinical laboratory tests, PCR assays for HBV DNA, and serologic tests for HBV were performed at SRL, Inc. (Tokyo, Japan), the central clinical laboratory designated by the trial sponsor. On-treatment testing for resistance was carried out using a direct-sequencing PCR method.

## Safety analyses

Safety analyses include the incidence of adverse events, serious adverse events, laboratory abnormalities, and discontinuations due to adverse events on-treatment throughout treatment in study ETV-060. On-treatment ALT flares were defined as ALT $>2 \times$ baseline and $>10 \times$ ULN.

## Resistance analysis

Resistance testing was performed using a direct-sequencing PCR method. Paired samples from all patients with HBV DNA $\geq$400 copies/mL were analyzed for substitutions associated with entecavir or lamivudine resistance at week 96 (72 weeks of entecavir therapy) or week 120 (96 weeks of entecavir therapy). Patients who discontinued therapy prior to week 120 had their last on-treatment sample analyzed. All patients with virologic breakthrough ($\geq$1 $\log_{10}$ increase from nadir on two consecutive measurements) were also tested for resistance.

# Results

## Study population

Of the 34 patients in ETV-047 who received treatment with lamivudine 100 mg once daily for 24 weeks, 33 entered ETV-060 and received treatment with entecavir 0.5 mg once daily. Two patients discontinued treatment during ETV-060: one due to an adverse event (depression) and the other due to insufficient effect. In addition, one patient completed treatment at week 76 (52 weeks of entecavir therapy) after meeting the criteria for protocol-defined complete response (undetectable HBV DNA by PCR assay, undetectable HBeAg and normal serum ALT).

Baseline demographic and disease characteristics for the switch cohort are presented in Table 1. The majority of patients (82%) in the cohort were male with a mean age of 43 years. The mean duration of entecavir therapy was 105.9 weeks (range 25–141 weeks). Baseline mean HBV DNA and ALT levels were 7.9 $\log_{10}$ copies/mL and 184 IU/L, respectively. Ninety-one percent of patients were HBeAg-positive and 88% had HBV genotype C infection.

## Virologic end points

After completion of 24 weeks of lamivudine treatment in ETV-047, 21% (7/33) of patients in the switch cohort had achieved HBV DNA <400 copies/mL (Fig. 1). Following the switch to entecavir, the proportion of patients achieving HBV DNA <400 copies/mL increased to 82% (27/33) by week 48 (24 weeks of entecavir therapy). Viral suppression

**Table 1** Baseline (pretreatment) demographics and disease characteristics: switch cohort

| Characteristic | ETV-047/-60 lamivudine to entecavir switch cohort ($n = 33$) |
|---|---|
| Age, mean (years) | 42.7 |
| Male, $n$ (%) | 27 (82) |
| Ethnicity Japanese, $n$ (%) | 33 (100) |
| Entecavir treatment periods, mean (range) (weeks) | 105.9 (25–141) |
| HbeAg-positive, $n$ (%) | 30 (91) |
| HBV DNA by PCR, mean $\log_{10}$ copies/mL (SD) | 7.9 (0.80) |
| ALT (IU/L), mean (SD) | 184.8 (132.9) |
| HBV genotype, $n$ (%) | |
| A | 2 (6) |
| B | 2 (6) |
| C | 29 (88) |
| Others | 0 |

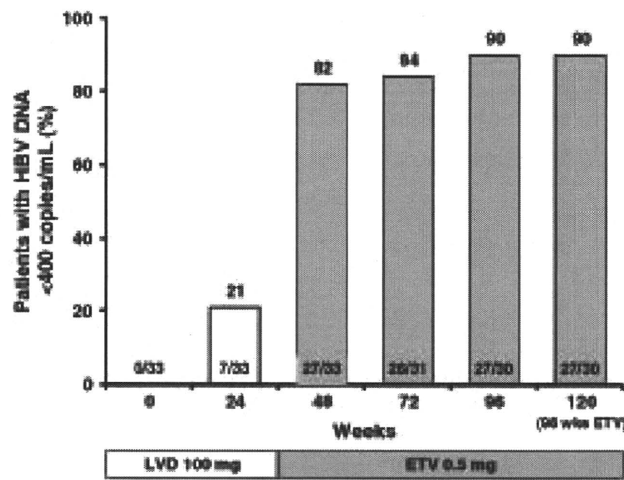


Fig. 1 Proportion of patients with HBV DNA <400 copies/mL through 120 weeks of therapy (ETV-047 to ETV-060). *Denominators* represent patients with available samples. *ETV* entecavir, *HBV* hepatitis B virus, *LVD* lamivudine

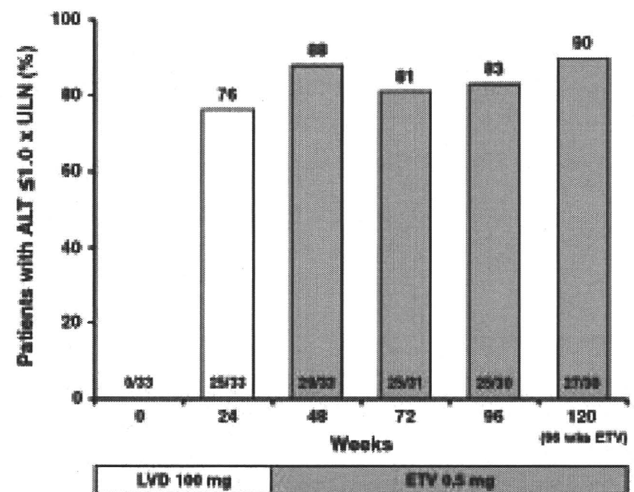


Fig. 3 Proportion of patients with ALT normalization (≤1.0 × ULN) through 120 weeks of therapy (ETV-047 to ETV-060). *Denominators* represent patients with available samples. *ALT* alanine aminotransferase, *ETV* entecavir; *LVD* lamivudine, *ULN* upper limit of normal

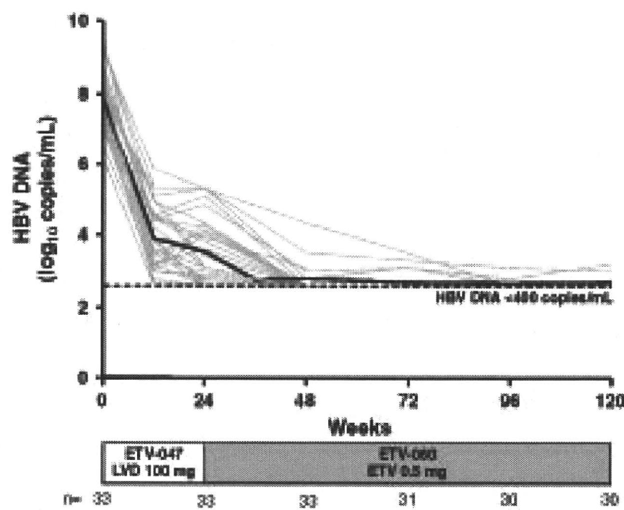


Fig. 2 HBV DNA suppression through week 120 (96 weeks of entecavir therapy). Individual patient HBV DNA profiles are plotted in *gray*. Mean HBV DNA levels are represented by the *solid black line*. *ETV* entecavir, *HBV* hepatitis B virus, *LVD* lamivudine

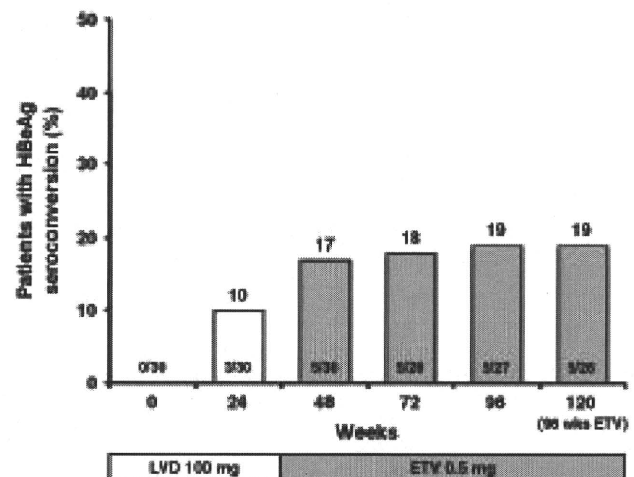


Fig. 4 Proportion of patients with HBeAg seroconversion through 120 weeks of therapy (ETV-047 to ETV-060). *Denominators* represent patients with available samples among the 30 patients HBeAg-positive at baseline. *ETV* entecavir, *HBeAg* hepatitis B e antigen, *LVD* lamivudine

was maintained with longer entecavir treatment, with 84% (26/31) and 90% (27/30) achieving HBV DNA <400 copies/mL at weeks 72 and 120, respectively (48 and 96 weeks of entecavir therapy). Mean HBV DNA levels decreased from a baseline of 7.90 to 3.52 $log_{10}$ copies/mL after 24 weeks of lamivudine therapy in ETV-047, and reached 2.69 $log_{10}$ copies/mL after 96 weeks of entecavir therapy in ETV-060 (week 120; Fig. 2). No viral breakthrough was observed during entecavir therapy.

Biochemical end points

ALT normalization (≤1.0 × ULN) was demonstrated in 76% (25/33) of patients after 24 weeks of lamivudine therapy in ETV-047 (Fig. 3). Following treatment with entecavir in ETV-060, ALT normalization was maintained in 90% (27/30) of patients achieving this end point by week 120. Minor fluctuations in the proportion of patients achieving ALT normalization were attributed to patients discontinuing entecavir therapy during the course of study ETV-060.

Serologic end points

HBeAg seroconversion was assessed among the 30 patients in the switch cohort who were HBeAg-positive at baseline in ETV-047 (Table 1; Fig. 4). Three patients (10%)

achieved HBeAg seroconversion during the initial 24-week lamivudine treatment period in ETV-047 (Fig. 4). Following switch to entecavir in ETV-060, two additional patients developed HBeAg seroconversion by week 120 (96 weeks of entecavir therapy). None of the patients in the switch cohort experienced HBsAg loss during treatment in ETV-047 or ETV-060.

### Resistance

Four of the 33 patients who received entecavir therapy in ETV-060 had HBV DNA ≥400 copies/mL either at treatment discontinuation or at week 120. One patient discontinued therapy at week 68 (44 weeks of entecavir therapy) due to insufficient effect. HBV DNA prior to treatment discontinuation was 3.1 $\log_{10}$ copies/mL, however, resistance testing revealed no substitutions associated with entecavir resistance. The remaining three patients had HBV DNA ≥400 copies/mL at weeks 96 and 120; however, only two patients had samples available for testing. Neither patient's samples had substitutions associated with entecavir or lamivudine resistance either at weeks 96 or 120.

### Safety

Entecavir was well tolerated during long-term treatment and the safety profile of patients in the switch cohort was consistent with that previously reported for patients who received continuous entecavir therapy in studies ETV-047 and ETV-060 (Table 2). Serious adverse events (Meniere's disease, subcutaneous abscess and ALT flare) were reported in three patients (9.1%). The most frequently reported adverse events during treatment in ETV-060, occurring in ≥10% of patients, were nasopharyngitis (76%), diarrhea (21%), back pain (18%), influenza (18%), and allergic rhinitis (15%). One patient discontinued entecavir therapy due to depression, which the investigator considered was possibly related to entecavir therapy. An ALT flare (ALT >2 × baseline and >10 × ULN) occurred in one patient at week 18, and was judged a serious adverse event by the investigator, but was not associated with a change in HBV DNA. No deaths were reported during the study.

**Table 2** Summary of safety in ETV-060: switch cohort

| On-treatment in ETV-060 | Patients, n (%) |
|---|---|
| Any adverse events | 33 (100) |
| Clinical adverse events | 33 (100) |
| Laboratory adverse events | 33 (100) |
| Grade 3/4 clinical adverse event | 1 (3) |
| Grade 3/4 laboratory adverse event | 5 (15) |
| Clinical serious adverse event[a] | 3 (9) |
| Discontinuations due to adverse events | 1 (3) |
| Deaths | 0 |
| ALT flares[b] | 1 (3) |

[a] Including ALT flares

[b] ALT >2 × baseline and >10 × ULN

## Discussion

Profound long-term suppression of HBV DNA is required for patients to meet the goals of CHB therapy, which are to prevent cirrhosis, hepatic failure, HCC and liver-related death [14–16]. A major concern with long-term therapy is the increasing risk of selecting resistance mutations, especially for therapies with a low-genetic barrier to resistance, such as lamivudine. The current analysis presents results for a cohort of Japanese patients who were switched directly from lamivudine to long-term entecavir therapy. The results show that this switch cohort achieved additional HBV DNA suppression after the switch to entecavir. The proportion of patients with HBV DNA <400 copies/mL increased from 21% after 24 weeks of lamivudine treatment to 82% following an additional 24 weeks of entecavir treatment. Mean HBV DNA decreased from 3.52 $\log_{10}$ copies/mL at week 24 to 2.80 $\log_{10}$ copies/mL at week 48. Rates of HBV DNA suppression were maintained in this cohort, with 90% of patients achieving HBV DNA <400 copies/mL through 96 weeks of entecavir therapy (week 120). These results are comparable to those achieved by the cohort of patients who received entecavir 0.5 mg once daily in the Japanese Phase II studies and the rollover study ETV-060 [17]. At baseline in ETV-060, 56% of this cohort had achieved HBV DNA <400 copies/mL, increasing to 83% through 96 weeks of entecavir therapy. Among patients with abnormal ALT levels at ETV-060 baseline, 88% of patients in the entecavir 0.5 mg cohort achieved normalized ALT levels at week 96 as compared to 90% of patients in the switch cohort. Rates of HBeAg seroconversion at week 96 in ETV-060 were also similar (20 vs. 19%, respectively). These rates of viral suppression also show comparison favorably to those reported for the global nucleoside-naïve cohorts treated for a similar period of time [18, 19]. The potent antiviral activity of entecavir and its high genetic barrier to resistance is expected to minimize the potential for resistance in the switch cohort, allowing long-term therapy for patients. Liver biopsies were not obtained from patients in the switch cohort; however, the histologic benefits of long-term entecavir therapy have been recently reported for a cohort of naïve Japanese patients in the ETV-060 rollover study [20]. Following treatment with entecavir 0.5 mg daily for 3 years, all patients experienced histologic improvement and 57% experienced improvement in fibrosis score. In

addition, the results from a separate global study have confirmed the histologic benefits of long-term entecavir treatment [21].

Previous Japanese (ETV-052/-060) and global (ETV-026) studies have examined the efficacy of entecavir in lamivudine-refractory patients. In these studies, entecavir demonstrated efficacy, with 54% of Japanese patients achieving HBV DNA <400 copies/mL through 3 years' treatment [10, 22]. However, as a result of the lower genetic barrier in these patients, a major drawback of entecavir therapy in this population is the development of resistance. The cumulative probabilities of genotypic entecavir resistance among lamivudine-refractory patients were 33% through 3 years' treatment in Japanese patients and 51% through 5 years' treatment among patients in the global cohort [12, 22]. In the current study, no entecavir- or lamivudine-associated resistance substitutions were detected after 96 weeks of entecavir treatment. However, in contrast to the previous studies where the majority of patients had high baseline HBV DNA and documented lamivudine resistance [10, 23], patients in the switch cohort received entecavir after achieving variable degrees of HBV DNA suppression with 24 weeks of lamivudine therapy. Therefore, the fact that no resistance has been observed in this cohort to date is not unexpected. This observation is consistent with an analysis of lamivudine-refractory patients enrolled in the worldwide lamivudine-refractory study ETV-026. Patients with baseline HBV DNA $<7 \log_{10}$ copies/mL had a higher probability of achieving HBV DNA <300 copies/mL as compared to those who had baseline HBV DNA $\geq 7 \log_{10}$ copies/mL (73 vs. 16%) [24]. Furthermore, among the 42 entecavir-treated patients in ETV-026 who achieved HBV DNA <300 copies/mL through 96 weeks of therapy, only one patient subsequently developed entecavir resistance.

Current recommendations on the treatment of patients with documented lamivudine resistance suggest that patients should receive a second drug without cross resistance. The combination of lamivudine and adefovir has been shown to be superior to adefovir monotherapy for the treatment of lamivudine resistance, especially in preventing the selection of adefovir resistance [25–27]. Although only short-term clinical data are available for tenofovir, rates of viral suppression among lamivudine-experienced or -resistant patients who received tenofovir monotherapy do not differ significantly from those of treatment-naïve patients [28, 29]. Small studies have also shown pegylated interferon alpha-2a to be a safe and beneficial treatment option for lamivudine-experienced patients [30]. However, the treatment options for Japanese lamivudine-resistant patients are more limited, since neither tenofovir nor pegylated interferon alpha-2a are currently approved in Japan.

The Japanese guidelines recommend that patients with detectable YMDD mutations should receive treatment with a combination of lamivudine and adefovir [13]. However, the guidelines also allow patients who have received <3 years of lamivudine therapy, have HBV DNA <400 copies/mL, and no breakthrough hepatitis or YMDD mutations to switch directly to entecavir. The results presented in this analysis suggest that the strategy of switching to entecavir is an effective one that may avoid the additional cost and potential toxicity of combination treatment with lamivudine and adefovir. Among patients in the switch cohort, 96 weeks of entecavir treatment was well tolerated and the safety profile was comparable with previous experience in Japanese patients. One patient experienced an ALT flare (ALT >2 × baseline and >10 × ULN) 18 weeks after initiating entecavir, which was not associated with a change in HBV DNA. This low rate of ALT flares is consistent with previous findings and demonstrates that lamivudine-treated patients can be switched safely to entecavir with a minimal risk of such flares [10, 22].

In summary, the data from the switch cohort presented in this analysis demonstrate that CHB patients can be switched from lamivudine to long-term entecavir. The treatment with entecavir resulted in increased rates of virologic suppression with no evidence of resistance through 2 years of therapy. These findings support recommendations in the current Japanese treatment guidelines that patients on stable lamivudine therapy with no YMDD mutations should be switched to entecavir.

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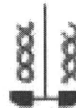

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# Semi-quantitative discrimination of HBV mutants using allele-specific oligonucleotide hybridization with Handy Bio-Strand


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**The analysis of hepatitis B virus (HBV) mutations is important for understanding HBV progression and for deciding on appropriate clinical treatments. However, it is difficult to determine the quantitative abundance of various mutants in heterogeneous mixtures by conventional methods such as direct sequencing or the TaqMan assay. In this study, we investigated the possibility of using both allele-specific oligonucleotide hybridization (ASOH) and allele-specific oligonucleotide competitive hybridization (ASOCH) with the Handy Bio-Strand system for the quantitative identification of three well-defined HBV variants: the basal core promoter (BCP) mutations (nt1762 and nt1764), the pre-core (PC) mutation (nt1896), and variance at nt1858. Using standardized mixtures of wild-type and mutant DNA, optimal hybridization conditions for ASOH and ASOCH were determined. Next, the performance of these methods was evaluated using actual serum DNAs from HBV patients. Excellent reproducibility was obtained both in the analysis of internal positive controls and in the semi-quantitative categorization of heterogeneous viral mixtures into five abundance groups (0%, 25%, 50%, 75%, and 100% mutant virus). Combined with real-time PCR to determine the HBV viral load, this hybridization method offers a new tool with applications both in HBV clinical research and treatment.**



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[**Key words:** ASOH (allele-specific oligonucleotide hybridization); ASOCH (allele-specific oligonucleotide competitive hybridization); Basal core promoter; HBV (hepatitis virus type B); Pre-core; Handy Bio-Strand]


Hepatitis B virus (HBV) causes transient and chronic infection of the liver, and is one of the most widespread infectious diseases in the world. Due to frequent mutations, HBVs in patients are not uniform, and this diverse mutant population can affect the progression of HBV. Thus, the analysis of HBV mutations is important for understanding HBV progression and determining the appropriate clinical treatment (1–4). Understanding HBV mutations, HBV loads and other biomarkers will provide new approaches for predicting HBV progression.

A variety of HBV mutations that affect HBV virus activity and disease progression have been reported (5). Among these, the basal core promoter (BCP) mutation (6, 7), the pre-core mutation (PC) (8–11), and nt1858 variance (12) are the most well-defined. The PC mutation (G to A, nt1896) introduces a stop codon (TAG) into the ORF and aborts the translation of the precursor of HBeAg (13), resulting in seroconversion from HBeAg (+) to Anti-HBe Ab (+). Stem-loop RNA structures around nt1896 often pair with nt1858. Six HBV genotypes (A–F) have been defined for nt1858. Genotypes A and F have the base C at nt1858, which forms a stable bond with the base G at position 1896 in wild-type PC, and maintains the wild-type phenotype. The other genotypes (B, C, D and E) have T at position 1858. A double mutation in the BCP (nt1762 and nt1764) frequently occurs in chronic HBV patients. These BCP mutations increase viral replication and enhance disease activity.

A number of methods have been proposed to detect HBV mutations, including direct sequencing, RFLP (14), point mutation assays (15), INNO-LiPA Line Probe Assays (16), reverse dot blots (17), genotype-specific probe assays (GSPA) (12), DNA arrays (18), mass spectrometric assays (19), Molecular-beacon (20), and real-time PCR (21). Except for Molecular-beacon and real-time PCR, all of these are qualitative methods and do not provide quantitative assessments of heterogeneous mutant mixtures.

Previously, we developed a three-dimensional DNA array (Bio-Strand), as well as a total three-dimensional DNA array system (Handy Bio-Strand). This system consists of a DNA array (the Bio-Strand Tip), a spotter, a scanner, and analysis software (Hy-soft) (22). The per-

*Abbreviations:* ASOH, allele-specific oligonucleotide hybridization; ASOCH, allele-specific oligonucleotide competitive hybridization; HBV, Hepatitis B virus; BCP, basal core promoter; PC, pre-core; GSPA, genotype-specific probe assay; RFLP, restriction fragment length polymorphism; SNP, single-nucleotide polymorphism; FI, fluorescence intensity.


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formance of Bio-Strand was first demonstrated with SNP genotyping (22–25). In this study, we developed a Handy Bio-Strand method that uses allele-specific oligonucleotide hybridization (ASOH) and allele-specific oligonucleotide competitive hybridization (ASOCH) to quantitatively discriminate HBV mutants. Our preliminary assessment of this method's ability to quantify specific HBV mutations in heterogeneous viral mixtures is described below.

## MATERIALS AND METHODS

**Materials and DNA extraction** All PCR primers, Cy5 probes, and competitors (Table 1) were synthesized by SIGMA Genosys (Ishikari, Japan). Oligo5 software (Molecular Biology Insights, Cascade, USA) was used to design PCR primers and to estimate the free energy of internal stability of each probe. Positive control DNAs (D, E, F) and sample DNAs for blind test (C1–C10 and G1–G10) were purified from whole blood sera obtained from HBV patients using the SMITEST EX-R&D Nucleic Acid Extraction Kit (Genome Science Laboratories, Fukushima, Japan). Total nucleic acids were precipitated with 99% ethanol, dried, and resuspended in 50 μl sterile water. Using AMPLICOR HBV monitor (F. Hoffmann-La Roche, Basel, Switzerland), HBV loads of G3, G4, G5, C4, C5, and C6 were estimated to be $10^{3.0}$, $10^{3.0}$, $10^{7.0}$, $10^{4.3}$, $10^{6.3}$, and $10^{3.3}$ copies/ml, respectively. The Ethics Committee of the associated institution approved this study, and written informed consent was obtained from each patient.

**Amplification of the target fragment** The target DNA fragment (304 bps) containing the BCP and PC mutation sites and nt1858 was amplified by nested PCR. Amplification reaction mixtures (50 μl) containing 3 μl of total nucleic acid solution, 1× TAKARA Ex Taq buffer, 10 nmol dNTPs, 10 pmol HBV 1601-S, 10 pmol HBV 1974-AS, and 2.5 U TAKARA Ex Taq (TAKARA BIO, Shiga, Japan) were prepared, heated to 94 °C for 3 min, subjected to 25 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and then kept at 4 °C in a Thermal Cycler Dice (TAKARA BIO). An aliquot of the first PCR solution (1 μl) was further amplified using the primers HBV 1653-S and HBV 1959-AS under the same conditions. The PCR products were resolved on a 3% agarose gel and purified using a PCR clean-up kit and the Magtration System 12GC (Precision System Science, Chiba, Japan). The concentrations of the PCR products were determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and then diluted to 100 ng/μl with sterile water.

**Preparation of standards** The standard DNA fragments (Table 2) were amplified from total DNAs prepared from patient sera by nested PCR, as described above. The amplified DNA fragments were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, USA) and their DNA sequences were determined using PCR primers by an ABI3730XL sequencer (Invitrogen). After sequence confirmation, three PCR-amplified HBV fragments were selected and denoted as D, E, and F. The PCR fragments were diluted to 100 ng/μl with sterile pure water and used to prepare standardized mixtures: D only, D/E = 75%/25%, D/E = 50%/50%, D/E = 25%/75%, E only, and F only, which were denoted P1 to P6, respectively (Table 3). These mixtures were spotted and fixed on the Bio-Strand and were used to determine optimal hybridization conditions.

**Immobilization of DNA on Bio-Strand** A three-dimensional DNA array (Bio-Strand Tip, Precision System Science), was prepared as described previously (22). A 5-μl aliquot of purified DNA solution (100 ng/μl) was mixed with an equal volume of 2 M NaOH and the mixture was spotted onto a thread using a spotting tool. After drying for a few minutes, the Bio-Strand was prepared by wrapping the thread around a cylindrical core (core pin). After fixing the denatured DNA onto the Bio-Strand by ultraviolet irradiation (wavelength 280 nm, 120 mJ), the Bio-Strand was inserted into a transparent plastic tip (Bio-Strand Tip).

**ASOH and ASOCH** Oligonucleotide hybridization and washing of the Bio-Strand Tip for ASOH and ASOCH were carried out using the Magtration System 12GC as described previously (22). The Bio-Strand Tip was immersed twice in 450 μl of the hybridization buffer [2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate) with 200 μg/ml salmon sperm DNA (Invitrogen)], left for 10 min, and then incubated for 5 min in 450 μl of the hybridization buffer containing 10 nM of the Cy5 probes listed in Table 1. For ASOCH, non-labeled opposing oligonucleotides (10–100 nM) were added as competitors. After hybridization, the tip was subjected to successive washings in 450 μl of wash buffer (2× SSC with 0.1% SDS, 1× SSC with 0.1% SDS, and 0.1× SSC with 0.1% SDS) for 2 min, and then soaked in 450 μl of 2× SSC. The Cy5 fluorescent hybridization signals were detected using a Handy Bio-Strand scanner (Precision System Science). To determine signal intensity, the average value was calculated from eight different spots. The fluorescence intensities (FIs) were calculated using Hy-soft software (Precision system science).

**Blind test for BCP, PC, and nt1858 variance using patient DNA** Blind tests for BCP, PC, and nt1858 variance were carried out using DNAs obtained from patients (C1 to C10 and G1 to G10). All samples and positive controls (P1–P6) were spotted and fixed on eight different areas of the same Bio-Strand. Each Bio-strand Tip was hybridized using either the ASOH or ASOCH method. After washing, the Handy Bio-Strand scanner was used to detect the Cy5 fluorescence signals on the Bio-Strand Tips. The signal intensity was calculated as the average value from the eight different spots. To determine the ratios of wild-type to mutant in the samples, the fluorescence intensities from the wild-type and mutant probes were compared with those of the standard mixture.

After the blind test, SNP types of patient DNA sequences were also determined by direct sequencing. Target DNA fragments were amplified using the primers HBV 1601-S and HBV 1974-AS PCR. After treatment with ExoSAP-IT (GE HealthCare Bioscience, Buckinghamshire, UK), they were applied for sequencing at Macrogen (Rockville, USA) with the primers HBV 1653-S or HBV 1959-AS PCR (22). The percent abundance or ratio at target site was determined by visual inspection of the electropherogram by two trained investigators with no knowledge of the HBV classifications of the samples.

**TABLE 1.** Sequences of PCR primers, Cy5 probes, and non-labeled competitors.

| Name | Target | Use | DNA sequence (5' → 3') |
|---|---|---|---|
| HBV 1601-S | External | 1st PCR | acgtcgcatggagaccaccg |
| HBV 1974-AS | | | ggaaagaagtcagaaggcaaa |
| HBV 1653-S | Internal | 2nd PCR | cataagaggactcttggact |
| HBV 1959-AS | | | ggcaaaaaagagagtaactc |
| Cy5-CPR1 | BCP | Cy5 Probes | Cy5-ggttaaaggtcttg |
| Cy5-CPR2 | | | Cy5-ggttaatgatctttg |
| Cy5-PC2 | PC | | Cy5-gctttggggca |
| Cy5-PC3 | | | Cy5-ggctttaggggca |
| Cy5-GA3 | nt1858 | | Cy5-atgtcccactgtt |
| Cy5-GA5 | | | Cy5-tgtcctactgt |
| CP-GA2 | | Competitors | catgtcctactgtt |
| CP-GA3 | | | atgtcccactgtt |

The target sites of the HBV genomic sequence were denoted as bold characters.

**TABLE 2.** DNA Sequences at each mutation site in the standard PCR DNA fragments (D, E and F).

| Standard PCR DNA Fragment | BCP | | PC | | nt1858 |
|---|---|---|---|---|---|
| | nt1762/nt1764 | Type | nt1896 | Type | nt1858 |
| D | A/G | wild | G | wild | C |
| E | T/A | mutant | A | mutant | T |
| F | A/G | wild | G | wild | T |

## RESULTS

**Optimization of ASOH and ASOCH conditions for determining the relative abundance of the mutants BCP, PC and T-1858** Fig. 1 shows a schematic of the proposed method for determining the relative abundance of the targeted HBV species using the Handy Bio-Strand system. Viral DNA was isolated from patient sera by conventional methods, and the targeted DNA fragments were amplified by nested PCR. After purifying the DNA and determining its concentration, amplicons were fixed on the surface of microporous nylon thread (Bio-Strand) by ultraviolet irradiation. The ASOH and ASOCH reactions were carried out at room temperature with Cy5-labeled oligonucleotide probes that were designed to detect the clinically important HBV mutations at BCP and PC and the base at nt1858. Fluorescence signals were detected using a Handy Bio-Strand scanner, and the abundance of each mutant was determined.

**TABLE 3.** Percent abundance of each species in the standard mixture.

| STD | Mixing (%) | | | Percent abundance (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | BCP | | PC | | nt1858 | |
| | D | E | F | Wild | Mutant | Wild | Mutant | C | T |
| P1 | 100 | 0 | 0 | 100 | 0 | 100 | 0 | 100 | 0 |
| P2 | 75 | 25 | 0 | 75 | 25 | 75 | 25 | 75 | 25 |
| P3 | 50 | 50 | 0 | 50 | 50 | 50 | 50 | 50 | 50 |
| P4 | 25 | 75 | 0 | 25 | 75 | 25 | 75 | 25 | 75 |
| P5 | 0 | 100 | 0 | 0 | 100 | 0 | 100 | 0 | 100 |
| P6 | 0 | 0 | 100 | 100 | 0 | 100 | 0 | 0 | 100 |

STD shows the name of the standard mixture which was prepared by mixing three standard PCR DNA fragment (D, E and F).

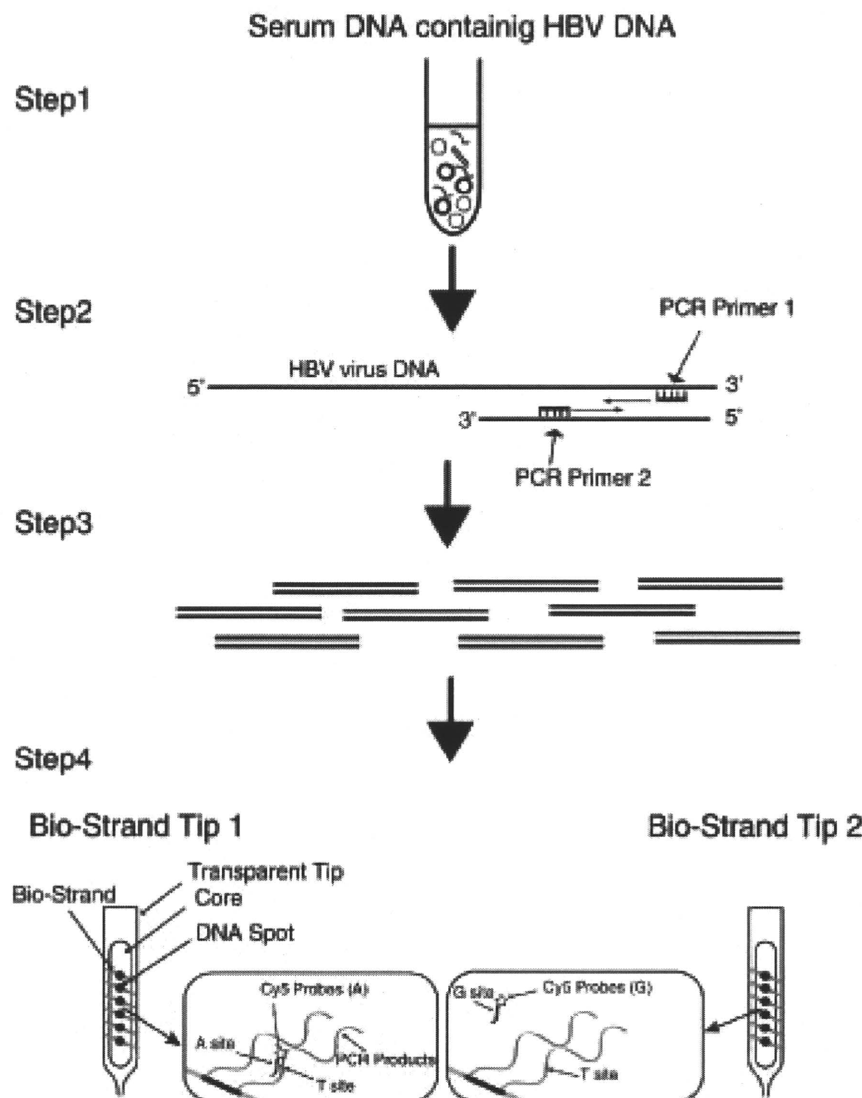


FIG. 1. Schematics of the ASOH and ASOCH assays using the Handy Bio-Strand system. Step1: Virus DNAs and RNAs are prepared from patient serum. Step2: HBV fragments (304 bps) are amplified using nested PCR. Step 3: The HBV fragments are purified, denatured, spotted and then fixed on Bio-Strand. Step 4: Two automatic hybridizations are separately carried out using two Bio-Strand Tips for a target site. Each Bio-Strand Tip contains different Cy5 probes. The stars and small circles show the Cy5 molecules and the target sites, respectively. The perfect-matching Cy5 probes bind to the SNP sites, but mismatching ones don't.

Fig. 2A shows the results of ASOH for the quantitative discrimination of the BCP mutation using the Cy5-CPR1 (wild-type) and Cy5-CPR2 (mutant) probes. The fluorescence intensities (FIs) of P1, P2, P3, P4, and P5 accurately reflected the abundance of the BCP mutants as 0%, 25%, 50%, 75%, and 100%, respectively. P1 gave a result similar to that of P6, demonstrating the high reproducibility of this assay. Unlike the previous results for SNP genotyping (22), accurate results were obtained without using competitors (non-labeled probes containing opposing sites). Double mutations (nt1762 and nt1764) in the center of the Cy5 probes are likely to be the reason for the high specificity and also the low background signals.

Fig. 2B shows the results obtained using ASOH with the Cy5-PC2 (wild-type) and Cy5-PC3 (mutant) probes to detect the PC mutation. Each FI increased in proportion to the increase in the amount of the PC mutant. The FIs of the standard solutions (P1, P2, P3, P4, and P5) allowed samples to be categorized into five groups according to the mutant content. In contrast to the results for the BCP mutant, the Cy5-PC2 and Cy5-PC3 probes did not give equal FIs for P3, which contained equal amounts of wild-type and mutant DNA. The FI for the wild-type DNA was about 27% higher than for the mutant DNA. This is probably due to non-specific binding of the Cy5-PC2 probe (wild-type, base G) to the mutant amplicon. To improve the specificity of Cy5-PC2, we added competitive unlabeled probe (CP-PC3) to the hybridization solution. Despite our expectations, this approach (ASOCH) did not improve the specificity of the Cy5 probes, and the total signal intensity was lower than that of ASOH (data not shown).

Concerning nt1858, it was difficult to distinguish between fragments E or F (T-1858) and fragment D (C-1858) by ASOH, due to the non-specific binding of the Cy5 probes (data not shown). We applied ASOCH using two different sets of probes, Cy5-GA3/CP-GA2 and Cy5-GA5/CP-GA3. Fig. 2C shows the ASOCH results for nt1858 with Cy5 probes and their unlabeled competitors (base C: 10 nM Cy5-GA3 and 100 nM CP-GA2; base T: 10 nM Cy5-GA5 and 100 nM CP-GA3). The background was moderately high, but the standard mixtures (P1–P6) were clearly distinguishable.

Our previous study estimated that the Handy Bio-Strand system hybridization signal error, the coefficient of variation (CV), was 4.6–11.3% (22). To obtain reliable classifications, standard mixtures were prepared within 2–3 fold of the previously determined CV, at 25% intervals and used as internal controls (Table 3). We also confirmed that standard mixtures prepared at 20% intervals worked well as for internal control (data not shown).

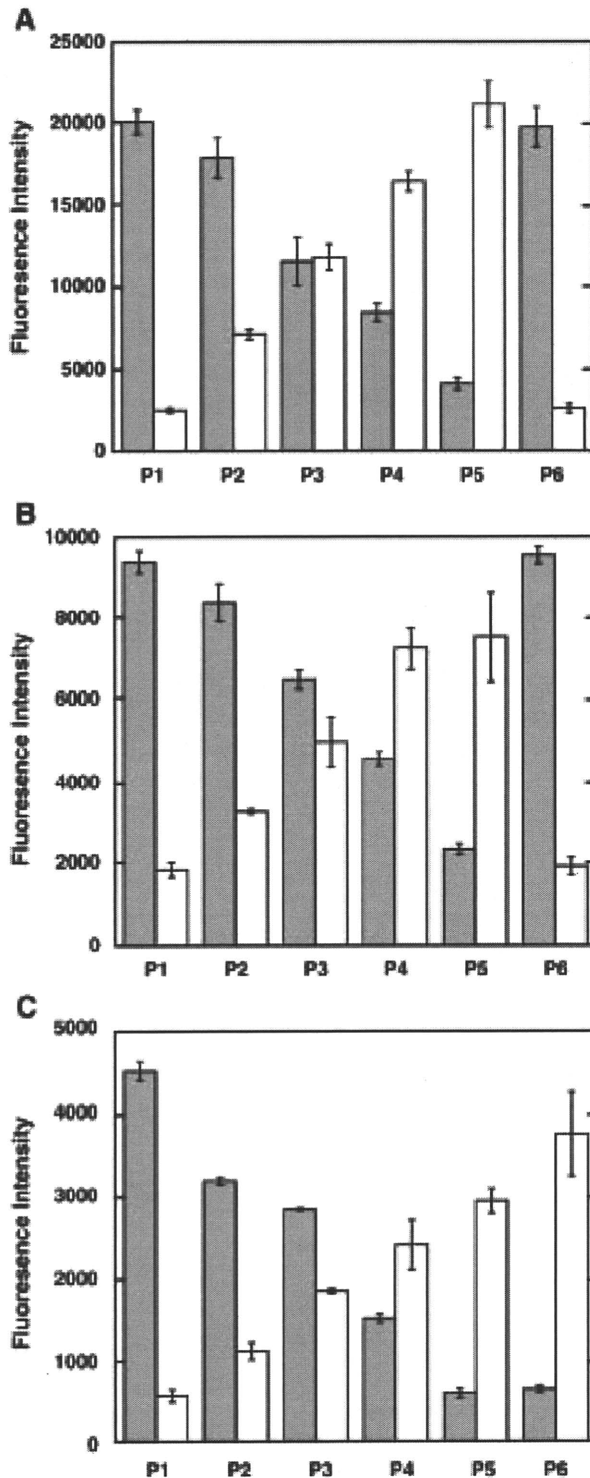


FIG. 2. Optimization of the ASOH and ASOCH assays using positive controls containing each mutation site. (A) Each BCP mutant sample was hybridized using ASOH with 10 nM Cy5-CPR1 (wild-type, A/G) or 10 nM Cy5-CPR2 (mutant, T/A). Black bars and white bars represent the FI of wild-type (A/G) and mutant (T/A), respectively. (B) Each PC mutation was hybridized using ASOH with 10 nM Cy5-PC2 (wild-type, G) or 10 nM Cy5-PC3 (mutant, A). Black bars and white bars represent the FI of wild-type (G) and mutant (A), respectively. (C) The nt1858 was hybridized using ASOCH with 10 nM Cy5-CA3 (C) and 100 nM CP-CA2 (T) or 10 nM Cy5-CA5 (T) and 100 nM CP-CA3 (C). Black bars and white bars represent the FI of C base and T base at nt1858, respectively. A series of standardized mixtures (mixtures of amplicons D, E, or F) were fixed onto the Bio-Strand. All data shown as the mean ± standard deviation of the FI of the different spots ($n = 8$).

**Determination of the relative abundance of BCP and PC mutants and nt1858T in patient serum DNA in a blind test** To determine the accuracy for quantification of mutations by ASOH and ASOCH, blind tests were carried out using DNAs from patient sera.

Fig. 3A shows the results of using ASOH to detect BCP sequences in ten patients (C1–C10). The patient samples were classified into three groups. The first group (C1, C2, C3, C8, and C9) showed the same pattern as P1 (0% mutant BCP). The second group (C4, C6, C7, and C10) showed the same pattern as P5 (100% mutant BCP). The last group, consisting only of patient C5, was similar to P3 (50% mutant BCP). All of the results, except for that of C5, were consistent with those obtained by direct sequencing. Direct sequencing of C5 exhibited that it contained 25% mutant BCP, which is lower that that observed by our method (Table 4).

Fig. 3B shows the results of classification of the same ten patient serum DNA samples on PC mutant using ASOH. The ten samples were classified into three groups. The first group (C1, C2, C4, C5, and C8) resembled P1 (0% mutant PC). The second group (C3, C6, C9, and C10) showed a pattern similar to P5 (100% mutant PC). The FI pattern of C7 was similar to that of P2 (25% mutant PC). All the results were consistent with the results from direct sequencing.

Fig. 3C shows the percent abundance of T-1858 in the ten patient samples (G1–G10) determined by the ASOCH method. The patient samples were classified into four groups. The first group (G4, G5, G6, G7, G9, G10) exhibited a similar pattern to P5 and P6 (100% T-1858). This group belonged to HBV genotypes B, C, D, and E. The second group included G2 and G8, and had FI patterns similar to that of P1 (0% T-1858). The second group belonged to HBV genotypes A and F. The third group (only G1) showed a similar FI pattern to P2 (25% T-1858). The last group (only G3) showed an FI pattern similar to P4 (75% T-1858). All the results, except for those of G1 and G3, were consistent with direct sequencing. Minor T base peak was not recognized at the direct sequencing for G1. G3 showed the different ratio between our method and direct sequencing. These inconsistencies should be due to the difficulty in recognizing a minor peak derived from nt1858 at our direct sequences.

**Estimation of the internal stability of the probes** Under optimized hybridization conditions, competitive hybridization (ASOCH) was not effective as a PC mutation (nt1896) assay. To determine the reason for this unexpected result, we estimated the free energy of internal stability ($\Delta G$) of each probe by the neighbor method using the Oligo5 program (Molecular Biology Insights, Cascade, USA), because a difference in internal stability at this site might have a significant impact on the ASOCH reaction. Figs. 4A–C show the calculated $\Delta G$ of the probes for BCP, PC, and nt1858, respectively. The $\Delta G$ of the PC mutation probe was determined to be very low (−12 kcal/mol), whereas the $\Delta G$s of the BCP probe and the nt1858 probe had moderate values (−6 kcal/mol). The internal stability of the PC mutation probes was approximately twice as high as that of the BCP or nt1858 probes. Thus, it appears that the high internal stability of the PC mutation probe may interfere with the exchange between the Cy5 probes and their non-labeled competitors, providing a possible explanation for the inability of ASOCH to increase probe specificity.

## DISCUSSION

Using the Handy Bio-Strand system, we have demonstrated that ASOH and ASOCH can semi-quantitatively determine population differences of HBV mutants. Our method is very reliable and applicable for determining the relative abundance of mutant species in sera from actual HBV patients. As shown in Table 4, while there were no differences between two methods used in the classification of major species, but contradictory results were observed for C5 (BCP mutation), G1 (nt1858), and G3 (nt1858). This inconsistency is likely caused by inaccurate quantification of minor peaks by direct sequence. It was speculated that our method was able to estimate the actual percent abundance of mixture species roughly (~25%). That is because the internal controls and the same patient samples always show similar hybridization patterns at the repeated experiments, and it was demonstrated that our method shows more reliable ratio than direct sequencing at the previous SNP genotyping study for hetero-type samples (22) (Data not shown).

Compared with other methods, the Handy Bio-Strand method has some advantages. It may have higher compatibility than other methods with regards to the quantification of various mutations, because the design of the Cy5 probes and their competitors are much simpler than the design of TaqMan and Molecular-beacon probes. Conventional methods such as PCR-RFLP (14, 15) and direct sequencing are very simple, but they pose difficulties as quantitative assays. Direct sequencing can only detect major HBV species and is unlikely to identify minor mutants. Waltz et al. also reported that direct sequencing could not detect minor populations (<20%) of HBV

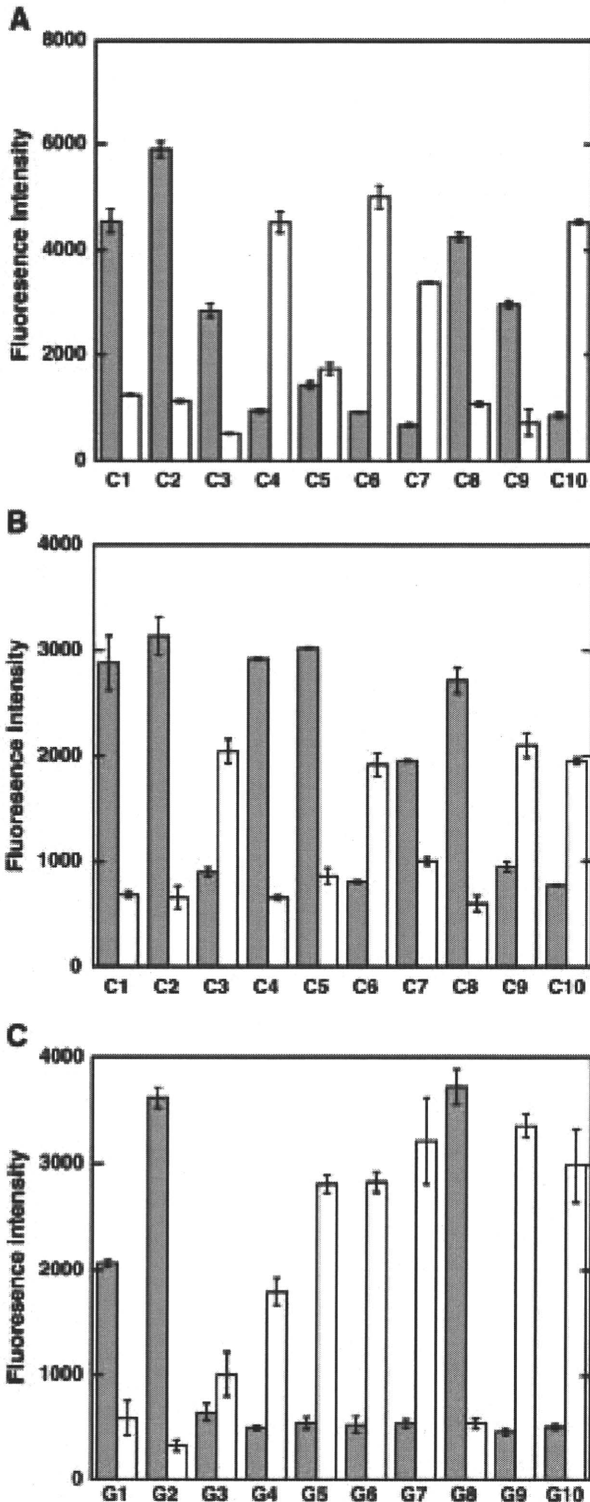


FIG. 3. Blind quantification of the BCP and PC mutations and nt1858T in DNA from patient serum. (A) The BCP mutation was analyzed by ASOH. Black bars and white bars represent the FI of wild-type (A/G) and mutant (T/A), respectively. (B) The PC mutation was analyzed by ASOH. Black bars and white bars represent the FI of wild-type (G) and mutant (A), respectively. (C) The nt1858 site was analyzed by ASOCH. Black bars and white bars represent the FI of C base and T base at nt1858, respectively. A series of standardized mixtures (P1–P6) and patient samples (C1–C10 or G1–G10) were separately fixed on each Bio-Strand. Conditions for both hybridization and internal positive controls were the same as those in Fig. 2. All data are shown as the mean ± standard deviation of the FI of the different spots ($n = 8$).

TABLE 4. Abundance determined by Handy Bio-Strand or direct sequencing.

| Target site | Sample | Handy Bio-Strand | Direct sequencing |
|---|---|---|---|
| BCP | C1 | W | W |
| | C2 | W | W |
| | C3 | W | W |
| | C4 | M | M |
| | C5 | W = M (1:1) | W > M (3:1) |
| | C6 | M | M |
| | C7 | M | M |
| | C8 | W | W |
| | C9 | W | W |
| | C10 | M | M |
| PC | C1 | W | W |
| | C2 | W | W |
| | C3 | M | M |
| | C4 | W | W |
| | C5 | W | W |
| | C6 | M | M |
| | C7 | W > M (3:1) | W > M (3:1) |
| | C8 | W | W |
| | C9 | M | M |
| | C10 | M | M |
| nt1858 | G1 | C > T (3:1) | C |
| | G2 | C | C |
| | G3 | C < T (1:3) | C = T (1:1) |
| | G4 | T | T |
| | G5 | T | T |
| | G6 | T | T |
| | G7 | T | T |
| | G8 | C | C |
| | G9 | T | T |
| | G10 | T | T |

W and M represent wild type and mutant type, respectively. Concerning to species mixtures, the ratios of wild type to mutant type (or C to T at nt1858) are shown under the abundance results.