

Prevalence of Hepatitis B Virus Subgenotypes and Basal Core Promoter, Precore Variants in Patients with Acute Hepatitis B in Central Vietnam

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Key Words

Hepatitis B virus · Acute hepatitis · Genotype · Phylogenetic analysis

Abstract

Objective: Hepatitis B virus (HBV) has been classified into 8 genotypes that have different geographic distributions. The clinical outcomes of acute hepatitis are dependent on genotype. The aim of this study was to investigate the distribution of HBV subgenotypes and basal core promoter (BCP)/pre-core (PC) regions in acute hepatitis patients in Central Vietnam to clarify the distributions and the clinical and virological differences. **Methods:** 27 patients with acute hepatitis B were studied. HBV subgenotypes and BCP/PC variants were determined by direct sequencing of the preS, BCP/PC regions, respectively. **Results:** HBV subgenotypes B4/Ba (n = 22) and C1/Cs (n = 5) were detected. Of the 27 patients, 3 developed fulminant hepatic failure, and all were infected with B4/Ba. Three patients had a BCP mutation, and 10 patients had a PC mutation in subgenotype B4/Ba. Three patients with C1/Cs had a BCP mutation. Two of 3 patients who progressed to fulminant hepatic failure had T1762, A1764, and A1896 simultaneously. None of the patients with acute, self-

limited hepatitis carried these triple mutations. **Conclusion:** The prevalent HBV subgenotypes in patients with acute hepatitis B in Central Vietnam were B4/Ba and C1/Cs. BCP/PC variants have an association with the development of fulminant hepatic failure in subgenotype B4/Ba.

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Introduction

There are approximately 350 million people who are infected with hepatitis B virus (HBV) worldwide [1]. HBV infection has a variable clinical course, including self-limited acute hepatitis, fulminant hepatic failure, chronic hepatitis, and progression to cirrhosis and hepatocellular carcinoma [2]. Therefore, HBV infection is one of the world's most important health problems. HBV has been classified into 8 major genotypes on the basis of divergence of 8% in the full-length sequence, and it can be further classified into several subgenotypes [3, 4]. Each subgenotype has a unique geographic distribution and virological characteristics. Vietnam is one of the most endemic areas of HBV infection, and the prevalence of HBV subgenotypes in patients with chronic hepatitis in

Vietnam has been reported [5–8]. HBV subgenotypes C1 and B4 are frequently found in Vietnam. Recently, genotype I, a recombination with genotypes A and C, has been reported from Northern Vietnam [8]. Vietnam has a long and narrow shape, and it has been traditionally divided into North, South, and Central regions. Most studies dealing with HBV infection were conducted in Northern and Southern Vietnam and HBV subgenotypes in patients with chronic hepatitis. Therefore, the distribution of HBV subgenotypes in patients with acute hepatitis in Central Vietnam remains unknown. Basal core promoter (BCP)/precore (PC) region variants may be associated with fulminant hepatic failure in acute hepatitis [9]. However, the clinical roles of BCP/PC variants in acute hepatitis are still controversial [10, 11]. Relationships between BCP/PC region variants and clinical course would depend on HBV subgenotypes and geographic distribution. The effects of BCP/PC variants in Vietnamese patients with acute hepatitis are little known. The aim of this study was to investigate the distribution of HBV subgenotypes and BCP/PC regions in patients with acute hepatitis B in Central Vietnam to clarify their distributions, as well as clinical and virological differences.

Patients and Methods

Twenty-seven Vietnamese patients (14 men, 13 women) with acute hepatitis B who were treated at Hue University Hospital were enrolled in this study. The patients' mean age was 31.0 ± 8.5 years (range 19–49). Each patient had high titers of hepatitis B surface antigen (HBsAg) and IgM class antibody against HBV core antigen, elevated serum levels of alanine aminotransferase, and absence of antibodies against hepatitis A virus and hepatitis C virus. Fulminant hepatic failure was defined as the development of hepatic encephalopathy and prolongation of the prothrombin time less than 40% during the course of acute hepatitis [12]. It was necessary to discriminate between initial HBV infection and acute exacerbation of asymptomatic HBV carrier. To exclude acute exacerbation of asymptomatic HBV carrier, the patients with high risks for chronic HBV infection such as family history of HBV infection, past history of blood transfusion in childhood were excluded. Written informed consent was obtained from each patient participating in the study, and the study was carried out in accordance with the 1975 Helsinki Declaration.

DNA Amplification and Sequencing

HBV viral loads and direct sequencing of the preS, polymerase, and PC/core regions were performed using serum samples taken within 2 days after admission at hospital. HBV DNA was isolated from peripheral blood with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The HBV-DNA quantitative viremia load was determined using real-time PCR [13]. Nested polymerase chain reaction (PCR) analysis and direct sequencing of the preS,

polymerase, and PC/core regions were performed as reported previously [14]. In brief, each 50- μ l PCR reaction contained 100 nM each primer, 1 ng template DNA, 5 μ l GeneAmp 10 \times PCR buffer, 2 μ l dNTP, and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, Calif., USA). Primers for the preS region were sense (TCACCTATTCTTGGGAACAAGA) and antisense (GGCAC-TAGTAAACTGAGCCA), and primers for the PC/core region were sense (GTTGCATGGAGACCACCGTGAAC) and antisense (CTGACTACTAATTCCTGGATGCTGGGTCT). Amplification conditions consisted of 5 min at 94° followed by 40 cycles of 94° for 30 s, 55° for 30 s, and 72° for 1 min in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The second PCR was done in the same reaction buffer with the first-round PCR product as template and the following sets of primers: for the preS region, sense (TCACCTATTCTTGGGAACAAGA) and antisense (AGAAGATGAGGCATAGCAGC), and for the PC/core region, sense (ATGTCGACAACCGACCTTGA) and antisense (GTATGGTGAGGTGAACAATG). The PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under UV light. The PCR products were purified and then sequenced with the second-round PCR primers with a dye terminator sequencing kit (Big Dye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems) by an ABI 310 DNA Sequencer (Applied Biosystems). The neighbor-joining method [15] was used for phylogenetic analysis of the preS region to classify HBV into subgenotypes. Bootstrap analysis (1,000 replicates) was performed [16].

Statistical Analyses

The data are expressed as mean \pm SD. Contingency table analysis with Fisher's exact probability test was used for comparisons between groups; $p < 0.05$ was considered statistically significant. The statistical software used was SPSS (SPSS Inc., Chicago, Ill., USA).

Results

The results of the phylogenetic analyses of the HBV subgenotypes of the 27 patients with acute hepatitis B are shown in figure 1. 22 patients had subgenotype B4/Ba, and 5 patients had subgenotype C1/Cs. There were 24 patients with acute self-limited hepatitis and 3 patients who developed fulminant hepatic failure. In patients with acute self-limited hepatitis, HBV subgenotypes B4/Ba ($n = 19$) and C1/Cs ($n = 5$) were found. All patients who progressed to fulminant hepatic failure had HBV subgenotype B4/Ba. There was no distinctive clustering among patients who progressed to fulminant hepatic failure defined by phylogenetic analyses of preS region, and no specific mutation in preS region was found among patients with fulminant hepatic failure. Clinical characteristics according to clinical course and subgenotypes are shown in table 1. The results of the BCP/PC sequence according to HBV subgenotypes and clinical course are

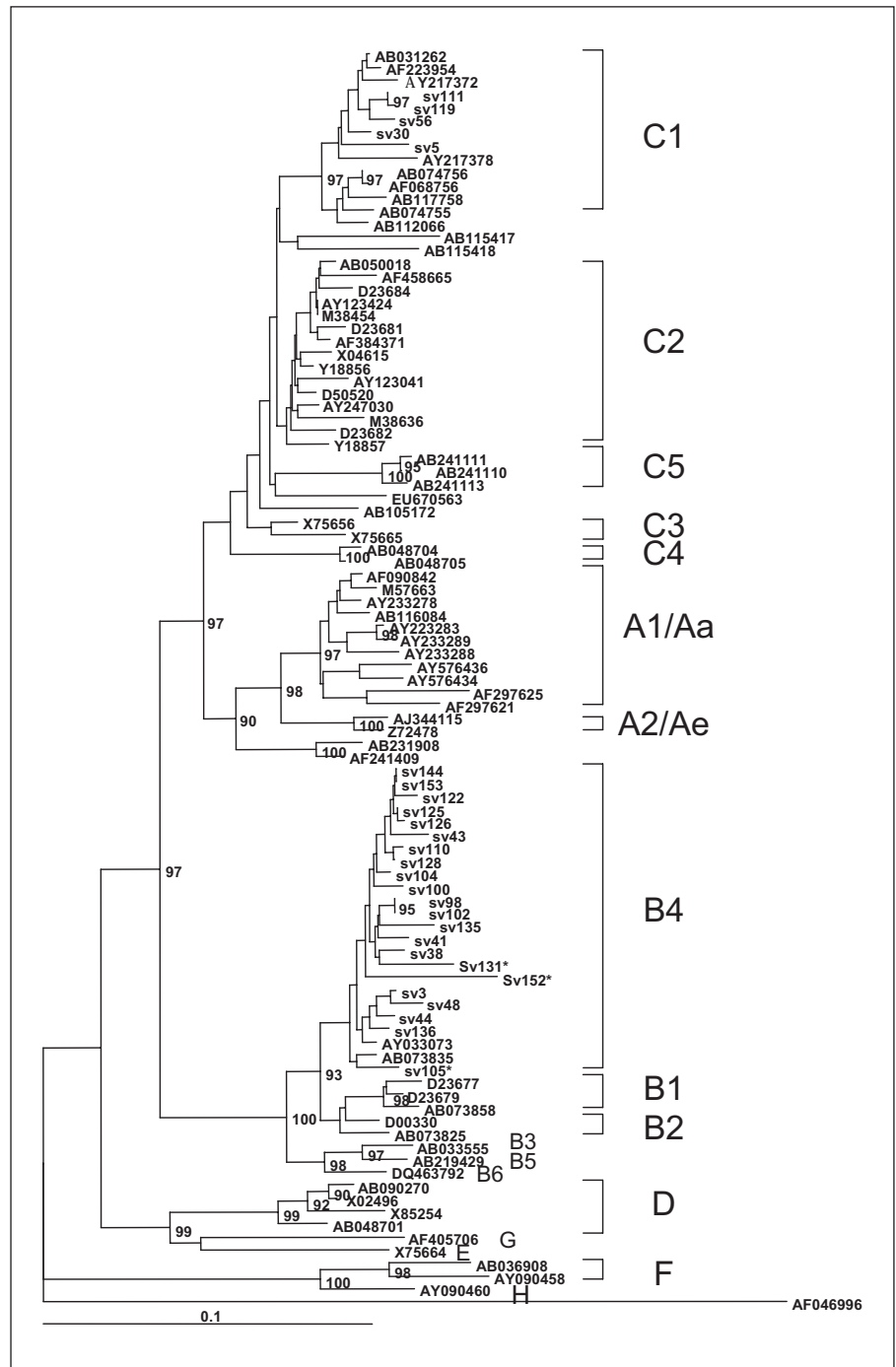


Fig. 1. Phylogenetic analysis of 27 sequences from the preS region of HBV of acute hepatitis patients and 70 reference strains from a database shown by accession number. Asterisks indicate the strain that progressed to fulminant hepatic failure. Phylogenetic analysis was performed by the neighbor-joining method with Woolly monkey HBV (AF046996) as the outgroup. The scale bar indicates genetic distance.

shown in figure 2. Mutations of BCP and PC regions were frequently found in nt 1754, 1762, 1764, and 1896. Six B4/Ba subgenotypes had a G1754 mutation, but all C1/Cs subgenotypes had a T1754 mutation. BCP variants in nt1762 and nt 1764 appeared to be more frequently found in subgenotype C1/Cs than in subgenotype B4/Ba (OR

0.105; 95% CI 0.012–0.917; $p = 0.056$). The majority of C1/Cs subgenotypes had the C1858 variant, and 1 patient had the T1858 variant. However, all B4/Ba subgenotypes had the T1858 variant. C1/Cs subgenotypes, which carry the C1858 variant, did not have the A1896 mutation and 10 of 22 B4/Ba subgenotypes with T1858 had the A1896 mu-

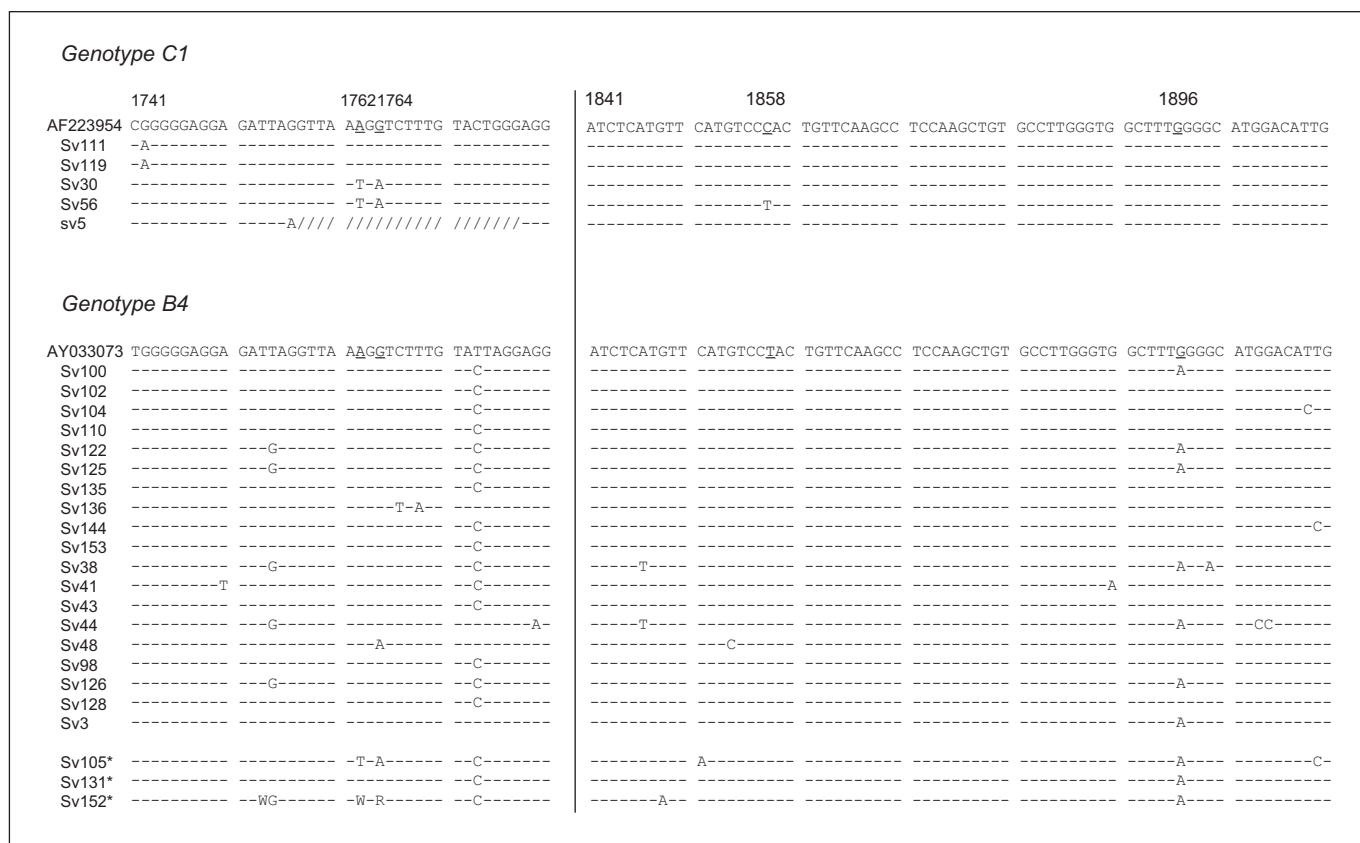


Fig. 2. Alignment of the nt sequence of 1741–1780 and 1841–1910 is shown. In the sequence alignment, dashes indicate nt identical to consensus sequence AF223954 for C1 and AY033073 for B4, respectively. Asterisks indicate the strains that progressed to fulminant hepatic failure.

Table 1. Clinical characteristics

	Acute hepatitis (B4) (n = 19)	Acute hepatitis (C1) (n = 5)	Fulminant hepatitis (B4) (n = 3)	p value
Age, years	31.6 ± 8.7	27.4 ± 8.4	33.0 ± 9.0	NS
Male/female	9/10	3/2	2/1	NS
AST, IU/l	861.7 ± 436.2	808.6 ± 202.4	547.6 ± 267.3	NS
ALT, IU/l	1,633.6 ± 1,526.4	1,667.6 ± 843.6	845.0 ± 91.9	NS
Total bilirubin, µmol/l	187.9 ± 125.7	112.7 ± 72.7	262.3 ± 158.8	NS
Prothrombin time, %	80.1 ± 14.4	87.1 ± 12.9	35.5 ± 19.6	<0.05
HBeAg: positive/negative*	9/2	1/3	3/0	NS
HBV DNA levels, copies/ml	2.8 × 10 ⁷	1.4 × 10 ⁷	1.2 × 10 ⁷	NS

Data are expressed as mean ± SD.

AST = Aspartate aminotransferase; ALT = alanine aminotransferase; HBV = hepatitis B virus.

* Presence at admission and 9 patients were not available.

tation. There were no significant differences in the other BCP and PC variants between the C1/Cs and B4/Ba subgenotypes. For subgenotype B4/Ba, T1762 and A1764 were detected more frequently in patients with fulminant hepatic failure (66.7%) than in patients with acute self-limited hepatitis (5.3%) (OR 0.024; 95% CI 0.001–0.544; $p = 0.029$). The patients with fulminant hepatic failure more frequently had A1896 (100%) than those with acute self-limited hepatitis (36.8%) ($p = 0.052$). Two of 3 patients with fulminant hepatic failure and no patients with acute self-limited hepatitis carried the T1762, A1764, and A1896 mutations simultaneously ($p = 0.010$).

Discussion

The prevalence of HBV infection in Vietnam has been reported to be 19.0%, and Vietnam is considered to be a high endemic area of HBV infection [7]. Subgenotypes B4/Ba and C1/Cs have been found in patients with acute hepatitis B in Central Vietnam. This distribution was similar to previous studies of patients with chronic HBV infection from Northern and Southern Vietnam, which indicates no significant regional differences in Vietnam.

The distributions of the HBV subgenotypes are changing gradually due to international interchange of patients. The study of HBV genotypes in Japanese immigrants and natives in Bolivia indicated that transmission of HBV genotypes was easy and widespread [17]. The prevalence of HBV BCP/PC variants has changed in the USA because of immigrants from endemic areas [18]. Studies from Japan and Ireland reported that the prevalence of HBV subgenotypes that were rare in Japan and Ireland appears to be increasing in patients with acute hepatitis B [19, 20]. However, Central Vietnam was still a conservative area and did not have the same tendency. Surveillance of HBV subgenotypes will need to be continued, because HBV may easily spread to the general population within a short period [17, 21].

The predominant HBV subgenotype in Central Vietnam was the B4/Ba subgenotype. Genotype B of HBV was classified into 6 subgenotypes. Each subgenotype has a different distribution. HBV subgenotype B1 has only been found in Japan. Subgenotype B2 has been found throughout Asia. Subgenotype B3 has been found in Indonesia, B4 in Vietnam, and B5 in the Philippines. Subgenotype B6 was recently reported from the Arctic area [22]. Virological differences between subgenotypes have been demonstrated, and HBV genotype B could be subdivided into two major subtypes based on recombination

with genotype C over the core promoter, PC region and core gene [23]. Subgenotypes without this recombination are B1 (also known as Bj) and B6. Subgenotypes with recombination are B2, B3, B4, and B5, which used to be called Ba. Insufficient information is available about the clinical differences of acute hepatitis with different B subgenotypes. However, one multicenter study reported that subgenotype B1(Bj) is an independent factor associated with the development of fulminant hepatic failure [24]. In the present study, 3 patients who developed fulminant hepatic failure were infected with subgenotype B4 (Ba), but the number of patients was very small. Further studies are needed to clarify the association between subgenotype B4 and the development of fulminant hepatic failure.

With respect to BCP/PC variants, A1896 mutation is associated with fulminant hepatic failure [9]. HBV genotype B was commonly found with the A1896 mutation [25]. Seven patients with acute self-limited hepatitis had the A1896 mutation. Determination of a single mutation at 1896 has a limited ability to predict the development of fulminant hepatic failure. Two of 3 patients who progressed to fulminant hepatic failure had both T1762 and A1764 in BCP and A1896 in PC. None of the patients with acute self-limited hepatitis B carried T1762, A1764, and A1896 mutations simultaneously. These findings suggest that subgenotype B4 and BCP/PC variants have an association with progression to fulminant hepatic failure. Therefore, investigation of BCP/PC variants in subgenotype B4 will be useful for predicting fulminant hepatic failure and developing treatment protocols, as has been previously reported for subgenotype C2/Ce [14].

Seroconversion from HBeAg to anti-HBe antibody occurs early in patients who progressed to fulminant hepatic failure which related to A1896 in PC. However, 3 patients who developed fulminant hepatic failure with A1896 in PC were positive for HBeAg at the first day of admission. HBeAg seroconversion might be delayed in patients with HBV subgenotype B4. Monitoring the HBeAg was not performed and the number of patients was insufficient. Then further studies will be needed to conclude this issue.

The other genotype that was found in the present study was HBV subgenotype C1. Genotype C is classified into 5 subgenotypes, designated C1–C5. Subgenotype C1 has been reported among patients in Vietnam, Thailand, and southern areas of China, including Hong Kong. Subgenotype C1 frequently has C1858 in the PC region. nt 1858 and nt 1896 were the pairs in the hairpin loop of the encapsidation sequence, and pairing of C1858 and G1896

is a stable structure. As a result, C1858 prevents the emergence of mutation A1896 in PC [26]. There were no patients with A1896 in subgenotype C1. This result also confirmed the virological features of nt 1858 and nt 1896 in the PC region. There were too few patients to conclude that subgenotype C1, especially with C1858, has a low potential for progressing to fulminant hepatic failure. However, C1858 may be one reason why subgenotype C1 strains were not detected in fulminant hepatic failure patients. The mutation at nt1896 in subgenotype B4 was useful for predicting fulminant hepatic failure, but mutation at nt1896 could not predict the progression to fulminant hepatic failure in subgenotype C1. The relationship of nt1896 in the PC region with fulminant hepatic failure needs to be considered with respect to the HBV subgenotypes. nt1896 mutation depends on the HBV subgenotype. Most A genotypes have C1858, and, infrequently, they have A1896, because A1896 pairs with T1858. However, some studies reported that genotype A, which emerged with a T1858 mutation, developed to an A1896 mutation. HBV easily develops several mutations, and these minor variants were taken into consideration.

Subgenotype C1 with TCC at nucleotides 1856–1858 caused more aggressive liver disease in patients with chronic hepatitis [27], but the association between this mutation and patients with acute hepatitis is unknown. There were no patients with TCC at codon 15 of the PC

region, and it was difficult to investigate the relationship between TCC at codon 15 and clinical features in patients with acute hepatitis in the present study. The difference in virological features between each subgenotype has been reported, but knowledge of the effect on the clinical course is limited. Thus, further studies are needed to reveal the impact of HBV subgenotypes on the clinical course of patients with acute hepatitis B.

The complete distinction between patients with acute exacerbation of asymptomatic HBV carrier from those with initial HBV infection who progressed to fulminant hepatic failure was the limitation of this study. One of the best ways to discriminate between initial HBV infection and acute exacerbation of asymptomatic HBV carrier was to confirm the negativity of HBsAg levels before onset of acute hepatitis by previous medical records such as blood donation screening, labor and delivery screening, or employment health screening. We could not get this information from all patients, thus some patients with acute exacerbation of asymptomatic carrier might be included in this study.

In conclusion, the prevalent HBV subgenotypes in patients with acute hepatitis B in Central Vietnam were B4/Ba and C1/Cs. The progression to fulminant hepatic failure in patients with subgenotype B4 was closely associated with the simultaneous mutations of T1762 and A1764 in the BCP and A1896 in the PC region.

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