MAJOR ARTICLE

Hepatitis B Virus Genotypes in Alaska Native People with Hepatocellular Carcinoma: Preponderance of Genotype F

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(See the editorial commentary by Tanaka and Mizokami, on pages 1–4.)

Background. The development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis B virus (HBV) infection has been associated with specific HBV genotypes and the presence of specific mutations.

Methods. From a cohort of Alaska Native people with chronic HBV infection, we genotyped 47 patients with HCC and 1129 patients without HCC, and we tested patients with HCC and control patients for mutations in the basal core promoter and precore regions.

Results. Genotype F was found in 68% of patients with HCC, versus 18% of those without HCC ($P < .001$). For patients with genotype F, the median age at diagnosis of HCC was lower than that for patients with other genotypes (22.5 vs. 60 years, respectively; $P = .002$). Overall, there were no significant differences in the number of basal core promoter and precore region mutations between patients with HCC and control patients.

Conclusions. We found a significant association between genotype F and the development of HCC among Alaska Native people with chronic HBV infection but no significant association between HCC and basal core promoter or precore mutations in genotype F.

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer-related death [1]. Chronic hepatitis B virus (HBV) infection is a major risk factor for the development of HCC, and a high rate of HBV-associated HCC has been found among Alaska Native people. In a 1990 study of chronic carriers of HBV among Alaska Native people, the annual incidence rate of HCC was 387/100,000 for men and 63/100,000 for women [2], whereas the worldwide age-adjusted incidence rates per 100,000 were 14.67 for men and 4.92 for women [3].

To date, 8 HBV genotypes (A–H) with specific geographic distributions have been identified worldwide [4, 5]. There has been much interest in the association of these genotypes with adverse outcomes for persons with chronic HBV infection. An association between genotypes B and C and the development of HCC has been found, with HCC occurring more frequently among those with genotype C than among those with genotype B [6–9]. The presence of an A→T mutation at nt 1762 in the basal core promoter region, coupled with a G→A mutation at nt 1764, has been associated with the development of HCC regardless of whether infection is with HBV genotype B or C [10]. A mutation in the precore region—most commonly a G→A stop codon mutation at nt 1896—has been implicated in the development of HCC [11–13]. However, the precise role of these mutations in the progression to HCC remains controversial, and only 2 or 3 HBV genotypes are found in most regions of the world, thereby limiting genotype comparisons.

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The purpose of this study was to determine the distribution of HBV genotypes among Alaska Native people with chronic HBV infection with HCC and among those without HCC. We also sought to determine the prevalence of mutations in the basal core promoter and precore regions in patients with HCC and in matched control patients.

PATIENTS AND METHODS

Patients with HCC

Between 1969 and 2003, HCC was diagnosed in 52 patients with chronic HBV infection, which was defined as being positive for hepatitis B surface antigen for at least 6 months. These patients were identified from a registry of 1536 Alaska Native people with chronic HBV infection and, since 1982, have been undergoing semiannual surveillance for HCC by means of testing for serum α-fetoprotein (AFP) levels.

For 49 of the 52 patients with HCC, stored serum samples were available from the Alaska Area Native Health Services/Arctic Investigations Program of the Centers for Disease Control and Prevention (CDC) serum bank in Anchorage. One patient declined consent; the remaining patients were screened for hepatitis C virus (HCV) infection, and 4 (8.3%) of the 48 patients were anti-HCV positive. One patient was found to be HCV RNA positive by polymerase chain reaction (PCR) and was excluded from the study, whereas 2 patients who were HCV RNA negative were included. Although no serum samples from the fourth patient were available for HCV RNA testing, this patient was retained in the study because the HCC diagnosis was made when the patient was 22 years of age and because it was believed that the HCC was unlikely to be secondary to HCV infection. Thus, 47 patients with chronic HBV infection and HCC were included in the final study group.

A tissue diagnosis of HCC was made for 44 patients by liver biopsy (13 patients), at the time of surgical resection (28 patients), or at autopsy (3 patients). For 3 patients, abnormal imaging study results and elevated AFP levels led to the diagnosis of HCC. At the time of diagnosis, these patients had abdominal ultrasound and computed tomography scans showing at least 2 lesions of 3–13 cm in diameter and AFP levels of 1637–15,251 ng/mL. All 3 patients died within 2 years of diagnosis.

This study was approved by the institutional review boards (IRBs) of the Alaska Area Indian Health Services and the CDC in Atlanta and by 2 Alaska Native boards, the Alaska Native Tribal Health Consortium and Yukon-Kuskokwim Health Corporation. Informed consent was obtained from all patients with HCC. Informed consent was obtained from all living patients without HCC, and information for deceased patients was used with IRB permission.

Non-HCC Cohort and Matched Control Patients

Designated the "non-HCC cohort," 1129 patients with chronic HBV infection but without HCC were genotyped. To determine whether there was an association between patient age and specific genotype, we divided the HCC and non-HCC cohorts into 2 birth groups. We tested persons in the non-HCC cohort born on or after 1 January 1940, 1 January 1960, and 1 January 1980. Between these 3 groups, differences in genotype did not affect the development of HCC. We arbitrarily chose 1 January 1960 as our cutoff point, since approximately one-half of the non-HCC cohort was born before that date.

To ensure that the comparison group actually reflected persons who did not have HCC, an additional comparison was made to a matched control group of carriers of HBV. Control patients were randomly selected from within the non-HCC cohort and were matched by sex, date of birth within 5 years of that of the case patient, and geographic region; control patients were known to be carriers of HBV at the time of HCC diagnosis in the case patient. Records for control patients were reviewed to ensure that none had an AFP level >10 ng/mL within 5 years before or after the date of diagnosis in the case patient. An exception was made for elevated AFP levels due to pregnancy, as long as the level subsequently decreased to <10 ng/mL. All control patients had AFP levels measured at least once during the 5 years before or after the date of diagnosis of HCC in the case patient. These patients were designated the matched control group.

Determination of HBV Genotype

HBV genotype testing was done for the 47 patients with HCC and for the entire non-HCC cohort.

DNA extraction. Viral DNA was extracted from 200 μL of serum by use of the Roche High Pure Viral Nucleic Acid Kit (Roche Diagnostics), in accordance with the manufacturer's instructions.

Nested PCR. Primers specific to the S gene of the HBV genome were used for nested PCR. The first-round PCR mixture was amplified with primers 5′-CTA GGA CCC CTG CTC GTG TT-3′ (outer sense, position 179–198) and 5′-CGA ACC ACT GAA CAA ATG GCA CT-3′ (outer antisense, position 681–704). For the second round of PCR amplification, the internal primers 5′-CTA GAC TCG TGG TGG ACT TCT CT-3′ (inner sense, position 248–270) and 5′-GAC TGA GGC CCA CTC CCA CCA TA-3′ (inner antisense, position 639–658) were used. To avoid false-positive results, the precautions and procedures suggested by Kwok and Higuchi [14] were strictly followed.

DNA sequencing and genotyping. PCR products from the second-round reaction were sequenced by use of the ABI Prism BigDye Terminator cycle-sequencing ready-reaction mixture (version 3.0; Applied Biosystems) and the internal antisense

primer. The sequencing products were analyzed on an ABI Prism 310 genetic analyzer (Applied Biosystems). The sequences obtained were aligned with GenBank sequences corresponding to HBV genotypes A–H. The GenBank accession numbers were as follows: genotype A—V00866, X70185, S50225Z35717, AJ309371, and X51970; genotype B—D23679, D00330, M54923, D00331, X75660, and D23678; genotype C—X52939, X75665, D23683, D23682, M38636, D00630, X14193, X04615, X75656, X75665, AY040627, and AB026811; genotype D—Z35716, M12393, X85254, X75668, J02203, X02496, X72702, M32138, X77310, X68292, X75668, L27106, X59795, X75662, and AF280817; genotype E—X75657, X75664, L29017, and X75664; genotype F—AB036920, X69798, X75658, X75663, X69798, and X75658; genotype G—AB056516, AB064313, AB064315, and AB064316; and genotype H—AY090457, AY090460, and AY090454. Sequencher software (version 4.1; Gene Codes Corporation) was then used to determine genotype identity by means of sequence similarity.

The GenBank accession numbers for patients with HCC were DQ845349–DQ845364, DQ845366–DQ845382, DQ845384, DQ845385, DQ849340, DQ849428, DQ862566, DQ862574, DQ862577, DQ862592, DQ862599, DQ862618, DQ862640, DQ862674, DQ862735, and DQ873422. Patients without HCC who gave consent were selected from GenBank accession numbers DQ849336–DQ849572, DQ849575–DQ849588, DQ849591–DQ849606, DQ849616–DQ849624, DQ852741–DQ852921, DQ852924–DQ852984, DQ852988–DQ852989, DQ859277–DQ859378, DQ859408–DQ859607, DQ859610–DQ859622, DQ859626–DQ859721, DQ862555–DQ862769, DQ862772–DQ862778, DQ873413–DQ873421, and DQ873423–DQ873500.

Detection of Basal Core Promoter and Precore Mutations

Mutations and/or wild-type sequences in the basal core promoter and precore regions were detected with the INNO-LiPA HBV precore line probe assay (Innogenetics), in accordance with the manufacturer's instructions. Serum samples for the line probe assay were available for 45 of the original 47 patients with HCC.

Genotype-matched control patients were selected for case patients with genotype F by matching them with persons with genotype F in the non-HCC cohort; control and case patients were matched one to one by age, sex, and geographic region. Twenty-four of the 32 case patients with genotype F were matched to a control patient. HBV DNA was not found in the tested specimens from 4 of the control patients; thus, results were obtained for 20 control patients. Control patients were selected for case patients with genotypes A, C, and D by 3-to-1 matching by age, sex, geographic region, and HBV genotype. Eight case patients were matched to 3 control patients each, 1 case patient was matched to 2 control patients, 2 case patients were matched to 1 control patient, and 3 case patients were not matched to any control patients, for a total of 28 control patients. HBV DNA was not found in the tested specimens from 5 of the control patients, yielding results for 23 control patients with genotypes other than F.

HBV DNA was extracted as described previously and was amplified by use of the 5′ biotinylated primers provided by the manufacturer of the line probe assay (Innogenetics). After nested PCR, 10 μL of each sample with detectable HBV DNA was applied to the line probe assay strips in accordance with the manufacturer's instructions. The presence or absence of mutations was determined on the basis of the pattern of reactive bands. Basal core promoter nt 1762/1764 and precore nt 1896 were classified as entirely wild type, mixed (mixture of wild-type and mutated sequences), or entirely mutated.

Statistical Analysis

Comparisons of categorical variables within the group of case patients were done by use of the χ^2, Fisher's exact, or randomization test, as appropriate. Comparisons of median age were done with nonparametric tests. For comparison of genotypes of case patients with those of other carriers, χ^2 tests were used, with Mantel-Haenszel stratification when appropriate. Multiple logistic regression analysis was done with case patients or non-HCC cohort members as an outcome variable and with region, sex, and birth cohort as predictor variables. Models with all 2-way interactions were examined. A cutoff of $P < .05$ (2-sided) was used to determine statistical significance. For assessment of family relationships, first-degree relatives were defined as biological parents, children, or siblings of a patient with HCC. Analyses of case patients and matched control patients were done by use of McNemar's test. The significance of mutations was assessed with a χ^2 test for trend.

RESULTS

Characteristics and genotypes of patients with HCC. Thirty-five (74%) of the 47 patients with HCC were male. The median age at diagnosis of HCC was 31 years (mean, 39.1 years; range, 8–80 years). Four HBV genotypes were found: genotypes A, C, D, and F. Genotype F was found in 32 (68%) of the 47 patients, genotype A in 5 (11%), genotype C in 5 (11%), and genotype D in 5 (11%) (figure 1). Genotype B was not found in any of the patients with HCC. The median age at diagnosis of HCC in persons infected with genotype F was 22.5 years, compared with a median age of 60 years for those infected with genotypes A, C, and D (combined; $P = .002$).

HBV genotypes in the non-HCC cohort. Genotype D was found in 660 (58%) of the 1129 patients in the non-HCC cohort, genotype F in 204 (18%), genotype A in 143 (13%), genotype C in 75 (7%), and genotype B in 46 (4%) (figure 1). Genotype H, found in 1 patient, was omitted from further

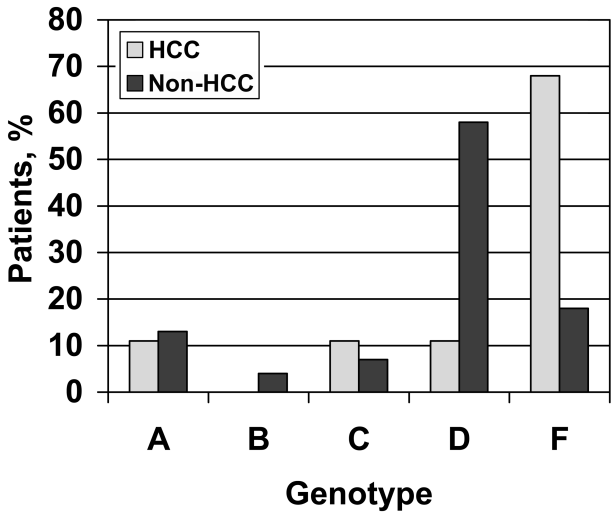

Figure 1. Frequency of hepatitis B virus (HBV) genotypes among patients with chronic HBV infection and hepatocellular carcinoma (HCC), versus those without HCC.

analysis. Genotypes were found to be clustered by region, with most persons in a community having the same genotype. Of 88 communities with >1 person tested, 16 (18%) had only 1 genotype. In 26 additional communities (30%), ⩾75% of persons tested had the same genotype. There were significant differences in genotype across regions ($P < .001$).

Comparison of genotype frequency among patients with HCC versus the non-HCC cohort. Genotype F was found in HBV-infected patients with HCC significantly more frequently than in the non-HCC cohort ($P < .001$; odds ratio [OR], 9.67 [95% confidence interval {CI}, 4.95–19.1]). In the non-HCC cohort, the proportion of persons infected with genotype F varied by the 6 geographic regions in Alaska ($P < .001$) (figure 2). After controlling for region, the association between HCC and genotype F remained significant ($P < .001$; OR, 7.73 [95% CI, 3.69–16.4]).

The proportion of persons in the non-HCC cohort who were born on or after 1 January 1960 varied significantly by genotype ($P < .001$). Twelve (48%) of 25 patients with HCC who were born before 1 January 1960 were infected with HBV genotype F, compared with 71 (14%) of 505 patients in the non-HCC cohort, and 20 (91%) of 22 patients with HCC who were born on or after 1 January 1960 were infected with HBV genotype F, compared with 133 (21%) of 624 patients in the non-HCC cohort. When adjusted for birth cohort, the association between HCC and genotype F was still significant ($P < .001$; OR, 10.63 [95% CI, 5.4–21.3]). The frequency of genotype F among patients with HCC, versus those in the non-HCC cohort, remained significant when adjusted for both region and birth cohort ($P < .001$; OR, 8.88 [95% CI, 4.27–18.5]). After region, sex, and birth cohort were adjusted for in multiple logistic regression analysis, this association with genotype F remained significant ($P < .001$; OR, 8.9 [95% CI, 4.4–17.8]). After inclusion of genotype C as another possible predictor in the multiple logistic regression analysis, both genotypes F and C were significantly associated with HCC, compared with the other genotypes (genotype F: $P < .001$; OR, 11.7 [95% CI, 5.40–25.4]; genotype C: $P = .012$; OR, 4.74 [95% CI, 1.40–16.0]).

Adjustment for first-degree relatives with HCC. We sought to determine whether there was an association between HBV genotype and familial history of HCC. Thirteen persons with HCC were first-degree relatives of other persons with HCC; genotype F was found in 4 family groups (11 persons), and genotype C was found in 1 family (2 persons). After omission of patients within the same family who developed HCC after the index case in each family, 39 patients with HCC were included in the analysis. Most case patients (31/39 [79%]) were male, with a median age at diagnosis of 37 years (mean, 40.9 years; range, 8–80 years). The distribution of HBV genotypes was as follows: genotype F, 25 patients (64%); genotype A, 5 patients (13%); genotype D, 5 patients (13%); and genotype C, 4 patients (10%). The median age at diagnosis of HCC among persons with genotype F remained significantly lower than the median age among those with genotypes A, C, and D combined (23 vs. 58.5 years, respectively; $P < .009$). When first-degree relatives were removed from the analysis, the association

Table 1. Prevalence of the hepatitis B virus (HBV) basal core promoter T1762/A1764 mutation among patients with hepatocellular carcinoma (HCC), versus matched control patients from the non-HCC cohort.

Genotype, patient group	No. (%) of patients[a]			P[b]
	Wild type	Mutated	Mixture	
F				
HCC	19 (59)	1 (3)	12 (38)	.009
Control	6 (30)	5 (25)	9 (45)	
A				
HCC	0	2 (50)	2 (50)	.05
Control	5 (71)	1 (14)	1 (14)	
C				
HCC	1 (25)	2 (50)	1 (25)	.047
Control	6 (67)	0	3 (33)	
D				
HCC	1 (20)	1 (20)	3 (60)	.03
Control	6 (86)	0	1 (14)	
All				
HCC	21 (47)	6 (13)	18 (40)	.68
Control	23 (53)	6 (14)	14 (33)	

NOTE. Control patients were matched by genotype, sex, age at diagnosis, and region of residence.

[a] Results were classified by those patients with wild-type HBV only, those with mutated virus only, or those with both wild-type and mutated virus (mixture).

[b] Determined by the χ^2 test for trend.

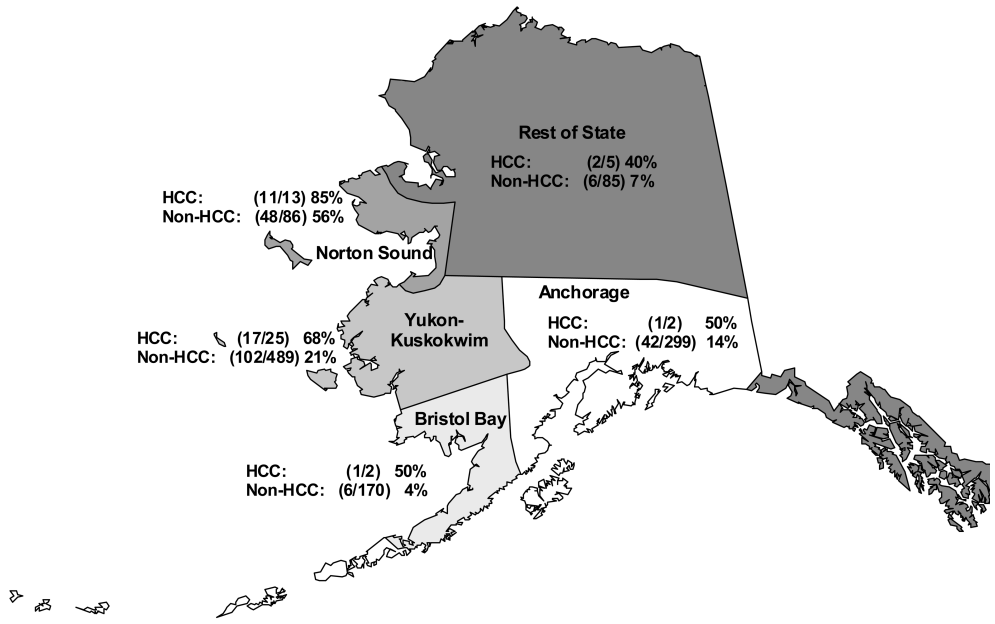

Figure 2. Percentage of patients with hepatitis B virus (HBV) genotype F infection and hepatocellular carcinoma (HCC), versus those without HCC, by region in Alaska.

of genotype F with HCC in this population remained statistically significant after adjustment for region ($P < .001$; OR, 6.52 [95% CI, 2.97–14.5]) and for region and birth cohort ($P < .001$; OR, 7.16 [95% CI, 3.26–15.7]). After region, sex, and birth cohort were adjusted for in multiple logistic regression analysis, this association with genotype F remained significant ($P < .001$; OR, 7.5 [95% CI, 3.57–15.6]).

Matched analysis of case and control patients. When compared with the matched control group, the association between genotype F and HCC remained highly significant ($P < .001$; OR, 9.5 [95% CI, 2.21–40.8]). The OR dropped slightly, to 8.0 ($P = .001$; 95% CI, 1.84–34.8), when familial cases other than the index case were omitted.

Adjustment for HCV-positive patients. When the 3 anti-HCV–positive patients with HCC were excluded from the analysis, the association between genotype F and HCC remained significant for the remaining 44 patients, compared with the non-HCC cohort, by multiple logistic regression analysis in which region, sex, and birth cohort were adjusted for ($P < .001$; OR, 8.3 [95% CI, 4.0–17.1]). The association also remained significant in the matched analysis of case and control patients ($P < .001$; OR, 18 [95% CI, 2.4–134]) and when familial cases other than the index case were omitted ($P = .001$; OR, 15 [95% CI, 2.0–114]).

Genotypes in cirrhosis. Cirrhosis was found in 15 (50%) of the 30 patients with HCC for whom nontumerous liver tissue was available. Cirrhosis was found in 9 (43%) of the 21 patients infected with genotype F and in 6 (67%) of the 9 patients infected with other HBV genotypes ($P = .427$).

Basal core promoter and precore mutations. The prevalences of basal core promoter and precore mutations among patients with HCC and the genotype-matched control patients are shown in tables 1 and 2, respectively. Thirty-three (73%) of 45 patients with HCC and 30 (70%) of 43 control patients were male; the mean age was 38.4 years (SD, 22.1 years) for patients with HCC and 37.0 years (SD, 18.9 years) for the control patients. Overall, the proportions of case and control patients with basal core promoter mutations were similar ($P = .684$). However, when separated by genotype, fewer patients infected with genotype F were found to have basal core promoter mutations ($P = .009$), whereas the mutation was found more frequently in patients infected with the other genotypes, compared with the genotype-matched control patients (for genotype A, $P = .05$; for genotype C, $P = .047$; for genotype D, $P = .030$). Overall, the number of precore mutations found in patients with HCC was not significantly different from that found in the control patients ($P = .128$), and no significant difference was found when results were stratified by genotype.

DISCUSSION

Chronic HBV infection in Alaska Native people is characterized by 5 genotypes (A, B, C, D, and F), with genotype F significantly associated with the development of HCC. This association remained significant after adjustment for regional differences in

Table 2. Prevalence of the hepatitis B virus (HBV) precore G1896A mutation among patients with hepatocellular carcinoma (HCC), versus matched control patients from the non-HCC cohort.

Genotype, patient group	No. (%) of patients[a]			P[b]
	Wild type	Mutated	Mixture	
F				
HCC	25 (78)	0	7 (22)	.73
Control	12 (60)	2 (10)	6 (30)	
A				
HCC	4 (100)	0	0	NA
Control	7 (100)	0	0	
C				
HCC	1 (25)	0	3 (75)	.83
Control	4 (44)	1 (11)	4 (44)	
D				
HCC	2 (40)	1 (20)	2 (40)	.73
Control	3 (43)	2 (29)	2 (29)	
All				
HCC	32 (71)	1 (2)	12 (27)	.13
Control	26 (60)	5 (12)	12 (28)	

NOTE. Control patients were matched by genotype, sex, age at diagnosis, and region of residence. NA, not applicable.

[a] Results were classified by those patients with wild-type HBV only, those with mutated virus only, or those with both wild-type and mutated virus (mixture).

[b] Determined by the χ^2 test for trend.

genotype distribution and date of birth. The median age at diagnosis of HCC for patients who were infected with genotype F was significantly lower than that for patients with HCC who were infected with the other genotypes combined.

Because 13 patients with HCC were first-degree relatives of other patients with HCC, we investigated whether the increased frequency of genotype F could be due to an unrelated genetic predisposition for HCC development. After omission of patients who developed HCC after the index case in each family with multiple cases of HCC, the relationship of genotype F to HCC remained highly significant. This relationship remained significant when we excluded the 3 anti-HCV–positive patients in this study.

Since initial random sampling of the study population showed that HBV genotypes tended to be clustered by region, we genotyped 89% of the consenting patients with chronic HBV infection in this population. The large number of persons tested suggests that the increased frequency of genotype F among patients with HCC versus control patients is unlikely to be due to sampling error. More than 95% of this population was screened for hepatitis B vaccine in the early 1980s, indicating a true population-based cohort. Furthermore, the increased frequency of genotype F among patients with HCC, compared with that among patients in the non-HCC cohort, was found to be similar across regions.

Genotype F is considered to be the indigenous genotype of Amerindians and is the most divergent strain, compared with genotypes prevalent in other parts of the world [15–18]. Studies of genotype F are limited, and the published results do not show an association with HCC, although one study found that death related to liver disease was more frequent among patients infected with genotype F than among those infected with genotype A or D [19].

As reported in Asia [9], we also found a significant association between genotype C and HCC, although this association was less than that for genotype F. Some differences have been found in genotype distribution by age at diagnosis of HCC: younger persons in Taiwan were more likely to be infected with genotype B than genotype C, and younger persons in India were more likely to be infected with genotype D than genotype A [6, 7, 20, 21]. Factors such as genotypic subtypes or environmental influences could play a role. We cannot rule out the latter in Alaska Native people, although high levels of aflatoxin B_1, which previously has been associated with HCC, were not found in a study of traditional foods [22]. The median age in our study population was 40.85 years. However, the median age at diagnosis for our 5 patients with HCC and genotype C and our 5 patients with genotype A was 60 years; thus, the frequency of HCC among persons with genotypes other than F may increase in the future as this population ages. The timing of the introduction of the 5 different genotypes might affect different rates of HCC by genotype, but no studies have been done in this population to demonstrate this effect.

In this study population, we found no association between basal core promoter mutations and the development of HCC, most likely because these mutations were found less frequently in genotype F but more frequently in other genotypes. Several studies have found the double mutation in both liver tissue and serum from patients with HCC and genotype A, B, or C [8, 23, 24]. In fact, in genotypes A, C, and D, we found significantly more basal core promoter mutations in those with HCC than in the control patients. Thus, this relationship may be valid for some genotypes but not for genotype F.

We found a similar frequency of G1896A precore mutations among case and control patients, regardless of genotype. Other studies have found that the prevalence of the mutation did not differ between patients with HCC and asymptomatic carriers [11, 25], and our data provide further evidence that this mutation is not associated with HCC.

Our study found a strong association between genotype F and HCC among Alaska Native people. This study population offered a unique opportunity to examine the role played by 5 of the 8 HBV genotypes in the development of HCC. The additional findings that HCC occurred at a significantly younger age and that basal core promoter and precore mutations were not found in those with genotype F suggests that unique characteristics of this HBV genotype might play a role in HCC

development. In future studies, we plan to investigate differences in genotype F viral sequences in HCC- and non-HCC–associated HBV and to search for unique mutations in the genotype F genome isolated from HCC tissue.

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