RSA/04/2540 นพ.ดร. อิศรางค์ นุชประยูร

รายงานฉบับสมบูรณ์

โครงการ

"อณูชีววิทยาของมะเร็งตับ"

Molecular carcinogenesis of Hepatocellular Carcinoma

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

รหัสโครงการ: RSA/04/2540

ชื่อโครงการ: "อณูชีววิทยาของมะเร็งตับ"

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ระยะเวลาโครงการ: 3 ปี

บทคัดย่อ

มะเร็งตับเป็นมะเร็งที่พบบ่อยที่สุดในชายไทย ไวรัสตับอักเสบบี และ อัฟลาทอกซินเป็นสาเหตุที่สำคัญที่ทำให้เกิดมะเร็งตับ แต่กลไกที่ทำให้เกิดมะเร็งยังไม่ชัดเจน งานวิจัยนี้เป็นการศึกษาระดับอณูชีววิทยา โดยศึกษาบทบาทของยีน C/EBP alpha และ ยีนของไวรัสตับอักเสบบีในมะเร็งตับ เปรียบเทียบกับตับส่วนที่ยังไม่เป็นมะเร็ง และในเลือดของผู้ป่วย ด้วยวิธี Sourthern และ Northern blot ตัวอย่างมะเร็งจากผู้ป่วย 6 ราย และ เลือดจากผู้ป่วยมะเร็งตับ 36 รายได้รับการตรวจหา ยีน HBx และ HBs ของไวรัสตับอักเสบ และ HBsAg, HBcAg ด้วยวิธี ELISA

ผลการตรวจยีน C/EBP alpha ไม่พบความผิดปกติของยีนนี้ในมะเร็งตับ สามารถตรวจพบ RNA จากยีน C/EBP alpha ในระดับต่ำทั้งในมะเร็งและในตับส่วนที่มิได้เป็นมะเร็ง ไม่แตกต่างกัน ดังนั้น C/EBP alpha จึงไม่น่าจะเป็นยีนที่มีความสำคัญในการเกิดมะเร็งตับ การตรวจยีนโนมของไวรัสตับอักเสบบีในยีโนมของมะเร็งตับไม่พบสัญญาน ซึ่งอาจแปลว่าไม่มีการแทรกของไวรัสตับอักเสบบี แต่การตรวจหายีน HBx และ HBs พบในมะเร็งตับของผู้ป่วยที่มีการติดเชื้อไวรัสตับอักเสบบีมาก่อน นอกจากนี้ยังตรวจ HBx พบในตับ และเลือดจากผู้ป่วยรายหนึ่งซึ่ง HBsAg ในเลือดได้ผลลบ การตรวจเลือดของผู้ป่วยมะเร็งตับ 36 รายเพื่อหายีน HBs และ HBx พบว่าใน 15 ราย HBsAg ในเลือดได้ผลบวกสามารถพบ HBx DNA 11 ราย และสามารถตรวจพบ HBx DNA 10 จาก 15 รายที่เคยติดเชื้อไวรัสบี (Anti-HBc ได้ผลบวก) แต่ HBsAg ในเลือดได้ผล HBV แต่ไม่พบ HBx ผู้ที่ไม่เคยติดเชื้อไวรัสบีเลย (6 ราย) หรือในผู้บริจาคโลหิตทั่วไป (20 ราย) การศึกษานี้ทำให้น่าเชื่อว่า HBx มีความสำคัญในการเกิดมะเร็งตับในผู้ที่เคยติดเชื้อไวรัสตับอักเสบบี แม้จะตรวจไม่พบ HBsAg ในภายหลัง

นอกจากนี้ยังได้ทำการศึกษาหาชนิดของความผิดปกติในระดับยีนของภาวะพร่องเอนซัย์ม กลูโคส-6-ฟอสเฟตดีฮัยโดรจิเนส (G6PD) ในประชากรไทย โดยการตรวจเลือดจากรก พบว่าภาวะพร่องเอนซัย์ม G6PD พบได้ร้อยละ11.1 ในชายไทย การตรวจทางอณูชีววิทยาของเลือดจากรกที่พร่อง G6PD 49 รายพบว่า เอนซัย์ม G6PD เป็นชนิด G6PD เวียงจันทน์ (ลำดับเบสที่ 871 เปลี่ยนจาก G เป็น A) 28 ราย (ร้อยละ 57), G6PD กวางตุ้ง (1376 G เป็น T) 4 ราย (ร้อยละ 8), G6PD มหิดล (487 G เป็น A) 3 ราย (ร้อยละ 6), G6PD ไกผิง (1388 G เป็น A) 2 ราย, G6PD ยูเนียน (1360 C เป็น T) และ G6PD จีนชนิดที่ 5 (1240 C เป็น T) อย่างละ 1 ราย ส่วนอีก 8 ราย (17%) ยังไม่ทราบชนิด

Project Code: RSA/04/2540

Project Title: Molecular carcinogenesis of Hepatocellular Carcinoma

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Project Period: 3 years

Abstract

Hepatocellular carcinoma (HCC) is the most common cancer in Thai men. Although it has been well known that hepatitis B virus (HBV) and toxins are important causative factors, pathway of carcinogenesis is still unclear. The specific aims of this study were to assess the role of a human transcription factor C/EBPα, to assess the integration of Hepatitis B virus (HBV) genome in HCC genome, and to assess the role of HBV-X gene in HCC patients.

There was no rearrangement of C/EBPα gene in HCC specimens by Southern assay. The expression of C/EBPα were low in HCC and non-tumorous cirrhotic liver from the same patients by Northern blot. HBs and HBx gene PCR products were present in 4 of 6 HBV-seropositive HCC samples. However, in one HBsAg-negative HCC sample, HBx but not HBs gene was present., and HBx but not HBs gene was detected in his PBMC. In search for more evidence, HBx and HBs genes were amplified from sera and PBMC of 36 patients, 23 of whom were known HBsAg negative. We found that HBx DNA was detectable in 11 of 15 HBsAg-positive patients. Interestingly, HBx DNA was also detectable in 10 of 15 patients who had had previous HBV infection (Anti-HBc positive) but had cleared HBsAg from circulation (HBsAg negative in serum) but not in 6 HBsAg and anti-HBc negative patients, nor from 20 random volunteer blood donors (negative controls). These findings suggest that HBx may be important in pathogenesis of HCC in previously HBV-infected patients, even in patients who subsequently clear HBsAg.

Another unrelated study under this funding was to search for mutations responsible for glucose-6-phosphate dehydrogenase (G6PD) deficiency in Thai population. Of 529 cord blood samples, 49 (11.2%) were G6PD deficient. We found that the three most common G6PD-deficient mutations were G6PD Viangchan(871G→A , 57%), followed by G6PD Canton (1376 G→T, 8%), and G6PD Mahidol (487 G→A, 6%). The less common mutations identified were G6PD Kaiping (1388 G→A, 4%), and 1 case each (2%) for G6PD Union (1360 C→T) and "Chinese-5" (1024 C→T) and 8 cases (18%) of G6PD deficient mutation remained unidentified.

Keywords: Hepatocellular carcinoma, C/EBP alpha, Hepatitis B virus, HBX, Glucose-6-phosphate dehydrogenase deficiency, G6PD mutations

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Molecular carcinogenesis of Hepatocellular Carcinoma

1. Introduction

Hepatocellular carcinoma (HCC) is a malignancy of hepatocytes. HCC is a uniformly fatal disease. While most cancers in adult and children had an improved survival with multimodality therapy during the past decade, survival of patients with HCC remains unchanged during the past four decades. The incidence of HCC in Thailand is far higher than that is Western countries. This correlates the high incidence of hepatitis B viral infection in Thai population.

Pathogenesis of HCC remains unclear. It has been shown that HCC is epidemiologically associated with Hepatitis B virus (HBV) infection, and aflatoxin. However, it remains unclear how HBV causes hepatocytes to become cancer. Progress in human hepatoma research has been slow due to a relative lack of specimens and incidence compared to other cancer in Western countries.

Cancer is a multi-step process, resulting from a serie of accumulating genetic mutations. Each type of cancer is probably results from a different set of gene mutations. A majority of patients who were infected with HBV never dies of HCC, suggesting that there are other critical steps in hepatic carcinogenesis. Hepatitis B viral genes might be important in introduction of mutations or inactivation of human genes in liver cells. Identification of inactivated human genes involved and the role of HBV gene expression and integration will provide important clues to the pathogenesis of liver cancers.

Treatment of HCC has been difficult. HCC are often resistant to chemotherapy and radiation. Knowledge in pathogenesis of liver cancer is important for the development of treatment strategies. Establishment of hepatocellular carcinoma cell culture will allows in-vitro testing of cancer cells for chemosensitivity and test of novel therapy. Knowledge of molecular pathogenesis may indicate preventive intervention of future cases of hepatoma in patients with hepatitis virus infection.

A few oncogenes and tumor suppressor genes have been investigated in HCC. The protooncogene c-Ha-ras has been shown to be mutated early in carcinogen-induced murine hepatocellular carcinoma (reviewed in Pascale et al, 1993), but not in rat HCC where it rare and late events (Watatani et al, 1989). Ras mutations are rare and uncommon in human (Tada et al, 1990; Tsuda et al, 1989). The c-Myc amplification is very common in woodchuck HCC

(Moroy et al, 1986), but not in human HCC (Buenda, 1992). The p53 gene, the most commonly mutated tumor suppressor genes in any human cancer, was shown to be lost in human HCCs and HCC-derived cell lines (Slagle et al, 1991; Fujimori et al, 1991). It is thought to be a late event, important in progression into poorly differentiated HCC (Tsuda et al, 1990). There may be other uncharacterized or uninvestigated genes that are important in the development of carcinoma.

Partial hepatectomy studies rodents have identified several transcription factors that are important during hepatic cell regeneration (Xanthopoulos and Mirkovitch, 1993). Members of transcription factors C/EBP has been known to regulate liver-, adipose-, and myeloid-specific genes (Friedman et al, 1989; MacDougald and Lane, 1995; Nuchprayoon et al, 1997). In liver, C/EBP alpha is highly expressed in a terminally differentiated hepatocytes (Birkenmeyer et al, 1989), and sharply declined in regenerating liver and in culture (Mischoulon et al, 1992). Although C/EBP alpha has not been implicated to be an oncogene or tumor suppressor gene, its activity correlates with hepatocyte growth and differentiation. Several experiments in hepatoma cell lines have indicated that C/EBP alpha have antiproliferative effect (Watkins et al, 1996). C/EBP alpha gene transfer using recombinant adenoviral vector reduce the growth of HCC cell line in vitro (Diehl et al, 1996), suggesting tumor suppressor effect. Thus it is reasonable to study whether C/EBP alpha might be inactivated in HCC. We will therefore plan to study the expression of C/EBP alpha in HCC fresh cancerous tissue as well as non-cancerous neighboring liver tissues.

Tumor suppressors gene inactivation can be from several mechanisms. Deletions and point mutations are the most common. Recently abnormal methylation was also identified as a mechanism for gene activation. Examples are p15 and p16 gene inactivation in leukemia (Herman et al, 1996).

Hepatitis B viral genes have been investigated. Integration of woodchuck hepatitis viral DNA is a prominent finding in the woodchuck HCC model (reviewed in Tenant and Gerin, 1994). These rare integration events seem to activate a gene at a common site on a chromosome (Fourel et al., 1990) or are likely to cause genomic instability leading to neoplasia. Only a few reports about the frequency of viral DNA integration in human HCC exist (Nagaya et al., 1987). HBV integration sites in human HCC have been reported in involve chromosome 11p13 near Wilm's tumor gene (Rogler et al, 1985), chromosome 17q21 near BRCA1 gene (Bowcock et al, 1993), a site near cyclin A gene (Wang et al, 1990), a site near retinoic acid receptor beta gene (deThe et al, 1987), a chromosomal translocation t(17;18)(q21;q11) (Hino et al, 1986), These reports are from a variety of source of HCC, it is an important question to ask whether

HBV that are prevalent in Thailand frequently cause HCC by integration, and if so, do they integrate at a common site.

2. ระเบียบวิธีวิจัย Materials and Methods

I. Specimen collection and DNA extraction

Hepatocellular carcinoma sample will be collected from surgical hepatectomy. Under sterile condition, the sample will be grossly examined, part of the tumor will be isolated from the neighboring liver tissue, cut into 0.5x0.5x0.5 cm cubes, and frozen in liquid nitrogen as soon as possible. Normal liver tissue will also be collected from the same patient. The rest will be sent to pathologist for histology confirmation.

DNA extraction from liver tissue was performed by grinding frozen HCC or liver tissue in liquid nitrogen with mortars and pestles to fine powder. The tissue were suspended in Extraction buffer (10 mM Tris pH7.4, 1% SDS, 10 mM proteinase K) and allowed digestion overnight at 50°C, followed by phenol-chloroform extraction, and ethanol precipitation. After dissolution in 10 mM Tris-EDTA, DNA was quantitated and used for Southern blot assays or PCR reactions.

Peripheral blood samples were obtained from 36 patients diagnosed with HCC. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque gradient, and frozen before DNA extraction. Serum was separated and frozen at -70°C for later DNA extraction.

II. Southern blot for C/EBP alpha in hepatic tissue.

Human C/EBP alpha genomic DNA-containing plasmid was obtained from Dr. Alan D. Friedman (Johns Hopkins University Oncology Center, Baltimore, Maryland, U.S.A.) A 5-kb *BamH1* digest of C/EBPα (Figure 1), was labeled with α-^{32}P-dCTP by using nick translation method, and used as a DNA probe in Southern blot assays. Ten microgram of DNA from HCC and non-cancerous liver tissue from the same patient (liver control) were digested overnight with *BamH1* or *HindIII,* resolved on 1% agarose gel at 30V for 12 hours, transferred to Nylon membrane (Hybond-N+) under 10xSSC, dried and baked at 80°C for 2 hours, then hybridized with DNA probe at 42°C for 24 hours. The blot was then washed and exposed to phosphor-imager screen and scanned with Storm T-3 ® densitometer.

III. Southern blot for Hepatits B- s gene in hepatic tissue.

Hepatitis B virus- s gene -containing plasmid (pCMV-HBs) was obtained from Dr. Watchara Krisanasin (Chiangmai University). A 2.5-kb *HindIII* digest of pCMV-HBs (Figure 2) was labeled with α-^{32}P-dCTP by nick translation and used as a DNA probe in Southern blot assays.

IV. Reverse transcriptase- polymerase assay for C/EBP RNA

A primer pairs CEBPA512F (5'-GGAGCGCACGGGCCCCCC-3') and CEBPA1114R (5'-CGGTCATTGTCACTGGTC-3') primers were used to amplify a C/EBP alpha plasmid (Fig. 1) as a probe for Northern blot below and also as positive control under various PCR conditions.

V. Northern blot for C/EBP alpha in hepatic tissue.

PCR amplification of C/EBP alpha gene was labeled using North-2-South® non-radioactive labelling kit (Pierce). Ten microgram of total RNA from HCC and non-cancerous liver tissue from the same patient (liver control) were resolved on 1% -agarose formaldehyde gel at 30V for 4 hours, transferred to Nylon membrane (Hybond-N+) under 10xSSC, dried and baked at 80°C for 2 hours, then hybridized with DNA probe at 42°C for 3 hours. The blot was then washed and exposed to North-2-South® substrate solution according to manufacturer's recommendation, and exposed to X-ray film (Kodak XR) for 10 minutes to 4 hours, and developed by automated developer.

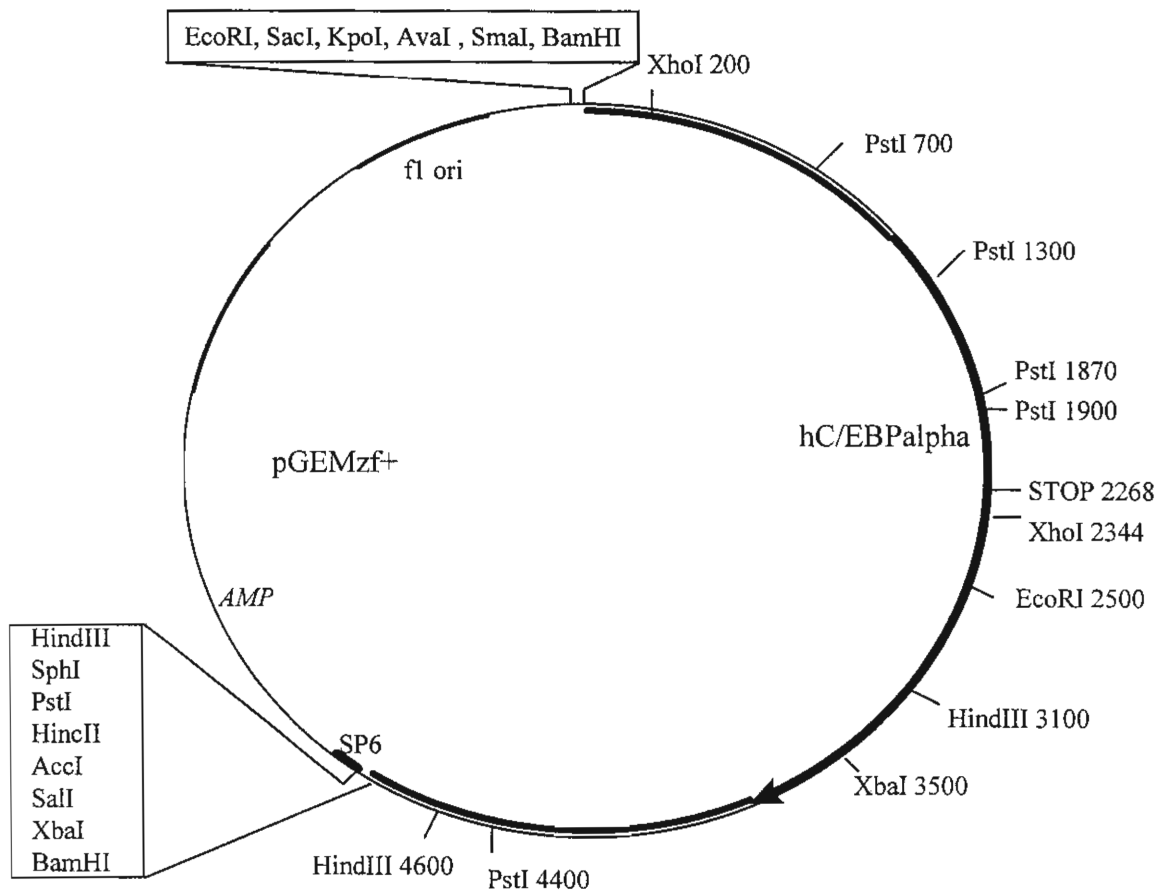

Figure 1. Map of human C/EBP alpha containing plasmid

VI. Polymerase chain reaction (PCR) assay for HBs-DNA and HBx DNA

For DNA amplification by nested (PCR) 10-μl aliquots of the re-suspended DNA samples were added to 40 μl of a reaction mixture containing 1.5 U of Taq polymerase (Pharmacia, Uppsala, Sweden), each of four deoxynucleotide triphosphates (Promega Corp., Madison, WI, USA) at a concentration of 200 μM, primer pairs (Biosynthesis, Lewisville, Texas) of F_1, R_6 (s gene) or Xo_1, Pc_1 (x gene) (first round) and F_2, R_5 (s gene) or Xi_1, Xi_3 (x gene) (second round) 1 μM each, 10 mM Tris/Cl buffer prepared with the required $MgCl_2$-concentration and sterile H_2O *ad* 40 μl in 0.2 ml PCR tubes. The reaction mixtures were spun in a microcentrifuge for 2 sec before being placed in the thermocycler (Perkin Elmer Cetus, Branchburg, NJ, USA).

The primer sequences were:

F_1 : 5'-GGA GCG GGA GCA TTC GGG CCA-3' (nucleotide position 3022-3042).

R_6 : 5'-GGC GAG AAA GTG AAA GCC TG-3' (nucleotide position 1103-1084).

Xo_1: 5'-CTC TGC CGA TCC ATA CTG C-3' (nucleotide position 1254-1272).

Pc_1: 5'-GGA AAG AAG TCA GAA GGC-3' (nucleotide position 1974-1956).

F_2 : 5'-CAT CCT CAG GCC ATG CAG TGG A-3' (nucleotide position 3193-3214).

R_5 : 5'-AGC CCA AAA GAC CCA CAA TTC-3' (nucleotide position 1015-995).

Xi_1 : 5'-AGC TTG TTT TGC TCG CAG C-3' (nucleotide position 1285-1305).

Xi_3: 5'-GGC ACA GCT TGG AGG CTT-3' (nucleotide position 1883-1866).

The reaction was then performed using both the first and second round s- and x-gene primer pairs consecutively for 30 cycles each, preceded by a denaturation cycle at 94° C for 1 min, 55° C for 1 min, and 72° C for 1 min, continued at 94° C for 30 sec, 55° C for 30 sec, and 72° C for 1 min, and concluded by an extension cycle at 94° C for 1 min, 55° C for 2 min, and 72° C for 10 min, respectively.

Upon electrophoresis in a 2% Nusieve agarose gel (FMC Bioproducts, Rockland, ME, USA) stained with ethidium bromide at 90 V for 80 minutes, the bands indicating the presence of HBV s and/or x DNA became visible under UV light at 1037 and 596 bp, respectively, after the second amplification round.

VII. HBx mutation analysis

Those PCR products revealing the presence of HBx gene were subjected to restriction fragment length polymorphism (RFLP) analysis using the restriction endonuclease *Sau 3AI* to investigate the core promoter sites at codons 1762-1764 for potential point mutations (16, 17). To that end, 15 U of *Sau 3AI* (New England Biolabs, MA, USA) were added to 10 μl of the respective 2nd round PCR products in a reaction buffer supplied by the manufacturer, and incubated at 37° C for 4 hours. The RFLP products were analyzed by electrophoresis on a 2% Nusieve gel and their respective sizes compared to those of a suitable nucleotide size marker (100 bp DNA ladder, Promega Corp., Madison, WI, USA). The sizes expected were 483 and 113 bp for the 1762/1764 wild type, and 362 and 121 bp for the 1762/1764 mutant, respectively. (Figure 2)

3. ผลที่ได้รับ Results

3.1 Hepatits B (HB) s DNA & HB-x DNA in HCC and non-cancerus liver tissue

We assessed the presence of HBs & HBx gene in HCC and liver control in six of our patient samples using a nested-PCR technique.

HBs and HBx gene PCR products were present in 3 of 5 HCC samples (H1, 2, 8, and 9) and both genes were absent in one HCC samples (#HCC3). In one remaining HCC sample (HCC6), HBx gene was present albeit weak, but HBs gene was absent (Fig. 1, the lower band is non-specific). We examined serum and PBMC of this patient (#HCC6) for presence of HBs and HBx DNA. We found that HBs was absent in serum and PBMC; however, HBx gene was present in PBMC, but not in his serum (data not shown). His serum anti-HBc was positive indicating that he had been previously infected with HBV virus, but had cleared HBsAg and HBs-DNA from his circulation and liver. Therefore, it is possible that HBx gene remained and might contribute to development of HCC.

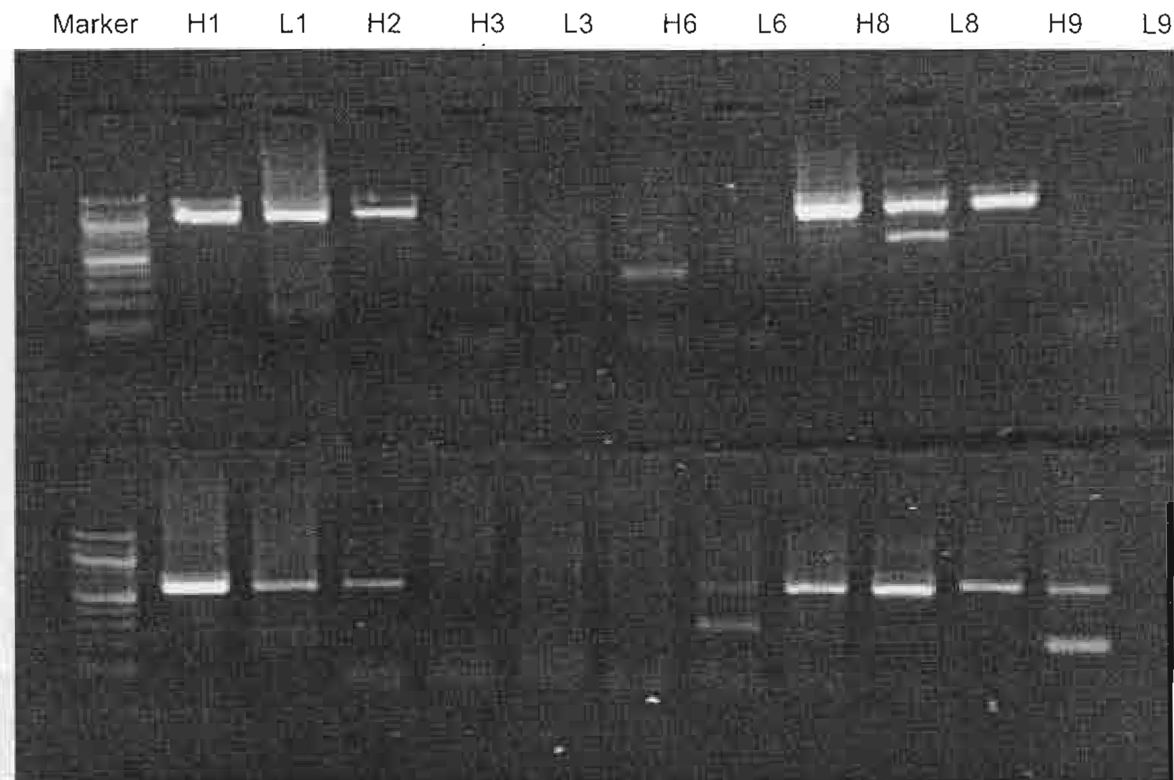

Figure 1. HBs gene (upper panel) and HBx gene (lower panel) in hepatocellular carcinoma cells (H) and non-cancerous liver from the same patient (L). The expected 1037-bp HBs and 550-bp HBx PCR products were shown.

Table 1. Hepatitis B and C serology in patients with hepatocellular carcinoma (HCC) and correlation of HBs and HBx genome in their HCC (tumor) tissue and non-carcinomatous liver tissue. + = Presence, - = Absence, N/A = data not available.

Speci-men ID#	serum HBs-Ag	serum Anti-HCV	HBs DNA in tumor	HBs DNA in liver	HBx DNA in tumor	HBx DNA in liver
HCC1	+	N/A	+	+	+	+
HCC2	+	N/A	+	N/A	+	N/A
HCC3	-	-	-	-	-	-
HCC6	-	-	-	-	-	+
HCC8	+	N/A	+	+	+	+
HCC9	+	+	+	+	+	+

3.2 Hepatits B (HB) s DNA & HB-x DNA in serum and PBMC of patients with HCC

More evidences are required to confirm whether HBx is relevant to pathogenesis of HCC; however, the liver specimens are scarce. Therefore, we turned to a more readily available source of samples, the peripheral blood samples, from patients with HCC, most of whom were not treated with surgical resection but was confirmed by liver biopsies.

Hepatitis B has previously known to infect only liver cells. However, recent studies has demonstrated the presence of hepatitis B virus DNA in the sera and PBMC of infected patients as well. It is not known how viral DNA in PBMC is related to pathophysiology of HBV infection, but it appears to be a marker of HBV infection.

We assessed the presence of HBs & HBx gene in the sera and PBMC of patients with HCC. Thirty-five samples were classified into 3 groups according to HBsAg and Anti-hepatitis B - core antibody (Anti-HBc). Patients who is HBsAg and Anti-HBc reactive is considered persistent to HBsAg infection. Patients who is HBsAg negative but anti-HBc positive is considered previously infected, but the patient has subsequently cleared HBsAg, probably by immune mechanism. Patients who are negative for anti-HBc are considered uninfected; however, it is plausible that the altered immune response to HBV renders anti-HBc negative.

We found that patients who had persistent HBV infection, HBs-DNA and HBx-DNA were detectable in PBMC as well as in their serum. In this study, over half of the patients had been previously infected with HBV but became HBsAg negative and subsequently developed HCC. This group of patients, despite HBsAg negative, HBx DNA was detectable in a majority of cases (10 of 15), more frequently in PBMC than in serum (Table 2). All three patients with HBx-DNA

in serum has detectable HBx-DNA in PBMC as well. Thus these findings support the hypothesis that HBx may be important for pathogenesis of HCC, regardless of HBsAg or HBs-DNA status.

Table 2. Hepatitis B serology, HBs (s-DNA) and HBx (x-DNA) genome in serum or peripheral blood mononuclear cell (PBMC) of patients with hepatocellular carcinoma. N = number examined.

Status	N	Serum HBsAg	Serum Anti-HBc	Number of Positives		Number of Positives	
				s-DNA in serum	s-DNA in PBMC	x-DNA in serum	x-DNA in PBMC
No HBV infection	6	Neg	Neg	0	0	0	0
Previous HBV infection	15	Neg	Pos	0	1	3	10
Persistent HBV infection	15	Pos	Pos	11	10	9	6

3.3 Hepatits B (HB) s DNA & HB-x DNA in serum and PBMC of patients with chronic hepatitis

Chronic hepatitis can be an important predisposition to HCC. As a comparison to HCC, we examine 31 chronic hepatitis patients, and found that 16 were positive for both HBsAg and anti-HBc, the remaining 15 were negative for HBsAg (Table 3). In this HBsAg negative group, 9 were positive for anti-HBc and 4 positive for anti-HCV. Both HBs and HBx DNA were detectable in most (14 of 16) HBsAg-positive patients, but less commonly (4 of 9) in HBsAg-negative/ anti-HBc-positive group. As a negative control, blood samples from 20 volunteer blood donors assayed for HBV genome in similar condition. All were negative for HBsAg, with 7 positive for anti-HBc, and none of which were positive for anti-HCV or has detectable HBV (s or x) DNA. There were no patients or normal volunteer who were positive for HBsAg but not anti-HBc.

Among chronic hepatitis patients who are HBsAg positive, most (14 of 16) patients had detectable HBs gene, usually in both serum and PBMC (11 cases) (Table 3). Of 9 chronic hepatitis patients who were previously infected with HBV (anti-HBc positive), but were HBsAg negative, only four cases had detectable serum HBs DNA, and only one case has detectable serum HBx DNA.

Table 3. HBV serology, HBs (s-DNA) and HBx (x-DNA) genome in serum or PBMC of patients with chronic hepatitis. N = number examined.

Status	N	Serum HBsAg	Serum Anti-HBc	Number of Positives		Number of Positives	
				s-DNA in serum	s-DNA in PBMC	x-DNA in serum	x-DNA in PBMC
No HBV infection	6	Neg	Neg	0	0	0	0
Previous HBV infection	9	Neg	Pos	4	2	1	1
Persistent HBV infection	16	Pos	Pos	13	12	11	8

Analysis of HBx mutation among patients HCC and chronic hepatitis

We analysed the RFLP pattern of HBx from HCC and chronic hepatitis patients who has detectable HBx DNA in serum and/or PBMC by PCR using restriction enzyme Sau3A/. (Figure 2.) Most (9 of 11) HBx-positive HCC sera were wild type, while the remaining 2 were mutant HBx genes. Similarly, most (16 of 17) HBx from PBMC preparations were also of wild type, only one was mixed type. On the contrary, HBx DNA from sera of chronic hepatitis patients revealed that 2 were wild type, 5 were mutant, and 6 were mixed type, while 9 PBMC preparations derived from the same source showed that 1 was wild type, 3 were mutant and 5 were mixed type. (Table 4)

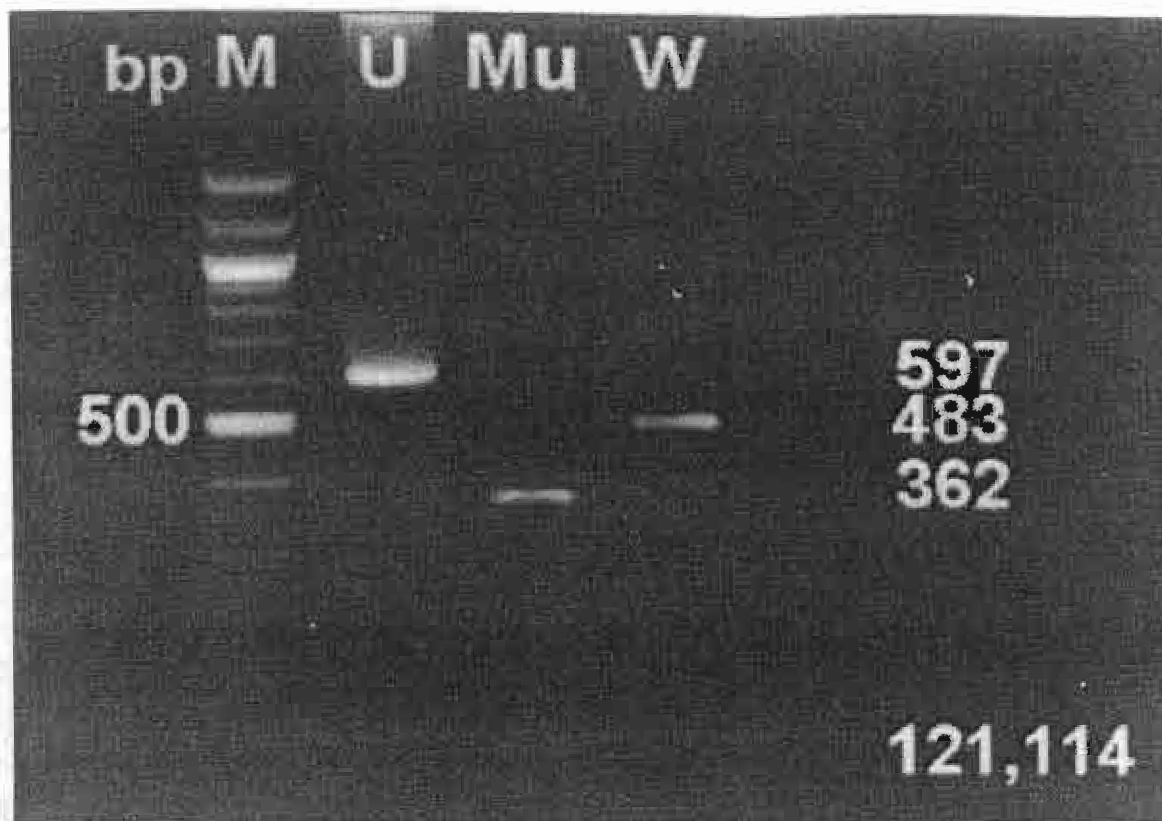

Figure 2. HBx gene RFLP analysis. Result of PCR amplification of HBx region from PBMC or serum (U), digested with *Sau*3A (Mu, W). Mutation of HBx at amino acid 130 and 131 (Mu) will result in a 362 bp + 121 bp bands, while wild type HBx (W) will result in 483 bp + 114 bp bands. M= 100-bp ladder marker.

Table 4. Frequency of HBx mutation among HBx-positive case

Disease Group	Presence of HBx in	Number of HBx DNA positive	HBx RFLP Type		
			Wild	Mutant	Mixed
Chronic hepatitis	serum	13	2	5	6
	PBMC	9	1	2	5
HCC	serum	11	9	2	0
	PBMC	17	16	0	1

3.3 <u>The role of C/EBP alpha gene in pathogenesis of HCC</u>

C/EBP alpha gene has been cloned by two groups of investigators, Antonson and Xanthopoulos in 1995, and Swart *et al* in 1997. C/EBP alpha gene sequence is available from Genbank and there are some difference in the DNA sequence from both groups, mostly in non-

coding regions. The genomic DNA sequence is the same as that of cDNA. The location on the chromosome is on chromosome 19 q13.1-13.2

We have been studying C/EBP alpha gene rearrangement using Southern blot technique in HCC and liver control samples. The major obstacle to this part of the study was lack of signals after hybridization. We have excluded the technical hybridization failure by using a dot blot experiment to which various DNA probe has been used as well as human genomic DNA, which was successful although human genomic DNA gave very weak signal. We will make an attempt to modify probe labeling technique, such as using a random primer labeling to improve signal detection.

As an alternative way to assess RNA expression, we also designed a primer pair that wiould amplify specifically C/EBP alpha gene. We are currently optimizing PCR condition using C/EBP alpha plasmid (between nucleotide 512 and 1114 of C/EBP alpha gene) as positive control. However, so far we have been unable to obtain a 602-bp predicted amplicon from this primer pair despite several attempts to optimize the PCR condition.

Northern blot showed low signal in HCC as well as in non-cancerous liver from the same patient. There is no evidence of differential expression in HCC compared to non-cancerous liver. These findings make our original hypothesis that C/EBP alpha is an important tumor suppressor gene unlikely. Therefore further investigation of this gene is unwarranted.

4.

4. วิจารณ์ Discussion

C/EBP alpha has been shown to expressed in differentiated liver cells but not regenerating liver cells. Despite evidences from gene transfer studies that expression of C/EBP alpha in hepatic cancer cell lines can inhibit cell growth. Our findings did not support hypothesis that C/EBP alpha is an important tumor suppressor gene involved in hepatocarcinogenesis. C/EBP alpha gene is not rearranged in cancer cells compared to non-cancerous liver form the same person. There was no evidence of differential expression of C/EBP alpha between cancer cells compared to non-cancerous liver. In addition, other studies on loss of heterozygosity (LOH) in hepatocellular carcinoma have not reported significant LOH on chromosome 19p13, the site of C/EBP alpha gene.

We found that C/EBP alpha RNA is detectable at low level in HCC as well as in non-cancerous liver from the same patient. There is no evidence of differential expression in HCC compared to non-cancerous liver. These findings make our original hypothesis that C/EBP alpha is an important tumor suppressor gene unlikely. Therefore further investigation of this gene is unwarranted.

In most patients, the non-cancerous part of liver specimens are often cirrhotic. The Although cirrhotic liver often accompany chronic hepatitis B infection, it is by no means precancerous lesions. The lack of pre-cancerous lesion make dissection of multi-step tumor suppressor gene inactivation difficult.

Due to unexpectedly small number of specimens acquisition, further search for more evidence of C/EBP alpha inactivation or mutation appear not worthwhile. The scarcity of specimen is due to a change in clinical practice for patients with hepatocellular carcinoma. Because of the low cure rate, the current recommendation for surgical excision of HCC in our institution is limited to small tumor size (less than 5 cm). However, this is a relatively uncommon event, and a few cases turned out not to be HCC, but other metastatic tumor. When tumor size is greater than 5 cm, transarterial-oily chemoembolization (TOCE) had become a popular method of treatment to reduce the size of the tumor, and could prolong survival.

Alternative specimens have been considered, liver biopsies with 'true cut' needle biopsy could yield 1-2 gm of liver specimens. However, since HCC is highly vascular tumor, there is a significant risk of bleeding with this procedure. In addition, because the consistency of tumor is not well formed, liver biopsy do not yield a good solid sample, but rather a mushy sample. Because of these risks, fine needle biopsies (FNA) had become a popular method for tissue

diagnosis of liver mass, especially in inoperable cases. FNA is safer, because of less risk of bleeding. However, FNA yield too small amount of cells and tissue to perform molecular studies as planned in this project. Therefore, we lost opportunity to study a majority of clinical cases.

Despite our careful attempt to procure specimen by immediately freeze the cancer sample in liquid nitrogen as soon as it was excised from the patient in operating room, RNA degradation was present. It is possible that the time taken to dissect cancer and excision from the normal liver, ranging from 2-4 hours, can cause infarction and necrosis of many cancer cells in surgical specimens by the time sample is frozen. In this way, a true cut biopsy sample might have been better, but this method of diagnosis has been abandoned for fear of bleeding. It is unlikely that the safe and popular method will yield enough samples for RNA analysis using traditional RNA blot.

Traditionally, it was believed that people who are chronic carriers of hepatitis B, defined by the presence of HBsAg, are at risk to develop hepatocellular carcinoma. Our results support the recent findings that in people who are previously infected with HBV, HBV genome can persist despite clearance of HBsAg (Brechot et al, 1985). This phenomenon could be explained by mutation of HBs antigen rendering it undetectable by commercially available ELISA method (Weinberger, Mangold et al 1993). Alternatively, serum HBV DNA in HBsAg-negative individuals may have resulted from masking of HBsAg in HBsAg – anti-HBs immunocomplexes (Joller-Jemelka et al 1994). Lastly, HBsAg-expressing liver could have been cleared by host immunity, while occasional integration of HBV into host genome without HBs-expression could escape clearance and regenerated.

HBx antigens have been detected in many HCC specimens obtained from patients infected with HBV (Wang et al 1991, Greenblatt et al 1997). HBx gene has been found to integrate more commonly than S gene (Matsubara et al 1990). After integration, HBx protein may be involved in liver cell transformation as it has been described as transforming cultured cells (Shirakata et al 1989, Hohne et al 1990) as well as transgenic mice (Kim et al 1991). HBx RNA transcript, rather than s- or c-transcript, is found in HCC from HBsAg-negative patients (Paterlini et al, 1994, Paterlini et al 1995). Because of limited number of our liver specimens, we can not pursue further study.

In addition to liver and serum, peripheral blood mononuclear cells, particularly lymphocytes, have been shown to harbor HBV (Lamelin et al 1995), in patients with acute and chronic hepatitis (Trippler et al 1999) where it has been shown most heavily in

monocytes and B-cell. HBV can replicate and transcribe HBx gene in PBMC of patients with active hepatitis (Stoll-Becker et al 1997). HBV DNA has been found in PBMC of chronic HBV carriers (Catterall et al 1994). In searching for evidence of HBV genome in serum and PBMC using nested PCR, special care were taken not to introduce cross-contamination between samples. Our results were valid because our negative controls: serum and PBMC from healthy HBsAg-negative volunteer blood donor samples, as well as anti-HBc negative patient samples, has undetectable HBV DNA. We found that hepatitis B genome, particularly HBx gene, was detectable more frequently in PBMC than in serum of HCC patients, while HBs DNA was present more frequently in serum of chronic hepatitis patients. Although there was no relationship between HBx in PBMC and HBx in hepatic cells, this HBV gene in PBMC could be a useful marker of persistence of HBV genome in the HBsAg negative patient who were previously infected with HBV. The relevance of HBx DNA in PBMC as a marker to identify people at high risk of HCC should be determined in a prospective study.

HBV DNA was not detectable in blood samples of all HBsAg-positive patients. HBV DNA is probably not related to the pathophysiology of HCC, but rather a marker of persistence of HBV DNA in these patients. While previous reports noted relationship between HBx DNA in liver of with HBsAg-negative, HCV positive patients (8), there are too few cases of HCV positive cases in our study to make any meaningful conclusion. A larger study should be conducted to determine whether HBx or HCV is a more important contributor to hepatocellular carcinoma in HBsAg-negative individuals.

HB x mutation has been frequently reported in HCC and non-cancerous tissue in patients with HBsAg (Catterall et al 1994) or without detectable HBsAg. The double mutations T^{1762} and A^{1764} changes codon 130 and 131 of X protein at the region which bind to p53 protein in the cytoplasm, and prevent apoptosis. This same region also functions as the core promoter of HBV DNA for precore mRNAs and the pregenome mRNAs (Lopez-Cabrera et al 1990) which codes for Hbe antigen. The mutation of this region has been shown to decrease transcription of the HBeAg precursor (Okamoto et al 1994) While HBx T^{1762}/A^{1764} mutation in often found in the carcinomatous liver (Tamori et al 1999), we found that most HBx from PBMC of our HCC patients were of wild type. However, HBx from PBMC of chronic hepatitis patient has higher prevalence of the T^{1762}/A^{1764} double mutation, as well as mixed populations of wild type and mutant among the chronic hepatitis patients. The significance of this finding is unclear at present.

We have attempted to assess the role of HBV integration by using a 2.5 kb DNA fragment, that covers HBs gene as a probe, to detect integrated HBV genome, by Southern blot analysis of HCC and liver control. However, we were unable to obtain a signal from this experiment. It is possible that there is no integration of HBV, but it is also possible that it is a technical failure.

In conclusion, our results has shown that presence or absence of HBsAg is an insufficient indicator with regard to a given individual's risk to proceed towards chronic liver disease. People who were previously infected by HBV may also be at risk in developing hepatocellular carcinoma despite HBsAg clearance.

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5. บรรณานุกรม References

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Molecular Genetics of Glucose-6-phosphate Dehydrogenase Deficiency in Thai Population

1. Introduction

Glucose – 6 phosphate dehydrogenase (G6PD) deficiency is the most common disease-producing enzyme disorder of human being (WHO, 1989). The enzyme G6PD was originally discovered to be related to the hemolytic anemia in primaquine-sensitive individuals (Carson et al, 1956, Beutler, 1959). Oxidative drugs, infections and other disorders can induce hemolysis in G6PD-deficient person as well (Miller 1995). The offending drugs or conditions generate free radicals and peroxides that attack unsaturated phospholipids of the lipid membrane and sulfhydryl groups of cellular proteins, which leads to denaturation, precipitation of the hemoglobin to form Heinz bodies, and eventual hemolysis (Miller 1995). The enzyme G6PD catalyses the first step in the hexose monophosphate pathway within the cell metabolism. This will generate NADPH, the reduced form of the cofactor NADP (nicotinamide-adenine dinucleotide phosphate), which is necessary for protection against oxidative damage of the cell.

In other cell, NADPH can be generated through other alternative system, which does not totally depend on G6PD, but G6PD is the only source in the erythrocyte (WHO, 1989). Thus G6PD is vital for life for red blood cells. Only 2% of G6PD is functioning in the normal red cell. This provides a large scale of reserve for the red cell to cope with the oxidative stress. The level of the-G6PD decreases as the cell ages, but this reduction does not affect the red cell life cycle of the normal individual. In contrary to the G6PD deficient cell, the level of the enzyme, which is low to begin with, diminishes even further, making it inefficient in protection against the oxidation. Clinically G6PD deficient person may not manifest any catastrophic complication until encountering oxidative stress that shortening of the red blood cell life span and hemolysis occurs.

The distribution of G6PD deficiency is worldwide with concentration in the countries around the Mediterranean, Africa and the Far East (Luzzato, 1975) Migration and intermarriage have allowed penetration of G6PD deficiency into different geographic areas.

Four clinical syndromes associated with G6PD deficiency have been identified: oxidative stress-induced hemolysis (Carson et al, 1956), favism (Beutler, 1959, Luzzato 1975), neonatal jaundice (Miller, 1995), chronic congenital hemolytic anemia Luzzato 1975).

Oxidative stress - induced hemolysis. Certain drugs (Beutler, 1994), infections (Chan 1972, Lampe et al 1975) and other conditions (Gellady & Greenwood, 1972), inflict oxidative injury on the red cell and precipitate hemolysis. The clinical expression of hemolytic anemia varies among ethnic groups. The variability may be dictated by G6PD variants, genetic difference of the red blood cell and other environmental triggering factors (WHO working group, 1989).

Favism is the condition of acute hemolytic anemia induced by ingestion of the fava bean or inhaling the pollen of the bean flower. The clinical spectrum ranges from mild to severe. Most for the cases of favism occurs in the G6PD Mediterranean individuals (Kattamis et al 1969). Sporadic cases have been observed including the Chinese (Miller 1995) and the person with G6PDA- (Galiano et al 1990). Sensitivity to fava bean requires G6PD deficiency and some other factors as well such as genetics (Stammatoyannopoulos et al 1966) and the metabolism of the active ingredients in the bean.

Neonatal jaundice occurs mostly in the Mediterranean and Asian G6PD deficient infants. In Thai, 65% of severe jaundice infants had G6PD deficiency (Phornphutkul et al, 1969). Hyperbilirubinemia may be severe that kernicterus (Brown & Boon 1968) occurs. Other exogenous factors as well as genetics affect the risk and severity of jaundice.

Chronic non-spherocytic hemolytic anemia (CNSHA) occurs in person who has inherited rare mutations. It is manifested by lifelong mild to moderately severe hemolysis. Sixty percent of the patients are of northern European ancestry, 22% Mediterranean, 16% oriental and 6% are black (Miller 1995).

G6PD has been studied biochemically, and more than 400 variants have been identified. Since the molecule was cloned (Persico et al, 1986, Takizawa, 1986) and the sequence of the entire gene was known (Chen et al, 1991), it is possible to characterize accurately the gene mutation. Different biochemical variants may be caused by a single gene mutation, at the same time one variant may result from several gene mutations (Beutler 1991). Analysis at the

molecular level has expanded the knowledge about G6PD extensively and allowed correlation of the enzyme property, function and clinical phenotype.

The gene of G6PD deficiency is located on the distal long arm of the X-chromosome and is inherited as X-linked. The male hemizygote (X'Y) and female homozygote (X'X') have diminished G6PD activity and are clinically apparent. The female heterozygote (X'X) has two populations of red blood cell, one of normal G6PD and the other of G6PD deficiency. The enzyme level is usually intermediate, but the amount of the activity is determined by the inactivation of the X chromosome bearing either normal or deficient gene. Therefore some of this heterozygote have normal activity, while some have defect as severe as the male hemizygote or the female homozygote (Miller 1995).

The frequency of G6PD deficiency is usually expressed defined as the proportion of males that is found to be hemizygotes. This figure is considered to be equal to the gene frequency. The gene frequency varies among different ethnic groups. It is about 0.5 in Chinese (Luzzato 1975), 0.10 to 0.11 among black Americans (Heller et al 1979), 0.4 of male and 0.19 of female Greek infants (Mission-Tsragaraki 1991), and as high as 0.7 in Kurdish Jews[7].

With the advance in molecular study, it is now possible to characterized definitely the nature of the mutation. More than 60 mutations or combination of mutations have been brought to light and more is yet to come. Population in the same geographic area shares certain gene mutation. G6PD Mediterranean (563 C→ T) is prominent in Southern Europe, the middle East and in the Indian subcontinent[7]. In Asia, several mutations were discovered. G6PD Gaohe (95 A→ G, Chao et al, 1991), Chinese-4 (392 G→T, Chiu et al, 1993), Chinese-5 (1024 C→T, Chiu et al, 1993), Union (1360 C→T, Hsia et al, 1993, Beutler et al, 1992), Canton (1376 G→ T, Stevens et al, 1992, Chin et al, 1991), Mahidol (487 G→A, Vulliamy et al 1989), Viangchan (871 G→A, Beutler et al, 1991), Kaiping (1388 G→A, Chin et al 1991), Chinese-3 (493 A→G, Tang et al, 1992) and Chatam (1003 G→A, Vulliamy et al, 1988) are found in China and South East Asia, while G6PD Kerala and Kalyan (949 G→A, Ahluwalia et al, 1992), Viangchan, and G6PD Orisa (131 G→C, Kotea et al, 1999) are found in India and G6PD Konan and Ube (241 C→T, Hirono et al, 1993a), Union and Nara (Hirono et al, 1993b) are identified in Japan.

G6PD Mahidol, a common G6PD variant among South East Asian countries named after the university where it was identified biochemically in 1972, was thought to be the most common G6PD variant in Thailand (Panich et al, 1972). The DNA analysis reveals the position of the point mutation at nucleotide 487 with substitution of G to A which changed translation of amino acid 163 from glycine to serine (Vulliamy et al, 1989). With the different ethnic background and controversial historical root of Thai people, various type of Southeast Asian gene mutations are yet to be identified.

The heterogenicity of G6PD deficiency prevalence in Thai has been documented (Wasi et al, 1972), 6-8% in the central, 10-15% in the north and 8-24% in the north east and 2.8 – 9.3% in the south of Thailand. In Bangkok the prevalence is as high as 12% (Sasanakul et al, 1989, Tanphaichitr et al, 1995).

Neonatal jaundice has been recognized as the result of G6PD deficiency. The prevalence and severity are different among different G6PD variants and vary even in the same ethnic groups (Valaes 1994). Genetic, environmental (Miller 1995) and background level of neonatal hyperbilirubinemia in the population (Valaes 1994) are the possible factors. The use of moth ball (Valaes et al 1963, Dawson et al, 1958), herbal remedy (Valaes 1994), a dye mixture to sterilizing the cord (Olowe and Ransome-Kuti, 1980) and Vitamin K analogues (Lucey and Dolan, 1959) has played an important role.

Hemolysis as the cause of hyperbilirubinemia has been a controversial issue. The absence of anemia and red blood cell morphology characteristic of hemolysis (Beutler, 1994) do not support the assumption of erythrocyte destruction as the pathogenesis of hyperbilirubinemia. Measuring end tidal breath carbonmonoxide, a more accurate and sensitive method to determine erythrocyte breakdown (Vreman et al, 1995) did not reveal any significant difference between the G6PD deficient and normal groups (Seidman et al, 1995). Jaundice is suggested to be caused by the impairment of hepatic function, presumable from the G6PD deficiency (Beutler, 1994). On the other hand, association between the level of carboxyhemoglobin, a product resulted from the catabolism of heme, and jaundice-related morbidity and mortality were shown in G6PD deficient Nigerian infants (Slusher et al, 1995). Many factors that determined the neonatal red blood cell mass and hemoglobin concentration were suggested to produce variability that masked the effect of mild hemolysis on the hemoglobin concentration and reticulocytosis (Valese, 1994).

Severe neonatal hyperbilibubinemia is well known to cause kernicterus and death (Maisels, 1994). In Thailand, 19.7% of hyperbilirubinemia is caused by G6PD deficiency (Tanphaichitr et al, 1995). Phototherapy, exchange transfusion, education and surveillance have shown to reduce these complications (WHO, 1989).

In summary, a wealth of information is known about the prevalence of G6PD deficiency in Thailand. However, the details on genetics is lacking. We therefore study G6PD at the molecular genetics level to define variants in Thailand.

2. ระเบียบวิธีวิจัย **Methods**

Umbilical cord blood samples were randomly obtained in delivery room of Chulalongkorn University Hospital. Five ml of cord blood were mixed with acid-citrate-dextrose (ACD) and stored at $4^{\circ}C$ until assay was done within 3 days of collection. Peripheral blood samples were obtained from jaundiced newborn from the nursery within the first 7 days of life. Serum total bilirubin was determined by its optical property using Reichert-Jung unistat bilirubinometer.

DNA was extracted using Qiaquick® Blood DNA extraction kit (Qiagen, Germany) according to manufacturer's recommendation.

G6PD activity assay

G6PD activity assay were performed according to WHO-recommended standard test (Betke et al, 1967) with minor modification. Two ml of citrated blood were washed with cold normal saline 3 times with removal of buffy coat. Washed red cell were assayed for hematocrit, then 50 μl of washed red cell were mixed with 950 μl ddH_20, mixed and frozen at $-20\ ^{\circ}C$ for 40 minutes. Lysed red cell were centrifuged at 3000 rpm (5000g) for 20 minutes, hemolysate supernatant were used for G6PD enzyme assay. Enzyme activity was quantitated by adding 50 μl of hemolysate to a 950 μl assay containing buffer (0.1 M Tris-HCl pH 8.0, 0.01 M $MgCl_2$), Glucose-6-phosphate (0.6 mM, Sigma), and NADP (0.2 mM, Sigma). The rate of NADPH generation was measured at 340 nm at $30\ ^{\circ}C$ over 10 minutes. The average change of optical density per minute was calculated to determine activity of the G6PD enzyme

Identification of G6PD mutations

The nine mutations chosen for the study is shown in figure 1. These mutation s are similar to the study by Huang (1996).

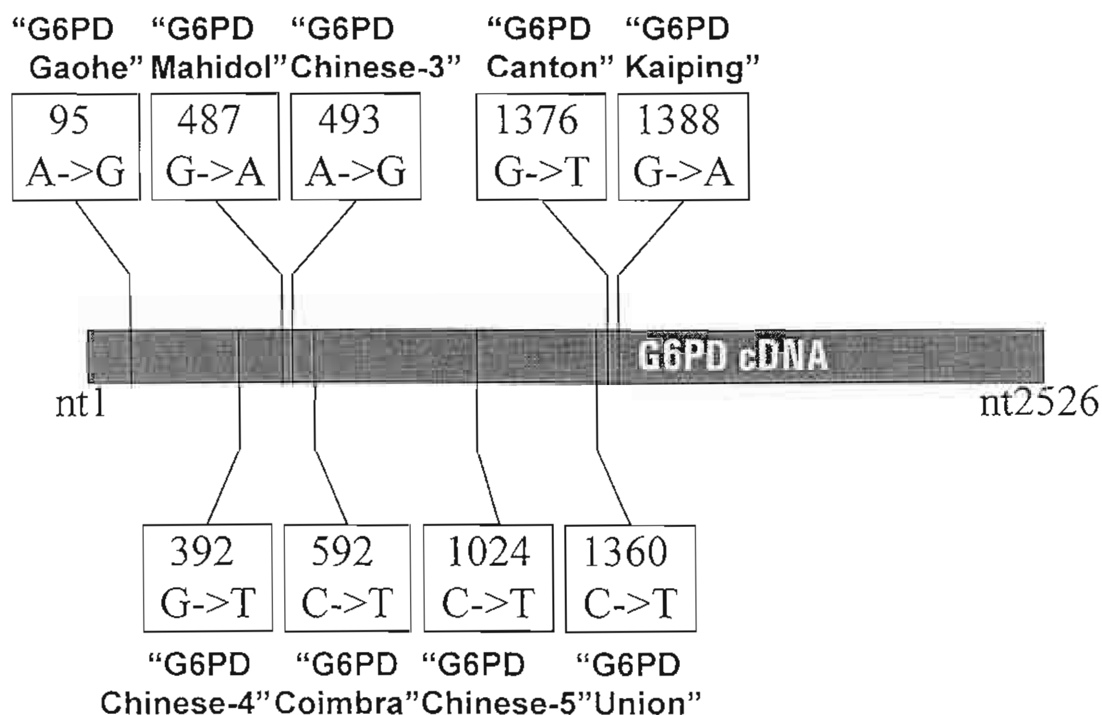

Figure 1. Schematic representation of mutations studied. Numbers indicated nucleotide (nt) position on G6PD cDNA coding sequence. Common names are also given.

For G6PD Viangchan mutation assay, a mutagenic primer pair 5'-TGGCTTTCTCTCAGGTCTAG-3' and 5'-GTCGTCCAGGTACCCTTTGGGG-3' were used in a polymerase-chain reaction (PCR). One microliter of purified DNA from blood were mixed, in 50 µl, with 50 ng of each primer, 200 M each dNTP, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.5 U of *Taq* polymerase (Promega). The PCR amplification was performed on the DNA thermal cycler for 1 cycle of 95°C for 5 minutes, then 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 1 min at 72 °C, and final extension at 72 °C for 10 minutes. In 30 µl reaction, 25µl of PCR product was digested with *XbaI* (Gibco BRL) for 2 hours, then resolved on 3% agarose gel (Metaphore, FMC Bioproduct, Rockland, ME) containing ethidium bromide.

For nt1311 polymorphism, three primers, G6P10F2 (5'-ATGATGACCAAGAAGCC GGGC-3'), 1311TR (5'-CGTCCAGGATGAGGCGCTCA-3') and G6P12R (5'-CTGCCATA AATATAGGGGATGGG-3') were used in a PCR reaction at the same condition above. The PCR amplification was performed on the DNA thermal cycler for 1 cycle of 95°C for 5 minutes, then 35 cycles of 1 min at 95 °C, 1 min at 68 °C, 1 min at 72 °C, and final extension at 72 °C for 10

minutes. Twenty-five µl of PCR product resolved on 3% agarose gel (Gibco BRL, Grand Island, NY) containing ethidium bromide.

Except for G6PD Viangchan (871 G→A), 9 oligonucleotides with natural or mutagenesis primer set (Table 1) were used for detection of the nine known G6PD mutations (NSTDA BIOTEC, Bangkok, Thailand). One microliter of purified DNA from blood were mixed, in 50 µl, with 50 ng of each primer, 200 M each dNTP, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.5 U of *Taq* polymerase (Promega). The PCR amplification was performed on the DNA thermal cycler for 1 cycle of 95°C for 5 minutes, then 35 cycles of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C, and final extension at 72 °C for 10 minutes. The PCR product was digested with appropriate restriction enzyme digestion (New England BioLab) according to manufacturer's recommendation, then resolved on 3% agarose gel (NuSieve 3:1, FMC Bioproduct, Rockland, ME) containing ethidium bromide.

Table 1: Primer sets, restriction enzymes, and the results for G6PD mutations

Position (cDNA)	Primer names	Sequence	Restriction enzymes		Results (bp)
95	95F	5'CTCTAGAAAGGGGCTAACTTCTCA3'	*Mlu* I	N	198
A→G	95R	5'GATGCACCCATGATGATGAATACG3'		M	174 + 24
392	392F	5'GGACTCAAAGAGAGGGGCTG3'	*BstE* II	N	188+15
G→T	392R	5'GAAGAGGCGGTTGGCCGGTGAC3'		M	203
487	487F	5'GCGTCTGAATGATGCAGCTCTGAT3'	*Hind* III	N	104
G→A	487R	5'CTCCACGATGATGCGGTTCAAGC3'		M	82+22
493	493F	same as 487F	*Ava* II	N	120+11
A→G	493R	5'CTCTGCAGGTCCCTCCCGAAGGGC3'		M	87+33+11
592	592F	5'GAGGAGGTTCTGGCCTCTACTC3'	*Pst* I	N	157+83
C→T	592R	5'TTGCCCAGGTAGTGGTCGCTGC3'		M	157+63+20
871	871F	5'-TGGCTTTCTCTCAGGTCTAG-3'	*Xba* I	N	124
G→A	G6P9R	5'-GTCGTCCAGGTACCCTTTGGGG-3'		M	104+20
1024	1024F	5'GTCAAGGTGTTGAAATGCATC3'	*Mbo* II	N	187
C→T	1024R	5'CATCCCACCTCTCATTCTCC3'		M	150+37
1360	1360F	5'ACGTGAAGCTCCCTGACGC3'	*Hha* I	N	142+45+27
C→T	1360R	5'GTGAAAATACGCCAGGCCTTA3'		M	187+27
1376	1376F	same as 1360F	*Afl* II	N	214

G→T	1376R	same as 1360R		M	194+20
1388	1388F	same as 1360F	*Nde* I	N	227
G→A	1388R	5'GTGCAGCAGTGGGGTGAACATA3'		M	206+21

3. ผลที่ได้รับ **Results**

PREVALENCE OF G6PD MUTATIONS

All blood samples from G6PD deficient newborn were extracted for genomic DNA and assessed for specific G6PD mutations using PCR followed by restriction enzyme digestions. The first mutation assessed in this study is 487 G→A, causing G6PD Mahidol, because it was thought to be the most common variant among Thai G6PD deficient individuals. The pattern of PCR amplification and restriction enzyme digestion is shown in Figure 4. Mutant PCR band will be digested by restriction enzyme *Hind*III and become smaller (82 bp) than normal (104 bp) band (figure 2, lane 2-4). Of 49 G6PD-deficient cord blood samples, only 3 (6%) was found to be causing G6PD Mahidol (figure 5).

1	2	3	4	5	6	7	8	9	10
	N	N	487m	N	N	1376m	N	N	1360m
Marker	-	*Hind*III	*Hind*III	-	*Afl*II	*Afl*II	-	*Hha*I	*Hha*I

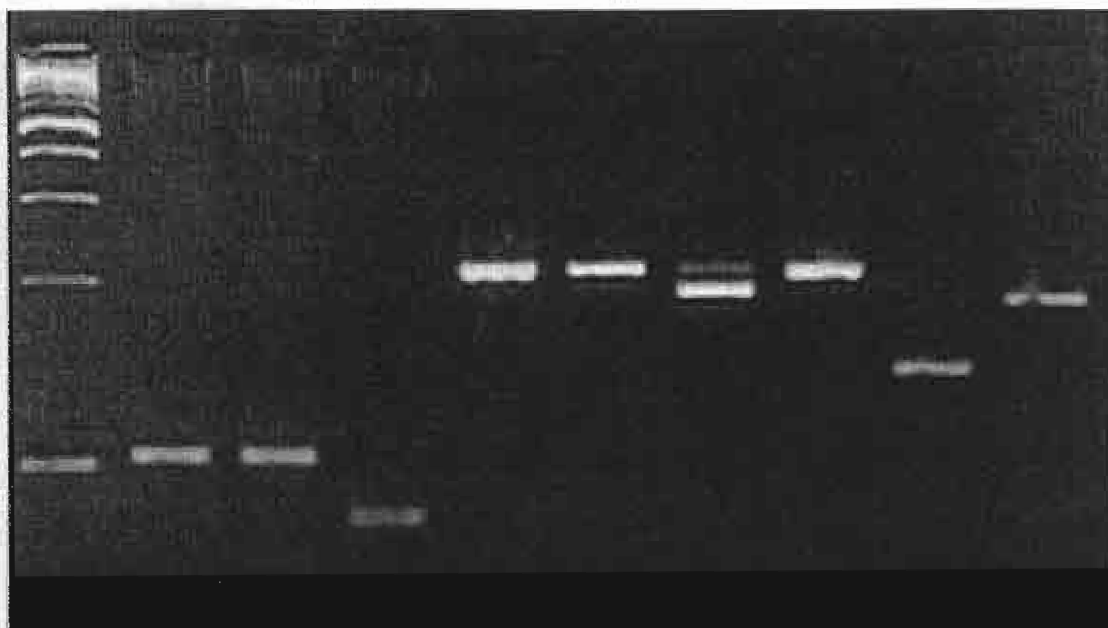

Figure 2. G6PD mutational assay by PCR-restriction enzyme methods. Using 100-bp ladder as molecular weight marker (lane 1) with lowest band corresponding to 100-bp, PCR was performed on genomic DNA from normal non-G6PD deficient control (N) or known G6PD mutant (m) at various nucleotide (only 487, 1376, and 1360 mutant was shown) using PCR primers 487F and 487R (lane 2-4), 1376F and 1376R (lane 5-7), 1360F and 1360R (lane 8-10), followed by restriction enzyme digestion.

primers 487F and 487R (lane 2-4), 1376F and 1376R (lane 5-7), 1360F and 1360R (lane 8-10), followed by restriction enzyme digestion.

Figure 3. PCR-restriction enzyme assay for G6PD Viangchan. PCR was performed on genomic DNA from normal known G6PD Viangchan (control, lane 2& 3) showed a 126-bp band that reduce to 106 bp after XbaI digestion. Female heterozygote (lane 4 & 5) show both 126- and 106-bp bands after XbaI digestion.

To search for mutations in remaining samples, we first PCR amplify exon 9 and determined the sequence. We found a point mutation at nt 871 (A→G) in this sample. The point mutation at nt871 has been reported and named G6PD Viangchan and was found in Laotians & Filipinos in Hawaii (Hsia et, 1993). Therefore this could be common mutation among Thais. We therefore developed a PCR-based assay for G6PD Viangchan using a mutagenic 5'-primer (871F) and a reverse primer (G6PD9R) to amplify exon 9, which will result in 126-bp amplicon. Restriction enzyme *XbaI* digestion will cleave mutant, but not wild-type amplicon, to 106-bp (Figure 3) To distinguish G6PD Viangchan from G6PD Jammu, which differs at a polymorphism at nt 1311, an allele-specific oligonucleotide primers set was used (see materials and methods). We found that in all samples with 871 G→ A, nt 1311 was T, consistent with G6PD Viangchan. (Figure 4) Using this PCR-based assay, G6PD Viangchan was identified in

21 of 39 male cord blood samples (53.8%) (Figure 5, Table 2) Approximately half of G6PD Viangchan has undetectable cord blood G6PD activities.

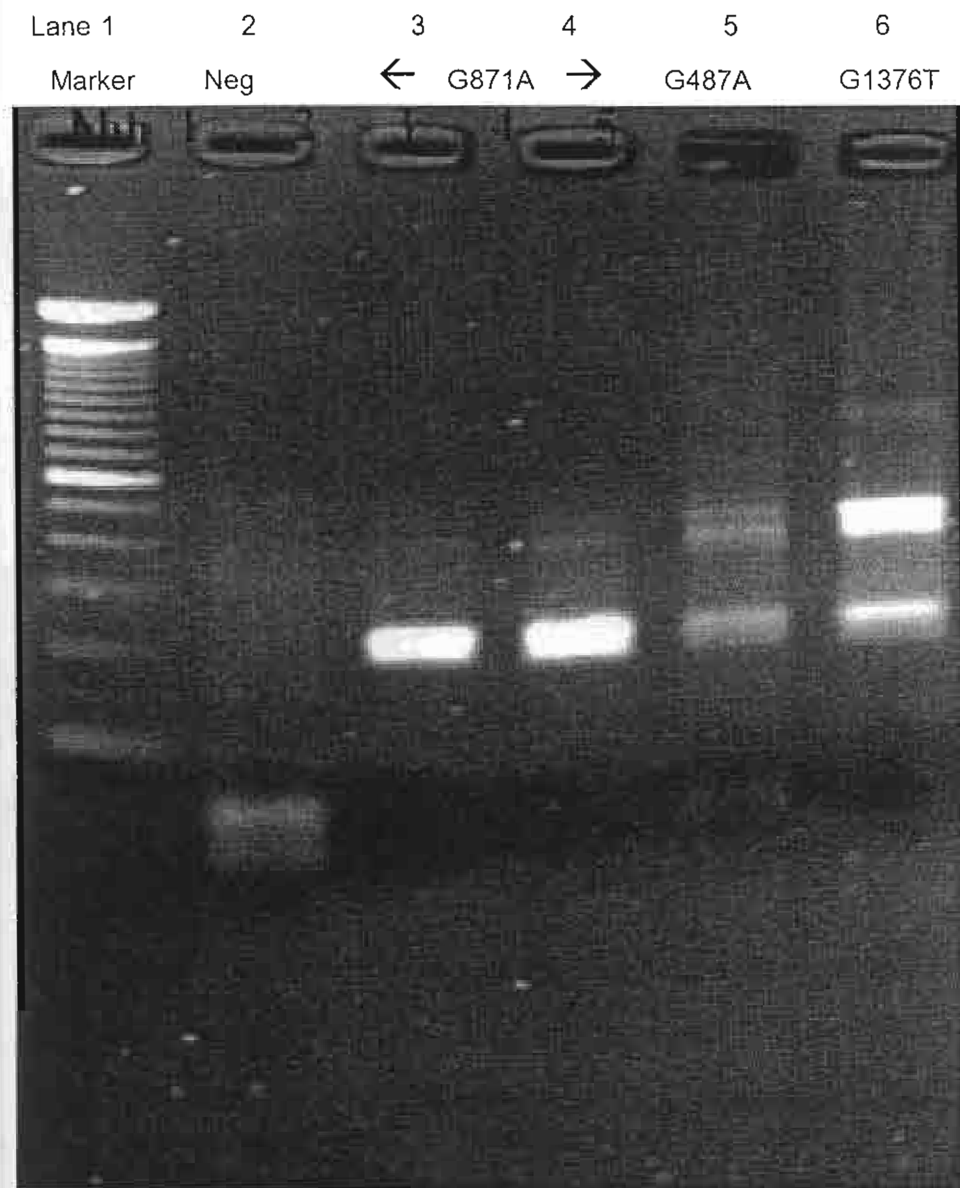

Figure 4. Assay for nt1311 polymorphism. PCR was performed on genomic DNA from G6PD Viangchan (lane 3&4) showed a 160-bp band from 1311T-allele specific primer pairs while other G6PD mutations showed a 360-bp band from outside primer pairs.

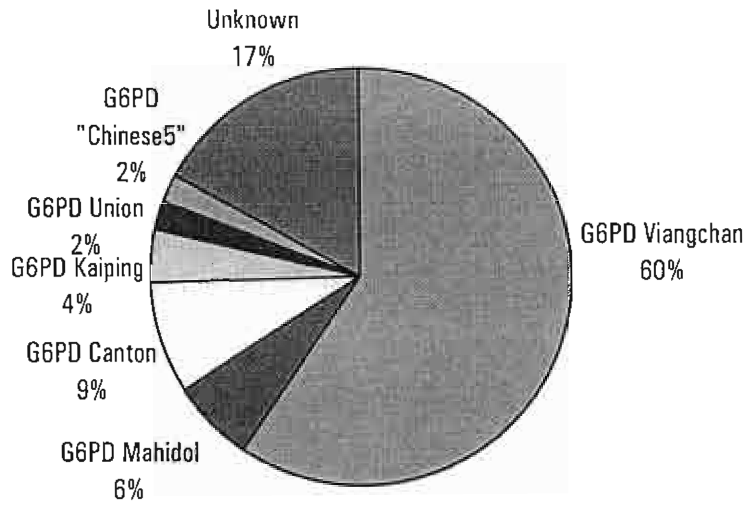

Figure 5. Prevalence of each G6PD mutation among 49 G6PD-deficient cord blood sample.

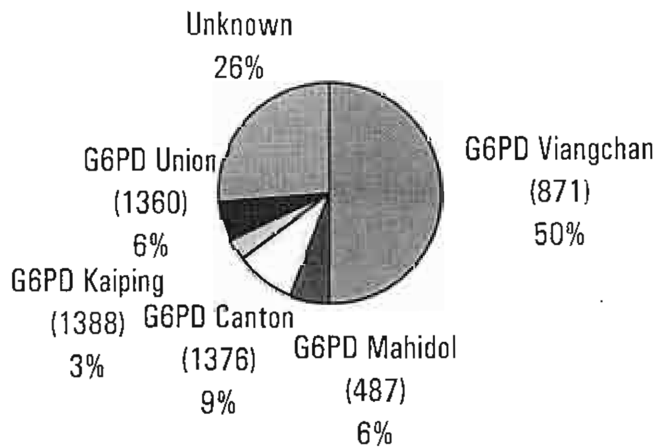

Figure 6. Prevalence of each G6PD mutation among 34 G6PD-deficient jaundiced newborns.

Table 2. Prevalence of G6PD mutations and their activity from male G6PD-deficient cord blood samples or peripheral blood sample from male with neonatal jaundice.

Mutation	Cord blood			Neonatal jaundice		
	Number (%)	G6PD activity (IU/ 100 ml RBC) Median	G6PD activity (IU/ 100 ml RBC) Range	Number (%)	G6PD activity (IU/ 100 ml RBC) Median	G6PD activity (IU/ 100 ml RBC) Range
871 (G6PD Viangchan)	21 (53.8%)	3.9	0.0 - 34.2	12 (60.0%)	4.7	0.0 - 53.5
1376 (G6PD Canton)	4 (10.3%)	12.8	4.8 - 51.7	2 (10.0%)	9.7	7.1 - 12.3
487 (G6PD Mahidol)	3 (7.7%)	3.6	0.0 - 14.5	1 (5.0%)	2.9	-
1388 (G6PD Kaiping)	2 (5.1%)	3.1	0.0 - 6.1	1 (5.0%)	37.7	-
1360 (G6PD Union)	1 (2.6%)	0.0	-	1 (5.0%)	0.0	-
1024 (G6PD "chinese-5")	1 (2.6%)	16.4	-	0	-	-
Unknown	7 (17.9%)	0.0	0.0 - 31.5	3 (15.0%)	0.0	0.0 - 5.7
Total	39 (100%)			20 (100%)		

Of 10 G6PD-deficient female cord blood sample, 6 were G6PD Viangchan, while 4 remained unidentified. All female samples were most likely heterozygote since residual G6PD activity were found, ranging from 19.8 to 55.8 IU/100 ml RBC and amplicon was partially digested (Figure 2.) Similarly, among 7 samples from G6PD-deficient female with neonatal jaundice, 4 were G6PD Viangchan.

4. วิจารณ์ Discussion

We have identified G6PD Viangchan as the most common variant in Thai population. With 21 cases identified among 350 male cord blood samples, the gene frequency of G6PD Viangchan in Thai population is calculated to be 0.06. Consistent with this finding, heterozygous deficient female are also found in 6 out of 172, indicating that some female heterozygote for G6PD Viangchan is not in deficient range.

G6PD Viangchan was first characterized biochemically in 1988 from patients in Laos. This G6PD variant was found to be a WHO class 2, or severely deficient, variant. G6PD Viangchan was subsequently defined molecularly to be a nucleotide 871G→A, predicting an amino acid 291 substitution from Val to Met. Nucleotide substitution 871G→A is also found in G6PD Jammu which was found in patient from India. These two variant differs at a nucleotide 1311 polymorphism, where it is C in G6PD Jammu, and T in G6PD Viangchan. (Beutler et al, 1991)

G6PD Viangchan has been reported to be a common variant among Laotian people (5 of 9 G6PD-deficient subjects) based on a small transplanted population in Hawaii (Hsia et al, 1993). The finding that gene frequency of G6PD Viangchan is high in Thais and Laotians support the common ancestry of these to ethnic group. In contrast, G6PD Viangchan is found in 10% of Filipinos (6 of 53) (Hsia 1993), and only rarely in Chinese population, only 1 in 112 G6PD-deficient male neonate (Huang et al, 1996).

In contrast to previous study (Panich 1972), we did not find G6PD Mahidol to be the most common G6PD variant in Thailand. G6PD Mahidol was named after the university where it was identified biochemically in 1972, and assessed to be a mild (WHO class 3) variant. Among 22 patients with acute hemolysis, G6PD Mahidol was identified in most cases. Subsequently, DNA analysis identified point mutation at nucleotide 487 with substitution of G to A which changed translation of amino acid 163 from glycine to serine (Vulliamy et al 1989). Based on molecular analysis used in our study, we found G6PD Mahidol in less than 10% of G6PD-deficient population. It remains possible that G6PD Mahidol is associated with only episodic hemolysis. The ease of PCR-based asssay would allow us to study this mutation in hemolytic patients in the subsequent study.

G6PD Canton was the most prevalent (50%) in chinese, and is found to be the second most common variant (10%) in our study. This subpopulation could be descendant of chinese

immigrant in to Thai population. The other less common chinese variants, G6PD Kaiping, G6PD Union, and G6PD "chinese-5" were also identified in the proportionately smaller number.

In contrast to G6PD Canton, which was shown to be related to severe hyperbilirubinemia (Huang et al, 1996), there is no trend toward a relationship between G6PD Viangchan and hyperbilirubinemia. The proportion of this to other mutations in G6PD deficient jaundiced newborn is similar to that found in general population, implied by cord blood study. Similar to G6PD deficiency at large, G6PD Viangchan contribute to a relatively late onset of hyperbilirubinemia. The level of bilirubin and date of onset is indistinguishable from other mutations.

5. บรรณานุกรม References

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กิจกรรมอื่น ๆ ที่เกี่ยวข้อง **Related Activities**

1. **ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ** International Publications

   1.1. Tangkijvanich, P., Hirsch, P., Theamboonlers, A., **Nuchprayoon, I.,** and Poovorawan, Y. (1999) Contribution of Hepatitis Viruses to Hepatocellular Carcinoma in Thailand. J Gastroenterol, 34:227-233.

   1.2. **Nuchprayoon I**, Jantaradsamee P, Tangkijvanich P, Suwanagool P, Kullavanijaya P, Janchai A, Hirsch P, Poovorawan Y. (2001) Presence of HBV DNA in Serum and PBMC of HBsAg-Negative Patients with Hepatocellular carcinoma and Chronic Liver Disease, submitted to Hepatology Gastroenterology.

   1.3. **Nuchprayoon I,** Sawatpanich A, Kittikalayawong A, Ungbumnet W, Sawatpanich A, Nuchprayoon S, Sanpawat S. (2000) G-6-PD Viangchan (871G→A) is the most common G-6-PD deficient variant in Southeast Asia. Blood 96 (11 suppl 1): 8b

   1.4. **Nuchprayoon I,** Sanpawat S. Triteeraprapab, S, (2001) G6PD Viangchan (871G→A) is the most common G6PD deficient variant in in Thai population. submitted to Human Mutation (Online)

2. **การนำผลงานวิจัยไปใช้ประโยชน์**

   2.1. **<u>เชิงพาณิชย์</u>** -

   2.2. **<u>เชิงนโยบาย</u>** -

   2.3. **<u>เชิงวิชาการ</u>**

   งานวิจัยเรื่อง HBV DNA ในผู้ป่วยมะเร็งตับอาจนำไปสู่การความรู้ใหม่ว่าในผู้เคยติดเชื้อไวรัสตับอักเสบบี แต่ดูเหมือนว่าสามารถกำจัดไวรัสได้ เพราะตรวจไม่พบ HBsAg นั้น อาจยังมีความเสี่ยงที่จะเกิดมะเร็งตับได้ แต่จำเป็นต้องได้รับการพิสูจน์ในระยะยาวต่อไป

   งานวิจัยเรื่อง G6PD นี้เป็นการค้นพบองค์ความรู้ใหม่ เกี่ยวกับลักษณะ (mutation) ของเอนซัยม์ G6PD ในประชากรไทย ซึ่งเปลี่ยนความเชื่อเดิม และมีการพัฒนาวิธีการตรวจหา mutation ชนิดนี้ ทำให้สามารถตรวจได้โดยสะดวก แต่ยังมิได้มีการเผยแพร่ให้สาธารณะชนได้ทราบ

   2.4. **<u>เชิงสาธารณะ</u>**

   จากงานวิจัยที่พบว่า G6PD เวียงจันทน์ เป็น ความผิดปกติที่พบได้เป็นส่วนใหญ่ของประชากรไทย ซึ่งก็พบได้ในประชากรลาว แต่พบได้น้อยมากในชาวจีน นับเป็นหลักฐานอีกประการหนึ่งที่สนับสนุนทฤษฎีที่ว่า ประชากรในเขตแหลมทองน่าจะตั้งหลักแหล่งในบริเวณนี้นานแล้ว และประชากรไทย-ลาว ล้วนมีบรรพบุรุษเดียวกัน

   2.5. **<u>เชิงวิชาการแบบอื่น</u>**

      2.5.1. <u>การพัฒนาการเรียนการสอน</u>

กำลังอยู่ในระหว่างการเขียนตำราเพื่อเพิ่มความรู้ใหม่ในเรื่องมะเร็งตับ และอณูชีววิทยาของ G6PD ในชาวไทย

2.5.2. การสร้างนักวิจัยใหม่ในลักษณะอื่น ๆ

ก. ระดับปริญญาตรี ไม่มี

ข. ระดับปริญญาโทที่กำลังศึกษาอยู่ 2 ราย

ค. ระดับปริญญาเอกที่กำลังศึกษาอยู่ 1 ราย

ง. ระดับปฏิบัติการ: อบรมเจ้าหน้าที่หน่วยโลหิตวิทยาเพื่อให้เพิ่มศักยภาพในการทำงานระดับอณูชีววิทยา ทำให้สนใจศึกษาต่อในระดับปริญญาโทต่อไป

3. **อื่น ๆ** Invited speaking activities

3.1. **ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการในประเทศ**

(1) Sanpawat S, Nuchprayoon I, Kittikalayawong A, Ungbumnet W. (2001) Methemoglobin Reduction test as screening test for neonatal glucose-6-phosphate dehydrogenase deficiency. J Med Assoc Thai (submitted)

3.1. **การเสนอผลงานในประชุมวิชาการ**

(1) Sanpawat S, Nuchprayoon I, Kittikalayawong A, Ungbumnet W. Prevalence of G-6-PD deficiency in normal newborn and neonatal jaundice. 41st Annual meeting, Faculty of Medicine, Chulalongkorn University, March 22-26, 2000

(2) Nuchprayoon I., Sawatpanich, A., Kittikalayawong A. Triteeraprapab S. and Sunpawat S. Molecular Genetics of G-6-PD Deficiency in Thai Newborn. 41st Annual meeting, Faculty of Medicine, Chulalongkorn University, March 22-26, 2000

(3) Nuchprayoon I., Sawatpanich, A., Kittikalayawong A. Triteeraprapab S. and Sunpawat S. G-6-PD Viangchan (871G→A) is the most common variant in G-6-PD deficiency in Thailand. ในการประชุมวิชาการประจำปี สมาคมโลหิตวิทยาแห่งประเทศไทย ในวันที่ 22 มกราคม 2544 ณ โรงแรมสยามซิตี้ กรุงเทพ

3.3. **หนังสือ / คู่มือ**

ยังไม่มี

ภาคผนวก

HBV DNA in Serum and PBMC of Patients with Hepatocellular carcinoma and Chronic hepatitis

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Running head:- HBV DNA in serum and PBMC in HBsAg-negative patients

Key words:- HBV DNA, PBMC, HBsAg, hepatocellular carcinoma, chronic liver disease.

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Background/Aims: Most patients with hepatocellular carcinoma (HCC) in Thailand have been infected with hepatitis B virus. Some of these patients are HBsAg negative, but it is not known whether hepatitis B viral genome is still present in these patients and contribute to hepatic carcinogenesis.

Methodology: We investigated sera and peripheral blood mononuclear cells (PBMC) of 36 HCC and 31 chronic hepatitis patients, by serology and by nested PCR of HBV DNA: hepatitis B- s gene (HBs) and x gene (HBx), and RFLP for the T 1762 / A 1764 mutation in the core promoter.

Results: In HBsAg-negative patients who has positive anti-HBc, HBx DNA were detectable detectable in PBMC or serum of 10 of 15 HCC, but only 1 of 9 chronic hepatitis patients. HBx DNA were detectable in PBMC in some (6 of 15) HBsAg-positive, and most readily (10 of 15) HBsAg negative anti-HBc positive HCC. RFLP analysis showed that most HBx were also of wild type, only one was mixed wild type and mutant.

Conclusion: Presence or absence of HBsAg is an insufficient indicator with regard to a given individual's risk to proceed towards chronic liver disease. People who were previously infected by HBV may also be at risk in developing hepatocellular carcinoma despite HBsAg clearance.

INTRODUCTION

Hepatitis B virus (HBV) infection constitutes a major public health burden on a global scale as it has been found responsible for chronic liver disease such as chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (1). It is believed that people who acquired HBV infection during infancy (2) or in the course of delivery are at high risk of progression towards chronic carriage and subsequent cirrhosis and/or HCC (3).

The worldwide carrier rate of HBV has been conservatively estimated at 5%, or 350 million individuals, with highest prevalence in Southeast Asia and sub-Saharan Africa where it has been established at between 5 and 35% (4). The high carrier rate in these countries is most likely due to vertical (perinatal) transmission or early horizontal transmission within extended families or among pre-school children (5).

In Europe and Japan, where most HCC are negative for HBsAg, hepatitis C virus (HCV) is most frequently found as the causative agent of chronic liver disease (6, 7). However, recent report showed that in HBsAg negative, HCV positive patients, HBV genome could be demonstrated in the cancerous liver of many patients (8). Among HBV genes, the X protein (HBx) is most important in hepatic carcinogenesis (9). HBx protein is highly conserved among all known HBV subtypes, is multifunctional and is capable of transactivation (10). The HBx protein can stimulate the promoter of the HBV genome itself, as well as numerous other viral promoters and an array of cellular genes (9,10). HBx has also been shown to impair *p53* function (11,12), as well as stimulating cellular gene expression through activation of several protein kinases (14). Moreover, the oncogenic potential of HBx has become evident by its capacity to transform rodent cells *in vitro* (11) and, as a transgene, to induce hepatocellular carcinoma in mice (12).

The current method of choice to detect HBV infection is serum hepatitis B surface antigen (HBsAg) along with serum antibody to hepatitis B core protein (anti-HBc). It is commonly interpreted that clinically asymptomatic individuals found to express anti-HBc but lacking HBsAg have been infected with HBV in the past but succeeded in clearing the virus, whereas those positive for both anti-HBc and HBsAg were considered chronic carriers. However, recent study

using polymerase chain reactions (PCR) could detect HBV DNA in blood from a large number of hemodialysis patients even if they are HBsAg-negative, suggesting that the viral DNA may persist for several years after acute hepatitis B has been resolved (13).

In contrast to Western countries, HCC is the most common cancer among Thai male (14). In a previous study aimed at determining the major etiologic factors responsible for HCC in Thailand, an area endemic for HBV infection, our group has established the prevalence of HBV at 65%, contrasted by that of HCV 14% (14). The role of hepatis B viral genome has not been investigated in patients with hepatocellular carcinoma, particular in HBsAg negative HCC cases. We therefore investigate presence of HBs and HBx DNA in our HCC patients. Because of liver specimen scarcity, we searched for evidence of HBV genome in the serum and PBMC in patients with chronic liver disease ranging from chronic hepatitis to HCC, and along with their HBsAg and anti-HCV serology. We detected HBV genome in many HBsAg-negative sera and PBMC, and investigated mutation of HBx in these specimens.

MATERIALS AND METHODS

Population Studied

Two groups of patients admitted to Chulalongkorn University Hospital between August 1997 and October 1999 were included in the study. The first group comprised 36 patients with HCC diagnosed on the basis of histopathology and/or serum α-fetoprotein levels above 400 U/ml with liver tumor evidenced on scintigraphy. The second group included 31 chronic hepatitis patients diagnosed on the basis of histopathology from liver biopsy and/or persistently elevate serum aminotransferases. Twenty healthy voluntary blood donors presented at the National Blood Center between October and November, 1999 were randomly selected and blood specimens served as negative controls.

All individuals were informed the objective of the study and provided their consent. Peripheral blood was obtained during examinations, administering ethylenediaminetetraacetic acid (EDTA) as anticoagulant for PBMC separation, as well as clotted blood for serum analysis.

Laboratory Methods

Serology

All sera were subjected to enzyme linked immunosorbent assays (ELISA) for detection of HBsAg, anti-HBc (Human Gesellschaft fur Biochemica und Diagnostica mbH, Germany) and anti-HCV (third-generation test, Abbott Laboratories, North Chicago, Ill.) using commercially available test kits according to the manufacturer's specifications.

PBMC separation

Sera were obtained by centrifugation of the clotted blood at 1,500 rpm for 10 minutes (Beckman refrigerated centrifuge). PBMC were separated by spinning the EDTA-treated blood on a Ficoll-Hipaque (Pharmacia, Uppsala, Sweden) gradient at 2500 rpm at 4° C for 15 minutes (Beckman refrigerated centrifuge), followed by four consecutive washing steps with phosphate buffered saline (PBS) at 2500 rpm at 4° C for 15 minutes each. In previous studies the washing buffer remaining after the final washing step had also been subjected to PCR in order to examine the plasma for HBV DNA contamination (15,16). Based on the negative results then obtained in the present study we did not perform PCR on this buffer but instead, kept it at -70° C. The PBMC thus obtained were suspended in 1 ml PBS and after staining with methylene blue, their respective concentration was determined in an improved Neubauer ruling chamber. All specimens were kept at -70° C until further analysis.

Liver tissue DNA extraction

Liver tissue was obtained from surgical resections. As soon as liver tissue is removed from the patient, carcinomatous part and non-carcinomatous part were dissected and frozen separately in aliquots and kept in liquid nitrogen. DNA extraction from liver tissue was performed by grinding frozen HCC or liver tissue in liquid nitrogen with mortars and pestles to fine powder. The tissue were suspended in extraction buffer (10 mM Tris pH7.4, 1% SDS, 10 mM proteinase K) and allowed to digest overnight at 50°C, followed by phenol-chloroform extraction, and ethanol precipitation. After dissolution in 10 mM Tris-EDTA, DNA was quantitated and used for PCR reactions.

HBV DNA extraction

DNA was extracted from sera and PBMC by incubating the respective samples in Tris/SDS-buffer containing proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. Pellets were re-suspended in 20 μl sterile water each and directly subjected to the polymerase chain reaction (PCR).

For DNA amplification by nested (PCR) 10-μl aliquots of the re-suspended DNA samples were added to 40 μl of a reaction mixture containing 1.5 U of *Taq* polymerase (Pharmacia, Uppsala, Sweden), each of four deoxynucleotide triphosphates (Promega Corp., Madison, WI, USA) at a concentration of 200 μM, primer pairs (Biosynthesis, Lewisville, Texas) of F_1, R_6 (s gene) or Xo_1, Pc_1 (x gene) (first round) and F_2, R_5 (s gene) or Xi_1, Xi_3 (x gene) (second round) 1 μM each, 10 mM Tris/Cl buffer prepared with the required $MgCl_2$-concentration and sterile H_2O *ad* 40 μl in 0.2 ml PCR tubes. The reaction mixtures were spun in a microcentrifuge for 2 sec before being placed in the thermocycler (Perkin Elmer Cetus, Branchburg, NJ, USA).

The details of primer sequences used in this study were derived from HBV DNA sequence as follows:

F_1 : 5'-GGA GCG GGA GCA TTC GGG CCA-3' (nucleotide position 3022-3042).

R_6 : 5'-GGC GAG AAA GTG AAA GCC TG-3' (nucleotide position 1103-1084).

Xo_1: 5'-CTC TGC CGA TCC ATA CTG C-3' (nucleotide position 1254-1272).

Pc_1: 5'-GGA AAG AAG TCA GAA GGC-3' (nucleotide position 1974-1956).

F_2 : 5'-CAT CCT CAG GCC ATG CAG TGG A-3' (nucleotide position 3193-3214).

R_5 : 5'-AGC CCA AAA GAC CCA CAA TTC-3' (nucleotide position 1015-995).

Xi_1 : 5'-AGC TTG TTT TGC TCG CAG C-3' (nucleotide position 1285-1305).

Xi_3: 5'-GGC ACA GCT TGG AGG CTT-3' (nucleotide position 1883-1866).

The reaction was then performed using both the first and second round s- and x-gene primer pairs consecutively for 30 cycles each round, at 94° C for 1 min, 55° C for 1 min, and 72° C for 1 min, for the first round, then continued at 94° C for 30 sec, 55° C for 30 sec, and 72° C for 1 min, for the second round, then concluded by an extension cycle at 94° C for 1 min, 55° C for 2 min, and 72° C for 10 min, respectively. Upon electrophoresis in a 2% Nusieve agarose gel (FMC Bioproducts, Rockland, ME, USA), stained with ethidium bromide, at 90 V for 80 minutes, the bands indicating the presence of HBs and/or x DNA became visible under UV light at 1037 and 596 bp, respectively.

Restriction Fragment Length Polymorphism (RFLP)

Those PCR products revealing the presence of HBx gene were subjected to restriction fragment length polymorphism (RFLP) analysis using the restriction endonuclease *Sau 3AI* to investigate the core promoter sites at codons 1762-1764 for potential point mutations (16, 17). To that end, 15 U of *Sau 3AI* (New England Biolabs, MA, USA) were added to 10 μl of the respective 2nd round PCR products in a reaction buffer supplied by the manufacturer, and incubated at 37° C for 4 hours. The RFLP products were analyzed by electrophoresis on a 2% Nusieve gel and their respective sizes compared to those of a suitable nucleotide size marker (100 bp DNA ladder, Promega Corp., Madison, WI, USA). The sizes expected were

483 and 113 bp for the 1762/1764 wild type, and 362 and 121 bp for the 1762/1764 mutant, respectively. (Figure II)

RESULTS

Hepatitis X gene is present in an HBsAg negative HCC

Using PCR for HBx and HBs gene to amplify DNA from hepatocellular carcinoma (H) and non-cancerous (L) liver from the same patient (Fig I), we demonstrated HBs and x gene in known HBsAg positive patients (case 1, 2, 8, 9). In two HBsAg negative patient, one has no detectable HBV genome (case 5), the other patient (case 6) has no detectable HBs DNA but detectable HBx DNA in the non-cancerous liver. In this patient, whose serum and peripheral blood mononuclear cell (PBMC) was available for study, HBx but not HBs DNA was detectable in DNA extract from PBMC (data not shown), but not serum. The finding of HBx DNA in liver and PBMC of this patient suggested that HBV DNA may persist in liver and blood samples of some HBsAg-negative patient who developed HCC.

HBV DNA in blood samples of patients with HCC and chronic hepatitis

Because of scarcity of HCC liver specimens, we further searched for evidence of HBV genome in blood samples of HCC patients who were not qualified for surgical treatment, compared with a group of patient with chronic hepatitis, as well as blood samples from volunteer donor as a control group. Blood samples were separated into serum and peripheral blood mononuclear cells and assayed for HBs and HBx DNA separately. Serum from each patients were also tested for HBsAg, anti-HBc antibody, and anti-HCV, and patients are classified into three groups according to their serological results: chronic hepatitis B carriers (HBsAg-positive, anti-HBc positive), previous HBV infected patients (HBsAg-negative, anti-HBc positive), and patients with no HBV infection (HBsAg-negative, anti-HBc negative).

Among 36 HCC patient, 15 were attributed to HBV as they were serologically positive for both HBsAg and anti-HBc, with one patient also positive for anti-HCV. In this group, HBV- s and/or x gene were detectable in all but two cases (Table I). In the remaining 21 HBsAg-negative HCC cases, 15 were previously infected with HBV (positive for anti-HBc). Among these HCC patients with previous HBV infection, HBV-X gene was detectable in two-third of their blood specimens despite HBsAg negativity. Evidence of hepatitis C infection (anti-HCV) is found in 7 cases of this HBsAg-negative group and seems to be an independent finding (data not shown).

Among the 31 chronic hepatitis patients, 16 were positive for both HBsAg and anti-HBc, the remaining 15 were negative for HBsAg (Table I). In this HBsAg negative group, 9 were positive for anti-HBc and 4 positive for anti-HCV. Both HBs and HBx DNA were detectable in most (14 of 16) HBsAg-positive patients, but less commonly (4 of 9) in HBsAg-negative/ anti-HBc-positive group. All the 20 volunteer blood donors were negative for HBsAg, with 7 positive for anti-HBc, and none of which were positive for anti-HCV or has detectable HBV (s or x) DNA. There were no patients or normal volunteer who were positive for HBsAg but not anti-HBc.

Hepatitis X gene is prevalent in PBMC of HBsAg-negative patients with HCC but not chronic liver diseases.

Among the 15 serologically HBsAg positive HCC patient samples, HBs DNA is detectable by PCR, either in the sera only (2 cases), PBMC only (1 case) or both (9 cases). HBx DNA is detectable mostly in the sera only (6 cases), PBMC only (3 cases) or both (3 cases) (Table II). Of 15 HCC patient who were previously infected with HBV but were HBsAg negative, HBs DNA were detectable in PBMC preparation from only one case. In contrast, HBx DNA, were readily detectable in sera and PBMC in 3 cases, and PBMC only in 7 cases.

Among chronic hepatitis patients who are HBsAg positive, most (14 of 16) patients had detectable HBs gene, usually in both serum and PBMC (11 cases) (Table II). Of 9 chronic hepatitis patients who were previously infected with HBV (anti-HBc positive), but were HBsAg negative, only four cases had detectable serum HBs DNA, and only one case has detectable serum HBx DNA.

Analysis of HBx mutation among patients HCC and chronic hepatitis

We analysed the RFLP pattern of HBx from HCC and chronic hepatitis patients who has detectable HBx DNA in serum and/or PBMC by PCR using restriction enzyme *Sau3AI*. Most (9 of 11) HBx-positive HCC sera were wild type, while the remaining 2 were mutant HBx genes. Similarly, most (16 of 17) HBx from PBMC preparations were also of wild type, only one was mixed type. On the contrary, HBx DNA from sera of chronic hepatitis patients revealed that 2 were wild type, 5 were mutant, and 6 were mixed type, while 9 PBMC preparations derived from the same source showed that 1 was wild type, 3 were mutant and 5 were mixed type. (Table III)

DISCUSSIONS

Traditionally, it was believed that people who are chronic carriers of hepatitis B, defined by the presence of HBsAg, are at risk to develop hepatocellular carcinoma. Our results support the recent findings that in people who are previously infected with HBV, HBV genome can persist despite clearance of HBsAg (18). This phenomenon could be explained by mutation of HBs antigen rendering it undetectable by commercially available ELISA method (19,20). Alternatively, serum HBV DNA in HBsAg-negative individuals may have resulted from masking of HBsAg in HBsAg – anti-HBs immunocomplexes (21). Lastly, HBsAg-expressing liver could have been cleared by host immunity, while

occasional integration of HBV into host genome without HBs-expression could escape clearance and regenerated.

HBx antigens have been detected in many HCC specimens obtained from patients infected with HBV (22,23). HBx gene has been found to integrate more commonly than S gene (24). After integration, HBx protein may be involved in liver cell transformation as it has been described as transforming cultured cells (25,26) as well as transgenic mice (27). HBx RNA transcript, rather than s- or c-transcript, is found in HCC from HBsAg-negative patients (28-29). Because of limited number of our liver specimens, we can not pursue further study.

In addition to liver and serum, peripheral blood mononuclear cells, particularly lymphocytes, have been shown to harbor HBV (30), in patients with acute and chronic hepatitis (31) where it has been shown most heavily in monocytes and B-cell. HBV can replicate and transcribe HBx gene in PBMC of patients with active hepatitis (32). HBV DNA has been found in PBMC of chronic HBV carriers (33) as well as in some HBsAg-negative hemodialysis patients (13). In searching for evidence of HBV genome in serum and PBMC using nested PCR, special care were taken not to introduce cross-contamination between samples. Our results were valid because our negative controls: serum and PBMC from healthy HBsAg-negative volunteer blood donor samples, as well as anti-HBc negative patient samples, has undetectable HBV DNA. We found that hepatitis B genome, particularly HBx gene, was detectable more frequently in PBMC than in serum of HCC patients, while HBs DNA was present more frequently in serum of chronic hepatitis patients. Although there was no relationship between HBx in PBMC and HBx in hepatic cells, this HBV gene in PBMC could be a useful marker of persistence of HBV genome in the HBsAg negative patient who were previously infected with HBV. The relevance of HBx DNA in PBMC as a marker to identify people at high risk of HCC should be determined in a prospective study.

HBV DNA was not detectable in blood samples of all HBsAg-positive patients. HBV DNA is probably not related to the pathophysiology of HCC, but rather a marker of persistence of HBV DNA in these patients. While previous reports noted relationship between HBx DNA in liver of with HBsAg-negative, HCV positive patients (8), there are too few cases of HCV positive cases in our study to make any meaningful conclusion. A larger study should be conducted to determine whether HBx or HCV is a more important contributor to hepatocellular carcinoma in HBsAg-negative individuals.

HB x mutation has been frequently reported in HCC and non-cancerous tissue in patients with HBsAg (34) or without detectable HBsAg (8). The double mutations T^{1762} and A^{1764} changes codon 130 and 131 of X protein at the region which bind to p53 protein in the cytoplasm, and prevent apoptosis (35). This same region also functions as the core promoter of HBV DNA for precore mRNAs and the pregenome mRNAs (36,37) which codes for Hbe antigen. The mutation of this region has been shown to decrease transcription of the HBeAg precursor (38) While HBx T^{1762}/A^{1764} mutation in often found in the carcinomatous liver (8), we found that most HBx from PBMC of our HCC patients were of wild type. However, HBx from PBMC of chronic hepatitis patient has higher prevalence of the T^{1762}/A^{1764} double mutation, as well as mixed populations of wild type and mutant among the chronic hepatitis patients. The significance of this finding is unclear at present.

In conclusion, our results has shown that presence or absence of HBsAg is an insufficient indicator with regard to a given individual's risk to proceed towards chronic liver disease. People who were previously infected by HBV may also be at risk in developing hepatocellular carcinoma despite HBsAg clearance.

ACKNOWLEDGEMENTS

We would like to express profound gratitude to the staff of the Gastroenterology Unit, Department of Internal Medicine, and the Department of Radiology for collecting the specimens required, and to the National Blood Center, Thai Red Cross for providing the control samples. We would also like to thank the entire staff of the Viral Hepatitis Research Unit, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University & Hospital for their tireless effort in conducting the present study. This project has been supported by the Molecular Biology Project, Office of Research Affairs, Faculty of Medicine, Chulalongkorn University & Hospital. IN is supported by The Thailand Research Fund.. YP is a Senior Research Scholar supported by the Thailand Research Fund.

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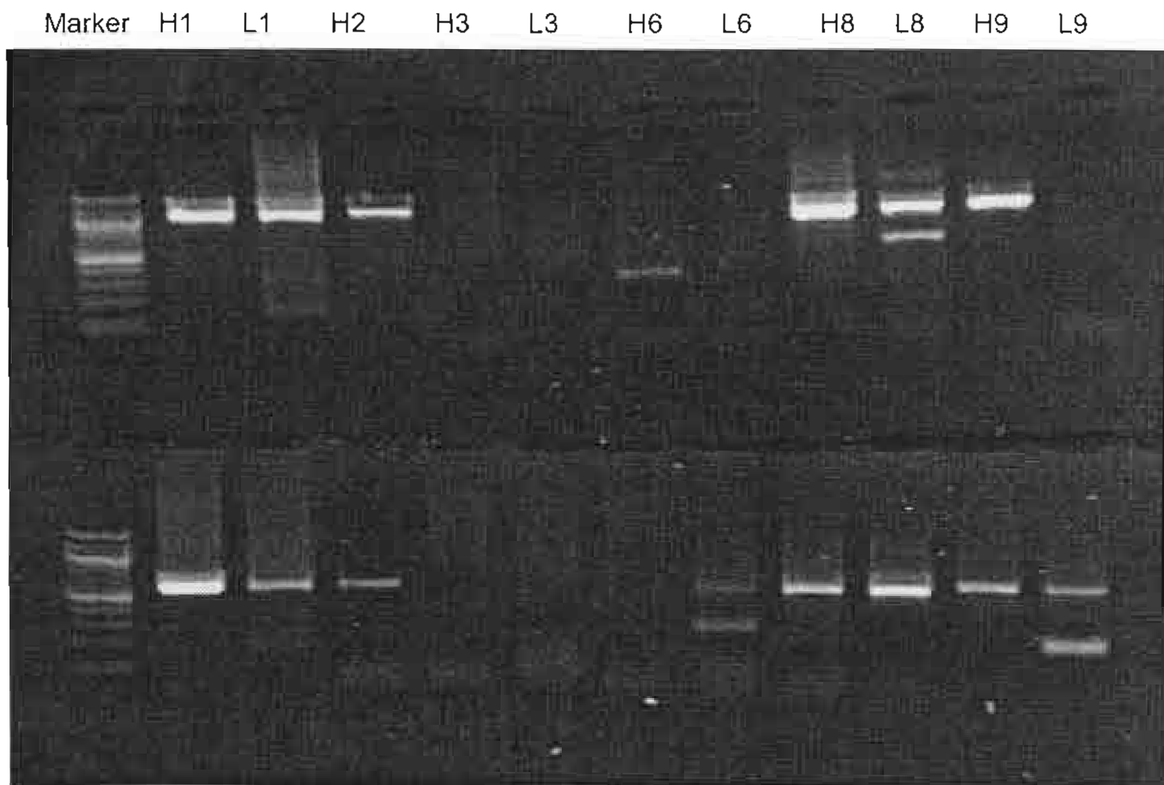

Figure I. Gel electrophoresis of HBs- (upper lanes) and HBx-DNA (lower lanes) in hepatocellular carcinoma (H) and non-carcinomatous liver from the same patient (L). Number indicate case number. PCR-amplified HBs band is 1037-bp, and HBx band is 596 bp.

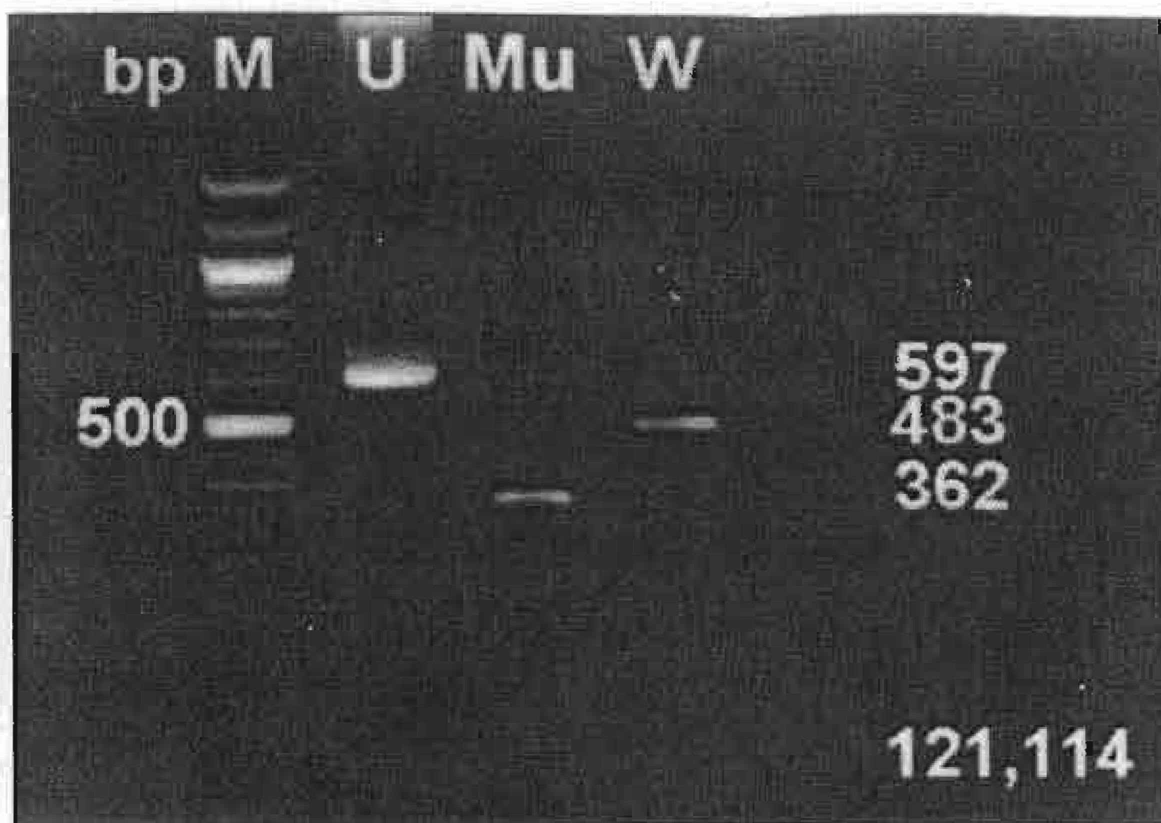

Figure II. Metaphor gel electrophoresis of the HBx DNA from samples of hepatitis B virus, after restriction-fragment-length-polymorphism (RFLP) analysis using *Sau*3A I. The photograph shows *Sau*3A I. restriction patterns of PCR products. [Molecular weight markers (M), undigested PCR product *(U), mutant type* T^{1762} A^{1764} *(Mu) and wild type* A^{1762} G^{1764} *(W)]*

Table 1. Detection of hepatitis B viral DNA in chronic liver disease patients compared with voluntary blood donors, classified by disease and hepatitis B serology. The total (N) and number of cases with detectable HB-s DNA (S only), HB-x DNA (X only), HBs- and HBx DNA (Both) or undetectable (None) HBV DNA by PCR, or positive for anti-hepatitis C virus antibody (Anti-HCV) are presented.

		Number of cases with HBV DNA				Anti-HCV
Group	N	None	S only	X only	Both	+ve
Hepatocellular carcinoma						
HBsAg+ve, Anti-HBc+ve	15	2	1	1	11	1
HBsAg-ve	21					8
and Anti-HBc +ve	15	4	1	10	0	7
and Anti-HBc -ve	6	0	0	0	0	1
Chronic Hepatitis						
HBsAg+ve, Anti-HBc+ve	16	2	0	0	14	0
HBsAg-ve	15					4
and Anti-HBc +ve	9	5	3	0	1	3
and Anti-HBc -ve	6	6	0	0	0	1
Blood donor HBsAg-ve	20	0	0	0	0	0

Table II. Detection of hepatitis B viral DNA in patients who has previous HBV infection (Anti-HBc positive), in the serum or peripheral blood mononuclear cell (PBMC).

Group	N	Presence of HBs-DNA in			Presence of HBx-DNA in		
		Serum	PBMC	Both	Serum	PBMC	Both
Hepatocellular carcinoma							
HBsAg+ve	15	2	1	9	6	3	3
HBsAg-ve	16	0	1	0	0	7	3
Chronic Hepatitis							
HBsAg+ve	16	2	1	11	6	3	5
HBsAg-ve	9	2	1	1	0	0	1

Table III. Frequency of core promoter mutants among HBx-DNA positive chronic liver disease patients.

	Number of HBx-DNA positive	HBx-DNA RFLP type		
		Wild	Mutant	Mix
Chronic hepatitis (n=31)				
Serum HBx-DNA	13	2	5	6
PBMC HBx-DNA	9	1	3	5
HCC (n=36)				
Serum HBx-DNA	11	9	2	0
PBMC HBx-DNA	17	16	0	1

Abstracts for the
42nd Annual Meeting
of the

AMERICAN SOCIETY OF HEMATOLOGY

Abstract# 3682

COBALAMIN (VITAMIN B12) DEFICIENCY IDENTIFIED IN YOUNG, CAUCASIAN WOMEN. Eric J. Norman* (Intr. by Robert [illegible]) *Norman Clinical Laboratory, Inc., Cincinnati, OH, USA*

[illegible] anemia usually occurs after age forty, although there are reports of cases among young African-American women. We report six young, non-vegetarian Caucasian women (Table) with initially low cobalamin (Cbl) who have been treated with regular Cbl [illegible]. Three of the six had pretreatment urinary methylmalonic acid (UMMA) levels [illegible] were increased for this indicator of tissue Cbl deficiency. Although these laboratory [illegible], most of these women had significant neurological [illegible] which first brought them to medical attention. Subjects 1 and 2 experienced [illegible] spinal cord problems causing debilitation of arms and legs. After nine years of [illegible] IM, Subject 1 has improved but still can only walk short distances [illegible], and has severe [illegible] pain. After two years of Cbl IM, Subject 2 is [illegible] recovered. After two months of Cbl IM Subject 3 has more energy and [illegible] and tingling have resolved. Subject 4 has a 18 month history of pain, [illegible] and balance problems. Subject 5 has no reported symptoms. Subject 6 has pain and numbness in hands, legs, and face. Subjects 4 and 6 are too early in therapy to assess improvement. Multiple Sclerosis was suspected and ruled out in Subjects 1, 2, and 4. [illegible] tests were performed in Subjects 1, 2, and 6 and were normal. This population [illegible] further evaluation since it has not been considered prone to Cbl deficiency.

[illegible]	Serum Cbl (pg/ml) (normal)	UMMA (µg/mg creatinine pre / post Cbl IM, normal < 4)
[illegible]	[illegible]	NA / NA (not available)
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	NA / [illegible]
[illegible]	[illegible]	[illegible]
[illegible]	NA	[illegible] / NA
[illegible]	[illegible]	[illegible]

Abstract# 3683

G6PD VIANGCHAN (871G→A) IS THE MOST COMMON G6PD DEFICIENT VARIANT IN SOUTHEAST ASIA. Issarang Nuchprayoon,[1] [illegible]aya Kittikalayawong*,[1] Waraporn Ungbumnet*,[1] A. Sawatpanich*,[1] Tita [illegible]chset*,[1] Surang Nuchprayoon*,[1] Suwimol Sanpawat*.[1] [1]*Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.*

Glucose-6-phosphate dehydrogenase (G6DP) deficiency is the most common inherited disorder in human. The prevalence of G6PD deficiency in Thailand and Southeast Asia is particularly high, and is a common cause of neonatal hyperbilirubinemia. We studied the prevalence of G6PD deficiency in 522 randomly selected cord blood and 229 peripheral blood from neonates with hyper-billirubinemia, and developed a PCR-restriction enzyme [illegible] method to identify G6PD Viangchan (871 G→A), and searched for this and 9 other mutations in DNA extract from G6PD deficient blood samples. We found that the prevalence of G6PD deficiency is 11.1% in Thai male (N=350) and 5.8% in female (N=172). G6PD Viangchan (871 G→A) is the most common mutation identified (57%, 28 of 49 samples) followed by G6PD Canton (1376 G→T, 4 cases, 8%), G6PD Mahidol (487 G→A, 3 cases, 6%), G6PD Kaiping (1388 G→A, 2 cases), and 1 each was G6PD Union (1360 C→T) and "Chinese-5" (1024 C→T). G6PD deficient mutation remained unidentified in [illegible] cases (20%). Among newborns with neonatal jaundice, the prevalence of G6PD deficiency is 22.1% in male (N=140) and 10.1% in female (N=89). G6PD Viangchan is the most commonly identified (50%, 17 of 34 samples), followed by G6PD Canton (3 cases), G6PD Mahidol (2 cases), and G6PD Kaiping (1 case), and 11 cases remained unidentified. No case of G6PD Gaohe (95 A→G), "Chinese-4" (392 G→T), "Chinese-3" ([illegible] A→G), or G6PD Coimbra (592 C→T) was identified. In conclusion, G6PD Viangchan is the most common mutation in Thai population. This mutation, together with G6PD Mahidol and G-6PD Canton, are responsible for over 70% of G6PD deficient variants in [illegible]. There is no demonstrable relationship between any mutation and neonatal hyperbilirubinemia. Together with data from other Southeast Asian ethnic group such as [illegible]ians, G6PD Viangchan (871 G→A) is the most common variant in Southeast Asian population.

Abstract# 3684

A NOVEL ASSAY METHOD FOR THE MEASUREMENT OF HOLO-TRANSCOBALAMIN IN HUMAN SERUM. Lars Orning*,[1] [illegible] Ulleland*,[1] Ingar Eilertsen*,[1] Erling Sundrehagen*,[1] Edward V. [illegible]*,[2] Sheldon P. Rothenberg,[2] Sergey N. Fedosov*,[3] Torben E. [illegible]sen*.[3] [1]*Axis-Shield ASA, Oslo, Norway;* [2]*Division of Hematology/[illegible], State University of New York Health Science Center, New [illegible], USA;* [3]*Protein Chemistry Laboratory, University of Aarhus, [illegible], Denmark.*

We have developed a novel radioimmunoassay that measures the concentration of [illegible] B12 bound to the carrier protein transcobalamin (holo-transcobalamin) in human serum or plasma. The method utilizes monoclonal antibodies specific for human [illegible] with Ka > 10[illegible], immobilized onto magnetic microspheres, to isolate and [illegible] the transcobalamin in the sample. The concentration of holo-transcobalamin [illegible] sample is determined from a standard curve constructed from standards of [illegible]. [illegible]

Abstract# 3685

DETECTION OF FOLATE DEFICIENCY. WHICH IS MORE USEFUL, SERUM FOLATE OR RED CELL FOLATE? Angel Remacha,[1] Pilar Sarda*,[1] Josep Cadafalch*,[1] Montserrat Barcelo*,[2] Montserrat Fuster*.[1] [1]*Hematology, Hospital Sant Pau, Barcelona, Spain;* [2]*Internal Medicine, Hospital Sant Pau, Barcelona, Spain.*

Folate deficiency induces many metabolic changes, including an increase in homocysteine (HC). In routine practice serum folate (SF) and red cell folate (RCF) measurements are utilised for evaluating folate status. RCF levels are related to folate stores and need more time than SF to decrease in case of folate deficiency. However, SF undergoes rapid changes reflecting the folate balance at a given moment. In a [illegible] hospital with a lot of seriously ill patients, SF is often found to be decreased.

In [illegible] patients from a universitary hospital, SF, RCF and HC were determined simultaneously. Forty-five patients had a real folate deficiency (HC > 17.5 micromol/l) and in 74 cases HC was lower than 17.5 micromol/l (normal folate status). In all cases serum vitamin B12 and renal function were normal.

Overall agreement between RCF and HC was [illegible]% and the kappa index [illegible] (very good). RCF sensitivity was [illegible]% and specificity [illegible]%. In contrast, overall agreement between SF and HC was [illegible]%, the kappa index [illegible] (low), sensitivity [illegible]% and specificity [illegible]%.

In conclusion, RCF is superior to SF for evaluating folate status in a universitary hospital, owing to the low specificity of SF (false positives).

Abstract# 3686

ANALYSIS OF HFE-CODON 63/282 (H63D/C282Y) GENE VARIANTS IN MEXICAN MESTIZOS: BLOOD DONORS AND PATIENTS WITH HEREDITARY HEMOCHROMATOSIS. G.J. Ruiz-Argüelles, J. Garcés-Eisele*, T. Gelbart*, M. Monroy-Barreto*, V. Reyes-Núñez*, J.L. Juárez-Morales*, M.L. González-Garrido*, F.J. Ramírez-Cisneros*. *Centro de Hematología y Medicina Interna de Puebla, Laboratorios Clínicos de Puebla, Universidad de las Américas-Puebla; Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA; Universidad Popular Autónoma del Estado de Puebla, Puebla, México.*

The analysis of HFE codon 63/282 (H63D/C282Y) gene variants was performed in Mexican Mestizos. In 153 blood donors [illegible] heterozygotes for the C282Y mutation [illegible] were found, whereas 19 heterozygotes and one homozygote for the H63D mutation were identified ([illegible]% and [illegible]%, respectively). [illegible] compound heterozygote for the two gene mutations. These data result in allelic frequencies of [illegible] respectively. On the other hand, in six patients with hereditary hemochromatosis (HH), two were heterozygous for C282Y and one heterozygous for H63D. It is concluded that the prevalence of the C282Y and H63D HFE mutations in Mexican Mestizos is similar to that informed from other populations. In addition, other gene mutations responsible for HH in México should be investigated, since in 3 of 6 individuals with HH, none of these mutations was identified.

Abstract# 3687

USE OF SOLUBLE TRANSFERRIN RECEPTOR FOR THE DIFFERENTIAL DIAGNOSIS OF IRON DEFICIENCY ANEMIA AND ANEMIA OF CHRONIC DISEASE. J. Rybski*,[1] S. Heikkinen*,[1] V. Hanninen*,[2] A. Jain*,[1] S. Hull*,[1] A. Novamo*.[2] (Intr. by A. Sterrukza) *R&D, Nichols Institute Diagnostics, San Juan Capistrano, CA, USA;* [2]*Orion Diagnostica, Oulu, Finland.*

We have developed an automated Soluble Transferrin Receptor (sTfR) Chemiluminescence Assay for the Nichols Advantage Specialty System to assist in the diagnosis of Iron Deficiency Anemia and for the differential diagnosis of Iron Deficiency Anemia and Anemia of Chronic Disease. The assay range is [illegible] nmol sTfR/L, with an analytical sensitivity of [illegible] nmol/L. Since the estimated reference range for sTfR is dependent upon the altitude of the study group's domicile, we established the range for two populations. The first cohort consisted of eighty eight (n=88) apparently healthy free-living Southern California community dwelling adults. Based upon the 95% confidence intervals of the mean, the estimated reference range for this group was [illegible] nmol sTfR/L. The second group was a Finnish cohort of eighty-four (n=84) apparently healthy free-living community dwelling adults. The estimated reference range for this group was [illegible] nmol sTfR/L. We analyzed the sTfR and Ferritin results for a group of eighty-nine (n=89) subjects for which Bone Marrow Iron (BMI) staining data were available. These samples were derived from patients diagnosed with Anemia of Chronic Disease (ACD, n=[illegible]), Iron Deficiency Anemia (IDA, n=[illegible]), the combination of ACD and IDA (Combi, n=[illegible]) and a control group (n=[illegible]). Univariate non parametric ANOVA with multiple comparisons of the sTfR results segregated the groups as follows: (Controls = ACD) ≠ (IDA = Combi). The ACD and IDA groups were further separated by analyzing the combination of sTfR and Ferritin results (P < [illegible], Multivariate ANOVA). The Nichols Advantage Soluble Transferrin Receptor Assay offers a fully automated method for the quantitation of sTfR in serum or plasma as an adjunct in the diagnosis of IDA and for the differential diagnosis of IDA and ACD.

G6PD Viangchan (871G>A) is the most common glucose-6-phosphate dehydrogenase deficient variant in Thai population

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Contract grant sponsors: Thailand Research F und to IN and Asahi Glass Foundation to SS; Contract grant number: RSA/04/2540.
Short Title: G6PD Viangchan in Thai population
Communicated by <Please don't enter>

Glucose-6-phosphate dehydrogenase (G6PD)deficiency is the most common inherited disorder in human. G6PD deficiency is a common cause of neonatal hyperbilirubinemia. We conducted a population study for G6PD deficiency using cord blood quantitative G6PD assay in Bangkok, Thailand and found that the prevalence of G6PD deficiency is 11.1% in Thai male (N=350) and 5.8% in female (N=172) cord blood samples. Among neonates with hyperbilirubinemia, the prevalence of G6PD deficiency is 22.1% in male (N=140) and 10.1% in female (N=89). We developed a PCR-restriction enzyme-based method to identify G6PD Viangchan (871G>A), and searched for this and 9 other mutations in DNA extract from G6PD deficient blood samples. We found that G6PD Viangchan (871G>A) is the most common mutation identified (54%, 21 of 39 males) followed by G6PD Canton (1376G>T, 4 cases, 10%), G6PD Mahidol (487G>A, 3 cases, 8%), G6PD Kaiping (1388G>A, 2 cases), G6PD Union (1360C>T) and "Chinese-5" (1024C>T, 1 case each) and 8 cases (17%) remained unidentified. Among neonates with hyperbilirubinemia, G6PD Viangchan is also most commonly identified (60%, 12 of 20 males), followed by G6PD Canton (2 cases), G6PD Mahidol, G6PD Union, G6PD Kaiping (1 case each), and 3 cases remained unidentified. In conclusion, G6PD Viangchan is the most common mutation in Thai population. This mutation, together with G6PD Mahidol and G6PD Canton, are responsible for over 70% of G6PD deficient variants in Thais. Together with data from other Southeast Asian ethnic group such as Laotians, G6PD Viangchan (871G>A) is the most common variant in non-chinese Southeast Asian population. © 2001 Wiley-Liss, Inc.

KEY WORDS: G6PD mutation, Thai

INTRODUCTION

Glucose – 6 phosphate dehydrogenase (G6PD, MIM # 305900) is an enzyme in hexose monophosphate pathway. G6PD deficiency is the most common disease-producing enzyme disorder of human (WHO, 1989). Four clinical syndromes associated with G6PD deficiency have been identified: oxidative stress-induced

hemolysis (Carson et al, 1956, Beutler 1959), favism, neonatal jaundice, chronic non-spherocytic hemolytic anemia (Beutler, 1994). Neonatal jaundice occurs mostly in the Mediterranean and Asian G6PD deficient infants (Maisel, 1994). In Thai, 65% of severe jaundice infants had G6PD deficiency (Sasanakul et al, 1989)

Severe neonatal hyperbilirubinemia is well known to cause kernicterus and death (Brown and Boon, 1968). In Thailand, 19.7% of hyperbilirubinemia is caused by G6PD deficiency (Tanpaichitr et al, 1995). Phototherapy, exchange transfusion, education and surveillance have been shown to reduce these complications (WHO, 1989).

G6PD has been studied biochemically, and more than 400 variants have been identified. To date, more than 68 mutants has been characterized at the DNA level (Beutler, 1994). Specific G6PD variant is found in people of different ethnic groups. In Asian region, G6PD Canton was found to be the most common variant among chinese in Taiwan (Huang et al, 1996), China, and Malaysia (Ainoon et al, 1999). There are few population studies published on G6PD mutation in other Asian ethnic groups. In Thailand, G6PD Mahidol (487G>A) was believed to be the most common G6PD variant in Thailand (Panich et al, 1992), but it has not been confirmed in population studies. In this report, we conducted a population screening for G6PD deficiency from cord blood samples, and identified G6PD deficient mutations in Thai population as well in newborns with neonatal jaundice.

MATERIAL AND METHODS

Five hundred and twenty nine umbilical cord blood samples were randomly obtained in delivery room at Chulalongkorn University Hospital. Five ml of cord blood were mixed with acid-citrate-dextrose (ACD) and stored at 4°C until assayed within 3 days from collection. Peripheral blood samples were obtained from jaundiced newborns from nursery in the first 7 days of life. Serum total bilirubin was determined by its optical property using Reichert-Jung unistat bilirubinometer. Neonates with serum bilirubin above 13 mg/dl were included assessed for G6PD deficiency.

G6PD activity assay

G6PD activity assays were performed according to the WHO-recommended standard test (Betke et al, 1967) with minor modification. Two ml of citrated blood were washed with cold normal saline 3 times with removal of buffy coat. Washed red cells were assayed for hematocrit, then 50 μl of washed red cells were mixed with 950 μl ddH_20, mixed and frozen at -20 °C for 40 minutes. Lysed red cells were centrifuged at 3000 rpm (5000g) for 20 minutes, hemolysate supernatant was used for G6PD enzyme assays. Enzyme activity was quantitated by adding 50 μl of hemolysate to a 950 μl assay containing buffer (0.1 M Tris-HCl pH 8.0, 10 mM $MgCl_2$), Glucose-6-phosphate (0.6 mM, Sigma), and NADP (0.2 mM, Sigma). The rate of NADPH generation was measured at 340 nm at 30 °C over 10 minutes. The average change of optical density per minute was calculated to determine activity of the G6PD enzyme. G6PD activity was calculated and reported as IU per gram hemoglobin (g Hb).

In our laboratory, the normal value of cord blood G6PD activity is 7.39 ± 2.57 IU/g Hb from normal male (mean ± standard deviation, S.D.), and 6.94 ± 2.51 IU/g Hb in normal female. G6PD deficiency was diagnosed when activity was less than 1.5 IU/g Hb (WHO, 1967).

DNA was extracted from G6PD-deficient blood samples using Qiaquick® Blood DNA extraction kit (Qiagen, Germany) according to manufacturer's recommendation.

Identification of G6PD mutations

For G6PD Viangchan mutation assay, a mutagenic primer pair 871F (5'-TGGCTTTCTCTCAGGTCTAG-3') and G6PD10R (5'-GTCGTCCAGGTACCC TTTGGGG-3') were used in a polymerase-chain reaction (PCR). One microliter of purified DNA from blood were mixed, in 50 μl, with 50 ng of each primer, 200 M each dNTP, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.5 U of *Taq* polymerase (Promega). The PCR amplification was performed on the DNA thermal cycler for 1 cycle of 95°C for 5 minutes, then 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 1 min at 72 °C, and final extension at 72 °C for 10 minutes. In 30 μl reaction, 25μl of PCR product was digested with *Xba*I (Gibco BRL) for 2 hours, then resolved on 3% agarose gel (Metaphore, FMC Bioproduct, Rockland, ME) containing ethidium bromide.

For 95A>G, 392G>T, 487G>A, 493A>G, 592C>T, 1024C>T, 1360C>T, 1376G>T, and 1388G>A, 9 oligonucleotides with natural or mutagenic primer set

(Huang et al, 1996) were used for detection of the nine known G6PD mutations (NSTDA BIOTEC, Bangkok, Thailand). The PCR amplification conditions were similar to G6PD Viangchan except for annealing temperature was 55 °C for 1 min. The PCR product was digested with appropriate restriction enzyme digestion set (Huang et al, 1996) (Gibco BRL) according to manufacturer's recommendation.

For nt1311 polymorphism, three primers, G6P10F2 (5'-ATGATGACCAAGA AGCCGGGC-3'), 1311TR (5'-CGTCCAGGATGAGGCGCTCA-3') and G6P12R (5'-CTGCCATAAATATAGGGGATGGG-3') were used in a PCR reaction at the same condition above. The PCR amplification was performed on the DNA thermal cycler for 1 cycle of 95°C for 5 minutes, then 35 cycles of 1 min at 95 °C, 1 min at 68 °C, 1 min at 72 °C, and final extension at 72 °C for 10 minutes. Twenty-five µl of PCR product resolved on 3% agarose gel (Gibco BRL, Grand Island, NY) containing ethidium bromide. Presence of a 200-bp band indicates C1311T. Presence of 400-bp but not 200-bp band indicates wild-type nt1311.

DNA Sequencing

PCR product from G6PD exon 9 was amplified using G6PD9F (5'-AGCTGCA GGCCAACAATGTGGT-3') and G6PD10R. The 360 bp amplicon was used as template and DNA sequence was determined using ABI prism 310 Genetic Analyser (Perkin-Elmer, Norwalk, CT) following manufacturer's recommendation using G6PD9F as primer.

RESULTS

Prevalence of G6PD deficiency

Of 522 cord blood samples, we identified G6PD deficiency in 11.1% of Thai male (N=350) and 5.8% of female (N=172). Among neonates with hyperbilirubinemia, the prevalence of G6PD deficiency is 22.1% in male (N=140) and 10.1% in female (N=89).

Prevalence of G6PD mutations

G6PD deficient DNA samples were assayed for G6PD known mutations. The first mutation assessed in this study is G6PD Mahidol (487G>A) because it was

thought to be the most common variant among Thai G6PD deficient individuals. Of 39 G6PD-deficient male cord blood samples, only 3 (7.7%) was found to be G6PD Mahidol. (Table 1) We then searched for eight other mutations and identified 4 (8%) cases of G6PD Canton (nt1376 mutation), 2 (4%) cases of G6PD Kaiping (nt1388 mutation), 1 case (2%) of G6PD Union (nt 1360 mutation), and case (2%) of "G6PD chinese-5" (nt 1024 mutation). No case of G6PD Gaohe (95A>G), "Chinese-4" (392G>T), "Chinese-3" (493A>G), or G6PD Coimbra (592C>T) was identified.

We then performed a PCR of exon 9 on one of the unidentified G6PD-deficient DNA sample and determined the DNA sequence. A mutation at nt871 from G to A was found. We then developed the assay for G6PD Viangchan using a mutagenic 5'-primer (871F) and a reverse primer (G6PD9R) to amplify exon 9, which will result in 126-bp amplicon. Restriction enzyme *Xba*I digestion will cleave mutant, but not wild-type amplicon, to 106-bp (Figure 1) To distinguish G6PD Viangchan from G6PD Jammu, which differs at a non-coding nt 1311, two allele-specific oligonucleotide primers sets were used. We found that in all samples with 871G>A, nt 1311 was T, consistent with G6PD Viangchan. (data not shown). Using this PCR-based assay, G6PD Viangchan was identified in 21 of 39 male cord blood samples (53.8%) as well as 12 of 20 peripheral blood samples from jaundiced newborn (60%). (Table 1) Approximately half of G6PD Viangchan has undetectable cord blood G6PD activities.

Of 10 G6PD-deficient female cord blood sample, 6 were G6PD Viangchan, while 4 remained unidentified. All female samples were most likely heterozygote since residual G6PD activity were found, ranging from 0.57 to 1.60 IU/g Hb and amplicon was partially digested (Figure 1). Similarly, among 7 samples from G6PD-deficient female with neonatal jaundice, 4 were G6PD Viangchan.

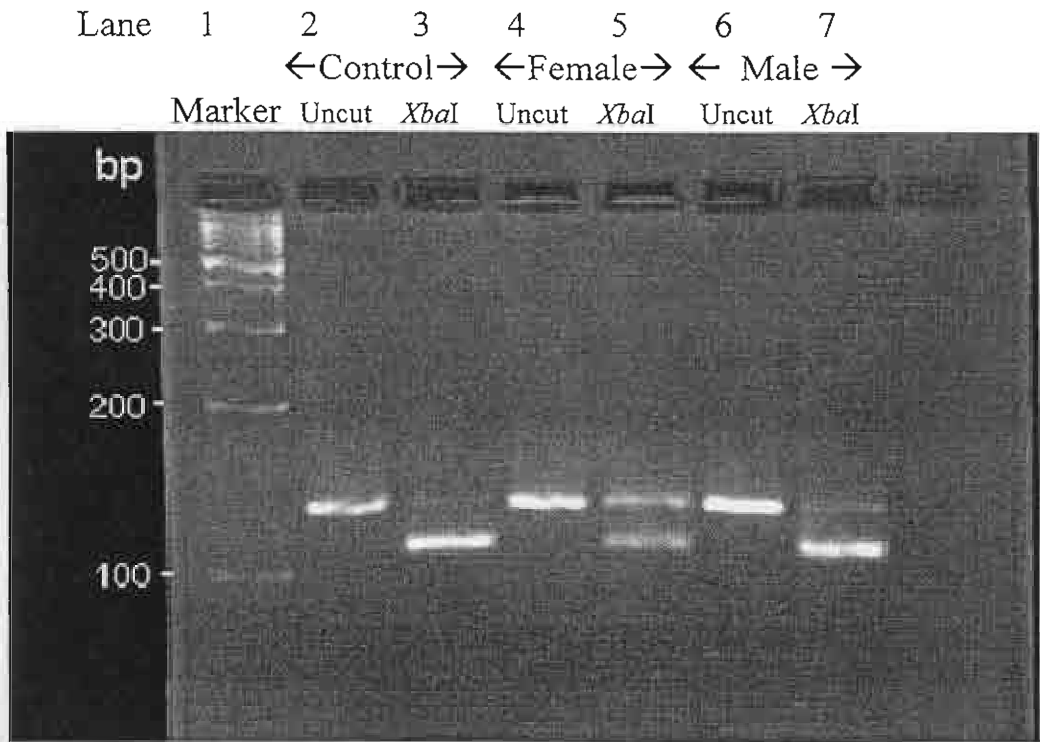

Figure 1. PCR-restriction enzyme assay for G6PD Viangchan. PCR was performed on genomic DNA from normal known G6PD Viangchan (control) showed a 126-bp band that reduce to 106 bp after *Xba*I digestion. Female heterozygote show both 126- and 106-bp bands after *Xba*I digestion.

Table 1. Prevalence of G6PD mutations and their activity from male G6PD-deficient cord blood samples or peripheral blood sample from male neonatal jaundice.

Mutation	Cord blood Number (%)	Cord blood G6PD activity (IU / g Hb) Median	Cord blood G6PD activity (IU / g Hb) Range	Neonatal jaundice Number (%)	Neonatal jaundice G6PD activity (IU/ g Hb) Median	Neonatal jaundice G6PD activity (IU/ g Hb) Range
871 (G6PD Viangchan)	21 (53.8%)	0.12	0.00 - 1.03	12 (60.0%)	0.14	0.00 -1.50
1376 (G6PD Canton)	4 (10.3%)	0.39	0.16 - 1.44	2 (10.0%)	0.29	0.21, 0.37
487 (G6PD Mahidol)	3 (7.7%)	0.11	0.00 - 0.44	1 (5.0%)	0.09	-
1388 (G6PD Kaiping)	2 (5.1%)	0.09	0.00, 0.17	1 (5.0%)	1.13	-
1360 (G6PD Union)	1 (2.6%)	0.00	-	1 (5.0%)	0.00	-
1024 (G6PD "chinese-5")	1 (2.6%)	0.50	-	0	-	-
Unknown	7 (17.9%)	0.00	0.00 - 1.05	3 (15.0%)	0.00	0.0 - 0.17
Total	39 (100%)			20 (100%)		

DISCUSSIONS

We have identified G6PD Viangchan as the most common variant in Thai population. With 21 cases identified among 350 male cord blood samples, the gene frequency of G6PD Viangchan in Thai population is calculated to be 0.06. Consistent with this finding, heterozygous deficient female are also found in 6 out of 172, indicating that some female heterozygote for G6PD Viangchan is not in deficient range.

G6PD Viangchan (MIM # 305900.0026) was first characterized biochemically in 1988 from a Laotian G6PD-deficient patient in Canada (Poon et al, 1988). This G6PD variant was found to be a WHO class 2, or severely deficient, variant. G6PD Viangchan was subsequently defined molecularly to be a nucleotide substitution at nt871 from G to A, predicting an amino acid 291 substitution from Val to Met. Nucleotide substitution 871G>A is also found in G6PD Jammu (Beutler et al, 1991) which was found in patient from India. These two variant differs at a nucleotide 1311 polymorphism, where it is C in G6PD Jammu, and T in G6PD Viangchan.

G6PD Viangchan has been reported to be a common variant among Laotian people (5 of 9 G6PD-deficient subjects) based on a small transplanted population in Hawaii (Hsia et al, 1993). The finding that gene frequency of G6PD Viangchan is high in Thais and Laotians support the common ancestry of these to ethnic group. In contrast, G6PD Viangchan is found in 10% of Filipinos (6 of 53) (Hsia et al, 1993), and only rarely in Chinese population, only 1 in 112 G6PD-deficient male neonate (Huang et al, 1996).

In contrast to previous study (Panich et al, 1972), we did not find G6PD Mahidol to be the most common G6PD variant in Thailand. G6PD Mahidol was named after the university where it was identified biochemically in 1972, and assessed to be a mild (WHO class 3) variant. Among 22 patients with acute hemolysis, G6PD Mahidol was identified in most cases. Subsequently, DNA analysis identified point mutation at nucleotide 487 with substitution of G with A, which changed translation of amino acid 163 from glycine to serine (Vulliamy et al, 1989). Based on molecular analysis used in our study, we found G6PD Mahidol in less than 10% of G6PD-deficient population. It remains possible that G6PD Mahidol is associated with only episodic hemolysis. The ease of PCR-based asssay would allow us to study this mutation in hemolytic patients in the subsequent study.

In contrast to multi-ethnic Malaysia and Singapore, Thai population consists of native Thai and assimilated Chinese. Similar to Malaysia and Singapore, Chinese immigrants were mostly from Guangdong province around 2 generations earlier. The proportion of Chinese ethnic is uncertain because of assimilation with the Thai population. G6PD Canton was the most prevalent (50%) in chinese, and is found to be the second most common variant (10%) in our study. This subpopulation could be descendant of chinese immigrant in to Thai population. The other less common chinese variants, G6PD Kaiping, G6PD Union, and G6PD "chinese-5" were also identified in the proportionately smaller number.

In contrast to G6PD Canton, which was shown to be related to severe hyperbilirubinemia (Huang et al, 1996), there is no trend toward a relationship between G6PD Viangchan and hyperbilirubinemia. The proportion of this to other mutations in G6PD deficient jaundiced newborn is similar to that found in general population, implied by cord blood study. Similar to G6PD deficiency at large, G6PD Viangchan contribute to a relatively late onset of hyperbilirubinemia. The level of bilirubin and date of onset is indistinguishable from other mutations.

In summary, our finding suggests that G6PD Viangchan is a marker of Thai ethnic, similar to of hemoglobin E allele in this population (Wasi, 1967). The high prevalence of G6PD Viangchan and hemoglobin E allele could be a result of malaria selection pressure in this region.

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