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.

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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 นับเป็นหลักฐานอีกประการหนึ่งที่สนับสนุนทฤษฎีที่ว่าประชากรในเขตแหลมทองน่าจะตั้งหลักแหล่งในบริเวณนี้นานแล้ว และประชากรไทย-ลาว ล้วนมีบรรพบุรุษเดียวกัน

2.5. <u>**เชิงวิชาการแบบอื่น**</u>

2.5.1. <u>การพัฒนาการเรียนการสอน</u>

กำลังอยู่ในระหว่างการเขียนตำราเพื่อเพิ่มความรู้ใหม่ในเรื่องมะเร็งตับ และอณูชีววิทยาของ G6PD ในชาวไทย

2.5.2. <u>การสร้างนักวิจัยใหม่ในลักษณะอื่น ๆ</u>

ก. ระดับปริญญาตรี ไม่มี

ข. ระดับปริญญาโทที่กำลังศึกษาอยู่ 2 ราย

ค. ระดับปริญญาเอกที่กำลังศึกษาอยู่ 1 ราย

ง. ระดับปฏิบัติการ: อบรมเจ้าหน้าที่หน่วยโลหิตวิทยาเพื่อให้เพิ่มศักยภาพในการทำงานระดับอณูชีววิทยา ทำให้สนใจศึกษาต่อในระดับปริญญาโทต่อไป

3. **อื่น ๆ** Invited speaking activities

3.1. <u>**ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการในประเทศ**</u>

(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. <u>**การเสนอผลงานในประชุมวิชาการ**</u>

(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. <u>**หนังสือ / คู่มือ**</u>

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 Sau3A*I*. 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.

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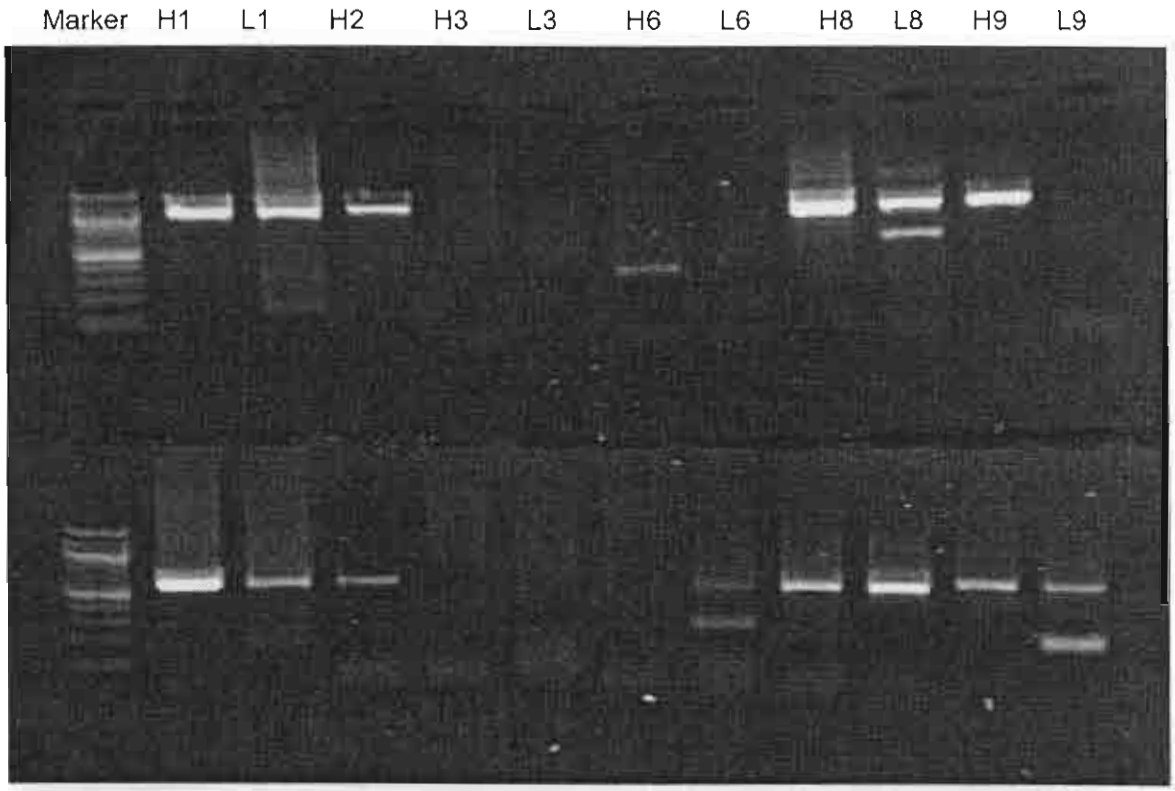

Figure I. Gel electrophoresis of HBs- (upper lanes) and HBx-DNA (lower lanes) in hepatocellular carcinoma (H) and non-carcinomatous liver from the same patient (L). Number indicate case number. PCR-amplified HBs band is 1037-bp, and HBx band is 596 bp.

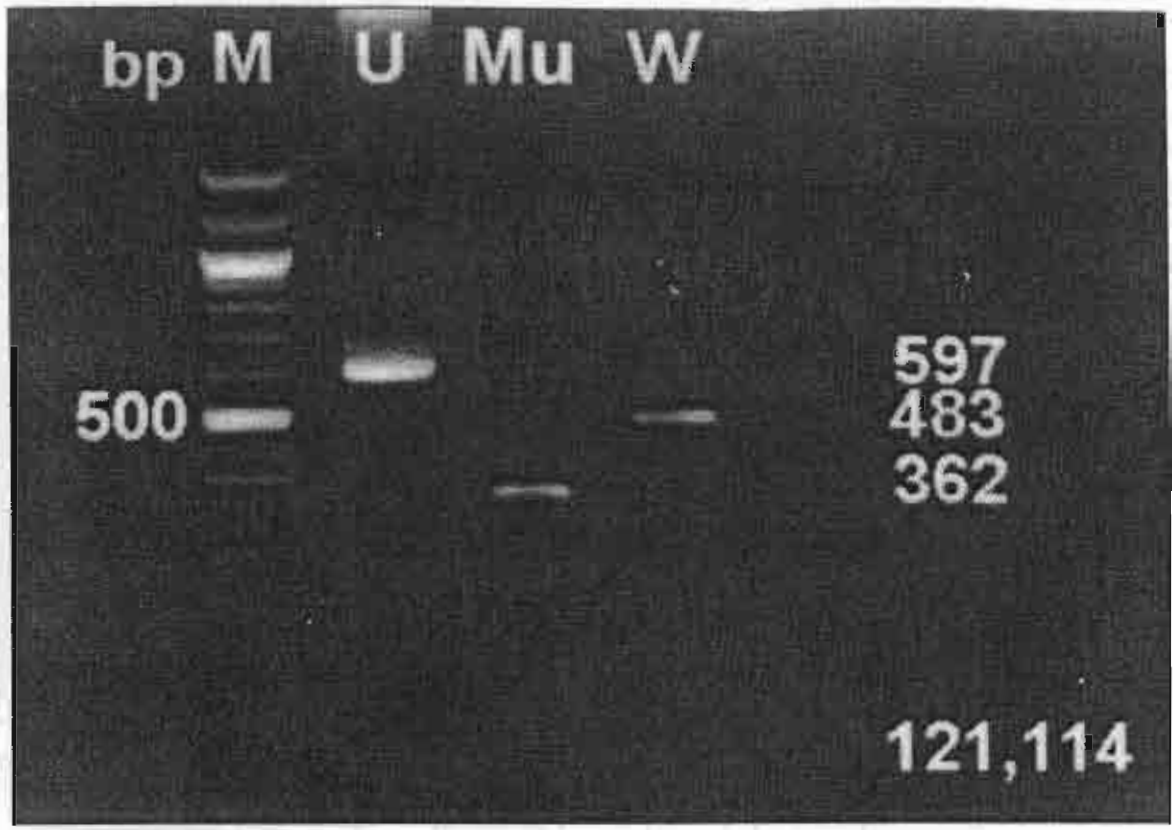

Figure II. Metaphor gel electrophoresis of the HBx DNA from samples of hepatitis B virus, after restriction-fragment-length-polymorphism (RFLP) analysis using *Sau*3A I. The photograph shows *Sau*3A I. restriction patterns of PCR products. [Molecular weight markers (M), undigested PCR product *(U), mutant 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.

Group	N	Number of cases with HBV DNA				Anti-HCV
		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
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AMERICAN SOCIETY OF HEMATOLOGY

Abstract# 3682

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

Pernicious 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 injections. Three of the six had pretreatment urinary methylmalonic acid (UMMA) levels that were abnormal for this indicator of tissue Cbl deficiency. Although these laboratory abnormalities were of modest magnitude, most of these women had significant neurological findings which first brought them to medical attention. Subjects 1 and 2 experienced increasing spinal cord problems causing debilitation of arms and legs. After nine years of Cbl IM, Subject 1 has improved but still can only walk short distances (socks, finger [illegible]), and has severe waist and pelvic pain. After two years of Cbl IM, Subject 2 is [illegible] completely 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 < [illegible])
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

Abstract# 3683

G6PD VIANGCHAN (871G→A) IS THE MOST COMMON G6PD DEFICIENT VARIANT IN SOUTHEAST ASIA. Issarang Nuchprayoon,[1] [illegible]ya 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 based 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" (493 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 Thais. There is no demonstrable relationship between any mutation and neonatal hyperbilirubinemia. Together with data from other Southeast Asian ethnic group such as Laotians, 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/Oncology, State University of New York Health Science Center, New York, NY, USA;* [3]*Protein Chemistry Laboratory, University of Aarhus, [illegible], Denmark.*

We have developed a novel radioimmunoassay that measures the concentration of vitamin B12 bound to the carrier protein transcobalamin (holo-transcobalamin) in human serum or plasma. The method utilizes monoclonal antibodies specific for human transcobalamin with $Ka > 10^{[illegible]}$, immobilized onto magnetic microspheres, to isolate and measure the transcobalamin in the sample. The concentration of holo-transcobalamin in the sample is determined from a standard curve constructed from standards of [illegible] human holo-transcobalamin. The assay is simple to perform, accurate, reliable, with an analytical sensitivity less than 10 pM. Serum samples from 57 apparently healthy volunteers of age below 20 or above 80 were measured for holo-transcobalamin by the method and for total vitamin B12 concentrations using a commercial radiodilution assay. The range of holo-transcobalamin was 22 to 196 pM, with the majority falling between [illegible] and [illegible] pM. The correlation between concentrations of holo-transcobalamin and total vitamin B12 was low, r=0.46.

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 university hospital with a lot of seriously ill patients, SF is often found to be decreased.

In 119 patients from a university 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 91% and the kappa index 0.8 (very good). RCF sensitivity was 91% and specificity 90.5%. In contrast, overall agreement between SF and HC was 63%, the kappa index 0.27 (low), sensitivity [illegible]% and specificity 43%.

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

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 4 heterozygotes for the C282Y mutation (2.6%) were found, whereas 19 heterozygotes and one homozygote for the H63D mutation were identified (12.4% and 0.6%, respectively). There was one compound heterozygote for the two gene mutations. These data result in allelic frequencies of 1.3% (0.2% - 3.05) and 6.2% (3.9% - 9.05) 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 recorded.

Abstract# 3687

USE OF SOLUBLE TRANSFERRIN RECEPTOR FOR THE DIFFERENTIAL DIAGNOSIS OF IRON DEFICIENCY ANEMIA AND ANEMIA OF CHRONIC DISEASE. J. Rybski*,[1] S. Heikura*,[2] V. Hanninen*,[2] A. Jain*,[1] S. Hall*,[1] A. Novamo*[2] (Intr. by A. Sterrusza). [1]*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 4 - 130 nmol sTfR/L with an analytical sensitivity of 0.1 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 6.4 - 25.7 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 8.6 - 25.4 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=28), Iron Deficiency Anemia (IDA, n=26), the combination of ACD and IDA (Combi, n=10) and a control group (n=25). Univariate one parameter ANOVA with multiple comparison 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 = 2.50E-21, 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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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.

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.

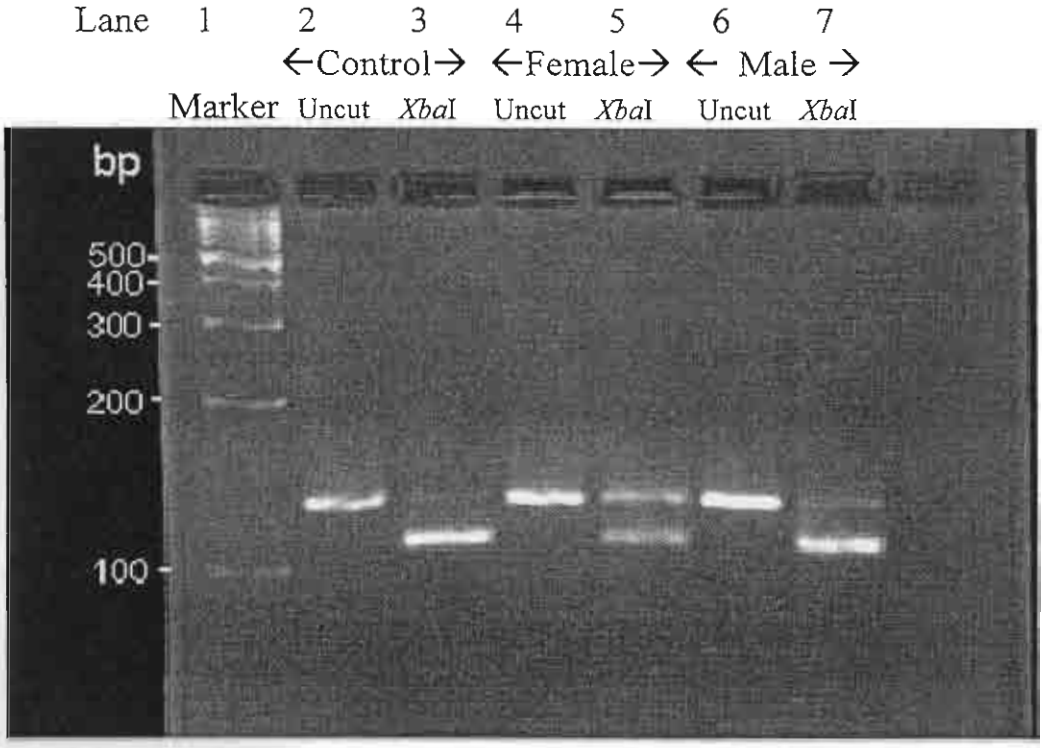

Figure 1. PCR-restriction enzyme assay for G6PD Viangchan. PCR was performed on genomic DNA from normal known G6PD Viangchan (control) showed a 126-bp band that reduce to 106 bp after *Xba*I digestion. Female heterozygote show both 126- and 106-bp bands after *Xba*I digestion.

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			Neonatal jaundice		
	Number (%)	G6PD activity (IU / g Hb) Median	Range	Number (%)	G6PD activity (IU/ g Hb) Median	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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