รายงานวิจัยฉบับสมบูรณ์

โครงการ การผันแปรของสภาวะเหนือพันธุกรรมในความชราของเซลล์ภายใต้ภาวะพร่องเอนไซม์จีซิกพีดี

โดย ผู้ช่วยศาสตราจารย์ ดร.ชาลิสา หลุยเจริญ ชีพสุนทร

เดือน กรกฎาคม ปี 2561 ที่เสร็จโครงการ

สัญญาเลขที่ TRG5880209

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(Epigenomic alterations in aging influenced by G6PD deficiency)

Investigator : ผู้ช่วยศาสตราจารย์ ดร.ชาลิสา หลุยเจริญ ชีพสุนทร

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Global methylation (Alu, LINE-1) มีบทบาทในกระบวนการชราของเซลล์ ทั้งการชราและการเปลี่ยนแปลงสภาวะเหนือธรรมชาตินั้นมีความเกี่ยวข้องกับความเครียดจากออกซิเดชั่นที่เพิ่มขึ้นโดยมีเอนไซม์ glucose 6-phosphate dehydrogenase (G6PD) เป็นตัวควบคุม ซึ่งพบว่าการทำงานของเอนไซม์ G6PD ลดลงตามอายุที่เพิ่มขึ้น ดังนั้นจึงมีข้อสันนิษฐานว่าภาวะพร่องเอนไซม์ G6PD อาจนำไปสู่การเสียสมดุลของภาวะ redox ที่จะส่งผลกระทบต่อการเปลี่ยนแปลง epigenomic ตามมา ด้วยเหตุนี้ในการศึกษาครั้งนี้จึงแสดงให้เห็นถึงความสัมพันธ์ระหว่างการทำงานของเอนไซม์ G6PD และระดับ methylation ของ Alu, LINE-1 ที่เกี่ยวข้องกับอายุที่เพิ่มขึ้น ทั้งในผู้สูงอายุที่มีภาวะขาด G6PD และในการทดสอบโมเดล *in vitro* ผลการทดสอบไม่พบความแตกต่างของระดับ methylation ของ Alu และ LINE-1 ในผู้ป่วยที่พร่องและไม่พร่องเอนไซม์ G6PD แต่พบระดับ methylation ของ Alu ในผู้ป่วยโรค neurodegeneration เพิ่มขึ้นอย่างมีนัยสำคัญในรูปแบบ mC และ uCmC (p = 0.001 และ 0.017) และลดลงใน uCuC ของ LINE-1 (p = 0.001) ภายหลังจากการปรับค่า OR ด้วยอายุ เพศ และภาวะพร่องเอนไซม์ G6PD แล้ว พบเพียงการลดลงของ uCuC ใน LINE-1 ที่มีความเกี่ยวข้องกับ NDs จากการศึกษาในระบบ *in vitro* พบความสัมพันธ์ระหว่างการลดลงของการทำงานของเอนไซม์ G6PD กับระดับ methylation ของ LINE-1 ที่เพิ่มขึ้นในเซลล์ประสาท SK-N-SH นอกจากนี้ยังพบว่าจำนวนเซลล์ประสาทที่แก่มีเพิ่มมากขึ้นอย่างมีนัยสำคัญในกลุ่มเซลล์ที่ knockdown G6PD ดังที่ได้กล่าวมาแล้วภาวะพร่องเอนไซม์ G6PD ทำให้เซลล์เกิดความไม่สมดุลของสถานะ redox ซึ่งอาจนำไปสู่ความเสียหายของดีเอ็นเอและ genomic integrity ดังนั้นภาวะพร่องเอนไซม์ G6PD อาจมีส่วนเกี่ยวข้องกับการชราของเซลล์ด้วยการทำให้เกิดความไม่เสถียรของจีโนมโดยการเปลี่ยนแปลงระดับ DNA methylation ในช่วงที่เกิดออกซิเดชัน

Abstract

Project Code : TRG5880209

Project Title : การผันแปรของสภาวะเหนือพันธุกรรมในความชราของเซลล์ภายใต้ภาวะพร่องเอนไซม์จีซิกพีดี
(Epigenomic alterations in aging influenced by G6PD deficiency)

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Global methylation (Alu, LINE-1) plays a role in the development of aging. Both aging & epigenomic alterations are associated with increased oxidative stress, which is counteracted by glucose 6-phosphate dehydrogenase (G6PD). G6PD activity decreases with age. Therefore, it is hypothesized that deficiency in G6PD activity could lead to the disruption of redox homeostasis and subsequently increased susceptibility to epigenomic alteration. Here, we demonstrated the association of G6PD activity & age-related methylation levels of Alu, LINE-1 in G6PD deficient elders & *in vitro* model. Alu and LINE-1 methylation were not changed between G6PD deficient patients and controls. Methylation in patients with neurodegenerative diseases (NDs) was also significantly increased in mC and uCmC of Alu (p=0.001 and 0.017) and decreased in uCuC of LINE-1 (p=0.001). After adjusting OR with age, gender and G6PD deficiency status, only a decrease of uCuC of LINE-1 was associated with NDs. *In vitro* system, we found a significant correlation between reduction of G6PD activity and LINE-1 hypermethylation in SK-N-SH neuronal cells. Cellular senescence was also significantly increased in G6PD knockdown neurons. As mentioned earlier, G6PD deficiency causes an imbalance of cellular redox state, which might lead to DNA damage and subsequently genomic integrity. Therefore, G6PD deficiency might be involved in cellular senescence by inducing genomic instability via alteration of DNA methylation during oxidation.

Keywords : G6PD deficiency, epigenomic alteration, LINE-1 methylation, aging

Objectives

1. To study the correlation between the levels of Alu, LINE-1 methylation and blood G6PD activity in elders

2. To examine the role of G6PD in regulation of cellular aging via methylation of Alu and LINE-1 in aged human neuronal cell line

Methodology

***IN VIVO* STUDY:** To study the correlation between the levels of Alu, LINE-1 methylation and blood G6PD activity in elders

Subjects

Ethical permission was approved by Research Ethics Committee of the National Blood Centre of Thai Red Cross Society, Bangkok (COA No. NBC 7/2016) and by Research Ethic committee of the Faculty of Medicine, Ramathibodi Hospital. One hundred and fourteen elders (60-90 years old) consisted of 66 volunteers (28 males and 38 females) from Division of Neurology, Department of Medicine, Ramathibodi Hospital and 48 volunteers (30 males and 18 females) from Thai Red Cross were enrolled in this study. All subjects agreed to be assessed for socio-demographic variables and lifestyle risk factor such as smoking and alcohol consumption.

Blood collection and G6PD activity assay

Peripheral blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tube and stored at 2-8 $^{\circ}$C. Hemoglobin (Hb) concentrations were measured using CBC machine Mindray BC-5150 (Mindray, CPR). Quality controls (QC) of Hb measurement were daily measured and recorded. Hb concentrations were used to calculate U/gHb for all G6PD tests. All bloods samples were then analysed for G6PD activity within 7 days using quantitative spectrophotometric method (Trinity Biotech, Ireland), according to the manufacturer's instructions (Procedure No.345-UV). The kinetic of G6PD activity at 30°C was monitored using spectrophotometer (Shimadzu Corp., Japan) at 340 nm with 5 min of time intervals from 10 to 15 min. Lyophilized hemolysates of normal and deficient G6PD controls were run with every test of samples (G6888, G5888) (Trinity Biotech, Ireland). The result was expressed as U/g Hb. The results were valid, only if G6PD activities of the controls were within the reference range.

***G6PD* mutation analysis**

DNAs from all samples were extracted using Nucleospin® Blood kit (MACHEREY-NAGEL, Duren, Germany). Samples with deficient and intermediate status were genotyped *G6PD* mutations; G6PD Mahidol (487G>A), G6PD Viangchan (871G>A), G6PD Canton (1376G>T), G6PD Union (1360C>T), and G6PD Kaiping (1388G>A) using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). An appropriate set of primers has been described in previous study (1). The PCR reaction was carried out in 10 μl reaction containing 1x PCR buffer (RBC Bioscience, Taiwan), 0.5 U of Taq polymerase, 200 nM of each primer (New England Biolabs, USA), 1.5 mM $MgCl_2$, 200 μM of each dNTPs (RBC RBC Bioscience, Taiwan) and approximately 50 ng DNA templates. After incubation at 94 ºC for 5 min, amplification was carried out for 35 cycles with the following temperature cycling parameters: 94 ºC for 30 sec, appropriate temperature for each mutation (56ºC: Mahidol, 60ºC: Viangchan, 65ºC: Kaiping, Canton) for 30 sec, 72ºC for 30 sec, and final extension at 72ºC for 7 min. Five microliters of each PCR product were digested with 1 U of an appropriate restriction enzyme digestion set as shown in previous report (16) according to manufacturer's protocols (Fermentas, Thermo Fisher Scientific, USA). The digestion was incubated overnight, followed by separation on 8% polyacrylamide gel (Bio-Rad Laboratories, UK), stained with loading buffer (GeneDirex novel juice, UK) and then visualized under UV light (Gel Doc XR System, Bio-Rad, California, USA). For all unknown mutation samples, PCR direct sequencing was performed for all coding exons (exon 3-13) with primers in **Table 1**. PCR reaction was carried out in 100 μl containing the mixture described above. Thermal cycles for amplification which were different in annealing temperature and extension times were presented in **Table 1**. PCR products were directly sequenced (Macrogen, South Korea) and the sequencing data were analyzed using BioEdit software with reference G6PD sequence (X55448.1) from GenBank.

Table 1 Primer, PCR product size and condition for PCR reaction

Exon	Primer	Sequence	Product size (bp)	Annealing temperature (ºC)	Extension Time (sec)
3-4	Forward	5'-TGTCCCCAGCCACTTCTAA-3'	400	62	30
	Reverse	5'-GGAGAGGAGGAGAGCATCC-3'			
5	Forward	5'-GTGTGTCTGTCTGTCCGTGTC-3'	320	65	30
	Reverse	5'-CACGCTCATAGAGTGGTGGG-3'			
6-7	Forward	5'-ACACAAGGCACGGGAGGT-3'	697	67	45
	Reverse	5'-GAGGAGCTCCCCCAAGATAG-3'			
8	Forward	5'-CATGCCCTTGAACCAGGTGA-3'	241	62	30
	Reverse	5'-GCATGCACACCCCAGCTC-3'			
9-10	Forward	5'-TTCTCTCCCTTGGCTTTCTC-3'	612	60	45
	Reverse	5'-CACACTGCTCCTTCTCTGTA-3'			
11-13	Forward	5'-GAAGCCGGGCATGTTCTTCAAC-3'	856	60	45
	Reverse	5'-GTCAATGGTCCCGGAGTC-3'			

Analysis of genomic Alu and LINE-1 methylation distribution pattern and levels

Combined bisulfite restriction analysis (COBRA) consisted of a standard sodium bisulfite PCR treatment followed by restriction digestion and quantitation. Bisulfite modification of 200 ng genomic DNA was performed using EZ DNA methylation^{TM} kit (Zymo research, California, US), according to the manufacturer's instructions. Bisulfite-treated DNAs were stored at -20^{O}C until use. The primers corresponding to the nucleotides in the regulatory region of the *Alu* sequence were F: 5'-GGTGGTTTAMGTTT GTAATTTTAGTATTT-3' and R: 5'-ATTTCACCATATTAACCAAACTAATC-3'. The PCR reactions were performed as followed: 35 cycles of 95^{O}C for 45 sec, 63^{O}C for 45 sec, and 72^{O}C for 45 sec. Then, PCR products were subsequently digested with 2 U *Taq*I (Fermentas, Thermo Fisher Scientific, USA) at 65^{O}C overnight, followed by separation on an 8% polyacrylamide gel. The gels were stained with loading buffer (GeneDirex novel juice, UK), and band intensities were measured by phospholmager with Image Quant software (Molecular Dynamics, GE Healthcare, Waukesha, WI, USA). The primer sequences corresponding to the nucleotides in the regulatory region of the LINE-1 sequence (GenBank: M80343) were F: 5'-CGTAAGGGCTTAGGGAGTTTTT-3' and R: 5'-(AG)TAAAACCCTCC(AG)AACCAAAT ATAAA-3'. PCR reactions consisted of 35 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min. PCR products were subsequently digested with 2 U of *Taq*I (Fermentas, Thermo Fisher Scientific, USA) and 2 U of *Tas*I (Fermentas, Thermo Fisher Scientific, USA) in TE buffer 3 at 65°C overnight and were separated on an 8% polyacrylamide gel. Gels were stained with loading buffer (GeneDirex novel juice, UK), and band intensities were measured using a

phospholmager with Image Quant software (Molecular Dynamics, GE Healthcare, Waukesha, WI, USA). Each COBRA was performed two to four times. LINE-1 PCR product was 160 bp and the digested product was 80 bp. Unmethylated bands of LINE-1 were 97 and 63 bp. Methylation levels were calculated as the intensity of methylated bands divided by the sum of the methylated and unmethylated bands. *Alu* loci were divided into 4 groups depending on the methylation status of the 2 CpG sites: 1) hypermethylated loci: mCmC, complete methylation at both CpG sites, yields three fragments of 37, 32, and 29 bp; 2) hypomethylated loci: uCuC, fully unmethylated sequence, yields 1 uncut amplicon with size of 98 bp; and 3) and 4) partially methylated loci: uCmC and mCuC. The unmethylated 37 bp CpG and methylated 69 bp CpG sequence yields 2 fragments of 69 and 29 bp. By contrast, the methylated 37 bp CpG and unmethylated 69 bp CpG sequence yields 2 fragments of 37 bp and 69 bp. *Alu* methylation distribution pattern combine with the percentage of methylation were determined. Each product was analyzed as a percentage of product intensity divided by the double strand length. The % number of hypomethylated loci (%mCmC) will be %32/30, of hypomethylated loci (%uCuC) will be %98/98, of partially methylated loci (%uCmC) will be %69/68, and of partially methylated loci (%mCuC) will be %61/60.

***IN VITRO* STUDY:** To examine the role of G6PD in regulation aging process via methylation of Alu and LINE-1 in aged human neuronal cell line

Construction of G6PD deficient neuroblastoma cell line

Human neuroblastoma SK-N-SH cell line (ATCC, USA) was cultured in MEM/EBSS (Hyclone, USA) containing 10% fetal bovine serum (Merck Millipore, USA). Cells were maintained at 37°C with a 5% CO_2 atmosphere in 95% humidified incubator (BINDER, Germany) and were sub-cultured every 3 days. Cells were plated at a density of 4×10^4 cells/well into 96-well plates for 24 h to approximately 80% confluence prior treatment.

Cells were transfected with Lentivirus containing *G6PD* shRNAs. There are 4 constructions of *G6PD* shRNA; A, B, C, D and control (scramble shRNA) (Origene, Maryland, USA). *G6PD* knockdown cells showed green fluorescence (525 nm) and were selected by puromycin culture system.

MTT Assay

MTT reagent (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was added directly to the cells until crystals formed. The media was carefully removed from the plate, leaving the cells intact and the cells were resuspended in DMSO. Absorbance was read at 570 nm to determine cell proliferation.

G6PD activity assay in cultured cells

G6PD activity was measured using spectrophotometrically at 340 nm by the reduction of NADP in the presence of G6P as described preciously (2).

DNA Methyltransferases (*DNMTs*) mRNA expression

Human neuroblastoma SK-N-SH cell line containing G6PD scramble shRNA (control), G6PD shRNA#A (shRNA#1) and G6PD shRNA#C (shRNA#2) were seeded in 25 cm^3 culture flask at a density of $2.5x10^6$ cells/well and allowed to grow for 24 h. Cells were trypsinized and the pellets were collected by centrifuging at 3,500 g for 5 min. Total RNA was isolated using Trizol reagent. The amount of RNA was determined by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). RNA solutions were stored in -80°C until use. cDNA was synthesized using 1 µg RNA added into a reaction mixture containing 5X Reaction Buffer, 10 mM dNTP Mix, Oligo(dT)18 Primer, RiboLockRNase Inhibitor, RevertAid RT and nuclease-free water. Each sample was incubated at 42°C for 60 min followed by inactivation at 99°C for 5 min. All samples were stored at -20°C until use. All qPCR reactions were performed using 10 µL reaction mixture containing 1 µg cDNA, 5 µL of 2X maxima SYBR Green, nuclease free water, and 200 nM of forward and reverse primers (5'-›3'): ß-Actin, (Forward: ACTCTTCCAGCCTTCCTTC and Reverse: ATCTCCTTCTGCATCCTGTC) DNMT1 (Forward: AGGTGAAAAGGCCCTCATCG and Reverse: CGGCCTCGTCATAAC TCTCC) and DNMT3a (Forward: TGATGGAATCGCTACAGGGC, and Reverse: CCTCT TGTCACTAACGCCCA). All reactions were run on the Real-Time Thermocycler (Thermo Fisher Scientific Inc., USA) using the following conditions: pre-denaturation 10 min incubation at 95 °C, 40 cycles of denaturation at 95°C for 10 sec, annealing at 57°C for 30 second extension at 72°C for 30 sec. ß-actin was used as an internal control. Relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and normalized with ß-actin expression.

Cell senescence detection

Cell senescence was detected using β-gal straining. Human neuroblastoma SK-N-SH cell line with G6PD scramble shRNA (control), G6PD shRNA#A (shRNA#1), and G6PD shRNA#C (shRNA#2) were plated at a density of $1x10^5$ cells/well into 12-well plates for 24 h to get approximately 80% confluence prior study. In brief, cells were fixed for 5 min at room temperature with 2% formaldehyde, then cells were washed with 1XPBS twice and stained with 1 mg/ml 5-bromo 4-chloro-3-indolyl β-D-galactoside (X-Gal) dissolved in a solution containing 40 mM citric/sodium phosphate; pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl and 2 mM $MgCl_2$. After incubation at 37°C (no CO_2) for 14 h, photographs were taken under phase microscope.

Statistical analysis

Data was analysed using SPSS statistical software version 22.0 (IBM Corp., Armonk, N.Y., USA). A descriptive data was performed on clinical and demographic characteristics. The distribution of continuous variables was assessed using Kolmogorov-Smirnov/Shapiro-Wilk tests. Mean and standard deviation (SD) were reported for continuous variables presenting normal distribution, median and interquartile range (IQR) for continuous variables with skewness. G6PD activity reference values (mean, SD, median, IQR and range) and adjusted male median (AMM) were analysed for the entire study population and by gender (3). AMM was the median G6PD activity of all male subjects after excluding male samples with severe G6PD deficiency (less than 10% of the overall median value for all males in the study) (3). AMM was aimed to eliminate the skewness from depleted values of population median during determination the cut-off points of various G6PD deficient levels (3). The cut-off points of deficiency and intermediate G6PD deficiency were determined as less than 30% and 30 to less than 60% G6PD activity of the AMM, respectively (3). Subjects with G6PD activity more than 60% of the AMM was defined as normal (3). Proportion of male and female and prevalence of G6PD deficiency between patients and blood donors were analysed. Median methylation levels of Alu and LINE-1 was nonparametrically compared between G6PD deficient and normal participants and between dementia and non-dementia participants using Mann-Whitney U-test. Correlation between the Alu, LINE-1 methylation pattern in all participant was assessed with Pearson correlation coefficient test. Logistic regression model was performed to evaluate the association between G6PD activity as a dependent variable specified as a binary outcome, normal and G6PD deficiency, and

%mC, %mCmC loci, %uCmC loci, %mCuC loci, %uCuC loci, and age as independent predictors. All the independent variables enter the model at the same time.

The results from cell culture; G6PD activity, methylation and ROS were expressed as the means±SEM. Data was analysed by ANOVA to compare the mean values of groups at a *p*-value <0.05.

Results

Characteristics of study subjects

A total of 114 elder participants in this study, 6 patients were G6PD deficiency (5 males and 1 female). There were no significant differences in age or gender between G6PD deficient and normal. However, female in group of normal and G6PD deficient had significantly higher age than groups of males (p=0.042 and p=0.005, respectively) (**Table 2**). Demographic characteristics of participants in this analysis were listed in **Table 3-4**. Mean age of patients was significantly higher than that of blood donors (p<0.001). G6PD activity in patients was also significantly higher than that of blood donors (p=0.006). Only neurodegenerative diseases (NDs) such as AD, IPD, dementia, VaD, and MCI were analysed further in detail (**Table 4**). Mean age of control was significantly lower than that of NDs patients (p<0.001).

Table 2 Demographic data of G6PD deficient and G6PD normal

Sample group	Number (case)	Age (year) (mean±SD)	*P*-value	G6PD activity (IU/gHb)	*P*-value
Normal	Total: 108	68.6±8.2	0.667[a]	8.1±2.7	
	Male: 53	**66.9±6.8**	**0.042** [b]	8.3±3.0	0.312[b]
	Female: 55	**70.1±9.2**		7.8±2.3	
G6PD deficiency	Total: 6	67.8±10.0	0.667[a]	1.3±0.6	
	Male: 5	**64.0±3.8**	**0.005** [b]	1.2±0.6	0.417[b]
	Female: 1	**87.0**		1.8	

[a] compared between normal and G6PD deficiency using Mann-Whitney U test, [b] compared between male and female in the same sample group using student T-test

Table 3 Demographic data of the blood donor and all patient samples

Sample group	Number (case)	Age (year) (mean±SD)	*P*-value	G6PD deficiency (case)	G6PD activity (IU/gHb)	*P*-value
Blood donors	Total: 48	**62.6±2.1**	**<0.001**[a]			
	Male: 30	62.9±2.0	0.091[b]	Normal: 26	**Normal: 7.28±1.32**	**0.006**[c]
				Deficiency: 4	**Deficiency: 0.95±0.19**	
	Female: 18	62.1±2.1		Normal: 18	Normal: 6.95±1.28	0.053[d]
Patients	Total: 66	**72.9±8.4**	**<0.001**[a]			
	Male: 28	70.8±7.4	0.157[b]	Normal: 27	**Normal: 9.27±3.75**	**0.006**[c]
				Deficiency: 1	**Deficiency: 2.22**	
	Female: 38	74.4±8.8		Normal: 37	Normal: 8.22±2.55	0.053[d]
				Deficiency: 1	**Deficiency: 1.79**	

[a] compared between patients and blood donors using Mann-Whitney U test, [b] compared between male and female in the same group using student T-test, [c] compared between patient and blood donor of G6PD normal males using Mann-Whitney U test, [d] compared between patient and blood donor of G6PD normal females using student T test

Table 4 Disorders in patients (Several disorders could be found in one patient.)

Sample group	Number (case)	Sample group	Number (case)
Dementia	4	Chronic kidney disease (CKD)	12
Vascular dementia (VaD)	25	Benign prostate hypertrophy (BPH)	8
Mild cognitive impairment (MCI)	2	Chronic obstructive pulmonary (COPD)	4
Idiopathic Parkinson's disease (IPD)	6	Obstructive sleep apnea (OSA)	1
Alzheimer's disease (AD)	17	Osteoarthritis (OA)	1
Ischemic heart disease (IHD)	9	Atrial fibrillation (AF)	2
- Single vessel disease (SVD)	4	PSP	1
- Double vessel disease (DVD)	2	Myelodysplastic syndrome (MDS)	1
- Triple vessel disease (TVD)	3	Transient global amnesia (TGA)	1
Stroke	11	Asthma	1
- Ischemic stroke	10	Dyslipidemia	35
- Haemorrhagic stroke	1	Impair fasting glucose (IFG)	3
Diabetic mellitus (DM)	16	Hypertension	41

Table 5 Demographic data of neurogenerative diseases (NDs) patients and non-NDs

Sample group	Number (case)	Age (year) (mean±SD)	*P*-value	G6PD deficiency (case)	G6PD activity (IU/gHb)	*P*-value
Non-NDs	Total: 68	**64.5±5.0**	**<0.001**[a]			
	Male: 43	65.0±4.8	0.368 [b]	Normal: 39	Normal: 8.02±3.13	0.055[c]
				Deficiency: 4	**Deficiency: 0.95±0.19**	
	Female: 25	63.8±5.4		Normal: 25	**Normal: 7.03±1.27**	**0.018**[d]
NDs Patients	Total: 46	**74.4±8.6**	**<0.001**[a]			
	Male: 15	71.5±8.6	0.113 [b]	Normal: 14	Normal: 9.09±2.52	0.055[c]
				Deficiency: 1	**Deficiency: 2.22**	
	Female: 31	75.8±8.4		Normal: 30	**Normal: 8.48±2.74**	**0.018**[d]
				Deficiency: 1	**Deficiency: 1.79**	

[a] compared between NDs patients and controls using Mann-Whitney U test, [b] compared between male and female in the same group using student T-test, [c] compared between NDs patients and controls of G6PD normal males using Mann-Whitney U test, [d] compared between patient and blood donor of G6PD normal females using student T test

Alu, LINE1 Methylation in G6PD deficiencies and controls

To study the correlation between the level of Alu, LINE-1 methylation and blood G6PD activity of elders, we measured Alu, LINE-1 methylation in peripheral blood leukocytes of elder G6PD deficiency and controls. Only mCuC of Alu was significantly increased in G6PD deficiency (p=0.043) (**Figure 1**). This finding contrasted to other reports that found Alu hypomethylation in elders. Association between Alu, LINE-1 methylation and G6PD deficiency were then analysed using odd ratio (OR). G6PD deficiency was not associated with Alu and LINE-1 methylation. This may be due to a small number of G6PD deficient cases in this study.

Alu, LINE1 Methylation in NDs patients and controls

To investigate global methylation in NDs patients, we measured Alu, LINE-1 methylation in peripheral blood leukocytes of NDs patients and non-NDs. Alu and LINE-1 methylation in NDs patients were also increased and significantly increased in mC and uCmC of Alu (p=0.001 and 0.017) and decreased in uCuC of LINE-1 (p=0.001) (**Figure 2)**. For unadjusted OR, increasing of mC of Alu and decreasing of uCuC of LINE-1 were

associated with risk of NDs. After adjusted OR with age, gender and G6PD deficiency status, only decreasing of uCuC of LINE-1 was associated with NDs (**Table 6**). However, association between G6PD deficiency and NDs was limited to analyse because of number of G6PD deficient cases in this study. Moreover, there are many confounding factors in patients including hypertension, DM and other diseases, which may interfere with data interpretation.

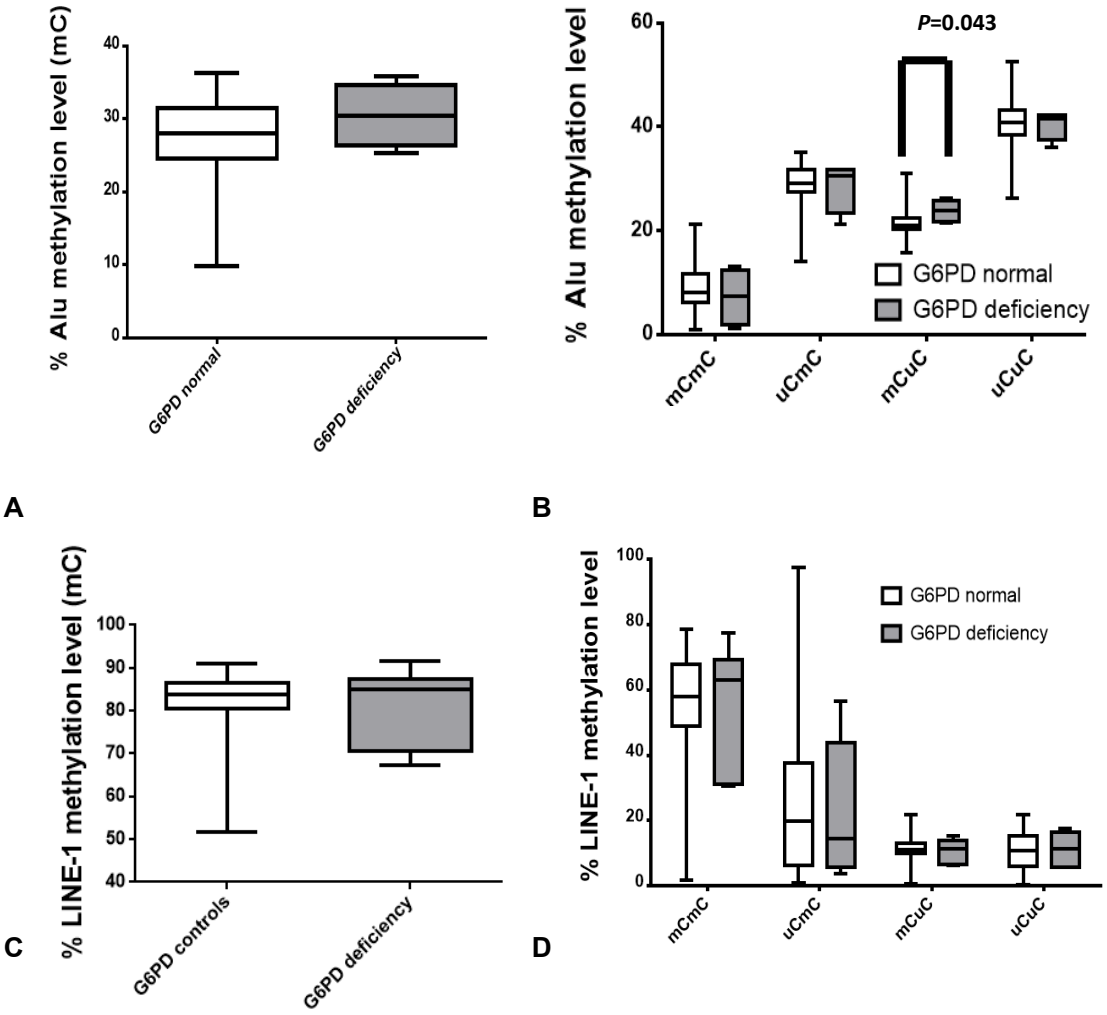


Figure 1 Methylation levels and patterns of Alu (upper panel: A-B) and LINE-1 (lower panel: C-D) in G6PD deficiency and normal.

Table 6 Odd ratio (OR) of global methylation and NDs

Methylation pattern	Unadjusted OR OR (95% CI)	*p*-value	Adjusted OR OR (95% CI)	*p*-value
Alu: mC	**1.065-1.340**	**0.002**	0.899-1.223	0.542
Alu: uCmC	0.961-1.229	0.186	0.882-1.258	0.563
LINE-1: uCuC	**0.738-0.920**	**0.001**	**0.863-0.988**	**0.032**

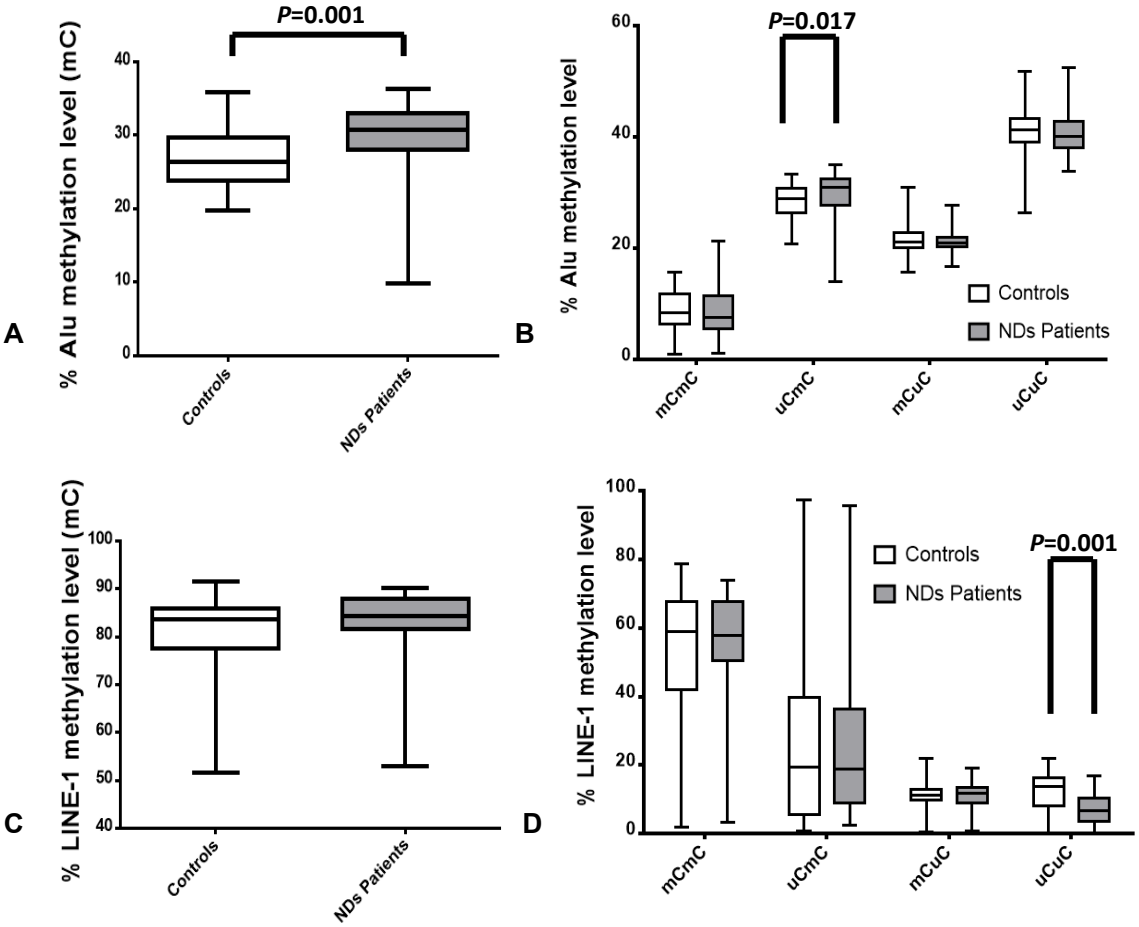
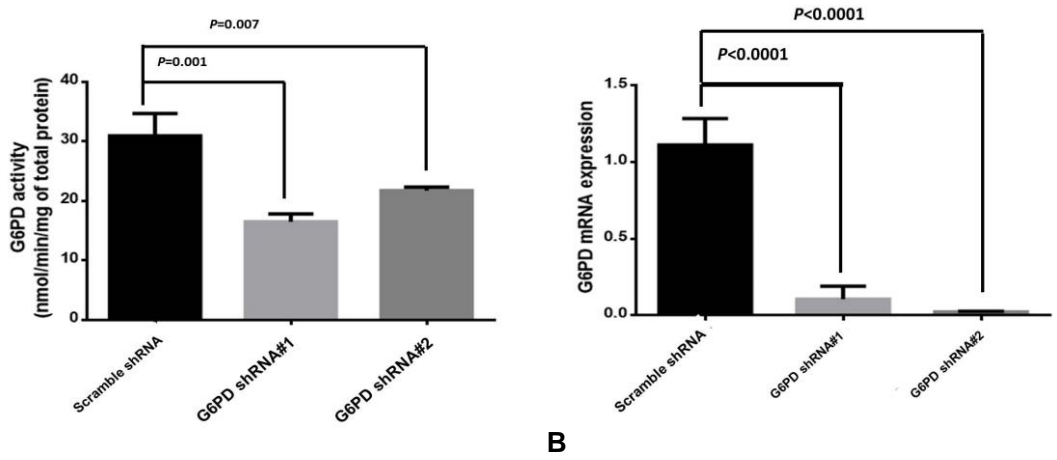


Figure 2 Methylation levels and patterns of Alu (upper panel: A-B) and LINE-1 (lower panel: C-D) in NDs patients and non-NDs (controls).

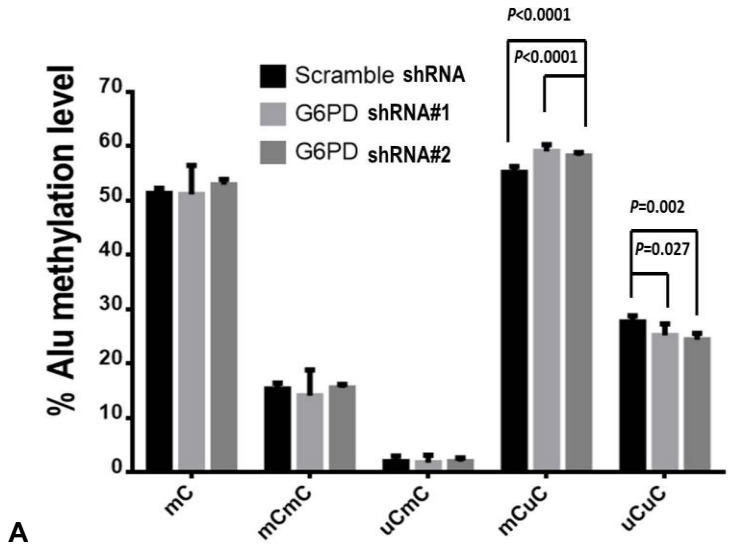
Correlation between G6PD deficiency and global methylation in cell culture

G6PD activities and mRNA expressions in G6PD shRNA#A (shRNA#1) and shRNA#C (shRNA#2) neuroblastoma cell lines were significantly decreased (**Figure 3A-B**). We found a significant correlation between reducing of G6PD activity and Alu and LINE-1 hypermethylation in these cells (**Figure 4A-B**).

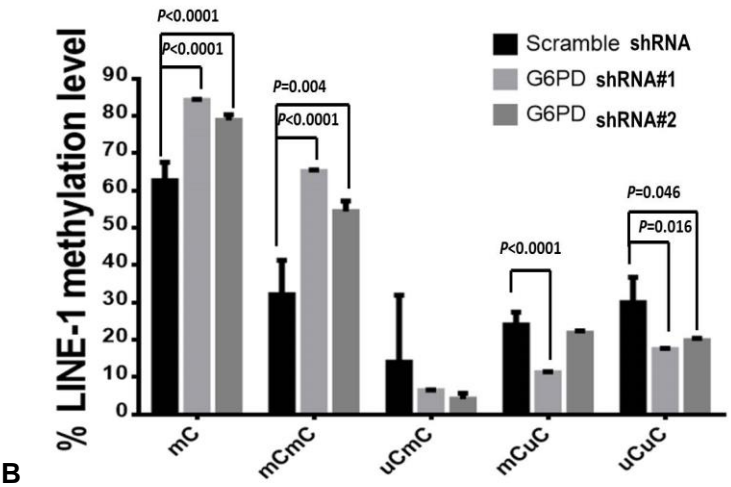


A B

Figure 3 A G6PD activity of scramble shRNA, shRNA#1 (#A) and #2(#C) neuronal cells **B** G6PD mRNA expression of scramble shRNA, shRNA#1 (#A) and #2 (#C) neuronal cells



A



B

Figure 4 A Alu methylation of scramble shRNA, shRNA#1 (#A) and #2 (#C) neuronal cells **B** LINE-1 methylation of scramble shRNA, shRNA#1 (#A) and #2 (#C) neuronal cells

DNA Methyltransferases (*DNMTs*) mRNA expression

The expression of *DNMT1* and *DNMT3a* in G6PD shRNA#1 (#A) and shRNA#2 (#C) neuroblastoma cell lines were significantly decreased when compared with scramble control (**Figure 5A and B**).

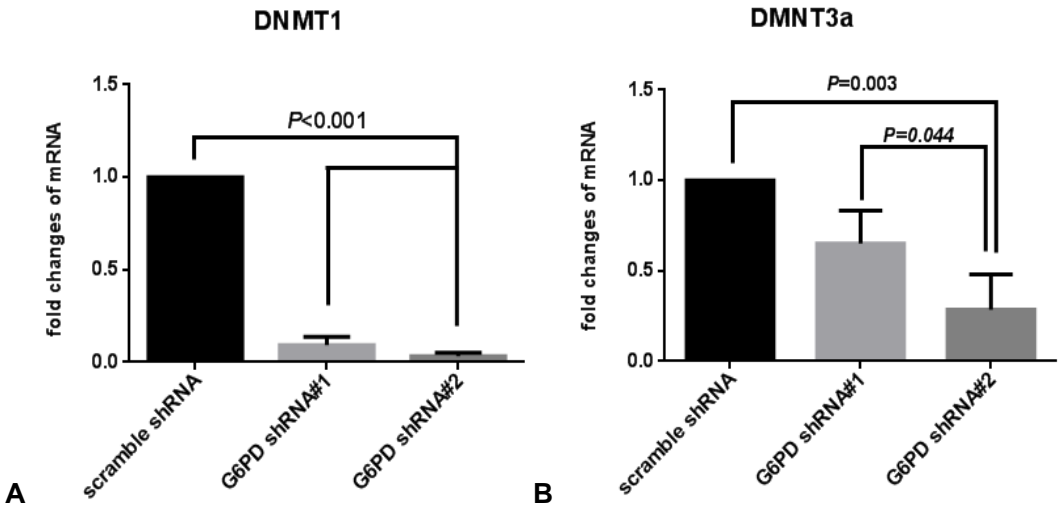


Figure 5 A Fold changes of *DNMT1* mRNA expression in scramble shRNA, shRNA#1 (#A) and #2 (#C) neuronal cells **B** Fold changes of *DNMT3a* mRNA expression scramble shRNA, shRNA#1 (#A) and #2 (#C) neuronal cells

Cell senescence detection

Increasing β-gal straining for cell senescence were detected in G6PD shRNA#1 (#A) and shRNA#2 (#C) (**Figure 6C and D**) when compared with SK-N-SH cell and scramble control (**Figure 6A and B**).

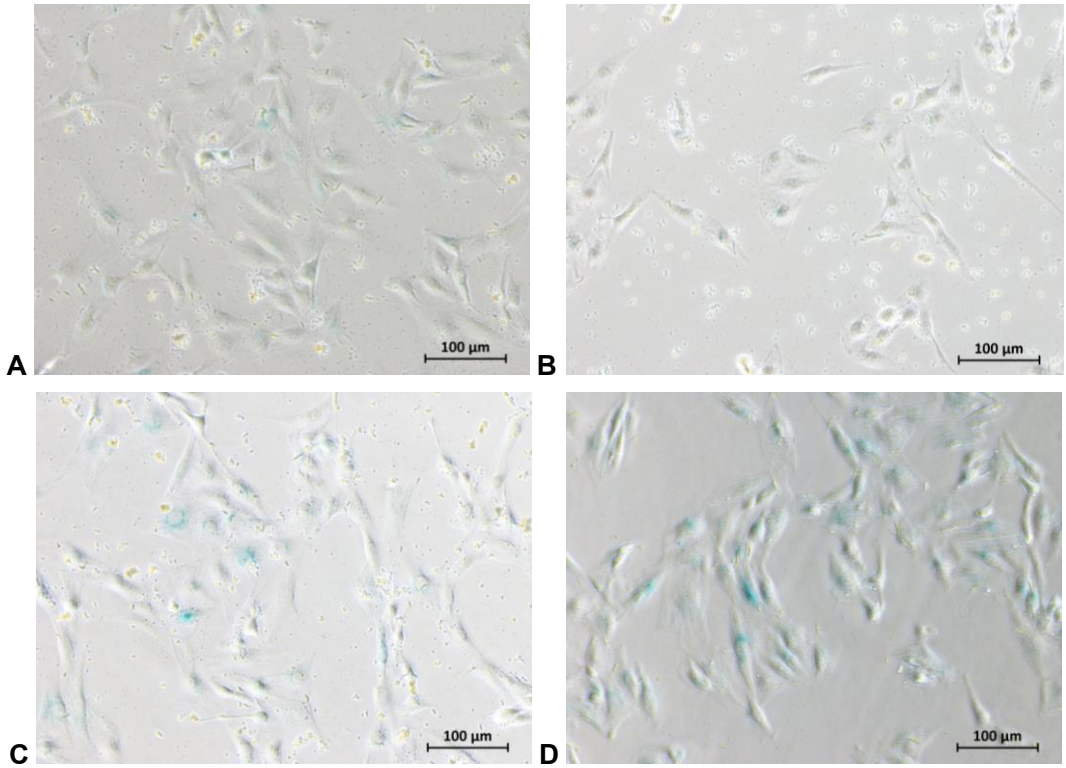


Figure 6 β-gal straining for cell senescence in SK-N-SH (**A**), scramble shRNA (**B**), G6PD shRNA#1 (#A) (**C**) and shRNA#2 (#C) (**D**) cells

Discussion

This study investigated the correlation between levels of Alu, LINE-1 methylation and G6PD activity in peripheral blood of elders and in *G6PD* knockdown neuronal cell line. Although there was no significant difference of Alu and LINE-1 methlyation levels between G6PD deficiency and normal blood samples, we found LINE-1 hypermethylation in *G6PD* knockdown neuron cell lines. Inability to detect LINE-1 hypermethylation *in vivo* system may be because there were only 6 cases of G6PD deficiency in the study. However, this is the first study to report that a decrease of G6PD activity associates with LINE-1 hypermethylation.

It has been reported that G6PD activity was significantly lower in elder patients with Parkinson's disease (PD), which is a common neurodegenerative disorder (5). It has been reported that G6PD activity decreased with age in erythrocytes, fibroblasts and lens cells (5-6). However, we could not find decreasing of G6PD activity in neurodegenerative disorder patients. The association between G6PD depletion and aging was detected in only *in vitro* system. Uncorrelation between G6PD deficiency and neurodegenerative disorders *in vivo* system may be caused by the number of case with coincidence of G6PD deficiency and neurodegenerative disorders was hard to enroll in

the study. As we reported above, both of their phenomenons have not been detected in our patients. Almost our elder patients confronted with several diseases associated with epigenetic alteration eg. hypertension, chronic kidney disease and diabetes which reduced number of cases after adjusted confounder factors. It has been reported that Alu hypomethylation and LINE-1 hypermethylation were significantly observed in aging cells and associated with degenerative diseases including Alzheimer disease (AD) (7-9). G6PD deficiency causes an imbalance of cellular redox state, which might lead to DNA damage and cell senescence.

Conclusion

This finding supported that depletion of G6PD leads to cellular aging. The mechanism of G6PD deficiency in control of cell senescence will be further investigated.

Acknowledgements

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References

1. Kanchanavithayakul A, Prasittisa K, Kiat-Amornrak P, Chanda M, KittiwatanasarnP, Nuchprayoon I, Cheepsunthorn CL. Prevalence of glucose 6-phosphte dehydrogenase deficiency and genetic mutations among Karen and Lao populations in Thailand. Southeast Asian J Trop Med Public Health. 2017;48(6):1308-17.
2. García-Nogales P, Almeida A, Fernández E, Medina JM, Bolaños JP. Induction of Glucose-6-Phosphate Dehydrogenase by Lipopolysaccharide Contributes to Preventing Nitric Oxide-Mediated Glutathione Depletion in Cultured Rat Astrocytes. J Neurochem. 1999;72(4):1750-8.
3. LaRue N, Kahn M, Murray M, Leader BT, Bansil P, McGray S, Kalnoky M, Zhang H, Huang H, Jiang H, Domingo GJ. Comparison of Quantitative and Qualitative Tests for Glucose-6-Phosphate Dehydrogenase Deficiency. Am. J. Trop. Med. Hyg. 20104;91(4):854–61.
4. Abraham S, Soundararajan CC, Vivekanandhan S, Behari M. Erythrocyte antioxidant enzymes in Parkinson's disease. Indian J Med Res 2005; 121: 111-5.
5. Maurya PK, Kumar P, Chandra P. Age-dependent detection of erythrocytes glucose-6-phosphate dehydrogenase and its correlation with oxidative stress. Arch

Physiol Biochem, 2016;122(2):61–66.

6. Biagiotti E, Malatesta M, Capellacci S, Fattoretti P, Gazzanelli G, Ninfali P. Quantification of G6PD in small and large intestine of rat during aging. acta histochem. 2002;104(3):225–34.
7. Bollati V, Galimberti D, Pergoli L, Dalla EV, Barretta F, Cortini F, Scarpini E, Bertazzi PA, Baccarelli A. DNA methylation in repetitive elements and Alzheimer disease. Brain Behav Immun. 2011;25(6):1078-83.
8. Lange NE, Sordillo J, Tarantini L, Bollati V, Sparrow D, Vokonas P, Zanobetti A, Schwartz J, Baccarelli A, Litonjua AA, DeMeo DL. Alu and LINE-1 methylation and lung function in the normative ageing study. BMJ Open. 2012;2.
9. Neven KY, Piola M, Angelici L, Cortini F, Fenoglio C, Galimberti D, Pesatori AC, Scarpini E. Bollati V. Repetitive element hypermethylation in multiple sclerosis patients. BMC Genetics. 2016;17:84.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. การนำผลงานวิจัยไปใช้ประโยชน์

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ภาคผนวก

Evaluating the performance of automated UV enzymatic assay for screening of glucose 6-phosphate dehydrogenase deficiency

Running Head: Evaluating the performance of automated UV enzymatic assay for screening of glucose 6-phosphate dehydrogenase deficiency

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Abstract

Introduction: A precise and reliable screening assay for G6PD deficiency would greatly help avoiding unwanted outcomes due to bilirubin neurotoxicity in neonatal jaundice and antimalarial induced hemolytic anemia in malaria patients. Currently, available assays are laborious and require sophisticated laboratory expertise. This study aimed to evaluate the performance of a recently introduced automated screening assay for G6PD deficiency by comparing with a routine spectrophotometric assay.

Methods: An automated UV-based enzymatic (Mindray, PRC) and spectrophotometric assays were performed simultaneously in parallel to determine G6PD activity in 251 blood samples from the subjects.

Results: The median G6PD activity value from spectrophotometric assay was significantly lower than that of from the automated assay. The mean difference was -2.0 U/g Hb (-7.3 to 3.2; $p<0.0001$). The mean activity values of both assays were strongly correlated with Pearson's correlation coefficient of r = 0.8. Cohen's Kappa statistics between assays was 0.77 (0.70-0.83). The sensitivity, specificity, positive and negative predictive values of the automated assay were 85.7%, 99.2%, 85.7%, 99.2%, respectively. The sensitivity and positive predictive value of the automated assay for identifying intermediate G6PD activity levels were 40.0% and 25.0%, respectively. Genotyping was performed to confirm G6PD deficient and intermediate samples. The turnaround time for 40 samples was 60 min for the automated assay and 300 min for spectrophotometric assay.

Conclusion: The automated assay for the detection of G6PD deficiency is comparable to a routine spectrophotometric assay and help reducing sample handling time. However, the assay shows limitation in identifying individuals with G6PD intermediate.

Keywords: G6PD, G6PD deficiency, quantitative method, automated UV enzymatic assay.

Introduction

Glucose 6-phosphate dehydrogenase (G6PD) is a key enzyme in the pentose phosphate pathway (PPP). It generates nicotinamide adenine dinucleotide phosphate (NADPH) to maintain the levels of reduced glutathione (GSH), which is an important cellular antioxidant.[1] G6PD deficiency is the most common inherited enzymopathy, affecting more than 400 million people worldwide.[2] Mosaicism of X chromosome inactivation in heterozygous females results in population based large distribution of enzymatic activities.[3] Individuals with G6PD deficiency are asymptomatic and prone to acute hemolytic anemia (AHA), hemoglobinuria and favism after exposure to triggers, such as high levels of oxidative free radicals, infections, fava bean and antimalarial drugs.[1]

G6PD deficiency is associated with neonatal hyperbilirubinemia and jaundice, commonly found in Mediterranean and Asian populations.[4] In Thailand, it has been reported that up to 65% of severe neonatal jaundice and 22.1% of hyperbilirubinemia are G6PD deficiency.[5] Delayed phototherapy in jaundice newborns due to unaware of G6PD deficiency can cause extreme hyperbilirubinemia, bilirubin neurotoxicity and kernicterus.[4] The World Health Organization (WHO) has recommended screening for G6PD deficiency in newborns in countries where G6PD deficiency is affected 3–5% among males to early recognize and to prevent the unwanted outcomes in a timely manner.[6]

Both qualitative and quantitative screening assays for G6PD activity are available. The fluorescent spot test (FST) is widely used for qualitative screening of G6PD deficiency. However, its power in discrimination between intermediate (30-60% of normal G6PD activity levels) and normal is limited.[3,7] The gold standard for the determination G6PD activity is based on a quantitative measurement of the amount of NADPH by UV spectrophotometer or flow cytometer, which make it possible to

resolutely distinguish intermediate from normal activity levels.[7,8] Nonetheless, performing these assays is manual and time consuming and requires experienced hands.

Mindray Medical International has recently introduced BS-240, an automated UV-based G6PD activity measurement system capable of quantifying G6PD activity in 40 venous blood samples simultaneously. The aim of this study was to evaluate the performance of this system by comparing side by side with spectrophotometric assay. All G6PD deficient and intermediate samples were subjected to genotypic analysis by PCR-RFLP and direct sequencing to confirm the assay results.

Materials and Methods

Subjects and sample collection

Research Ethics Committee, National Blood Centre, Thai Red Cross Society (COA No. NBC 7/2016) and Research Ethics Committee, Faculty of Medicine Ramathibodi Hospital have approved the protocol of this study. Two hundred and fifty-one subjects were randomly recruited from blood donors and volunteers from National Blood Centre, Thai Red Cross Society, Bangkok and from Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. The subjects were 117 males and 134 females who signed their informed consent forms before participating in this cross-sectional study in 2016. Whole blood samples were collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA), stored at 2-8 °C and analyzed for G6PD activity within 72 hours after the collection. The assays were conducted on the same day for each sample. Hemoglobin (Hb) concentrations measured by auto hematology analyzer BC-5150 (Mindray Medical International, PRC) were used to calculate the activity level of U/gHb for quantitative G6PD assays.

Quantitative spectrophotometric assay

G6PD activity levels in whole blood sample were determined using G6PD quantitative assay kits, Procedure No.345 (Trinity Biotech, Ireland) according to the manufacturer's instructions as the reference assay for all tests. Lyophilized hemolysates of normal and deficient G6PD controls (G6888, G5888; Trinity Biotech, Ireland) were run with every duplicate samples. Five microliters of whole blood samples were added into 0.5 mL reagent and incubated at room temperature for 5 min. Then, 1 mL of substrate was added into the mixture. One milliliter of sample and control mixtures aliquoted into cuvettes were incubated at 37°C for 10 min. Then, the absorbance was immediately measured at 340 nm and exactly 5 min later in the temperature-regulated spectrophotometer (Shimadzu UV-1800) (Shimadzu, Japan). Results were expressed as U/gHb. The results were validated, if the control G6PD activity was well within the reference range.

Automated UV enzymatic assay

Packed red blood cells (PRBCs) were separated from the whole blood samples by centrifugation at 3000 rpm for 5 min. Then, 20 µL of PRBCs were mixed with 1 mL of distilled water in a new tube for 2-5 min. Six microliters of the hemolysate was mixed with 120 µL of reagent 1 and incubated at 37°C for 4 min. Then, 30 µL of reagent 2 was mixed with sample mixture and incubated for 1 min at 37°C. G6PD activity was measures at 340 nm for 2 min of time intervals after incubated at 37°C by clinical chemistry analyzer (BS-240, Mindray Medical International, PRC). The rate of absorbance change was calculated by the following equation, $\Delta A/min = [\Delta A/min\text{-}sample]\text{-}[(\Delta A/min\ blank]$. Results were valid, if the control activity was well within the reference range.

PCR-RFLP and Direct Sequencing

Genomic DNA from all deficient and intermediate samples was extracted using Nucleospin® Blood kit (Macherey Nagel, Germany). Four common mutations

in Thailand including G6PD Mahidol (487G>A), G6PD Viangchan (871G>A), G6PD Kaiping (1388G>A) and G6PD Canton (1376G>T) were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique with appropriate primer sets, reported previously.[9] The typical PCR reaction was carried out in 20 µl containing 1X PCR buffer (RBC Bioscience, Taiwan), 0.5 U of Taq polymerase (RBC Bioscience, Taiwan), 200 nM of forward and reverse primers (New England Biolabs, USA), 1.5 mM $MgCl_2$, 200 µM dNTPs (RBC Bioscience, Taiwan) and 50 ng of DNA template. After incubation at 94ºC for 5 min, reaction was carried out for 35 cycles with the following temperature cycles: 94ºC for 30 sec, appropriate temperature for each mutation (56ºC: Mahidol; 60ºC: Viangchan; 65ºC: Kaiping, Canton) for 30 sec, 72ºC for 30 sec, and final extension at 72ºC for 7 min. Ten microlitters of each PCR product was digested with 1 U of appropriate restriction enzyme sets reported previously[10], according to manufacturer's protocols (Fermentas, USA). The digestion mixture was incubated overnight, subjected to electrophoresis on 8% acrylamide gel (Bio-Rad Laboratories, UK), mixed with loading buffer (GeneDirex, USA) and then visualized under UV light using Gel Doc XR System (Bio-Rad, USA).

All unknown mutant samples were subjected to direct sequencing for all coding exons (exon 3-12).[9] The purified PCR products were directly sequenced by Macrogen (Korea) and the sequencing data were analyzed using BioEdit software with the *G6PD* reference sequence (GenBank accession number X55448.1).

Statistical analysis

All statistical analyses were performed using SPSS software version 22 (IBM Corp., Armonk, N.Y., USA). The normal distribution of age was assessed using Kolmogorov-Smirnov/Shapiro-Wilk tests. G6PD activity reference values (mean, SD, median, interquartile range (IQR) and range) and adjusted male median (AMM)

of both quantitative G6PD assays were analyzed for the entire study population and by gender.[7,11] The spectrophotometric assay was used as the reference assay. AMM was the median G6PD activity of all male subjects after excluding male samples with severe G6PD deficiency (less than 10% of the overall median value for all males in the study).[11] AMM was aimed to eliminate the skewness from depleted values of population median during determination the cut-off points of various G6PD deficient levels.[12] The cut-off points of deficiency and intermediate were determined as less than 30%, and 30 to less than 60% G6PD activity of the AMM, respectively.[12] Subjects with G6PD activity more than 60% of the AMM was defined as normal.[12] G6PD activity was presented as median and range. Mean G6PD activity difference between two quantitative assays was analysed by related samples Wilcoxon signed rank test. Pearson correlation, Bland-Altman method, and Cohen's Kappa statistic were used to evaluate the relationship or the degree of agreement between spectrophotometric and automated UV enzymatic assays. Sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs) of automated UV enzymatic assay were evaluated.

Results

From a total of 251 Thai subjects, the sex ratio of male and female was 1:1.1. The median age in males was 44 years (interquartile range (IQR): 17.75-71.25 years, range: 20-83 years) and in females was 37 years (IQR: 15.5-57.5 years, range: 18-73 years). Spectrophotometric and automated UV enzymatic assays were performed to determine G6PD activity in all enrolled participants.

Quantitative measurement of G6PD activity

G6PD activities measured by spectrophotometric and automated UV enzymatic methods presented bimodal distribution in males and normal distribution in

females (**Figure 1A-B**). In spectrophotometric assay, the median G6PD activity of females (11.4 U/g Hb; interquartile range (IQR): 8.0-14.8 U/g Hb) was slightly higher than that of adjusted males (10.7 U/g Hb; IQR: 7.3-14.1 U/g Hb) (**Table 1)**. In automated UV enzymatic assay, the median G6PD activity of females (14.2 U/g Hb; IQR: 10.0-18.4 U/g Hb) was also higher than that of adjusted males (12.2 U/g Hb; IQR: 8.6-15.8 U/g Hb) (**Table 1**). The median absolute activity of automated UV enzymatic assay was higher than that of spectrophotometric assay. The mean G6PD activities of difference between the two assays was -2.0 U/g Hb (95% limit of agreement (95% LoA): -7.3 to 3.2) (**Figure 2A**), with significant difference (p<0.0001). However, the correlation of G6PD activity levels measured by spectrophotometric and automated UV enzymatic assays was moderately strong with Pearson's correlation coefficient of 0.80 (**Figure 2B**). Cohen's Kappa statistics of agreement between the two assays was 0.77 (95%CI: 0.70-0.83) (**Table 2**). According to the manufacturer instructions, the reference cut-off point for G6PD deficiency of automated UV enzymatic assay is 3.8 U/g Hb in line with 30% (3.7 U/g Hb) G6PD activity of the adjusted male median (AMM) (12). The sensitivity, specificity, positive predictive value (PPV) and negative prediction value (NPV) of automated UV enzymatic method were 85.7% (95%CI:79.4-92.0), 99.2% (95%CI:98.8-99.6), 85.7% (95%CI:79.4-92.0) and 99.2% (95%CI:98.8-99.6), respectively.

Genotyping of *G6PD* mutations

All *G6PD* coding regions from 23 G6PD deficient and intermediate samples (10 males and 13 females) identified by spectrophotometric and automated UV enzymatic assay were genotyped. *G6PD* mutations and their activities were shown in **Table 3**. Eight mutations were observed among deficient and intermediate samples; G6PD Viangchan (871G>A) (4/10 males, 7/13 females), G6PD Mahidol (487G>A)

(1/10 males), G6PD Union (1360C>T) (1/10 males, 2/13 females), G6PD Valladolid (406C>T) (1/10 males, 1/13 females), G6PD Aures (143T>C) (1/10 males), G6PD Kaiping (1388G>A) (1/10 males), G6PD Canton (1376G>T) (1/13 females), and G6PD Chinese-5 (1024C>T) (1/13 females). There were 2 samples that no mutations could be found in the coding regions of *G6PD*. One of these was identified as G6PD deficiency. Another sample was identified as normal by spectrophotometric assay and as intermediate by automated UV enzymatic assay.

Discussion

The precision and reliability of automated UV enzymatic assay in determining G6PD activity in blood samples of adult Thai volunteers with various ages was evaluated by comparing with the gold standard spectrophotometric assay. Since male and female distribution patterns of blood G6PD activity were different, normal G6PD activity reference values for males and females were reported separately. It was shown that bimodal distribution of male G6PD activity was affected by obvious enzymatic deficient values of mutated hemizygous males, whereas normal distribution of female G6PD activity showing wide range of enzymatic activities resulted from lyonization in heterozygous females.[3] After minimizing the impact of severe G6PD deficient activity (below 10% of median value) on the reference normal value by excluding 5 males from spectrophotometric data set, the standardized normal reference values of female and adjusted male median G6PD activity (AMM) from spectrophotometric and automated UV enzymatic method of a study population were established. The normal reference values of females from both spectrometric and automated UV enzymatic assays are slightly higher than that of males, which was consistent with a previous report.[13]

Comparative analysis showed that the absolute activity values of the same samples obtained from automated UV enzymatic assay were significantly higher than that of from spectrophotometric assay, despite the fact that both assays had a moderately strong Pearson's correlation. This can be explained in term of the amount of G6PD proteins which directly affecting the kinetics of enzymatic reactions in both assays. Considered per sample unit volume as described in Materials and Methods, G6PD is expected to be much richer in packed red cells used in automated UV enzymatic assay than in whole blood used in spectrophotometric assay. Also, both assays used the same Hb value to calculate G6PD activity levels and the other reaction components are present in excess.

From Bland-Altman plot, mean G6PD activity of automated UV enzymatic assay was significantly different from than that of spectrophotometric assay. This might due to the presence of outliers, which appeared to be anaemic, as determined by complete blood count (CBC). These individuals may produce more immature red blood cells which have high levels of G6PD to compensate with mature red cell loss, a condition known as reticulocytosis.[14] Such blood samples would affect the analysis of G6PD activity in this study. Thus, when using packed red cells to measure G6PD activity, a reticulocyte count should be performed to prevent false negative result.

In this study, we set the cut-off points for G6PD deficiency and intermediate at <30% and 30%-<60% of their AMM, as reported previously.[11] According to the manufacturer's instruction, the cut-off point for G6PD deficiency of automated UV enzymatic assay is 3.8 U/g Hb, which is in range with our setting cut-off 3.7 U/g Hb (30% of AMM). We obtained a relatively high performance from the automated assay in the detection of G6PD deficiency, compared to the gold standard. The precision of the assay was confirmed by molecular analysis of *G6PD* mutations. Nonetheless, the assay was less effective in the detection of intermediate cases.

In molecular analysis of *G6PD* mutation, some cases of deficiency and intermediate were unable to identify mutation which found similar results with other study.[15] It may be because the mutation in *cis* acting elements or non-coding regions may interfere with *G6PD* gene expression.[16]

In term of the population genetics of G6PD deficiency, we found that G6PD Viangchan was the most common mutation in Thai population consistent with previous reports.[5,17] Other less common mutations in Southeast Asian and Chinese including G6PD Mahidol, G6PD Union, G6PD Kaiping, G6PD Canton and G6PD Chinese-5 were also detected.[5,17] In addition, we also detected G6PD Valladolid and G6PD Aures in our samples. G6PD Valladolid (406 C > T, Arg136Cys) is a single point mutation located in exon 5, previously reported in Spanish and Burmese.[18,19] G6PD Aures (143T>C, Ile48Thr) located in exon 3 and originally reported in Algeria and Tunisia is associated with favism and neonatal jaundice.[20,21] To our knowledge, this may be the first report of G6PD Valladolid and Aures in Thai population.

In conclusion, our study demonstrated that the automated assay is comparable to a routine gold standard spectrophotometric assay for the detection of G6PD deficiency with the detection limit of G6PD-intermediate individuals. The assay system helps reducing sample handling time. Therefore, the assay should benefit the screening for G6PD deficiency in population at risk of jaundice, hemolytic anemia and other diseases that G6PD deficiency could be a risk factor including malaria, diabetes and cancer.

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References

1. Cappellini MD, Fiorelli G. Glucose-6-phosphate dehydrogenase deficiency. Lancet. 2008;371(9606):64-74
2. Howes RE, Piel FB, Patil AP, Nyangiri OA, Gething PW, Dewi M, et al. G6PD deficiency prevalence and estimates of affected populations in malaria endemic countries: a geostatistical model-based map. PLoS Med. 2012;9(11):e1001339.
3. Thielemans L, Gornsawun G, Hanboonkunupakorn B, Paw MK, Porn P, Moo PK, et al. Diagnostic performance of the fluorescent spot test for G6PD deficiency in newborns along the Thailand-Myanmar border cohort study. Wellcome Open Research. 2018;3:1-11.
4. Bhutani VK. Kaplan M, Glader B, Cotton M, Kleinert J, Pamula V. Point-of-care quantitative measure of glucose-6-phosphate dehydrogenase enzyme deficiency. Pediatrics. 2015;136:e1268-75.
5. Nuchprayoon I, Sanpavat S, Nuchprayoon S. Glucose-6-phosphate dehydrogenase (G6PD) mutations in Thailand: G6PD Viangchan (871G>A) is the most common deficiency variant in the Thai population. Hum Mutat. 2002;19:185.
6. World Health Organization: Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group. *Bull World Health Organ.*1989;67(6):601–11.

7. LaRue N, Kahn M, Murray M, Leader BT, Bansil P, McGray S, et al. Comparison of quantitative and qualitative tests for glucose-6-phosphate dehydrogenase deficiency. Am. J. Trop. Med. Hyg. 2014;91:854-61.
8. Beutler E, Blume KG, Kaplan JC, Lohr GW, Ramot B, Valentine WN. International Committee for Standardization in Haematology: recommended screening test for glucose-6-phosphate dehydrogenase (G6-PD) deficiency. Brit J Haematol. 1979;43:465-7.
9. Nuchprayoon I, Louicharoen C, Charoenvej W. Glucose-6-phosphate dehydrogenase mutations in Mon and Burmese of southern Myanmar. J Hum Genet. 2008;53:48-54.
10. Huang CS, Hung KL, Huang Mj, Li YC, Liu TH, Tang TK. Neonatal jaundice and molecular mutations in glucose-6-phosphate dehydrogenase deficient newborn infants. Am J Hematol. 1996;51:19-25.
11. Domingo GJ, Satyagraha AW, Anvikar A, Baird K, Bancone G, Bansil P et al. G6PD testing in support of treatment and elimination of malaria: recommendations for evaluation of G6PD test. Malar J. 2013;12:391.
12. Ley B, Alam MS, O'Donnell JJ, Hossain MS, Kibria MG, Jahan N, et al. A comparison of tree quantitative methods to estimate G6PD activity in the Chittagong hill tracts, Bangladesh. Plos One. 2017;12:e0169930.
13. Fu C, Luo S, Li Q, Xie B, Yang Q, Geng G, et al. Newborn screening of glucose-6-phosphate dehydrogenase deficiency in Quangxi, China: determination of optimal cutoff value to identify heterozygous female neonates. Sci Rep. 2018;8:833.
14. Barcellini W, Fattizzo B. Clinical Applications of Hemolytic Markers in the Differential Diagnosis and Management of Hemolytic Anemia. Dis Markers. 2015;2015:1-7.

15. Nadarajan V, Shanmugam H, Sthaneshwar P, Jayaranee S, Sultan KS, Ang C, Arumugam S. Modification to reporting of qualitative fluorescent spot test results improves detection of glucose-6-phosphate dehydrogenase (G6PD)-deficient heterozygote female newborns. Int J Lab Hematol. 2011;33:463-470.
16. Samilchuk E, Al-Suliman I, Usanga E, Al Awadi S. Glucose-6-phosphate dehydrogenase (G6PD) mutations and UDP-glucuronosyltransferase promoter polymorphism among G6PD deficient Kuwaitis. Blood Cells Mol Dis. 2003;31:201-205.
17. Minucci A, Morasdkhani K, Hwang MJ, Zuppi C, Giardina B, Capoluongo E. Glucose-6-phosphate dehydrogenase (G6PD) mutations database: review of the "old" and update of the new mutations. Blood cells Mol Dis. 2012;48:154-65.
18. Zarza R, Pujades A, Rovira A, Saavedra R, Fernandez J, Aymerich M, et al. Two new mutations of the glucose-6-phosphate dehydrogenase (G6PD) gene associated with haemolytic anaemia: clinical biochemical and molecular relationships. Br J Haematol. 1997;98:578-82.
19. Nuchprayoon I, Louicharoen C, Charoenwej W. Glucose-6-phosphate dehydrogenase mutations in Mon and Burmese of southern Myanmar. J Hum Genet. 2008;53:48-54.
20. Nafa N, Reghis A, Osmani N, Baghli L, Benabadji M, Kaplan JC, et al. G6PD Aures: a new mutation (48Ile→Thr) causing mild G6PD deficiency is associated with favism. Hum Genet. 1993;2:81-2.
21. Daound BB, Mosbehi I, Prehu C, Chaouachi D, Hafsia R, Abbes S. Molecular characterization of erythrocyte glucose-6-phosphate dehydrogenase deficiency in Tunisia. Pathol Biol. 2008;56:260-7.

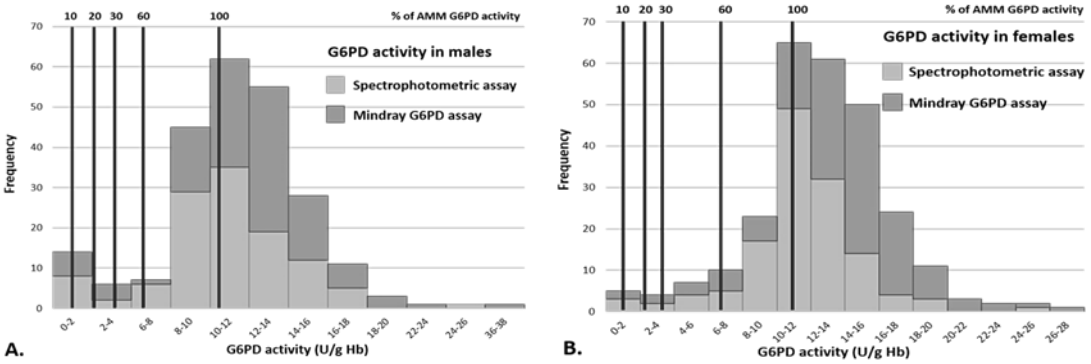

Figure 1 Comparative distribution of G6PD activities between spectrophotometric and Automated UV enzymatic assays in males (**A**) and females (**B**); 10%, 20%, 30%, 60%, and 100% of the adjusted median activity for males (**A**) and of normal activity for females (**B**) described in Table 2 are indicated on the graph.

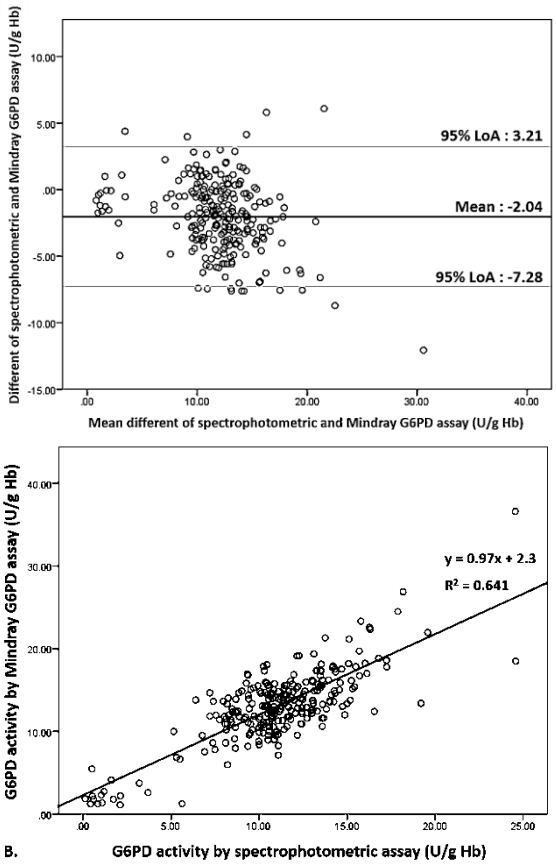

Figure 2 A. Tukey-mean difference plot (Bland-Altman plot) of spectrophotometric and Automated UV enzymatic methods. **B.** Correlation of G6PD activities measured by spectrophotometric and automated UV enzymatic methods with Pearson correlation coefficient is 0.80 (N = 251).

Table 1 G6PD activity reference values of spectrophotometric and automated UV enzymatic methods in the study population

Reference values (U/g Hb)	Spectrophotometric assay				Automated UV enzymatic method			
	Total (N = 251)	Female (N = 134)	Male (N = 117)	Adjusted male (N = 112)	Total (N = 251)	Female (N = 134)	Male (N = 117)	Adjusted male (N = 117)
Mean	11.0	11.4	10.4	10.9	13.0	13.9	12.0	12.0
SD	3.7	3.5	3.8	3.3	4.5	4.2	4.5	4.5
Median	10.9[*, **]	11.4[*, **]	10.7[*, **]	10.7[*, **]	13.1[*, **]	14.2[*, **]	12.2[*, **]	12.2[*, **]
IQR	3.7	3.4	3.7	3.4	4.2	4.2	3.6	3.6
Range	0.1-24.6	0.5-24.6	0.1-24.6	1.1-24.6	1.1-36.6	1.1-26.9	1.2-36.6	1.2-36.6

U: International Unit; Hb: hemoglobin

* Wilcoxon signed rank test between spectrophotometric and Automated UV enzymatic methods (p<0.0001)

** Pearson correlation test between spectrophotometric and Automated UV enzymatic methods (p<0.0001)

Table 2 Proportion of subjects with different G6PD activities determined by spectrophotometric and automated UV enzymatic methods

		Automated UV enzymatic method			
	G6PD activity	**Deficiency (<30%)** (M: <3.7 U/g Hb) (F: <4.3 U/g Hb)	**Intermediate (30-<60%)** (M: 3.7-<7.3 U/g Hb) (F: 4.3-<8.5 U/g Hb)	**Normal (>=60%)** (M: >=7.3 U/g Hb) (F: >=8.5 U/g Hb)	**Total**
Spectro-photometric assay	**Deficiency (<30%)** (M: <3.2 U/g Hb) (F: <3.4 U/g Hb)	**12** **M: 9** **F: 3**	2 M: 1 F: 1	0	14 M: 10 F: 4
	Intermediate (30-<60%) (M: 3.2-<6.4 U/g Hb) (F: 3.4-<6.8 U/g Hb)	2 F: 2	**2** **F: 2**	1 F: 1	5 F: 5
	Normal (>=60%) (M: >6.4 U/g Hb) (F: >6.8 U/g Hb)	0	4 F: 4	**228** **M: 107** **F: 121**	232 M: 111 F: 121
	Total	14 M: 9 F: 5	8 M: 1 F: 7	229 M: 107 F: 122	251 M: 117 F: 134
	Cohen's Kappa: 0.77 (95%CI: 0.70-0.83)				
Performance of Automated UV enzymatic method	**Sensitivity % (95% CI)**	**85.7** **(79.4-92.0)**	40.0 (25.2-54.8)	**98.3** **(97.7-98.9)**	
	Specificity % (95% CI)	**99.2** **(98.8-99.6)**	**97.6** **(96.9-98.2)**	**94.7** **(91.3-98.2)**	
	PPV % (95% CI)	**85.7** **(79.4-92.0)**	25.0 (14.7-35.3)	**99.6** **(99.3-99.9)**	
	NPV% (95% CI)	**99.2** **(98.8-99.6)**	**98.8** **(98.3-99.2)**	**81.8** **(76.3-87.4)**	

PPV: positive predictive value; NPV: negative predictive value

Table 3 Types of *G6PD* mutations in G6PD deficient and intermediate samples and their corresponding enzymatic activities determined by spectrophotometric assay

Mutation	Base substitution	Amino acid substitution	Class (14)	Zygosity	N	G6PD acitivity (U/g Hb)	
						Mean±SD	**Range**
G6PD Viangchan	871G>A	Val291Met	II	Hemizygote	4	1.4±0.7	0.4-2.1
				Heterozygote	5	5.4±2.9	0.5-8.2
				Homozygote	2	5.4±4.6	2.1-8.7
G6PD Mahidol	487G>A	Gly163Ser	III	Hemizygote	1	1.0	-
G6PD Union	1360C>T	Arg454Cys	II	Hemizygote	1	0.1	-
				Heterozygote	2	3.4±2.5	1.6-5.2
G6PD Valladolid	406C>T	Arg136Cys	II	Hemizygote	1	3.2	-
				Heterozygote	1	3.7	-
G6PD Aures	143T>C	Ile48Thr	III	Hemizygote	1	1.1	-
G6PD Kaiping	1388G>A	Arg463His	II	Hemizygote	1	0.6	-
G6PD Canton	1376G>T	Arg459Leu	II	Heterozygote	1	0.5	-
G6PD Chinese-5	1024C>T	Leu342Phe	III	Heterozygote	1	5.3	-
Unknown mutation	-	-		Male	1	0.8	-
				Female	1	11.1	-