Detection of BCL11A and HMIP Polymorphisms among Anemic Patients with Elevated HbF in Hospital Universiti Sains Malaysia

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Abstract
Anemia is a condition that is usually associated with a variety of diseases. For normal adults, the level of fetal hemoglobin (HbF) is less than 1.0%, which is influenced by several genetic loci.

Objectives: To determine the association between elevated HbF level and the presence of rs1186868 and rs9376090 polymorphism in anemic patients due to acquired causes.

Methods: This study involved 144 anemic patients with HbF level ≥1.0%. High-Performance Liquid Chromatography (HPLC) was used to determine the HbF and HbA2 level. Multiplex ARMS-PCR and Gap-PCR were performed for those samples with high HbA2 level (>3.2%) and normal HbA2 level (≤3.2%) to detect mutation and deletion at β-globin gene cluster, respectively. Allelic discrimination for rs1186868 and rs9376090 were performed using real-time PCR for samples with no mutation and deletion.

Results: The mean age of patients is 19.99±1.64 year with 61.1% female predominance. The majority of the patients were Malays (99.3%). There was a moderate negative correlation and statistically significant difference between HbF level and Hb level, (r=-0.348, P<0.05) while the correlation between HbF level with MCV and MCH showed weak negative correlation but was not statistically significant, (r=-0.079, P>0.05) (r=-0.072, P>0.05). The minor allele frequency (MAF) in both rs1186868 and rs9376090 for HbF>1.0% patients showed similarity with East Asian (EAS) population.

Conclusion: This finding shows the presence of rs1186868 and rs9376090 SNPs in Malaysian population and emphasising the need for further functional studies to confirm the association between these SNPs with HbF hence, could provide better approach in management of anemic patients.

Keywords: Acquired anemia, SNPs genotyping, allele frequency.

Introduction
Anemia is a global public health problem that affects both developing and developed countries. Anemia is usually associated with a variety of diseases. There are several factors for anemia that are associated with elevated level of fetal hemoglobin (HbF). HbF level is increased in inherited conditions, such as in hereditary persistence of HbF (HPFH), hereditary spherocytosis, sickle cell crisis, and thalassemia.

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e carried out using the automated
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maturation pathway. Previous study has shown
ontogenesis as well as erythropoiesis [13].

BCL11A
Xmn1
-2021
Vol
HMIP
-2021
HMIP
http://

Another QTL regulating HbF levels is
HMIP which codes elongation factors and regulates
multiple cellular processes and MYB gene which
encodes transcription factors, and participates in
ontogenesis as well as erythropoiesis [13].

HMIP is known to participate in the erythroid-
maturation pathway. Previous study has shown

that there was a correlation between HMIP and
HbF level in Indian β-thalassemia patients [13].
In another study, Indian female population with
β-thalassemia was found to have higher HbF
concentration in homozygous HMIP (24%) compared to non-homozygous HMIP (10%) [14]. Similar correlation was observed among
the healthy population in the same study which
showed that HbF level in homozygous HMIP is
3% compared to 1% in non-homozygous HMIP
[9]. Therefore, this study aimed to determine the
association between elevated HbF level with
hematological parameters and the presence of
the BCL11A (rs1186868) and HMIP (rs9376090) single nucleotide polymorphisms (SNPs) in anemic patients due to acquired
causes.

Methods
Patient recruitment and sample collection

A cross-sectional study was carried out at Hospital Universiti Sains Malaysia (USM), by
recruiting 144 subjects with anemia and elevated
HbF (>1.0%). Peripheral blood samples were
taken after informed written consents were
obtained from participating patients. This study
was approved by the Human Research Ethics
Committee, Universiti Sains Malaysia (USM/ JEPeM/ 16090283). All blood samples were
collected into blood collection tube containing
ethylenediaminetetraacetic acid (EDTA).

Routine full blood counts (FBCs) and Hb
analysis were carried out on all samples. The
FBCs were carried out using the automated
hematology analyzer (Sysmex XN-1000™,
USA). The Hb analyses were performed using
cation exchange high performance liquid
chromatography (CE-HPLC) (Bio-rad Variant II
System, USA). Subjects were considered when
red cell indices were below normal value as
reported by previous study [15].

Multiplex Amplification Refractory Mutation
System (ARMS)-PCR and Gap-PCR

Genomic DNA was extracted from peripheral
blood using DNA Blood Kit (Macherey Nagel,
Germany) according to the manufacturer’s
instructions. Multiplex ARMS-PCR and Gap-
PCR were performed for those samples with

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high HbA₂ level (>3.2%) and normal HbA₂ level (≤3.2%) to detect mutation and deletion at β-globin gene cluster, respectively [16]. Multiplex ARMS-PCR is used to detect β-globin gene mutations which include IVS 1–5, Cd 41/42, Cd 17, Cd 26, IVS 1-1 (G>T), Cd 8/9 and ~28 mutations [17]. Meanwhile multiplex Gap-PCR detect Siriraj J GY (γαΔβ)²-thalassemia (~118 kb deletion), Thai (δβ)²-thalassemia (~12.5 kb deletion), HPFH-6 and Hb Lepore [18]. All these primers were commercially purchased from Integrated DNA Technologies (Malaysia). All amplifications were carried out using 96-Well Thermal Cycler (Applied Biosystem, USA). Electrophoresis of PCR products was carried out on agarose gel pre-stained with Florosafe DNA Stain (Apical Scientific, Malaysia), and the results were observed under ultraviolet light. Allelic discrimination for rs1186868 and rs9376090 were performed using real-time PCR on samples from multiplex ARMS-PCR and Gap-PCR with no mutation and deletion.

Single Nucleotide Polymorphisms (SNPs) genotyping

The samples with no mutation and deletion from multiplex ARMS-PCR and Gap-PCR were further analyzed using TaqMan allelic discrimination to characterize single nucleotide polymorphisms (SNPs). Briefly, ready-made TaqMan SNP Genotyping Assay (Taqman MGB probes, FAM and VIC dye-labelled) (Thermofisher Scientific, USA) was used. Subjects were genotyped for rs1186868 polymorphism (G>A) and rs9376090 polymorphism (T>C) by using CFX96 real-time PCR (Bio Rad Laboratories, USA). Allelic discrimination analysis was performed using CFX96 Manager Software (Bio Rad Laboratories, USA) [19].

Statistical analyses

Qualitative data of populations was expressed as frequency and percentage while quantitative data on the red blood cell parameters such as Hb, RBC, MCV, MCH, HbF and HbA₂ level of patients with HbF more than 1.0% was expressed as mean, ± SEM and median. Chi square and odds ratio were determined by Prism 7.0 software (GraphPad, USA) to evaluate the allele associations. The Mann-Whitney U test was used to compare the data between two groups. A P-value less than 0.05 is statistically significant.

Results

The mean age of 144 patients is 19.99±1.64 years with female predominance which was 61.1% compared to male (38.9%). The Majority of patients were Malays (99.3%). Summarized data on the hematological profiles which include MCV, MCH and quantifications of HbA₂ and HbF level are shown in Table 1. Table 2 shows the correlation coefficients (r) between HbF and hematological parameters including red blood cells count (RBC), hemoglobin level (Hb), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH).

From 144 anemic samples with HbF>1.0%, 87 samples were analyzed using multiplex ARMS-PCR. As a result, 65 (74.7%) mutation detected which comprises of 49 heterozygous Cd 26 (%), 10 heterozygous Cd 41/42 (%), 3 compound heterozygous Cd 26 and Cd 41/42 (%) and 3 heterozygous IVS 1–1 (%), while 22 patients were not detected with any β-globin mutations. Meanwhile, 57 samples which were analyzed using multiplex Gap-PCR, only 1 (1.8%) mutation was detected which was the δβ-Thai deletion while 56 samples did not show any mutations.

We have successfully performed allelic discrimination using real-time PCR for rs1186868 polymorphism (G>A) and rs9376090 polymorphism (C>T) which are located in the BCL11A gene and the HMIP respectively. Allelic discrimination plot shows the horizontal axis as allele 1 while the vertical axis as allele 2 labelled with FAM dye and VIC dye respectively. Figure 1 shows the allelic discrimination plot for representative samples and two no template controls (NTCs) for rs9376090 polymorphism. Homozygous TT genotypes were auto assigned to samples containing allele 1. Samples containing allele 2 were genotyped as homozygous CC. Heterozygous genotypes were assigned to samples containing both allele 1 and allele 2 (TC).

In this study, the frequency of HbF-
promoting alleles across the two loci is similar from that reported for South East Asian as shown in Table 3. The minor allele frequency (MAF) for rs1186868 and rs93760890 obtained from this study were compared to other populations including East Asian (EAS), African (AFR), American (AMR), European (EUR) as well as South Asian (SAS). This minor allele frequency (MAF) in both rs1186868 and rs93760890 shows similar with East Asian (EAS) population. The comparison of HbF levels between genotypes are tabulated in Table 4. In this study, there was no significant difference of HbF level between genotypes containing the minor allele of rs9376090 (TC and CC) when compared to genotype TT (P > 0.05).

Discussion
Analyses of the hematological data on the study subjects showed that the mean for MCV was 68.72 ± 0.83 fL and MCH was 21.81 ± 0.30 pg which were relatively low compared to the normal range. The mean of HbF was 4.6 ± 9.6% with the observed range was from 1.1 to 68.1%. HbA2 displayed a mean value of 5.51 ± 0.85%, which further from the standard normal range. Data obtained from our study were similar to those previously reported on low mean values of MCV and MCH observed in HPFH-6 and δβ-thalassemia Thais patients, together with high HbF (>5%), but normal HbA2 level (2.2%) [20]. People with blood disorders related to an elevated HbF level may not have symptoms but may have varying degrees of anemia [21]. Samples with high HbF > 1.0%, microcytosis (MCV < 80 fL) and/or hypochromia (MCH < 27 pg) could be an indicator for thalassemia or other genetic modifier such as SNPs [1]. The allele-specific real-time PCR method was found to be an accurate method for SNPs genotyping and can identify germline mutants through either threshold cycle (Ct) or end-point fluorescence reading. The allele-specific qPCR utilised allele-specific primers, a locus-specific reverse primer, universal fluorescent probes and quenchers, and hot start DNA polymerase [24]. By Using the allele-specific real-time PCR technique, we were able to determine the genotypes of the samples based on relative fluorescence units (RFU) that employs fluorescence detection and quantitation cycle (Cq) value. CFX Manager software automatically sets the threshold lines for discriminating alleles by the value of Cq or RFU.

Allele A of rs1186868 was associated with increased HbF levels and genotypes containing the minor allele exhibited significantly higher HbF levels. In addition, only 5% of the world population genetic was reported to have A allele at rs1186868 [25]. In this study, the distribution of the rs1186868 allele was similar to Ensembl databases which reported that East Asian (EAS) population does not have allele A. However, more samples are required for identification of allele A of rs1186868 in a wider population.

Allele C of rs9376090 was associated with increased HbF levels and genotypes containing the minor allele exhibited significantly higher HbF levels. In this study, the HbF-promoting ‘C’ allele frequency of rs9376090 is 0.25 which was similar to East Asian (EAS) population. In addition, 15% of the world population genetics and 25% of the South East Asia population were reported to have C allele at rs9376090 [26].

Conclusion
In conclusion, this is the first study on SNPs analysis, specifically the BCL11A and HMIP
polymorphisms among anemic patients with elevated HbF in Kelantan. Although data collection could be a challenge in a multi-ethnic country like Malaysia, by using our method, the genotyping is not impossible. Furthermore, data obtained from this study could add more knowledge that can be used as a guide for better treatment and management of anemic patients.

**Acknowledgements**

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**Authors’ disclosures of potential conflicts of interest**

The authors declare no conflict of interest.

**Tables and Figure**

**Table 1:** Summary of the hematological profiles of patients

<table>
<thead>
<tr>
<th>Red Cell indices (Unit)</th>
<th>Range</th>
<th>Mean ± SEM</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>4.1-15.1</td>
<td>10.22 ± 0.19</td>
<td>10.6</td>
</tr>
<tr>
<td>RBC</td>
<td>1.86-7.97</td>
<td>4.74 ± 0.08</td>
<td>4.91</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>48.2-95.4</td>
<td>68.72 ± 0.83</td>
<td>69.1</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>14.7-31.7</td>
<td>21.81 ± 0.30</td>
<td>21.8</td>
</tr>
<tr>
<td>HbF (%)</td>
<td>1.1-68.1</td>
<td>5.51 ± 0.85</td>
<td>2.20</td>
</tr>
<tr>
<td>HbA2 (%)</td>
<td>1.1-87.5</td>
<td>15.72 ± 1.78</td>
<td>4.85</td>
</tr>
</tbody>
</table>

Abbreviations: Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; g/dL, gram per deciliter; fL, femtoliter; pg, picogram; %, percentage

**Table 2:** Pearson correlation coefficients (r) between HbF and hematological parameters RBC, Hb level, MCV and MCH

<table>
<thead>
<tr>
<th></th>
<th>HbF</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>r</td>
<td>P-value</td>
</tr>
<tr>
<td>RBC</td>
<td>-0.3767</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>-0.3480</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>-0.0794</td>
<td>0.3440</td>
<td></td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>-0.0726</td>
<td>0.3870</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: RBC, red blood cells count; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin
Table 3: Distribution of allelic frequencies of SNPs

<table>
<thead>
<tr>
<th>SNPs (Position)</th>
<th>Genotype frequency (%)</th>
<th>Observed Allele Frequency</th>
<th>Minor allele frequency of each population*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EAS</td>
<td>AFR</td>
</tr>
<tr>
<td>rs1186868 G&gt;A</td>
<td>100.0 (GG)</td>
<td>0.00</td>
<td>0.113</td>
</tr>
<tr>
<td>(2p16)</td>
<td>0 (GA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (AA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs9376090 T&gt;C</td>
<td>56.4 (TT)</td>
<td>0.246</td>
<td>0.015</td>
</tr>
<tr>
<td>(6q23)</td>
<td>37.2 (TC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.4 (CC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.750/0.250</td>
<td></td>
<td></td>
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</tbody>
</table>

*Population for minor allele frequency comparison were the East Asian (EAS), African (AFR), American (AMR), European (EUR), South Asian (SAS) [25,26].

Table 4: Comparison of HbF levels between genotypes

<table>
<thead>
<tr>
<th>Locus</th>
<th>SNP/genotypes</th>
<th>N</th>
<th>Mean HbF level (%)</th>
<th>Mean rank</th>
<th>Mann–Whitney U</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL11A</td>
<td>rs1186868</td>
<td>78</td>
<td>3.13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td></td>
<td>3.13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GA + AA</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HMIP</td>
<td>rs9376090</td>
<td>44</td>
<td>3.46</td>
<td>41.98</td>
<td>639.5</td>
<td>0.276</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td></td>
<td>3.46</td>
<td>41.98</td>
<td>639.5</td>
<td>0.276</td>
</tr>
<tr>
<td></td>
<td>TC + CC</td>
<td>34</td>
<td>2.71</td>
<td>36.32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Allelic discrimination real-time PCR genotyping of rs9376090. Genotype clusters TT (circle), CC (square), TC (triangle) and NTCs (diamond) are distant from each cluster.

Abbreviations: NTCs; no template control
References


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الكشف عن (868668) و (rs9376090) BCL11A و (rs1186868) HMIP في مستشفى جامعة العلوم الطبية الماليزية

فقر الدم الذين يعانون من ارتفاع HbF

سيتي نور اشهدا مت غني، روزياتي محمد صالح، سويتانا وان عبادر الرحمن، محمد نزري حسن، زهيد عبد الله، زهيد ذو الكفلي، مريم ازل، زفرينى ذو الكفلي

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الملخص

الدم هو حالة ترتبط عادة بمجموعة متنوعة من الأمراض، بالنسبة للبالغين العاديين، ويتون مستوى الهيموجلوبين الجنيني (HbF) أقل من 1% الذي يتتأثر بالعديد من المواضع الجينية. والهدف: تحديد العلاقة بين ارتفاع مستوى الهيموجلوبين السكري ووجود تعدد الأشكال rs1186868 و rs9376090 في مرضى فقر الدم لأسباب متتسلبة.

الطريقة: شملت هذه الدراسة 011 مريضاً بفقر الدم بمستوى HbF 1.0% و HbA2 > 2.3% و HbA2 ≤ 2.3% للتشخيص بنظام HPLC و PCR و Multiplex ARMS - PCR. تم إجراء التمييز الأليلي لـ rs1186868 و rs9376090 باستخدام PCR RT-PCR و PCR و 함께ً في مجموعة الجينات HbA2 و HbF و HbA2 و HbF لتحديد مستوى HbA2 و HbF في مجموعة الأشخاص المختبرين، و يمكن ملاحظة اختلاف من المرضى بين مجموعة العمليات (EAS)، و تخصيص هذه النتائج و HbF من المرضى و SNPs في السكان الماليزيين و تأكيد على الحاجة إلى زيادة من الدراسات الوظيفية لتأكيد الارتباط بين هذه النيوكولوتايد مع HbF و تأكيد النهج أفضل في إدارة مرضى فقر الدم.