ORIGINAL ARTICLE

# *LARP2* DNA Methylation in Transfusion-dependent Haemoglobin E-Beta (HbE/β) and β-Thalassaemia Major Patients

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**ABSTRACT**

**Introduction:** The large clinical spectrum of haemoglobin E-beta (HbE/β) thalassaemia leads to the investigation of complex mechanisms involved in erythropoiesis. DNA methylation in *LARP2* is one of the potential epigenetic modifiers not fully explored in HbE/β and β-thalassaemia major. This study aimed to analyse DNA methylation profile and gene expression of *LARP2* using peripheral blood (PB) in nucleated red blood cells (NRBCs) for the source of DNA of HbE/β- and β-thalassaemia major patients. **Methods:** PB were collected from 33 transfusion-dependent thalassemia patients from Hospital USM and Hospital RPZII, Kelantan, Malaysia. DNA methylation profile and gene expression of *LARP2* were examined by bisulphite sequencing PCR and quantitative real-time PCR respectively. **Results:** Partial DNA methylation of *LARP2* was observed in 43% (9/21) HbE/β- and 17% (2/12) β-thalassaemia major patients. *LARP2* expression (1.49±26.60) in HbE/β-thalassaemia was not significant against normal controls and β-thalassaemia major ($p>0.05$). In contrast, *LARP2* expression (6.8±16.42) in β-thalassaemia major showed a significant up-regulation against normal controls ($p<0.05$). The association of *LARP2* expression and DNA methylation profile was statistically significant ($p<0.001$). *LARP2* expression was down-regulated in 75% (3/4) HbE/β-thalassaemia patients with CD26/IVS1-5, in contrast to up-regulation of 80% (4/5) IVS1-5/IVS1-5 β-thalassaemia major patients. DNA methylation of *LARP2* in these patients were either partially methylated or unmethylated in CD26/IVS1-5 and IVS1-5/IVS1-5 respectively. **Conclusion:** DNA methylation of *LARP2* may act as an additional modifier to gene mutation especially involving IVS1-5 in HbE/β-thalassaemia. Homozygous IVS1-5 in β-thalassaemia major may contribute to different disease presentation compared to those involving CD26 in HbE/β-thalassaemia.

**Keywords:** HbE/β, thalassaemia, methylation, *LARP2*, IVS1-5

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## INTRODUCTION

HbE/β-thalassaemia is an inherited haemoglobin disorder that is the most prevalent severe form of β-thalassaemia in Southeast Asia including Malaysia (1, 2). HbE/β-thalassaemia is a combination of β-thalassaemia, a quantitative defect in β-globin chain synthesis and the structural variant, HbE, point mutation G>A substitution at codon 26 in exon 1 of the β-globin gene (HBB:c.79G>A). In contrast to β-thalassaemia major, the clinical manifestations of HbE/β-thalassaemia are more heterogeneous (3, 4).

The large clinical spectrum of HbE/β-thalassaemia, ranging from mild anaemia to severe manifestations remains unexplained with several data suggesting primary, secondary and tertiary genetic modifiers. These genetic modifiers include the types of HBB mutation, co-inheritance of Alpha (α)-thalassaemia, regulation of fetal haemoglobin (HbF), Xmn1-Gγ polymorphism and other genetic and environmental factors (5, 6). Lack of pathophysiology understanding of the disease caused high economic burden to the country as thalassaemia management requires lifelong blood transfusion and iron chelation therapy (7). HbE/β-thalassaemia is the second highest cases (33%) of transfusion-dependent (TDT) thalassaemia from the data of 4678 registered cases in Malaysian registry not far from the β-thalassaemia major (38%) TDT (Malaysian Thalassaemia Registry, 2015).

Epigenetic is one of the potential modifiers that may involve in contributing to the large clinical spectrum of HbE/β-thalassaemia patients. Epigenetic is the process by which a phenotype is modified without changes in genotype or DNA sequence. DNA methylation is one of the epigenetic mechanism that has been reported to play a role in regulating the activation and switching of globin gene transcription during the globin chain synthesis (8, 9). Gene expression of HBB particularly with CD26

mutation could be altered by DNA methylation and may result in the modification of the disease severity. Thus, DNA methylation has the potential to promote silencing or overexpression of the gene expressions resulting in abnormal genes regulation (10, 11).

La ribonucleoprotein domain family, member 1 gene (*LARP2*) is one of the significant components of ribonucleoprotein complexes. *LARP2* acts as RNA binding protein (RBP), mediating the correct process of RNA polymerase III maturation that is essential for gene transcription. It also functions in initiating the process of protein translation. *LARP2* was reported to interact with genes in the regulation network of globin expression and hypermethylation of *LARP2* disrupts the synthesis of globin chains (12, 13).

To study the role of *LARP2* DNA methylation in regulating the *LARP2* function is hindered since erythrocytes as the target source of DNA are mostly anucleate in peripheral blood (PB) due to enucleation process during erythropoiesis in marrow. Due to ineffective erythropoiesis in thalassemia major, nucleated red blood cells (NRBCs) are commonly found in the PB of the patients. Therefore, DNA sources from these circulating nucleated DNA can be used to analyse the *LARP2* DNA methylation profiles and to observe the relationship between *LARP2* DNA methylation with the degree of the gene expression.

## MATERIALS AND METHODS

### Blood sample collection and preparation

PB were collected from 33 TDT thalassaemia patients recruited either from Klinik Pakar Perubatan (KPP) or Paediatric Day Care, Hospital USM and Medical Day Care, Hospital RPZII after obtaining informed consent. The haematological parameters were taken before patients undergone blood transfusion. These patients were screened for β-thalassaemia and β-thalassaemia mutation in our previous study (14) with no co-inheritance of β-thalassaemia reported. The approval and ethical clearance were obtained from the Human Research Ethics Committee, Universiti Sains Malaysia (USM/JEPeM/17100421) and Medical Research Ethics Committee, Ministry of Health, Malaysia [NMRR-12-980-13829 (IIR)].

### Isolation of Nucleated Red Blood Cells (NRBCs)

PB samples were diluted (1:2) with phosphate buffer saline (PBS) (Gibco, CA, USA), layered on lymphoprep (Alere Technologies AS, Oslo, Norway) and separated through density gradient centrifugation at 600 x g for 20 mins. RBCs sediment at the bottom of the tube was saved for reticulocyte enrichment for gene expression analysis. PBMCs suspended at the middle layer between plasma and lymphoprep were collected for the isolation of NRBCs by magnetic-activated cell sorting (MACS) for DNA methylation study. NRBCs were tagged with anti-CD71 microbeads (Macs Miltenyl Biotec, CA, USA) and validated by BD FACSCantoTM II flow cytometry (Becton-Dickson, NJ, USA) as described previously (15).

### DNA extraction and bisuphite modification

DNA was extracted from isolated NRBCs using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The concentration of DNA was quantified by NanoDrop spectrophotometer ND-1000 (Thermo Scientific, MA, USA) with ranged of 1.7-1.9 ratio at A260/280 is considered high purity of DNA. The DNA samples were subsequently subjected to bisulphite modification following the manufacturer's protocol (EZ DNA methylation-gold Kit, Zymo Research, CA, USA).

### Bisulphite sequencing PCR (BSP)

Bisulphite modified DNA was used to analyse the DNA methylation profile of the promoter region of *LARP2* in HbE/β- and β-thalassaemia patients. *LARP2* primers; 5′ GGGGTAAAGGTTTTTTAAATAGTTAT 3′ (forward) and 5′ CCCTTAAACCCTAAAACTAAATC 3′ (reverse) were designed using MethPrimer software (http://www.urogene.org/methprimer). Commercialized human methylated and non-methylated DNA (Zymo Research, CA, USA) subjected to bisulphite modification were used as positive and negative controls respectively. Amplification of BSP was performed in 25 µL final volume comprising of 50 ng bisulphite-converted DNA, 1X MyTaq Red Mix (Bioline Ltd, London, UK), 0.8 µM forward and reverse primers and nuclease-free water in TC-5000 thermocycler (Techne, MN, USA). The reaction conditions were; initial denaturation at 95°C for 60 sec, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 59°C for 15 sec and extension at 72°C for 10 sec, with a final extension at 72°C for 5 min. Amplicons were electrophoresed on 2.5% agarose gels, and visualized by ethidium bromide staining under ultraviolet transillumination for confirmation. The CpG sites methylation was confirmed by automated DNA sequencing. The BSP amplicons were purified using GeneAll ExpinTM (GeneALL Biotechnology, Seoul, Kr) and sequenced on an automated DNA sequence analyzer (MWG, Ebersberg, Germany). Sequences were compared with wild-type sequence, viewed and analysed in Finch TV software Version 1.4.0 (Geospiza, Inc., USA) and EMBOSS Needle (Pairwise Sequence Alignment (Nucleotide) (EMBL-EBI, Cambridgeshire, UK).

### Reticulocyte enrichment

The saved RBCs sediment from density centrifugation was diluted with 9 mL PBS (Gibco, CA, USA) and loaded into a 10 mL syringe layered with mixed cellulose (1g sigmacell cellulose type 50, 50 µM (Sigma-Aldrich, MO, USA), α-cellulose powder (Sigma-Aldrich, MO, USA) and 100 mL PBS (Gibco, CA, USA)) for reticulocytes enrichment. The cells were centrifuged at 3000 rpm for 10 min at 4°C and homogenized with 1 mL TRIzol®

reagent (Invitrogen, Thermo Fisher Scientific, USA). The enriched reticulocytes were stored at -80°C before used for RNA extraction.

### RNA extraction and cDNA synthesis

RNA was extracted from the enriched reticulocytes using TRIzolTM Reagent (Invitrogen, Thermo Fisher Scientific, USA) and concentrated by isopropanol precipitation following the manufacturer's protocol. RNA was quantified by NanoDrop spectrophotometer ND-1000 (Thermo Scientific, MA, USA) and the purity at A260/280 ratio ranged from 1.8 to 2.2. One microgram of total RNA was used to synthesize cDNA using SensiFASTTM cDNA synthesis kit (Bioline Ltd, London, UK). The other components for cDNA synthesis include 4 µL 5x TransAmp buffer, 1 µL reverse transcriptase and 5 µL nuclease-free water. The thermocycler was set-up for primer annealing at 25°C for 10 sec, reverse transcription at 42°C for 15 sec and inactivation at 85°C for 5 sec.

### Quantitative real-time PCR (qPCR)

The cDNA was subjected for the quantitative real-time (qPCR) amplification using SYBR Green QuestTaq qPCR PreMix (Zymo Research, CA, USA). The qPCR amplification was performed in Stratagene Mx3000P (Thermo Scientific, MA, USA) with reaction conditions; initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 7 min. The reactions were performed in triplicates. The primers used for this assay were as described (7) (Table I). Relative gene expression of *LARP2* was calculated by $2^{-\Delta\Delta CT}$ method ($\Delta\Delta CT=\Delta CT_{Thalassaemia}-\Delta CT_{Normal}$, $\Delta CT=Ct_{LARP2}-Ct_{\beta\text{-actin}}$).

### Statistical analysis

Data were analysed using SPSS version 22 (SPSS Inc., Chicago, IL). Statistical significance was determined at $p\leq0.05$. Descriptive analysis was reported in percentages, means (SD) for normal distribution data while in non-normal distribution data, median (IQR) were reported. Fisher's exact test and Mann-Whitney test were used to compare the DNA methylation profile and gene expressions of *LARP2* between the two groups of patients respectively.

**Table I: Primers for qPCR**

| Gene | Primer sequence (5′→ 3′) | Product size (bp) | Reference |
|---|---|---|---|
| *LARP2*-Forward | CACAATCCAAT-TACCCCACT | 361 | (Gao *et al.*, 2012a) (12) |
| *LARP2*-Reverse | GCAGGTTTTA-AATTACTCGTG | | |
| β-*actin*-Forward | TGACAGGATC-GAGAAGGAGA | 75 | |
| β-*actin*-Reverse | CGCTCAGGAG-GAGCAATG | | |

## RESULTS

### Patients' demographic

Subjects comprised of 21 HbE/β-thalassaemia and 12 β-thalassaemia major (18 males and 15 females; 31 Malays and 2 Chinese; 9-39 years old). All patients were not infected with hepatitis C nor HIV and 12 were splenectomised. Patients' demographic showed that the patients were among the same cohort with no significant difference in the haematological and clinical findings (Table II). The mutations previously found within the patients were comparable in both HbE/β- and β-thalassaemia major groups. There were seven CD26/IVS1-1 and three homozygous IVS1-1, four CD26/IVS1-5 and five homozygous IVS1-5, five CD26/CD41/42 and three heterozygous IVS1-5/CD41/42, and three CD26/CD19 and one heterozygous CD19 patients.

### DNA methylation in HbE/β- and β-thalassaemia major

Eighteen CpGs were analysed for both groups of patients at *LARP2* promoter region. Fig. 1 demonstrates the BSP results of 8/18 CpG sites. In methylated control, BSP showed methylation of all CpG dinucleotides by lack of conversion of cytosines within the amplified region. In contrast, unmethylated control and fully unmethylated patients contained converted cytosine molecules (to tyrosine) at CpG dinucleotides. In partially methylated patients, converted cytosine were seen together with methylation of CpG dinucleotides. The overall *LARP2* methylation profile of HbE/β- and β-thalassaemia major patients were summarized in Fig. 2. Partial methylation was identified in 43% (9/21) of HbE/β- and 17% (2/12) of β-thalassaemia major patients with CpGs 3 and 5 at the highest frequency. Five from the 7 subjects of CD26/IVS1-1 and all four CD26/IVS1-5 HbE/β-thalassaemia patients together with one of five IVS1-5/IVS1-5 and one of three IVS1-1/IVS1-1 β-thalassaemia major patients were partially methylated in at least two CpGs at the promoter region of *LARP2*. *LARP2* promoter region was fully unmethylated in 57% (12/21) HbE/β- and 83% (10/12) β-thalassaemia major patients with CpGs 1, 2, 4, 7, 10, 11, 12, 14, 15 and 16 at the highest frequency. Two from the 7 subjects of CD26/IVS1-1, all five CD26/CD41/42, all three CD26/CD19 and all two CD26/CD8/9 HbE/β-thalassaemia patients together with four of five IVS1-5/IVS1-5, two of three IVS1-1/IVS1-1, all three IVS1-5/CD41/42 and one IVS1-5/CD19 β-thalassaemia major patients were fully unmethylated at the promoter region of *LARP2*.

### Association between DNA methylation and expression of *LARP2*

*LARP2* expression (1.49±26.60) in HbE/β-thalassaemia was not significant against normal controls and β-thalassaemia major patients ($p>0.05$). In contrast, *LARP2* expression (6.8±16.42) in β-thalassaemia major showed a significant up-regulation against the normal controls ($p<0.05$) (Fig.3). *LARP2* expressions between fully unmethylated (16.15±13.98) and partially

**Table II: Patients' demographic and clinical data**

| Variables | Types of Thalassaemia n (%) | | Reference Range | Z statistics/ (df)[b] | *p*-value* |
|---|---|---|---|---|---|
| | HbE/β-thalassaemia 21 (64) Mean (SD)[a] | β-thalassaemia major 12 (36) Mean (SD)[a] | | | |
| **Gender, n (%)** | | | | | |
| Male | 11 (52.4) | 7 (58.3) | | | |
| Female | 10 (47.6) | 5 (41.7) | | | |
| **Age, mean (SD)** | 19 (12.35) | 17(8.75) | | | |
| **Races, n (%)** | | | | | |
| Malay | 19 (90.5) | 12 (100) | | | |
| Chinese | 2 (9.5) | 0 (0) | | | |
| **Splenectomized, n (%)** | | | | | |
| Yes | 6 (28.6) | 6 (50) | | | |
| No | 15 (71.4) | 6 (50) | | | |
| ***β-mutation, n (%)*** | | | | | |
| CD26/IVS1-1 | 7 (33.3) | - | | | |
| CD26/CD41/42 | 5 (23.8) | - | | | |
| CD26/IVS1-5 | 4 (19.0) | - | | | |
| CD26/CD19 (A-G) | 3 (14.3) | - | | | |
| CD26/CD8/9 | 2 (9.5) | - | | | |
| IVS1-5/IVS1-5 | - | 5 (41.7) | | | |
| IVS1-5/CD41/42 | - | 3 (25.0) | | | |
| IVS1-1/IVS1-1 | - | 3 (25.0) | | | |
| IVS1-5/CD19(A-G) | - | 1 (8.3) | | | |
| **Clinical data** | | | | | |
| Hb (g/dL)[a] | M 7.7 (1.84)<br>F 8.1 (0.77) | M 7.6 (1.59)<br>F 7.4 (1.47) | M 13.5-17.4<br>F 11.6-15.1 | -1.667 | 0.096 |
| MCV (fL)[a] | M 72.4 (6.32)<br>F 72.0 (4.83) | M 69.0 (3.99)<br>F 74.0 (3.96) | M 78.90-95.74<br>F 77.49-94.49 | 0.105 (31)[b] | 0.917 |
| MCH (pg)[a] | M 22.9 (2.14)<br>F 22.9 (2.16) | M 20.5 (1.39)<br>F 23.8 (2.21) | M 25.38-31.10<br>F 24.75-31.23 | 1.310 (31)[b] | 0.200 |
| NRBCs ($10^3$/uL)[a] | 6.7 (15.67) | 17.1 (19.34) | 0.0 | -2.545 | 0.011* |
| Reticulocytes (%)[a] | 3.8 (4.66) | 6.0 (6.19) | 0.4-1.6 | -2.134 | 0.033* |

Source: Reference range <60 years old, adopted from (Ambayya *et al.*, 2014).
MCV, Mean corpuscular volume; MCH, Mean corpuscular haemoglobin; NRBCs, Nucleated red blood cells.
*Mann-Whitney test, *Independent *t* test[a].

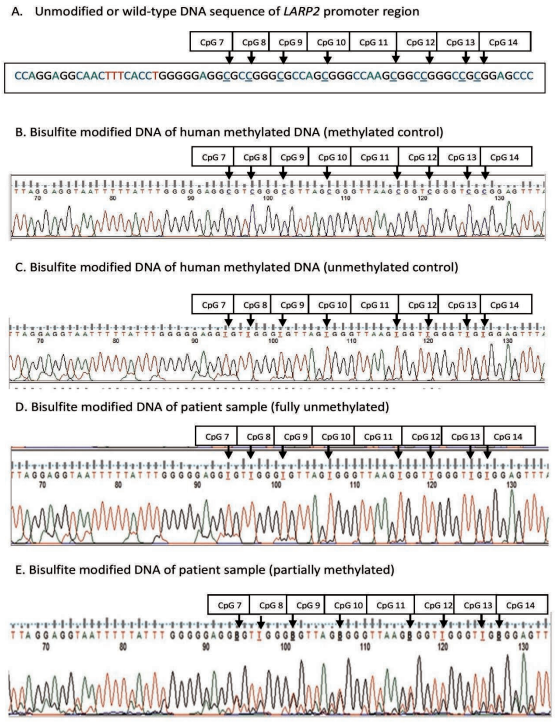


**Figure 1: Representative of BSP result analysing 8 out of 18 CpG sites at promoter region of *LARP2* (arrows).** (A). Unmodified or wild-type DNA sequence of *LARP2*, nucleotides -125 to -55 (Accession number NM_018078.3). (B). Modified DNA of methylated control showed all cytosine converted to thymine except in CpG sites which remained as cytosine (underlined in blue). (C). Modified DNA of unmethylated control showed all cytosine converted to thymine (underlined in red). (D). Modified DNA of selected β-thalassaemia major patient representing fully unmethylation showed all cytosine converted to thymine (underlined in red). (E). Modified DNA of selected HbE/β-thalassaemia patient representing partial methylation showed all cytosine converted to thymine except in CpGs 7, 9, 10, 11, and 14 with double peaks of cytosine and thymine (underlined in black).

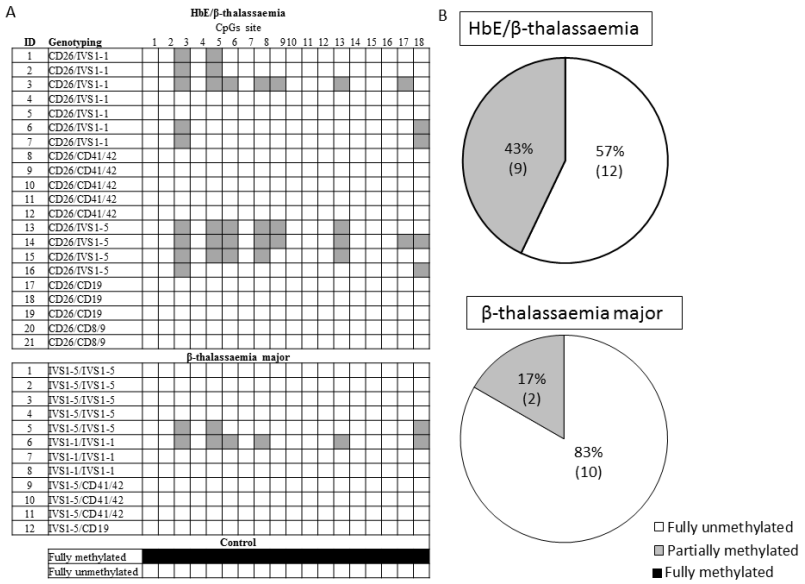


**Figure 2:** (A). DNA methylation profile of 18 CpG sites at promoter region of LARP2 in HbE/β- and β-thalassaemia major patients. (B). Percentage of *LARP2* DNA methylation in HbE/β- and β-thalassaemia major patients, Fisher's Exact Test ($p>0.05$). (White: unmethylated; gray: partially methylated, black: methylated).

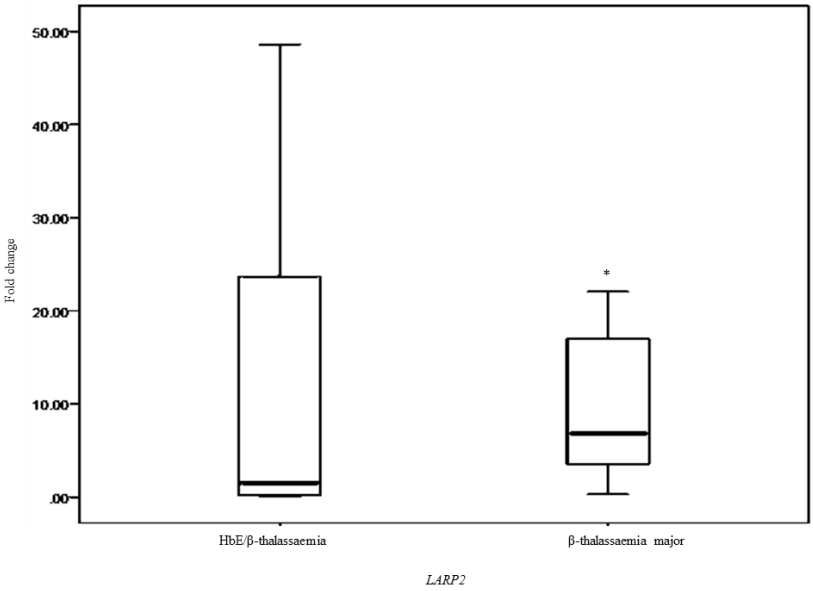


**Figure 3: Mean fold change of *LARP2* expression of 21 HbE/β (1.49±26.60) and 12 β-thalassaemia major patients (6.8±16.42) examined by qPCR against normal control normalised with β-actin.** Error bars represent IQR (Mann-Whitney test *, $p<0.05$).

methylated (0.57±0.49) profiles were statistically significant ($p<0.001$) (Independent t test). 48% (10/21) HbE/β- and 75% (9/12) β-thalassaemia major patients were at least twofold increased in *LARP2* expression against normal control (Fig. 4). The *LARP2* promoter region was unmethylated in these patients. Relative to control sample, the expression of *LARP2* was down-regulated in 75% (3/4) HbE/β-thalassaemia with CD26/IVS1-5 but up-regulated in 80% (4/5) IVS1-5/IVS1-5 β-thalassaemia major patients (Fig. 4). The promoter *LARP2* methylation profile assessment revealed that all CD26/IVS1-5 HbE/β-thalassaemia patients were partially methylated whilst all IVS1-5/IVS1-5 β-thalassaemia major patients were unmethylated.

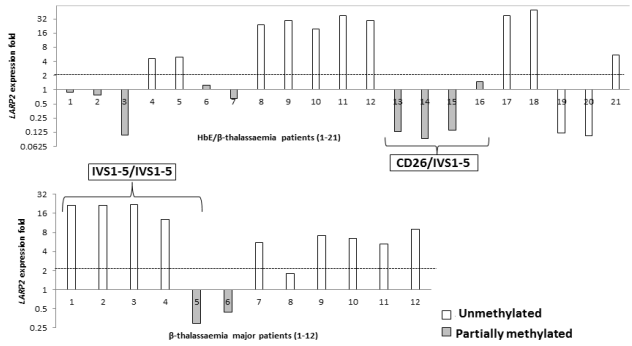


**Figure 4: Relative expression of *LARP2* in reticulocytes of HbE/β and β-thalassaemia major patients. T**he fold change of qPCR was calculated using logarithmic scale of 2-ΔΔCt value. Dotted lines represent two-fold increase in mRNA level against the normal control. HbE/β-thalassaemia patients with IVS1-5 showed down-regulation of *LARP2* expression compared to up-regulation in homozygous IVS1-5 in β-thalassaemia major.

## DISCUSSION

DNA methylation is an epigenetic mechanism that can change the functional state of regulatory regions without any modification in the DNA sequence. The activation and inactivation of genes at one time and transcription

of different genes that occur at different stages of development are controlled by DNA methylation (8). DNA methylation of promoter region has the potential to lead to transcriptional silencing of the associated genes (8, 9). Alterations in DNA methylation were denoted in the previous study contributing to the various phenotypes of diseases (10). This suggested the possibility of large clinical spectrum heterogeneity in HbE/β-thalassaemia could be explained through *LARP2* DNA methylation activity.

*LARP2* was previously identified as epigenetic markers for β-thalassemia (13). *LARP2* DNA methylation is associated with its expression and plays an important role in gene regulation. *LARP2* is an RNA binding protein (RBPs) that plays a significant role in mediating mRNA transcription and translation. It ensures the correct processing and maturation of RNA polymerase III that is essential for transcription initiation. This is important for the major regulatory element of the β-globin locus, the LCR to regulate the transcription of different β globin genes for haemoglobin synthesis throughout the development (16). RBPs are also vital for post-transcriptional regulators of gene expressions in various human tumours which promotes cell migration, invasion and tumorigenesis (17).

The results indicated that the *LARP2* promoter region was partially methylated in HbE/β-thalassaemia whilst unmethylated in β-thalassaemia major. This finding was supported by previous study in which partial methylation was reported in foetal tissue and adult blood DNA (18). DNA methylation is not uniform and different methylation profile was denoted across the analysed CpGs (19). Partial methylation was frequently reported in the aberrant methylation in cancer (20). There are two forms of partial methylation; uCmC and mCuC. Both partial methylation forms may represent hypomethylation, in contrast to hypermethylation represented by only mCuC (21). Both hypomethylation and hypermethylation may result in genome instability and alteration in gene expressions (21). Most of the partial methylation shown in *LARP2* of HbE/β-thalassaemia is associated with down-regulation of *LARP2* expression. However, up-regulation of *LARP2* expression also observed in several HbE/β-thalassaemia patients with partial methylation profile of *LARP2* promoter region. This altered gene expression in *LARP2* of HbE/β-thalassaemia patients could lead to different phenotypic consequences.

The *LARP2* DNA methylation profile is different between HbE/β- and β-thalassaemia major although it was not statistically significant. This may be due to the limitation in patients' selection in which the cohort of the cases studied was all TDT HbE/β- and β-thalassaemia major patients. A significant result may be obtained in a larger cohort study with a wider clinical variability of patients. In addition, the biological factor may ameliorate the epigenetic role in thalassaemia as all patients had been transformed to TDT as well as the involvement of other genes or hidden modifiers. Similarly, environment exposure can change epigenetic patterns and affect the gene expression and phenotype presentations (22).

In contrast, DNA methylation result for β-thalassaemia major patients was not consistent with previous study (7). This may be due to the used of different DNA source and different CpGs location analysed. In this study, DNA was extracted from NRBCs isolated from the PB which was the primary source of methylated DNA of interest thus more specific for further molecular characterisation on DNA methylation roles on phenotypes of thalassaemia. Previous study extracted the DNA for methylation analysis from whole PB. This composed of various cell types that derived from hematopoietic stem cells (HSCs) including the white blood cells (13). These various cell types would confound the result as DNA methylation is specific by cell types (22).

Furthermore, the analysed region for DNA methylation analysis in this study was located at the promoter region of *LARP2*. DNA methylation would involve in distinct gene region of a cell or tissue and act according to their specific manner. The promoter region is enriched with potential methylated transcription factor binding sites (23). Thus epigenetic alteration at this site may affect the gene transcription and eventually the gene expression. *LARP2* was reported to be associated with histone deacetylase (HDAC) to influence the transcription status of the LCR (13). It is exerted that hypomethylation of *LARP2* activates HDAC. This caused removal of acetyl group from the histone proteins making the DNA compact and less in contact with transcription factors, thus down-regulation of the β-globin gene production in β-thalassaemia.

The gene expression study was performed to support the DNA methylation results. *LARP2* hypomethylation profile at the promoter region was associated with an up-regulation in *LARP2* expression. This finding was consistent with previous study reported that active *LARP2* expression was related to hypomethylation of the promoter region (17). In hypomethylation, chromatin is "open" and the promoter is turned on, promoting the binding of RNA polymerase and the accumulation of specific transcription factors for gene transcription (24). Aberrant upregulation of *LARP2* was also identified in several diseases such as cervical, lung and colorectal cancers which related to cancer progression and poor prognosis (17, 25). In this study, it was postulated that overexpression of *LARP2* may cause down-regulation of β-globin gene resulted in a decrease in β-globin production, causing or ameliorate the presentation of β-thalassemia.

On the other hand, partial methylation in HbE/β-thalassaemia patients was significantly associated with

a down-regulation of *LARP2* expression. In this finding, CpG sites 3 and 5 were mostly partially methylated. It is suggested that both CpGs may be vital for the overall effect of *LARP2* promoter methylation on gene expression.

DNA methylation profile and gene expression of *LARP2* were different between HbE/β- and β-thalassaemia major patients. The up-regulation and down-regulation in *LARP2* expression among HbE/β-thalassaemia patients could explain the complex phenotypic of HbE/β-thalassaemia patients. Down-regulation of *LARP2* expression shown in partially methylated HbE/β-thalassaemia patients with CD26/IVS1-5 compared to up-regulation in unmethylated IVS1-5/IVS1-5 β-thalassaemia major patients. Both HbE and IVS 1-5 have β+ phenotype with a minimal amount of HbA synthesis (2.7-5.8%) (1, 26). This proposed that the combination of CD26 (HbE) with IVS1-5 mutation results in different *LARP2* expression that may lead to different pathological in HbE/β-thalassaemia patients compared to homozygous β-thalassaemia major patients.

This study was based on the assumption that epigenetic activities rely on the same finding in BM and PB. Limitation arises where the study comparing epigenetics activities of NRBCs in BM and PB was unavailable. Furthermore, DNA methylation profile of thalassaemia patients cannot be compared with normal individuals due to unavailability of DNA as NRBCs are not present in normal PB. This cause limitation of the small sample size as the inclusion criteria of the subjects were already TDT. Moreover, the used of BSP for DNA methylation analysis was limited to qualitative data. Although BSP analysed the intermediary CpG of the targeted amplicons (27), the degree of methylation at specific CpG sites were not quantified (20, 28). Notwithstanding, these data are important for a better comparison between cases although in future it is suggested to use pyrosequencing or DNA microarray technology to provide genome-wide methylation profiles. These precise measurements of *LARP2* methylation level may provide a potential indicator in monitoring HbE/β-thalassaemia patients before converting patients to TDT.

In addition, it is worth to explore other gene and protein expressions that may epigenetically involve in TDT thalassaemia to correlate clinical pathological parameters including disease severity. Epigenetic is a reversible process, thus identification of aberrant DNA methylation may provide insight towards the development of new therapeutic strategies for thalassaemia treatment and disease management.

## CONCLUSION

Different DNA methylation profile of *LARP2* between HbE/β- and β-thalassaemia major may suggest a different pathophysiological process. Homozygous IVS1-5 in β-thalassaemia major may contribute to different disease presentation compared to those involving CD26 in HbE/β-thalassaemia. DNA methylation of *LARP2* may act as an additional modifier to the clinical presentation of HbE/β-thalassaemia besides other known factors such as specific IVS1-5 beta globin mutation.

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