รายงานวิจัยฉบับสมบูรณ์

โครงการ การกลายพันธุ์ของยีน KLF1 และการแสดงออกของฮีโมโกลบินเอฟในผู้ที่มีฮีโมโกลบินอีชนิดต่างๆ

โดย ดร. สุภาวดี แย้มศรี

พฤษภาคม 2559

สัญญาเลขที่ TRG5780067

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ผู้วิจัย ดร. สุภาวดี แย้มศรี
สังกัด คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ: TRG5780067

ชื่อโครงการ: การกลายพันธุ์ของยีน KLF1 และการแสดงออกของฮีโมโกลบินเอฟในผู้ที่มีฮีโมโกลบินอีชนิดต่างๆ

ชื่อนักวิจัย: ดร. สุภาวดี แย้มศรี คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น

E-mail Address: Supawadee@kku.ac.th

ระยะเวลาโครงการ: 2 มิถุนายน 2557 - 2 มิถุนายน 2559

Erytroid Kruppel-Like Factor (EKLF หรือ KLF1) เป็นกลุ่ม DNA-binding transcriptional regulators ที่พบว่าสำคัญต่อการพัฒนาของเซลล์เม็ดเลือดแดง พบว่ามีความสัมพันธ์เกี่ยวกับการเพิ่มขึ้นของฮีโมโกลบินเอฟ การศึกษาครั้งนี้จึงต้องการศึกษาความชุกของ KLF1 mutation และความสัมพันธ์กับระดับฮีโมโกลบินเอฟในกลุ่มผู้ที่มีฮีโมโกลบินอีชนิดต่างๆ โดยส่วนแรกทำการศึกษาการกลายพันธุ์ในยีน KLF1 จำนวน 4 ชนิด ดังนี้ G176AfsX179, T334R, R238H, และ -154 (C-T) ด้วยวิธี PCR ที่ได้พัฒนาขึ้น ในผู้ที่เป็น homozygous Hb E จำนวน 461 ราย, คนปกติ จำนวน 100 ราย โดยผลการดำเนินการพบว่าในผู้ที่เป็น homozygous Hb E มีอัตราการตรวจพบการกลายพันธุ์ ชนิด G176AfsX179 เท่ากับ 3.9%, ชนิด T334R เท่ากับ 2.0%, ชนิด R238H เท่ากับ 1.7%, ในขณะที่คนปกติ จำนวน 100 รายตรวจไม่พบการกลายพันธุ์ดังกล่าวเลย โดยผู้ที่เป็น homozygous Hb E ที่มีปริมาณ Hb F $\geq$ 5 g/dl จะตรวจพบการกลายพันธุ์ในยีน KLF1 ในสัดส่วนที่สูงกว่ากลุ่มที่มีปริมาณ Hb F < 5 g/dl อย่างมีนัยสำคัญทางสถิติ ผลการศึกษาแสดงให้เห็นว่าการกลายพันธุ์ในยีน KLF1 มีความสัมพันธ์กับการเพิ่มขึ้นของปริมาณฮีโมโกลบินเอฟ ส่วนที่สองทำการศึกษา KLF1 จำนวน 4 ชนิด ดังนี้ G176AfsX179, T334R, R238H, และ -154 (C-T) ในผู้ที่เป็นโรค Hb E - β-thalassemia ที่มีอาการไม่รุนแรงจำนวน 73 ราย ผลการศึกษาพบการกลายพันธุ์ชนิด G176AfsX179 ในผู้ป่วย 1 รายซึ่งมีระดับฮีโมโกลบินเอฟ 50.4% จากการศึกษาทั้งสองส่วนแสดงให้เห็นว่า KLF1 เป็นหนึ่งในปัจจัยทางพันธุกรรม (genetic factor) ที่มีความสำคัญต่อการเพิ่มของระดับ Hb F

คำหลัก: KLF1, EKLF, Thalassemia, Hb E related disorders, Hb F

Abstract

Project Code : TRG5780067

Project Title : KLF1 mutations and Hb F level in Hb E related disorders

Investigator : Dr. Supawadee Yamsri, Faculty of Associated Medical Sciences, Khon Kaen University

E-mail Address: Supawadee@kku.ac.th

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Erytroid Kruppel-Like Factor (EKLF or KLF1) is a group of DNA-binding transcriptional regulators which is essential for maturation of erythroid cell. KLF1 is associated with increased of Hb F. This study aims to study the prevalence of KLF1 mutation and its association with increasing of Hb F level among homozygous Hb E subjects. Four KLF1 mutations including G176AfsX179, T334R, R238H, and -154 (C-T) were screened among 461 homozygous Hb E subjects and 100 normal controls using developed PCR methods. The result found that none of these four mutations were observed in 100 normal controls. Among 461 subjects with homozygous Hb E, KLF 1 mutations were identified in 37 cases including the G176AfsX179 mutation (3.9%), T334R mutation (2.0 %), -154 (C-T) mutation (1.7 %), and R328H mutation (0.4%). The higher proportion of KLF1 mutations in the homozygous Hb E with high Hb F group (Hb F $\geq$ 5 g/dl) as compared to homozygous Hb E with low Hb F group (Hb F < 5 g/dl) was observed, the data indicating the association of KLF1 mutations and high Hb F phenotype in the homozygous Hb E syndrome. When 4 KLF1 mutations including G176AfsX179, T334R, R238H, and -154 (C-T) were screened in 73 Hb E-β-thalassemia patients with non-transfusion dependent thalassemia (NTDT). Due to the small sample size, it was found only 1 patient positive with G176AfsX179 mutation. However, the Hb F level in this patient is quite high (50.4%). All these studies confirm the role of KLF1 as another modulator of Hb F expression in the Hb EE disease and indicate that regulation of Hb F expression in homozygous Hb E is controlled by several genetic factors and complex interactions.

Keywords: KLF1, EKLF, Thalassemia, Hb E related disorders, Hb F

Executive summary

โดยทั่วไปแล้วผู้ที่เป็น homozygous Hb E มักมีปริมาณฮีโมโกลบินเอฟน้อยกว่า 5 % หากปริมาณฮีโมโกลบินเอฟมีปริมาณมากกว่าปกติ อาจทำให้วินิจฉัยผิดเป็นโรคธาลัสซีเมียชนิดฮีโมโกลบินอี/เบต้าธาลัสซีเมียได้ และปัจจัยทางพันธุกรรมที่มีผลต่อระดับฮีโมโกลบินเอฟสูงในกลุ่มตัวอย่างเหล่านี้ยังไม่ทราบแน่ชัด ในการศึกษาครั้งนี้จึงต้องการศึกษาปัจจัยทางพันธุกรรมที่มีผลต่อการเพิ่มขึ้นของฮีโมโกลบินเอฟ โดยทำการศึกษาการกลายพันธุ์ของยีน KLF1 จำนวน 4 ชนิด ดังนี้ G176AfsX179, T334R, R238H, และ -154 (C-T) โดยวิธีพีซีอาร์ที่ได้พัฒนาขึ้นใหม่ในผู้ที่เป็นhomozygous Hb E จำนวน 461 ราย, คนปกติ จำนวน 100 ราย พบว่าผู้ที่เป็น homozygous Hb E มีอัตราการตรวจพบการกลายพันธุ์ KLF1 เท่ากับ 8% โดยชนิดที่พบได้บ่อยคือ ชนิด G176AfsX179 เท่ากับ 3.9%, รองลงมาคือชนิด T334R, ชนิด -154 (C-T) และชนิด R238H ตามลำดับ ในขณะที่คนปกติไม่พบการกลายพันธุ์ทั้ง 4 ชนิดดังกล่าว และเมื่อวิเคราะห์ร่วมกับปริมาณฮีโมโกลบินเอฟ พบว่าในผู้ที่เป็น homozygous Hb E ที่มีปริมาณฮีโมโกลบินเอฟสูงจะพบการกลายพันธุ์ของยีน KLF1 ในสัดส่วนที่สูงกว่าผู้ที่มีระดับฮีโมโกลบินเอฟต่ำ เมื่อวิเคราะห์ร่วมกับปัจจัยทางพันธุกรรมอื่นๆ แสดงให้เห็นว่า KLF1 mutation เป็นปัจจัยทางพันธุกรรมตัวหนึ่งที่มีผลต่อระดับฮีโมโกลบินเอฟที่สูงขึ้นในผู้ที่เป็น homozygous Hb E

เนื้อหางานวิจัย

วัตุประสงค์ของโครงการ

1. ศึกษาความชุกของ KLF1 mutation ซึ่งประกอบด้วย G176AfsX179, T334R, R238H, และ -154 (C-T) ในผู้ที่ผู้ที่มีฮีโมโกลบินเอฟสูงที่ไม่สัมพันธ์กับจีโนไทป์ของธาลัสซีเมีย ที่มารับบริการตรวจวินิจฉัยธาลัสซีเมีย ที่หน่วยบริการธาลัสซีเมีย ศูนย์พัฒนาการตรวจวินิจฉัยทางห้องปฏิบัติการทางการแพทย์ คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น
2. ศึกษาความสัมพันธ์ของระดับของฮีโมโกลบินเอฟ ในผู้ที่มีและไม่มี KLF1mutation ดังกล่าวในกลุ่มผู้ที่มีฮีโมโกลบินเอฟสูงที่ไม่สัมพันธ์กับจีโนไทป์ของธาลัสซีเมีย
3. พัฒนาการวิธีการตรวจ KLF1 mutation อย่างง่ายโดยใช้เทคนิคพีซีอาร์

วิธีการศึกษา

1. ตรวจยืนชนิดของธาลัสซีเมียในตัวอย่างที่นำมาศึกษา ซึ่งเป็นตัวอย่างดีเอ็นเอ ที่เหลือจากงานบริการจากหน่วยบริการธาลัสซีเมีย ศูนย์วิจัยและพัฒนาการตรวจวินิจฉัยทางห้องปฏิบัติการทางการแพทย์ คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น
2. ตรวจ XmnI polymorphism ในตัวอย่างที่นำมาศึกษา
3. เลือกตัวอย่างที่มีปริมาณฮีโมโกลบินเอฟที่สูงมากๆ ตรวจ DNA sequencing ในส่วน promoter ของยีน KLF1
4. พัฒนาการตรวจวินิจฉัย KLF1 mutation ด้วยวิธีพีซีอาร์ โดยพัฒนาการตรวจ KLF1 mutation ที่เคยมีรายงานแล้ว 3 ชนิด คือ G176AfsX179, T334R, และ R238H รวมไปถึง KLF1 mutation ชนิดอื่น ที่พบจากการตรวจด้วยวิธี DNA sequencing
5. ตรวจหา KLF1 mutation ด้วยวิธีที่พัฒนาขึ้น ในกลุ่มตัวอย่างทุกราย
6. วิเคราะห์ข้อมูล เปรียบเทียบระดับฮีโมโกลบินเอฟ ระหว่างผู้ที่มีและไม่มี KLF1 mutation รวมไปถึงวิเคราะห์เปรียบเทียบกับ genetic modifier ชนิดอื่น

ผลการศึกษา

เมื่อทำการศึกษาการกลายพันธุ์ในยีน KLF1 จำนวน 4 ชนิด ดังนี้ G176AfsX179, T334R, R238H, และ -154 (C-T) ในผู้ที่เป็นhomozygous Hb E จำนวน 461 ราย, คนปกติจำนวน 100 ราย พบว่าผู้ที่เป็น homozygous Hb E มีอัตราการตรวจพบการกลายพันธุ์ ชนิด G176AfsX179 เท่ากับ 3.9%, ชนิด T334R เท่ากับ 2.0%, ชนิด R238H เท่ากับ 1.7%, และชนิด -154 (C-T) เท่ากับ 0.4%,ในขณะที่คนปกติ จำนวน 100 รายตรวจไม่พบการกลายพันธุ์ทั้ง 4 ชนิด และเมื่อทำการแบ่งกลุ่มผู้ที่เป็น homozygous Hb E ตามปริมาณฮีโมโกลบินเอฟ พบว่าผู้ที่เป็น homozygous Hb E ที่มีปริมาณ Hb F $\geq$ 5 g/dl จะตรวจพบการกลายพันธุ์ในยีน KLF1 ในสัดส่วนที่สูงกว่ากลุ่มที่มีปริมาณ Hb F < 5 g/dl อย่างมีนัยสำคัญทางสถิติ

และนอกจากนี้เมื่อทำการศึกษา KLF1 จำนวน 4 ชนิด ดังนี้ G176AfsX179, T334R, R238H, และ -154 (C-T) ในผู้ที่เป็นโรค Hb E - β- thalassemia ที่มีอาการไม่รุนแรงจำนวน 73 ราย ผลการศึกษาพบการกลายพันธุ์ชนิด G176AfsX179 ในผู้ป่วย 1 รายซึ่งมีระดับฮีโมโกลบินเอฟ 50.4% ซึ่งอยู่ในแนวโน้มที่สูง

สรุปและวิจารณ์ผลการทดลอง

จากการศึกษาความชุกของ KLF1 mutation ในผู้ที่เป็น homozygous Hb E พบอัตราการตรวจพบการกลายพันธุ์ KLF1 mutation เท่ากับ 8.0% (37/461 ราย) แยกเป็นชนิด G176AfsX179 3.9%, ชนิด T334R 2.0%, ชนิด -154 (C-T) 1.7% และ ชนิด R238H 0.4% ซึ่งสัดส่วนการตรวจพบ KLF1 mutation ในผู้ที่มีฮีโมโกลบินเอฟสูง (Hb F $\geq$ 5 g/dl) แตกต่างจากผู้ที่มีระดับฮีโมโกบินเอฟต่ำ (Hb F < 5 g/dl) อย่างมีนัยสำคัญทางสถิติ ชนิดที่พบบ่อยในประชากรไทยคือชนิด G176AfsX179 ซึ่งเป็นความผิดปกติที่ทำให้เกิด frame shift mutation ทำให้ได้โปรตีนในส่วนที่เป็น zinc finger domain ที่ผิดปกติไป ซึ่งผลการศึกษาเป็นไปในแนวทางเดียวกันกับที่เคยมีรายงานมาก่อน และเมื่อทำการศึกษาในผู้ที่เป็นโรค Hb E - β- thalassemia ที่มีอาการไม่รุนแรง แม้จะพบผู้ที่มี KLF1 mutation 1 ราย คือพบชนิด G176AfsX179 แต่ระดับฮีโมโกลบินเอฟในผู้ป่วยรายนี้ก็อยู่ในแนวโน้มที่สูง จากการศึกษาทั้งสองส่วนแสดงให้เห็นว่า KLF1 เป็นหนึ่งในปัจจัยทางพันธุกรรม (genetic factor) ที่มีความสำคัญต่อการเพิ่มของระดับ Hb F และสามารถใช้อธิบายการเพิ่มขึ้นของ Hb F ในประชากรได้

ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

แม้ว่าในการศึกษาครั้งนี้ แสดงให้เห็นว่า KLF1 มีความสำคัญต่อการเพิ่มของระดับ Hb F แต่ยังขาดในส่วนที่เป็น functional study หากมีการวิจัยในอนาคต การศึกษาเกี่ยวกับ functional study ของ KLF1 mutation ที่ตรวจพบ จะทำให้การอธิบายกลไกของ KLF1 ต่อการเพิ่มขึ้นของฮีโมโกลบินเอฟ ได้ชัดเจนและถูกต้องยิ่งขึ้น

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1) Krüppel-like factor 1 mutations and expression of hemoglobins F and A2 in homozygous hemoglobin E syndrome. Tepakhan W, *Yamsri S*, Fucharoen G, Sanchaisuriya K, Fucharoen S. Ann Hematol. 2015 Jul; 94(7):1093-8 (impact factor 2.634)

2) Nine known and five novel mutations in the erythroid transcription factor *KLF1* gene and phenotypic expression of fetal hemoglobin in hemoglobin E disorder. Tepakhan W, Yamsri S, Sanchaisuriya K, Fucharoen G, Xu X, Fucharoen S. Blood Cells, Molecules and Diseases. 2016 (Accepted). (impact factor 2.646)

3) Manuscript “Molecular understanding of non-transfusion dependent thalassemia associated with Hb E - beta - thalassemia in northeast Thailand. Yamsri S, Pakdee N, Fucharoen G, Sanchaisuriya K, Fucharoen S.“ *(Submitted)*

2. การนำผลงานวิจัยไปใช้ประโยชน์

การนำผลงานวิจัยไปใช้ประโยชน์

☑ **ด้านวิชาการ**

โดยใคร *(กรุณาให้ข้อมูลเจาะจง)*

☐ หน่วยงาน (ภาครัฐ/เอกชน) ☑ **สถาบันการศึกษา** ☐ ผู้ประกอบการ ☐ เกษตรกร ☑ **อื่น ๆ**

- คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น
- ผู้รับบริการ หน่วยบริการธาลัสซีเมีย ศวป. คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น

มีการนำไปใช้อย่างไร *(กรุณาให้ข้อมูลเจาะจง)*

- ผลที่ได้จากการศึกษาของโครงการ “การกลายพันธุ์ของยีน KLF1 และการแสดงออกของฮีโมโกลบินเอฟในผู้ที่มีฮีโมโกลบินอีชนิดต่าง ๆ” ได้สร้างองค์ความรู้ใหม่ (journal paper) และได้มีการนำองค์ความรู้ที่ได้ไปใช้ในการสอนวิชา Molecular Pathology and Analysis of Thalassemia คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น และนอกจากนี้ยังใช้องค์ความรู้ที่ได้ ในงานบริการวิชาการที่หน่วยบริการธาลัสซีเมีย ศวป. คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น โดยทำการตรวจ KLF1 mutation ในตัวอย่างประจำวันที่พบว่าระดับฮีโมโกลบินเอฟสูงที่ไม่สัมพันธ์กับจีโนไทป์ของธาลัสซีเมีย เพื่อให้ได้ข้อมูลที่สมบูรณ์ประกอบการวินิจฉัยธาลัสซีเมีย

3. อื่น ๆ นำเสนอผลงานวิจัยในการประชุมระดับนานาชาติ

1) เรื่อง KLF1 mutations and variability of hemoglobin F expression in homozygous Hb E. Wanicha Tepakhan, Supawadee Yamsri, Goonnapa Fucharoen, Kanokwan Sanchaisuriya, Supan Fucharoen. ในการประชุม Pan-Asian Biomedical Sciences Conference ระหว่างวันที่ 11-12 พฤศจิกายน พ.ศ. 2557 ณ Hong Kong Sciences Park, Hong Kong.

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ORIGINAL ARTICLE

Krüppel-like factor 1 mutations and expression of hemoglobins F and A_2 in homozygous hemoglobin E syndrome

Wanicha Tepakhan · Supawadee Yamsri · Goonnapa Fucharoen · Kanokwan Sanchaisuriya · Supan Fucharoen

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Abstract The basis for variability of hemoglobin (Hb) F in homozygous Hb E disease is not well understood. We have examined multiple mutations of the Krüppel-like factor 1 (KLF1) gene; an erythroid specific transcription factor and determined their associations with Hbs F and A_2 expression in homozygous Hb E. Four KLF1 mutations including G176AfsX179, T334R, R238H, and −154 (C-T) were screened using specific PCR assays on 461 subjects with homozygous Hb E and 100 normal controls. None of these four mutations were observed in 100 normal controls. Among 461 subjects with homozygous Hb E, 306 had high (≥5 %) and 155 had low (<5 %) Hb F. DNA analysis identified the KLF1 mutations in 35 cases of the former group with high Hb F, including the G176AfsX179 mutation (17/306=5.6 %), T334R mutation (9/306=2.9 %), −154 (C-T) mutation (7/306=2.3 %), and R328H mutation (2/306=0.7 %). Only two subjects in the latter group with low Hb F carried the G176AfsX179 and −154 (C-T) mutations. Significant higher Hb A_2 level was observed in those of homozygous Hb E with the G176AfsX179 mutation as compared to those without KLF1 mutations. These results indicate that KLF1 is among the genetic factors associated with increased Hbs F and A_2, and in combination with other factors could explain the variabilities of these Hb expression in Hb E syndrome.

Keywords Homozygous hemoglobin E · Hemoglobin F · Hemoglobin A_2 · KLF1 mutations

Introduction

Hemoglobin (Hb) E is the most common Hb variant in Southeast Asian populations. The high prevalence of Hb E is especially observed in northeastern part of Thailand where the frequency could be as high as 50 % [1]. It is resulted from a single nucleotide base substitution at codon 26 (*G*AG to *A*AG) of β-globin gene leading to a replacement of lysine for glutamic acid at this position. The mutation could also activate a cryptic splice site in exon I leading to abnormal splicing and a reduction of β^E globin synthesis. It is therefore considered as a β^+-thalassemia allele [2]. In the region, Hb E may be presented as heterozygous, homozygous (Hb EE disease), or in combination with other forms of thalassemia leading to Hb E/β-thalassemia, AEBart's, and EF Bart's diseases [3–7]. Heterozygous Hb E is symptomless, whereas Hb EE disease usually has mild anemia with reduced mean corpuscular volume (MCV) and mean corpuscular Hb (MCH) values [2]. In Hb E heterozygote, the level of Hb F is usually less than 1 %, s but in Hb E homozygote this can be varied from less than 1 % to more than 10 %. Hb A_2 can be within normal range or elevated due to a β-thalassemic nature of Hb E mutation [8]. Accordingly, at routine setting, a misdiagnosis of Hb E/β-thalassemia disease for a homozygous Hb E with elevated Hbs F and A_2 could happen.

Multiple single nucleotide polymorphisms (SNPs) in several genes such as *BCL11A* and *HBS1L-MYB* have also been shown to be associated with Hb F expression in β-thalassemia

Wanicha Tepakhan and Supawadee Yamsri contributed equally to this work.

W. Tepakhan
Biomedical Science Program, Graduate School, Khon Kaen University, Khon Kaen 40002, Thailand

W. Tepakhan · S. Yamsri · G. Fucharoen · K. Sanchaisuriya · S. Fucharoen (✉)
Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand
e-mail: supan@kku.ac.th

and sickle cell diseases [9, 10]. In addition, the Krüppel-like factor 1 (KLF1) (or Erythroid Krüppel-like factor, EKLF), an essential erythroid transcription factor, which plays multifunctional role during erythropoiesis and Hb switching between fetal and adult states, has also been found to be involved [11, 12]. Mutations in KLF1 gene have been shown to be associated with increased Hb F in adults in diverse populations [13–16]. It has been shown recently that KLF1 mutations could ameliorate the clinical and hematological severity of β-thalassemia in Chinese patients [17]. In this study, we have examined the association of KLF1 mutations with variability in Hb F and Hb A_2 in homozygous Hb E disease.

Materials and methods

Subjects

Ethical approval of the study protocol was obtained from the Institutional Review Board (IRB) of Khon Kaen University, Thailand (HE 562140). The study was done on 100 normal control subjects and 461 Thai subjects with homozygous Hb E. Leftover DNA samples were selectively recruited from our ongoing thalassemia screening program at Khon Kaen University in northeast Thailand.

Hematological and DNA analyses

Hematological parameters were obtained using the Coulter T series automated blood cell counter (Beckman Coulter Co., USA). Hb analysis was done using capillary zone electrophoresis (Capillarys 2; Sebia Co., Lisses, France) because this method can report the amount of Hb A_2 in the presence of Hb E [8]. Identifications of the β^E-mutation, α^0-thalassemia (SEA and THAI deletions) and α^+-thalassemia (3.7 and 4.2 kb deletions), Hb Constant Spring and Hb Paksé mutations are performed routinely in our laboratory using PCR methods described elsewhere [18]. The −158 (C-T) $^G\gamma$ *Xmn*I polymorphism was additionally identified by PCR amplifications of γ-globin gene promoter using primers γ4 (5′-GGCCTAAAAC CACAGAGAGT-3′) and γ5 (5′-CCAGAAGCGAGTGTGT GGAA-3’) followed by digestion with *Xmn*I restriction enzyme [19].

Allele-specific PCR assays for rapid screening of KLF1 mutations

Screening for four common KLF1 mutations including −154 (C-T), G176AfsX179 in exon 2, R328H, and T334R in exon 3 was done using allele specific PCR with four sets of PCR primers as shown in Fig. 1. The −154 (C-T) and T334R mutations were detected in a multiplex PCR manner. The −154

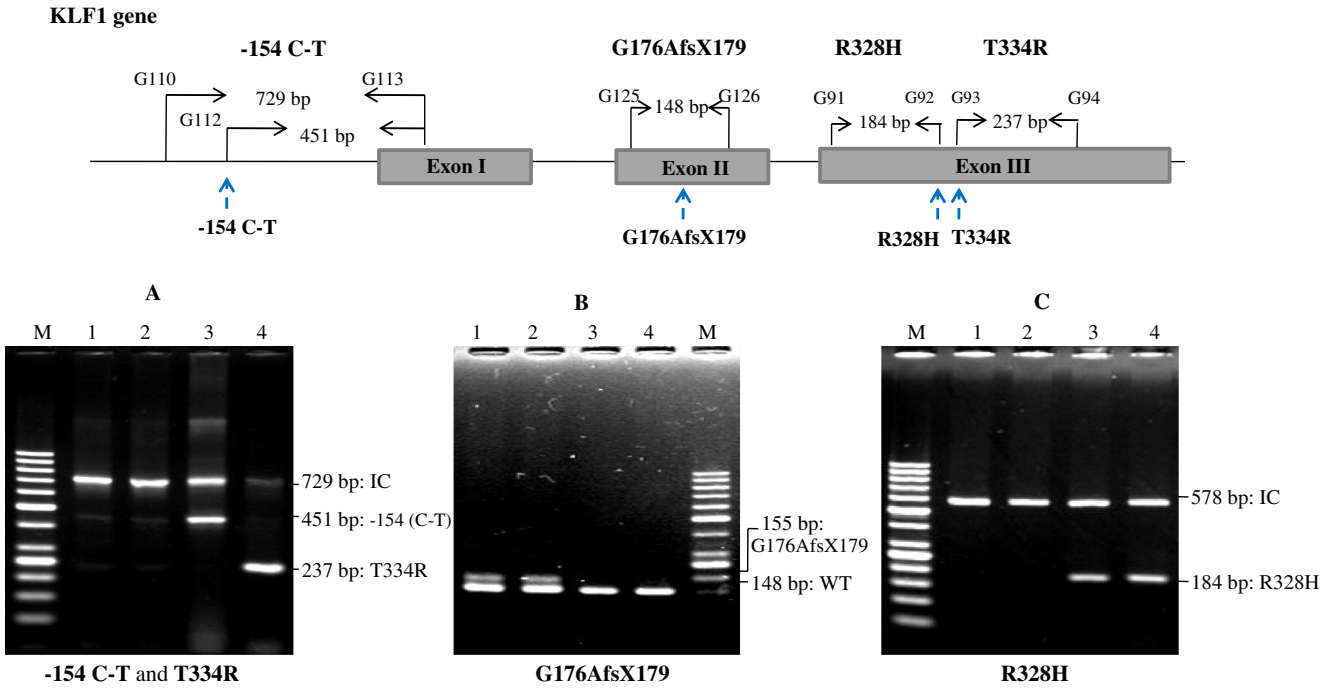

Fig. 1 Identification of four KLF1 mutations, −154 (C-T), T334R, G176AfsX179, and R328H, by allele specific PCR. Schematic diagram showing the locations and orientations of primers used as well as sizes of the amplified fragments. **a** M is 50 bp DNA ladder, *1* and *2*: normal controls *3*: carrier of the −154 (C-T) and *4*: carrier of T334R mutation. **b** *1* and *2*: carriers of G176AfsX179 mutation, *3* and *4*: normal controls. **c** 1 and 2 are normal controls, 3 and 4: carriers of R328H mutation

(C-T)- and T334R-specific primers, G112 (5′-GTGCCCCA GAAACAGTGCT-3′) and G93 (5′-CGCCACTACCGGAA ACACAG-3′), were used with primers G113 (5′-ACCTCA AA CCCCTAGACCACC-3′) and G94 (5′-TCCAGGAGAG GGTCCATTCGT-3′), respectively, to produce fragments of 451 and 237 bp specific for these two mutations. The 729-bp fragment generated from primer G110 (5′-CGGTTGTT GCTGTTTACTGGG-3′) and G113 was used as an internal control (Fig. 1a). The PCR reaction (50 μl) contained 50 ng DNA, 0.48 pmol of primers G93 and G94, 1.2 pmol of primers G110 and G113, 0.3 pmol of primer G112, 200 mM dNTPs and 1 Unit *Taq* DNA polymerase in a PCR buffer provided by the manufacturer (New England Biolabs Inc., MA, USA). The G176AfsX179 mutation is characterized by 7-bp insertion. In a PCR using primers G125 (5′-GCTCCCGACGCCTTCGT-3′) and G126 (5′-ACGCCGCAGGCACTGAAA-3′), a normal fragment with 148 bp in length is changed to 155 bp with the G176AfsX179 mutation (Fig. 1b). Each PCR reaction (50 μl) contained 50 ng DNA, 0.9 pmol of primer G125 and G126, 200 mM dNTPs, 100 mM Tris-HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 5 % DMSO, and 1 Unit *Taq* DNA polymerase (New England Biolabs Inc., MA, USA). As shown in Fig. 1c, the R328H mutation is detected by allele-specific PCR with specific primer G92 (5′-CCGTGT GTTTCCGGTAGTGGT-3′) and common primer G91 (5′-CAGCCCAGGCTGAGTAAAGGG-3′) which produces a 184-bp specific fragment. In the same reaction, the 578-bp internal control fragment is generated using primers γ4 and γ5 mentioned above. Each PCR reaction (50 μl) contained 50 ng DNA, 0.06 pmol of primer G91, 0.12 pmol of primer G92, 0.18 pmol of primer G94, 200 mM dNTPs, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, and 1 Unit *Taq* DNA polymerase (New England Biolabs Inc., MA, USA). PCR was performed with initiation heating step at 94 °C for 3 min followed by 30 cycles of (94 °C, 30 s–65 °C, 30 s–72 °C, 45 s) using the TProfessional Standard Thermocycler (Biometra, GmbH, Goettingen, Germany). The amplified product was separated on 2 % agarose gel electrophoresis, stained with ethidium bromide (0.5 μg/ml) and visualized under UV-light. Each mutant-specific fragment was reconfirmed by DNA sequencing on an ABI PRISM™ 3130 XL analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Mean and standard deviation were used to describe hematological parameters of subjects in each polymorphic group. Difference in mean Hbs F and A_2 levels of two polymorphic groups was analyzed by the Mann–Whitney *U* test. A *P* value of less than 0.05 was considered statistically significance.

Table 1 The hematological characteristics of 461 homozygous Hb E subjects (EE) and 100 normal subjects (AA) according to KLF1 mutations

Genotype	KLF1 mutations	Number	HbA_2 (%)	Hb E (%)	Hb F (%)	Rbc ($\times 10^{12}$)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (%)	RDW (%)
EE	None	424	4.6±2.6	89.8±6.9	8.7±5.8	5.4±0.8	11.4±1.5	34.0±4.7	63.8±5.8	21.4±2.3	33.4±2.4	16.5±2.4
	G176AfsX179	18	5.4±0.7[a]	82.2±8.2	14.6±6.4[b]	5.8±0.7	12.3±1.5	36.5±6.3	62.7±4.6	21.2±0.6	34.1±2.6	17.3±1.5
	T334R	9	5.0±1.0	79.4±5.7	15.9±6.3[b]	6.3±0.8	12.8±0.7	37.9±3.3	59.8±4.5	19.6±1.3	32.5±1.1	20.8±0.1
	−154 (C-T)	8	4.6±0.6	85.3±9.0	10.7±4.1	5.9±0.6	11.7±1.7	35.1±5.5	67.8±6.0	16.5±8.1	33.3±2.8	19.5±0.8
	R328H	2	5.0, 4.6	75.2, 81.1	13.6, 17.4[b]	6.4, 6.7	14.5, 13.7	42.2, 38.5	65, 57.3	22.7, 20.5	35.6	20.4, 22.4
AA	None	100	2.6±0.4	–	0.4±0.4	4.7±0.8	12.3±1.5	37.8±4.7	78.4±10.0	26±3.2	32.8±1.3	14.3±1.8

Values are presented as mean ± SD or as raw data where appropriate

[a] Significant difference from the Hb A_2 levels of those without KLF1 mutations ($P<0.05$; Mann–Whitney *U* test)

[b] Significant difference from the Hb F levels of those without KLF1 mutations ($P<0.05$; Mann–Whitney *U* test)

Table 2 Distribution of subjects with KLF1 mutations according to high (≥5.0 %) and low (<5 %) Hb F among 37 subjects with homozygous Hb E

KLF1 mutations	Hb F levels							
	High (≥5%) (n=306)				Low (<5 %) (n=155)			
	No.	Proportion (%)	Median	(Min, max)	No.	Proportion (%)	Median	(Min, max)
With mutation	35	11.4[a]	13.3	(5.6, 27.0)	2	1.3[a]	–	4.3, 3.5
G176AfsX179	17	5.6	14.2	(5.6, 27.0)	1	0.6	–	4.3
T334R	9	2.9	13.3	(8.4, 26.9)	–	–	–	–
−154 (C-T)	7	2.3	10.8	(8.5, 16.0)	1	0.6	–	3.5
R328H	2	0.7	–	(13.6, 17.4)	–	–	–	–
Without mutation	271	88.6	10.8	(5.2, 31.9)	153	98.7	1.9	(0, 4.8)

[a] Significant difference in proportion of cases with KLF1 mutation between high and low Hb F groups (P<0.001)

Results

Allele-specific PCR for β^E-mutation identified that all of the 461 subjects carried the homozygosity for Hb E mutation. PCR assays developed as shown in Fig. 1 detected the KLF1 mutations in 37 of 461 subjects with homozygous Hb E including G176AfsX179 (n=18), T334R (n=9), −154 (C-T) (n=8), and R238H (n=2), representing proportions 5.0, 2.4, 1.8 %, and 0.6 %, respectively. In contrast, no any KLF1 mutation was detected among 100 normal control subjects. Hematological findings of all these subjects were shown in Table 1 according to the mutations found. As shown in the table, no significant difference of most of these hematological features between homozygous Hb E subjects with or without KLF1 mutations was observed. However, significant higher levels of Hbs F and A_2 were noted for those with KLF1 mutations. When these subjects with homozygous Hb E were further classified into those with high Hb F (Hb F ≥5 %; n=306) and those with low Hb F (Hb F <5 %; n=155), we observed higher proportion of KLF1 mutations in the former group (35 of 37) as compared to the latter group (2 of 37) as shown in Table 2, the data indicating the association of KLF1 mutations and high Hb F phenotype in the Hb EE syndrome.

In Table 3, we further examined the combined effect of KLF1 mutations with the −158 (C-T) $^G\gamma$ *Xmn*I polymorphism known to be associated with high Hb F phenotype. Apparently, the presence of −158 $^G\gamma$ *Xmn*I polymorphism could lead to a higher Hb F expression. Additive effects of the −158 (C-T) $^G\gamma$ *Xmn*I polymorphism (either +/+ or +/−) for KLF1 mutations were observed for the G176AfsX179 and T334R but not for the −154 (C-T) mutation. This could not be confirmed for the R328H mutation since both two subjects with this mutation had −158 (C-T) $^G\gamma$ *Xmn*I polymorphism (+/+) pattern.

Discussion

The hematological findings of the 461 subjects with homozygous Hb E shown in Table 1 confirm that they had mild anemia and reduced MCV and MCH values. Hb A_2 and Hb F are elevated. As shown in Table 1, screening for the four known KLF1 mutations in these subjects identified 37 of 461 (8.0 %) cases with the KLF1 mutations. Interestingly, we detected no KLF1 mutations among 200 chromosomes of 100 normal control subjects investigated. It has been shown recently that KLF1 mutations are significantly more prevalent in the Chinese β-thalassemia patients in the regions with high incidence of β-thalassemia and correlate with a milder phenotype. This study on the Chinese patients has also demonstrated that KLF1 mutations are associated with elevation of Hb F and Hb A_2 levels and a decrease in CD44 expression, which can form a basis to screen for KLF1 mutation in the patient [17, 20]. A higher prevalence of KLF1 mutations in Hb EE disease as compared to normal subjects in our study is therefore not unexpected. Of interest is the finding that these KLF1 mutations were identified much more commonly in those with high

Table 3 Hb F levels of homozygous Hb E subjects according to KLF1 mutations and $^G\gamma$ *Xmn*I polymorphism

KLF1 mutations	Number	$^G\gamma$ *Xmn*I	Hb F levels	
			Median	(Min, max)
G176 AfsX179	1	−/−	–	5.6
T334R	1	−/−	–	12.1
−154 (C-T)	1	−/−	–	12
G176 AfsX179	10	+/+	14.8	(10.8, 25.6)
G176 AfsX179	6	+/−	13.1	(7.9, 27.0)
−154 (C-T)	5	+/+	10.8	(9.0,16.0)
−154 (C-T)	1	+/−	–	8.5
T334R	8	+/+	15	(8.4, 26.9)
R328H	2	+/+	–	13.6, 17.4

Hb F (Hb F ≥5 %) as compared to those with low Hb F expression (Hb F <5 %) as shown in Table 2. It is conceivable as for β-thalassemia that these KLF1 mutations are associated with high Hb F expression in Hb EE disease. For expression of Hb A_2, however, we observed a significant effect only on the G176AfsX179 mutation in which the levels of 5.4±0.7 % Hb A_2 was detected as compared to the levels of 4.6±2.6 % found in those without this mutation. This might be explained by the fact that elevated Hb A_2 is the general characteristic of all β-thalassemia mutation regardless of KLF1 mutation. In contrast, variable Hb A_2 levels ranging from less than 1 % to around 12 % have been documented in Hb EE disease [8]. It is noteworthy that apart from these levels of Hb A_2 and Hb F, we observed no difference in other hematological findings between homozygous Hb E subjects with and without KLF1 mutations. This suggests that the four KLF1 mutations which located respectively in the promoter region (−154; C-T), the exon 2 (G176AfsX179), and within the second zinc finger motif in exon 3 (T334R and R328H) of the KLF1 gene may not have much affect on the erythropoiesis. This is in contrast to another missense mutation; the E325K located within the second zinc finger motif of KLF1 which had a dominant effect leading to a congenital dyserythropoietic anemia [21]. This phenotypic diversity of KLF1 mutations might reflect difference in the altered DNA binding affinity of each mutation to the KLF1 regulated genes [22–24].

It is noteworthy that variation of the Hb F levels in individuals with homozygous Hb E can lead to a misdiagnosis of Hb E–β-thalassemia, the common thalassemic disease in routine practice in the region [8]. Our results demonstrated that the four KLF1 mutations including G176AfsX179, T334R, R328H, and −154 (C-T) are associated with high Hb F phenotype in this disorder. However, the G176AfsX179 seems to be the major one in Thai population. This G176AfsX179 frame shift mutation leading to a premature termination and an ablation of protein prior to the zinc finger domain has been shown to be associated with increased Hb F in Koreans and Chinese populations [16, 25]. The R328H was firstly reported to be the cause of In (Lu) phenotype in an individual from the UK [23], whereas both T334R and R328H have been detected in two sporadic cases with increased Hb F [14]. It is postulated that amino acid changes in these two mutations could lead to an ablation of DNA binding site and reduction of DNA binding affinity to the β-globin gene and the BCL11A gene which encode the γ-globin gene suppressor. Thus, increased expression of γ-globin gene can be found in adult with these mutations [12, 24]. It has been documented that compound heterozygote for the −154 (C-T) and A298P mutations of KLF1 gene could lead to a transfusion-dependent hemolytic anemia with elevated Hb F and persistence of embryonic globin gene expression [26].

The observation on variability of Hb F ranging from 4.3 to 27.0 % in Hb EE disease with the G176AfsX179 mutation in this study points to a combined effect of this mutation and other genetic modifying factors in modulating Hb F levels in Hb EE disease. Among these are the −158 (C-T) $^{G}\gamma$ *Xmn*I polymorphism as shown in Table 3 and several other SNPs in the BCL11A and HBS1L-MYB genes recently documented [27]. All these studies confirm the role of KLF1 as another modulator of Hb F expression in the Hb EE disease and indicate that regulation of Hb F expression in homozygous Hb E is controlled by several genetic factors and complex interactions.

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Conflict of interest The authors declare that they have no conflict of interest.

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Nine known and five novel mutations in the erythroid transcription factor *KLF1* gene and phenotypic expression of fetal hemoglobin in hemoglobin E disorder

Wanicha Tepakhan,[a, b] Supawadee Yamsri,[b] Kanokwan Sanchaisuriya,[b] Goonnapa Fucharoen,[b] Xiangmin Xu,[c] Supan Fucharoen [b, *]

[a] *Biomedical Science Program, Graduate School, Khon Kaen University, Khon Kaen, Thailand*
[b] *Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand*
[c] *Department of Medical Genetics, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong, China*

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ABSTRACT

Hemoglobin E is the most common Hb variant found in South East Asia. Variation of Hb F expression in Hb E syndrome is associated with several genetic modifiers. We report several single nucleotide polymorphisms (SNPs), including nine known and five novel mutations of the Krüppel-like factor 1 (*KLF1*; an erythroid specific transcription factor) gene and determine their associations with phenotypic expression of Hb F in Hb E disorders. *KLF1* mutations were examined using high resolution melting (HRM) assay and DNA sequencing in 575 homozygous Hb E, 278 heterozygous Hb E and 100 normal subjects. Fourteen mutations were mostly observed in subjects with elevated Hb F, including nine known mutations (G176AfsX179, T334R, R238H, − 154 (C > T), A298P, S270W, R301H, − 148 (G > A) and G335R and five novel mutations (Q217X, Q223X, Y290_S293del, K307N, and M358I). None of them, but the − 148 (G > A), were observed in normal controls to have Hb F < 1%. Combined *KLF1* mutations with other SNPs including ^{G}γ-*Xmn*I, *BCL11A* and *HBS1L-MYB* were associated with higher Hb F levels. *KLF1* is therefore an important genetic factor associated with increased Hb F and in combination with other modifying factors could explain the phenotypic variation of Hb F expression in this common hemoglobinopathy.

1. Introduction

Hemoglobin (Hb) E is the most common Hb variant found in Southeast Asia [1]. It is caused by a single nucleotide substitution at codon 26 (*G*AG to *A*AG) of β-globin gene. This mutation creates an abnormal splicing within the exon 1 of β-globin gene leading to a β^{+}-thalassemia phenotype [2]. Hb E is particularly important in the region, especially in northeast Thailand, where the frequency of Hb E could reach 50% in some ethnic minorities [3,4]. Association of Hb E with β-thalassemia leads to Hb E-β-thalassemia disease, the most common thalassemia encountered in the region [1]. Hb E could be presented as a heterozygous state with asymptomatic and minor hematological change or as a homozygote form with mild hypochromic microcytosis with reduced mean corpuscular volume (MCV) and mean corpuscular Hb (MCH) and elevated Hb F [7–10]. The level of Hb F in normal adult is usually < 1% of the total Hb. However, among those with homozygous Hb E, a variable level ranging from < 1% to > 10% can be observed [11]. This could lead to difficulties in differential diagnosis by protein analysis of homozygous Hb E and Hb E-β^{0}-thalassemia, both of which are common in the region [12]. It has been shown that a number of single nucleotide polymorphisms (SNPs) at different loci, e.g. HBG2: − 158 C > T on 11p15.4 namely the ^{G}γ-*Xmn*I polymorphism, *HBS1L-MYB* intergenic region on 6q23.3 and the *BCL11A* on 2p16.1, are associated with high Hb F expression in patients with β-thalassemia, sickle cell disease and homozygous Hb E as well as in healthy subjects in various populations [13–15]. In addition, an erythroid transcription factor, Krüppel-like factor 1 (*KLF1*) or Erythroid Krüppel-like factor (EKLF), also plays an important role in erythropoiesis and fetal to adult Hb switching by direct activation of β-globin gene expression. This indirectly reduces γ-globin gene expression via activation of γ-globin gene suppressor, *BCL11A* [16,17]. Multiple SNPs in the *KLF1* gene have been found to be associated with high Hb F expression in diverse populations [18–22]. Here we reported several known and novel mutations in *KLF1* gene and determined their association with phenotypic expression of Hb F in Hb E disorder. Additional information on the combined effect of these *KLF1* mutations with other genetic modifying factors, including the ^{G}γ-*Xmn*I (rs7482144), *BCL11A* (rs4671393), *HBS1L-MYB* (rs4895441) and (rs9399137) [15] are also presented.

2. Materials and methods

2.1. Subjects

The study was done on 953 Thai subjects, including 575 homozygous Hb E, 278 heterozygous Hb E and 100 normal control subjects recruited from our ongoing thalassemia screening program at Khon Kaen University, Khon Kaen, Thailand. Ethical approval of the study

* Corresponding author at: Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand.
Email address: supan@kku.ac.th (S. Fucharoen)

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protocol was obtained from the Institutional Review Board (IRB) of Khon Kaen University (HE 562140).

2.2. Hematological and DNA analyses

Routine hematological parameters were collected on the Coulter T series automated blood cell counter (Beckman Coulter Co., USA). Hb analysis was performed using capillary zone electrophoresis (Capillarys 2; Sebia Co., Lisses, France) because this method could report the amount of Hb A_2 in the presence of Hb E [23]. In our laboratory, identifications of the β-thalassemia and β^E-mutations, α^0-thalassemia (SEA & THAI deletions) and α^+-thalassemia (3.7 & 4.2 kb deletions), Hb Constant Spring and Hb Paksé are performed routinely using PCR methods described elsewhere [24].

2.3. High-resolution melting (HRM) assay for screening of KLF1 mutations and other SNPs genotyping

Screening of *KLF1* mutations was performed using HRM assay described elsewhere [25]. Each mutation was then confirmed by DNA sequencing. Functional prediction for the novel *KLF1* genes observed was determined using the SIFT (http://sift.jcvi.org) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) softwares. Genotyping of other known SNPs, including the $^G\gamma$ *Xmn*I (rs7482144) and *HBS1L-MYB* (rs9399137) polymorphisms were performed using PCR-RFLP and HRM analysis described elsewhere [15,25–27]. In addition, PCR-HRM assays for *BCL11A* (rs4671393) and *HBS1L-MYB* (rs4895441) genotyping were developed using primers **rs467-F** (5′CACAACACTCCAGGGAGGCAG3′) and **rs467-R** (5′GGAGGCAGGGGGAATCTTAAT3′) specific for *BCL11A* (rs4671393) and primers **rs489-F** (5′GTGGCTGGGGAAAGACTCTT3′) and **rs489-R** (5′TTATCTCCCTCACTTA CTCAG3′) specific for *HBS1L-MYB* (rs4895441). Each PCR reaction includes 1 × KAPA HRM FAST PCR Kit (Kapa Biosystems Inc., MA, USA), 2.5 mM Mg^{2+}, 0.2 pmol/μl of forward primer and reverse primer, 0.05 pmol/μl of interior controls. The PCR was started at 95 °C for 5 min followed by 45 cycles of 95 °C for 30 s, 64 °C (rs4671393) or 60 °C (rs4895441) for 15 s, and 72 °C for 15 s. This was followed by incubation at 95 °C for 1 min and held at 16 °C before HRM analysis was performed.

2.4. Statistical analysis

Median and 95% CI were used to describe hematological parameters of subjects in each group. Difference in the median values of Hbs F and A_2 was analyzed by the Mann–Whitney *U* test. A *P* value of < 0.05 was considered statistically significant.

3. Results

Fig. 1 depicts 14 *KLF1* mutations identified in this study. Thirteen of those mutations were identified with heterozygous or homozygous Hb E with high Hb F levels. These included eight known mutations (G176AfsX179, T334R, R238H, − 154 (C > T), A298P, S270W, R301H and G335R) and five novel mutations, i.e. two nonsense mutations in exon 2 prior to the zinc finger domain (Q217X & Q223X), two mis-sense mutations in exon 3 (K307N, & M358I) and a deletion of 12 nucleotides (ACACCAAGAGCT) within the zinc finger domain of exon 2 (a deletion of codons 290–293; Y290_S293del). The remaining known mutation, − 148 (G > A) was found in 2 control subjects but not in those with Hb E.

As shown in Table 1, among 575 homozygous Hb E, *KLF1* mutations were identified in 48 of them (8.3%). These included heterozygosity for G176AfsX179 ($n = 19$, 3.3%), T334R ($n = 8$, 1.4%), − 154 (C > T) ($n = 7$, 1.2%), R328H ($n = 4$, 0.7%), S270W ($n = 3$, 0.5%), G335R ($n = 3$, 0.5%), A298P ($n = 1$, 0.5%), K307N ($n = 1$, 0.5%), M358I ($n = 1$, 0.5%) and a compound heterozygote for Q217X and − 154 (C > T) ($n = 1$, 0.5%). Among 278 subjects with heterozygous

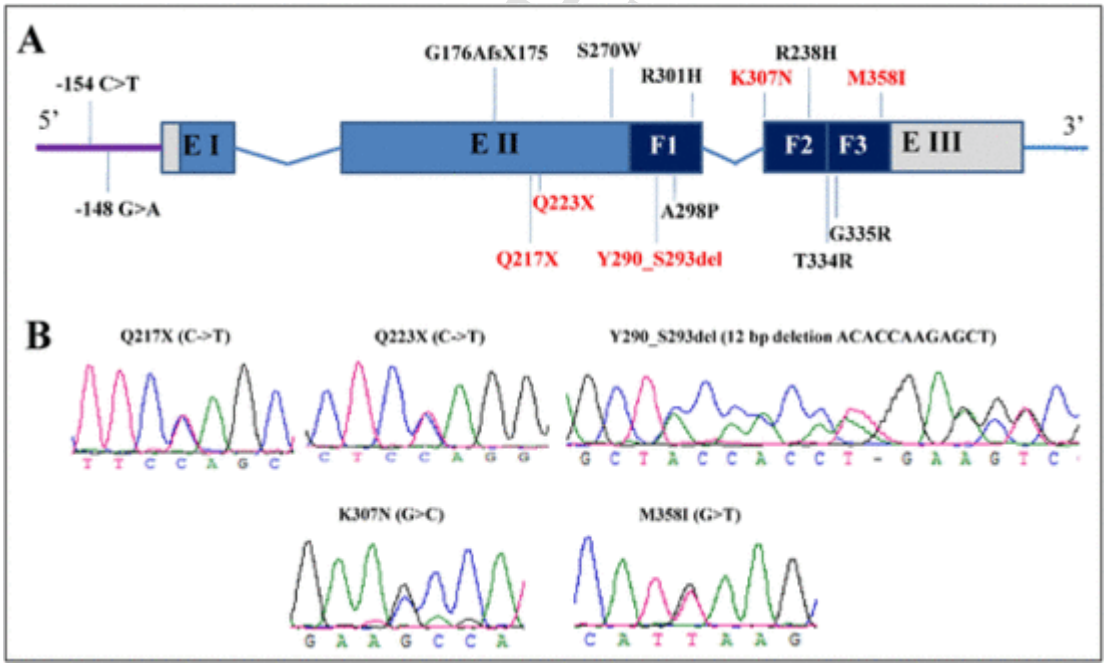

Fig. 1. Schematic of the *KLF1* gene illustrating 14 *KLF1* mutations identified in this study, including 9 known mutations and 5 novel mutations (shown in red). EI, EII & EIII indicate exons 1, 2 and 3, respectively. F1, F2 & F3 are the three zinc finger domains (A). DNA sequencing profile of each novel mutation is shown in B.

Table 1
Hematological characteristics of subjects with homozygous Hb E ($N = 575$), heterozygous Hb E ($N = 278$) and 100 normal control ($N = 100$) according to *KLF1* mutations. Values are presented as median (95% CI) or as raw data where appropriate.

β genotype	*KLF1* mutation	n	(%)	Hb F		Hb A_2		Hb E		Rbc	Hb	Hct	MCV	MCH	MCHC	RDW
				g/l	%	g/l	%	g/l	%	$\times 10^{12}$	g/l	%	fl	pg	g/dl	%
β^E/β^E (575)	G176AfsX179	19	(3.3)	15.4	13.1	7.0	5.4	105.7	84.1	6.2	126.0	38.4	62.0	21.0	34.5	16.7
				(12.3–22.8)	(10.9–18.1)	(5.5–7.3)	(5.0–5.6)	(90.9–112.4)	(72.3–86.8)	(5.0–6.6)	(105.0–135.5)	(30.3–42.7)	(59.2–65.9)	(20.5–21.5)	(32.4–36.1)	(15.5–19.1)
	T334R	8	(1.4)	24.5	15.0	6.0	5.3	107.3	79.8	6.3	128.5	39.1	57.8	19.0	32.5	17.2
				(17.0–27.6)	(12.0–23.3)	(5.2–8.1)	(3.7–6.0)	(86.9–111.0)	(73.1–86.6)	(5.7–6.8)	(119.0–136.4)	(33.0–40.3)	(57.0–66.4)	(18.2–20.8)	(31.7–33.2)	(16.3–20.0)
	− 154 C > T	7	(1.2)	13.9	10.8	5.0	4.7	94.0.3	89.2	6.0	120.5	35.3	69.0	21.0	33.0	19.3
				(6.1–20.0)	(7.2–15.9)	(4.3–7.1)	(3.8–5.2)	(78.5–118.7)	(72.7–92.1)	(5.2–6.5)	(96.2–132.0)	(29.1–41.5)	(61.6–72.3)	(20.2–23.2)	(30.0–36.6)	(18.8–20.3)
	R328H	4	(0.7)	19.7	13.8	6.5	5	111.1	83.6	6.6	137.1	39.8	57.3	20.5	34.8	21.4
				(18.2–23.8)	(8.8–17.4)	(6.1–7.2)	(4.6–7.8)	(109.0–112.8)	(75.2–91.2)	(6.4–6.7)	(131.6–145.3)	(38.5–42.0)	(50.2–65.0)	(16.5–22.7)	(33.9–35.6)	(20.4–22.4)
	S270W	3	(0.5)	12.2	10.8	5.4	4.4	95.3	88.2	5.6	113.2	33.0	64.6	21.7	32.9	17.6
				(10.6–16.6)	(9.9–11.8)	(4.7–5.0)	(4.0–4.8)	(91.8–122.6)	(81.2–89.1)	(5.4–5.8)	(107.5–139.6)	(31.0–34.0)	(57.9–66.0)	(18.6–22.7)	(32.5–35.1)	(14.5–20.7)
	G335R	3	(0.5)	15.8	14.4	5.5	5.7	85.7	85.6	4.6	101.5	30.2	58.5	18.9	31.9	17.1
				(15.0–16.6)	(11.3–17.0)	(5.1–6.3)	(4.8–5.8)	(73.0–98.4)	(83.0–88.7)		(88.7–115.2)	(27.9–32.5)	(57.2–61.1)	(18.5–19.2)	(31.5–32.2)	
	A298P	1	(0.2)	NA	9.1	NA	4.9	NA	90.9				58.8			
	K307N*	1	(0.2)	NA	8.4	NA	NA	NA	86.7							
	M358I*	1	(0.2)	11.5	10.4	5.0	4.8	99.8	89.6	5.5	111.0	32.3	58.6	20.1	34.3	16.7
	Q217X*, − 154 C > T	1	(0.2)	NA	9	NA	5.3	NA	91							
	None	527	(91.6)	4.9	5.9	5.0	4.5	104.4	89.8	5.6	116.4	34.0	62.2	21.0	33.7	
				(3.9–6.0)	(4.6–6.8)	(4.9–5.0)	(4.4–4.5)	(101.6–108.3)	(88.9–90.7)	(5.5–5.8)	(113.8–118.5)	(33.4–35.0)	(61.6–62.7)	(20.9–21.1)	(33.5–34.0)	
β^E/β^A (278)	G176AfsX179	20	(7.2)	11.6	9.1	5.5	4.1	31.9	25.8	5.0	123.6	37.8		25.5		
				(9.0–14.8)	(8.0–11.6)	(4.8–5.9)	(3.7–4.5)	(30.5–34.6)	(22.8–27.3)	(4.7–5.8)	(121.3–134.2)	(36.1–41.1)		(24.5–26.0)		
	T334R	11	(4.0)	8.9	10.2	5.5	3.0	33.0	25.0	5.2	130.2	39.4		24.7		
				(7.1–15.3)	(6.1–11.7)	(3.7–6.6)	(3.4–4.6)	(25.0–34.9)	(23.9–28.5)	(4.3–5.6)	(107.8–138.3)	(33.5–45.1)		(23.6–25.0)		
	Q217X*, − 154 C > T	8	(2.9)	10.3	7.7	4.4	3.8	27.4	22.9	5.6	122.0	37.9		24.3		

Table 1 (*Continued*)

β genotype	*KLF1* mutation	n	(%)	Hb F		Hb A_2		Hb E		Rbc	Hb	Hct	MCV	MCH	MCHC	RDW
				g/l	%	g/l	%	g/l	%	$\times 10^{12}$	g/l	%	fl	pg	g/dl	%
				(6.1–14.1)	(5.7–10.4)	(3.7–4.8)	(3.4–3.8)	(19.6–36.9)	(17.2–27.3)	(4.4–5.9)	(103.5–138.6)	(30.6–42.5)		(17.8–24.7)		
	R301H	2	(0.7)	22.6	14.7–15.5	6.8	3.2	33.70	21.9–16.9	6.0	154.0	45.0	76.0			12.7
	Y290_S293del*	2	(0.7)	7.6.11.4	8.0–5.5	7.0	4.9	34.9–37.9	26.5, 25.3	5.5	143.0, 138.0	42.0, 43.0	75.8, 78.0	25.0		13.4
	G335R	2	(0.7)	12.6.2.1	9.3–1.5	4.7–6.8	3.5, 4.8	34.0–36.5	25.2, 25.9	5.0, 5.3	135.0, 141.0	39.2, 43.0	79.2, 81.4	27.0, 26.8	32.9	13.6, 12.7
	− 154 C > T	1	(0.4)	11.1	7.4	NA	NA	41.0	27.3	5.9	150.0	45.0	76.0	25.6		15.5
	Q217X*	1	(0.4)	24.3	18.3	5.2	3.9	32.19	24.2		133.0	39.8	75.7			
	Q223X*	1	(0.4)	NA	7.4	NA	NA	NA	25.9							
	R328H	1	(0.4)	17.1	10.6	NA	NA	37.7	23.4		161.0	48.3	76.0	25.4	33.3	
	None	229	(82.2)	0.0	0.0	5.1	3.7	38.6	28.4	5.2	136.2	41.0	78.0	26.1	33.5	13.2
				(0.0–0.0)	(0.0–0.3)	(4.9–5.2)	(3.7–3.8)	(37.2–36.9)	(28.1–28.5)	(5.2–5.4)	(133.1–138.9)	(39.2.42.0)	(77.3–78.7)	(25.9–26.4)	(33.4–33.6)	(13.1–13.6)
β^A/β^A (100)	− 148 G > A	2	(2)	0.0.0.6	0.0–0.5	4.4–3.3	2.7.2.7	–	–	5.8, 4.1	152, 123	45.0, 35.4	77.0, 87.1	25.7, 30.3	34.8	14.0, 13.8
	None	98	(98)	0.4	0.4	3.2	2.7	–	–	4.6	122.0	37.0	79.2	26.6	32.8	14.0
				(0.3–0.5)	(0.3–0.5)	(3.0–3.4)	(2.6–2.8)			(4.5–4.8)	(118.2–125.6)	(36.0–37.8)	(77.4–81.3)	(25.8–26.9)	(32.3–33.3)	(13.6–14.3)

* Novel mutation, NA; not available.

Hb E, *KLF1* mutations were identified in 49 of them (17.6%), including heterozygous for G176AfsX179 ($n = 20$, 7.2%), T334R ($n = 11$, 4.0%), − 154 (C > T) ($n = 1$, 0.4%), R301H ($n = 2$, 0.7%), Y290_S293del ($n = 2$, 0.7%), G335R ($n = 2$, 0.7%), Q217X ($n = 1$, 0.4%), Q223X ($n = 1$, 0.4%), R328H ($n = 1$, 0.4%) and a compound heterozygous for the − 154 (C > T) and Q217X ($n = 8$, 2.9%). These *KLF1* mutations were not detected in 100 normal control subjects with Hb F < 1%. However, the 2 subjects in this control group who had Hb F of 0% and 0.5%, were found to carry the − 148 (G > A) mutation. Therefore, in this study population with Hb E, the six most common *KLF1* mutations were the G176AfsX179 (39.4%) followed by the T334R (19.2%), − 154 (C > T) (17.2%), Q217X (9.1%), R328H (5.1%) and G335R (5.1%).

Hematological characteristics of all subjects according to *KLF1* mutations are listed in Table 1. In both Hb E heterozygote and homozygote, it was found that those with *KLF1* mutations had apparently higher Hb F levels as compared to those without the mutation. Since co-inheritance of α-thalassemia could modify the level of Hb F in Hb E [5,6], we therefore excluded cases with α-thalassemia for further analysis. Hematological findings of the remaining 252 subjects with homozygous Hb E and 145 heterozygous Hb E, according to *KLF1* mutations, are summarized in Table 2. 29 Subjects with homozygous Hb E and *KLF1* mutations had significantly higher Hb F levels as compared to those of 223 subjects without *KLF1* mutation, i.e. [16.0 (12.1–19.4 g/l) vs. 6.0 (4.9–7.4 g/l)]. This was also the case for heterozygous Hb E whose Hb F levels were 11.3 (9.6–13.1) g/l and 0 g/l for 33 subjects with and 112 subjects without *KLF1* mutations, respectively. This was clearly observed for those with the G176AfsX179, T334R, − 154 (C > T), S270 W and compound heterozygous for − 154 (C > T) & Q217X mutations. In addition, slight increase in Hb A_2 levels were also noted for those with *KLF1* mutations as compared to those without the mutations, i.e. 6.0 (5.1–6.9) g/l vs. 5.1 (4.8–5.3) g/l in homozygous Hb E and 5.2 (4.7–5.6) g/l vs. 5.0 (4.8–5.2) g/l in heterozygous Hb E.

We further examined the combined effect of *KLF1* mutations and other SNPs, including $^{G}\gamma$ *Xmn*I (rs7482144), *BCL11A* (rs4671393) and *HBS1L-MYB* (rs4895441) & (rs9399137) known to be involved in Hb F expression in our population [9,15]. In Table 3, a significantly higher Hb F levels were observed for homozygous Hb E, who had the same patterns of 4 other SNPs [(rs7482144), (rs4671393), (rs4895441) & (rs9399137)], but with *KLF1* mutations. For example, the level of Hb F of 9.6 (8.7–10.7) g/l was found for homozygous Hb E with SNPs at (rs46713931/rs4895441/rs9399137) and $^{G}\gamma$-XmnI +/+, but no *KLF1* mutation. This was 15.9 (10.8–23.3) g/l for those with similar SNPs and $^{G}\gamma$-XmnI +/+, who had *KLF1* mutation. Likewise, in homozygous Hb E who had the $^{G}\gamma$ *Xmn*I (+/+) but not the 3 other SNPs, elevated Hb F was noted in those with *KLF1* mutation, i.e. Hb F 11.1 (8.8–14.1) g/l as compared to those without *KLF1* mutation [Hb F 2.3 (1.9–3.0) g/l].The pattern of KLF1 mutation and 4 other SNPs as shown in supplementary Table1 and 2. Box plots of Hb F levels in both homozygous and heterozygous Hb E according to all SNPs are presented in Fig. 2. In all the categories, those with *KLF1* mutations had higher Hb F levels.

4. Discussion

KLF1, an erythroid-specific transcription factor plays important roles in hematopoiesis and globin gene switching. *KLF1* mutations have been reported to be associated with hematological disorders, including hereditary persistence of fetal hemoglobin (HPFH), congenital dyserythropoietic anemia type IV, transfusion dependent hemolytic anemia, borderline Hb A_2 and amelioration of clinical severity of β-thalassemia disease [20,22,25,28–32].

We examined *KLF1* mutations and determined their association with phenotypic expression of Hb F, Hb A_2 and other hematological parameters in Hb E disorder. As shown in Table 1, scanning for *KLF1* mutations in 853 Thai subjects with Hb E identified that 48 out of 575 (8.3%) homozygous Hb E and 49 out of 278 (17.6%) heterozygous Hb E had *KLF1* mutations. Interestingly, all of these *KLF1* mutations were not observed among 100 normal control subjects. In contrast, 2 of the 100 subjects in a normal control group were carriers of the − 148 G > A mutation. This *KLF1* promoter mutation was originally found to be associated with atypical HPFH in an adult female of Serbian origin with elevated Hb F (Hb F = 11.0%). The mutation resides in the Sp1 binding site and alters Sp1 binding to *KLF1* promoter, leading to a decreased gene transcription [21]. As shown in Table 1, we found that this mutation was relatively rare and was not associated with increased Hb F and Hb A_2 in Thai population. The two cases with this mutation had normal Hb F (0–0.5%) and Hb A_2 (2.7%) levels. This might reflect the multifunctional role of *KLF1* during erythropoiesis [16,17].

Unlike in normal subjects, *KLF1* mutations are relatively common among Thai individuals with Hb E and elevated Hb F levels. This finding has important clinical implication because the association of Hb E with β-thalassemia endemic in the region, leads to Hb E-β-thalassemia disease with diverse clinical presentations [33,34]. The presence of *KLF1* mutation can likely modify the phenotype of the patient. *KLF1* mutations and increased Hb F production can ameliorate the clinical and hematological features of β-thalassemia disease in Chinese patients [25]. As for Chinese and Korean populations [18,25], the most common *KLF1* mutation in Thai population is the G176AfsX179. It seems likely that this mutation is the most common *KLF1* mutation among Asian population. Interestingly, in addition to nine known mutations, we unexpectedly identified five novel mutations, which were predicted to the loss of gene function in Thai individuals with Hb E (Table 4). These included two nonsense mutations, Q217X and Q223X, which caused premature termination and a haploinsufficient outcome. The two missense mutations, K307N and M358I, located within the conserve region of the zinc finger domains 2 and 3, could result in structurally impaired proteins. The Y290_S293del caused by 12 nucleotides deletion resulted in an in frame deletion of 4 amino acid residues between codons 290–293 and a structurally impaired protein. These results indicated a diverse molecular heterogeneity of *KLF1* gene in Thai population.

The results presented in Table 3 clearly confirmed that in addition to the $^{G}\gamma$ *Xmn*I (rs7482144) and 3 other SNPs, namely *BCL11A* (rs4671393) and *HBS1L-MYB* (rs4895441) & (rs9399137), *KLF1* mutation was an additional genetic modifying factor associated with increased Hb F levels in Hb E disorder. In both homozygous and heterozygous Hb E, those with the formerly known SNPs had higher Hb F levels with the presence of *KLF1* mutation (Fig. 2). Screening of *KLF1* mutations may be useful for routine differential diagnosis of Hb E/β^0-thalassemia and homozygous Hb E with elevated Hb F level [35]. A study in southern China has also revealed that *KLF1* mutations are common in a thalassemia endemic region and can ameliorate the severity of β-thalassemia. The mutations are associated with increased Hbs F and A_2 and decreased CD44 expression [25]. Furthermore, it has been reported that *KLF1* mutations are associated with borderline MCV and MCH values in non-thalassemic individuals and lower MCV and MCH values in α-thalassemia carriers as compared to those without *KLF1* mutations [27]. We also observed lower MCV and MCH values in Hb E heterozygote and homozygote with the *KLF1* T334R and compound heterozygote for − 154 (C > T) and

Table 2
Hematological parameters among 252 homozygous Hb E and 145 heterozygous Hb E without α-thalassemia according to KLF1 mutations. Values are presented as median (95% CI) or as raw data where appropriate.

β genotype	KLF1 mutation	n	Hb F		Hb A2		Hb E		Rbc	Hb (g/dl)	Hct	MCV	MCH	MCHC	RDW
			g/l	%	g/l	%	g/l	%	$x10^{12}$	g/l	%	fl	pg	g/dl	%
β^E/β^E (252)	With mutation	29	16*[c]	12*[c]	6*[b]	5.2*[b]	105.2	84.1	5.8	129.5	36.2	60.9	20.8	33.6	17.2
			(12.1–19.4)	(10.7–14.2)	(5.1–6.9)	(4.7–5.3)	(93.9–112.3)	(81.1–88.3)	(5.5–6.4)	(112.3–132.1)	(33.4–39.4)	(58.2–64.2)	(20.1–21.6)	(32.8–35.4)	(16.1–19.3)
	G176AfsX179	10	16*[c]	13.1*[c]	7.5*[c]	5.4*[b]	108.6	83.2	6.2	133.6	38.4	61.5	21.2	33.6	16.7
			(7.9–26.5)	(9.9–19.0)	(6.6–7.6)	(4.6–6.0)	(96.1–118.0)	(79.6–87.6)	(5.0–6.6)	(113.8–139.3)	(32.5–43.3)	(58.2–64.5)	(20.5–21.7)	(31.4–35.9)	(15.1–17.2)
	T334R	5	21.4.27.5	16.6*[b]	6.8–5.1	5.3*[a]	102.6	80.9	6.8.5.7	129.0117.0	38.8.33.0	57.0.58.0	18.9.20.8	33.2	20.8.20.8
				(12.1–26.9)		(4.3–5.8)	(86.9–119.2)	(73.0–87.9)							
	− 154 C > T	5	15.8*[a]	12*[a]	5.3	4.3	99.0	83.0	6.0	129.6	35.4	68.7	21.0	33.5	19.3
			(4.6–20.6)	(3.5–16.0)	(3.9–6.9)	(3.8–5.2)	(75.4–123.8)	(69.4–93.2)	(5.2–6.5)	(102.8–132.5)	(31.0–44.0)	(57.1–76.0)	(14.2–23.2)	(30.0–36.6)	(18.8–20.3)
	S270W	3	12.2*[a]	10.8*[a]	5.4	4.4	95.3	88.2	5.6	113.2	33.0	64.6	21.7	32.9	17.6
			(10.6–16.6)	(9.9–11.8)	(4.7–5.0)	(4.0–4.8)	(91.8–122.6)	(81.2–89.1)	(5.4–5.8)	(107.5–139.6)	(31.0–34.0)	(57.9–66.0)	(18.6–22.7)	(32.5–35.1)	(14.5–20.7)
	R328H	2		8.8, 13.9	7.2		117.1	86.1.91.2		136.0	39.8	50.2	16.5	33.9	
	K307N	1	NA	8.4	NA	NA	NA	76.7							
	M358I	1	11.5	10.4	5.328	4.8	99.5	89.6		111.0	32.3	58.6	20.1	34.3	16.7
	A298P	1	NA	9.1	NA	4.9	NA	90.9				58.8			
	− 154 C > T, Q217X	1	NA	9	NA	5.3	NA	91.1							
	Without mutation	223	6	6.8	5.1	4.4	103.6	90.4	5.6	113.2	33.4	62	20.9	33.9	17.6
			(4.9–7.4)	(5.7–8.0)	(4.8–5.3)	(4.3–4.5)	(99.1–107.2)	(89.5–91.7)	(5.3–5.8)	(108.6–117.1)	(32.1–35.0)	(61.2–62.7)	(20.6–21.1)	(33.5–34.1)	(14.5–20.7)
β^E/β^A (145)	With mutation	33	11.3*[c]	8.2*[c]	5.2*[a]	4.0*[a]	32.8	25.8	5.0	131.2	39.2	76.0	25.0	32.8	14.4
			(9.6–13.1)	(7.5–10.3)	(4.7–5.6)	(3.8–4.3)	(31.2–35.7)	(24.2–26.9)	(4.7–5.3)	(122.0–135.4)	(36.4–41.8)	(75.2–78.6)	(24.6–25.8)	(32.1, 33.4)	(13.8–15.2)
	G176AfsX179	15	11*[c]	8.2*[c]	5.4*[a]	4.1*[a]	31.9	25.8	5	123	38	78.8	25.7	32.5	15
			(8.4–15.4)	(7.5–11.1)	(4.7–5.9)	(3.7–4.5)	(30.9–35.7)	(22.1–27.1)	(4.7–5.8)	(121.5–135.2)	(36.1–42.3)	(75.6–81.4)	(24.6–26.0)	(31.7, 33.6)	(13.7–15.3)
	T334R	7	12.3*[b]	10.6*[b]	5.8*[a]	4.0*[a]	32.2	27.4	4.6	123.4	34.0	74.6	24.8	33.0	14.5
			(7.1–16.0)	(7.7–11.9)	(4.9–6.6)	(3.8–4.3)	(25.2–43.2)	(23.9–28.6)	(4.3–4.9)	(105.6–157.1)	(33.0–47.0)	(70.0–77.1)	(23.7–25.0)	(31.22, 34.1)	(13.9–15.0)

Table 2 (*Continued*)

β genotype	KLF1 mutation	n	Hb F		Hb A2		Hb E		Rbc	Hb (g/dl)	Hct	MCV	MCH	MCHC	RDW
			g/l	%	g/l	%	g/l	%	$x10^{12}$	g/l	%	fl	pg	g/dl	%
	− 154C > T, Q217X	5	11.2*[b]	8.2*[b]	4.5	3.8	31.6	25.3	5.2	123.0	37.9	75.2	24.5	33.2	14.4
			(10.2–15.2)	(6.3–13.9)	(3.7–4.8)	(3.0–3.8)	(23.9–39.2)	(21.9–27.8)	(4.4–5.9)	(109.8–144.3)	(31.8–44.0)	(72.4–75.7)	(24.3–25.0)	(32.3, 34.1)	(14.2–16.9)
	G335R	2	NA	9.5.1.5	NA	3.5, 4.8	NA	25.2.25.9				79.2, 81.4	27.0, 26.8		
	− 154C > T	1	11.1	7.4	NA	NA	41.0	27.3	5.9	150.0	45.0	76.0	25.6		15.5
	Q217X	1	24.3	18.3	5.2	3.9	32.2	24.2		133.0	39.8	75.7			
	Q223X	1	NA	7.4	NA	NA	NA	25.9							
	Y290_S293del	1	11.4	8.0	7	4.9	37.9	26.5		143.0	42.0	75.8			
	Without mutation	112	0	0	5.0	3.7	38.6	28.6	5.2	136.0	41.0	77.3	26.0	33.5	14.3
			(0.0–0.1)	(0.0–0.4)	(4.8–5.2)	(3.6–3.8)	(36.9–40.2)	(28.4–28.8)	(5.1–5.3)	(132.3–138.1)	(39.0–42.0)	(76.6–78.4)	(25.7–26.2)	(33.3, 33.7)	(13.9.15.6)

NA; not available

* Significant difference from those without *KLF1* mutation (a; $P < 0.05$, b; $P < 0.01$, c; $P < 0.001$; Mann–Whitney *U* test).

Table 3
Hb F levels of 252 subjects with homozygous Hb E and 145 subjects with heterozygous Hb E without α-thalassemia, presented according to *KLF1* and other known genetic polymorphisms.

β genotype	*KLF1*	$^{G}\gamma$-*Xmn* I	BCL11A (rs4671393),	n	Hb F levels			
		(rs7482144)	HBS1L-MYB (rs4895441),		%		g/l	
			HBS1L-MYB (rs9399137)		median	(95% CI)	median	(95% CI)
β^E/β^E (252)	No (223)	−/−	−	5	1.3	(0.5–2.3)	1.4	(0.7–2.8)
			+	5	2.4	(0.7–8.1)	4.3	(0.6–11.6)
		+/−	−	21	2.2	(1.1–4.6)	2.3	(1.1–5.5)
			+	47	7.0	(4.8–9.4)	6.0	(4.9–8.2)
		+/+	−	38	2.3	(1.9–3.0)	2.4	(2.1–3.3)
			+	107	9.6	(8.7–10.7)	9.8	(8.0–11.7)
	Yes (29)	−/−	−	1	12.1	−	NA	−
			+	1	12.2	−	15.8	−
		+/−	−	3	7.9	(4.3–13.0)	11.1	(4.5–17.6)
			+	6	13.5*[a]	(8.9–23.0)	18.2*[b]	(13.2–22.4)
		+/+	−	10	11.1*[b]	(8.8–14.1)	14.2*[b]	(6.7–19.6)
			+	8	15.9*[a]	(10.8–23.3)	16.1*[a]	(11.9–27.8)
β^E/β^A (145)	No (112)	−/−	−	12	0.0	(0.0–0.0)	0.0	(0.0–0.0)
			+	12	0.0	(0.0–0.0)	0.0	(0.0–0.1)
		+/−	−	37	0.0	(0.0–0.0)	0.0	(0.0–0.0)
			+	36	0.4	(0.0–2.4)	0.5	(0.0–2.1)
		+/+	−	3	1.3	(0.6–3.9)	1.9	(0.8–4.5)
			+	12	7.7	(2.8–10.8)	9.0	(1.8–13.9)
	Yes (33)	−/−	+	3	13.9	(7.4–18.3)	19.8	(15.2–24.3)
		+/−	−	1	3.7	−	5.0	−
			+	23	9.0*[b]	(7.7–10.3)	11.6*[b]	(9.7–15.2)
		+/+	+	6	8.1	(7.2–11.3)	9.9	(8.5–11.4)

NA; not available

* Significantly higher than those without *KLF1* mutations (a; $P < 0.01$, b; $P < 0.001$; Mann–Whitney *U* test).

Q217X mutations (Table 2). This result confirmed that some *KLF1* mutations could affect RBC indices. Therefore, screening of *KLF1* mutations in a β-thalassemia patient with unusual phenotype is recommended [25].

It has been found that compound heterozygosity for *KLF1* mutations can cause diverse clinical symptoms such as transfusion-dependent hemolytic anemia and persistence of embryonic globin gene expression [29], microcytic hypochromic anemia with increased Hb F in children [31] and hydrops fetalis in neonates [36]. We also detected compound heterozygosity for the − 154 (C > T) and a novel Q217X mutations in 8 subjects with heterozygous Hb E and a subject with homozygous Hb E (Table 1). However, apart from elevated Hb F and Hb A_2, all of them were generally healthy and had no clinical symptom. Similar findings have been reported in other populations for compound heterozygote of other mutations, i.e. (G176AfsX179/L51R) and (S270X/K332Q) [18,19]. These findings have suggested that compound heterozygote for *KLF1* mutations could be associated with both benign and severe clinical conditions. The phenotypic variation may be related to the age of the patient, type of *KLF1* mutations and the amount of *KLF1* protein synthesized. Further study is required to clarify this matter.

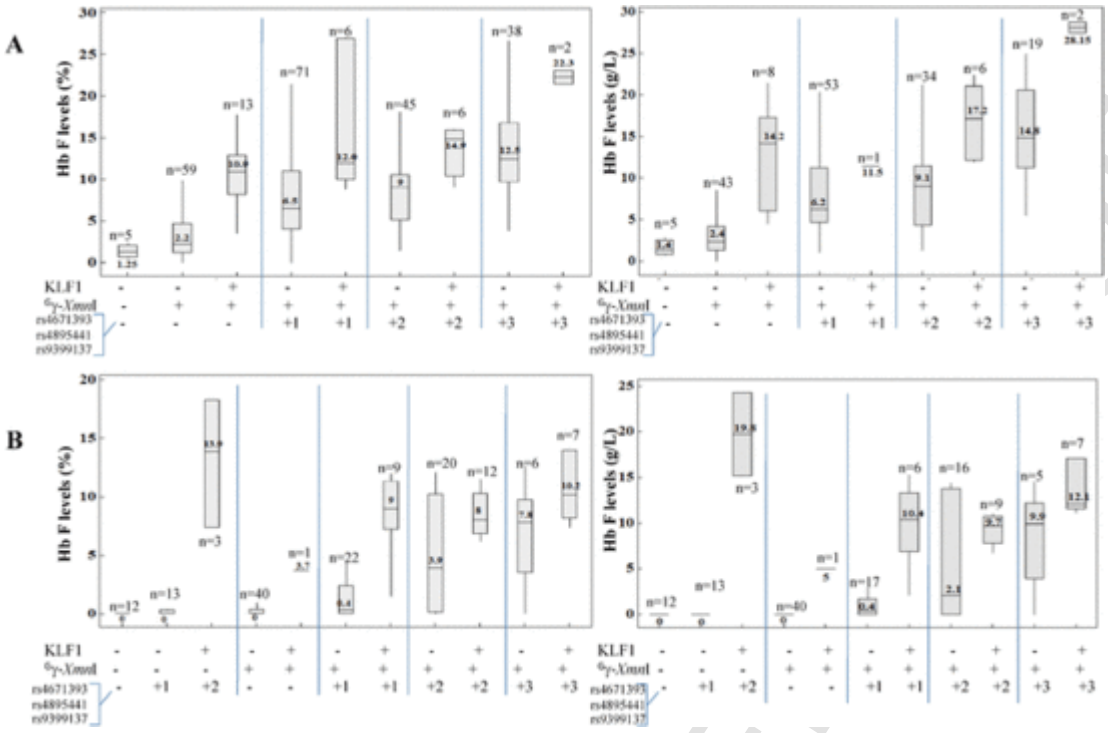

Fig. 2. Box plots showing Hb F levels (% & g/l) of 252 homozygous Hb E (A) and 145 heterozygous Hb E (B) without α-thalassemia according to genetics polymorphisms, including *KLF1* mutation, ^{G}γ-*Xmn*I polymorphism (rs7482144) and the 3 SNPs; *BCL11A* (rs4671393), *HBS1L-MYB* (rs4895441) and (rs9399137). + and − indicate the presence and absence of each polymorphism, respectively.

Table 4
Functional predictions of five novel *KLF1* mutations.

KLF1 mutation	Description	Functional prediction
Q217X	*C*AA-*T*AA, premature termination	Haploinsufficiency
Q223X	*C*AA-*T*AA, premature termination	
K307N	Amino acid substitution (lysine to asparagine) in a conserve region between the zinc finger domains 2 and 3	Structurally impaired *KLF1* proteins
M358I	Amino acid substitution (methionine to isoleucine) in a conserve region of the zinc finger domain 3	
Y290_S293del	In frame deletion of 4 amino acid residues (YTKS) within the zinc finger domain 1	

The wide range of Hb F expression from 3.5–27.6% observed among homozygous Hb E points to further study on the combined effect of *KLF1* mutations and other genetic modifying factors. These include a T allele of the ^{G}γ *Xmn*I (rs7482144), A allele of the *BCL11A* (rs4671393), G allele of the *HBS1L-MYB* (rs4895441) and C allele of the *HBS1L-MYB* (rs9399137) [15]. Fig. 2 demonstrates as expected the additive effects to these 4 genetic modifiers of *KLF1* mutations for higher expression of Hb F in both homozygous and heterozygous Hb E. The results from this study confirm that *KLF1* is an essential modulator of Hb F expression in Hb E and *KLF1* in combination with other genetic modifiers could explain the variability of Hb F expression of this common hemoglobinopathy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bcmd.2016.04.010.

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Molecular understanding of non-transfusion dependent thalassemia associated with Hb E - beta - thalassemia in northeast Thailand.

Supawadee Yamsri[1], Naruwat Pakdee[2], Goonnapa Fucharoen[1], Kanokwan Sanchaisuriya[1], Supan Fucharoen[1]

[1] Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand

[2]Department of Thai Traditional Medicine, Faculty of Natural resources, Rajamangala University of Technology Isan, Sakon Nakhon Campus, Sakon Nakhon, Thailand

Address all correspondence to:

Dr. Supan Fucharoen

Centre for Research and Development of Medical Diagnostic Laboratories,

Faculty of Associated Medical Sciences,

Khon Kaen University, Khon Kaen, Thailand 40002

Tel/Fax +66-43-202083 e-mail: supan@kku.ac.th

Abstract

Background Hb E-β-thalassemia is the most common form of β-thalassemia syndromes in Thailand. The disease exhibits varied clinical expression from asymptomatic to severe β-thalassemia syndrome. Hemoglobin F levels also are variable. We studied the genetic modifying factors of Hb E-β-thalassemia patients with non-transfusion dependent thalassemia (NTDT) in northeast Thai.

Method Subjects were 73 adult Hb E-β^0-thalassemia patients with NTDT encountered at our prenatal diagnosis center at Khon Kaen University, Thailand. Hematological parameters were collected and Hb analysis was obtained from automated Hb analyzer based on HPLC or capillary electrophoresis techniques. The α- and β-thalassemia mutations, $^{G}\gamma$-*Xmn* I polymorphism and 7 single nucleotide polymorphisms (SNPs) including rs2297339, rs2838513, rs4895441 and rs9399137 in *HBS1L-MYB* gene, rs4671393 and rs11886868 in BCL11A gene, and G176AfsX179 in KLF1 gene were identified. Expression Hb F was present as Hb F level in g/L unit.

Results Six β^0-thalassemia mutations *in trans* to the β^E gene were identified including $\beta^{CD41/42}$ (n=38), β^{CD17} (n=26), $\beta^{CD71/72}$ (n=3), $\beta^{IVS1\#1}$ (n=3), β^{CD43} (n=2) and $\beta^{IVS1\#5}$ (n=1). No significant difference of hematological parameters was observed among each β^0-thalassemia genotype. As much as 42.5% (n=31) of the subjects were co-inherited with α-thalassemia. Hb F expressions of the patients who co-inherited with α-thalassemia were significant lower than those of normal α-globin genotype. The study of 8 SNPs demonstrated that 4 SNPs including rs7482144 of $^{G}\gamma$-*Xmn*I, rs2297339, rs4895441 and rs9399137 of HBS1L-MYB was significant associated with high Hb F level. The alleles of these 4 SNPs which responsible to high Hb F expression were T, T, G and T of rs7482144, rs2297339, rs4895441 and rs9399137, respectively.

Conclusion The results demonstrated that there are multiple factors associated with high Hb F expression including rs7482144 of $^{G}\gamma$-*Xmn*I, rs2297339, rs4895441 and rs9399137 of HBS1L-MYB in northeast Thai Hb E-β^0-thalassemia patients with NTDT.

Keywords: Genetic factors, Hb E-β^0-thalassemia, Hemoglobin F expression, HBS1L-MYB, BCL11A, KLF1

Introduction

Hemoglobin E-β-thalassemia is the most common thalassemia diseases found in northeast Thailand (1). The phenotypic expression of this condition is varying ranging from mild thalassemia intermedia to severe transfusion dependent (2-8). Type of β-thalassemia mutations were found to be the primary modifying factor of the disease severity. Hb E-β-thalassemia resulting from β^+-thalassemia mutations usually has milder phonotype when compared with those of β^0-thalassemia mutations (9, 10). However, it is difficult to predict the phenotype of Hb E-β^0-thalassemia patient based on type of β^0-thalassemia mutations (9). The study in patients with non-transfusion dependent (NTDT) was also demonstrated that β^0-thalassemia mutations are common among Hb E-β-thalassemia diseases. It is indicated that there are other genetic factors underlying for the clinical severity of these patients (11). The remarkable phenotypic diversity of Hb E-β-thalassemia patients is also associated with coinheritance of α-thalassemia or the presence of genetic determinants associated with increased production of γ-globin chains and consequent ability to produce functional fetal hemoglobin (Hb F) in adult life (12). The presence of variation in Hb F level in patient with affected disease is also reported (2). The level of Hb F is regulated by three major loci: HBG2:g.-158C>T on 11p15.4 namely $^{G}\gamma$ *Xmn* I polymorphism, HBS1L-MYB intergenic region on 6q23.3 and BCL11A on 2p16.1. Polymorphisms on these three loci are responsible for Hb F variation in patients with β-thalassemia or sickle cell disease and in healthy Europeans (13-17). The studies in sickle cell and β-thalassemia indicated that $^{G}\gamma$ *Xmn* I polymorphism are associated with high Hb F production (16). Additionally, polymorphisms in *HBS1L*-MYB gene are associated with severity and Hb F level in individuals of Chinese and Thai descent with Hb E-β-thalassemia (18). The associated of $^{G}\gamma$ *Xmn* I polymorphism, HBS1L-MYB and BCL11A with increased Hb F production was recently demonstrated in

homozygous Hb E syndrome in Thai population (19). Other resent study also demonstrated that KLF1 mutations play a role as modulators for Hb F expression among homozygous Hb E syndrome in northeast Thai subjects (20). However, there is no information about the effect of KLF1 mutations on Hb F production in Hb E-β^0-thalassemia patients with NTDT. To explain about the ameliorating factors for hematological phenotypic expression of Hb E-β^0-thalassemia patients with NTDT, the genetic factors as well as single nucleotide polymorphisms were focused. Here, we studied on single nucleotide polymorphisms including HBG2:g.-158C>T, HBS1L-MYB, BCL11A and KLF1 variants on Hb F expression among Hb E-β^0-thalassemia patients with NTDT in northeast Thailand.

Materials and methods

Subjects

Subjects were pregnant women and their husbands who were encountered at Thalassemia unit, the Centre for Research and development of Medical Diagnostic Laboratories, Khon Kaen University, for thalassemia prevention and control program. Total of 73 subjects (28 males and 45 females) with non transfusion dependent Hb E-β^0-thalassemia genotypes were recruited. Ethical approval of the study protocol was obtained from the Institution Review Board of Khon Kaen University, Thailand (HE451007).

Hematological analysis

Hematological parameters were collected using an automate blood cell counter (Coulter STKs; Beckman Coulter Co., Fullerton, CA, USA). Hb profiles were obtained from hemoglobin analysis which done using automated Hb analyzer based on high pressure liquid chromatography (HPLC) (Variant^{TM}, Bio-Rad Laboratories, Hercules, CA, USA) and capillary electrophoresis (Capillarys 2, Sebia, France). The Hb F level (g/L) was obtained from Hb F percentage multiplied by the total hemoglobin level (21).

α- and β-globin genotyping

Identification of common α-thalassemia including a including --^{SEA}, --^{THAI}, -$\alpha^{3.7}$, -$\alpha^{4.2}$, Hb Constant Spring and Hb Paksé were carried out using PCR related techniques as

previously described (22, 23). Screening of common β-thalassemia mutations and Hb E mutation were performed using ASPCR as described elsewhere (24).

SNPs gene genotyping

$^{G}\gamma$ *Xmn* I (rs7482144) genotyping was done using PCR-RFLP technique. The PCR amplification of $^{G}\gamma$-globin gene promoter was performed and followed by digestion with the *Xmn* I restriction enzyme (25). Identification of SNP (rs 2297339) in HBS1L gene located on chromosome 6q23 was performed using the PCR with primers G62 (5' GGC AAC ACT GAC GAG AAA C 3') and G63 (5' ATT CTA GGC GGC GGA TTT C 3'). The 465 bp amplicon was digested with *Apa*L I (New England Biolabs, Massachusetts, USA) restriction enzyme (18). Determining of the four SNPs consisting rs2838513 & rs4895441 in HBS1L gene and rs4671393 & rs11886868 in BCL11A gene were performed by real-time PCR method using LightCycler® 480 (Roche Diagnostics, USA). To investigate the SNP rs9399137, direct sequencing on ABI Prism 3130 XL (Applied Biosystems, Courtaboeuf, France) was performed. Identification for G176AfsX179 in exon 2 of KLF1 gene was carried out using allele specific PCR as described elsewhere (20)

Statistical analysis

Descriptive statistic including Mean and SD were used to describe hematological parameters of the subjects. To compare the differences, Mann-Whitney U test and Kruskal-Wallis test were done using the Minitab statistical software (Minitab Inc., State College, Pa., USA). A statistical significant was considered at P-value < 0.05. The observed genotype frequencies of each SNP were tested for the Hardy–Weinberg equilibrium (HWE) assumptions.

Results

According to molecular analysis of β-globin genotypes, six β^0-thalassemia mutations were identified including CD 41/42, CD 17, CD 71/72, CD 43, IVSI#1 and IVSI#5. The most common β^0-thalassemia mutations found was CD 41/42 which was detected in 52.1% of the subjects followed by CD 17 which was detected in 36.6%, CD 71/72 (4.1%), IVSI#1 (4.1%), D 43 (2.7%) and IVSI#5 (1.4%), respectively. Hematological parameters of the subjects were demonstrated mild thalassemia intermedia phenotypes, which the hemoglobin levels ranging from 6.9 to 8.9 g/dl. No significant difference of the hematological characteristics was observed among each β-globin genotype (Data not shown).

Among 73 Hb E-β^0-thalassemia patients, as much as 31 subjects (42.5%) were co-inherited with α-thalassemia. α-globin genotypes were categorized into 4 groups; normal, 1-α-gene defect, 2-α-gene defect, and 3-α-gene defect, as shown in **Table 1**. It is revealed that Hb level of Hb E-β^0-thalassemia patients which co-inherited with α-thalassemia is higher than those with normal α-globin genotypes (*P*-value = 0.008). MCV and MCH values of Hb E-β^0-thalassemia patients with 1-α-gene defect genotype were statistical significant lower than those with normal α-globin gene genotype. The significant different of Hb F level (g/L) among Hb E-β^0-thalassemia patients with 1-α-gene defect genotype and normal α-globin gene genotype was observed. It is demonstrated that Hb F level was decreased according to number of α-globin gene defects. The association of Hb F level among Hb E-β^0-thalassemia patients co-inherited with and without with α-thalassemia is illustrated in **Figure 1A**.

Hb E-β^0-thalassemia patients with normal α-globin gene genotype were selected for further SNPs analysis. Total of 8 SNPs including the rs7482144 of $^{G}\gamma$-*Xmn*I, 4 SNPs in HBS1L-MYB (rs2297339, rs2838513, rs4895441 and rs9399137), 2 SNPs in BCL11A (rs4671393 and rs11886868) and 1 SNP in KLF1 (G176AfsX179) were studied. As shown in **Table 2**, Seven out of 8 SNPs, excepted rs7482144 of $^{G}\gamma$-*Xmn*I, meet the assumption of Hardy–Weinberg Equilibrium (HWE) of the studied population (χ^2 test; $P > 0.05$). When compared Hb F level (g/L), it is interesting that significant differences in 4 out of 8 SNPs were observed including rs7482144 of $^{G}\gamma$-*Xmn*I, rs2297339, rs4895441 and rs9399137 of HBS1L-MYB (*P*-value = 0.015, 0.023, 0.045 and 0.020, respectively). The alleles of these 4 SNPs which responsible to high Hb F expression were T, T, G and T of rs7482144,

rs2297339, rs4895441 and rs9399137, respectively. The effect of these 4 SNPs on Hb F level (g/L) were also presented as interval plot as shown in **Figure1 B-E**.

Discussion

This study was performed on 73 Hb E-β^0-thalassemia patients with NTDT. As expected, the molecular analysis of β-thalassemia mutations revealed that CD 41/42 was the most common β^0-thalassemia mutations, followed by CD 17. There no different effect of these β^0-thalassemia mutations on the hematological phenotypes. And the data also indicated that these Hb E-β^0-thalassemia subjects exhibit thalassemia intermedia phenotype which Hb levels were ranged from 6.9 to 8.9 g/dL. When searching for genetic modulating factors in these patients such as coinheritance with α-thalassemia. It was demonstrated that as much as 31 subjects could be explained by these factors. The result demonstrated as expected that coinheritance of α-thalassemia could reduce severity of Hb E-β^0-thalassemia, similar to several previous studies (8, 26, 27). The mean Hb level of subject with normal, 1-α-gene defect, 2-α-gene defect, and 3-α-gene defect were 7.6, 8.7, 10.9 and 6.7 g/dl, respectively. These findings suggested that coinheritance of α-thalassemia resulted in reducing number of unpaired α-globin chain, which could be expected to lead to more balanced globin chain synthesis and milder phenotype (28). Other red blood cell parameters that could be altered by coinheritance of α-thalassemia were MCV and MCH values. MCV and MCH values in the patients with α-thalassemia were significantly decreased. Significantly reducing in Hb F level in association with the presence of α-thalassemia was also observed (Table 1), it might be due to the reduction of α-globin chain production resulting from the coinheritance of α-thalassemia.

The remaining 42 non-transfusion dependent Hb E-β^0-thalassemia subjects with normal α-globin genotype were selected further for identification of other genetic modifying factors. SNPs in HBS1L-MYB intergenic region, BCL11A and KLF1 were selected based on previous studies which showed the association with high Hb F levels (19-21, 29). The result demonstrated that rs7482144 of $^{G}\gamma$-*Xmn*I, rs2297339, rs4895441 and rs9399137 of HBS1L-MYB were associated with high Hb F expression. This study showed that *Xmn*I T allele was strongly associated with high Hb F production in these Hb E-β^0-thalassemia patients with NTDT as shown in Table 2 and Figure 1B. This result was similar to previous studies in various populations (15, 30, 31). The T, G and T of rs2297339, rs4895441 and

rs9399137 in HBS1L-MYB were also associated with high Hb F expression similar in our previous study in homozygous Hb E syndrome (19). The study was also found that one case which positive for KLF1 mutation seem to have high Hb F level. However, due to the small number of subjects investigated, the effect of these SNPs on Hb F expression could not be made. The larger numbers of subjects were required. It is concluded therefore that rs7482144 of $^{G}\gamma$-*Xmn*I, rs2297339, rs4895441 and rs9399137 of HBS1L-MYB were one of genetic factors that responsible for Hb F expression and could be explained about 93% of Hb E-β^0-thalassemia patients with NTDT in our study. However, our data suggests that there are other genetic modifying factors need to be investigated.

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Disclaimer statements

There is no conflict of interests to declare.

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Figure legends

Figure 1

Interval plot of Hb F level (g/L) among Hb E-β-thalassemia patients with NTDT according to genetic factors. A: α-globin genotypes, B: ^{G}γ-*Xmn*I; rs2297339, C: HBS1L-MYB; rs4895441 and D: HBS1L-MYB; rs9399137

Figure 2 The proportion of subjects with and without 4 informative SNPs including rs7482144 of ^{G}γ-*Xmn*I, rs2297339, rs4895441 and rs9399137 of HBS1L-MYB

Table 1 Hematological parameters among 73 Hb E - β^0 - thalassemia patients with NTDT according to α-globin genotypes

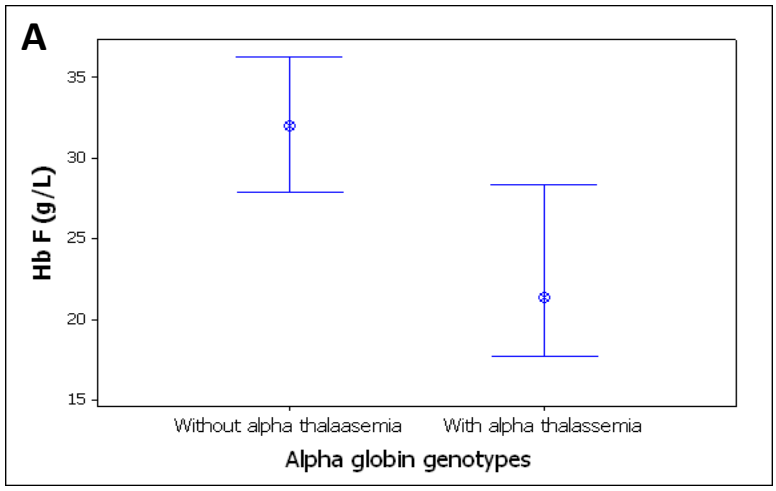
Parameters	α-globin genotypes				*P*-value[c]
	Normal	1-α-gene defect[a]	2-α-gene defect[b]	3-α-gene defect	
N (%)	42 (57.5)	26 (35.6)	4 (5.5)	1 (1.4)	-
(Male:Female)	(14:28)	(11:15)	(3:1)	(0:1)	-
RBC(x10^{12}/l)	3.7 ± 0.8	4.8 ± 0.9	5.9 ± 1.8	4.41	0.00001
Hb (g/dl)	7.6 ± 1.4	8.7 ± 1.5	10.9 ± 2.2	6.7	0.008
Hct (%)	23.7 ± 3.8	26.1 ± 4.5	32.1 ± 7.0	23.8	0.08
MCV (fl)	65.8 ± 8.3	56.7 ± 7.0	55.9 ± 5.2	54	0.0001
MCH (pg)	20.3 ± 3.0	18.2 ± 1.8	19.1 ± 2.3	15.1	0.0005
MCHC (g/dl)	31.2 ± 4.2	32.8 ± 2.7	34.2 ± 1.5	28.2	0.0569
RDW (%)	29.2 ± 4.6	29.5 ± 2.3	23.3 ± 5.1	23.3	0.8506
Hb E (%)	52.2 ± 11.1	66.8 ± 12.2	56.4 ± 21.9	73	0.00001
Hb F (%)	40.0 ± 12.4	25.7 ± 11.2	27.2 ± 19.8	15.4	0.0001
Hb F (g/L)	33.1 ± 10.6	24.4 ± 11.8	16.9 ± 14.5	10.3	0.009

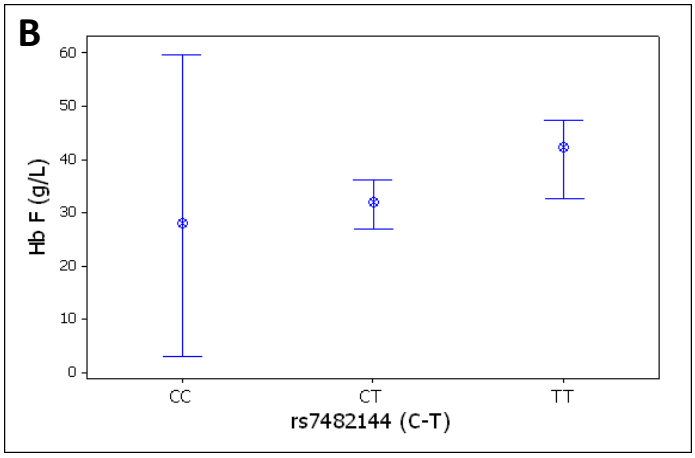
a: one α gene defect including - $\alpha^{3.7}/\alpha\alpha$ (n=20), - $\alpha^{4.2}/\alpha\alpha$ (n=2),and $\alpha^{CS}\alpha$ /αα (n=4)
b: two α genes defect including - $-^{SEA}/\alpha\alpha$ (n=3) and - $\alpha^{3.7}/-\alpha^{3.7}$ (n=1)
c: Significant different between normal and 1-α-gene defect genotypes (Mann-Whitney U-test)
Data are given as mean ± SD

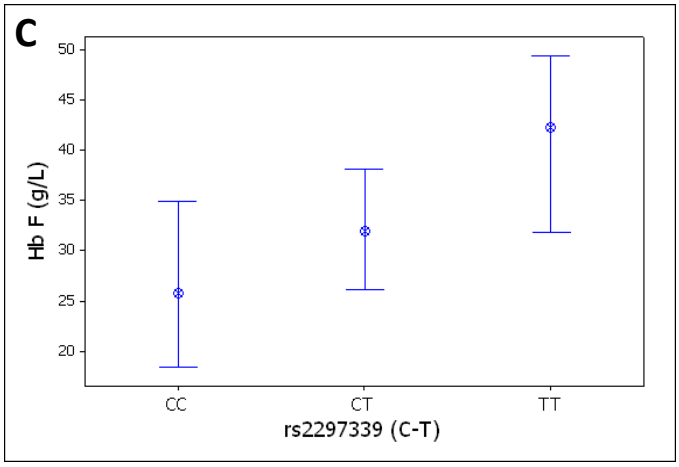
Table 2 Genotype frequencies and median of Hb F level (g/L) of each single nucleotide polymorphism (SNP) among 42 Hb E - β^0 - thalassemia patients with NTDT

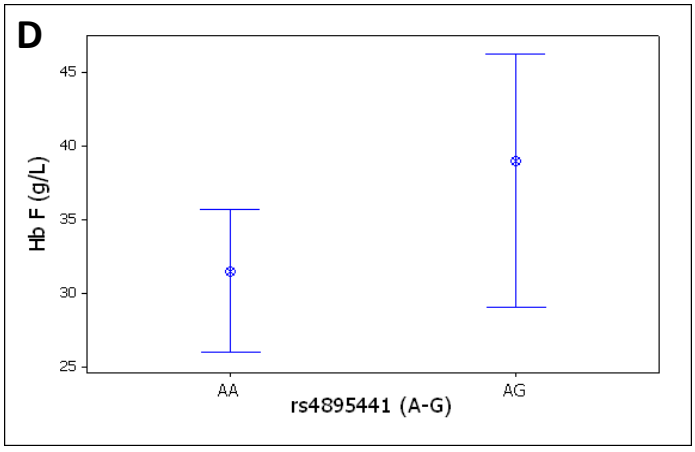
SNPs		Genotype	n	(%)	95% CI	HWE equilibrium[a]	Hb F (g/L)	*P-value*[b]
Gγ-XmnI	rs7482144 (C-T)	CC	4	9.5	2.7- 22.6	0.011	28	0.015
		CT	30	71.4	55.4- 84.3		32	
		TT	8	19.0	8.6- 34.1		42.3	
HBS1L-MYB	rs2297339 (C-T)	CC	11	26.2	13.9- 42.0	0.758	25.8	0.023
		CT	20	69.0	32.0- 63.6		31.5	
		TT	11	26.2	13.9- 42.0		42.3	
	rs2838513 (G-A)	GG	4	9.5	2.7- 22.6	0.382	23.1	0.265
		AG	12	28.6	15.7- 44.6		33.6	
		AA	26	61.9	45.6- 76.4		34.3	
	rs4895441 (A-G)	AA	26	61.9	45.6- 76.5	0.127	30.6	0.045
		AG	16	38.1	23.6- 54.4		39	
		GG	0	-	-		-	
	rs9399137 (T-C)	TT	25	59.5	43.3- 74.4	0.100	30.2	0.020
		CT	17	40.5	25.6- 56.7		39.1	
		CC	0	-	-		-	
BCL11A	rs4671393 (A-G)	AA	1	2.4	0.1- 12.6	0.853	44.5	0.514
		AG	12	28.6	15.7- 44.6		29.1	
		GG	29	69.0	52.9- 82.4		34.3	
	rs11886868 (C-T)	CC	36	85.7	71.5- 94.6	0.618	31	0.357
		CT	6	14.3	5.4- 28.5		37.8	
		TT	0	-	-		-	
KLF1	G176AfsX179 (7-bp insertion)	Wt/Wt	41	97.6	87.4- 99.9	0.998	31.4	na
		Wt/+7 bp	1	2.4	0.1- 12.6		50.4	
		+7 bp/+7 bp	0	-	-		-	

a: χ^2 test to check the Hardy–Weinberg Equilibrium (HWE). A p value>0.05 means that population meets the assumption the HWE
b: Kruskall–Wallis test between the median of Hb F level (g/L) and each genotype
Wt: wild type, +7 bp:7-bp insertion, na: not available









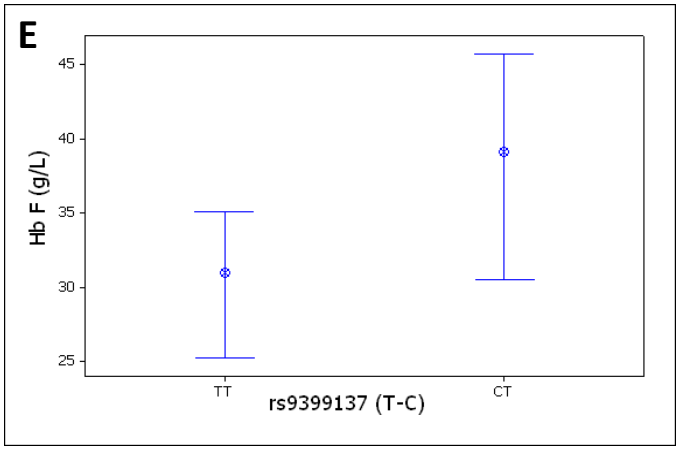


Figure 1

Interval plot of Hb F level (g/L) among Hb E-β-thalassemia patients with NTDT according to genetic factors. A: α-globin genotypes, B: Gγ-*XmnI; rs2297339, C: HBS1L-MYB; rs4895441 and D: HBS1L-MYB; rs9399137*

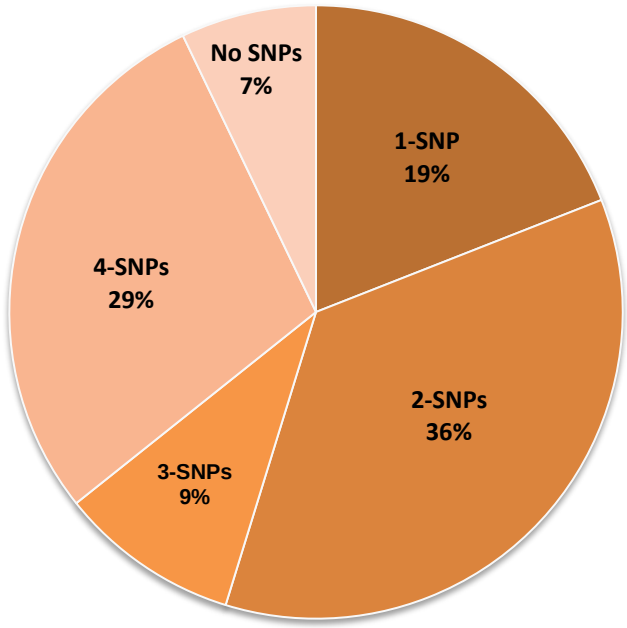


Figure 2 The proportion of subjects with and without 4 informative SNPs including rs7482144 of Gγ-*XmnI, rs2297339, rs4895441 and rs9399137 of HBS1L-MYB*

PS-18

KLF1 mutations and variability of hemoglobin F expression in homozygous Hb E

W. Tepakhan[1,2], S. Yamsri[2], G. Fucharoen[2], K. Sanchaisuriya[2], S. Fucharoen[2]

[1]Biomedical Science Program, Graduate School and [2]Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

Hemoglobin (Hb) E is the most common Hb variant found in Thailand and the frequency of 40-50% has been noted in northeastern part of the country. Heterozygous Hb E is asymptomatic and has low production of Hb F. Homozygous Hb E is however associated with mild hypochromic microcytic Rbc and variable Hb F expression. We have examined multiple single nucleotide polymorphisms (SNPs) in the KLF1; an erythroid specific transcription factor and determined their associations with Hb F expression in homozygous Hb E. Study was done on 462 homozygous Hb E subjects and 100 normal control subjects. Allele specific PCR assays were developed for identification of three KLF1 mutations including T334R, R238H and -154 (C>T). The results showed that none of these three mutations were observed among 100 normal control subjects. Among 462 subjects with homozygous Hb E, 307 had high Hb F levels (≥ 5%), 155 had lower Hb F levels (< 5%). DNA analysis identified the KLF1 mutations in 18 cases with high Hb F, including the T334R mutation (9/307 = 2.9%), -154 (C>T) mutation (7/307= 2.3%) and R328H mutation (2/307 = 0.7%). Only one subject in the low Hb F group carried the -154 (C>T) mutation. Although not exclusively, these KLF1 mutations might be one of the genetic factors associated with increased Hb F and in combination could explain the variation of Hb F expression in Hb EE disease in Thai population. Other genetic factors such as HBS1L-MYB and BCL11A regulating Hb F expression in this common genetic disorder remains to be elucidated.