ความถี่ของยีน JAK2 V617F ตรวจวิเคราะห์ด้วยวิธี Quantitative Allele Specific Amplification (QASA) ในภาคตะวันออกเฉียงเหนือ ประเทศไทย

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Abstract: Frequency of JAK2 V617F mutation detected by Quantitative Allele Specific Amplification (QASA) in Northeast Thailand

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(Received: 30 November, 2021; Revised: 18 March, 2022; Accepted: 25 May, 2022)

Background: The JAK2 V617F mutation has been described as a frequent genetic event among a majority of patients with Myeloproliferative neoplasms (MPNs) including Polycythemia Vera (PV), Essential Thrombocythemia (ET), and Primary Myelofibrosis (PMF). Its frequency varies in different populations, but there are no data from northeast Thailand. **Objective:** Therefore, we aimed to report the JAK2 V617F mutation frequency and laboratory correlation in northeast Thailand patients. **Methods:** This study included retrospective reviews of all hematologist patients requested to tested for JAK2V167F mutations from Srinagarind and Khon Kaen Hospitals, the main tertiary medical center in the northeastern region Thailand. Collected data from January 2017 to January 2021. 418 peripheral blood and bone marrow samples were analyzed by qRT-PCR using the Quantitative Allele Specific Amplification (QASA) JAK2V617F kit. **Results:** 418 patients were referred by physicians for JAK V617F mutation analysis. In this group, 101 (24.17%) were positive for the JAK2V617F mutation, whereas 317 (75.87%) patients were negative for the JAK2V617F mutation. The JAK2V617F mutation positive group (101 patients) included 46 (45.54%) patients with PV, 39 (38.61%) patients with ET, 11 (10.90%) patients with primary myelofibrosis (PMF), and 5 (4.95%) patients with unclassified MPNs. **Conclusions:** The frequency of the JAK2V617F mutation in our study is compatible with previous reports. JAK2V617F mutation screening can be incorporated in the initial evaluation of patients suspected of having MPNs. Detection of JAK2V617F is of diagnostic significance, and quantification of this mutation is also useful in monitoring patients as a residual disease marker.

Keywords: JAK2V617F, Quantitative Allele Specific Amplification (QASA), real-time quantitative polymerase chain reaction (qRT-PCR)

บทคัดย่อ

ภูมิหลัง: การกลายพันธุ์ของยีน JAK2V617F ได้รับการอธิบายว่าเป็นเหตุการณ์ทางพันธุกรรมที่เกิดขึ้นบ่อยในผู้ป่วยกลุ่ม Myeloproliferative neoplasms (MPNs) ได้แก่ Polycythemia Vera (PV), Essential Thrombocythemia (ET) และ Primary Myelofibrosis (PMF) ความถี่แตกต่างกันไปในแต่ละกลุ่มประชากร แต่ยังไม่มีข้อมูลจากภาคตะวันออกเฉียงเหนือของประเทศไทย **วัตถุประสงค์:** เพื่อรายงานความถี่ของการกลายพันธุ์ของยีน JAK2V617F และความสัมพันธ์กับผลทางห้องปฏิบัติการในผู้ป่วยภาคจากตะวันออกเฉียงเหนือของประเทศไทย **วิธีการ:** การศึกษาแบบย้อนหลัง ในผู้ป่วยทุกรายที่ได้รับการวินิจฉัยจากแพทย์เฉพาะทางสาขาโลหิตวิทยา และส่งทดสอบการกลายพันธุ์ของยีน JAK2V167F จากโรงพยาบาลศรีนครินทร์และโรงพยาบาลขอนแก่น จังหวัดขอนแก่น ซึ่งเป็นศูนย์การแพทย์ระดับตติยภูมิในภาคตะวันออกเฉียงเหนือของประเทศไทย รวบรวมข้อมูลตั้งแต่มกราคม 2560 ถึงมกราคม 2564 ตัวอย่างเลือดและไขกระดูกจำนวน 418 ราย ได้รับวิเคราะห์ด้วยเทคนิค real-time quantitative polymerase chain reaction (qRT-PCR). โดยใช้ชุดตรวจ

วิเคราะห์ Quantitative Allele Specific Amplification (QASA) JAK2 V617F **ผล:** ในผู้ป่วยจำนวน 418 ราย พบ 101 ราย (24.17%) มีผลบวกต่อการกลายพันธุ์ของยีน JAK2 V617F ในขณะที่ผู้ป่วย 317 ราย (75.87%) มีผลลบต่อการกลายพันธุ์ของยีน JAK2V617F กลุ่มผลบวกของการกลายพันธุ์ของยีน JAK2V617F เป็นผู้ป่วย PV 46 ราย (45.54%) ผู้ป่วย ET 39 ราย (38.61%) ผู้ป่วย PMF 11 ราย (10.90%) และผู้ป่วยผู้ป่วย MPN ที่ไม่จำแนกประเภท 5 ราย (4.95%) **สรุป:** ความถี่ของการกลายพันธุ์ยีน JAK2V617F ในการศึกษาครั้งนี้สอดคล้องกับรายงานก่อนหน้านี้ การตรวจคัดกรองการกลายพันธุ์ของยีน JAK2V617F สามารถใช้ในการประเมินเบื้องต้นและวินิจฉัยโรคในกลุ่ม Myeloproliferative neoplasms (MPNs) นอกจากนี้การหาปริมาณของการกลายพันธุ์นี้ยังมีประโยชน์ในการติดตามการรักษาผู้ป่วย

คำสำคัญ: JAK2 V617F, Quantitative Allele Specific Amplification (QASA), real-time quantitative polymerase chain reaction (qRT-PCR)

Introduction

Myeloproliferative neoplasms (MPNs) are a group of hematological neoplasms that share similar molecular and cellular abnormalities. However, these diseases differ in their phenotypes, clinical presentation, and therapy.[1-3] According to the WHO classification, MPNs are divided into two large groups: those that possess the BCR-ABL1 fusion protein, such as Chronic Myeloid Leukemia (CML), and those that are BCR/ABL1–negative[4] including Polycythemia Vera (PV), Essential Thrombocythemia (ET), Myelofibrosis (MF) macrocytosis, chronic neutrophilic leukemia, and chronic eosinophilic leukemia. The latter group of MPNs shares elements of pathogenesis and symptomatology that may be related to dysregulated Janus kinase (JAK) signaling.[2] PV, ET, and MF are Philadelphia (Ph) chromosome negative chronic MPNs by the definition of WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues 2008 revision.[4] PV is characterized by an increase in red cells, white cells and platelets and clinically a plethoric appearance, itch and splenomegaly. Thromboembolic phenomena and hemorrhage can complicate the disease and in the end stages can progress to myelofibrosis and acute leukemia. ET is characterized by an increased platelet count. Clinically it is frequently asymptomatic, but the thromboembolic events may detect to disease. There is a small propensity to progress to myelofibrosis and acute leukemia, which may be influenced by the treatment modalities used. MF is defined by a leukoerythroblastic blood picture, splenomegaly, and bone marrow fibrosis. The most common mutation was JAK2V617F mutation which can be detected at rates of about 23 – 95%[5-7] and higher in patients with PV (65 – 97%) than ET and PMF (20 – 65%).[8-9] In 2005, 4 independent research groups identified a single acquired mutation in the JAK2 gene on chromosome 9 that had a high incidence of occurrence in patients with PV, ET, or MF.[10-13] The point mutation in exon 14 of JAK2 alters codon 617 from valine to phenylalanine. This amino acid alteration in the JH2 domain of JAK2 causes constitutive activation of the tyrosine kinase, which is believed to confer erythropoietin hypersensitivity and erythropoietin-independent survival to the myeloid stem cell.[14] This mutation is a gain of function mutation, in that it releases the autoinhibitory action of JH2. It recruits STAT in the complete absence, or in the presence of only trace quantities, of hematopoietic growth factors[15-17] The detection of the JAK2V617F mutation provides a qualitative diagnostic parameter for the identification of the nonchronic myelogenous leukemia subgroup of MPNs. Different groups reported a variable frequency of JAK2 V617F mutation ranging from 65%–97% for PV, 23%–57% percent for ET, and 35%–%57 percent for MF.[18-21] Green's group[20] found the mutation in 57% of individuals with ET, 50% of individuals with MF and 97% of those with PV. It was detected in both granulocyte macrophage and erythroid colonies and intriguingly was present in all EECs (Endogenous Erythroid Colony), demonstrating a link with growth factor hypersensitivity. However, JAK2V617F mutation was also reported in lower frequencies in other hematologic malignancies,[22] and possible consequences of the JAK2V617F and the development of JAK2 in MPNs are still under active investigation.[18-19, 23] There are few studies about JAK2 V617F mutation in Thailand.[24] In this study, we evaluated the prevalence of the JAK2V617F mutation and its clinical and laboratory correlations in patients in northeast Thailand.

Materials and Methods

We designed a retrospective descriptive study of all patients requested to tested for JAK2V167F mutations from Srinagarind and Khon Kaen Hospitals, the main tertiary medical center in the northeastern region Thailand. Data has been collected from medical records for 4 years (January 2017 to January 2021). Included in this study were 418 patients referred by physicians for JAK2V617F mutation analysis. The necessary investigations, including measurements of white blood cell (WBC) count, platelet count (Plt) and hemoglobin (Hb), were performed

whenever required. The Khon Kaen University Ethic Committee approval for the study protocol was obtained (HE 631504), and written informed consent was taken from all patients. Genomic DNA was extracted using MagNAPure LC DNA Isolation Kit. DNA was quantified using spectrophotometric measurements. For the RNA quantity and quality was assessed using a NanoDrop spectro-photometer (Nano Drop Technologies, Wilmington, Delaware, USA) and if 260/280 ratio in the Nano Drop monitor less than 1.8, repeated RNA extraction can be carried out.[25] For quantitative analysis of the JAK2 V617F mutation, we performed qRT-PCR using Quantitative Allele Specific Amplification (QASA) JAK2V617F kit (Primerdesign quasa detection kits) according to the Manufacturer's instructions. Briefly, 5 µL of genomic DNA was added to 15 µL of the RQ-PCR premix solution (V617F or wild type) in each well. A 45-cycle PCR was performed on a Rotor-Gene 6000 real-time analyzer (Qiagen) according to the following cycling conditions, 5 cycling conditions: 10 seconds at 95°C, 15 seconds at 50°C and 15 seconds at 72°C. Followed by 40 cycles of 10 seconds at 95°C, 30 seconds at 60°C and 15 seconds at 72°C. This study protocol has been approved and reviewed by the Khon Kaen University Ethic Committee for Human Research based on the declaration of Helsinki and the ICH Good Clinical Practice Guideline, reference No. HE631504. In case of categorical data, the information was presented in the form of percentage, while two sets of statistical tools, median with range and mean, were applied to interpret continuous data.

Results

From January 2017 to January 2021. 418 peripheral blood and bone marrow samples were analyzed by qRT-PCR. In this group, 101 (24.17%) of the patients were positive for the JAK2V617F mutation, whereas 317 (75.87%) patients were negative for the JAK2 V617F mutation. The JAK2V617F mutation positive group (101 patients) included 46 (45.54%) patients with PV, 39 (38.61%) patients with ET, 11 (10.90%) patients with primary myelofibrosis (PMF), and 5 (4.95%) patients with unclassified MPNs. The mean ages of JAK2 V617F positive patients were 59.86 years (range: 21-88) in males and 57.14 years (range: 23-85) in females, and those of the JAK2 V617F negative patients were 43.29 years (range: 4-91) in males and 48.12 years (range: 6-82) in females as shown in table 1 and 2.

Table 1 Characteristics of patients

Parameter	JAK2V617F Positive patients	JAK2V617F Negative patients
Patients n (%)	101 (24.17%)	317 (75.87%)
Male mean age (range)	59.86 (21–88)	43.29 (4–91)
Female mean age (range)	57.14 (23–85)	48.12 (6–82)

Table 2 Characteristics of patients with the JAK2 V617F mutation

Disease	Number of patients (%)	Male/ Female	Male mean age (range)	Female mean age (range)
PV	46 (45.54)	28/18	59.80 (29–88)	56.28 (22-78)
ET	39 (38.61)	24/15	60.93 (22-85)	57.28 (22-79)
MF	11 (10.90)	5/6	60.39 (39-86)	55.22 (25-80)
Unclassified MPNs	5 (4.95)	5/0	50.17 (25-84)	-

Laboratory findings in the 46 PV patients showed a mean hemoglobin (Hb) level of 17.8 g/dL (14.1-21.6g/dL). For the 39 ET patients, mean Hb level was 14.30 g/dL (range 9.5-17.5 g/dL). The mean level of WBC was 12.15 x10^9/L (range 4.7-29.4 x10^9/L in PV patients and 10.2 x10^9/L (range 5.8-43 x10^9/L.) in ET patients. PV patients had a mean platelet count at 512.5 x10^9/L (range 151-1028 x10^9/L), and the mean platelet count was 803 x10^9/L (range 355-2571 x10^9/L) in ET patients as shown in Table 3.

Table 3 Hematological parameters in JAK2 V617F mutation positive patients

Parameter	PV n= 46	ET n=39	MF n=11
Hb (g/dL) Mean (range)	17.8 (14.1-21.6)	14.30 (9.5-17.5)	12.3 (5.5-15.6)
WBC x 10^9/L Mean (range)	12.15 (4.7-29.4)	10.2 (5.8-43.8)	12.6 (3.5-85.5)
Platelet x 10^9/L Mean (range)	512.5 (151-1028)	803 (355-2571)	293 (44-677)

Discussion

The percentage of SNP present in the sample is calculated using the delta Cq method. The proportions of SNP and WT in the sample are corrected by referencing to a positive control standard where the SNP is present at a known proportion of 1%. Sample data and calculations the calculation is performed in two stages. Firstly, the delta Cq values are used to calculate relative detection levels between the biological sample and the 1% control for both the Wild type and the mutant. These relative amounts are then converted into a percentage.

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Equations

JAK2WT Relative Amount = [2^ - (JAK2 WT Sample – JAK2 WT Control)] X Control Proportion of WT

JAK2V617F Relative Amount = [(2^ - (JAK2 V617F Sample – JAK2 V617F Control)] X Control

Proportion of SNP

$$\text{JAK2V617F Percentage conversion} = \frac{\text{JAK2 V617F Relative Amount X 100}}{\text{JAK2 WT Relative Amount + JAK2 V617F Relative Amount}}$$

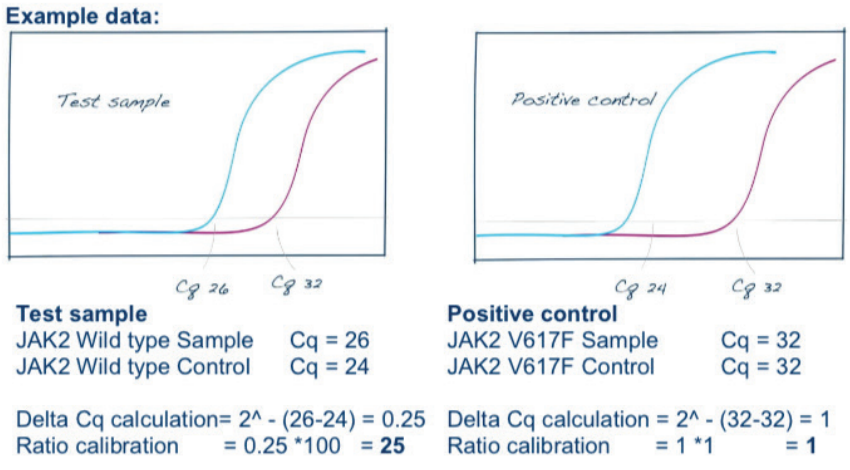

Figure 1 JAK2 V617F Percentage Conversion

Calculate the Percentages.

From the example set of data the Wild type to mutant ratio is 25:1, when calibrated against the 1% positive control. Calculating the percentage of either the Wild type or the mutant can be calculated from this ratio. For the mutant, the percentage conversion is as follows.

$$\text{JAK2 V617F Percentage conversion} = \frac{1 \times 100}{25 + 1}$$

$$= 3.85\%$$

Interpretation: "3.85% of the sample DNA is mutated in the background of 96.15% Wild type." The kit is sensitive down to detection levels of 0.1%. Results that report detection at a lower proportion than 0.1% should be considered as negative.

Qualitative uses of the kit

In some clinical scenarios, it is sufficient to know if a mutation is present or absent and the exact proportion of the mutant is not of diagnostic or therapeutic value. In these circumstances, the sample can be tested using a single test for the Wild type and mutant primer/probe mixes. Positive template containing 1% mutant sequence. The kit contains a positive control which includes blend of both Wild type and mutant sequences at a known copy number. The ratio of Wild type to mutant template is 100:1 which is typical of some biological samples which can contain mutant sequences at a very low level. The positive control, therefore, provides a template for both primer and probe mixes, and the quantification cycle (Cq) data from this control is used in the quantitative analysis.

Negative control

To confirm absence of contamination, a negative control reaction should be included every time the kit is used. In this instance the Rnase/Dnase free water should be used instead of the template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained, the results should be ignored and the test samples repeated. Possible sources of contamination should first be explored and removed.

Green's group[20] found the mutation in 57% of individuals with ET, 50% of individuals with IMF and 97% of those with PV. It was detected in both granulocyte macrophage and erythroid colonies and intriguingly was present in all EECs, demonstrating a link with growth factor hypersensitivity. However, JAK2V617F mutation was also reported in lower frequencies in other hematologic malignancies[22] and possible consequences of the JAK2V617F and the development of JAK2 in MPNs are still under active investigation.18-19,[23] The frequency of the JAK2 mutation in our study is compatible with previous reports. In adult, northeastern Thailand found the JAK2V617F mutation-positive group (101 patients) included 46 (45.54%) patients with PV, 39 (38.61%) patients with ET, 11 (10.90%) patients with primary myelofibrosis (PMF), and 5 (4.95%) patients with unclassified MPNs. The mean ages of JAK2V617F positive patients were 59.86 years (range: 21–88) in males and 57.14 years (range: 23-85) in females, and those of the JAK2 V617F negative patients were 43.29 years (range: 4-91) in males and 48.12 years (range: 6-82) in females.

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Acknowledgement

We are indebted to Mr. Prachub Chaimanee and Ms.Pimjai Ananta for their invaluable comments.

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