

**The Prevalence of *TET2*, *ASXL1*, *IDH1/2*, and *DNMT3A* Mutations in Thai Patients with Philadelphia-Negative Myeloproliferative Neoplasms**

**ความชุกของการกลายพันธุ์ของยีน *TET2*, *ASXL1*, *IDH1/2* และ *DNMT3A* ในกลุ่มโรคไขกระดูก  
ผลิตเม็ดเลือดมากผิดปกติชนิดที่ไม่มีฟิลาเดลเฟียโครโมโซมในผู้ป่วยชาวไทย**

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**ABSTRACT**

Somatic mutations associated with disease progression and increase risk of leukemic transformation in Myeloproliferative neoplasms (MPNs) as *TET2*, *ASXL1*, *IDH1/2*, and *DNMT3A* mutations have been reported differently in numbers based on a study population. This study aim to investigate the prevalence of these mutations in Thai patients with Philadelphia negative MPNs. Total of 50 MPNs were analysed by direct sequencing. The *TET2*, *ASXL1*, *IDH1/2*, and *DNMT3A* were detected in 13 (26%), 42 (84%), 3 (6%) and 0 (0%) of MPNs patients, respectively. The mutations found were 2 missense mutations of *TET2*, 1 missense and 1 silent mutation of *ASXL1* and 1 silent mutation of *IDH1*.

**บทคัดย่อ**

ในกลุ่มผู้ป่วยโรคไขกระดูกผลิตเม็ดเลือดผิดปกติที่มีการกลายพันธุ์ของยีน *TET2*, *ASXL1*, *IDH1/2*, และ *DNMT3A* ร่วมด้วยนั้นพบว่าการดำเนินโรคที่แยกลงรวมถึงมีความเสี่ยงสูงในการดำเนินโรคไปเป็นมะเร็งเม็ดเลือดขาวเฉียบพลัน อย่างไรก็ตามความชุกของการกลายพันธุ์ที่พบแตกต่างกันไปตามกลุ่มประชากรที่ศึกษา การศึกษานี้มีวัตถุประสงค์ที่สำรวจความชุกของการกลายพันธุ์ของยีนดังกล่าวในกลุ่มผู้ป่วยโรคไขกระดูกผลิตเม็ดเลือดผิดปกติชาวไทยโดยการตรวจวิเคราะห์ลำดับเบสของดีเอ็นเอในผู้ป่วยจำนวน 50 ราย การศึกษานี้พบการกลายพันธุ์ในยีน *TET2*, *ASXL1*, *IDH1/2*, และ *DNMT3A* เท่ากับ 13 ราย (26%), 42 ราย (84%), 3 ราย (6%) และ 0 ราย (0%) ตามลำดับ โดยการกลายพันธุ์ที่พบในยีน *TET2*, *ASXL1* และ *IDH1/2* เป็นชนิด missense และ silent

**Keywords:** Myeloproliferative Neoplasm, *TET2*, *ASXL1*

**คำสำคัญ:** โรคไขกระดูกผลิตเม็ดเลือดผิดปกติ ยีน *TET2* ยีน *ASXL1*

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## Introduction

Myeloproliferative neoplasms (MPNs) represent a group of diseases affect the clonal hematopoietic disorders caused by genetic mutations in the hematopoietic stem cells result in expansion, overproduction and accumulation of erythrocytes, granulocytes and platelets. The philadelphia negative MPNs called classical MPNs are the most frequent disease among the myeloproliferative disorder compose of Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF). These neoplasms have common clinical features, laboratory findings, and pathogenetic similarities They have related complication such as thrombosis, hemorrhages and transformation to acute myeloid leukemia (AML) (Walenga, 2015). The discovery of mutations activating JAK-STAT signaling in the majority of patients with MPNs led to identification of tyrosine kinase activation as mechanism driving MPNs pathogenesis. The MPNs driver mutations are *Janus Kinase2 (JAK2)*, *Calreticulin (CALR)* and *Thrombopoietin receptor gene (MPL)*. They are high specificity for MPNs and considered as diagnostic criteria in WHO 2016 (Arber et al., 2016; Them & Kralovics, 2013). Despite this, other mutations in genes involved in epigenetic regulation as *TET2*, *ASXL1*, *IDH1/2*, and *DNMT3A*, also found in MPNs patients whose carry *JAK2*, *MPL*, or *CALR* mutations and play role in disease progression (Kim & Abdel-Wahab, 2013; Nangalia & Green, 2014; Skoda, Duek, & Grisouard, 2015; Vainchenker & Kralovics, 2017).

The two main mechanisms of epigenetic regulation are DNA methylation and chromatin modification. Genes involved in this process such as *TET2*, *ASXL1*, *IDH1/2* and *DNMT3A* have been described in MPNs patients in variable frequencies. *TET2* and *DNMT3A* play role in DNA methylation. *TET2* and *DNMT3A* mutations are a loss of function lead to increase self-renewal of hematopoietic stem cell and abnormal differentiation resulting in imbalance of leukemogenesis (Omar Abdel-Wahab, Tefferi, & Levine, 2012; Day & Sweatt, 2010; Pronier & Delhommeau, 2012; Stegelmann et al., 2011). Mutation of *TET2* and *DNMT3A* found 5%-20% and 1%-10% of MPNs, respectively. Moreover, *TET2* and *DNMT3A* mutations also found in MPNs evolved to AML 26% and 10%-15%, respectively (O. Abdel-Wahab et al., 2011; Nangalia & Green, 2014; Prick, de Haan, Green, & Kent, 2014; Saeidi, 2016). In case of *IDH1* (R132) and *IDH 2* (R140, R172) mutations, they produce 2-hydroxyglutarate (2-HG) which is metabolite that blocks hematopoietic differentiation (Langabeer et al., 2015; Yang, Ye, Guan, & Xiong, 2012). *IDH1/2* mutations present in 1%-5% of chronic MPNs and present in 20%-22% of leukemic transformation of MPNs (Kim & Abdel-Wahab, 2013; Shih, Abdel-Wahab, Patel, & Levine, 2012). *ASXL1* encode protein which is part of the polycomb repressor complex 2 (PRC2) that regulates chromatin modification are also frequently altered in MPNs. *ASXL1* mutaion resulting in loss of function lead to impair normal control of cell proliferation (Omar Abdel-Wahab et al., 2012; Gelsi-Boyer et al., 2012; Langabeer, et al., 2015; Them & Kralovics, 2013). *ASXL1* mutations are found in 2%-5% of PV and ET, but increased to 13%-23% in PMF. Furthermore, *ASXL1* mutations detected in 20%-27% of post-MPNs AML (Kim & Abdel-Wahab, 2013; Nangalia & Green, 2014; Saeidi, 2016).

Many studies showed these mutations involve disease progression and increased risk of leukemic transformation during the course of disease that result in worsen overall survival of patients. However, there is very few reports of these mutations in Thai MPNs patients. This information may lead to usefulness as prognostic marker in MPNs.

### **Objective of the study**

The aim of this study was to determine the prevalence of *TET2*, *ASXL1*, *IDH1/2* and *DNMT3A* mutations in Thai patients with Philadelphia Negative MPNs.

### **Materials and methods**

#### **Subject**

Whole blood samples of 50 cases who diagnosed for PV, ET and PMF according to WHO 2016 criteria include 18 of PV, 22 of ET and 10 of PMF were collected at Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. All patients provided written an informed consent before entering the study. This study was approved by Ethical Committee for Human Research, Faculty of Medicine Siriraj Hospital, Mahidol University. (Approval number 078/2560 (EC4))

#### **DNA preparation**

DNA was extracted from cells using Genra Puregene blood kit (QAIGEN, Germany) according to the manufacture's instruction. Briefly, red blood cells were lysed and removed firstly then white blood cells were lysed with an anionic detergent in the presence of a DNA stabilizer. Other contaminants such as protein were removed by salt precipitation. Finally, genomic DNA was precipitated with alcohol and dissolved in hydration solution. Purity of DNA was affirmed by optical density (OD) measurement and safely stored at -20 °C.

#### **Polymerase Chain Reaction (PCR) protocol**

PCR were performed using primers targeting of *TET2* (Exons 11), *ASXL1* (Exon 12), *IDH1* (Exon 4), *IDH2* (Exon 4) and *DNMT3A* (Exon 23) to amplify PCR product (Brecqueville et al., 2012). The concentration of  $Mg^{2+}$ , *Taq* DNA polymerase, dNTP, primers, cycling conditions and annealing temperature of each primer was optimized. Each reaction was done in total volume of 50 uL. PCR mixture contained 5 uL of 1x PCR buffer, 1.5 uL of 1.5mM  $MgCl_2$ , 1 uL of 0.2mM dNTP mix, 0.25 U Platinum *Taq* DNA polymerase (Invitrogen), 1 uL of 0.2 uM of each forward primer and reverse primer, 50 ng genomic DNA and distilled water to obtained a 50 uL final volume. PCR reactions were performed on Applied biosystems ProFlex PCR system. In this study, the cycling was 10 minutes at 95 °C for 1 cycle, followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at variable annealing temperature of each primer, 1 minute at 72 °C, followed by final extension at 72 °C for 10 minutes. All PCR products were confirmed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

#### **Purification of PCR product**

PCR clean up procedure was performed to concentrate DNA fragment form PCR product and to remove excess primers and nucleotides that could interfere the sequencing. PCR clean up using GenePHlow™ Gel/PCR kit (Geneaid) was done follow to the manufacture's instruction. Briefly, PCR buffer facilitated DNA binding to the spin

column, contaminants were removed with a wash buffer and the purified DNA fragments were eluted by elution or water. Gel electrophoresis was performed to check the concentration of purified DNA fragment to ensure that it was in range according the requirement of sequencing protocol.

### **DNA sequencing**

The purified fragment DNA was used as template for the subsequent sequencing reaction. All DNA samples were sequenced using the Bigdye terminator 3.1v cycle sequence kits on ABI's 3730XL DNA Analyzers. The data from the analysis were compared to the references obtained from NCBI (<http://www.ncbi.nlm.nih.gov/>). References for nucleotide and protein sequences are respectively for *TET2*: NM\_1127208.2, NP\_001120680.1, *ASXL1*: NM\_015338.5, NP\_056153.2; for *IDH1*: NM\_005896.3, NP\_005887.2; for *IDH2*: NM\_002168.3, NP\_002159.2 and for *DNMT3A*: NM\_022552.4, NP\_022552.3.

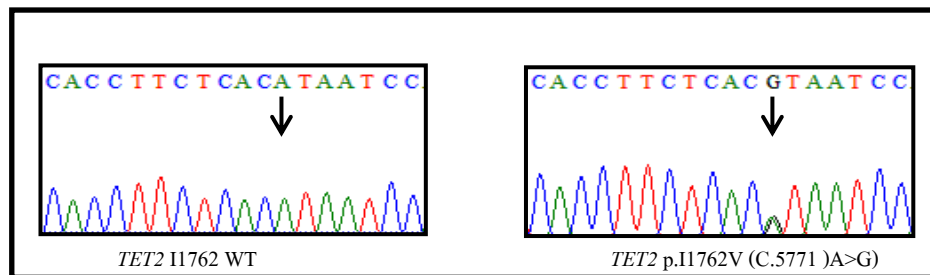
### **Result**

In this study, we investigated the mutational profiles of 5 genes, *TET2* (exon11), *ASXL1* (exon12), *IDH1/2* (exon4), and *DNMT3A* exon23, in 50 patients of Philadelphia negative MPNs. We found 2 different missenses of *TET2* mutations in 13 cases (26%) of patients. The most frequent mutation was *TET2* I1762V which found in 12 cases (24%) of MPNs patients, comprised of 3 cases of PV, 5 cases of ET and 4 cases of PMF. The *TET2* L1721W was observed in 1 case (2%), the case was ET patient. For *ASXL1* mutations, 1 missense mutation and 1 silent mutation were observed in 42 cases (84%) of patients. The most frequent mutation was *ASXL1* S1253S which is silent mutation, detected in 39 cases (78%) of MPNs patients, comprised of 15 cases of PV, 18 cases of ET and 6 cases of PMF whereas missense mutation, *ASXL1* G652S found in 3 cases (6%) of MPN patients, comprised of 1 case of PV, 1 case of ET and 1 case of PMF. In case of *IDH1* mutation, we found *IDH1* G105G which is silent mutation in 3 cases (6%) of MPNs patients, including 2 cases of PV and 1 case of PMF. Regarding the mutational analysis of *IDH2* (exon4) and *DNMT3A* (exon23), no mutations were detected in any of the 50 patients. All mutations found in the current study summarized on the Table 1. The example of missense and silent sequencing chromatogram of *TET2* I1762V and *ASXL1* S1253S were shown in Figure 1 and 2, respectively.

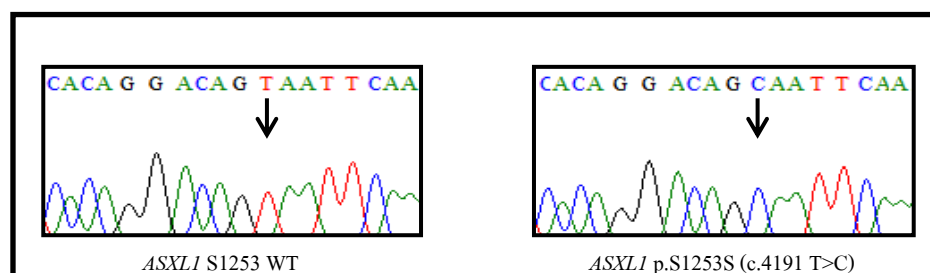
**Table 1** Summary of Missense and Silent mutations detected in *TET2* exon11, *ASXL1* exon12 and *IDH1* exon4 of MPNs patients in this study

Variants	Type	Nucleotide change	Amino acid change	Frequency (%)	Type of MPNs
<i>TET2</i> I1762V	missense	A to G	Isoleucine to Valine	12 (24%)	PV(3), ET(5) PMF(4)
<i>TET2</i> L1721W	missense	T to G	Leucine to Tryptophan	1 (2%)	ET
<i>ASXL1</i> S1253S	silent	T to C	Not change	39 (78%)	PV(15), ET(18) PMF(6)
<i>ASXL1</i> G652S	missense	G to A	Glycine to Serine	3 (6%)	PV(1), ET(1) PMF(1)
<i>IDH1</i> G105G	silent	C to T	Not change	3 (6)%	PV(1), PMF(2)

A=Adenine; G=Guanine; T=Thymine; C=Cytosine



**Figure 1** Sequencing chromatogram of the I1762V detected in *TET2* exon11. A is sequence of wild type and B is sequence of I1762V mutation in patients. The arrows indicate mutation sites.



**Figure 2** Sequencing chromatogram of the S1253S detected in *ASXL1* exon12. A is sequence of wild type and B is sequence of S1253S mutation in patients. The arrows indicate mutation sites.

## Discussion

In the present study, PCR were performed using primers targeting of *ASXL1* (Exon 12), *DNMT3A* (Exon 23), *IDH1* (Exon 4), *IDH2* (Exon 4) and *TET2* (Exons 11) followed by direct sequencing to investigated the mutational profiles in 50 Thais patients with Philadelphia negative MPNs. Pathogenic mutations of *TET2* gene have been reported in various variants and normally found in conserved domains, result in truncated *TET2* protein. Missense mutations are predominantly found in the conserved domains of the protein which suggests that they could interfere with the catalytic activity of the protein (Delhommeau et al., 2009; Delhommeau, Jeziorowska, Marzac, & Casadevall, 2010). As the result shown, we found 2 missense mutations which are located in conserved domain. *TET2* I1762V and L1721W variants were found in various myeloid disorders including MPN (Chou et al., 2011; Kutny et al., 2010; Li et al.; Nibourel et al., 2010). Martinez-Aviles et al. (Martínez-Avilés et al., 2012) was reported *TET2* I1762V and L1721W in 45% (28/62) and in 19.3% (12/62) in MPNs, respectively, and also detected in control samples. In the present study, we found *TET2* I1762V in 24% (12/50) and L1721W in 2% (1/50) that were lower than the prior study. These 2 variants were documented as single nucleotide polymorphisms (SNPs) in single nucleotide polymorphisms database (dbSNP) as rs2454206 and rs34402524, respectively, suggesting that these variants were considered as non-pathogenic variants (<http://www.ncbi.nlm.nih.gov/snp/>) (Langemeijer et al., 2009; Martínez-Avilés et al., 2012; Scopim-Ribeiro et al., 2016).

For *ASXL1* mutations, the most common pathogenic mutation is p.Gly646TrpfsX12 in exon 12, which account more than 50% of *ASXL1* mutations (Gelsi-Boyer et al., 2012). Moreover, Brecqueville et al. (Brecqueville, et al., 2012) demonstrated that *ASXL1* mutation influence low hemoglobin level and in PMF patients have a significant worsened overall survival compared with wild type. In this study, we did not detect any of *ASXL1* mutation in exon 12 other than S1253S which was silent mutation and G652S variant. The missense G652S was reported as SNP rs3746609 in dbSNP and has been described as rare polymorphism in myeloid disorders in the range 0.37%-2% (Schnittger et al., 2011; West, Hsu, Holland, Cuellar-Rodriguez, & Hickstein, 2014) while we found 6% that was quite higher than the previous study.

In case of *IDH1* mutation, the most common pathogenic mutation was *IDH1* R132. We did not detect pathogenic mutation of *IDH1* in our cases that was similar to the prior study in Thai (Chotirat, Thongnoppakhun, Wanachiwanawin, & Auewarakul, 2015). However, *IDH1* G105G was detected in 6% in our cases which was documented as SNP rs 11554137 in dbSNP. It has been reported at rate of 2.3% and 3.7% in MPNs patients (Chotirat et al., 2015; Soyer et al., 2017). The frequency of *IDH1* G105G in our study was quite high from previous studies. We also looked for the mutations of *IDH2* (exon4) and *DNMT3A* (Exon 23), no mutations were detected in any of our cases.

However, we analyzed the mutations only in targeted regions which commonly found pathogenic mutations so others mutations in the remaining exons might be missed from this study. Moreover, the difference of the prevalence of mutation might be varied by ethics background of population, criteria of study samples or the sensitivity of detection methods. Another one limitation of this study is the small samples size, the result from this

study might not represent the prevalence of the mutations in Thai population. The larger sample size is required to assess more accurate data.

### Conclusion

Our data suggest that mutations of *TET2* (exon11), *ASXL1* (exon12), *IDH1* (exon4), *IDH2* (exon4) and *DNMT3A* (exon23) may be rare in Thai MPNs patients. The prevalence of these mutations might be varies with population ethnicity. However, the larger sample sizes, the prevalence of these mutations in healthy control group including the association between mutations and clinical/laboratory characteristics of MPNs patients are needed for the further studies.

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