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鈣網蛋白突變在骨髓增生性腫瘤的角色

The Roles of *Calreticulin* Mutations in Myeloproliferative Neoplasms

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鈣網蛋白突變在骨髓增生性腫瘤的角色

The Roles of *Calreticulin* Mutations in Myeloproliferative Neoplasms

本論文係林建鴻君（學號 D00453004）在國立臺灣大學腫瘤醫學研究所完成之博士學位論文，於民國 106 年 5 月 24 日承下列考試委員審查通過及口試及格，特此證明

口試委員：　　周文堅　（簽名）

（指導教授）

所長　　（簽名）

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新約聖經羅馬書八章 28 節題到“萬有都互相效力，叫愛神的人得益處。”這節經文所說的相當符合我進修博士班的過程和經歷。我很感謝我所相信、我所愛、我所事奉並跟隨的救主耶穌，帶領我在博士班進修這段過程裡面有很多的學習，讓我在基礎與臨床醫學的研究上，立下了一個很好的根基，也在信仰上更多經歷祂這位又活又真的神。

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中文摘要

骨髓增生性腫瘤是克隆性造血幹細胞疾病，並且可以分類為“典型”和“非典型”骨髓增生性腫瘤。典型骨髓增生性腫瘤通常會在周邊的血液中表現出終端骨髓細胞的擴增，包括真性紅血血球增多症、原發性血小板增多症、原發性骨髓纖維化和慢性骨髓性白血病。近年來，在大約 30%的 *JAK2/MPL*-未突變的骨髓增生性腫瘤中發現鈣網蛋白(*Calreticulin, CALR*)突變。目前，鈣網蛋白突變已經成為診斷原發性血小板增多症和原發性骨髓纖維化的重要克隆標誌物。

這項研究的第一個目的是開發一種快速和敏感的篩選工具，用於檢測鈣網蛋白突變。我們使用 CFX Connect 即時系統成功開發了高分辨率熔解分析，以檢測原發性血小板增多症病人的鈣網蛋白第 9 外顯子突變。我們的高分辨率熔解分析系統在識別病人基因組 DNA 中的鈣網蛋白第 1 型和第 2 型突變體的最大敏感性為 2.5%。我們的高分辨率熔解分析系統假陽性率為 3%，並且無假陰性出現。

本研究的第二個目的是評估台灣原發性血小板增多症病人鈣網蛋白突變和 *JAK2 /CALR* 共突變的臨床和預後意義。我們在 92 例成年原發性血小板增多症病人中利用高分辨率熔解分析篩選了鈣網蛋白第 9 外顯子的改變，隨後並且進行了 TA 克隆。我們在 21 例（22.8%）病人中鑑定出典型的鈣網蛋白插入/刪除型突變。鈣網蛋白突變與年齡較輕（p = 0.025），血小板數較高（p <0.001）和較低的血紅素（p = 0.016）有相關。有趣的是，我們檢測到在 59 例 *JAK2* 突變的原發性血小板增多症病人中有 13 例（22%）的鈣網蛋白第 9 外顯子的改變。與這些具有 *JAK2 /CALR* 共突變的原發性血小板增多症病人有相關的因子包括年齡較大（p = 0.025），診斷後發生血栓事件較多（p = 0.048），診斷後主要動脈血栓事件較多（p = 0.022），且較多屬於血栓出血併發症高風險組病人（p = 0.023）。我們的研究顯示 *JAK2* 突變的原發性血小板增多症病人可以出現頻繁的鈣網蛋白第 9 外顯子的改變，並且界定出一群具有血栓事件風險增加的病人亞群。

本研究的第三個目的是要探討鈣網蛋白突變之原發性血小板增多症病人的 B 細胞免疫特徵。我們篩選了 54 例台灣成年原發性血小板增多症病人的鈣網蛋白突變並評估了其 B 細胞免疫特徵。在這 54 例原發性血小板增多症病人中有 19 例（35.2%）具有 8 種不同類型的鈣網蛋白第 9 外顯子突變，其中包括 4 例（7.4%）同時伴有 *JAK2*V617F 共突變的病人。經過

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年齡、性別、追蹤期和血液學參數校正後，我們進行的多變量分析證實，與健康成年人相比，活化的 B 細胞在 *JAK2* 突變，鈣網蛋白突變和三陰性原發性血小板增多症病人中都有增加的現象。因此，活化的 B 細胞增加在不同突變亞組的原發性血小板增多症病人中是一種普遍存在的現象。

本研究的第四個目的是使用斑馬魚動物模型研究鈣網蛋白突變的分子發病機制。我們確定了 3 種與人類鈣網蛋白直系同源的斑馬魚基因，稱為 *calr*，*calr3a* 和 *calr3b*。*CALR*-del52 和 *CALR*-ins5 突變體的表達使斑馬魚早期的造血幹/前驅細胞增加，並進而造成血小板球增多但不影響正常的血管生成。我們發現使用 morpholino 降低 *mpl* 但不是 *epor* 或 *csf3r* 可以顯著的減弱 CALR 突變體對血小板球增多的影響。此外，CALR 突變體的表達也活化 *jak*/*stat* 信息傳遞路徑，而使用 JAK 抑製劑（ruxolitinib 和 fedratinib）可以抑制此活化現象。這些研究結果說明 CALR 突變體通過 mpl 依賴機制活化 *jak* 信息傳遞路徑導致斑馬魚致病性之血小板球生成。我們研究的結果也說明與突變型 CALR 腫瘤發生相關的訊息傳遞機制在人與斑馬魚之間是保守的。

關鍵詞: B 細胞、鈣網蛋白、原發性血小板增多症、高分辨率融合分析、免疫、突變、斑馬魚

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Abstract

The myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders and can be classified into "classic" and "atypical" MPNs. Classic MPNs usually exhibit terminal myeloid cell expansion in the peripheral blood and include polycythemia vera, essential thrombocythemia (ET), primary myelofibrosis and chronic myeloid leukemia. *Calreticulin* (*CALR*) mutations have recently been discovered in about 30% *JAK2*/*MPL*-unmutated myeloproliferative neoplasms (MPN), and have become an important clonal marker for the diagnosis of essential thrombocythemia (ET) and primary myelofibrosis.

The first aim of this study is to develop a rapid and sensitive screening tool for the detection of *CALR* mutations. We successfully developed a high-resolution melting analysis (HRMA) with the CFX Connect real-time system to detect *CALR* exon 9 mutations in ET patients. The maximal sensitivity of our HRMA system in identifying both *CALR* type 1 and type 2 mutants from patients' genomic DNA was 2.5%. Our HRMA has a false positive rate of 3% and no false negative.

The second aim of this study is to evaluate the clinical and prognostic significance of *CALR* mutations and *JAK2*/*CALR* co-mutations in Taiwanese ET patients. We screened for *CALR* exon 9 alterations with HRMA followed by TA-cloning in 92 adult ET patients. We identified classic *CALR* indel mutations in 21 (22.8%) patients. *CALR* mutations were associated with younger age ($p=0.025$), higher platelet count ($p<0.001$) and lower hemoglobin level ($p=0.016$). Interestingly, we detected various *CALR* exon 9 alterations in 13 (22%) of 59 *JAK2*-mutated ET patients. *JAK2*-mutated ET patients with concomitant *CALR* alterations were associated with oldest age ($p=0.025$), higher thrombotic events after diagnosis ($p=0.048$), higher major arterial thrombotic events after diagnosis ($p=0.022$) and more patients being high risk group for thrombo-hemorrhagic complications ($p=0.023$). Frequent *CALR* exon 9 alterations in *JAK2*-mutated ET patients define a

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specific subgroup of patients with increased risk of thrombotic events.

The third aim of this study is to determine the B cell immune profiles in *CALR* mutated ET patients. We screened for *CALR* mutations and evaluated B cell immune profiles in a cohort of 54 adult Taiwanese ET patients. 19 (35.2%) of 54 ET patients harbored 8 types of *CALR* exon 9 mutations including 4 (7.4%) patients with concomitant *JAK2*V617F mutations. Multivariate analysis adjusted for age, sex, follow-up period and hematological parameters confirmed that increased activated B cells were universally present in *JAK2*-mutated, *CALR*-mutated and triple-negative ET patients when compared to healthy adults. In conclusion, increased B cell activation is present in ET patients across different mutational subgroups.

The fourth aim of this study is to investigate the molecular pathogenesis of *CALR* mutations using zebrafish animal models. We identified 3 zebrafish genes orthologous to human *CALR*, referred to as *calr*, *calr3a* and *calr3b*. Expression of the CALR-del52 and CALR-ins5 mutants caused an increase in the hematopoietic stem/progenitor cells followed by thrombocytosis without affecting normal angiogenesis. The expression of CALR mutants also perturbed early developmental hematopoiesis in zebrafish. Importantly, morpholino knockdown of *mpl* but not *epor* or *csf3r* could significantly attenuate the effects of mutant CALR. Furthermore, expression of mutant CALR caused jak-stat signaling activation in zebrafish that could be blocked by JAK inhibitors (ruxolitinib and fedratinib). These findings showed that mutant CALR activates jak-stat signaling through an mpl-dependent mechanism to mediate pathogenic thrombopoiesis in zebrafish, and illustrated that the signaling machinery related to mutant CALR tumorigenesis are conserved between human and zebrafish.

Keywords: B cell, *CALR*, essential thrombocythemia, high-resolution melting analysis, immune,

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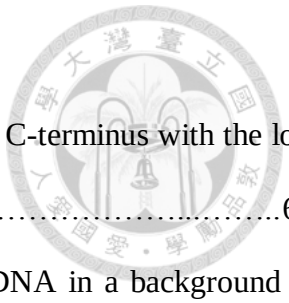

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Chapter 1

Classic *BCR-ABL*-negative myeloproliferative neoplasms

1. Myeloproliferative neoplasms

1.1 Introduction of myeloproliferative neoplasms

The myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders. According to the 2008 World Health Organization (WHO) classification, the MPNs are classified into "classic" and "atypical" MPNs.[1] The classic MPNs usually exhibit terminal myeloid cell expansion in the peripheral blood and include polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) and *BCR-ABL*-positive chronic myeloid leukemia (CML). The classic *BCR-ABL*-negative MPNs are heterogenous diseases with overlapping clinical and molecular characteristics.[2] PV is characterized by increased hematocrit/red blood cell mass accompanied by the presence of a *JAK2* mutation along with a decreased erythropoietin level. PMF is characterized by the presence of bone marrow fibrosis that cannot be attributed to another myeloid disorder such as CML, ET or myelodysplastic syndrome. ET is characterized by the presence of a clonal or autonomous thrombocytosis and the exclusion of other myeloid diseases. The chronic-phase PV and ET are associated with an increased risk of arterial and venous thrombosis, hemorrhagic complication, and sometimes with constitutional symptoms.[2,3] Myelofibrosis may be primary or may follow PV/ET, and may be considered as an accelerated-phase MPN with an increased risk of leukemic transformation and decreased survival.[4]

1.2 Driver mutations in MPNs

The *JAK2* V617F mutation was discovered in 2005, and has provided important diagnostic, therapeutic, and prognostic implications in MPNs. The frequency of *JAK2* V617F mutation is over 90% in PV, and about 60% in ET and PMF.[5-7] Besides, *MPL* mutations are identified in

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about 4-5% of *JAK2*-unmutated ET and PMF patients.[8] Following these seminal reports, other somatic mutations such as *LNK*, *TET2* and *DNMT3A* have also been detected in patients with MPN.[9] However, they are not mutually exclusive with *JAK2* and *MPL* mutations and also not specific to patients with MPN.[9,10] Despite many somatic mutations have been identified in patients with ET, clonal molecular marker is still not identified in ~40% of ET patients.

2. *CALR* mutations in MPNs

2.1 Calreticulin

Calreticulin (CALR) is a 46-kDa developmentally highly conserved, multicompartmental and multifunctional protein. Its gene is located on chromosome 19p13.2 and contains nine exons. Mature CALR consists of three structurally and functionally distinct domains:[11]

(i) Residues 1-180: the globular N-terminal domain is lectin binding and consists of a signal sequence for targeting to the ER.

(ii) Residues 181–290: the middle proline-rich or P-domain contains high affinity, low capacity, binding sites for Ca^{2+}.

(iii) Residues 291–400: the highly acidic C-terminal domain contains a number of high capacity, low-affinity Ca^{2+} binding sites, and terminates in a KDEL ER retention sequence, which can be used by KDEL receptors to retrieve CALR from the *cis*-Golgi back to the ER.

The N- and P-domains are mainly responsible for the protein's chaperone function, whereas the C-domain is principally involved in calcium regulation in the ER.

2.2 The function of CALR

CALR is best known for its role as a Ca^{2+} binding chaperone protein located primarily in the endoplasmic reticulum (ER).[12] Inside the ER, CALR plays an integral role in calcium

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homeostasis and protein folding. CALR can also be found in the nucleus, localize to cell surface and accumulate in extracellular compartments. Outside the ER, CALR can regulate integrin-mediated cell adhesion, gene nuclear transport, programmed cell removal, and immunogenic cell death.[12-14] CALR was also found to involve in the immune response to pre-apoptotic cancer cells, and early cell surface exposure of CALR is followed by expression and release of heat-shock proteins (e.g. HSP70), and high-mobility group I (HMGB1) protein.[15] Recombinant *CALR* fragment was shown to exhibit potent stimulatory activities against B cells.[16,17]

2.3 *CALR* mutations in *JAK2*/*MPL*-unmutated ET and PMF patients

In 2013, two research groups discovered a high frequency of somatic *CALR* mutations in 60–88% patients with *JAK2*/*MPL*-unmutated ET and PMF patients, but none in patients with PV.[18,19] The pattern of most *CALR* mutations in MPN is heterozygous base pair insertions or deletions (indels) in exon 9 causing one base pair (bp) reading frameshift. As a consequence, *CALR* exon 9 mutations generate a novel peptide sequence in the C-terminus with the loss of acidic domain and the KDEL ER retention sequence (Figure 1).[20]

More than 50 different mutational types in CALR have been detected. The most common types of *CALR* mutations are a 52 bp deletion (L367fs*46, type 1 mutation) and a 5 bp insertion of TTGTC (K385fs*47, type 2 mutation) accounting for more than 80% of all patients with mutant CALR. Rarely, *CALR* exon 9 point mutations have been reported in follicular lymphoma (E403X and E405Q) [21], PMF (E379D) [18] and chronic neutrophilic leukemia (E398D).[22] Importantly, *CALR* mutations are not only mutually exclusive with *JAK2* and *MPL* mutations, but they are also infrequently detected in other myeloid neoplasms such as myelodysplastic syndrome, chronic myelomonocytic leukemia and atypical chronic myeloid

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leukemia.[18,19] In addition, *CALR* mutations have not been found in other myeloid or lymphoid malignancies, solid tumors, or healthy controls. These findings indicate that *CALR* mutations are selective for ET and PMF. Based on these discoveries, *CALR* mutations have been included in the 2016 WHO classification system for the molecular diagnosis of ET and PMF.[23,24] The *JAK2* 46/1 haplotype does not predispose to *CALR*-mutated MPNs in one study.[25]

2.4 Screening for *CALR* mutations in MPNs

Several methods have been used to detect *CALR* exon 9 mutations including direct DNA sequencing, PCR followed by fragment analysis and immunostaining.[18,19,26,27] Although fragment analysis has a relatively high sensitivity for *CALR* mutations detection, it cannot discriminate point mutation from wild-type sequence. High-resolution melting analysis has also been used for the screening of *CALR* mutations.[28]

2.5 Clinical and and prognostic significance of *CALR* mutations

CALR mutations have been shown to have important clinical and prognostic significance in ET and PMF patients.[18,19,29] In ET patients, *CALR* mutations are associated with younger age, more frequently male, higher platelet counts, lower hemoglobin and leukocyte counts, and a lower risk of thrombosis than those with the *JAK2* mutation, with no difference in the rate of transformation to post-ET MF.[19,29-31] In PMF patients, *CALR* mutations are associated with younger age, higher platelet count, and lower DIPSS-plus score.[32] *CALR*-mutated PMF patients are also less likely to be anemic, require transfusions, or display leukocytosis. In an international study of 570 PMF patients, $CALR^{+}ASXL1^{-}$ patients had the longest survival (median 10.4 years) and $CALR^{-}ASXL1^{+}$ patients had shortest survival (median 2.3 years).[33] In another study demonstrating the additional value of the number of prognostically detrimental mutations in PMF, *CALR* mutations favorably affected survival, independently of both number

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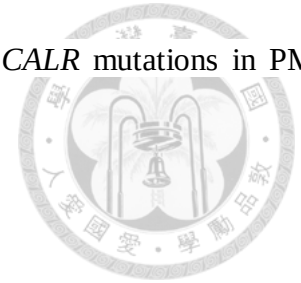
of mutations and IPSS/DIPSS-plus.[34] The prognostic advantage of *CALR* mutations in PMF patients might be confined to type 1 or type 1-like *CALR* variants.[35]



3. Molecular pathogenesis of *CALR* mutations in MPNs

3.1 Mutated *CALR* is an initiating event in MPNs

CALR mutations were acquired at the level of the hematopoietic stem cell (HSC) and clonal characterization of MPN samples has shown that mutated *CALR* is present in the earliest clone, which is consistent with it being an initiating event in MPNs.[18,19] Mutant CALR was shown to be highly expressed in the megakaryocyte lineage in bone marrow trephines immunostained with a polyclonal antibody against a 17-amino-acid peptide derived from mutant CALR's novel C-terminus.[26] In this study, wild-type CALR was also found to have a megakaryocyte-restricted expression pattern, which may explain why the mutant form is associated with MPNs characterized predominantly by abnormal megakaryopoiesis.[26]

3.2 Mutant *CALR* causes overactivation of JAK-STAT signaling

Although the C-terminus Golgi-to-ER retention signaling motif (KDEL) is lost in the CALR mutant protein, it appears that mutant CALR remains largely within the ER.[18,19] The characteristic basic and positively charged novel C-terminal peptide sequence has been predicted to alter the calcium-binding capacity of the protein.[36] How the novel mutant CALR C-terminus result in myeloid proliferation with a specificity for abnormal megakaryopoiesis remain unclear. Preliminary evidence suggests that mutant CALR may also lead to overactivation of JAK-STAT signaling. By using transcriptional studies, Rampal et al. have also shown that activated JAK2 signaling is seen in all MPN patients, including those with *CALR* mutations.[37] Besides, overexpression of the most frequent *CALR* deletion, but not wild-type *CALR* caused cytokine-independent growth in cell lines owing to the activation of

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signal transducer and activator of transcription 5 (STAT5) by means of an unknown mechanism.[18] Nevertheless, the MARIMO cell line which harbors a *CALR* mutation has been found to not dependent on JAK/STAT signaling and was more resistant to JAK inhibitors, in marked contrast to *JAK2*-mutated cell lines.[38] Upon addition of thapsigargin (which blocks ER Ca^{2+}-ATPase channels resulting in ER calcium depletion and increased cytosolic calcium levels), MARIMO cells also showed the slowest rate of increase of cytoplasmic calcium levels consistent with the concept that mutant CALR alters ER dependent calcium homeostasis.[38] This study raised the possibility that mutations of *CALR* may have activation of pathways other than the STATs.

3.3 *In vitro* and *in vivo* models of *CALR* mutations

Recent studies have focused on the underlying mechanism of *CALR* mutations in the pathophysiology of MPNs. With the use of *in vitro* cell lines and retroviral mouse models, *CALR* mutants were found to activate the JAK-STAT signaling in an MPL-dependent manner.[39-43] Araki and co-workers used the TPO-dependent megakaryocytic cell line UT-7/TPO to demonstrate that mutant CALR, but not wild-type, activates MPL and downstream signaling molecules including JAK2, STAT5, and extracellular signal-regulated kinase 1/2, and subsequently promotes the TPO-independent growth of UT-7/TPO cells.[39] Besides, they also showed that mutant CALR preferentially binds to MPL, and that the mutant-specific domain of CALR is required for this interaction. Marty *et al*. engrafted lethally irradiated recipient mice with bone marrow cells transduced with retroviruses expressing *CALR* type 1 and type 2 mutants.[40] In contrast to wild-type *CALR*, *CALR* type 1 and, to a lesser extent, *CALR* type 2 induced thrombocytosis due to a megakaryocyte hyperplasia. It is noteworthy that *CALR* type 1 mutation-expressing mice rapidly developed marked thrombocytosis and then progressed to a condition similar to human myelofibrosis. By contrast, *CALR* type 2

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mutation-expressing mice had a mild ET phenotype with low propensity to disease progression. In the report by Chachoua *et al.*, they found that pathogenic *CALR* mutants specifically activate the TPO receptor by a mechanism dependent on the presence of the extracellular N-glycosylation residues of MPL and the glycan-binding site at the new C-terminal tail of the mutant CALR.[41] They also found that *CALR* mutants can activate JAK2, and downstream STAT5/3/1, mitogen-activated protein kinase, and phosphatidylinositol-3 kinase signaling via MPL. Finally, Elf *et al*. demonstrated that expression of mutant *CALR* alone is sufficient to engender MPN in mice and recapitulates the disease phenotype of patients with *CALR*-mutant MPN.[42] They further showed that the thrombopoietin receptor MPL is required for mutant *CALR*-driven transformation through JAK-STAT pathway activation. Interestingly, Elf *et al*. also showed that the oncogenicity of mutant *CALR* is dependent on the positive electrostatic charge of the C-terminus of the mutant protein, which is necessary for physical interaction between mutant CALR and MPL.

4. Zebrafish animal models

4.1 Zebrafish as a valuable model to study hematopoiesis and leukemogenesis

Zebrafish has proven to be a useful vertebrate model in which to elucidate the molecular mechanisms of hematologic malignancies based on the high degree of genetic and morphological similarity in hematopoiesis between the zebrafish and human.[44,45] Over the last decade, studies using the zebrafish model have contributed to our understanding of vertebrate hematopoiesis, myelopoiesis, and leukemogenesis.[46-48] A high degree of similarity in the gene signatures of specific types of tumor cells in fish and humans have been demonstrated after comparisons of cancer-associated gene expression profiles indicating that the contributing genetic pathways leading to cancer are evolutionarily conserved.[49] Therefore, zebrafish can provide valuable knowledge about the mechanisms behind pathogenesis of leukemia.

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Furthermore, zebrafish animal model offers obvious advantages as a result of its rapid external development, small size and optical transparency of the embryos, which are permeable to small molecules and drugs. As a result, chemical screenings have been successfully conducted by using appropriated zebrafish lines.[50] The unique advantage of *in vivo* imaging in zebrafish also helps to dissect the molecular pathways underlying tumor initiation, progression and metastasis.

4.2 Genome editing tools in zebrafish

The zebrafish is an affordable, efficient, and genetically modifiable vertebrate model for studying hematopoiesis and leukemogenesis when compared to the traditional mammalian models. In addition, many molecular methods and models have been established to facilitate both forward and reverse genetic studies in zebrafish. For example, the expression of proteins can be "knocked down" transiently in the embryos by morpholino antisense oligonucleotides (MO) and microRNA or permanently "knockouted" by using the recently developed transcription activator-like effector nucleases (TALEN) technology or clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system.[51-59] Highly efficient *Tol2* transgenic tool is also available for use in the study of hematopoiesis and leukemogenesis in zebrafish.[46,60] The advance in technology has significantly improved the genetic tractability in zebrafish. One of the main limitations in zebrafish model is that antibody markers for hematopoiesis are mostly not available. However, this lack of markers can be compensated in some cases by the use of fluorescent transgenic blood cell reporter lines to identify hematopoietic cell types in zebrafish.[61]

5. B cell immune profiles in ET patients

5.1 Increased B cells activation in ET patients

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We have reported that activated B cells are increased in ET patients, and can facilitate platelet production mediated by cytokines, such as interleukin (IL)-1beta and IL-6 regardless *JAK2*V617F mutational status.[62] We found that increased production of B cell-activating factor (BAFF) by granulocytes and monocytes up-regulates toll-like receptor 4 (TLR4) expression on B cells of ET patients and promotes B cell activation, which play a pathogenic role augmenting thrombocytosis in ET by producing IL-1beta and IL-6. However, whether *CALR* mutations are also associated with activated B cells in ET patients requires further study.

6. Aims of the study

6.1 To develop a rapid and sensitive screening tool for the detection of *CALR* mutations

CALR mutations have become an important clonal maker for the diagnosis of MPNs especially in *JAK2*/*MPL*-unmutated ET and PMF. Our first aim is to develop a rapid and sensitive screening tool using HRMA for the detection of *CALR* exon 9 mutations.

6.2 To evaluate the clinical and prognostic significance of *CALR* mutations and *JAK2*/*CALR* co-mutations in Taiwanese ET patients

Although the clinical and prognostic significance of *CALR* mutations in Caucasian ET patients have been studied,[63,64] there is still a need to evaluate the clinical and prognostic significance of CALR mutations in Taiwanese ET patients. Besides, *JAK2* and *CALR* co-mutations have been reported in a few MPN patients in several studies. With the use of a highly sensitive HRMA, we have identified higher frequency of *JAK2* and *CALR* co-mutations in ET patients. We therefore want to evaluate the clinical and prognostic significance of *JAK2*/*CALR* co-mutations in Taiwanese ET patients.

6.3 To determine the B cell immune profiles in *CALR* mutated ET patients

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We have reported that activated B cells are increased in ET patients, and can facilitate platelet production mediated by cytokines, such as interleukin (IL)-1β and IL-6 regardless *JAK2*V617F mutational status.[62] The discovery of *CALR* mutations in *JAK2*/*MPL*-unmutated ET patients in December 2013 have prompted us to ask the question that whether increased B cell activation can also be found in ET with *CALR* mutations similar to that in *JAK2*V617F-mutated ET.[18,19,65]

6.4 To investigate the molecular pathogenesis of *CALR* mutations using zebrafish animal models

Although the expression of *CALR* mutants resulted in pathogenic thrombocytosis in adult mice, whether *CALR* mutants may disrupt normal hematopoiesis during early development remains unknown. We aim to evaluate the pathophysiologic effects of mutant CALR during embryonic hematopoietic development and to test the therapeutic effects of JAK inhibitors on mutant CALR using the *in vivo* zebrafish model.

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Chapter 2

High-resolution melting analysis as a rapid and sensitive screening tool for the detection of *CALR* mutations

1. Summary

Somatic *CALR* exon 9 mutations have recently been identified in patients with *JAK2*/*MPL*-unmutated myeloproliferative neoplasm, and have become an important clonal marker for the diagnosis of essential thrombocythemia (ET) and primary myelofibrosis. In the present study, we sought to use high-resolution melting analysis (HRMA) as a screening method for the detection of *CALR* mutations. 32 *JAK2*/*MPL*-unmutated ET patients were retrospectively enrolled and 8 healthy adults were used as wild-type control. *CALR* exon 9 mutation was independently screened by HRMA with the CFX Connect real-time system and Sanger sequencing. TA-cloning was used to detect *CALR* exon 9 mutations in patients suspected to have low mutant allele burden. The maximal sensitivity of HRMA in identifying both *CALR* type 1 and type 2 mutants from patients' genomic DNA was 2.5%. Twenty-two samples were found to have distinct melting curves from wild-type. The presence of *CALR* mutations in 16 of these 22 samples were confirmed by Sanger sequencing, while the other 6 samples were wild-type by sequencing. After TA-cloning, *CALR* mutations were detected in 5 of 6 patients from 1 (6%) of 16 clones to 1 (2%) of 50 clones. Therefore, HRMA identified *CALR* mutations in 21 (65.6%) of 32 ET patients compared to 16 (50%) patients by Sanger sequencing, with a false positive rate of 3% and no false negative. In conclusion, the HRMA developed in our system is a rapid and sensitive technique for the detection of *CALR* exon 9 mutations.

2. Introduction

The classic *BCL-ABL1*-negative chronic myeloproliferative neoplasm (MPN) is a clonal

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hematopoietic stem cell disorder and includes polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF).[2] ET is characterized by increased number of mature megakaryocytes in the bone marrow and sustained thrombocytosis in the peripheral blood. Although most ET patients have a normal life expectancy, some may encounter serious events such as thrombotic and hemorrhagic complications and leukemic transformation during their clinical course.[3] The *JAK2* V617F mutation was discovered in 2005, and has provided important diagnostic, therapeutic, and prognostic implications in MPNs. The frequency of *JAK2* V617F mutation is over 90% in polycythemia vera (PV), and about 60% in ET and PMF.[5-7] Besides, *MPL* mutations are identified in about 4-5% of *JAK2*-unmutated ET and PMF patients.[8] Following these seminal reports, other somatic mutations such as *LNK*, *TET2* and *DNMT3A* have also been detected in patients with MPN.[9] However, they are not mutually exclusive with *JAK2* and *MPL* mutations and also not specific to patients with MPN.[9,10] Despite many somatic mutations have been identified in patients with ET, clonal molecular marker is still not identified in ~40% of ET patients.

Recently, a high frequency (around 49-88%) of somatic calreticulin (*CALR*) mutations was identified in patients with *JAK2*/*MPL*-unmutated patients with ET and PMF.[18,19,32,66,67] Most *CALR* mutations in MPNs are heterozygous indels in exon 9 causing one base pair reading frameshift and resulted in the generation of a novel CALR protein C-terminus. The majority of the *CALR* exon 9 mutants were a 52 bp deletion of nt1172 to nt1223 (L367fs*46, type 1 mutation) and a 5 bp insertion of TTGTC (K385fs*47, type 2 mutation). Rarely, *CALR* exon 9 point mutations have been reported in follicular lymphoma (E403X and E405Q),[21] PMF (E379D)[18] and chronic neutrophilic leukemia (E398D).[22] Importantly, *CALR* mutations are not only mutually exclusive with *JAK2* and *MPL* mutations, but they

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are also infrequently detected in other myeloid neoplasms such as myelodysplastic syndrome, chronic myelomonocytic leukemia and atypical chronic myeloid leukemia.[18,19] These findings indicate that *CALR* mutations are quite specific for ET and PMF. Based on these discoveries, *CALR* mutations have been proposed to be included in the World Health Organization classification system for the molecular diagnosis of ET and PMF.[23] Therefore, the detection of *CALR* mutations with reliable and cost-effective methods in patients suspected to have ET or PMF is very important.

Several methods have been used to detect *CALR* exon 9 mutations including direct DNA sequencing, PCR followed by fragment analysis and immunostaining.[18,19,26,27] Although fragment analysis has a relatively high sensitivity for *CALR* mutations detection, it cannot discriminate point mutation from wild-type sequence. High-resolution melting analysis (HRMA) is a closed-tube and PCR-based technique for the detection of gene polymorphism and mutations by measuring changes in the melting of a DNA duplex.[68] HRMA is a well-established method for the detection of or prescreening for mutations both in a routine molecular laboratory and in a research setting. For example, HRMA has shown high sensitivity and specificity for the detection of *JAK2* V617F and *JAK2* exon 12 mutations in patients with MPN.[69-71] Recently, the feasibility of using HRMA for the detection of *CALR* mutations in ET and persistent thrombocytosis has been reported using the LightCycler 480 platform (Roche Diagnostics).[28] In this study, we sought to assess HRMA for rapid and sensitive detection of *CALR* exon 9 mutations in ET using the CFX Connect real-time system (Bio-Rad Laboratories, Hercules, CA, USA).

3. Patients and Methods

3.1 Patient samples and DNA extraction

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The screening for mutations in patients with hematologic neoplasms was approved by the Institutional Review Board of Mackay Memorial Hospital. 32 adult patients with *JAK2*/*MPL*-unmutated ET were retrospectively enrolled based on the 2008 World Health Organization classification and 8 healthy adults were used as wild-type control. Written informed consent was obtained from all patients. Patient genomic DNA was derived from bone marrow or peripheral blood by using EasyPure Genomic DNA Spin Kit (Bioman, Taipei, Taiwan).

3.2 Assay design and the HRMA technique

Oligonucleotide primers were designed by Primer3 software to flank all *CALR* exon 9 variants reported in MPN. The primers were used to amplify a 134 bp amplicon [GenBank: NM_004343]: forward 5' - GAAACAAATGAAGGACAAACAGG -3', and reverse 5' - CCTCATCCTCCTCATCCTCA -3'. PCR was performed in a 20 μl reaction volume containing precision melt supermix (Bio-Rad Laboratories, Hercules, CA, USA), 100 nM of each primer, and 25 ng genomic DNA. The 134 bp amplicon was run according to the following conditions: an initial denaturation step of 95˚C for 2 min, followed by 35 cycles of 95˚C for 10 sec, 58˚C for 30 sec, and 72˚C for 30 sec. After completion of amplification, DNA was heated at 95˚C for 30 sec, kept at 60˚C for 1 min, and then melted from 70 to 95˚C (increment 0.2˚C, dwell time 10 sec). The results were analyzed using the Bio-Rad Precision Melt Analysis software. Melting profiles were normalized, grouped and displayed as fluorescence-versus-temperature plots or subtractive difference plots (-df/dt vs T). All samples with distinguished melting curves from wild-type were confirmed by duplicate study. Both type 1 and type 2 *CALR* exon 9 mutant cDNA were obtained by direct DNA synthesis, and *CALR* wild-type cDNA was cloned from patient sample.

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3.3 Sanger sequencing

All patients were also independently screened for *CALR* exon 9 mutations spanning codons 352–417 by Sanger sequencing on an ABI 3730 sequencer based on previously described method.[19] All identified sequence variants were subjected to repeated bi-directional sequencing for confirmation. Mutations were identified using DNA Dynamo sequence analysis software (Blue Tractor Software Ltd, Conwy, UK). All patients had been screened for *JAK2*V617F and *MPL* exon 10 mutations as previously described.[7,9]

3.4 Sensitivity of HRMA in detecting *CALR* type 1 and type 2 mutations

To study the sensitivity of the methodology, we serially diluted two plasmids carrying *CALR* type 1 and type 2 mutations with wild-type plasmid DNA in different concentrations (100% mutant, 50% mutant, 25% mutant, 10% mutant, 7.5% mutant, 5% mutant, 2.5% mutant, 1.25% mutant, and 0% mutant). The sensitivity tests were carried out in triplicate samples. The sensitivity of HRMA was validated by serially diluting two patient samples carrying *CALR* type 1 and type 2 mutations with control DNA. Based on the relative peak areas of the mutant and wild-type PCR products, the mutant allele burden of these 2 patient samples was estimated to be ~50%. *CALR* type 1 and type 2 patients' DNA were also serially diluted by wild-type DNA in different concentrations (50% mutant, 25% mutant, 12.5% mutant, 5% mutant, 3.75% mutant, 2.5% mutant, 1.25% mutant, 0.625% mutant, and 0% mutant). We did not evaluate the sensitivity of HRMA for other types of *CALR* mutations because they are less frequently detected.

3.5 TA-cloning

The PCR products of *CALR* exon 9 of 6 ET patients suspected to have a low allele burden mutant were purified using a EasyPure High Pure PCR clean-up Kit (Bioman, Taipei,

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Taiwan) and cloned into a pGEMT-easy vector (Promega, Madison, CA, USA). We obtained at least 16 clones in each individual. The PCR product of each clone was checked on a 2% agarose gel by electrophoresis for the presence of mutant band. All selected clones were then sent for Sanger sequencing regardless the presence or absence of mutant band.

4. Results

4.1 Sensitivity of HRMA in identifying the *CALR* type 1 and type 2 mutants

We first evaluated the sensitivity of HRMA in detecting the *CALR* type 1 and type 2 mutant plasmid DNA with different concentrations of mutant DNA serially diluted by wild-type plasmid DNA. HRMA could distinguish *CALR* type 1 and type 2 mutants with the maximal sensitivity of 2.5% and 1.25%, respectively (Figure 2A and C). Whereas, the maximal sensitivity of Sanger sequencing for the detection of both *CALR* type 1 and type 2 mutants was at least 10% or higher (Figure 2B and D). Besides, the maximal sensitivity of HRMA was validated with 2 patient samples and was found to be 2.5% for both *CALR* type 1 and type 2 mutants (Figure 3A and B).

4.2 Detection of *CALR* exon 9 mutations in *JAK2*/*MPL*-unmutated ET patients

In this cohort of 32 ET patients, the normalized melting curves of 22 (68.8%) patient samples clearly showed a distinctive difference from that of wild-type group, and the representative normalized melting curves from 6 of the 22 patient samples were shown in Figure 4A. When the data were represented in difference plots, the individual nature of the mutant melting curves became more apparent as illustrated in Figure 4B. To determine the concordance between HRMA and Sanger sequencing, all 32 ET patients were also screening for *CALR* exon 9 mutations by Sanger sequencing. In the 22 ET patients with distinctive melting curves, Sanger sequencing could only detect *CALR* exon 9 mutations in

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16 patients. All the remaining 6 patients with distinctive melting curves as shown on Figure 5A had wild-type *CALR* exon 9 sequences by Sanger sequencing. The other 10 patients were determined to have wild-type *CALR* by both HRMA and Sanger sequencing. In the 6 patients with discordant results between HRMA and Sanger sequencing, we then performed TA-cloning to determine whether these 6 patients had low allele burden *CALR* mutations not detected by Sanger sequencing (Figure 5B and C).

After TA-cloning, *CALR* type 2 mutations were detected in 5 of 6 patients from 1 (6%) of 16 clones to 1 (2%) of 50 clones, and only 1 clone from each patient was tested positive for the *CALR* mutation (Figure 5). We did not identify *CALR* mutation in the last patient after screening for 100 clones. Therefore, HRMA identified *CALR* mutations in 21 (65.6%) of 32 ET patients compared to 16 (50%) by Sanger sequencing. The possible 3% false positive rate is low and no false negative was detected in our HRMA system.

In this study, 21 *JAK2*/*MPL*-unmutated ET patients were found to harbor 6 types of *CALR* exon 9 mutations: 5 type 1 (p.L367fs*46), 11 type 2 (p.K385fs*47), 1 type 3 (p.L367fs*48), 2 type 34 (p.K385fs*47), and 2 other types (p.L367fs*43 and p.E369fs*50). All *CALR* exon 9 mutations are indels causing +1 base-pair reading frameshift, with type 2 (11/21, 52.4%) being the most prevalent mutational type. In these 21 patients with *CALR* mutations, the number of female patients was slightly higher than male patients (57% vs 43%) (Table 1).

5. Discussion

The identification of *CALR* mutations is important in the molecular diagnosis of MPN especially in *JAK2*/*MPL*-unmutated patients. In addition, *CALR* mutational status was

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found to be one of the most significant risk factor for survival in PMF.[33] Sanger sequencing has been used to detect *CALR* exon 9 mutations in many studies, but it is rarely sensitive below a 10% mutant allele burden as illustrated in Figure 2B and D. Fragment analysis assay was also used and the sensitivity was estimated to be 5% or less for *CALR* exon 9 mutations.[27] Although fragment analysis assay is able to detect most indel mutations in *CALR*, it cannot discriminate point mutation from wild-type sequence. Recently, Bilbao-Sieyro et al. showed that HRMA is a feasible method for the detection of *CALR* mutations using the LightCycler 480 platform.[28] The amplicon size of their primer sequences was 265 bp, and the limit of detection for *CALR* type 2 (K385fs*47) mutant was of 3%. However, the ideal amplicon length for HRMA is usually less than 250 bp. In this study, the HRMA primers with an amplicon size of 134 bp were designed and are capable of detecting common *CALR* exon 9 mutations in myeloid neoplasms with satisfactory sensitivity.

Based on the dilution studies using patients' genomic DNA, the maximal sensitivity of our HRMA using CFX Connect real-time system for both *CALR* type 1 (L367fs*46) and type 2 (K385fs*47) mutants was of 2.5%. In addition to 16 *CALR* mutated samples that could be detected by both HRMA and Sanger sequencing, we were able to identify another 5 patients with low *CALR* mutant allele burden only by HRMA. In this situation, we used TA-cloning followed by Sanger sequencing to confirm the mutation suspected. Alternatively, fragmented analysis may be used for mutation detection because it also has a better sensitivity than Sanger sequencing. We were not able to detect *CALR* mutation in 1 of the 6 patients after screening for 100 clones. It is likely that this patient might still have low allele burden *CALR* mutation which, by chance was missed by random selection of clones (Figure 5A). However, we counted the result as a possible 3% false positive rate to

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avoid overestimation of our data. Importantly, no false negative was found in our HRMA system and this is critical in regard to its role as a screening tool.

HRMA developed in this study can be utilized for rapid, sensitive and reliable detection of *CALR* mutations. Although a total of 5 SNPs (rs201971744, rs143880510, rs370029737, rs374121178 and rs150264068) are reported in the region covered by our amplicon, the minor allele frequency of 3 of them is reported to be less than 0.01%. Therefore, the influence of these 5 SNPs to our HRMA system will likely be very small. Nevertheless, one limitation to this HRMA methodology is that it will not be able to identify the 2 *CALR* exon 9 point mutations reported in follicular lymphoma (E403X and E405Q) because they are not covered by our 134 bp amplicon. The frequency and significance of these 2 *CALR* point mutations in follicular lymphoma are currently not yet clear. Therefore, our HRMA methodology is suitable for use in patients suspected to have myeloid neoplasms especially ET and PMF. By using HRMA, we detected a total of 6 different types of *CALR* mutations in ET patients. All the *CALR* mutations detected in this study resulted in a +1 base-pair shifting in the reading frame and generated the characteristic novel peptide sequence in the C-terminus. All the *CALR* exon 9 indel mutations likely contribute to a similar, yet not clearly understood molecular pathogenesis in ET and PMF. In addition, the number of female patients was slightly higher than male patients (57% vs 43%) in our 21 ET patients with *CALR* mutations, and this has also been observed in other study.[72]

HRMA, a close-tube method, is not only rapid as it is conducted immediately after PCR amplification, but is also cost effective because it can reduce the use of Sanger sequencing. By using HRMA, a medium-throughput screening for *CALR* mutations is also possible. Based on these advantages, our results clearly illustrated that HRMA is a more suitable and

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sensitive method over Sanger sequencing for the screening of *CALR* mutations in both clinical and research settings. Nevertheless, in samples with distinct melting curves, complimentary Sanger sequencing is still required to determine their exact genotypes because the pattern of melting curves does not correlate with specific *CALR* mutational types.

In conclusion, we have shown that HRMA is a rapid, sensitive, reliable and cost effective method for the detection of *CALR* mutations. Because *CALR* mutations have important diagnostic and prognostic significance in ET and PMF, HRMA can be a useful screening method for the identification of common *CALR* mutations.

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Chapter 3

The clinical and prognostic significance of *CALR* mutations and *JAK2*/*CALR* co-mutations in Taiwanese ET patients

1. Summary

Frequent *CALR* mutations have been discovered in patients with *JAK2*/*MPL*-unmutated essential thrombocythemia (ET) and primary myelofibrosis. We sought to screen for *CALR* exon 9 alterations with high-resolution melting analysis (HRMA) in 92 adult ET patients, and to determine the clinical and molecular correlates. In this cohort, 59 (64%) patients harbored *JAK2* V617F mutation and one (1%) harbored *MPL* W515K mutation. By HRMA followed by TA-cloning, we identified classic *CALR* indel mutations in 21 (22.8%) patients. Eleven (12%) patients were triple-negative. The 59 *JAK2*-mutated patients were also screened for *CALR* exon 9 alterations by HRMA, and 16 (27.1%) samples were found to have distinct melting curves from wild-type. In 2 of these 16 samples, one *CALR* type 3 mutation and one single nucleotide polymorphism (rs143880510) were detected by Sanger sequencing. Although the remaining 14 patients were wild-type by Sanger sequencing, *CALR* alterations were detected in 12 (85.7%) patients after TA-cloning: 3 harbored classic *CALR* indel mutations, 5 (8.5%) harbored 4 types of 3 bp inframe deletions, and 5 (8.5%) harbored 5 types of point mutations. Overall, various *CALR* exon 9 alterations were detected in 13 (22%) of 59 *JAK2*-mutated ET patients. In comparable to previous reports, *CALR* mutations were associated with younger age ($p=0.025$), higher platelet count ($p<0.001$) and lower hemoglobin level ($p=0.016$). *JAK2*-mutated ET patients with concomitant *CALR* alterations were associated with oldest age ($p=0.025$), higher thrombotic events after diagnosis ($p=0.048$), higher major arterial thrombotic events after diagnosis ($p=0.022$) and more patients being high risk group for thrombo-hemorrhagic

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complications (p=0.023). Frequent *CALR* exon 9 alterations in *JAK2*-mutated ET patients define a specific subgroup of patients with increased risk of thrombotic events.

2. Introduction

Essential thrombocythemia (ET) is a clonal hematopoietic stem cell neoplasm and one of the classic *BCL-ABL1*-negative chronic myeloproliferative neoplasm (MPN), which also includes polycythemia vera (PV) and primary myelofibrosis (PMF).[2] *JAK2* V617F mutation can be detected in more than 95% PV patients, and 50% to 60% of ET and PMF patients. *MPL* mutations at codon 515 are found in 3% to 5% of *JAK2*-unmutated ET and PMF patients. The 2008 World Health Organization (WHO) classification has incorporated *JAK2* V617F and *MPL* mutations into the diagnostic criteria of MPN. Both *JAK2* V617F and *MPL* mutations cause activation of the Janus kinase/signal transducer and activator of transcription (STAT) signaling pathway leading to the development of JAK inhibitor therapy in MPN.

Recently, two seminal studies discovered a high frequency of somatic calreticulin (*CALR*) mutations in patients with *JAK2*/*MPL*-unmutated ET and PMF.[18,19] The pattern of most *CALR* mutations in MPN is heterozygous indels in exon 9 causing one base pair (bp) reading frameshift. *CALR* mutations have been shown to have important diagnostic and prognostic significance in ET and PMF patients,[18,19,29] and will likely be incorporated into the WHO diagnostic criteria for MPN. *In vitro* studies on the molecular pathogenesis of *CALR* mutations in MPN have shown controversial results in regard to the involvement and/or activation of the JAK/STAT signaling pathway,[18,19,38] and the exact pathogenesis of *CALR* mutations is not yet completely understood at the present time.

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Several techniques such as Sanger sequencing and polymerase chain reaction (PCR) followed by fragment analysis have been used to detect *CALR* mutations.[10,18,19,27] High-resolution melting analysis (HRMA) is a well-established method for the screening of mutations, and we have developed a rapid and sensitive HRMA for the detection of *CALR* exon 9 mutations.[73] In this study, we sought to screen a cohort of 92 Taiwanese ET patients for *CALR* exon 9 mutations with HRMA and Sanger sequencing independently, and to determine the clinical and molecular correlates.

3. Patients and Methods

3.1 Patients

The institutional review board of Mackay Memorial Hospital has approved the screening for mutations. All patients provided written informed consent. Diagnosis of ET was established based on the 2008 WHO criteria. The clinical and laboratory characteristics at the time of diagnosis or referral were collected. Genomic DNA derived from bone marrow, peripheral blood, and peripheral blood granulocytes and/or mononuclear cells were used for mutation screening.

3.2 Screening for *CALR* mutations

CALR mutations were screened by Sanger sequencing on an ABI 3730 sequencer as preciously described.[19] *CALR* exon 9 mutations were independently screened by HRMA using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) as previously described with a maximal sensitivity of 2.5% for both *CALR* type 1 and type 2 mutants.[73] Briefly, a pair of oligonucleotide primers were used to amplify a 134 bp amplicon [GenBank: NM_004343] which flanked all *CALR* exon 9 variants reported in

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MPN. All samples with distinguished melting curves from wild-type were confirmed by duplicate studies. Peripheral blood samples from 78 healthy adults were also used to validate the specificity of our HRMA. *JAK2* V617F mutation was screened by allele-specific PCR with an analytic sensitivity of 5% and *MPL* exon 10 mutation by Sanger sequencing as previously described.[7,9]

3.3 TA-cloning

TA-cloning was performed by pGEM-T easy vector system (Promega, Madison, CA, USA) as previously described.[73] At least 10 clones in each individual were randomly selected for the screening of *CALR* exon 9 alterations by Sanger sequencing. All novel single nucleotide variant that was only detected once was treated as artifact and excluded.

3.4 Statistical analysis

The correlation between clinical characteristics and mutational status was calculated by the chi-square test or Fisher's exact test. The comparison between continuous and categorical variables was performed by the Mann-Whitney U test or Kruskal-Wallis H test. SPSS Statistics software (IBM, New York, USA) was used for all calculations. P values <0.05 were considered significant.

4. Results

4.1 *CALR* exon 9 mutations

Among the 92 ET patients (median age 53 years; 58% females), 59 (64%) patients harbored *JAK2* V617F mutation and one (1%) patient harbored *MPL* W515K mutation. 32 *JAK2*/*MPL*-unmutated ET patients were utilized for the development of our HRMA platform.[73] Briefly, 22 (68.8%) samples were found to have distinct melting curves from

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wild-type. In 16 of these 22 samples, Sanger sequencing confirmed the presence of 6 types of *CALR* mutations: 5 type 1 (p.L367fs*46), 6 type 2 (p.K385fs*47), 1 type 3 (p.L367fs*48), 2 type 34 (p.K385fs*47), and 2 other types (p.L367fs*43 and p.E369fs*50). The other 6 samples were wild-type by sequencing, and *CALR* type 2 mutations were detected in 5 of 6 patients after TA-cloning indicating the presence of low allele burden *CALR* mutants in them. By using our HRMA platform, we identified *CALR* mutations in 21 (22.8% overall and 65.6% in *JAK2*/*MPL*-unmutated) ET patients and this frequency is comparable to other studies.[18,19,29] 11 (12%) ET patients were negative for *JAK2*, *CALR* and *MPL* mutations. In the 78 healthy adults, 2 samples were found by HRMA to have distinct melting curves from wild-type. One single nucleotide polymorphism (SNP, rs143880510) and one wild-type were found after Sanger sequencing in these 2 samples. Therefore, the false positive rate of our HRMA system was 1.3%.

4.2 *CALR* exon 9 alterations

After screening the 59 *JAK2* V617F-mutated ET patients for *CALR* alterations by HRMA, 16 (27.1%) samples were found to have distinct melting curves from wild-type (Figure 6). In 2 of these 16 samples, one *CALR* type 3 mutation (p.L367fs*48) and one SNP (rs143880510) were detected by Sanger sequencing. All the other 14 samples were wild-type by sequencing. Surprisingly, we detected a high frequency of *CALR* exon 9 alterations in 12 (85.7%) of these 14 patients after TA-cloning (Table 2). Three patients harbored the classic *CALR* indel mutations: one each of type 2 p.K385fs*47, p.E370fs*60 and p.E371fs*59. Hence, 4 (6.8%) ET patients had classic *CALR* indel and *JAK2* V617F co-mutations in this cohort. Five patients (8.5%) including the aforementioned patient (P520) with type 2 *CALR* mutation harbored 4 types of 3 bp inframe deletions all resulted in the deletion of a single amino acid of glutamic acid: two p.E381del, and one each of

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p.E371del, p.E378del and p.E396del (Figure 7). Another five patients (8.5%) harbored 5 types of point mutations: one each of p.E374X, p.E380X, p.K391X, p.E372G and p.E380G. The latter p.E380G has been reported as a SNP but might be a low allele burden somatic mutation in this patient because it was only detected after TA-cloning and not by Sanger sequencing on patient's genomic DNA. The remaining two patients were found to have wild-type *CALR* exon 9 after screening for 100 independent clones, and were counted as *CALR* wild-type. Overall, various *CALR* exon 9 alterations were detected in 13 (22%) of 59 *JAK2* V617F-mutated ET patients.

4.3 Clinical and molecular correlates

We then examined the clinical and molecular correlates in 91 ET patients excluding the one *MPL*-mutated patient (Table 3). *JAK2*-mutated ET patients with concomitant *CALR* alterations were associated with oldest age (p=0.025), higher thrombotic events after diagnosis (p=0.048), higher major arterial thrombotic events after diagnosis (p=0.022) and more patients being high risk group for thrombo-hemorrhagic complications (p=0.023). In comparable to previous reports, *CALR* mutations were associated with younger age (p=0.025), higher platelet count (p<0.001) and lower hemoglobin level (p=0.016). *JAK2* V617F-mutation was associated with leukocytosis (p=0.046).

5. Discussion

After the discovery of *CALR* mutations, it has been proposed to be mutually exclusive with *JAK2* and *MPL* mutations in MPN. However, *CALR* and *JAK2* V617F co-mutations have been reported in a few MPN cases across different ethnic groups and the frequency is usually below 1%.[10,32,74-76] In contrast to these reports, we detected a higher frequency of

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6.8% *CALR* indel and *JAK2* co-mutations in ET patients. Interestingly, 3 of these *CALR* mutations were low allele burden mutants not detected by Sanger sequencing. Nevertheless, the use of a sensitive HRMA technique has enabled us to detect these low allele burden *CALR* mutants in both *JAK2*-mutated and *JAK2*/*MPL*-unmutated ET patients.

In addition, we also detected several *CALR* exon 9 point mutations and inframe deletions in *JAK2*-mutated ET patients, but none in our *JAK2*/*MPL*-unmutated ET patients. Recently, point mutations in *CALR* were also reported in follicular lymphoma (E403X and E405Q), PMF (E379D) and chronic neutrophilic leukemia (E398D).[22] Two rare inframe deletions in *CALR* exon 9 (p.E393_E395del and p.E405del) have been reported in the National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project with undetermined significance. All the 5 inframe deletions we detected were 3 bp deletions similar to the latter one. Although the possibility of low allele burden germline sequence variations cannot be completely excluded, these 3 bp inframe deletions detected by HRMA were more likely to be low allele burden somatic mutations not detected by Sanger sequencing in our patients.

Interestingly, *CALR* point mutations (E381A and D373M) and inframe deletions (E381_A382>A, D397_D400>D, D400_K401>D and E405_V409>V) were also detected in patients with suspected MPN and *JAK2*-mutated MPN in another study albeit with a lower frequency.[77] These *CALR* alterations were also found to co-occur with *MPL*, *CSF3R*, *ASXL1* and *ZRSR2*. Currently, the role of these *CALR* point mutations and inframe deletions in the molecular pathogenesis of MPN is not yet clear. Because they frequently

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co-occurred with mutations involving the JAK-STAT pathway and affected disease phenotype in *JAK2*-mutated ET patients, these non-classic *CALR* mutant proteins are suspected to play a contributory role in the pathogenesis of MPN.[77] The frequency of these non-classic *CALR* mutations in PMF and other MPN requires further study.

In conclusion, we have detected a high frequency of both classic and non-classic *CALR* exon 9 alterations in *JAK2*-mutated ET patients by HRMA. The presence of *CALR* alterations in *JAK2*-mutated ET defines a specific subgroup of patients requiring careful follow-up and management for their increased risk of thrombotic events. Because our study is limited by small patient number, larger study is warranted to confirm our observation.

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Chapter 4

B cell immune profiles in *CALR* mutated ET patients

1. Summary

Essential thrombocythemia (ET) is a *BCL-ABL1*-negative myeloproliferative neoplasm. We have reported that increased activated B cells can facilitate platelet production mediated by cytokines regardless *JAK2* mutational status in ET. Recently, *calreticulin* (*CALR*) mutations were discovered in ~30% *JAK2*/*MPL*-unmutated ET and primary myelofibrosis. Here we sought to screen for *CALR* mutations and to evaluate B cell immune profiles in a cohort of adult Taiwanese ET patients. B cell populations, granulocytes/monocytes membrane-bound B cell-activating factor (mBAFF) levels, B cells toll-like receptor 4 (TLR4) expression and intracellular levels of interleukin (IL)-1β/IL-6 and the expression of CD69, CD80, and CD86 were quantified by flow cytometry. Serum BAFF concentration was measured by ELISA. 48 healthy adults were used for comparison. 19 (35.2%) of 54 ET patients harbored 8 types of *CALR* exon 9 mutations including 4 (7.4%) patients with concomitant *JAK2*V617F mutations. Compared to *JAK2*V617F mutation, *CALR* mutations correlated with younger age at diagnosis (p=0.04), higher platelet count (p=0.004), lower hemoglobin level (p=0.013) and lower leukocyte count (p=0.013). Multivariate analysis adjusted for age, sex, follow-up period and hematological parameters confirmed that increased activated B cells were universally present in *JAK2*-mutated, *CALR*-mutated and triple-negative ET patients when compared to healthy adults. *JAK2*- and *CALR*-mutated ET have significantly higher fraction of B cells with TLR4 expression when compared to triple-negative ET (p=0.019 and 0.02, respectively). *CALR*-mutated ET had significantly higher number of CD69-positive activated B cells when compared to triple-negative ET (p=0.035). In conclusion, increased B cell activation is present in ET patients across different mutational subgroups.

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2. Introduction

Essential thrombocythemia (ET) is a *BCL-ABL1*-negative myeloproliferative neoplasm (MPN), and is characterized by increased number of mature megakaryocytes (MKs) in the bone marrow and sustained thrombocytosis in the peripheral blood.[2] ET is associated with an increased risk of hemorrhagic and thrombotic complications and leukemic transformation.[2] Most ET patients can have a normal life expectancy but some may encounter serious events during their disease course. In 2005, the *JAK2*V617F mutation was discovered in MPNs including 50-60% patients with ET and primary myelofibrosis (PMF).[78-81] *JAK2*V617F mutation plays an important role in cytokine-independent hematopoietic stem cells (HSCs) proliferation in MPNs. Also, hypersensitivity of hematopoietic cells to cytokines stimulation is noted in MPNs through the interaction between *JAK2*V617F mutation and various cytokine receptors.[82] Recently, a high frequency of *calreticulin* (*CALR*) mutations was discovered in *JAK2*/*MPL*-unmutated ET and PMF.[18,19,65] We and others have reported that *CALR* mutations are associated with distinct clinical characteristics including higher platelet counts, lower leukocyte counts and hemoglobin levels, and a lower thrombosis risk when compared to *JAK2*-mutated ET patients.[18,19,29,65,83] Using *in vitro* and/or *in vivo* models, we and others have recently reported that mutant *CALR* can activate JAK-STAT signaling pathway through an MPL-dependent mechanism to mediate pathogenic thrombopoiesis.[39-43,84,85]

CALR is a 46-kDa Ca2+ binding chaperone protein located in the endoplasmic reticulum, but it can also localize to cell surface and accumulate in extracellular compartments.[14] In addition to ensuring proper protein and glycoprotein folding within the lumen of

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endoplasmic reticulum, CALR was also found to involve the immune response to pre-apoptotic cancer cells, and early cell surface exposure of CALR was followed by expression and release of heat-shock proteins (e.g. HSP70), and high-mobility group I (HMGB1) protein.[15] Recombinant *CALR* fragment was shown to exhibit potent stimulatory activities against B cells.[16,17] Recently, we reported that activated B cells are increased in ET patients, and can facilitate platelet production mediated by cytokines, such as interleukin (IL)-1β and IL-6 regardless *JAK2*V617F mutational status.[62] We found that increased production of B cell-activating factor (BAFF) by granulocytes and monocytes up-regulates toll-like receptor 4 (TLR4) expression on B cells and promotes B cell activation in ET patients. Consequently, these activated B cells play a pathogenic role in augmenting thrombocytosis by producing IL-1β and IL-6 in ET patients through cytokine-dependent thrombopoiesis in the bone marrow. However, ET with *CALR* mutations was not included in our previous study because *CALR* mutations have not yet been discovered in MPNs when we conducted our study in 2013. The discovery of *CALR* mutations in *JAK2*/*MPL*-unmutated ET patients in December 2013 have prompted us to ask the question that whether increased B cell activation can also be found in ET with *CALR* mutations similar to that in *JAK2*V617F-mutated ET.[18,19,65] Hence, we sought to screen for *CALR* mutations in a cohort of adult Taiwanese ET patients and to evaluate B cell immune profiles in *JAK2*V617F-mutated, *CALR*-mutated and triple-negative ET in this study.

3. Patients and Methods

3.1 Patient enrollment

The screening for mutations in patients with hematologic neoplasms was approved by the Institutional Review Board of MacKay Memorial Hospital (09MMHIS157 and

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12MMHIS034). 54 adult Taiwanese ET patients were enrolled and written informed consent was obtained. The clinical and laboratory characteristics at the time of diagnosis/referral and at testing were determined retrospectively by chart review. Parts of the clinical data of 48 patients in this cohort have been described in our recent publication.[83]

3.2 Mutation screening

Genomic DNA derived from bone marrow granulocytes, peripheral blood leukocytes, peripheral blood granulocytes or peripheral blood mononuclear cells were used for the screening of *CALR* exon 9 mutations spanning codons 352–417 [GenBank: NM_004343]. Oligonucleotide primers targeting *CALR* exon 9 were used to amplify a 285 bp product: (*CALR* Forward 5'-CCTGCAGGCAGCAGAGAAAC-3') (*CALR* Reverse 5'-ACAGAGACATTATTTGGCGCG-3'). The PCR were amplified using GoTaq Green Master Mix (Promega, CA, USA) on a Thermal Cycler® PCR System 2720 (Applied Biosystems, CA, USA). The final concentrations were as follows: 3 mM MgCl2 and 0.4 mM deoxyribo-nucleotide triphosphate, 2.5 μM each of forward and reverse oligo primer, 50 ng of DNA template and water to a final reaction volume of 20μl. Cycling parameters consisted of an initial denaturation at 94^{o}C for 5 min; 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 58^{o}C for 30s, and extension at 72^{o}C for 45s; and final extension at 72^{o}C for 10 min. The EXO-SAP reagent (USB, CA, USA) was used to clean up the PCR product prior to sequencing. Direct DNA sequencing was conducted using the same primers for amplification and a BigDye terminator v3.1 Cycle sequencing kit (Applied Biosystems, CA, USA) on an ABI 3730 sequencer. Mutations were identified using DNA Dynamo sequence analysis software (Blue Tractor Software Ltd, Conwy, UK). All identified sequence variants were subjected to repeated bidirectional sequencing for confirmation.

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CALR exon 9 mutations were also independently screened by high-resolution melting analysis (HRMA) and TA-cloning was used to detected low allelic burden mutants in selected samples as previously described.[73] *JAK2*V617F mutation was determined by allele-specific PCR as previously described and/or mutation-enrich high sensitive PCR method over *JAK2* exon 14 mutation hot spot area.[86,87] *MPL* exon 10 mutation was screened by nucleotide sequencing as previously described.[88] In order to exclude the influence of other possible mutations on B cell immune profiles, *DNMT3A* exon 23 and *IDH1*/*2* exon 4 mutations were also screened as previously described.[88]

3.3 B cell immune profiles

The quantification of B cell populations and various B cell subsets including T1, T2, pre-germinal center, memory, and plasmablast/plasma cells, based upon the surface expression of CD19, CD24, CD27, CD38, and IgD was assessed by flow cytometric analysis as previously described.[62] To quantify various B cell populations, peripheral blood mononuclear cells were isolated using Ficoll-Paque Plus (Amersham, Buckinghamshire, UK) gradient centrifugation. A total of 5×10^5 cells were stained with various combinations of fluorescence-conjugated monoclonal antibodies. Stained cells were fixed with 4% paraformaldehyde and examined by FACScalibur (BD, Franklin Lakes, NJ, USA). A total of 50,000 lymphoid events were acquired in each sample. Cells were stained for 20 minutes with directly conjugated anti-human monoclonal antibody. αCD24-PerCP and αCD38-APC were purchased from BD Biosciences, αCD19-FITC,-PE, αCD27-FITC, and αIgD-PE from BioLegend (San Diego, CA, USA), and αCD27-APC from eBioscience (San Diego, CA, USA). Peripheral blood mononuclear cells were incubated with Fc receptor (FcR) reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 minutes to prevent nonspecific staining. Granulocytes and monocytes membrane-bound BAFF

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(mBAFF) levels, TLR4 expression and the expression of CD69, CD80, and CD86 on B cells were also quantified by flow cytometry using appropriated antibodies.[62] To identify B cell activation, CD19-positive B cells were gated to examine the expression of CD80, CD86 and CD69. The FITC-conjugated monoclonal antibodies of αCD80, αCD86, and αCD69 were purchased from BD Biosciences. Positive staining populations were determined by comparison with isotype controls. For mBAFF determination, surface expression of BAFF of granulocytes or monocytes was examined by flow cytometry using PE-conjugated αBAFF monoclonal antibody (BioLegend). For surface TLR4 determination, PE-conjugated αTLR4 monoclonal antibody (eBioscience) was used. The B cell immune profiles of 38 patients in this cohort had been described in our previous publication.[62] B cell immune profiles from 48 healthy adults were used for comparison.

3.4 Intracellular cytokine detection

For analysis of human intracellular cytokine production, isolated B cells were cultured in RPMI-1640 media (Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-amphotericin B (Life Technologies) overnight.[62] For intracellular staining, cells were first stained with combinations of αCD19-PE and αCD27-APC monoclonal antibodies. Then, cells were washed, fixed, permeabilised, and stained for detection of intracellular cytokines with αIL-1beta-FITC (eBioscience) or αIL-6-FITC (BioLegend) monoclonal antibodies.

3.5 Serum BAFF determination

Serum BAFF concentrations were measured using an enzyme-linked immunosorbent assay kit from R&D Systems according to the manufacturer's instructions.[62] Briefly, appropriate sample volumes were transferred by pipette into the wells of antihuman BAFF-coated

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microtitre strips. Secondary monoclonal antibody-conjugated horseradish peroxidase was then added to the wells and incubated at room temperature for 90 min. After removing the excess secondary antibodies by washing, the samples were incubated with substrate solution to produce a color, which was directly proportional to the concentration of BAFF present in the sample.

3.6 Statistical analysis

The correlation between *CALR* mutational status and clinical characteristics was calculated by the chi-square test or Fisher's exact test. Kolmogorov-Smirnov test was used to test normality of numerical variables. The independent t-test and the one-way analysis of variance (ANOVA) were used to compare differences between two and three independent groups when the dependent variables were normally distributed, respectively. When the dependent variables were not normally distributed, non-parametric Mann-Whitney U test and Kruskal-Wallis H test were used to compare differences between two and three independent groups, respectively. Spearman's rank correlation coefficient was used to evaluate the relationship between two variables. Multivariate analysis was performed using linear regression model adjusted for age, sex, follow-up period and hematological parameters. Statistical significance was defined as a two-sided p value <0.05 and SPSS version 22.0/24.0 (IBM, New York, USA) was used for analyses.

4. Results

4.1 Mutational analysis

Among 54 ET patients (median age at diagnosis 54.5 years; 54% females; median follow-up 4.4 years), 27 (50%) patients harbored the *JAK2*V617F mutation and one (1.9%) patient harbored the *MPL* W515K mutation. By nucleotide sequencing and HRMA, 19

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(35.2% overall and 68.2% in *JAK2*/*MPL*-unmutated cases) patients harbored 8 types of *CALR* exon 9 mutations: 2 type 1 (p.L367fs*46), 10 type 2 (p.K385fs*47), 2 type 3 (p.L367fs*48), 1 type 34 (p.K385fs*47), and 4 other types (one each of p.L367fs*43, p.E370fs*60, p.E371fs*59 and p.E381del). Except p.E381del which is a 3 base-pair inframe deletion, all other *CALR* exon 9 mutations are indels causing +1 base-pair reading frameshift, with type 2 (10/19, 52.6%) being the most prevalent mutational type. One patient with *JAK2*V617F mutation harbored a single nucleotide polymorphism in *CALR* exon 9 (c.1142 A>C, rs143880510). Four (21%) of the 19 *CALR*-mutated patients had simultaneous *JAK2*V617F mutation; one each of type 3, p.E370fs*60, p.E371fs*59 and p.E381del, and the latter 3 *CALR* mutations were only detected by HRMA and required TA-cloning to confirm the presence of mutations indicating that they were low allelic burden mutants. Seven patients (13%) were triple-negative (TN) for *JAK2*, *CALR* and *MPL* mutations. No *DNMT3A* exon 23 or *IDH1*/*2* exon 4 mutation was detected in this cohort of ET patients. The only one *MPL*-mutated and the 4 *CALR*/*JAK2*V617F co-mutated ET patients were excluded from further clinical and molecular correlation analysis to avoid statistical bias.

4.2 Clinical and molecular correlates

In 49 ET patients used for analysis, there was no significant difference in gender among the three major mutational groups. In this cohort, ET patients with *CALR* mutations had statistically significant longer follow-up (median 6.2 year, p=0.031, Table 4), highest platelet count at the time of diagnosis (p=0.01), and lower hemoglobin level at the time of diagnosis (p=0.037). When compared with *JAK2*V617F-mutated ET patients, *CALR* mutations also correlated with younger age at diagnosis (p=0.04) and lower

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leukocyte count (p=0.013). *JAK2*V617F mutation was associated with leukocytosis (p=0.002) and white blood cell count was lowest in TN ET patients.

4.3 Distribution of B cells and B cell subsets

Among 49 ET patients in the three major mutational groups, there were no significant differences in the number of total B cells and all the B cell subset populations (Table 5). When compared to healthy adults, ET patients had significantly lower numbers of total B cells and naïve B cells, but had significantly higher number of plasmablast in all three mutational groups. The number of memory B cells was statistically lower in *CALR*- and *JAK2* mutated-ET patients when compared with healthy adults. There were no statistically significant differences in the numbers of early and late transitional B cells and pre-germinal center B cells between ET patients and healthy adults.

4.4 B cell immune profiles

Among 49 ET patients in the three major mutational groups, the B cell immune profiles in 34 (69.4%; 19 *JAK2*V617F-mutated, 9 *CALR*-mutated and 6 TN) patients had been previously described [62]. When compared with *JAK2*V617F-mutated and TN ET patients, *CALR* mutations correlated with significantly lower serum BAFF level (median 1.6 ng/mL, p=0.049) (Figure 8A) and higher fraction of B cells with TLR4 expression (median 11.3%, p=0.021) (Figure 9A). Besides, ET patients with *CALR* mutations had statistically higher number of CD69-positive activated B cells when compared to TN group (median: 20.8/μL vs 7.6/μL, p=0.048) (Figure 10A). There were no significant differences in mean fluorescence intensity (MFI) of mBAFF on both granulocytes and monocytes (Figure 8B and C, respectively), in the fraction of B cells with intracellular IL-1β or IL-6 expression (Figure 9B and C, respectively), and the numbers of CD80-positive and CD86-positive

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activated B cells among the three mutational groups of ET patients (Figures 10B and C, respectively, 11 and 12).

When compared to healthy adults, patients with ET had statistically significant higher serum BAFF level and higher MFI of mBAFF on both granulocytes and monocytes (Figure 8), and higher fraction of B cells with TLR4 expression and higher fractions of B cells with intracellular IL-1β and IL-6 expression irrespective of their genotypes (Figure 9) (Table 5). Although ET patients had significantly lower numbers of CD19-positive B cells and naïve B cells when compared to healthy adults, ET patients with *CALR* and *JAK2* mutations had statistically higher numbers of CD69-positive and CD86-positive activated B cells (Figure 10A and C, respectively). 38 (70.4%) ET patients were treated with hydroxyurea to lower their blood counts in this cohort. There were no significant differences in all the B cells immune profiles in ET patients with or without hydroxyurea treatment, except lower IL-1β expression level in B cells (median 6.9% vs 16.4%, p=0.014) was found in ET patients being treated with hydroxyurea (Tables 4 and 6).

In this study, platelet count at testing had moderately positive correlation with the fractions of B cells with intracellular IL-1β and IL-6 expression (Table 7). MFI of mBAFF on granulocytes had strong positive correlation with MFI of mBAFF on monocytes, and had moderately positive correlation with the fractions of B cells with intracellular IL-1β and IL-6 expression. In addition, serum BAFF levels had moderately positive correlation with the fraction of B cells with intracellular IL-6 expression. Interestingly, only MFI of mBAFF on granulocytes, but not MFI of mBAFF on monocytes or the serum BAFF levels, had weak positive correlation with the numbers of CD69-positive and CD86-positive activated B cells in our cohort. We also analyzed the correlation between platelet count,

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serum BAFF levels, and B cell immune profiles in the group of healthy controls. Platelet count of healthy controls only had moderately negative correlation with the MFI of mBAFF on monocytes (Spearman's rho= -0.625, p=0.013) suggesting that the platelet count of healthy controls did not have obvious correlation with their B cell immune profiles.

4.5 Multivariate analysis of B cell immune profiles in ET

The results of multivariate analysis using logistic regression model adjusted for multiple parameters confirmed that increased activated CD69+ B cells were universally present in *JAK2*-mutated, *CALR*-mutated and triple-negative ET patients when compared to healthy adults, although the number of total B- cells was significantly lower in ET patients (Table 8). Activated B cells were characterized by the expression of CD69 and CD86, increased intracellular IL-6 and IL-1β levels, and higher expression of TLR4. Interestingly, peripheral granulocytes and monocytes mBAFF expression was significantly higher in ET patients compared to healthy controls. *JAK2*-mutated and *CALR*-mutated ET patients had significantly higher number of B-cells expressing TLR4 and IL-6, and TN ET patients had significantly higher number of B-cells expressing IL-6 and IL-1β (Table 8). TN ET patients had significantly lower number of B-cells expressing TLR4 when compared to *CALR*-mutated and *JAK2*-mutated ET patients (Table 9). TN ET patients also had significantly lower number of CD69+ B-cells when compared to *CALR*-mutated ET patients.

5. Discussion

CALR mutations have been found to have phenotypic and prognostic significances in patients with ET from both Caucasian and Chinese populations.[18,19,29,64,89-91] In this cohort

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of adult Taiwanese ET patients, *CALR* mutations were found to have a similar phenotypic correlation with higher platelet count, lower hemoglobin level and younger age at diagnosis. However, we detected a higher frequency of type 2 *CALR* mutation (10 of 19 patients) in this study while there was only 2 type 1 *CALR* mutation detected. These results are contradictory to the vast majority of the reports in the literature. The possible explanations for this discrepancy in our results might be related to small sample size and selection bias cannot be excluded completely in this study.

In accordance with our previous report, a relatively high frequency of *CALR* and *JAK2*V617F co-mutations (21% in 19 *CALR*-mutated ET) was still found in this study. Several papers have reported the co-occurrence of *CALR* and *JAK2*V617F mutations in ET across different ethnic groups including one of our previous publication (Table 10). The frequency of *CALR* and *JAK2*V617F co-mutations ranges from 0.5 to 14.1%, 1.7 to 38%, and 0.8 to 22%, in ET, *CALR*-mutated ET, and *JAK2*V617F-mutated ET, respectively.[10,75,92-97] The cause of the difference in the frequency of *CALR* and *JAK2*V617F co-mutations in these studies might be related to the different methods used to detect *CALR* mutations. Higher frequency of *CALR* and *JAK2*V617F co-mutations was detected by using HRMA, whereas Sanger sequencing will likely miss to detect low allelic burden (<10%) *CALR* mutants. On the other hand, Usseglio *et al*. found that *CALR* mutations could be detected in low allelic burden (<4%) *JAK2*V617F-mutated ET suggesting that the frequency of *CALR* and *JAK2*V617F co-mutations might be further increased if a highly sensitive test was employed to detect *JAK2*V617F mutation in *CALR*-mutated ET.[97] Since both of our studies used a sensitive in-house developed HRMA followed by TA-cloning to detected *CALR* mutations, we were able to identify many low allelic burden *CALR* mutants resulting in the higher frequency of *CALR* and *JAK2*V617F

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co-mutations in our series. However, because our study was limited by small patient size, larger study using sensitive screening methods for the detection of both *CALR* and *JAK2*V617F mutations will be warranted to confirm our results.

Recently, we have shown that ET patients have quantitative and qualitative changes in their B cell immune profiles regardless of *JAK2*V617F mutational status.[62] In our previous report, we found that the number of CD19+ B cells did not differ between ET patients and age-matched healthy adults using univariate analysis. However, we found that ET patients had significantly lower numbers of total CD19+ B cells in univariate analysis (Table 5) and also in multivariate analysis adjusted for hematological parameters (Table 8) in this study. We believe that the results reported in this study are more accurate because *CALR*-mutated ET patients were not included in our previous report and the results from multivariate analysis are more reliable. In the present study, we found that ET patients with *CALR* mutations also had similar quantitative and qualitative changes in most of the B cell immune profiles when compared to healthy adults using univariate and multivariate analyses (Tables 5 and 8, respectively). Although the number of total B cells was lower in ET patients including those with *CALR* mutations when compared with healthy controls, the number of activated B cells was significantly increased in ET patients across all 3 genotypes that characterized by the expression of CD69 and CD86, increased intracellular IL-6 and IL-1β levels, and higher expression of TLR4.

Regarding to the mechanism of B cell activation in ET patients, it has been well documented that elevated serum levels of inflammatory cytokines are frequently detected in patients of MPN, especially PMF, and may correlate to their constitutional symptoms which could be effectively ameliorated by the use of JAK inhibitor.[98] Previous study has

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reported that cytokine levels were also significantly increased in ET and PV patients.[99] Therefore, it is reasonable to argue that B cell activation could only be an epiphenomenon in ET rather than a cause of thrombopoiesis. However, we found that increased B cell activation was only present in ET patients but not in PV patients when compared to healthy controls or patients with reactive thrombocytosis (Table 11). Although we did not evaluate B cell immune profiles in PMF patients due to difficulty in patient enrollment, our findings provided evidence to illustrate that increased B cell activation in ET patients could not be solely explained by the increased cytokine levels in MPN patients, and therefore might not be an epiphenomenon in these patients. Nevertheless, we had previously reported that some humoral factors such as endogenous toll-like receptor 4 (TLR4) ligands HSP70 and HMGB1 or other inflammatory cytokines, might participate in the activation of B cells in ET patients because peripheral B cells of ET patients could be stimulated by ET patients' sera to cause IL-1beta and IL-6 production.[62] In addition, we had also demonstrated that increased production of BAFF by granulocytes and monocytes up-regulates TLR4 expression on B cells and promotes B cell activation in ET patients. Consequently, these activated B cells play a pathogenic role in augmenting thrombocytosis by producing IL-1β and IL-6 in ET patients through cytokine-dependent thrombopoiesis in the bone marrow. Altogether, our data suggested that increased B cell activation in ET might be caused by the stimulation of specific humoral factors on B cells and the interaction of B cells with BAFF on granulocytes and monocytes. Importantly, our studies suggested that activated B cells in ET could play a role in mediating pathogenic thrombopoiesis in the bone marrow.

Besides, we had previously reported that TLR4 expression is upregulated in both naïve and memory B cell subsets, and BAFF receptor signaling has reciprocal effects on TLR interaction.[62] B cells are characterized by the expression of a clonally rearranged,

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antigen-specific B cell receptor (BCR) in combination with the expression of one or more members of the TLRs.[100] This dual expression feature allows B cells to integrate both antigen-specific signals and environmental danger signals via these key receptor systems. Since we did not measure or characterize the expression level of BCR on B cells, whether dual BCR and TLRs engagement may also play a role in the activation and/or affect the function of B cells in ET patients remains to be elucidated in future study.

Furthermore, we did not favor the paracrine effect of serum BAFF secreted by peripheral granulocytes and monocytes because its level was not different between ET patients and healthy controls in multivariate analysis. Rather, we hypothesized that the direct interaction between peripheral granulocytes and monocytes and B-cells might play a role in the activation of B-cells in ET patients since mBAFF expression was significantly higher in ET patients compared to healthy controls. Recently, mBAFF has been found to be a more potent stimulus for B cells than soluble BAFF thus supporting our view.[101] Our observation was also supported by the finding that mBAFF expression on peripheral granulocytes significantly correlated with higher number of IL-1β/IL-6-producing B cells and activated B-cells in ET patients (Table 7). In addition, higher number of TLR4-producing B cells in *JAK2*-mutated and *CALR*-mutated ET patients might also augment the production of IL-1β/IL-6 in B cells in these patients. We had previously shown that IL-1β and IL-6 play an important role in thrombopoiesis in ET patients, and hematopoietic stem cells of ET patients differentiated towards a megakaryocytic lineage after incubation with their own B cells.[62] Therefore, our data suggested that activated B-cells in ET patients might link to the pathogenic thrombopoiesis in these patients through the production of IL-1β/IL-6 in activated B cells regardless of their genotypes.

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It is possible that the use cytoreductive therapy might affect B cell immune profiles in ET patients. However, most B cell immune profiles in ET patients were not affected by the treatment of hydroxyurea in this study (Table 6). Therefore, we believed that the changes in B cell immune profiles may be more closely related to the underlying pathogenic mechanisms that could not be altered by non-specific cytoreductive therapy such as hydroxyurea.

Currently, the exact molecular mechanism of B cell activation in ET patients has not yet been fully elucidated. However, most of the changes in B cell immune profiles are independent of the three genotypes in ET patients, and the activation of JAK-STAT signaling pathway can be seen in most ET patients regardless of their molecular profiles.[37] *JAK2*V617F is a gain-of-function mutation resulting in the cytokine-independent growth of hematopoietic progenitors.[102] However, *JAK2*V617F mutation requires the presence of cytokine receptors (especially MPL) to be constitutively active.[103] *JAK2*V617F mutation can activate erythropoietin receptor, thrombopoietin receptor or granulocyte colony-stimulating factor receptor on progenitor cells to promote erythropoiesis, megakaryopoiesis, or granulopoiesis. Interestingly, *CALR* mutations are recently found to activate the JAK-STAT signaling through a MPL-dependent mechanism, and cause thrombocytosis both *in vitro* and *in vivo*. Hence, both *JAK2*V617F and *CALR* mutations can activate the JAK-STAT signaling in megakaryocytes. Although *CALR* mutations can be detected in hematopoietic stem/progenitor cells, it largely promotes the growth and the differentiation of megakaryocytic precursors resulting in the phenotype of ET and/or PMF. Therefore, *CALR* mutations are exclusively detected in around 25 % of ET or PMF, but not in PV. On the other hand, *JAK2*V617F mutation can be identified in about in 95% of PV and in around 60 % of ET or PMF. Several observations have suggested that

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megakaryocytes play a major role in the pathogenesis of MPNs.[104] There is evidence suggesting that MPN associated mutations could alter megakaryocyte differentiation, migratory ability, and proplatelet formation, leading to increased platelet production.[105] *JAK2*V617F mutation was also found to lead to intrinsic changes in both megakaryocyte and platelet biology in a mouse model of ET.[106] Recently, CALR mutations have been shown to activate essential MAPK signaling through MPL-dependent mechanism and facilitate megakaryocyte differentiation.[107] Current evidences suggest that both *JAK2*V617F and *CALR* mutations intrinsically play a major role in the pathogenesis of ET through the promotion of megakaryopoiesis and thrombopoiesis. Based on our findings, increased platelet production in ET patients may be resulted from activating mutations synergistic with bystander thrombopoietic cytokines produced by activated B cells. We believe that these results would help advance our understanding of the pathogenesis of ET.

Our study is limited by a total number of 54 ET patients. However, the distribution and the percentage of the 3 driver mutations in these 54 ET patients are comparable with most studies: 27 (50%) patients harbored the *JAK2*V617F mutation, 1 (1.9%) patient with the *MPL*W515K mutation, 19 (35.2% overall and 68.2% in *JAK2*/*MPL*-unmutated cases) patients with *CALR* exon 9 mutations, and 7 patients (13%) triple-negative. In this study, we detected a higher percentage of *CALR*/*JAK2*V617F co-mutations in 4 (7.4%) ET patients due to the use of a sensitive HRMA followed by TA-cloning to detect low allelic burden *CALR* mutants. To avoid statistic bias on the results, we excluded these 4 *CALR*/*JAK2*V617F co-mutated ET patients and the only one *MPL*-mutated ET patient from further analysis. We have also consulted our biostatistician for help with the analysis of our data. Our results showed that increased B cell activation is present in *JAK2*V617F-mutated, *CALR*-mutated and triple-negative ET, and these findings are consistent with our previous

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report. Although we believe that there was no statistical bias on the results, larger study is still warranted to confirm our findings. In conclusion, increased B cell activation is present in ET patients across different mutational subgroups.

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Chapter 5

The molecular pathogenesis of *CALR* mutations using zebrafish animal models

1. Summary

CALR mutations are identified in about 30% of *JAK2*/*MPL*-unmutated myeloproliferative neoplasms (MPNs) including essential thrombocythemia (ET) and primary myelofibrosis. Although the molecular pathogenesis of *CALR* mutations leading to MPNs has been studied using *in vitro* cell lines models, how mutant CALR may affect developmental hematopoiesis remains unknown. Here we took advantage of the zebrafish model to examine the effects of mutant CALR on early hematopoiesis and model human *CALR*-mutated MPNs. We identified 3 zebrafish genes orthologous to human *CALR*, referred to as *calr*, *calr3a* and *calr3b*. Expression of the CALR-del52 and CALR-ins5 mutants caused an increase in the hematopoietic stem/progenitor cells followed by thrombocytosis without affecting normal angiogenesis. The expression of CALR mutants also perturbed early developmental hematopoiesis in zebrafish. Importantly, morpholino knockdown of *mpl* but not *epor* or *csf3r* could significantly attenuate the effects of mutant CALR. Furthermore, expression of mutant CALR caused jak-stat signaling activation in zebrafish that could be blocked by JAK inhibitors (ruxolitinib and fedratinib). These findings showed that mutant CALR activates jak-stat signaling through an mpl-dependent mechanism to mediate pathogenic thrombopoiesis in zebrafish, and illustrated that the signaling machinery related to mutant CALR tumorigenesis are conserved between human and zebrafish.

2. Introduction

The *BCR-ABL*-negative classic myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders including polycythemia vera, essential thrombocythemia

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(ET) and primary myelofibrosis (PMF).[108] The *JAK2*V617F and *MPL* exon 10 mutations are two important driver mutations in MPNs and cause the activation of the JAK-signal transducer and activator of transcription (STAT) signaling that is central to the pathogenesis of MPNs.[109] Calreticulin (CALR) is a 46-kDa highly conserved, multicompartmental and multifunctional protein.[12] CALR plays its role as a Ca^{2+} binding chaperone protein and acts in concert with calnexin to ensure proper protein and glycoprotein folding in the endoplasmic reticulum (ER).[110] Recently, two research groups discovered *CALR* mutations in about 30% of *JAK2* and *MPL* unmutated ET and PMF patients.[18,19] All *CALR* mutations are indels mutations in exon 9 and cause +1 base frameshift generating a novel C-terminus characterized by the loss of the ER retention signal KDEL and the change from acidic to basic amino acid sequence. Although there are more than 50 *CALR* mutants identified in MPNs, the most prevalent types of *CALR* mutations are a 52 bp deletion (L367fs*46, type 1 mutation, CALR-del52) and a 5 bp insertion of TTGTC (K385fs*47, type 2 mutation, CALR-ins5) accounting for more than 80% of all patients with mutant *CALR*.[18,19] Most *CALR* mutations are mutually exclusive with the *JAK2* and *MPL* mutations, but some patients were found to have *JAK2* and *CALR* co-mutations.[83] ET and PMF patients with *CALR* mutations have been found to have different clinical characteristics such as younger age and higher platelet count and to carry a better prognosis than those patients with *JAK2*V617F mutation.[32,83,90,91]

Recent studies have focused on the underlying mechanism of *CALR* mutations in the pathophysiology of MPNs. With the use of *in vitro* cell lines and retroviral mouse models, *CALR* mutants were found to activate the JAK-STAT signaling in an MPL-dependent manner.[39-43] Although the expression of *CALR* mutants resulted in pathogenic thrombocytosis in adult mice, whether *CALR* mutants may disrupt normal hematopoiesis

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during early development remains unknown. The zebrafish is a useful disease model system and has been successfully utilized in studying hematopoiesis and leukemogenesis.[45,48,111-113] The early hematopoietic system in zebrafish involves two distinct primitive and definitive waves of development that is rapidly established within a few days after fertilization.[111] The developmental hematopoiesis of zebrafish also shows broad conservation with mammalian species and is regulated by conserved molecular pathways.[111] The transparency of zebrafish at the embryonic and larval stages has made it suitable for direct observation of the hematopoietic process. In addition, zebrafish can be used for *in vivo* high throughput screening due to its good permeability to chemical added to water during early developmental stages.[114-116] Here we aimed to evaluate the pathophysiologic effects of mutant CALR during embryonic hematopoietic development and to test the therapeutic effects of JAK inhibitors on mutant CALR using the *in vivo* zebrafish model.

3. Materials and Methods

3.1 Zebrafish husbandry

Wild-type AB strain of zebrafish (*Danio rerio*) and the transgenic lines Tg(*cd41*:GFP)[117] and Tg(*fli1*:EGFP)[118] were maintained and manipulated with standard measure as previously described.[119] The stages of embryonic development were determined based on Kimmel *et al.*[120] Pigmentation was blocked by using 0.003% 1-phenyl-2-thiourea in some experiments. For pharmacologic inhibition, embryos were incubated with ruxolitinib (Abmole Bioscience, Houston, TX, USA) or fedratinib (Abmole Bioscience) from 1-2 cells stage to 5 days post fertilization (dpf) with or without microinjection of *CALR* mRNA. The zebrafish experiments were approved by the MacKay Memorial Hospital Animal Care and Use Committee.

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3.2 Identification of zebrafish ortholog of human *CALR*

Human genes located in 19p13.11-13.2 were identified using the National Center for Biotechnology Information (NCBI) Map Viewer.[121] Genes surrounding the 3 zebrafish *calr* genomic regions were identified using Ensembl[122] and Synteny database.[123] Human CALR protein sequence was used to BLASTP against zebrafish GRCz10 using the Ensembl platform (Ensembl release 82).[122] Alignment and comparative analysis between protein sequences was performed using the Clustal Omega algorithm[124] and edited by GeneDoc.[125]

3.3 Human and zebrafish *CALR* cDNAs cloning and mRNA synthesis

Full-length wild-type *CALR* cDNA was cloned from K562 cells into T&A™ Cloning Vector (Yeastern Biotech Co., Taipei, Taiwan) according to the manufacturer's protocol (Forward primer: 5'-GATC**CTCGAG**ATGCTGCTATCCGTGCCGCTGC-3'; reverse primer: 5'-GATC**GAATTC**CTACAGCTCGTCCTTGGCCTGGC-3'; restriction enzyme sites in bold letters: *Xho*I-*Eco*RI). Human *CALR* type 1 (CALR-del52) and type 2 (CALR-ins5) mutated cDNAs were obtained by custom gene synthesis. Full-length *CALR* cDNAs were subcloned in the pCS2^{+} vector and into a bicistronic pSYC-102 T2A vector (a gift from Dr. Seok-Yong Choi) replacing the mCherry-CAAX reporter gene using the In-Fusion Cloning Kit (Clontech, Mountain View, CA, USA) (Figure 13).[126] All vector sequences were verified by sequencing. The mMessage mMachine SP6 kit (Ambion, Austin, TX, USA) was used for *in vitro* transcription of capped mRNAs from vectors according to the manufacturer's protocol. mRNAs from the bicistronic pSYC-102-CALR vectors were only used to express EGFP and CALR concurrently in wild-type zebrafish embryos and only embryos expressing green fluorescence were collected under fluorescence microscope for use in the reverse-transcription and real-time polymerase

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chain reaction.

3.4 Morpholinos and microinjection

Morpholinos (MOs) blocking splicing of *mpl* and *epor*, and translation (ATG/5'UTR) of *csf3r* were purchased from Gene Tools (Philomath, OR, USA) (MO sequences are listed in Table 12).[117,127,128] Standard control MO was used as negative control. Embryos at the 1-2 cells stage were injected with MO (1 ng) or mRNAs (100 pg). Co-injection of each MO and *CALR* mutant mRNA was performed in a subset of embryos.

3.5 Reverse-transcription and real-time polymerase chain reaction

Total RNA was extracted from embryos using miTotal Miniprep System (Viogene, New Taipei City, Taiwan) and reverse transcribed using a High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table 13. Fast SYBR® Green Master Mix (Applied Biosystems) was used for real-time quantitative polymerase chain reaction according to the manufacturer's instructions.

3.6 Western blotting

Total proteins were extracted from zebrafish embryos at 24 hours post fertilization (hpf). Equal amounts of protein were denatured and electrophoresed. Membranes were immunoblotted with the following primary antibodies: CALR (Abcam, Cambridge, UK; recognizing N-terminal sequences of both human and zebrafish wild-type CALR proteins), gapdh and customized mutant CALR (GeneTex, Hsinchu City, Taiwan; specifically recognizing human CALR exon 9 indel mutant protein sequence), STAT5 (Santa Cruz, Dallas, TX, USA), and phospho-STAT5 (Cell Signaling, MA, USA).

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3.7 Imaging

Live embryos were imaged using a Leica MSV269 fluorescence stereomicroscope and photographed using a Leica DFC425 C digital camera and Leica Application Suite software. GraphPad Prism 7 software and ImageJ (National Institutes of Health) were used to process images.

3.8 Statistical analysis

The Student *t* test or ANOVA test were used. Data were expressed as mean ± standard error of the mean (SEM). Significance was determined at $P<0.05$(*), $P<0.01$(**) and $P<0.001$(***).

4. Results

4.1 Zebrafish ortholog of human *CALR*

To search for the zebrafish ortholog of human *CALR* gene, human CALR protein sequence was used to BLASTP against zebrafish GRCz10 (Ensembl release 82). We identified 3 annotated zebrafish orthologs of the human *CALR* gene (ENSG000001792), *calr* (ENSDARG00000076290 at chromosome 8), *calr3a* (ENSDARG00000103979 at chromosome 22) and *calr3b* (ENSDARG00000102808 at chromosome 2). After comparative analysis using the Clustal Omega algorithm, the amino acid sequence of zebrafish calr, calr3a, and calr3b proteins shares an overall 75%, 71% and 70% identity to human CALR protein sequence, respectively. The 3 functional domains in CALR are conserved in all 3 zebrafish calr proteins, including the KDEL ER retention signal at the C-terminus (Figure 14A). In addition, the genomic loci surrounding human chromosome 19p13.2 containing the *CALR* gene are syntenic with the regions of zebrafish *calr* on chromosome 8, *calr3a* on chromosome 22 and *calr3b* on chromosome 2 based on the

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search in NCBI Map Viewer, Ensembl database and Synteny database (Figure 14B). These results indicated that the 3 zebrafish *calr* genes are likely true orthologs of human *CALR*.

4.2 Effects of mutant *CALR* expression on thrombopoiesis and angiogenesis in zebrafish

For the expression of mRNA in zebrafish embryo, we first performed a dose-finding study ranging from 50 to 200 pg *CALR* mRNA. Phenotype could be observed at dose of 100 pg mRNA per embryo which was compatible with normal development for most embryos. All CALR proteins were adequately expressed at comparable amount at dose of 100 pg (Figure 15A middle panel). The expression of CALR-del52 and CALR-ins5 mutant proteins was also confirmed by mutant CALR specific antibody (Figure 15A top panel). Therefore, 100 pg mRNA was injected throughout the study. To determine whether mutant CALR had an effect on hematopoietic stem and progenitor cells (HSPCs) in zebrafish, we injected the 3 mRNAs encoding *CALR* wild-type (CALR-wt), *CALR*-del52, and *CALR*-ins5 into 1-2 cells stage embryos of the *cd41*:GFP line, and the numbers of $cd41^+$ cells in the caudal hematopoietic tissue (CHT) at 3 dpf indicating the HSPCs were counted.[117] Expression of both *CALR*-del52 and *CALR*-ins5 mutant mRNA significantly increased the numbers of HSPCs in the CHT when compared with CALR-wt mRNA (Figure 15B). However, the numbers of HSPCs did not have statistically significant difference between *CALR*-del52 and *CALR*-ins5 mutant groups at this developmental stage. To ascertain that the increase of HSPCs was not affected by the change in angiogenesis during early development, mRNAs encoding *CALR*-wt, *CALR*-del52, and *CALR*-ins5 were injected into 1-2 cells stage embryos of the *fli1*:EGFP line. No obvious changes in the angiogenesis were visualized in CALR-wt and mutant CALR expressing embryos at 3 dpf when compared with uninjected control (Figure 15C). To determine whether CALR had an effect on mature thrombocyte,

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the numbers of cd41$^+$ thrombocytes in the *cd41*:GFP line were counted at 5 dpf after injection. Mutant *CALR*-del52 significantly increased the number of cd41$^+$ thrombocytes (mean 162.5±4.1 per embryo) when compared to *CALR*-wt (mean 117.1±3.1 per embryo, P<0.001), mutant *CALR*-ins5 (mean 128.3±6.1 per embryo, P<0.001) and uninjected control (mean 136.7±3.0 per embryo, P<0.001) (Figure 15D). Although mutant *CALR*-ins5 slightly increased the number of cd41$^+$ thrombocytes when compared to *CALR*-wt, there was no statistically significant difference. Together, our data demonstrated that the effect of mutant CALR on thrombopoiesis in zebrafish is dependent on the presence of the novel C-terminus and is also related to specific CALR mutant protein sequences.

4.3 Mutant CALR requires mpl to cause thrombocytosis in zebrafish

To test whether cytokine receptors are involved in the pathogenesis of thrombocytosis caused by mutant CALR in zebrafish, *mpl*, *epor* and *csf3r* MOs (1 ng) were injected in 1-2 cells stage embryos of *cd41*:GFP line and assayed for their effects on the number of cd41$^+$ thrombocytes at 5 dpf. Co-injection of *CALR*-del52 mutant mRNA (100 pg) with each MO was also performed in a subset of embryos. At 5 dpf, the number of cd41$^+$ thrombocytes significantly decreased upon mpl knockdown (mean 43.6±4.9 per embryo) when compared to the control MO group (mean 123.5±5.9 per embryo, P<0.001) and the mutant *CALR*-del52 group (P<0.001) (Figure 16). Importantly, co-injection of *CALR*-del52 mutant mRNA (mean 73.7±5.1 per embryo) can only partially reverse the knockdown effect of *mpl* MO. In contrast, the numbers of cd41$^+$ thrombocytes did not decrease significantly upon *epor* MO (mean 110.6±5.5 per embryo) or *csf3r* MO (mean 116.6±5.6 per embryo) knocked-down compared with the control MO group. When *CALR*-del52 mutant mRNA was co-injected with *epor* (mean 151.7±9.2 per embryo) or *csf3r* (mean 153.6±7.2 per embryo) MOs, the numbers of cd41$^+$ thrombocytes were comparable to those of

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CALR-del52–injected embryos (both *P*=0.3). Collectively, these findings indicated that the expression of mutant CALR causes thrombocytosis through an mpl-dependent mechanism in zebrafish.

4.4 Effects of CALR mutants on lineage-specific and cytokine gene expression

The increase in thrombopoiesis upon expression of mutant CALR prompted us to evaluate their effects on hematopoietic lineage-specific, thrombopoiesis,[129] cytokine and cytokine receptor gene expression in zebrafish embryos at 3 dpf. The expression of HSC gene *runx1* was significantly upregulated in CALR-ins5 group but was modestly downregulated in CALR-del52 group (Table 14). Also, the expression of *c-myb* and *scl* was only downregulated in CALR-del52 group. Although *gata1* was modestly downregulated in mutant CALR groups, the expression of *α-eHb* and *β-eHb* was not affected by both CALR mutants. The expression of early (*spi1b)* and late myeloid (*mpo*: granulocytic; *l-plastin*: macrophage) lineage genes, *epo* and *epor* showed no significant changes. However, the expression of lymphoid lineage genes (*rag1*, *rag2* and *lck*) was modestly downregulated in mutant CALR groups. While the expression of *mpl* was significantly downregulated in both mutant CALR groups, both *tpo* and *csf3r* expressions were only downregulated in CALR-del52 group. In the group of genes related to thrombopoiesis, only the expression of *nbeal2* was significantly downregulated in CALR-del52 group.

4.5 Effects of mutant CALR on jak-stat signaling in zebrafish

We then investigated whether the expression of mutant CALR can activate the jak-stat signaling in zebrafish. The expression of *CALR*-del52 mRNA significantly increased stat5 phosphorylation (Figure 17A, lane 2). Furthermore, treatment with ruxolitinib and fedratinib significantly ameliorated the enhanced stat5 phosphorylation induced by

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CALR-del52 mRNA (Figure 17A, lane 3, 4). In addition, treatment with ruxolitinib significantly decreased the numbers of cd41^{+} thrombocytes in uninjected control as well as CALR-del52–injected embryos in a dose-dependent manner (Figure 17B). Whereas, treatment with fedratinib only had minimal inhibitory effect on the number of cd41^{+} thrombocytes in uninjected control embryos, and had a modest and significant dose-independent inhibitory effect on mutant CALR induced thrombocytosis (Figure 17C). Our results demonstrated that mutant CALR mediated pathogenic thrombopoiesis involves jak-stat activation that can be blocked by JAK inhibitors.

5. Discussion

In this study, we have used the zebrafish animal model to examine the pathogenesis of mutant CALR in MPNs. We first identified 3 zebrafish orthologs for human *CALR* gene. We have shown that expression of the CALR-del52 mutant disturb thrombopoiesis and increase the number of HSPCs in the CHT followed by significant thrombocytosis in the zebrafish embryo. These findings are consistent with the myeloproliferative phenotype in retroviral mouse bone marrow transplantation models elicited by mutant CALR expression characterized by thrombocytosis and megakaryocytic hyperplasia recapitulating those seen in patients with ET and myelofibrosis.[40,42]

The highly conserve protein sequences between human *CALR* and the 3 zebrafish *calr* genes suggested functional conservation between human and zebrafish *CALR*. Ma *et al.* recently reported that MO knockdown of *calr* perturbs myeloid and HSCs lineages during zebrafish embryonic development including a decrease in the expression of genes associated with myeloid lineages at 24 hpf and an increase in the expression of *cmyb* at 48, 72 and 96 hpf.[130] We have also shown that the expression of genes involved in

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lineage-specific hematopoiesis, thrombopoiesis, cytokines and cytokine receptors could be perturbed by the expression of mutant CALR in zebrafish during early development. These data suggested that zebrafish *calr* genes play an important role in the regulation of vertebrae hematopoiesis. In addition, our data suggested that mutant CALR does not promote thrombopoiesis through the upregulation of *mpl* and *tpo* levels. Rather, the downregulation of *mpl* and *tpo* might represent a negative-feedback mechanism related to increased thrombopoiesis due to mutant CALR expression.

Based on the data from the murine and zebrafish animal models, the causative relationship between CALR mutations and thrombocytosis can be confirmed, and *CALR* mutations have been established as one of the driver mutations in MPNs. Furthermore, we demonstrated that expression of *CALR*-del52 (type 1 mutation) causes higher thrombocyte count than *CALR*-ins5 (type 2 mutation) at 5 dpf in zebrafish embryo. Similar finding has been reported in murine model that marked thrombocytosis was rapidly induced in *CALR*-del52-expressing mice and then progressed to myelofibrosis, and *CALR*-ins5-expressing mice only developed modest thrombocytosis resembling mild ET phenotype.[40] This is consistent with the clinical finding that *CALR*-del52 mutation is more frequently detected in PMF than in ET,[18] and also confirms the differential effects of *CALR* variants on thrombopoiesis and clinical phenotypes.[90,131,132]

To further elucidate the molecular pathogenesis of mutant in our zebrafish model, we have used MO knockdown experiments to show that only the *mpl* MO can significantly attenuate the effect of mutant CALR on thrombopoiesis. Both *epor* and *csf3r* MOs were not able to inhibit the effect of mutant CALR. These findings indicated that mpl has an essential and specific role required by mutant CALR to cause thrombocytosis in zebrafish.

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Because CALR is physiologically functioning as a chaperone for MPL, it is reasonable to speculate that mutant CALR may interact directly with mpl to cause thrombocytosis in zebrafish. Our data are consistent with those recently reported by several groups of researchers using *in vitro* cell line models.[39-43] In these studies, both the novel C-terminus of CALR mutants and the direct interaction of mutant CALR with MPL receptor are required to activate MPL and the downstream JAK-STAT signaling which in turn is responsible for cytokine-independent growth of Ba/F3-MPL and UT-7/TPO cell lines. Chachoua *et al.* reported that the specific activation of MPL receptor by mutant CALR required both the presence of extracellular N-glycosylation residues of MPL and the glycan-binding site at the novel C-terminus of mutant CALR.[41] In addition, Elf *et al.* reported that the physical interaction between mutant CALR and MPL is dependent on the positive electrostatic charge of the C-terminus of the mutant CALR but not dependent on specific novel C-terminal sequence.[42] Recently, Balligand *et al.* reported similar finding that highly similar but not identical murine *Calr* exon 9 frameshift mutants also require Mpl interaction to activate the JAK/STAT signaling.[133] Moreover, the positive charge predominant novel C-terminus of the mutant CALR results in different calcium binding capacity which may alter calcium homeostasis and signaling processes in mutant cells. All these structural differences and changes will likely contribute to the different clinical phenotypes seen in different *CALR* variants.[90,131,132,134]

We have also demonstrated that the expression of human CALR mutant is able to activate jak-stat signaling in zebrafish. In addition, jak-stat signaling in zebrafish can also be inhibited by JAK2 inhibitors used in clinical trials illustrating that the conserved signaling machinery in human and zebrafish.[135-137] Our data showed that ruxolitinib treatment results in a dose-dependent inhibitory effect on both normal thrombopoiesis and thrombocytosis

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caused by mutant CALR in zebrafish. By contrast, JAK2-selective inhibitor fedratinib has only minimal inhibitory effects on normal thrombopoiesis but has modest and dose-independent inhibitory effect on thrombocytosis caused by mutant CALR. Our data suggested that fedratinib can normalize the thrombocytosis caused by the expression of mutant CALR and does not cause significant thrombocytopenia in zebrafish model. These observations are comparable with the findings that both ruxolitinib and fedratinib have been demonstrated to have clinical responses in MPN patients harboring *CALR* mutations.[138-140] However, fedratinib has less hematological toxicities than ruxolitinib especially thrombocytopenia which is a dose-limiting toxicity of ruxolitinib.[135-137] Despite both JAK inhibitors are effective in the reduction of splenomegaly and the relief of clinical symptoms, they are not likely to substantially modify the natural history of the *BCR-ABL*-negative classic MPNs including *CALR*-mutated PMF. Importantly, these JAK inhibitors are not specifically designed for *JAK2*V617F mutation. However, the unique pathogenic mechanism of mutant CALR in MPNs has led to the possibility of new therapeutic approach targeting the interaction and binding between mutant CALR and MPL. In this regard, our results highlight the advantage and support the use of zebrafish as a relevant *in vivo* whole organism model for the testing and screening of therapeutic compounds targeting mutant CALR.[115]

In conclusions, we have used the zebrafish model to show that mutant CALR promotes the activation of jak-stat signaling through an mpl-dependent mechanism to mediate pathogenic thrombopoiesis during zebrafish early hematopoiesis. These findings are consistent with those observed in *in vitro* cell line and mouse models and illustrated that the signaling machinery related to mutant CALR tumorigenesis are conserved between human and zebrafish. Zebrafish has also been shown to be a relevant *in vivo* model for the

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development of novel therapeutic compounds targeting mutant CALR. Future studies using stable mutant CALR transgenic or knock-in zebrafish models for this purpose will be warranted.

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Chapter 6

Conclusions and future work

In this dissertation, we have examined the roles of *CALR* mutations in MPNs through clinical studies and basic research using the zebrafish animal models. *CALR* mutations have now been recognized as an important clonal marker in MPNs. Therefore, we developed a rapid and sensitive HRMA with the CFX Connect real-time system to detect *CALR* exon 9 mutations in ET patients. We have also evaluated the clinical and prognostic significance of *CALR* mutations and *JAK2*/*CALR* co-mutations in Taiwanese ET patients. We confirmed that *CALR* mutations were associated with younger age, higher platelet count and lower hemoglobin level in ET patients. Interestingly, we also detected various *CALR* exon 9 alterations in 22% *JAK2*-mutated ET patients, and *JAK2*-mutated ET patients with concomitant *CALR* alterations were associated with oldest age, higher thrombotic events after diagnosis, higher major arterial thrombotic events after diagnosis and more patients being high risk group for thrombo-hemorrhagic complications. Our data suggested that *JAK2*-mutated ET patients with concomitant *CALR* alterations probably define a specific subgroup of patients with increased risk of thrombotic events. After the evaluation of B cell immune profiles in a cohort of 54 adult Taiwanese ET patients, we confirmed that increased activated B cells were universally present in *JAK2*-mutated, *CALR*-mutated and triple-negative ET patients when compared to healthy adults. Finally, we used the zebrafish animal models to investigate the molecular pathogenesis of *CALR* mutations. Our findings showed that mutant CALR activates jak-stat signaling through an mpl-dependent mechanism to mediate pathogenic thrombopoiesis in zebrafish, and illustrated that the signaling machinery related to mutant CALR tumorigenesis are conserved between human and zebrafish.

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Although our results have shed lights on the roles of *CALR* mutations in MPNs, several experiments may still need to confirm and validate our findings in the future. For example, transgenic zebrafish neutrophil reporter line driven by *mpx* promoter can be used to investigate the role of BAFF expression in neutrophils and B cells activation. In addition, the generation of stable mutant *CALR* zebrafish lines using transgenic or knock-in methods will be need to validate our findings using MO knock-down zebrafish models. Moreover, stable mutant *CALR* zebrafish lines can also be used to conduct high throughput *CALR* mutant specific drug screening. It is also noteworthy that the role of JAK1-mediated signaling associated with mutant CALR can be studied because the JAK1/JAK2 inhibitor ruxolitinib had significantly higher inhibitory effect on mutant *CALR*-mediated thrombopoeisis in zebrafish than that of the JAK2-selective inhibitor fedratinib. We believe that the results of these translational researches will further advance our understanding on the roles of *CALR* mutations in MPNs and may also help to develop novel therapeutic compounds targeting mutant CALR in the future.

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Figures

Figure 1. *CALR* exon 9 mutations generate a novel peptide sequence in the C-terminus with the loss of acidic domain and the KDEL ER retention sequence. (Adapted from Nangalia J, Green TR. The evolving genomic landscape of myeloproliferative neoplasms. ASH Education Program Book 2014;2014:287-96.)

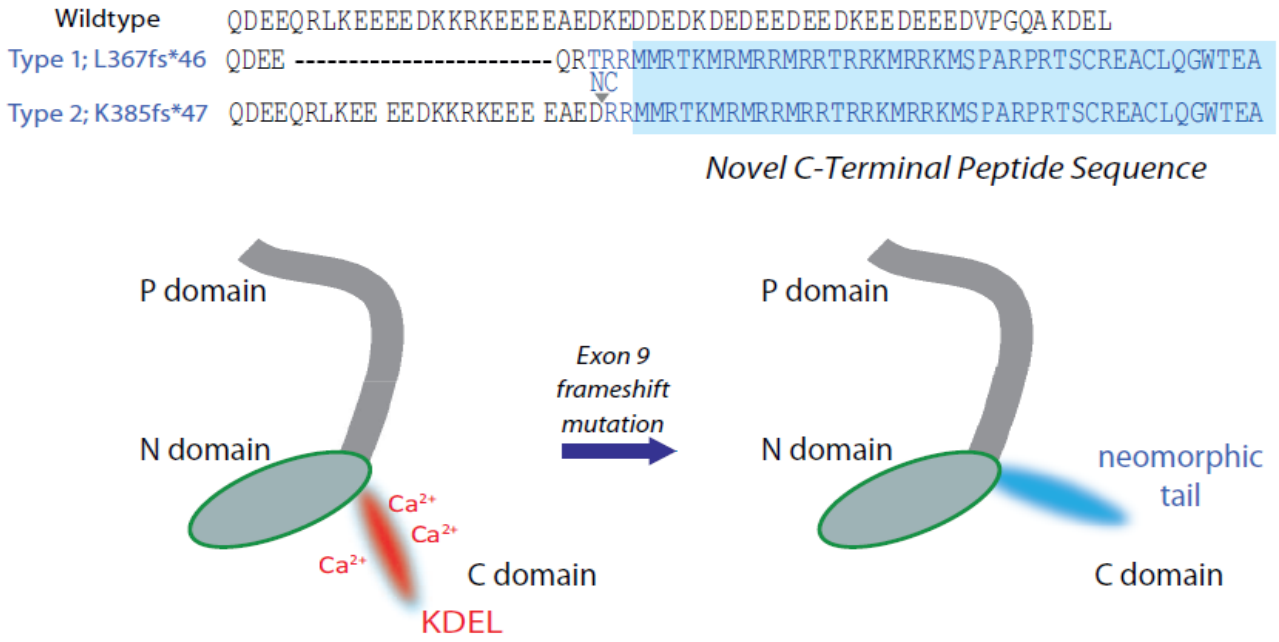

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Figure 2. Results of the dilution series of *CALR* mutants from plasmid DNA in a background of plasmid *CALR* wild-type DNA detected by HRMA in triplicate samples.

A and B. Normalized and temperature-shifted difference curves and corresponding Sanger sequencing of plasmid *CALR* type 1 mutant.

C and D. Normalized and temperature-shifted difference curves and corresponding Sanger sequencing of plasmid *CALR* type 2 mutant. HRMA can detect *CALR* type 1 and type 2 mutants with the maximal sensitivity of 2.5% and 1.25%, respectively, compared to the 10% or higher sensitivity obtained by Sanger sequencing. Each dilution percentage is indicated by corresponding arrow.

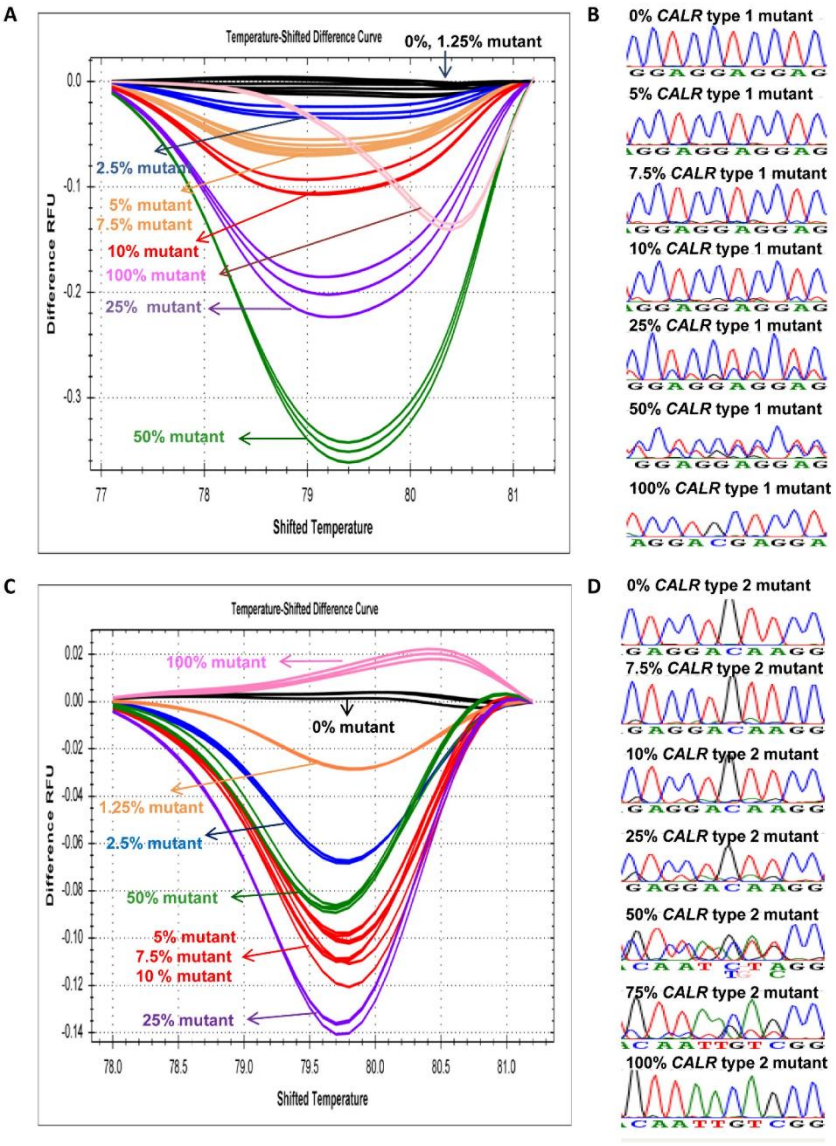

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Figure 3. Results of the dilution series of *CALR* mutants from patients' genomic DNA in a background of wild-type DNA from healthy control detected by HRMA in triplicate samples.

A and B. Normalized temperature-shifted difference curves of *CALR* type 1 and type 2 mutants from patients' genomic DNA, respectively. The maximal sensitivity of HRMA to detect both *CALR* type 1 and type 2 mutants from patients' genomic DNA was 2.5%. Each dilution percentage is indicated by corresponding arrow.

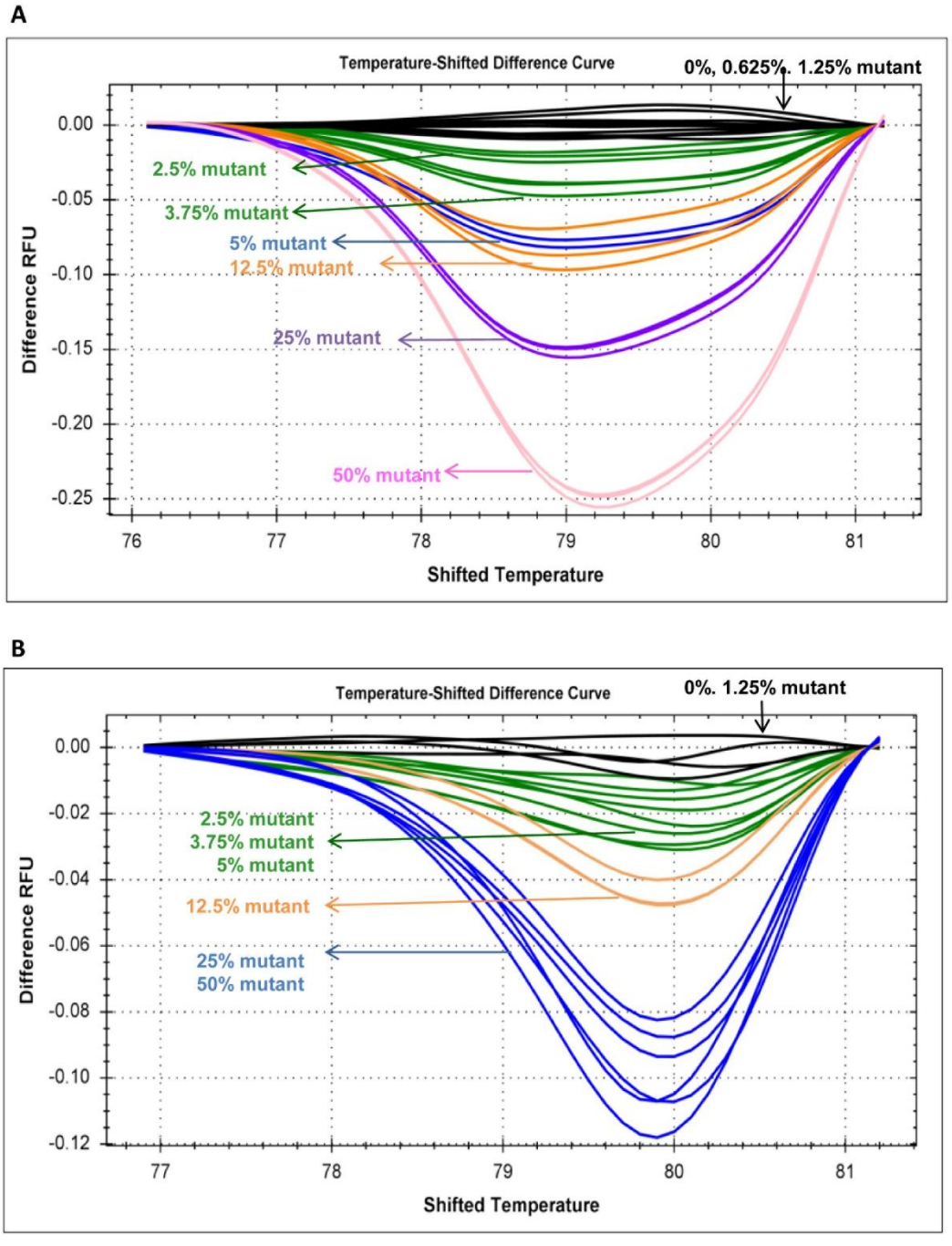

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Figure 4. Representative normalized melting curves and difference curves from the 22 patient samples.

A and B. Representative normalized melting curves and difference curves from 6 patient samples each with a specific type of *CALR* exon 9 mutations detected by HRMA in this study. The individual nature of the mutant melting curves became more apparent when the data were represented in normalized difference plots. *CALR* mutational types in A are indicated by arrows and labeled on the curves in B.

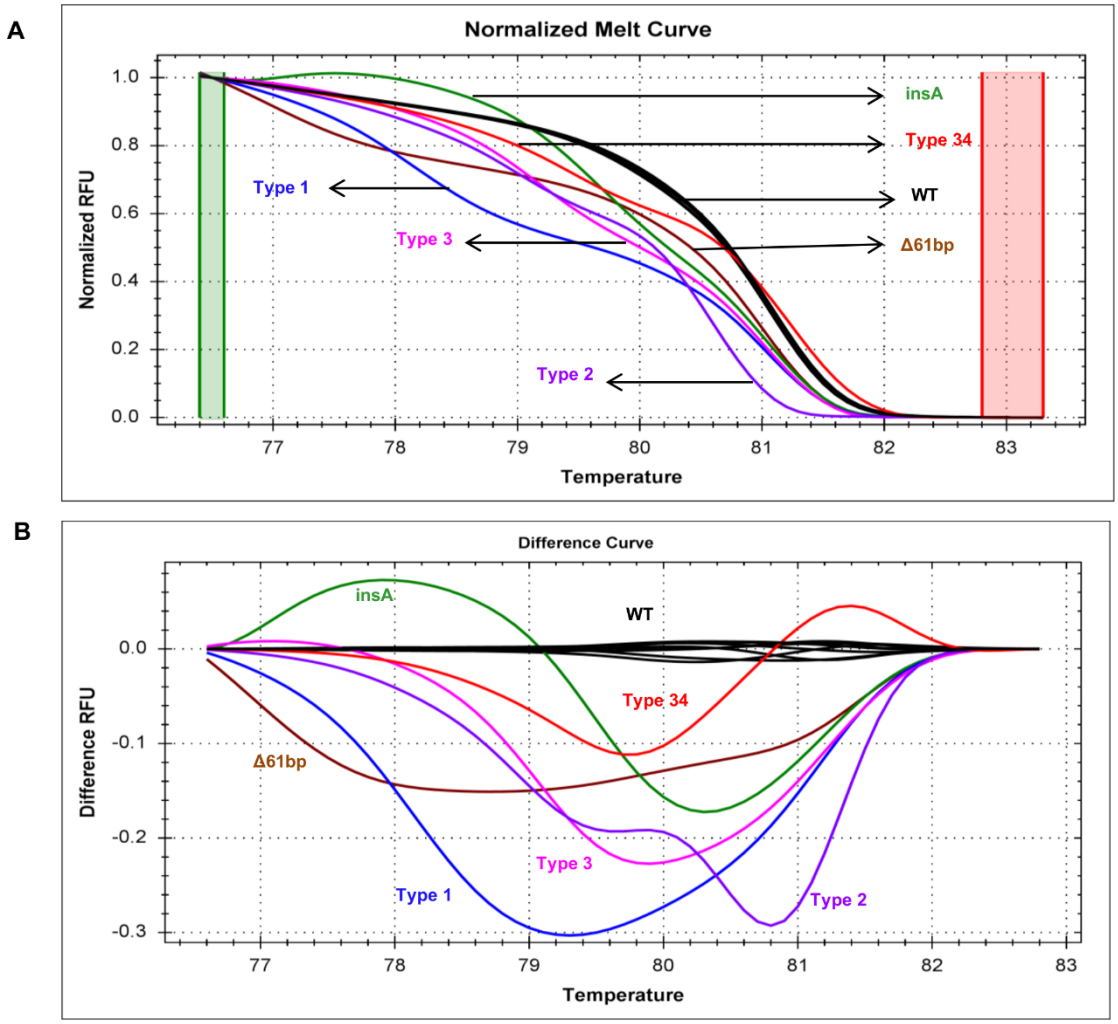

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Figure 5. The comparison of HRMA with Sanger sequencing in 6 patient samples with discordant results between HRMA and Sanger sequencing.

A. Distinctive normalized difference curves from the 6 patient samples. After TA-cloning, *CALR* type 2 mutations were detected in 5 of 6 patients. Only 1 clone from each patient was tested positive for the *CALR* mutation indicating that they all had low mutant allele burden. Patient number and the total number of clones selected for screening in each patient are indicated.

B. Representative Sanger sequencing from two patients P344 and J139 as indicated. All 6 patient samples had wild-type *CALR* exon 9 sequences by Sanger sequencing. C. Representative Sanger sequencing after TA-cloning showing wild-type *CALR* sequence from patient P344 and *CALR* type 2 mutation from patient J139.

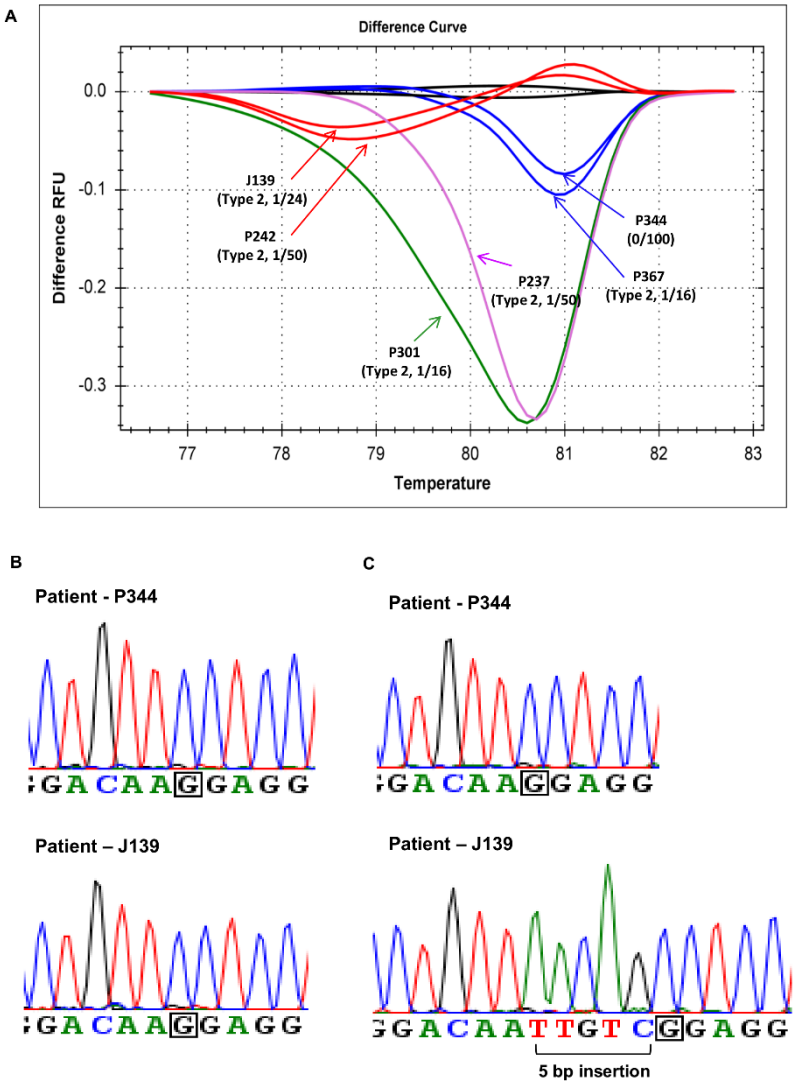

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Figure 6. Normalized difference curves of 16 *JAK2* V617F-mutated essential thrombocythemia patient samples showing distinct melting curves from *CALR* exon 9 wild-type samples (black color). Corresponding patient number, genotype and number of positive clone in TA-cloning of each curve is indicated by arrow.

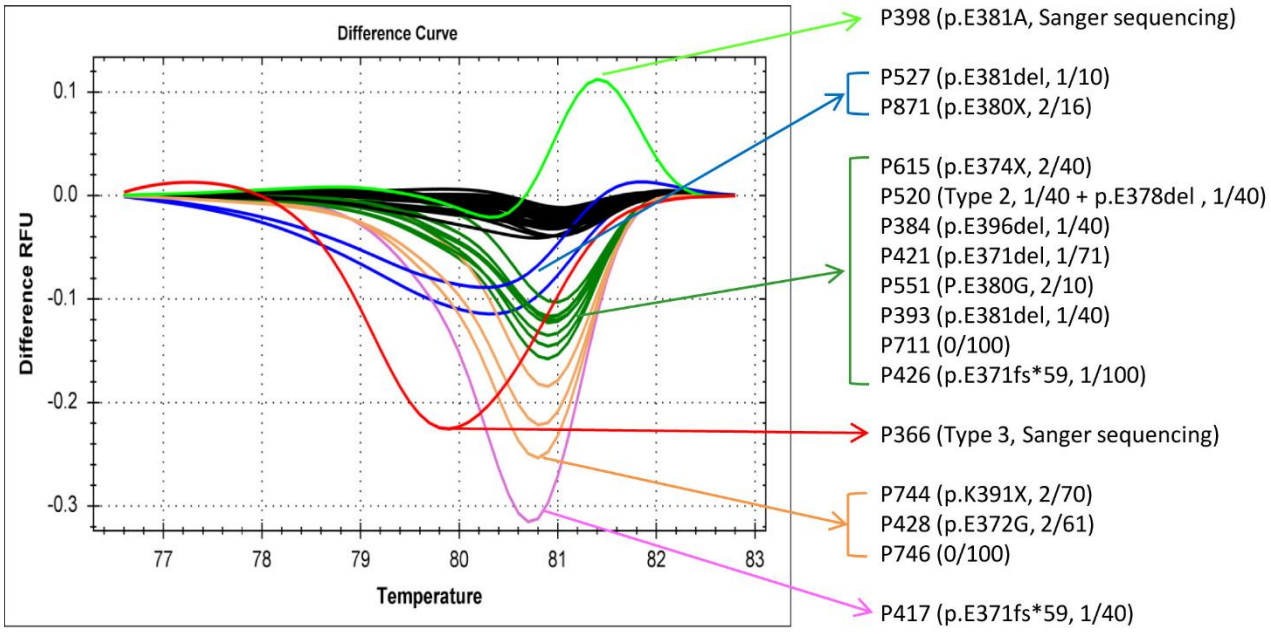

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Figure 7. Variants of *CALR* exon 9 alterations in essential thrombocythemia patients detected by high-resolution melting analysis followed by TA-cloning. WT, wild-type.

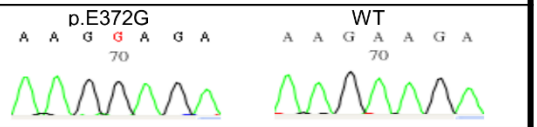
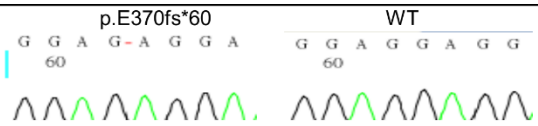
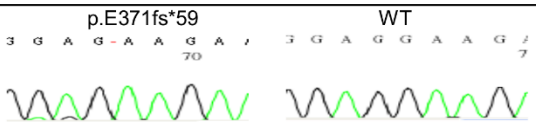
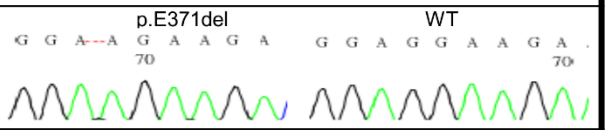
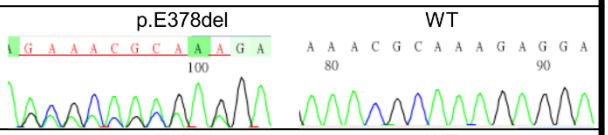
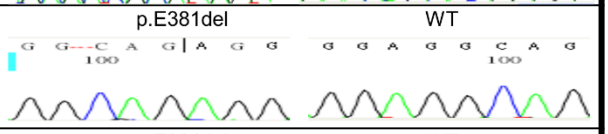
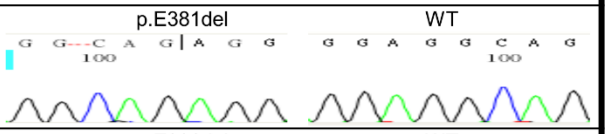
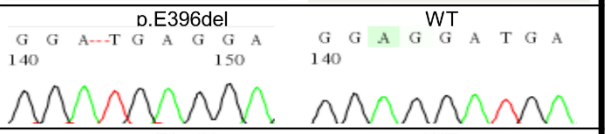
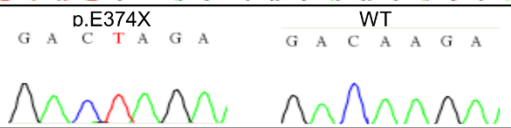
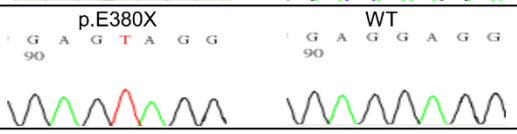
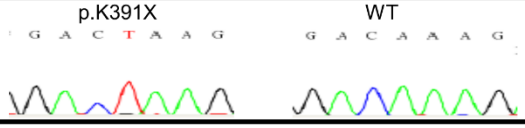
Variants of *CALR* exon 9 alterations in essential thrombocythemia patients		
Patient	Nucleotide change	*CALR* TA-cloning
P428	c.1115 A>G	
P426	c.1108delG (Δ1)	
P417	c.1111delG (Δ1)	
P421	c.1110_1112delGGA (Δ3)	
P520	c.1132_1134delGAG (Δ3)	
P393	c.1142_1144delAGG (Δ3)	
P527	c.1142_1144delAGG (Δ3)	
P384	c.1188_1190delGGA (Δ3)	
P615	c.1120 A>T	
P871	c.1138 G>T	
P744	c.1171 A>T	

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Figure 8. Elevated serum BAFF levels and higher membrane-bound BAFF expression in peripheral granulocytes and monocytes of ET patients.

A, Elevated serum BAFF levels are found in ET patients, regardless of genotypes compared to healthy adults. *CALR*-mutated ET patients have lowest serum BAFF levels compared to *JAK2*-mutated and triple-negative ET patients in univariate analysis.

B and C. Membrane-bound BAFF expression in peripheral granulocytes and monocytes is higher in ET patients, regardless of genotypes compared to healthy adults, respectively. Median values are indicated by the short horizontal bars. Asterisks represent significant differences between groups. *p< 0.05, **p<0.01, ***p< 0.001.

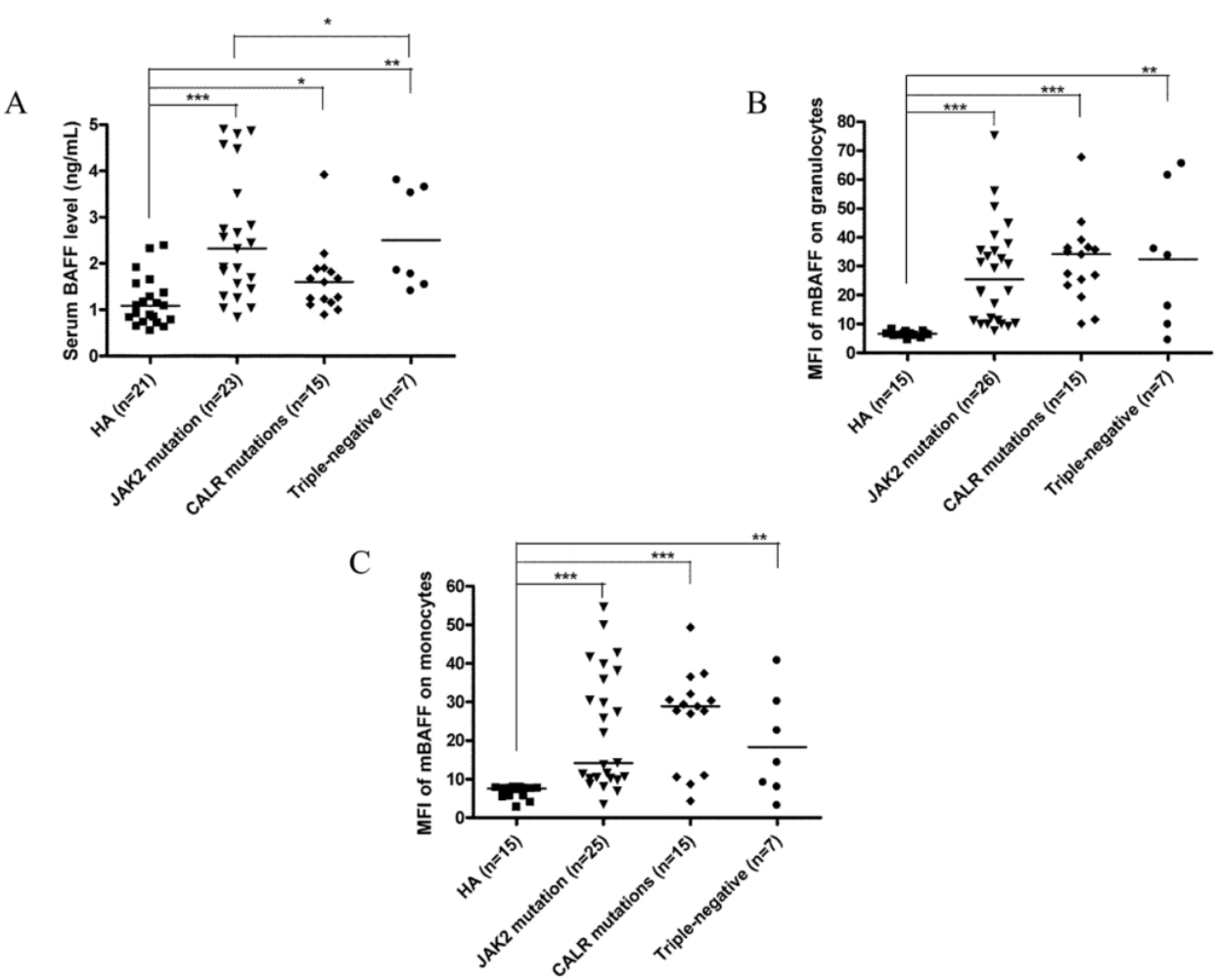

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Figure 9. Fractions of activated B cells with TLR4 expression, and IL-1β and IL-6 production are higher in ET patients.

A. B cells from patients with ET, regardless of genotypes express significantly higher levels of TLR4 compared to healthy adults. *CALR*-mutated ET patients have highest TLR4 expression compared to *JAK2*-mutated and triple-negative ET patients.

B and C. B cells from patients with ET, regardless of genotypes express significantly higher levels of IL-1β and IL-6 compared to healthy adults, respectively. Median values are indicated by the short horizontal bars. Asterisks represent significant differences between groups. *p< 0.05, **p<0.01, ***p< 0.001.

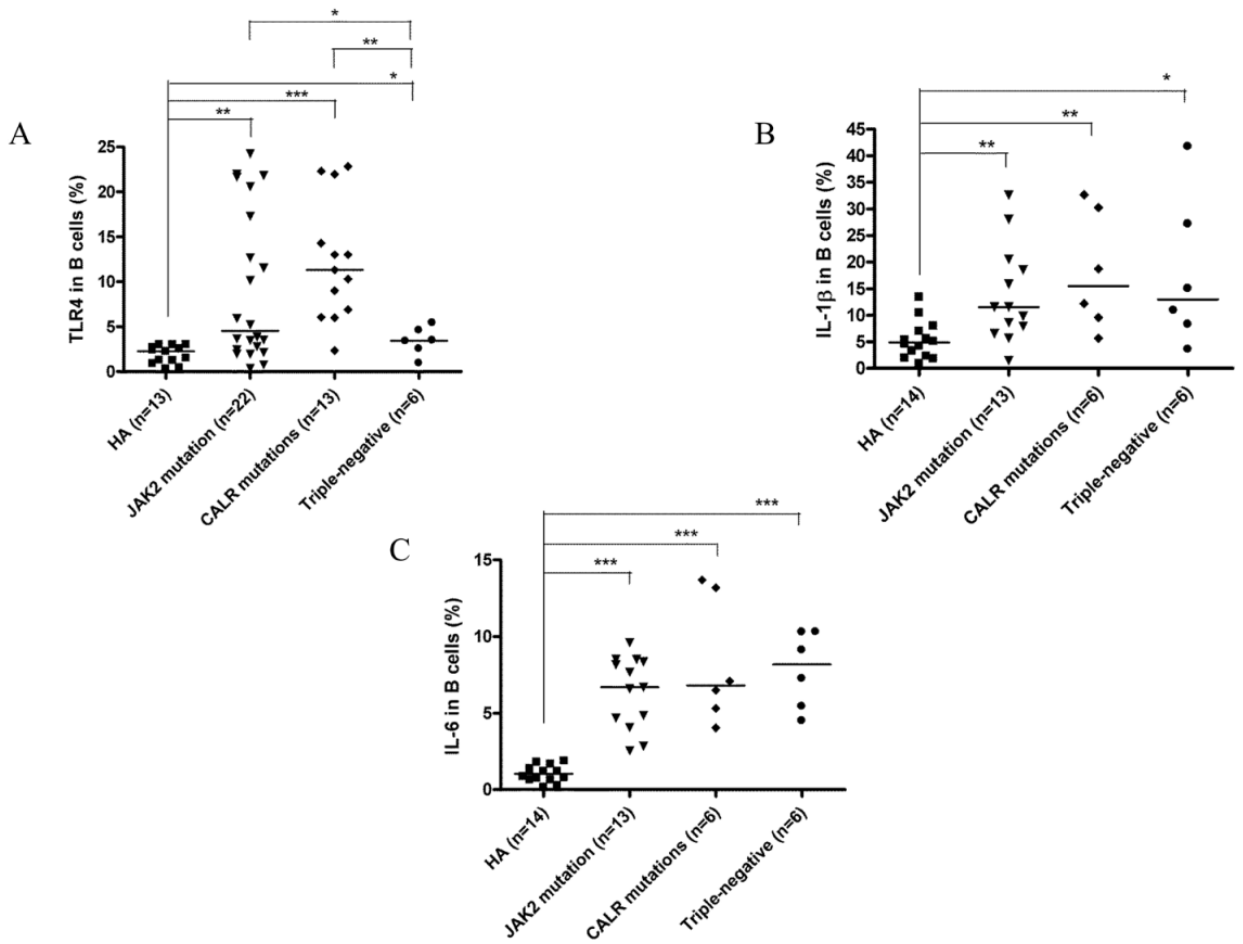

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Figure 10. *CALR* mutations are associated with activated B cells in patients with ET.

A, B and C. The number of activated B cells is higher in *CALR*-mutated ET patients, as evidenced by expression of CD69, CD80 and CD86, respectively. Data are presented as the number of B cells expressing these markers. Median values are indicated by the short horizontal bars. Asterisks represent significant differences between groups. *p< 0.05, **p<0.01, ***p< 0.001.

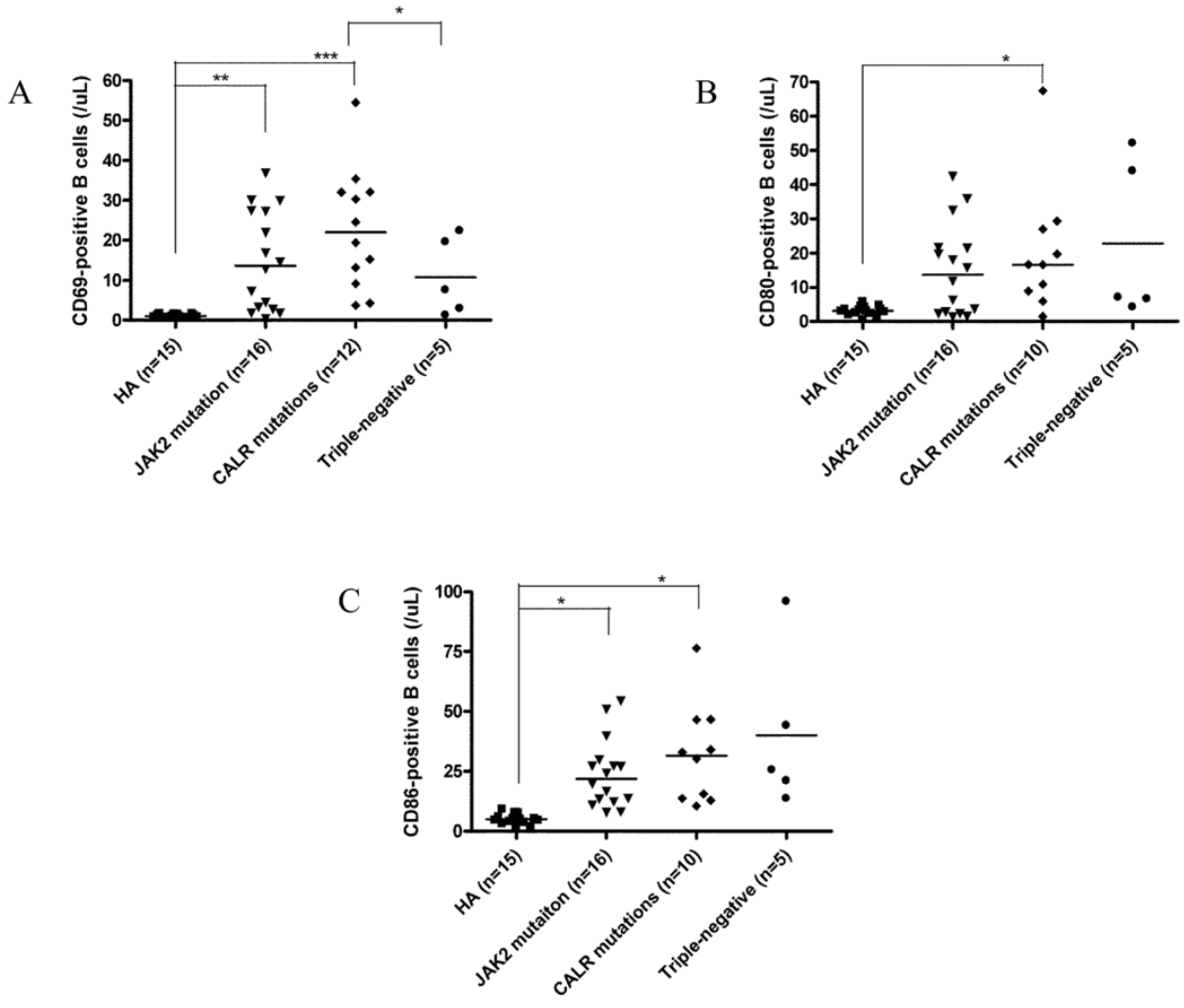

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Figure 11. Gated CD19+ B lymphocytes in whole blood cells using flow cytometry.

A. Lymphocytes were gated in whole blood cells depending on FSC and SSC. The gated lymphocytes were then used to select CD19+ cells in B and C.

B. No CD19+ cells was showed when using isotype control antibody.

C. Gated part showed CD19+ lymphocytes when using CD19 FITC antibody. The gated CD19+ B lymphocytes were then used to analyze the expression of CD69, CD80 and CD86 in Figure 12.

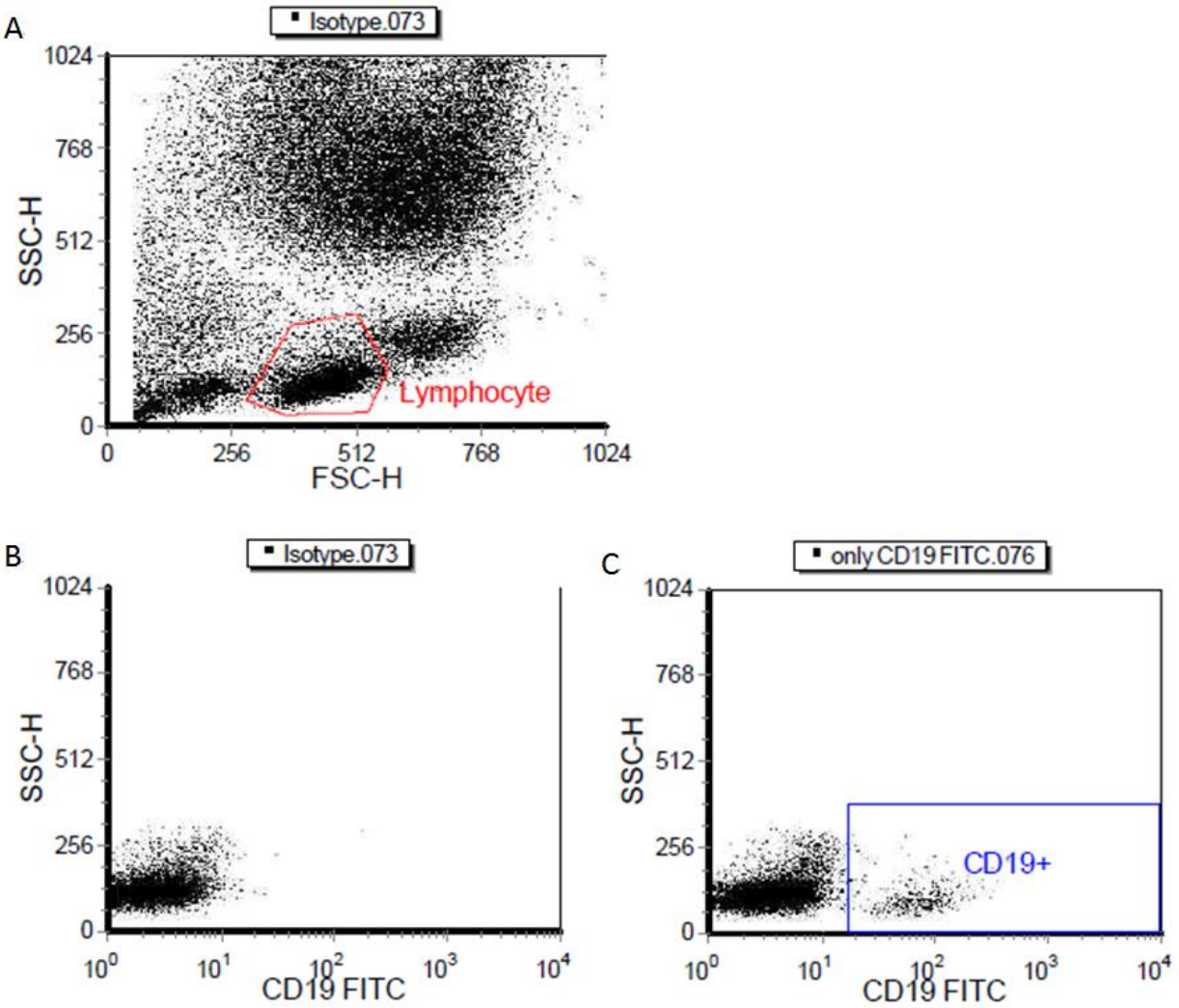

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Figure 12. Expression of CD69, CD80 and CD86 on CD19+ B lymphocytes using flow cytometry was showed in histograms.

A. CD69+ B lymphocytes from samples were showed and indicated in several colored lines.

B. CD80+ B lymphocytes from samples were showed and indicated in several colored lines.

C. CD86+ B lymphocytes from samples were showed and indicated in several colored lines.

Isotype control was indicated in black.

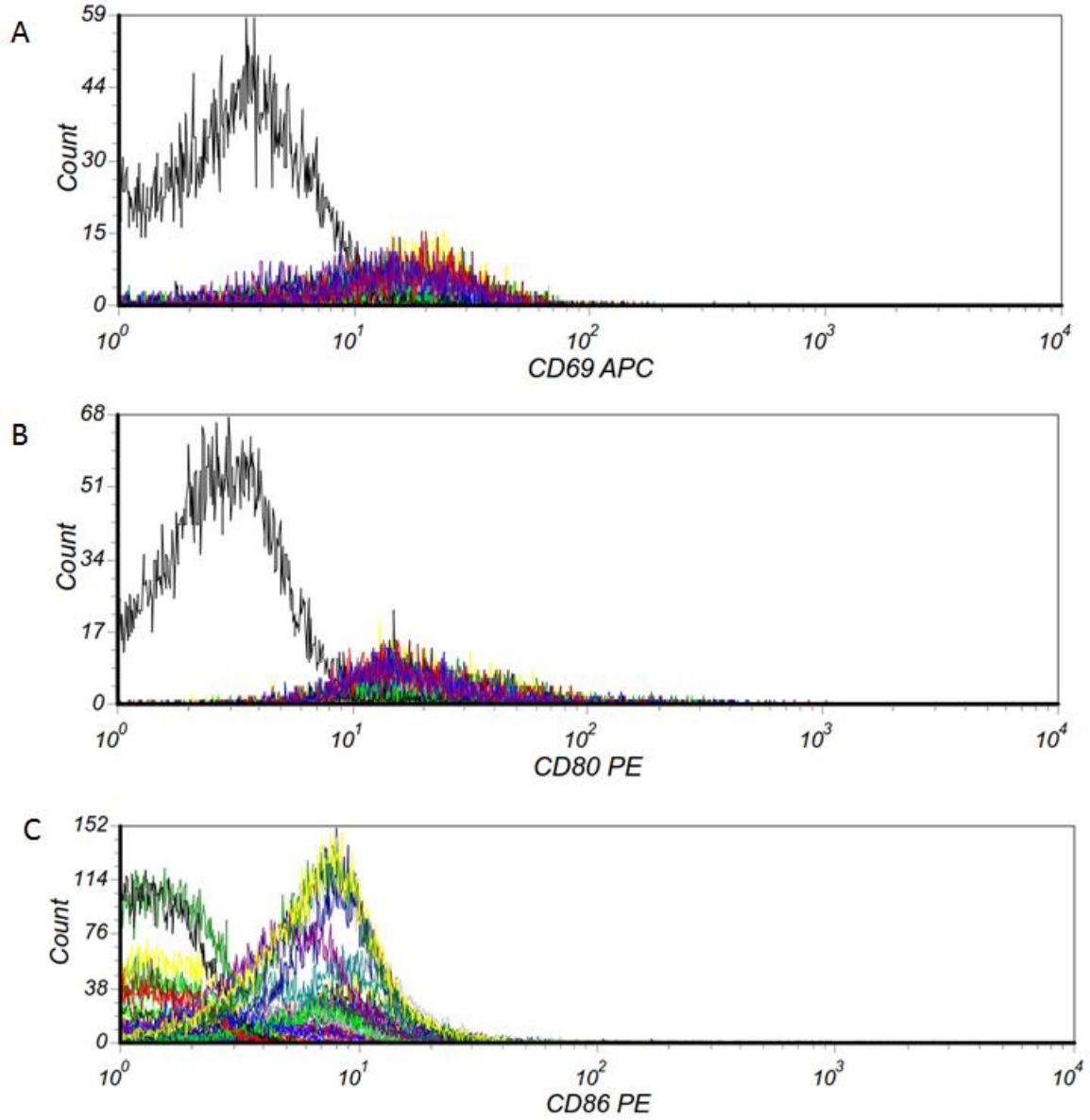

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Figure 13. The map for pSYC-102-CALR vectors.

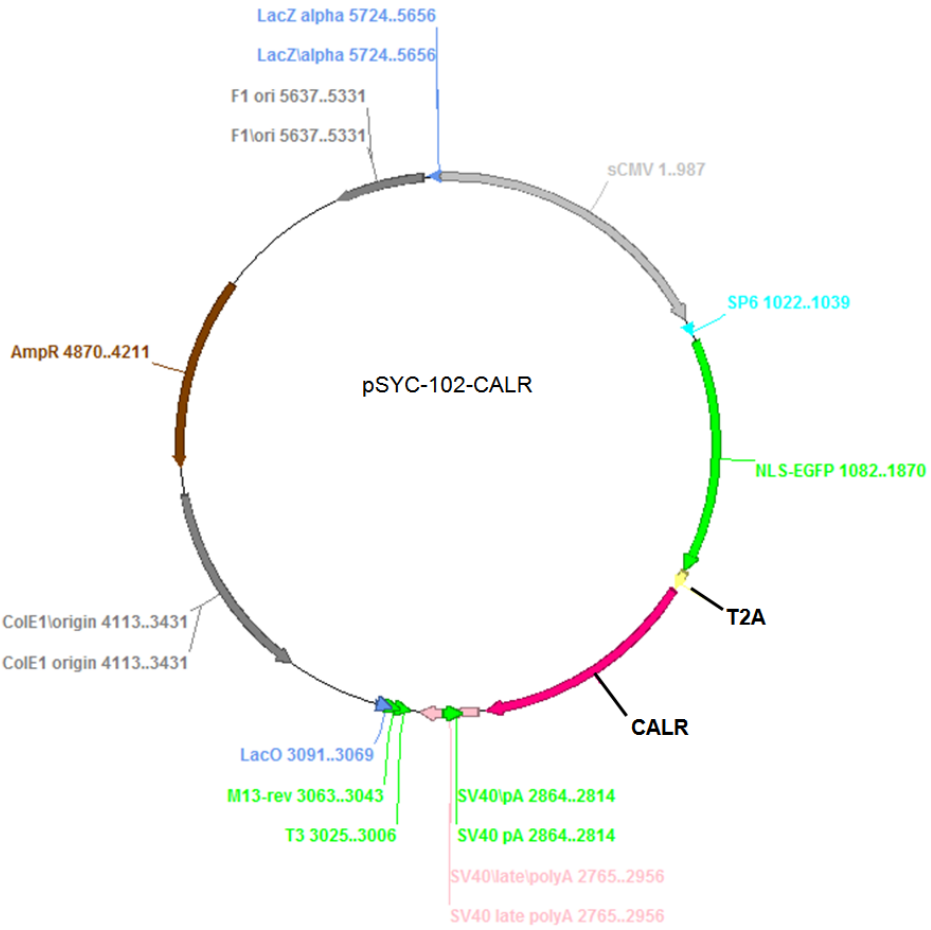

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Figure 14. Identification of 3 zebrafish *calr* genes.

A. Alignment of human CALR (top row), zebrafish calr (second row), calr3a (third row), and calr3b (bottom row) protein sequences. The regions of sequence identity in the four proteins are shaded.

B. The genomic loci surrounding human *CALR* on chromosome 19p13.2 (Hsa19) are syntenic with the regions where zebrafish *calr* (on chromosome 8, Dre8), *calr3a* (on chromosome 22, Dre22) and *calr3b* (on chromosome 2, Dre2) are located in the zebrafish genome, respectively.

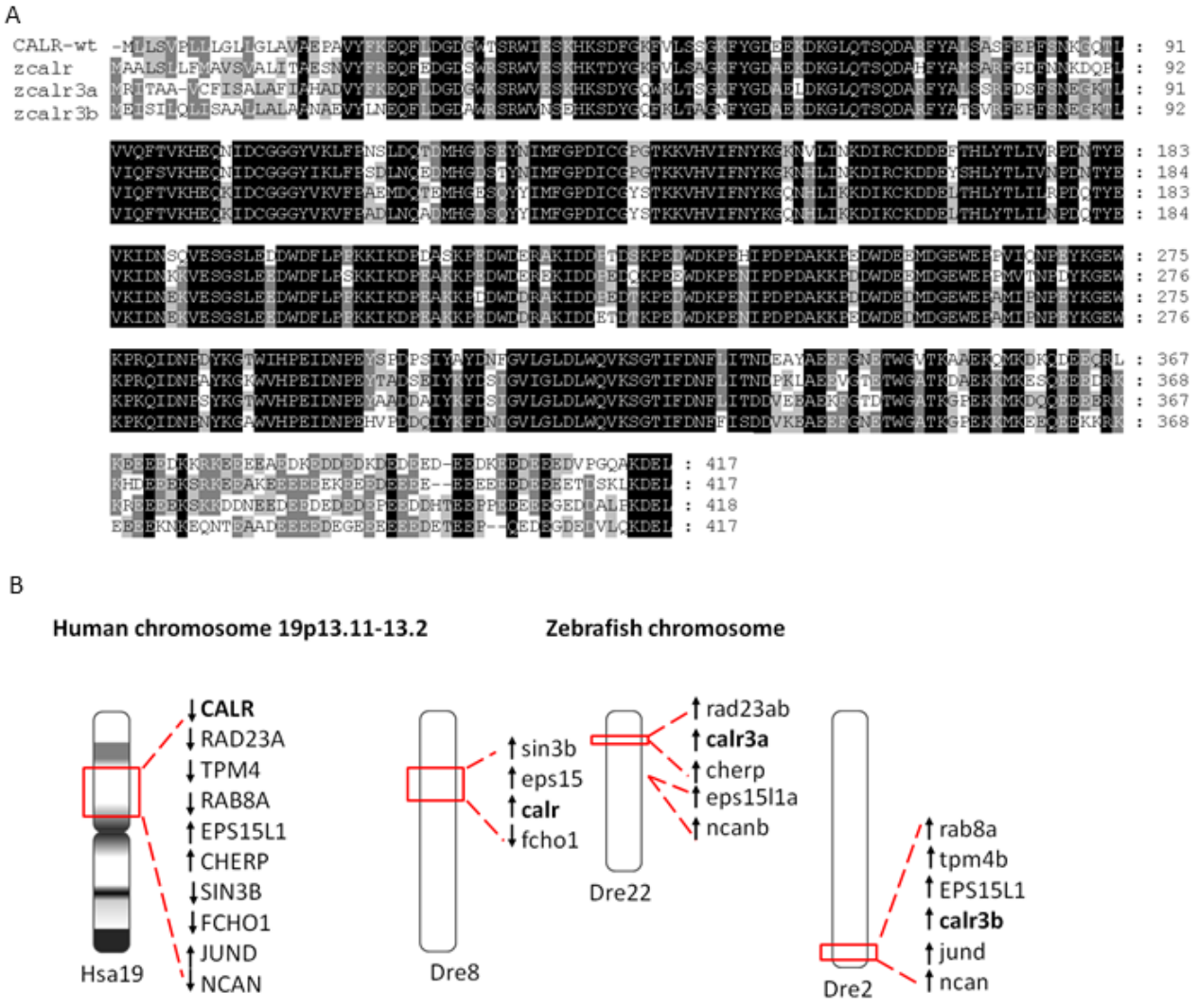

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Figure 15. Effects of the expression of mutant CALR on the number of hematopoietic stem/progenitor cells and angiogenesis.

A. CALR protein expression in uninjected, CALR-wt–, CALR-del52– or CALR-ins5–injected embryos at 24 hpf. gapdh was used to normalize the total amount of protein in each sample. The expression of human CALR mutant proteins was confirmed in CALR-del52– and CALR-ins5–injected embryos by a customized human CALR mutant-specific polyclonal antibody (top panel).

B. Brightfield image (left panel) of a 3 dpf embryo with a box area indicating caudal hematopoietic tissue where $cd41^{+}$ cells were counted. Green cells in the darkfield images (left panel) indicate expression of GFP under the control of the *cd41* promoter, and were counted and showed on the right panel.

C. The development of dorsal aorta (DA), dorsal vein (DV) and intersegmental vessel (ISV) as indicted by the red arrows (top panel) in uninjected, CALR-wt–, CALR-del52– or CALR-ins5–injected embryos of the Tg(*fli1*:EGFP) line at 3 dpf.

D. The total numbers of $cd41^{+}$ thrombocytes were counted in uninjected, CALR-wt–, CALR-del52– or CALR-ins5–injected embryos of the Tg(*cd41*:GFP) line at 5 dpf. (The direction of embryos was anterior to the left, dorsal upwards, lateral view) (n.s., not significant; **P*<0.05, ***P*<0.01, ****P*<0.001; Student *t* test). The number of embryos used in each experiment is indicated by "n" in figures.

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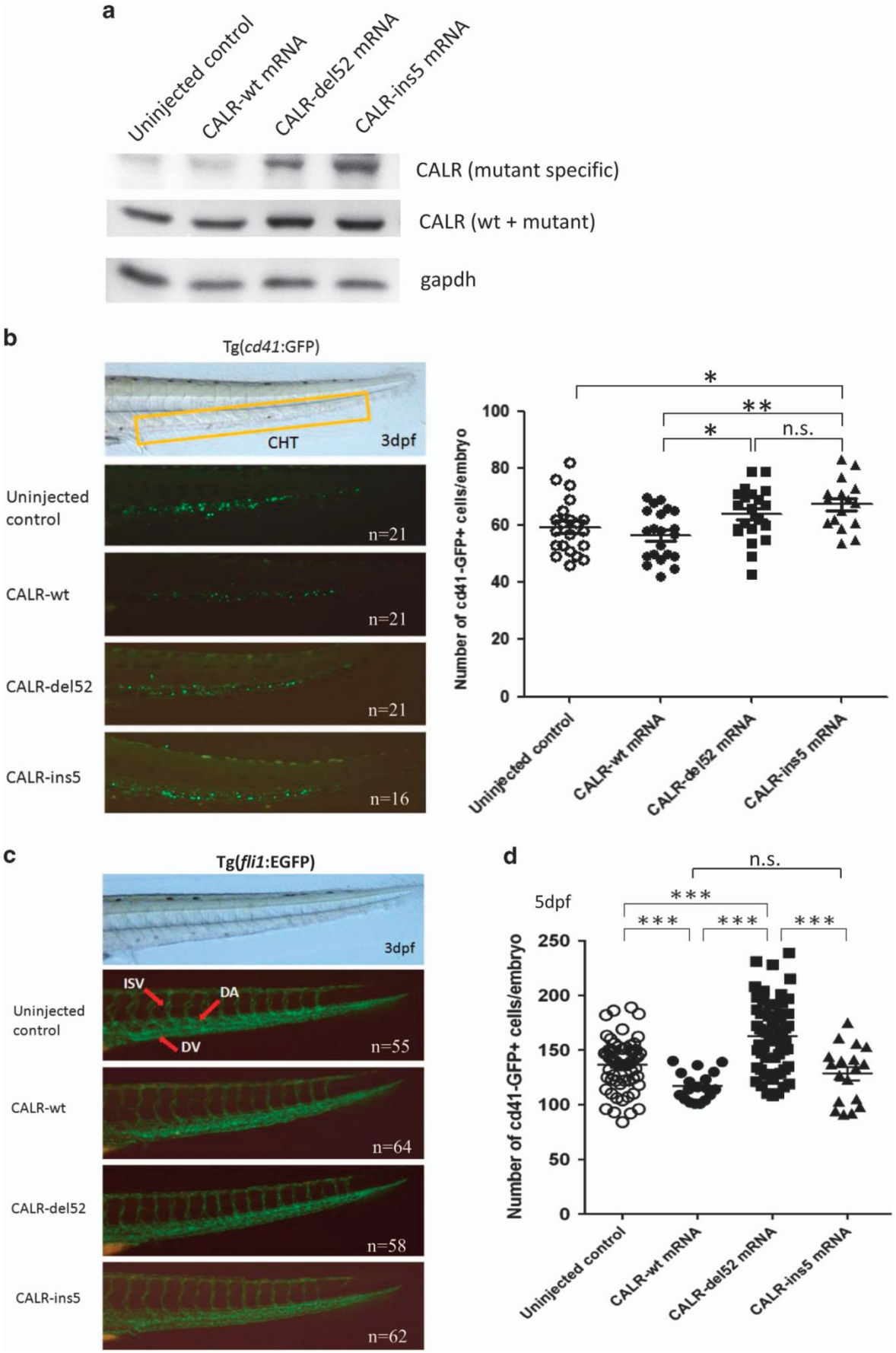

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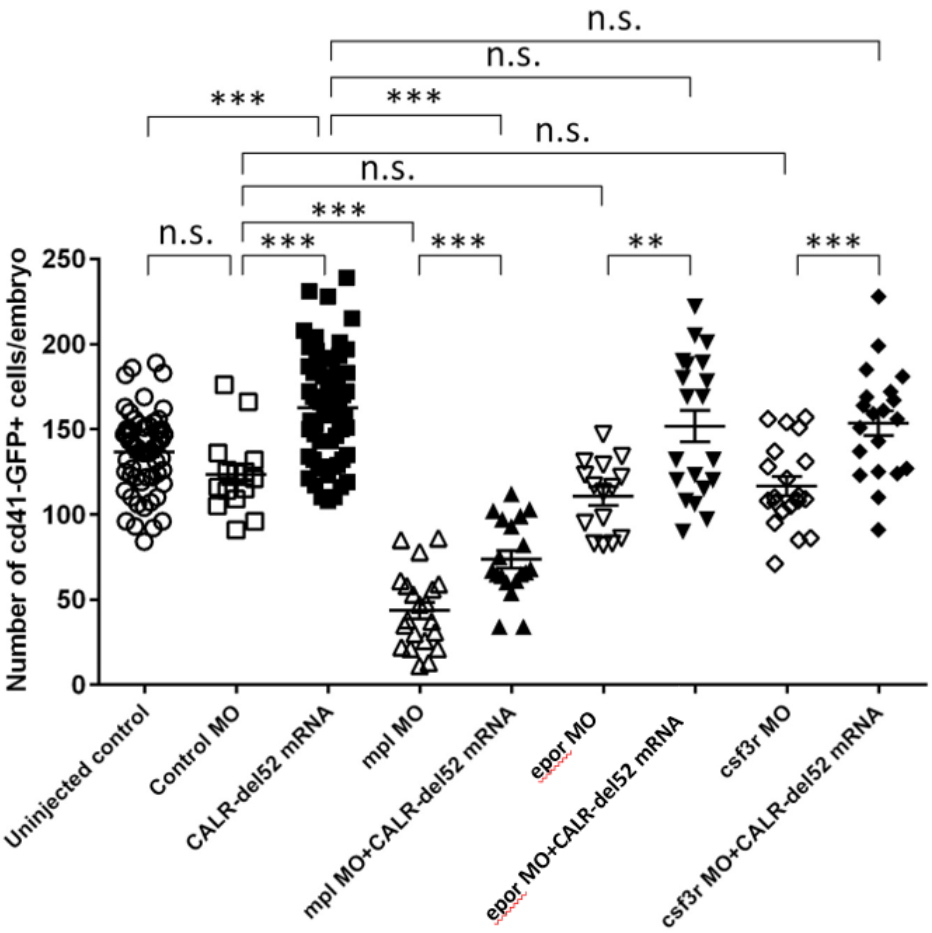
Figure 16. Mutant CALR requires mpl to cause thrombocytosis in zebrafish.

Morpholinos (MOs) targeting mpl, epor or csf3r (1 ng per embryo) were injected into 1-2 cells stage embryos of the Tg(*cd41*:GFP) line with or without co-injection of CLAR-del52 mRNA (100 pg). Standard control MO was used as negative control. The total numbers of cd41^{+} thrombocytes were counted at 5 dpf and compared as indicated. The number of cd41^{+} thrombocytes significantly decreases upon mpl knockdown when compared to the control MO group as well as the *CALR*-del52 group. Co-injection of *CALR*-del52 mRNA can only partially reverse the knockdown effect of mpl MO. When *CALR*-del52 mRNA was co-injected with epor MO or csf3r MO, the numbers of cd41^{+} thrombocytes are comparable to those of *CALR*-del52–injected embryos. (n.s., not significant; ***P*<0.01, ****P*<0.001; Student *t* test).



doi:10.6342/NTU201700938

Figure 17. The expression of mutant CALR activates jak-stat signaling in zebrafish.

A. Western blotting showing embryos of wild-type zebrafish injected with CALR-del52 mRNA (100 pg) have significantly increased signal transducer and activation of transcription (stat) 5 phosphorylation (lane 2) as compared with uninjected control embryos from the same batch at 24 hpf (lane 1). Pharmacologic treatment of embryos with a JAK2-selective inhibitor (fedratinib) and a dual JAK1/JAK2 inhibitor (ruxolitinib) significantly attenuated the enhanced stat5 phosphorylation induced by CALR-del52 mRNA stat5 phosphorylation (lane 3 and 4, respectively). Total amount of stat5 protein was not affected in all experiments.

B and C. Effects of the pharmacologic treatment with ruxolitinib and fedratinib (from 1 μM to 8 μM) on the numbers of cd41+ thrombocytes at 5 dpf with or without the injection of CALR-del52 mRNA. Treatment with ruxolitinib significantly decreases the numbers of cd41+ thrombocytes in uninjected control as well as CALR-del52–injected embryos in a dose-dependent manner. Whereas, treatment with fedratinib has minimal and insignificant inhibitory effect on the number of cd41+ thrombocyte in uninjected control embryos, and has a modest and significant dose-independent inhibitory effect on mutant CALR induced thrombocytosis. (n.s., not significant; $*P<0.05$, $**P<0.01$, $***P<0.001$; Student *t* test).

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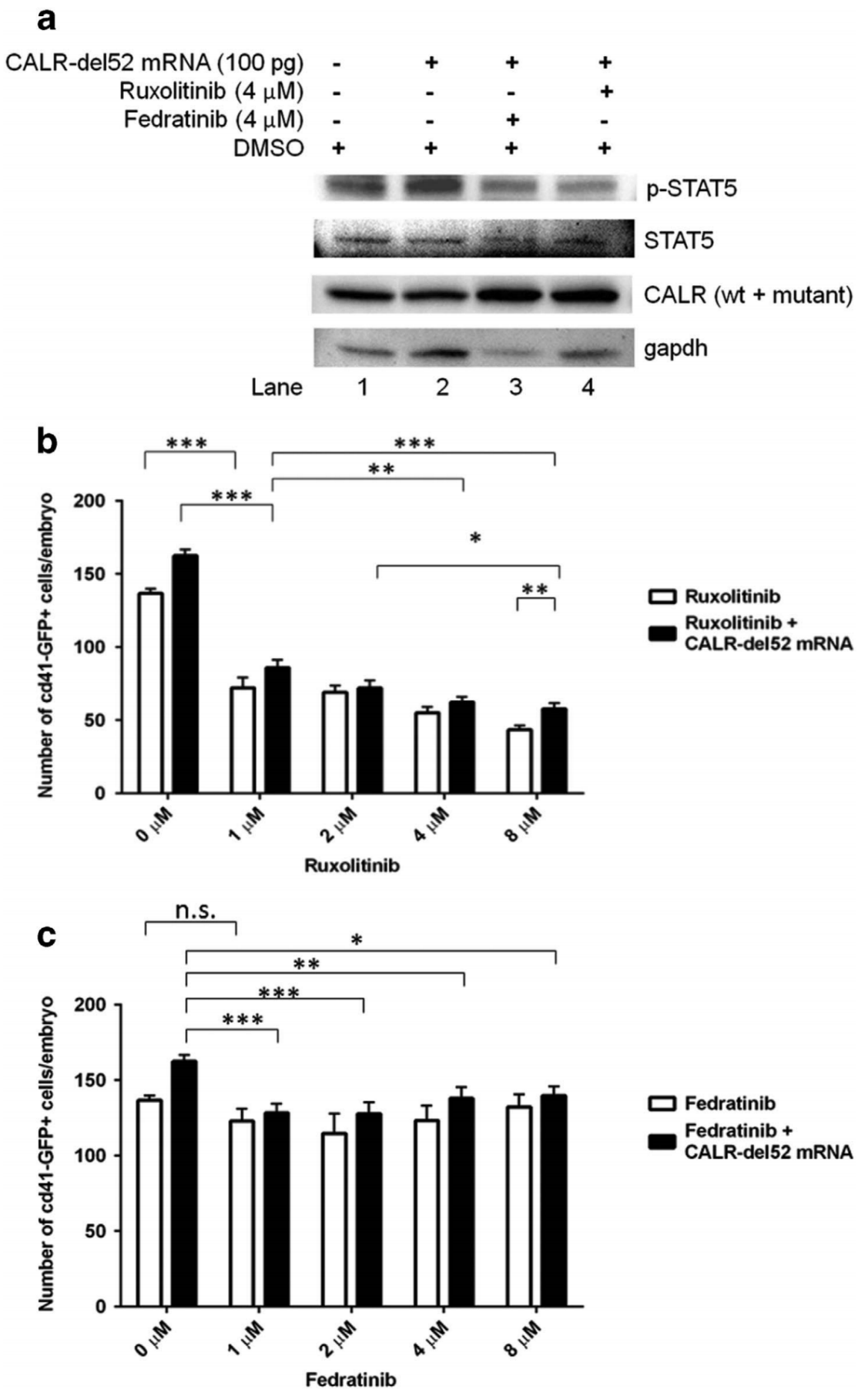

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Tables

Table 1. Clinical and laboratory characteristics at diagnosis or referral in 21 essential thrombocythemia patients with 6 types of *CALR* mutations.

		No. (%)	Male/Female gender, n (%)	Age at diagnosis (y), median (range)	Hemoglobin (g/dL), median (range)	WBC ($x10^3/\mu L$), median (range)	Platelets ($x10^9/L$), median (range)
CALR mutations		21 (65.6)	9/12 (43/57)	47 (22-76)	12.6 (8.5-15.2)	9.2 (4.9-27.9)	1351 (642-2834)
Type 1	p.L367fs*46	5 (23.8)	2/3 (40/60)	35 (25-63)	12.6 (11.7-14.6)	8.1 (7.1-14.4)	1351 (1095-1745)
Type 2	p.K385fs*47	11 (52.4)	4/7 (36/64)	47 (23-58)	11.6 (8.5-15.2)	10.6 (4.9-27.9)	1369 (642-2834)
Type 3	p.L367fs*48	1 (4.8)	0/1 (0/100)	22	12.8	9.2	1477
Type 34	p.K385fs*47	2 (9.5)	2/0 (100/0)	53 (30-76)	15.0 (14.9-15)	10.9 (9.3-12.4)	1362.5 (1072-1653)
Other	p.L367fs*43	1 (4.8)	1/0 (100/0)	61	9.3	7.9	1045
Other	p.E369fs*50	1 (4.8)	0/1 (0/100)	55	9.4	7.1	1235

Abbreviations: n, number; y, year.

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Table 2. *CALR* exon 9 alterations and single nucleotide polymorphism in 14 *JAK2* V617F-mutated essential thrombocythemia patients detected by high-resolution melting analysis.

Patient	*CALR* mutation	Nucleotide change	Protein change	Amino acid		Protein sequence*	*CALR* SEQ	CALR-TA clone number**	*JAK2* V617F allele burden#
NA	Wild-type	NA	NA	417	365	QRLKEEEEDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild-type	NA	NA
P520	Type 2	c.1154_1155insTTGTC	p.K385fs*47	430	365	QRLKEEEEDKKRKEEEEAEDNCRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	Wild-type	1/40	7%
P366	Type 3	c.1095_1140del(Δ46)	p.L367fs*48	413	365	QRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	Heterozygous	NA	83%
P426	New	c.1108delG (Δ1)	p.E370fs*60	428	365	QRLKERKKTRNAKRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	Wild-type	1/100	25%
P417	New	c.1111delG (Δ1)	p.E371fs*59	428	365	QRLKEEKKTRNAKRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	Wild-type	1/40	20%
P421	New	c.1110_1112delGGA (Δ3)	p.E371del	416	365	QRLKEE—EDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild-type	1/71	71%
P520	New	c.1132_1134delGAG (Δ3)	p.E378del	416	365	QRLKEEEEDKKRK—EEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild-type	1/40	7%
P393	New	c.1142_1144delAGG (Δ3)	p.E381del	416	365	QRLKEEEEDKKRKEEE—AEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild-type	1/40	5%
P527	New	c.1142_1144delAGG (Δ3)	p.E381del	416	365	QRLKEEEEDKKRKEEE—AEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild-type	1/10	4%
P384	New	c.1188_1190delGGA (Δ3)	p.E396del	416	365	QRLKEEEEDKKRKEEEEAEDKEDDEDKDEDE—DEEDKEEDEEEDVPGQAKDEL	Wild-type	1/40	27%
P615	New	c.1120 A>T	p.E374X	373	365	QRLKEEEED	Wild-type	2/40	23%
P871	New	c.1138 G>T	p.E380X	379	365	QRLKEEEEDKKRKEE	Wild-type	2/16	32%
P744	New	c.1171 A>T	p.K391X	390	365	QRLKEEEEDKKRKEEEEAEDKEDDED	Wild-type	2/70	13%
P428	New	c.1115 A>G	p.E372G	417	365	QRLKEEEGDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild-type	2/61	50%
P551	rs201971744	c.1139 A>G	p.E380G	417	365	QRLKEEEEDKKRKEEGEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild-type	2/10	41%
P398	rs143880510	c.1142 A>C	p.E381A	417	365	QRLKEEEEDKKRKEEEAAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Heterozygous	NA	26%

*Red and blue fonts indicate acidic and basic amino acids, respectively. Underline indicates the same C-terminal sequence changes after +1 base-pair reading frameshift.

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**TA clones of the *CALR* PCR products amplified from each colony were analyzed by Sanger sequencing. The total number of clones examined and the number of each genotype are listed in the table.

[#]Based on the relative peak areas of the mutant and wild-type PCR products in Sanger sequencing. All patients were tested positive by allele-specific PCR.

Abbreviations: NA, not available; SEQ, Sanger sequencing.

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Table 3. Clinical and laboratory characteristics at diagnosis or referral of 91 essential thrombocythemia patients stratified by mutation profiles.

Variables	All (n=91)	A. *JAK2* V617F mutation (n=46)	B. *CALR* mutations (n=21)	C. *JAK2*-mutated and *CALR* alterations (n=13)	D. Triple-negative (n=11)	A vs B vs C vs D p value	A vs B vs C p value	A vs C p value	B vs C p value
Male/Female gender, n (%)	39/52 (43/57)	21/25 (46/54)	9/12 (43/57)	5/8 (39/61)	4/7 (36/64)	NS	NS	NS	NS
Age at diagnosis (y), median (range)	53 (22-89)	54.5 (25-89)	47 (22-76)	60 (26-80)	52 (35-79)	0.025	0.012	NS	0.004
Follow-up (y), median (range)	3.7 (0.02-23.1)	3.6 (0.04-23.1)	5,4 (0.5-13.2)	3.8 (0.02-6.1)	3.1 (0.2-10.3)	NS	NS	NS	0.032
History of thrombosis, n (%)	19 (20.9)	9 (19.6)	3 (14.3)	5 (38.5)	2 (18.2)	NS	NS	NS	NS
Major thrombosis, n (%)	17 (18.7)	8 (17.4)	2 (9.5)	5 (38.5)	2 (18.2)	NS	NS	NS	NS
Thrombosis after diagnosis, n (%)	10 (11)	3 (6.5)	2 (9.5)	4 (30.8)	1 (9.1)	NS	0.048	0.036	NS
Major arterial thrombosis after diagnosis, n (%)	6 (6.6)	1 (2.2)	1 (4.8)	3 (23.1)	1 (9.1)	NS	0.022	0.03	NS

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History of hemorrhage, n (%)	25 (27.5)	13 (28.3)	9 (42.9)	2 (15.4)	1 (9.1)	NS	NS	NS	NS
Major hemorrhage, n (%)	17 (18.7)	9 (19.6)	6 (28.6)	2 (15.4)	0	NS	NS	NS	NS
High risk group for thrombo-hemorrhagic complications*, n (%)	43 (47.3)	22 (47.8)	6 (28.6)	10 (76.9)	5 (45.5)	NS	0.023	NS	0.012
Hemoglobin (g/dL), median (range)	13.3 (4.5-17.9)	14.0 (4.5-17.9)	12.6 (8.5-15.2)	13.3 (8.8-16.6)	12.8 (9.3-15.2)	0.016	0.016	NS	NS
WBC ($x10^3/\mu L$), median (range)	10.3 (4.8-29.9)	12.1 (4.8-29.9)	9.2 (4.9-27.9)	11.8 (6.0-24.2)	8.2 (5.3-25.5)	0.046	NS	NS	NS
Platelets ($x10^9/L$), median (range)	936 (335-2834)	942 (335-1496)	1351 (642-2834)	855 (547-1931)	708 (532-1374)	< 0.001	< 0.001	NS	0.001

*High risk group for thrombo-hemorrhagic complications: Age ≥60 years and/or a previous history of thrombosis.

Abbreviations: n, number; NS, not significant; WBC, white blood cell; y, year.

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Table 4. Clinical and laboratory characteristics in healthy adults and patients with essential thrombocythemia.

Variables	HA (n=48)	*JAK2* mutation *(n= 27)*	*CALR* mutations (n=15)	TN (n=7)	*CALR* mutations vs *JAK2* mutation vs TN p value	*CALR* mutations vs *JAK2* mutation p value	*CALR* mutations vs TN p value	*JAK2* mutation vs TN p value	*CALR* mutations vs HA p value	*JAK2* mutation vs HA p value	TN vs HA p value
Male/Female gender, n (%)	15/33 (31/69)	11/16 (41/59)	7/8 (47/53)	3/4 (43/57)	NS	NS	NS	NS	-	-	-
Age at diagnosis (y), median (range)	-	55 (25-89)	45 (21-76)	52 (20-79)	NS	0.04	NS	NS	-	-	-
Follow-up (y), median (range)	-	3.6 (0.1-20.8)	6.2 (0.8-13.4)	3.3 (0.1-10.3)	0.031	0.019	0.039	NS	-	-	-
Hemoglobin at diagnosis (g/dL), median (range)	-	13.5 (8.6-17.1)	11.9 (8.5-15.2)	12.9 (10.1-15.2)	0.037	0.013	NS	NS	-	-	-
WBC at diagnosis ($x10^9$/L), median (range)	-	12.3 (5.7-27.7)	8.7 (4.3-17.5)	7.8 (5.3-10.2)	0.002	0.013	NS	0.002	-	-	-
Platelet at diagnosis ($x10^9$/L),	-	948 (335-1437)	1275 (759-2606)	900 (608-1374)	0.01	0.004	0.039	NS	-	-	-

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median (range)											
Hemoglobin at testing (g/dL), median (range)	12.9 (10.3-16.7)	13.4 (7.2-15.9)	12.5 (9.1-15)	13.3 (10.1-15.6)	NS	NS	NS	NS	NS	0.039	NS
WBC at testing ($x10^9$/L), median (range), n=56	5.5 (3.9-7)	12.0 (6.8-21.8)	8.3 (4-13.8)	7.8 (4.6-8.6)	< 0.001	0.001	NS	0.001	0.005	< 0.001	0.034
Platelet at testing ($x10^9$/L), median (range)	241.5 (118-366)	842 (449-1227)	734(247-2 215)	824 (551-1127)	NS	NS	NS	NS	< 0.001	< 0.001	< 0.001
Cytoreductive therapy with hydroxyurea, n (%)	-	20 (74.1)	11 (73.3)	3 (42.9)	NS	NS	NS	NS	-	-	-

Abbreviations: HA, healthy adults; No. and n, number; NS, not significant; TN, triple-negative; WBC, white blood cell; y, year.

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Table 5. Univariate analysis of B cell immune profiles in healthy adults and patients with essential thrombocythemia.

Variables	HA (n=48)	*JAK2* mutation *(n= 27)*	*CALR* mutations (n=15)	TN (n=7)	*CALR* mutations vs *JAK2* mutation vs TN p value	*CALR* mutations vs *JAK2* mutation p value	*CALR* mutations vs TN p value	*JAK2* mutation vs TN p value	*CALR* mutations vs HA p value	*JAK2* mutation vs HA p value	TN vs HA p value
CD19+ B cells (/μL), median (range), n=71	230.5 (143-455)	129.0 (25.8-358)	121.8 (17.8-318)	97.2 (38.1-219.5)	NS	NS	NS	NS	0.001	< 0.001	0.001
Early transitional B cells (T1) (/μL), median (range), n=61	3 (1-11)	1.1 (0.0-15.5)	2 (0.0-19.4)	2.5 (1-10)	NS	NS	NS	NS	NS	NS	NS
Late transitional B cells (T2) (/μL), median (range), n=61	7 (3-15)	15 (1-46)	14 (0.4-29.5)	9.2 (2-46)	NS	NS	NS	NS	NS	NS	NS
Pre-germinal center B cells (/μL), median (range), n=61	4 (1-24)	7 (2-17.6)	6.6 (2-19)	4.0 (3-25)	NS	NS	NS	NS	NS	NS	NS

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Memory B cells (/μL), median (range), n=61	75.5 (32-181)	34 (6.8-108)	34 (3.4-145)	28.5 (20-165)	NS	NS	NS	NS	< 0.001	< 0.001	NS
Plasmablast (/μL), median (range), n=61	0.0 (0.0-1)	0.4 (0.0-4)	0.3 (0.0-3)	0.5 (0.0-2)	NS	NS	NS	NS	< 0.001	< 0.001	0.025
Naive B cells (/μL), median (range), n=61	134.5 (75-238)	65.6 (5.8-293)	45.8(5.8-223)	57.5 (16-591)	NS	NS	NS	NS	0.003	0.01	0.014
MFI of mBAFF on granulocytes, n=63	6.7 (4.6-8.3)	25.4 (7.8-75.2)	34.2 (10.2-67.7)	33.7 (4.5-65.4)	NS	NS	NS	NS	< 0.001	< 0.001	0.007
MFI of mBAFF on monocytes, n=62	7.6 (2.8-8.0)	14.2 (3.5-54.6)	28.9 (4.4-49.4)	14.3 (3.2-40.7)	NS	NS	NS	NS	< 0.001	< 0.001	0.005
Serum BAFF level (ng/mL), n=66	1.1 (0.6-2.4)	2.3 (0.8-4.9)	1.6 (0.9-3.9)	1.8 (1.4-3.8)	0.049	0.02	NS	NS	0.015	< 0.001	0.001
IL-6 in B cells (%), n=39	1.0 (0.2-1.9)	6.7 (2.6-9.6)	6.8 (4.0-13.7)	8.2 (4.5-10.3)	NS	NS	NS	NS	< 0.001	< 0.001	< 0.001
IL-1β in B	4.8	11.5	15.4	12.9	NS	NS	NS	NS	0.002	0.002	0.012

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cells (%), n=39	(0.9-13.4)	(1.4-32.5)	(4.6-32.6)	(3.6-41.7)							
TLR4 in B cells (%), n=54	2.3 (0.3-3.0)	4.5 (0.4-24.2)	11.3 (2.3-22.8)	3.4 (1.0-5.4)	0.021	NS	0.001	NS	< 0.001	0.001	0.02
CD69+ B cells (/μL), median (range), n=48	2.2 (1.0-4.6)	12.5 (0.6-39.1)	20.8 (2.5-51.4)	7.6 (1.8-18.6)	NS	NS	0.048	NS	< 0.001	0.002	NS
CD80+ B cells (/μL), median (range), n=46	9.2 (0.8-12.5)	9.0 (1.9-79.1)	13.8 (1.0-72.6)	10.0 (1.6-44.7)	NS	NS	NS	NS	0.036	NS	NS
CD86+ B cells (/μL), median (range), n=46	10.9 (3.0-41.9)	26.3 (4.3-101.6)	18.3 (5.9-63.4)	24.2 (8.0-82.3)	NS	NS	NS	NS	0.041	0.012	NS

Abbreviations: BAFF, B cell-activating factor; HA, healthy adults; IL, interleukin; mBAFF, membrane-bound B cell-activating factor; MFI, mean fluorescence intensity; No. and n, number; NS, not significant; TLR4, toll-like receptor 4; TN, triple-negative; WBC, white blood cell; y, year.

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Table 6. Laboratory characteristics and B cell immune profiles in essential thrombocythemia patients with and without treatment with hydroxyurea.

Variables	No	Yes	p value
Cytoreductive therapy with hydroxyurea, n (%)	16 (29.6)	38 (70.4)	-
Hemoglobin at testing (g/dL), median (range), n=54	13.4 (9.1-17.7)	13.4 (7.2-15.8)	NS
WBC at testing (x10^9/L), median (range), n=54	8.2 (4.0-29.3)	10.5 (4.4-19.7)	NS
Platelet at testing (x10^9/L), median (range), n=54	850.0 (333.0-1369.0)	728.5 (247.0-2215.0)	NS
CD19+ B cells (/μL), median (range), n=52	139.0 (17.8-358.0)	121.8 (25.8-2031.9)	NS
Early transitional B cells (T1) (/μL), median (range), n=37	2.0 (0.1-10.0)	2.5 (0.0-19.4)	NS
Late transitional B cells (T2) (/μL), median (range), n=37	11.4 (0.4-46.0)	15.0 (1.0-46.0)	NS
Pre-germinal center B cells (/μL), median (range), n=37	6.9 (2.4-25.0)	6.8 (2.0-19.0)	NS
Memory B cells (/μL), median (range), n=37	21.2 (6.0-165.0)	35.1 (3.4-145.0)	NS
Plasmablast (/μL), median (range), n=37	0.3 (0.0-2.0)	0.4 (0.0-4.0)	NS
Naive B cells (/μL), median (range), n=37	48.2 (5.8-591.0)	59.0 (5.8-367.0)	NS
MFI of mBAFF on granulocytes, n=52	35.3 (7.8-65.4)	23.5 (4.5-75.2)	NS
MFI of mBAFF on monocytes, n=51	27.7 (8.0-38.1)	18.1 (3.2-54.6)	NS
Serum BAFF level (ng/mL), n=49	1.7 (0.8-4.8)	1.9 (0.9-4.9)	NS
IL-6 in B cells (%), n=28	9.4 (2.7-13.6)	8.1 (2.8-13.6)	NS
IL-1β in B cells (%), n=28	16.4 (5.6-32.1)	6.9 (4.1-17.9)	0.014

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TLR4 in B cells (%), n=44	22.3 (13.1-103.3)	19.1 (6.9-134.4)	NS
CD69+ B cells (/μL), median (range), n=35	14.2 (2.5-35.8)	13.2 (0.5-943.4)	NS
CD80+ B cells (/μL), median (range), n=33	26.3 (3.0-72.6)	13.8 (1.0-449.7)	NS
CD86+ B cells (/μL), median (range), n=33	21.7 (5.9-82.3)	26.8 (4.3-191.7)	NS

Abbreviations: BAFF, B cell-activating factor; IL, interleukin; mBAFF, membrane-bound B cell-activating factor; MFI, mean fluorescence intensity; No. and n, number; NS, not significant; TLR4: toll-like receptor 4; WBC, white blood cell.

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Table 7. Correlation of platelet count at testing, serum BAFF levels, and B cell immune profiles in this study.

Variables	Platelet at testing		MFI of mBAFF on granulocytes		MFI of mBAFF on monocytes		Serum BAFF levels	
	Spearman's rho	p value	Spearman's rho	p value	Spearman's rho	p value	Spearman's rho	p value
MFI of mBAFF on granulocytes	0.377	0.002	-	-	0.786	<0.001	0.29	0.021
MFI of mBAFF on monocytes	0.312	0.011	0.786	<0.001	-	-	0.304	0.016
Serum BAFF levels	0.486	<0.001	0.29	0.021	0.304	0.016	-	-
IL-6 in B cells	0.61	<0.001	0.545	<0.001	0.398	0.009	0.572	<0.001
IL-1β in B cells	0.543	<0.001	0.55	<0.001	0.472	0.002	0.293	NS
CD69+ B cells	0.325	0.021	0.388	0.005	0.161	NS	0.225	NS
CD86+ B cells	0.26	NS	0.42	0.003	0.252	NS	0.141	NS

Abbreviations: BAFF, B cell-activating factor; IL, interleukin; mBAFF, membrane-bound B cell-activating factor; MFI, mean fluorescence intensity; NS, not significant.

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Table 8. Comparison of B cell immune profiles between healthy adults with patients with essential thrombocythemia using linear regression model adjusted for hematological parameters.

Variables	ET subgroups	Unstandardized Coefficients		p value	95% Confidence Interval for B	
		B	Std. Error		Lower Bound	Upper Bound
CD19+ B cells (/μL)	*JAK2*-mutated	-109.918	42.713	0.013	-195.846	-23.990
	CALR-mutated	-102.613	40.102	0.014	-183.289	-21.938
	TN	-148.166	46.096	0.002	-240.899	-55.433
Neutrophil mBAFF MFI	*JAK2*-mutated	25.969	9.115	0.006	7.642	44.297
	CALR-mutated	33.242	8.565	<0.001	16.020	50.463
	TN	34.018	9.410	0.001	15.099	52.938
Monocyte mBAFF MFI	*JAK2*-mutated	14.897	7.287	0.047	0.237	29.556
	CALR-mutated	23.159	6.779	0.001	9.522	36.796
	TN	16.591	7.446	0.031	1.612	31.571
Serum BAFF levels (ng/ml)	*JAK2*-mutated	0.915	0.625	NS	-0.343	2.173
	CALR-mutated	0.194	0.586	NS	-0.986	1.373
	TN	1.102	0.643	NS	-0.194	2.397
Total B IL-6 %	*JAK2*-mutated	4.488	1.912	0.027	0.551	8.425
	CALR-mutated	4.906	2.337	0.046	0.093	9.719
	TN	5.463	1.724	0.004	1.913	9.014
Total B IL-1β%	*JAK2*-mutated	11.566	7.807	NS	-4.513	27.644
	CALR-mutated	16.529	9.544	NS	-3.128	36.186
	TN	14.697	7.040	0.047	0.199	29.196
Total B TLR4 %	*JAK2*-mutated	14.090	4.281	0.002	5.439	22.742
	CALR-mutated	14.262	3.746	<0.001	6.692	21.832
	TN	5.888	4.378	NS	-2.961	14.737
CD69+ B cells (/μL)	*JAK2*-mutated	35.629	9.431	0.001	16.440	54.817
	CALR-mutated	34.581	7.583	<0.001	19.153	50.008
	TN	19.206	9.302	0.047	0.282	38.130
CD80+ B cells (/μL)	*JAK2*-mutated	18.875	15.117	NS	-11.956	49.705
	CALR-mutated	15.519	12.409	NS	-9.790	40.828
	TN	16.876	14.963	NS	-13.640	47.393
CD86+ B	*JAK2*-mutated	38.875	17.586	0.035	3.007	74.742

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cells (/μL)	*CALR*-mutated	27.556	14.437	NS	-1.888	57.000
	TN	33.042	17.407	NS	-2.460	68.545

Abbreviations: mBAFF, membrane-bound B cell-activating factor; IL: interleukin; MFI, mean fluorescence intensity; NS, not significant; Std., standard; TLR4: toll-like receptor 4; TN, triple-negative.

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Table 9. Comparison of B cell immune profiles among patients with essential thrombocythemia using linear regression model adjusted for age, sex, follow-up period and hematological parameters.

Variables	ET subgroups	*CALR*-mutated group as control					*JAK2*-mutated group as control				
		Unstandardized Coefficients		p value	95% Confidence Interval for B		Unstandardized Coefficients		p value	95% Confidence Interval for B	
		B	Std. Error		Lower Bound	Upper Bound	B	Std. Error		Lower Bound	Upper Bound
Serum BAFF levels (ng/ml)	*JAK2*-mutated	0.436	0.479	NS	-0.536	1.408	–	–	–	–	–
	CALR-mutated	–	–	–	–	–	-0.436	0.479	NS	-1.408	0.536
	TN	0.571	0.578	NS	-0.601	1.743	0.135	0.577	NS	-1.035	1.304
Total B TLR4 %	*JAK2*-mutated	0.437	2.759	NS	-5.183	6.057	–	–	–	–	–
	CALR-mutated	–	–	–	–	–	-0.437	2.759	NS	-6.057	5.183
	TN	-8.169	3.329	0.02	-14.951	-1.388	-8.606	3.467	0.019	-15.669	-1.543
CD69+ B cells (/μL)	*JAK2*-mutated	-1.303	7.025	NS	-15.801	13.196	–	–	–	–	–
	CALR-mutated	–	–	–	–	–	1.303	7.025	NS	-13.196	15.801
	TN	-18.480	8.265	0.035	-35.539	-1.421	-17.177	8.605	NS	-34.936	0.582
CD80+ B cells (/μL)	*JAK2*-mutated	6.135	11.382	NS	-17.470	29.740	–	–	–	–	–
	CALR-mutated	–	–	–	–	–	-6.135	11.382	NS	-29.740	17.470
	TN	3.273	13.175	NS	-24.051	30.597	-2.862	13.175	NS	-30.185	24.462
CD86+ B cells (/μL)	*JAK2*-mutated	16.243	13.696	NS	-12.161	44.647	–	–	–	–	–
	CALR-mutated	–	–	–	–	–	-16.243	13.696	NS	-44.647	12.161
	TN	11.584	15.854	NS	-21.294	44.463	-4.658	15.854	NS	-37.537	28.221

Abbreviations: BAFF, B cell-activating factor; NS, not significant; Std., standard; TLR4: toll-like receptor 4; TN, triple-negative.

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Table 10. Characteristics and the frequency of *CALR* and *JAK2*V617F co-mutations in patients with essential thrombocythemia.

Author	Population	Method to detect *CALR* mutations	*CALR* mutation	Frequency of *CALR* and *JAK2*V617F co-mutations		
				In ET no. (%)	In *CALR*-mutated ET no. (%)	In *JAK2*V617F-mutated ET no. (%)
Lundberg *et al.* [10]	Caucasian	Allele-specific PCR	p.K385fs*47	1/69 (1.4)	1/17 (5.9)	1/41 (2.4)
Fu *et al.* [75]	Chinese	Sanger sequencing	L367fs*46 c.997 C>T (arginine> tryptophan)	2/436 (0.5)	2/99 (2)	2/240 (0.8)
Shirane *et al.* [92]	Japanese	Fragment analysis and deep sequencing	p.E378fs*45	1/111 (0.9)	1/22 (4.5)	1/60 (1.7)
Ha and Kim [93]	Korean	Sanger sequencing	p.L367fs*46	1/114 (0.9)	1/25 (4)	1/68 (1.5)
Al Assaf *et al.*[94]	Caucasian	Sanger sequencing	p.K385fs*47	1/160 (0.6)	1/59 (1.7)	1/57 (1.8)
Lin *et al.*[95]	Chinese	Sanger sequencing	2 p.L367fs*46 2 p.K385fs*47	4/428 (0.9)	4/101 (4.0)	4/254 (1.6)
Lim *et al.* [96]	Taiwanese	HRMA and Sanger sequencing	p.L367fs*48 p.E381A p.K385fs*47 p.E370fs*60 p.E371fs*59 p.E371del p.E378del p.E396del p.E374X p.E380X p.K391X	13/92 (14.1)	13/34 (38)	13/59 (22)

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			p.E372G p.E380G			
Usseglio *et al*. [97]	Caucasian	HRMA	2 p.K385fs*47 p.L367fs*48 c.1125_1147del	4/103 (3.9)	4/48 (8.3)	4/56 (7.1)
Lim *et al*. (this study)	Taiwanese	HRMA and Sanger sequencing	p.L367fs*48 p.E370fs*60 p.E371fs*59 p.E381del	4/54 (7.4)	4/19 (21.1)	1/31 (3.2)

Abbreviations: ET, essential thrombocythemia; HRMA, high-resolution melting analysis; no., number.

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Table 11. Univariate analysis of B cell immune profiles in healthy adults, reactive thrombocytosis, polycythemia vera and essential thrombocythemia.

Variables	HA (n=15)	RT (n= 20)	ET (n=30)	PV (n= 15)	ET vs PV	ET vs RT	ET vs HA	PV vs RT	PV vs HA	RT vs HA
					p value	p value	p value	p value	p value	p value
CD69+ B cells (/μL), median (range)	1.0 (0.52-1.97)	1.03 (0.00-8.97)	4.36 (0.33-46.43)	1.51 (0.15-5.94)	<0.001	<0.001	<0.001	NS	NS	NS
CD80+ B cells (/μL), median (range)	3.11 (0.53-5.73)	4.36 (0.89-17.24)	8.99 (1.34-52.12)	3.77 (1.06-18.01)	0.006	0.010	0.001	NS	NS	0.047
CD86+ B cells (/μL), median (range)	5.04 (1.74-9.20)	6.78 (2.80-13.91)	17.65 (1.32-95.99)	5.99 (2.34-25.61)	<0.001	<0.001	<0.001	NS	NS	0.039

Abbreviations: ET, essential thrombocythemia; HA, healthy adults; n, number; NS, not significant; PV, polycythemia vera; RT, reactive thrombocytosis.

*Number=33.

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Table 12. Morpholino sequences for *mpl*, *epor* and *csf3r* knockdown.

Gene	Target	ZFIN identity	MO sequence
mpl	Intron1/ exon2 boundary of exon 2	ZDB-MRPHLNO-060421-1	5' - CAGAACTCTCACCCTTCAATTATAT - 3'
epor	Intron1/ exon2 boundary of exon 2	ZDB-MRPHLNO-080325-2	5' - AACTGGGCCACTGAACAATCAAATT - 3'
csf3r	ATG/5'UTR	ZDB-MRPHLNO-111213-1	5' - GAAGCACAAGCGAGACGGATGCCAT - 3'
Standard control	Human beta-globin intron mutation	NA	5' - CCTCTTACCTCAGTTACAATTTATA - 3'

Abbreviation: ZFIN, Zebrafish International Resource Center; NA, not applicable

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Table 13. Real-time quantitative polymerase chain reaction primer sequences for zebrafish hematopoietic lineage-specific, thrombopoiesis, cytokine and cytokine receptor genes.

ZF β-actin_qF	5'-ATTGCTGACAGGATGCAGAAG
ZF β-actin_qR	5'-GATGGTCCAGACTCATCGTACTC
ZF scl_qF	5'-CTATTAACCGTGGTTTTGCTGG
ZF scl_qR	5'-CCATCGTTGATTTCAACCTCAT
ZF lmo2_qF	5'-GGACGCAGGCTTTACTACAAAC
ZF lmo2_qR	5'-CCGGATCCTCTTTTCACAGGAA
ZF CD41_qF	5'-CTGAAGGCAGTAACGTCAAC
ZF CD41_qR	5'-TCCTTCTTCTGACCACACAC
ZF cmyb_qF	5'-AGGGAATCGTCTGCTCTTCCG
ZF cmyb_qR	5'-CAGCAGTTGAACACATGGGAAC
ZF runx1_qF	5'-CCGACAGAAGCCGGATGA
ZF runx1_qR	5'-TGGCACTTCGCCTCAACTG
ZF gata1_qF	5'-AAGATGGGACAGGCCACTAC
ZF gata1_qR	5'-TGCTGACAATCAGCCTCTTTT
ZF α-eHb_qF	5'-TGCTCTCTCCAGGATGTTGA
ZF α-eHb_qR	5'-TCACAGTCTTGCCGTGTTTC
ZF spi1_qF	5'-GGGCAGTTTTAACCAAAGATCA
ZF spi1_qR	5'-CCCAAGAGTGATCGTTCTGAC
ZF l-plastin exon9_qF	5'-CGAAAACCAGGACATCGACT
ZF l-plastin exon9_qR	5'-CCCCAGTGAGTTCATCCAGT
ZF rag1_qF	5'-TCTCCAGACGATTCCGTTATGA
ZF rag1_qR	5'-TGACCACCACAGTAAAGCCAGA
ZF rag2_qF	5'-CAAACAGCTCTCAGATTTCG
ZF rag2_qR	5'-CCAGGTCTAGTAAGGAGAAAC
ZF Lck_qF	5'-AACAACCGTCAAGTGGCTATC
ZF Lck_qR	5'-TGCGTGACCACAGCGAACAGT
ZF arhgef3_qF	5'-ACTGCAGCGTGGACGAGT
ZF arhgef3_qR	5'-CCCGAGACAGAGGCTTCAC
ZF emilin1a_qF	5'-AGGGCAAGACCTACTGGTGA
ZF emilin1a_qR	5'-CTCGAGTTGTCTAATCCTGTCG
ZF nbeal2_qF	5' GTGGACTTTCCACCATCTCG
ZF nbeal2_qR	5'-TGAGAGGGGCTGATACACG
ZF max_qF	5'-TTGCAAGGGGAAAAGCAA

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ZF max_qR	5'-TTCTGCCTCTTCAGGTCGTC
ZF csf3r_qF	5'-CAGCTGGAGTCTTTCGGAGA
ZF csf3r_qR	5'-CTTAACTGCACAATGAAGGTCAA
ZF mpl_qF	5'-ACCAACCTACCTTTAAGCAAAGAG
ZF mpl_qR	5'-CTGGAGAAGAAAGAGATGACTGC
ZF epor_qF	5'-TGTGGAGGACAGTCACATGG
ZF epor_qR	5'-CTGTAAACCACGCAAAACCA
ZF tpo_qF	5'-GCATCAGCATTCACAAAGCA
ZF tpo_qR	5'-CCCAAAGACAGCAGGATCTC
ZF epo_qF	5'-TGTTTTGCGAATGTTTCACG
ZF epo_qR	5'-TCCACTCCAGCACCATCAG
ZF mpo_qF	5'-GGGGCAGAAGAAGAAAGTCC
ZF mpo_qR	5'-CCCTTGCTAAACTCTCATCTCG
ZF β-eHb_qF	5'-AGGCTCTGGCAAGGTGTCTCA
ZF β-eHb_qR	5'-CATTGGGTTTCCCAGGAT
ZF fli_qF	5'-CAACGGATCCAGAGAGTCG
ZF fli_qR	5'-CCATGTAGCCAGTATAGTTCATCTG

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Table 14. Effects of *CALR* mutant mRNA injection on the expression of genes in zebrafish embryo.

Genes involved in lineage-specific hematopoiesis, thrombopoiesis, cytokines and cytokine receptors were examined based on real-time quantitative polymerase chain reaction of zebrafish embryos at 3 days post fertilization, with reference to that of *CALR* wild-type mRNA injection.

Category	Gene	*CALR*-wt*	*CALR*-del52 Mean ± SEM	*CALR*-ins5 Mean ± SEM	*CALR*-wt vs. *CALR*-del52 vs. *CALR*-ins5 *p* value[@]	*CALR*-wt vs. *CALR*-del52 *p* value[#]	*CALR*-wt vs. *CALR*-ins5 *p* value[#]	*CALR*-del52 vs. *CALR*-ins5 *p* value[#]
Hematopoietic stem cells	*cmyb*	1.00	0.73 ± 0.04	1.07 ± 0.02	< 0.001	0.02	NS	0.001
	runx1	1.00	0.78 ± 0.06	1.20 ± 0.06	0.003	NS	0.029	0.008
Hemangioblast	*scl*	1.00	0.75 ± 0.08	0.91 ± 0.05	0.043	NS	NS	NS
	lmo2	1.00	0.85 ± 0.06	0.99 ± 0.09	NS	NS	NS	NS
Erythropoiesis	*gata1*	1.00	0.87 ± 0.01	0.89 ± 0.02	0.002	0.012	0.036	NS
	α eHb	1.00	0.82 ± 0.11	0.89 ± 0.11	NS	NS	NS	NS
	β eHb	1.00	0.73 ± 0.12	0.96 ± 0.22	NS	NS	NS	NS
Vasculature	*fli1*	1.00	0.76 ± 0.06	0.84 ± 0.08	NS	0.017	NS	NS
Early myelomonocytic lineage	*spi1b*	1.00	1.13 ± 0.10	1.01 ± 0.10	NS	NS	NS	NS
Late myelomonocytic lineage	*L-plastin*	1.00	0.99 ± 0.17	0.94 ± 0.09	NS	NS	NS	NS
Lymphoid lineage	*rag1*	1.00	0.56 ± 0.07	1.01 ± 0.12	0.012	0.003	NS	0.033
	rag2	1.00	0.96 ± 0.07	0.84 ± 0.03	NS	NS	0.01	NS
	lck	1.00	0.50 ± 0.06	1.08 ± 0.13	0.006	0.015	NS	0.017

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Thrombopoiesis	*arhgef3*	1.00	0.80 ± 0.09	0.90 ± 0.07	NS	NS	NS	NS
	emilin1a	1.00	0.90 ± 0.03	0.91 ± 0.04	NS	0.036	NS	NS
	nbeal2	1.00	0.64 ± 0.10	1.08 ± 0.06	0.007	NS	NS	0.019
	max	1.00	0.88 ± 0.03	0.87 ± 0.06	NS	0.021	NS	NS
Cytokines and cytokine receptors	*tpo*	1.00	0.88 ± 0.05	1.12 ± 0.05	0.016	NS	NS	0.026
	mpl	1.00	0.57 ± 0.05	0.52 ± 0.06	0.001	0.014	0.002	NS
	epo	1.00	0.99 ± 0.10	1.12 ± 0.23	NS	NS	NS	NS
	epor	1.00	0.80 ± 0.10	0.96 ± 0.09	NS	NS	NS	NS
	mpo	1.00	0.85 ± 0.09	0.84 ± 0.06	NS	NS	NS	NS
	csf3r	1.00	0.62 ± 0.04	1.15 ± 0.14	0.01	0.001	NS	0.021

Data are from triplicate results. Abbreviation: NS, not significant; SEM, standard error of the mean; wt, wild-type.

* Data of *CALR*-wt was arbitrarily set to 1.00 in all cases. @ ANOVA test. # Student t-test.

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Appendices

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Rapid and sensitive detection of *CALR* exon 9 mutations using high-resolution melting analysis

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ABSTRACT

Background: Somatic *CALR* exon 9 mutations have recently been identified in patients with *JAK2*/*MPL*-unmutated myeloproliferative neoplasm, and have become an important clonal marker for the diagnosis of essential thrombocythemia (ET) and primary myelofibrosis. In the present study, we sought to use high-resolution melting analysis (HRMA) as a screening method for the detection of *CALR* mutations.
Methods: 32 *JAK2*/*MPL*-unmutated ET patients were retrospectively enrolled and 8 healthy adults were used as wild-type control. *CALR* exon 9 mutation was independently screened by HRMA with the CFX Connect real-time system and Sanger sequencing. TA-cloning was used to detect *CALR* exon 9 mutations in patients suspected to have low mutant allele burden.
Results: The maximal sensitivity of HRMA in identifying both *CALR* type 1 and type 2 mutants from patients' genomic DNA was 2.5%. Twenty-two samples were found to have distinct melting curves from wild-type. The presence of *CALR* mutations in 16 of these 22 samples was confirmed by Sanger sequencing, while the other 6 samples were wild-type by sequencing. After TA-cloning, *CALR* mutations were detected in 5 of 6 patients from 1 (6%) of 16 clones to 1 (2%) of 50 clones. Therefore, HRMA identified *CALR* mutations in 21 (65.6%) of 32 ET patients compared to 16 (50%) patients by Sanger sequencing, with a false positive rate of 3% and no false negative.
Conclusion: The HRMA developed in our system is a rapid and sensitive technique for the detection of *CALR* exon 9 mutations.

1. Introduction

The classic *BCL-ABL1*-negative chronic myeloproliferative neoplasm (MPN) is a clonal hematopoietic stem cell disorder and includes polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) [1]. ET is characterized by increased number of mature megakaryocytes in the bone marrow and sustained thrombocytosis in the peripheral blood. Although most ET patients have a normal life expectancy, some may encounter serious events such as thrombotic and hemorrhagic complications and leukemic transformation during their clinical course [2]. The *JAK2* V617F mutation was discovered in 2005, and has provided important diagnostic, therapeutic, and prognostic implications in MPNs. The frequency of *JAK2* V617F mutation is over 90% in polycythemia vera (PV), and about 60% in ET and PMF [3–5]. Besides, *MPL* mutations are identified in about 4–5% of *JAK2*-unmutated ET and PMF patients [6]. Following these seminal reports, other somatic mutations such as *LNK*, *TET2* and *DNMT3A* have also been detected in patients with MPN [7]. However, they are not mutually exclusive with *JAK2* and *MPL* mutations and also not specific to patients with MPN [7,8]. Despite many somatic mutations have been identified in patients with ET, clonal molecular marker is still not identified in ~40% of ET patients.

Recently, a high frequency (around 49–88%) of somatic calreticulin (*CALR*) mutations was identified in patients with *JAK2*/*MPL*-unmutated

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patients with ET and PMF [9–13]. Most *CALR* mutations in MPNs are heterozygous indels in exon 9 causing one base pair reading frameshift and resulted in the generation of a novel CALR protein C-terminus. The majority of the *CALR* exon 9 mutants were a 52 bp deletion of nt1172 to nt1223 (L367fs*46, type 1 mutation) and a 5 bp insertion of TTGTC (K385fs*47, type 2 mutation). Rarely, *CALR* exon 9 point mutations have been reported in follicular lymphoma (E403X and E405Q) [14], PMF (E379D) [9] and chronic neutrophilic leukemia (E398D) [15]. Importantly, *CALR* mutations are not only mutually exclusive with *JAK2* and *MPL* mutations, but they are also infrequently detected in other myeloid neoplasms such as myelodysplastic syndrome, chronic myelomonocytic leukemia and atypical chronic myeloid leukemia [9,10]. These findings indicate that *CALR* mutations are quite specific for ET and PMF. Based on these discoveries, *CALR* mutations have been proposed to be included in the World Health Organization classification system for the molecular diagnosis of ET and PMF [16]. Therefore, the detection of *CALR* mutations with reliable and cost-effective methods in patients suspected to have ET or PMF is very important.

Several methods have been used to detect *CALR* exon 9 mutations including direct DNA sequencing, PCR followed by fragment analysis and immunostaining [9,10,17,18]. Although fragment analysis has a relatively high sensitivity for *CALR* mutation detection, it cannot discriminate point mutation from wild-type sequence. High-resolution melting analysis (HRMA) is a closed-tube and PCR-based technique for the detection of gene polymorphism and mutations by measuring changes in the melting of a DNA duplex [19]. HRMA is a well-established method for the detection of or prescreening for mutations both in a routine molecular laboratory and in a research setting. For example, HRMA has shown high sensitivity and specificity for the detection of *JAK2* V617F and *JAK2* exon 12 mutations in patients with MPN [20–22]. Recently, the feasibility of using HRMA for the detection of *CALR* mutations in ET and persistent thrombocytosis has been reported using the LightCycler 480 platform (Roche Diagnostics) [23]. In this study, we sought to assess HRMA for rapid and sensitive detection of *CALR* exon 9 mutations in ET using the CFX Connect real-time system (Bio-Rad Laboratories, Hercules, CA, USA).

2. Materials and methods

2.1. Patient samples and DNA extraction

The screening for mutations in patients with hematologic neoplasms was approved by the Institutional Review Board of Mackay Memorial Hospital. 32 adult patients with *JAK2*/*MPL*-unmutated ET were retrospectively enrolled based on the 2008 World Health Organization classification and 8 healthy adults were used as wild-type control. Written informed consent was obtained from all patients. Patient genomic DNA was derived from bone marrow or peripheral blood by using EasyPure Genomic DNA Spin Kit (Bioman, Taipei, Taiwan).

2.2. Assay design and the HRMA technique

Oligonucleotide primers were designed by Primer3 software to flank all *CALR* exon 9 variants reported in MPN. The primers were used to amplify a 134 bp amplicon [GenBank: NM_004343]: forward 5′-GAAA CAAATGAAGGACAAACAGG-3′, and reverse 5′-CCTCATCCTCCTCATCCT CA-3′. PCR was performed in a 20 μl reaction volume containing precision melt supermix (Bio-Rad Laboratories, Hercules, CA, USA), 100 nM of each primer, and 25 ng genomic DNA. The 134 bp amplicon was run according to the following conditions: an initial denaturation step of 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s. After completion of amplification, DNA was heated at 95 °C for 30 s, kept at 60 °C for 1 min, and then melted from 70 to 95 °C (increment 0.2 °C, dwell time 10 s). The results were analyzed using the Bio-Rad Precision Melt Analysis software. Melting profiles were normalized, grouped and displayed as fluorescence-versus-temperature plots or subtractive difference plots (−df/dt vs T). All samples with distinguished melting curves from wild-type were confirmed by duplicate study. Both type 1 and type 2 *CALR* exon 9 mutant cDNA were obtained by direct DNA synthesis, and *CALR* wild-type cDNA was cloned from patient sample.

2.3. Sanger sequencing

All patients were also independently screened for *CALR* exon 9 mutations spanning codons 352–417 by Sanger sequencing on an ABI 3730 sequencer based on previously described method [10]. All identified sequence variants were subjected to repeated bi-directional sequencing for confirmation. Mutations were identified using DNA Dynamo sequence analysis software (Blue Tractor Software Ltd, Conwy, UK). All patients had been screened for *JAK2* V617F and *MPL* exon 10 mutations as previously described [5,7].

2.4. Sensitivity of HRMA in detecting CALR type 1 and type 2 mutations

To study the sensitivity of the methodology, we serially diluted two plasmids carrying *CALR* type 1 and type 2 mutations with wild-type plasmid DNA in different concentrations (100% mutant, 50% mutant, 25% mutant, 10% mutant, 7.5% mutant, 5% mutant, 2.5% mutant, 1.25% mutant, and 0% mutant). The sensitivity tests were carried out in triplicate samples. The sensitivity of HRMA was validated by serially diluting two patient samples carrying *CALR* type 1 and type 2 mutations with control DNA. Based on the relative peak areas of the mutant and wild-type PCR products, the mutant allele burden of these 2 patient samples was estimated to be ~50%. *CALR* type 1 and type 2 patients' DNA were also serially diluted by wild-type DNA in different concentrations (50% mutant, 25% mutant, 12.5% mutant, 5% mutant, 3.75% mutant, 2.5% mutant, 1.25% mutant, 0.625% mutant, and 0% mutant). We did not evaluate the sensitivity of HRMA for other types of *CALR* mutations because they are less frequently detected.

2.5. TA-cloning

The PCR products of *CALR* exon 9 of 6 ET patients suspected to have a low allele burden mutant were purified using a EasyPure High Pure PCR clean-up Kit (Bioman, Taipei, Taiwan) and cloned into a pGEMT-easy vector (Promega, Madison, CA, USA). We obtained at least 16 clones in each individual. The PCR product of each clone was checked on a 2% agarose gel by electrophoresis for the presence of mutant band. All selected clones were then sent for Sanger sequencing regardless the presence or absence of mutant band.

3. Results

3.1. Sensitivity of HRMA in identifying the CALR type 1 and type 2 mutants

We first evaluated the sensitivity of HRMA in detecting the *CALR* type 1 and type 2 mutant plasmid DNA with different concentrations of mutant DNA serially diluted by wild-type plasmid DNA. HRMA could distinguish *CALR* type 1 and type 2 mutants with the maximal sensitivity of 2.5% and 1.25%, respectively (Fig. 1A and C). Whereas, the maximal sensitivity of Sanger sequencing for the detection of both *CALR* type 1 and type 2 mutants was at least 10% or higher (Fig. 1B and D). Besides, the maximal sensitivity of HRMA was validated with 2 patient samples and was found to be 2.5% for both *CALR* type 1 and type 2 mutants (Fig. 2A and B).

3.2. Detection of CALR exon 9 mutations in JAK2/MPL-unmutated ET patients

In this cohort of 32 ET patients, the normalized melting curves of 22 (68.8%) patient samples clearly showed a distinctive difference from

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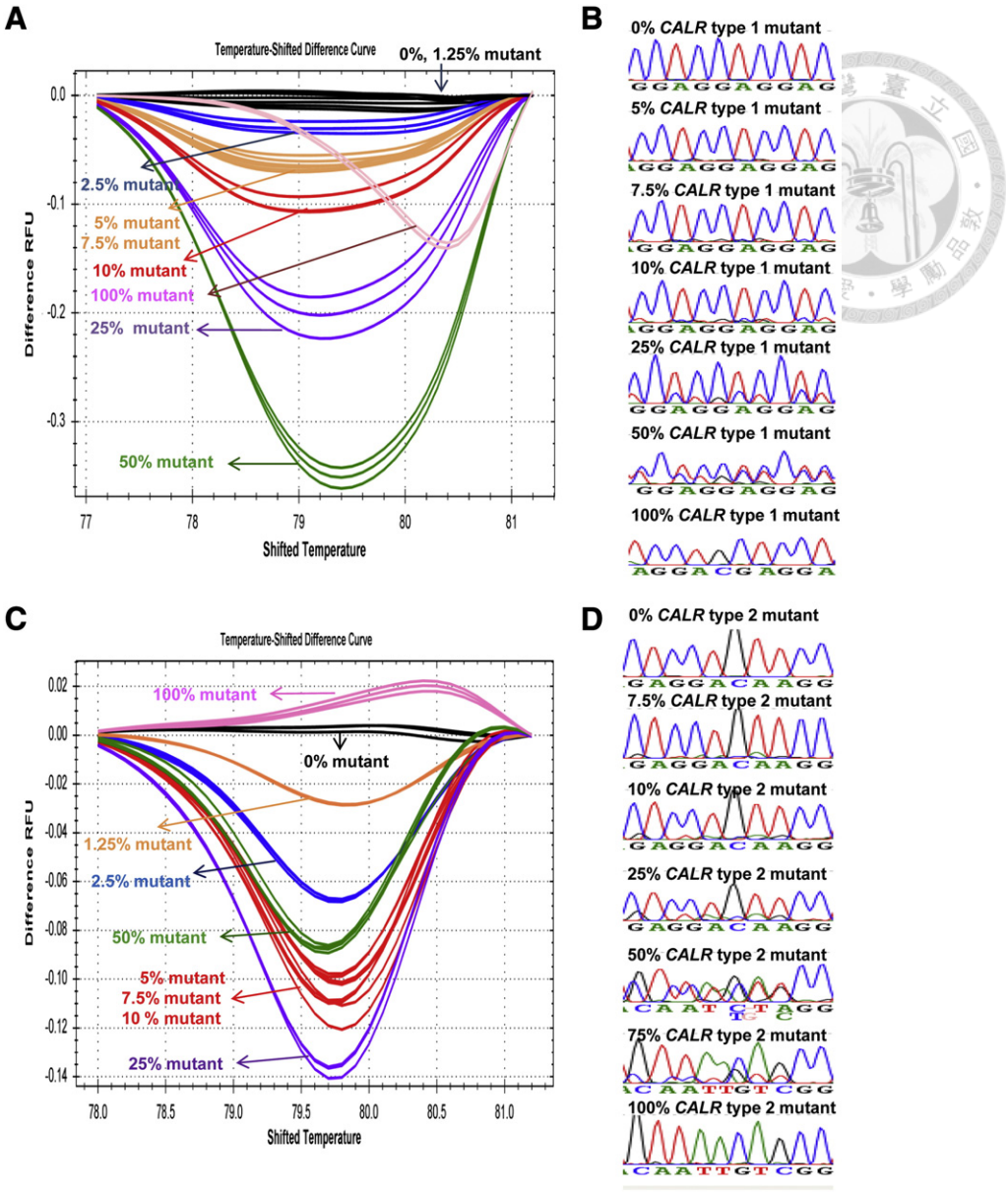

Fig. 1. Results of the dilution series of *CALR* mutants from plasmid DNA in a background of plasmid *CALR* wild-type DNA detected by HRMA in triplicate samples. A and B. Normalized and temperature-shifted difference curves and corresponding Sanger sequencing of plasmid *CALR* type 1 mutant. C and D. Normalized and temperature-shifted difference curves and corresponding Sanger sequencing of plasmid *CALR* type 2 mutant. HRMA can detect *CALR* type 1 and type 2 mutants with the maximal sensitivity of 2.5% and 1.25%, respectively, compared to the 10% or higher sensitivity obtained by Sanger sequencing. Each dilution percentage is indicated by corresponding arrow.

that of wild-type group, and the representative normalized melting curves from 6 of the 22 patient samples were shown in Fig. 3A. When the data were represented in difference plots, the individual nature of the mutant melting curves became more apparent as illustrated in Fig. 3B. To determine the concordance between HRMA and Sanger sequencing, all 32 ET patients were also screening for *CALR* exon 9 mutations by Sanger sequencing. In the 22 ET patients with distinctive melting curves, Sanger sequencing could only detect *CALR* exon 9 mutations in 16 patients. All the remaining 6 patients with distinctive melting curves as shown on Fig. 4A had wild-type *CALR* exon 9 sequences by Sanger sequencing. The other 10 patients were determined to have wild-type *CALR* by both HRMA and Sanger sequencing. In the 6 patients with discordant results between HRMA and Sanger sequencing, we then performed TA-cloning to determine whether these 6 patients had low allele burden *CALR* mutations not detected by Sanger sequencing (Fig. 4B and C).

After TA-cloning, *CALR* type 2 mutations were detected in 5 of 6 patients from 1 (6%) of 16 clones to 1 (2%) of 50 clones, and only 1 clone from each patient was tested positive for the *CALR* mutation (Fig. 4). We did not identify *CALR* mutation in the last patient after screening for 100 clones. Therefore, HRMA identified *CALR* mutations in 21 (65.6%) of 32 ET patients compared to 16 (50%) by Sanger sequencing. The possible 3% false positive rate is low and no false negative was detected in our HRMA system.

In this study, 21 *JAK2*/*MPL*-unmutated ET patients were found to harbor 6 types of *CALR* exon 9 mutations: 5 type 1 (p.L367fs*46), 11 type 2 (p.K385fs*47), 1 type 3 (p.L367fs*48), 2 type 34 (p.K385fs*47), and 2 other types (p.L367fs*43 and p.E369fs*50). All *CALR* exon 9 mutations are indels causing +1 bp reading frameshift, with type 2 (11/21, 52.4%) being the most prevalent mutational type. In these 21 patients with *CALR* mutations, the number of female patients was slightly higher than male patients (57% vs 43%) (Table 1).

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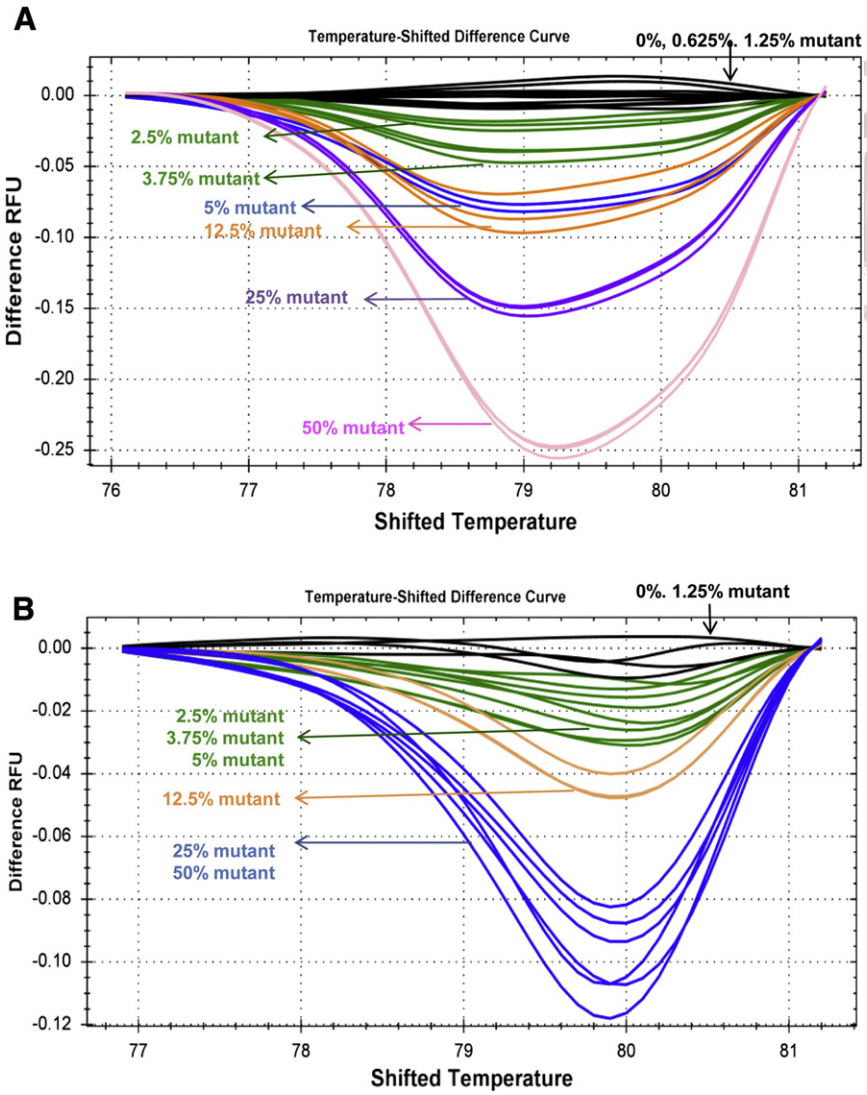

Fig. 2. Results of the dilution series of *CALR* mutants from patients' genomic DNA in a background of wild-type DNA from healthy control detected by HRMA in triplicate samples. A and B. Normalized temperature-shifted difference curves of *CALR* type 1 and type 2 mutants from patients' genomic DNA, respectively. The maximal sensitivity of HRMA to detect both *CALR* type 1 and type 2 mutants from patients' genomic DNA was 2.5%. Each dilution percentage is indicated by corresponding arrow.

4. Discussion

The identification of *CALR* mutations is important in the molecular diagnosis of MPN especially in *JAK2*/*MPL*-unmutated patients. In addition, *CALR* mutational status was found to be one of the most significant risk factors for survival in PMF [24]. Sanger sequencing has been used to detect *CALR* exon 9 mutations in many studies, but it is rarely sensitive below a 10% mutant allele burden as illustrated in Fig. 1B. and 1D. Fragment analysis assay was also used and the sensitivity was estimated to be 5% or less for *CALR* exon 9 mutations [18]. Although fragment analysis assay is able to detect most indel mutations in *CALR*, it cannot discriminate point mutation from wild-type sequence. Recently, Bilbao-Sieyro et al. showed that HRMA is a feasible method for the detection of *CALR* mutations using the LightCycler 480 platform [23]. The amplicon size of their primer sequences was 265 bp, and the limit of detection for *CALR* type 2 (K385fs*47) mutant was of 3%. However, the ideal amplicon length for HRMA is usually less than 250 bp. In this study, the HRMA primers with an amplicon size of 134 bp were designed and are capable of detecting common *CALR* exon 9 mutations in myeloid neoplasms with satisfactory sensitivity.

Based on the dilution studies using patients' genomic DNA, the maximal sensitivity of our HRMA using CFX Connect real-time system for both *CALR* type 1 (L367fs*46) and type 2 (K385fs*47) mutants was of 2.5%. In addition to 16 *CALR* mutated samples that could be detected by both HRMA and Sanger sequencing, we were able to identify another 5 patients with low *CALR* mutant allele burden only by HRMA. In this situation, we used TA-cloning followed by Sanger sequencing to confirm the mutation suspected. Alternatively, fragmented analysis may be used for mutation detection because it also has a better sensitivity than Sanger sequencing. We were not able to detect *CALR* mutation in 1 of the 6 patients after screening for 100 clones. It is likely that this patient might still have low allele burden *CALR* mutation which, by chance was missed by random selection of clones (Fig. 4A). However, we counted the result as a possible 3% false positive rate to avoid overestimation of our data. Importantly, no false negative was found in our HRMA system and this is critical in regard to its role as a screening tool.

HRMA developed in this study can be utilized for rapid, sensitive and reliable detection of *CALR* mutations. Although a total of 6 SNPs (rs151032910, rs201971744, rs143880510, rs370029737,

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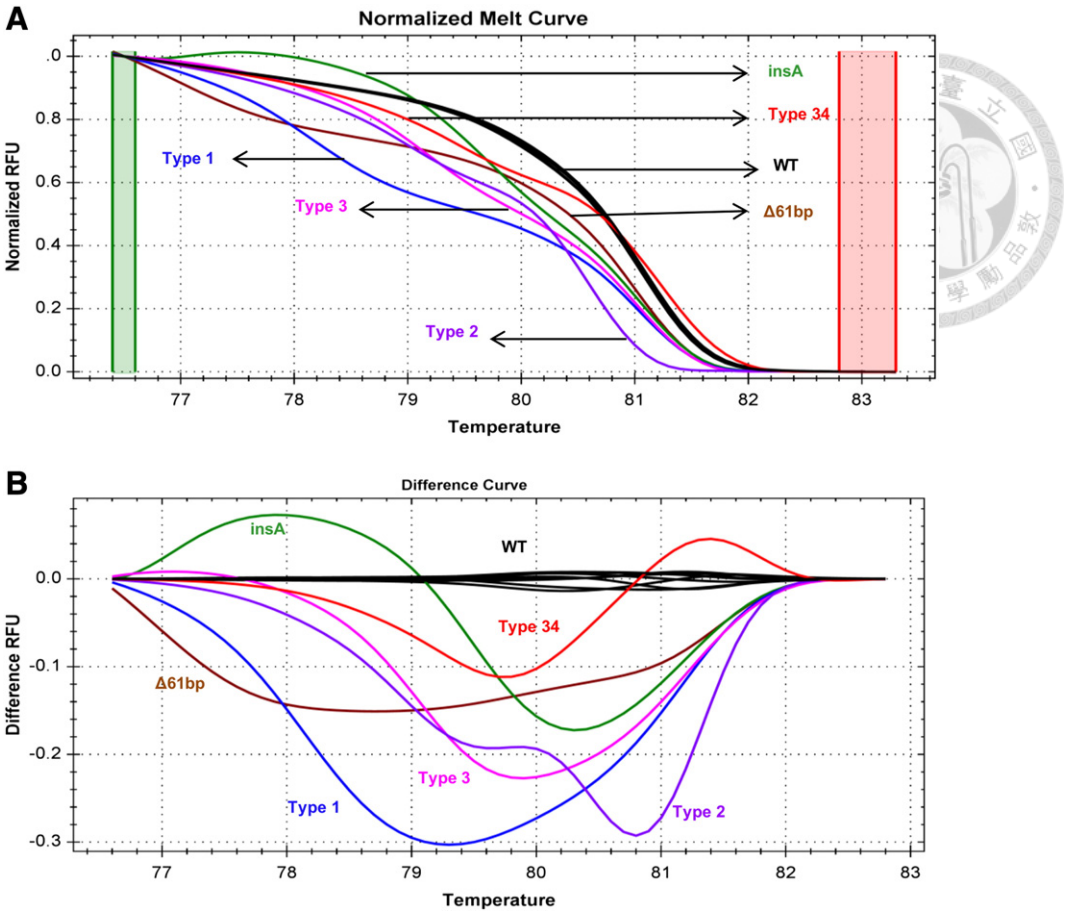

Fig. 3. Representative normalized melting curves and difference curves from the 22 patient samples. A and B. Representative normalized melting curves and difference curves from 6 patient samples each with a specific type of *CALR* exon 9 mutations detected by HRMA in this study. The individual nature of the mutant melting curves became more apparent when the data were represented in normalized difference plots. *CALR* mutational types in Fig. 3A are indicated by arrows and labeled on the curves in Fig. 3B.

rs374121178 and rs150264068) are reported in the region covered by our amplicon, the minor allele frequency of 3 of them is reported to be less than 0.01%. Therefore, the influence of these 6 SNPs to our HRMA system will likely be very small. Nevertheless, one limitation to this HRMA methodology is that it will not be able to identify the 2 *CALR* exon 9 point mutations reported in follicular lymphoma (E403X and E405Q) because they are not covered by our 134 bp amplicon. The frequency and significance of these 2 *CALR* point mutations in follicular lymphoma are currently not yet clear. Therefore, our HRMA methodology is suitable for use in patients suspected to have myeloid neoplasms especially ET and PMF. By using HRMA, we detected a total of 6 different types of *CALR* mutations in ET patients. All the *CALR* mutations detected in this study resulted in a +1 bp shifting in the reading frame and generated the characteristic novel peptide sequence in the C-terminus. All the *CALR* exon 9 indel mutations likely contribute to a similar, yet not clearly understood molecular pathogenesis in ET and PMF. In addition, the number of female patients was slightly higher than male patients (57% vs 43%) in our 21 ET patients with *CALR* mutations, and this has also been observed in other study [25].

HRMA, a close-tube method, is not only rapid as it is conducted immediately after PCR amplification, but is also cost effective because it can reduce the use of Sanger sequencing. By using HRMA, a medium-throughput screening for *CALR* mutations is also possible. Based on these advantages, our results clearly illustrated that HRMA is a more suitable and sensitive method over Sanger sequencing for the screening of *CALR* mutations in both clinical and research settings. Nevertheless, in samples with distinct melting curves, complimentary Sanger sequencing is still required to determine their exact genotypes because the pattern of melting curves does not correlate with specific *CALR* mutational types.

In conclusion, we have shown that HRMA is a rapid, sensitive, reliable and cost effective method for the detection of *CALR* mutations. Because *CALR* mutations have important diagnostic and prognostic significance in ET and PMF, HRMA can be a useful screening method for the identification of common *CALR* mutations.

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Conflict of interest

None.

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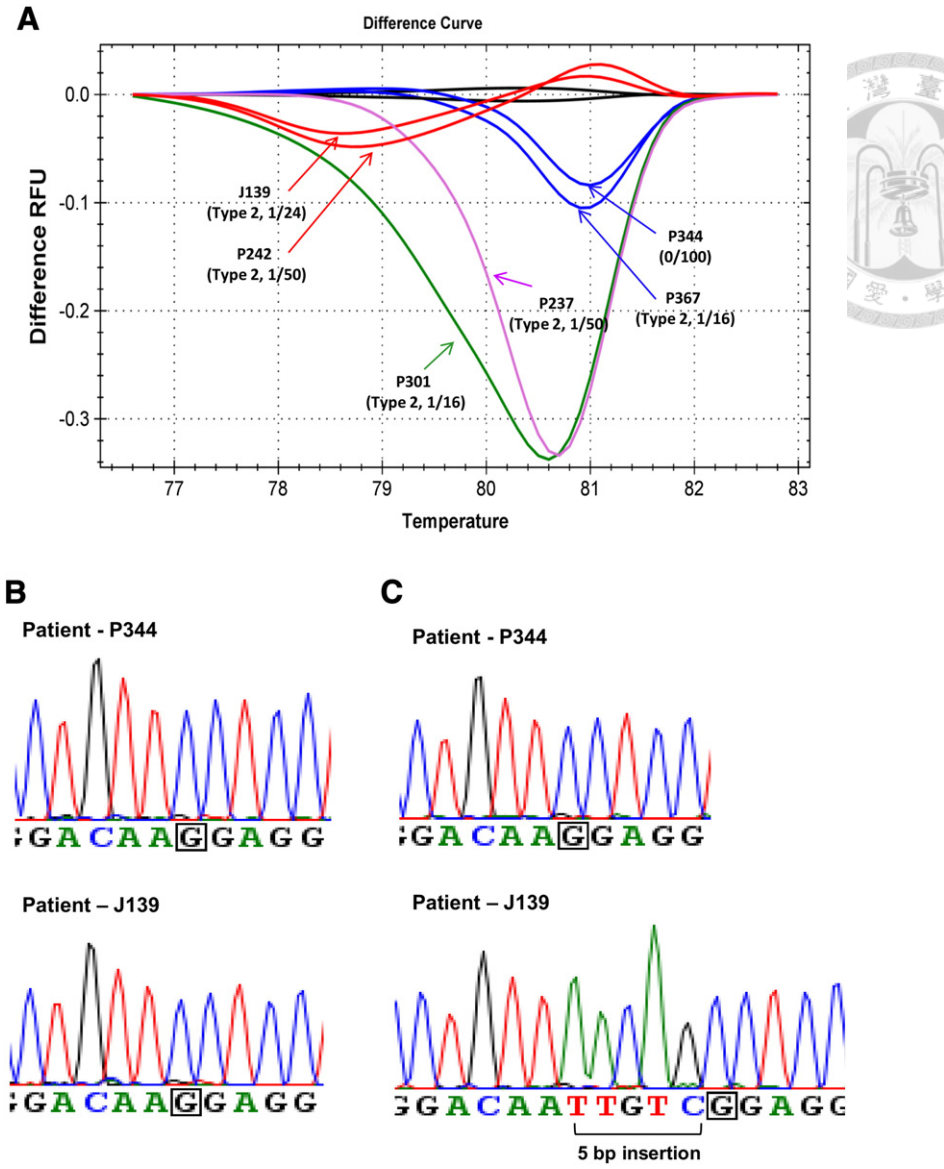

Fig. 4. The comparison of HRMA with Sanger sequencing in 6 patient samples with discordant results between HRMA and Sanger sequencing. A. Distinctive normalized difference curves from the 6 patient samples. After TA-cloning, *CALR* type 2 mutations were detected in 5 of 6 patients. Only 1 clone from each patient was tested positive for the *CALR* mutation indicating that they all had low mutant allele burden. Patient number and the total number of clones selected for screening in each patient are indicated. B. Representative Sanger sequencing from two patients P344 and J139 as indicated. All 6 patient samples had wild-type *CALR* exon 9 sequences by Sanger sequencing. C. Representative Sanger sequencing after TA-cloning showing wild-type *CALR* sequence from patient P344 and *CALR* type 2 mutation from patient J139.

Table 1
Clinical and laboratory characteristics at diagnosis or referral in 21 essential thrombocythemia patients with 6 types of *CALR* mutations.

		No. (%)	Male/female gender, n (%)	Age at diagnosis (y), median (range)	Hemoglobin (g/dL), median (range)	WBC ($\times 10^3$/μL), median (range)	Platelets ($\times 10^9$/L), median (range)
CALR mutations		21 (63.6)	9/12 (43/57)	47 (22–76)	12.6 (8.5–15.2)	9.2 (4.9–27.9)	1351 (642–2834)
Type 1	p.L367fs*46	5 (23.8)	2/3 (40/60)	35 (25–63)	12.6 (11.7–14.6)	8.1 (7.1–14.4)	1351 (1095–1745)
Type 2	p.K385fs*47	11 (52.4)	4/7 (36/64)	47 (23–58)	11.6 (8.5–15.2)	10.6 (4.9–27.9)	1369 (642–2834)
Type 3	p.L367fs*48	1 (4.8)	0/1 (0/100)	22	12.8	9.2	1477
Type 34	p.K385fs*47	2 (9.5)	2/0 (100/0)	53 (30–76)	15.0 (14.9–15)	10.9 (9.3–12.4)	1362.5 (1072–1653)
Other	p.L367fs*43	1 (4.8)	1/0 (100/0)	61	9.3	7.9	1045
Other	p.E369fs*50	1 (4.8)	0/1 (0/100)	55	9.4	7.1	1235

Abbreviations: n, number; WBC, white blood cell; y, year.

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LETTER TO THE EDITOR

Frequent *CALR* exon 9 alterations in *JAK2* V617F-mutated essential thrombocythemia detected by high-resolution melting analysis

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Essential thrombocythemia (ET) is a clonal hematopoietic stem cell neoplasm and one of the classic *BCL-ABL1*-negative chronic myeloproliferative neoplasm (MPN), which also includes polycythemia vera and primary myelofibrosis (PMF).[1] Recently, two seminal studies discovered a high frequency of somatic calreticulin (*CALR*) mutations in patients with *JAK2*/*MPL*-unmutated ET and PMF.[2,3] The pattern of most *CALR* mutations in MPN is heterozygous indels in exon 9 causing one-base pair (bp) reading frameshift. *CALR* mutations have been shown to have important diagnostic and prognostic significance in ET and PMF patients,[2–4] and will likely be incorporated into the World Health Organization (WHO) diagnostic criteria for MPN. *In vitro* studies on the molecular pathogenesis of *CALR* mutations in MPN have shown controversial results in regard to the involvement and/or activation of the JAK/STAT signaling pathway,[2,3] and the exact pathogenesis of *CALR* mutations is not yet completely understood at the present time.[5]

Several techniques such as Sanger sequencing and polymerase chain reaction (PCR) followed by fragment analysis have been used to detect *CALR* mutations.[2,3,6,7] High-resolution melting analysis (HRMA) is a well-established method for the screening of mutations, and we have developed a rapid and sensitive HRMA for the detection of *CALR* exon 9 mutations.[8] In this study, we sought to screen a cohort of 92 Taiwanese ET patients for *CALR* exon 9 mutations with HRMA and Sanger sequencing independently, and to determine the clinical and molecular correlates.

The institutional review board of Mackay Memorial Hospital has approved the screening for mutations. All patients provided written informed consent. Diagnosis of ET was established on the basis of the 2008 WHO criteria. The clinical and laboratory characteristics at the time of diagnosis or referral were collected. Genomic DNAs derived from the bone marrow, peripheral blood and peripheral blood granulocytes and/or mononuclear cells were used for mutation screening. *CALR* mutations were screened by Sanger sequencing on an ABI 3730 sequencer as preciously described.[3] *CALR* exon 9 mutations were independently screened by HRMA using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) as previously described with a maximal sensitivity of 2.5% for both *CALR* type 1 and type 2 mutants.[8] Briefly, a pair of oligonucleotide primers were used to amplify a 134-bp amplicon (GenBank: NM_004343), which flanked all *CALR* exon 9 variants reported in MPN. All samples with distinguished melting curves from wild type were confirmed by duplicate studies. Peripheral blood samples from 78 healthy adults were also used to validate the specificity of our HRMA. *JAK2* V617F mutation was screened using allele-specific PCR with an analytic sensitivity of 5% and *MPL* exon 10 mutation using Sanger sequencing as previously described.[9,10] TA-cloning was performed using the pGEM-T easy vector system (Promega, Madison, CA, USA) as previously described.[8] At least 10 clones in each individual were randomly selected for the screening of *CALR* exon 9 alterations by Sanger sequencing. All novel single-nucleotide variant that was only detected once was treated as artifact and were excluded. The SPSS Statistics software (IBM, New York, NY, USA) was used for all calculations. *P*-values < 0.05 were considered significant.

Among the 92 ET patients (median age 53 years; 58% females), 59 (64%) patients harbored *JAK2* V617F mutation and one (1%)

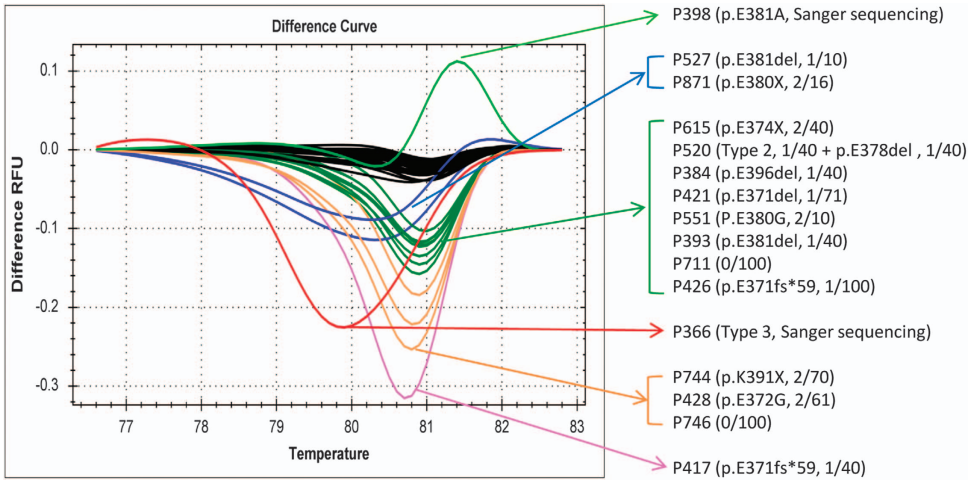

Figure 1. Normalized difference curves of 16 *JAK2* V617F-mutated essential thrombocythemia patient samples showing distinct melting curves from *CALR* exon 9 wild-type samples (black color). Corresponding patient number, genotype and number of positive clone in TA-cloning of each curve is indicated by arrow.

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patient harbored *MPL* W515K mutation. Thirty-two *JAK2*/*MPL*-unmutated ET patients were utilized for the development of our HRMA platform.[8] Briefly, 22 (68.8%) samples were found to have distinct melting curves from wild type. In 16 of these 22 samples, Sanger sequencing confirmed the presence of six types of *CALR* mutations: five type 1 (p.L367fs*46), six type 2 (p.K385fs*47), one type 3 (p.L367fs*48), two type 34 (p.K385fs*47) and two other types (p.L367fs*43 and p.E369fs*50). The other six samples were wild type by sequencing, and *CALR* type 2 mutations were detected in five of six patients after TA-cloning, indicating the presence of low-allele-burden *CALR* mutants in them. By using our HRMA platform, we identified *CALR* mutations in 21 (22.8% overall and 65.6% in *JAK2*/*MPL*-unmutated) ET patients and this frequency is comparable to other studies.[2–4] Eleven (12%) ET patients were negative for *JAK2*, *CALR* and *MPL* mutations. In the 78 samples from healthy adults, two were found with HRMA to have distinct melting curves from wild type. One single-nucleotide polymorphism (rs143880510) and one wild type were found after Sanger sequencing in these two samples. Therefore, our HRMA system has a low false-positive rate of 1.3%.

After screening the 59 *JAK2* V617F-mutated ET patients for *CALR* alterations by HRMA, 16 (27.1%) samples were found to have distinct melting curves from wild type (Figure 1). In 2 of these 16 samples, one *CALR* type 3 mutation (p.L367fs*48) and one single-nucleotide polymorphism (rs143880510) were detected using Sanger sequencing. All the other 14 samples were wild type by sequencing. Interestingly, we detected a high frequency of *CALR* exon 9 alterations in 12 (85.7%) of these 14 patients after TA-cloning (Table 1A). Three patients harbored the classic *CALR* indel mutations: one each of type 2 p.K385fs*47, p.E370fs*60 and p.E371fs*59. Hence, four (6.8%) ET patients had classic *CALR* indel and *JAK2* V617F co-mutations in this cohort. Five patients (8.5%) including the aforementioned patient (P520) with type 2 *CALR* mutation harbored four types of 3-bp inframe deletions all resulted in the deletion of a single amino acid of glutamic acid: two p.E381del and one each of p.E371del, p.E378del and p.E396del (Supplementary Figure 1). Another five patients (8.5%) harbored five types of point mutations: one each of p.E374X, p.E380X, p.K391X, p.E372G and p.E380G. The latter p.E380G has been reported as an single-nucleotide polymorphism but might be a low-allele-burden somatic mutation in this patient because it was only detected after TA-cloning and not by Sanger sequencing on patient's genomic DNA. The remaining two patients were found to have wild-type *CALR* exon 9 after screening for 100 independent clones, and were counted as *CALR* wild type. Overall, various *CALR* exon 9 alterations were detected in 13 (22%) of 59 *JAK2* V617F-mutated ET patients.

We then examined the clinical and molecular correlates in 91 ET patients excluding the one *MPL*-mutated patient (Table 1B). *JAK2*-mutated ET patients with concomitant *CALR* alterations were associated with oldest age ($P = 0.025$), higher thrombotic events after diagnosis ($P = 0.048$), higher major arterial thrombotic events after diagnosis ($P = 0.022$) and more patients being in the high-risk group for thrombohemorrhagic complications ($P = 0.023$). Consistent with previous reports, *CALR* mutations were associated with younger age ($P = 0.025$), higher platelet count ($P < 0.001$) and lower hemoglobin level ($P = 0.016$). *JAK2* V617F mutation was associated with leukocytosis ($P = 0.046$).

After the discovery of *CALR* mutations, it has been proposed to be mutually exclusive with *JAK2* and *MPL* mutations in MPN. However, *CALR* and *JAK2* V617F co-mutations have been reported in a few MPN cases across different ethnic groups and the frequency is usually below 1%.[7,11–13] In contrast to these reports, we detected a higher frequency of 6.8% *CALR* indel and *JAK2* co-mutations in ET patients. Interestingly, three of these *CALR* mutations were low-allele-burden mutants not detected using Sanger sequencing. Nevertheless, the use of a sensitive HRMA technique has enabled us to detect these low-allele-

Table 1A. *CALR* exon 9 alterations and single-nucleotide polymorphism in 14 *JAK2* V617F-mutated essential thrombocythemia patients detected using high-resolution melting analysis

Patient	*CALR mutation*	*Nucleotide change*	*Protein change*	*Amino acid*	*Protein sequence*[a]	*CALR SEQ*	*CALR-TA clone number*[b]	*JAK2 V617F allele burden*[c]
NA	Wild type	NA	NA	417	QRLKEEEEDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	NA	NA
P520	Type 2	c.1154_1155insTTGTC	p.K385fs*47	430	QRLKEEEEDKKRKEEEEAEDNCRRMMKTKMRMRRMRRTRRKMRRKMSPARRPRTSCREACLQGWTEA	Wild type	1/40	7%
P366	Type 3	c.1095_1140del (Δ46)	p.L367fs*48	413	QRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	Heterozygous	NA	83%
P426	New	c.1108delG (Δ1)	p.E370fs*60	428	QRLKERKKTRNAKRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	Wild type	1/100	25%
P417	New	c.1111delG (Δ1)	p.E371fs*59	428	QRLKEEKKTRNAKRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	Wild type	1/40	20%
P421	New	c.1110_1112delGGA (Δ3)	p.E371del	416	QRLKEI—EDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	1/71	71%
P520	New	c.1132_1134delGAG (Δ3)	p.E378del	416	QRLKEEEEDKKRK—EEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	1/40	7%
P393	New	c.1142_1144delAGG (Δ3)	p.E381del	416	QRLKEEEEDKKRKEEI—AEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	1/40	5%
P527	New	c.1142_1144delAGG (Δ3)	p.E381del	416	QRLKEEEEDKKRKEEI—AEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	1/10	4%
P384	New	c.1188_1190delGGA (Δ3)	p.E396del	416	QRLKEEEEDKKRKEEEEAEDKEDDEDKDEDE—DEEDKEEDEEEDVPGQAKDEL	Wild type	1/40	27%
P615	New	c.1120A>T	p.E374X	373	QRLKEEEED	Wild type	2/40	23%
P871	New	c.1138 G>T	p.E380X	379	QRLKEEEEDKKRKEE	Wild type	2/16	32%
P744	New	c.1171A>T	p.K391X	390	QRLKEEEEDKKRKEEEEAEDKEDDED	Wild type	2/70	13%
P428	New	c.1115A>G	p.E372G	417	QRLKEEEGDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	2/61	50%
P551	rs201971744	c.1139A>G	p.E380G	417	QRLKEEEEDKKRKEEGEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	2/10	41%
P398	rs143880510	c.1142A>C	p.E381A	417	QRLKEEEEDKKRKEEEAAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Heterozygous	NA	26%

Abbreviations: NA, not available; PCR, polymerase chain reaction; SEQ, Sanger sequencing. [a]Red and blue fonts indicate acidic and basic amino acids, respectively. Underline indicates the same C-terminal sequence changes after +1 base pair reading frameshift. [b]TA clones of the *CALR* PCR products amplified from each colony were analyzed using Sanger sequencing. The total number of clones examined and the number of each genotype are listed in the table. [c]Based on the relative peak areas of the mutant and wild-type PCR products in Sanger sequencing. All patients were tested positive for *JAK2* V617F mutation using allele-specific PCR.

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Table 1B. Clinical and laboratory characteristics at diagnosis or referral of 91 essential thrombocythemia patients stratified by mutation profiles

Variables	*All* (n = *91*)	*A. JAK2 V617F mutation* (n = *46*)	*B. CALR mutation* (n = *21*)	*C. JAK2-mutated and CALR alterations* (n = *13*)	*D. Triple-negative* (n = *11*)	*A vs B vs C vs D* P *value*	*A vs B* P *value*	*A vs C* P *value*	*B vs C* P *value*
Male/female gender, *n* (%)	39/52 (43/57)	21/25 (46/54)	9/12 (43/57)	5/8 (39/61)	4/7 (36/64)	NS	NS	NS	NS
Age at diagnosis (years), median (range)	53 (22–89)	54.5 (25–89)	47 (22–76)	60 (26–80)	52 (35–79)	0.025	0.012	NS	0.004
Follow-up (years), median (range)	3.7 (0.02–23.1)	3.6 (0.04–23.1)	5,4 (0.5–13.2)	3.8 (0.02–6.1)	3.1 (0.2–10.3)	NS	NS	NS	0.032
History of thrombosis, *n* (%)	19 (20.9)	9 (19.6)	3 (14.3)	5 (38.5)	2 (18.2)	NS	NS	NS	NS
Major thrombosis, *n* (%)	17 (18.7)	8 (17.4)	2 (9.5)	5 (38.5)	2 (18.2)	NS	NS	NS	NS
Thrombosis after diagnosis, *n* (%)	10 (11)	3 (6.5)	2 (9.5)	4 (30.8)	1 (9.1)	NS	0.048	0.036	NS
Major arterial thrombosis after diagnosis, *n* (%)	6 (6.6)	1 (2.2)	1 (4.8)	3 (23.1)	1 (9.1)	NS	0.022	0.03	NS
History of hemorrhage, *n* (%)	25 (27.5)	13 (28.3)	9 (42.9)	2 (15.4)	1 (9.1)	NS	NS	NS	NS
Major hemorrhage, *n* (%)	17 (18.7)	9 (19.6)	6 (28.6)	2 (15.4)	0	NS	NS	NS	NS
High-risk group for thrombohemorrhagic complications[a], *n* (%)	43 (47.3)	22 (47.8)	6 (28.6)	10 (76.9)	5 (45.5)	NS	0.023	NS	0.012
Hemoglobin ($g\,dl^{-1}$), median (range)	13.3 (4.5–17.9)	14.0 (4.5–17.9)	12.6 (8.5–15.2)	13.3 (8.8–16.6)	12.8 (9.3–15.2)	0.016	0.016	NS	NS
WBC ($x10^3\,\mu l^{-1}$), median (range)	10.3 (4.8–29.9)	12.1 (4.8–29.9)	9.2 (4.9–27.9)	11.8 (6.0–24.2)	8.2 (5.3–25.5)	0.046	NS	NS	NS
Platelets ($x10^9\,l^{-1}$), median (range)	936 (335–2834)	942 (335–1496)	1351 (642–2834)	855 (547–1931)	708 (532–1374)	< 0.001	< 0.001	NS	0.001

Abbreviations: *n*, number; NS, not significant; WBC, white blood cell. [a]High-risk group for thrombohemorrhagic complications: Age ⩾ 60 years and/or a previous history of thrombosis.

burden *CALR* mutants in both *JAK2*-mutated and *JAK2*/*MPL*-unmutated ET patients. In addition, we also detected several *CALR* exon 9 point mutations and inframe deletions in *JAK2*-mutated ET patients, but none in our *JAK2*/*MPL*-unmutated ET patients. Recently, point mutations in *CALR* were also reported in follicular lymphoma (E403X and E405Q), PMF (E379D) and chronic neutrophilic leukemia (E398D).[14] Two rare inframe deletions in *CALR* exon 9 (p.E393_E395del and p.E405del) have been reported in the National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project with undetermined significance. All the five inframe deletions we detected were 3-bp deletions similar to the latter one. Although the possibility of low-allele-burden germline sequence variations cannot be completely excluded, these 3-bp inframe deletions detected using HRMA were more likely to be low-allele-burden somatic mutations not detected using Sanger sequencing in our patients. Recently, *CALR* point mutations (E381A and D373M) and inframe deletions (E381_A382 > A, D397_D400 > D, D400_K401 > D and E405_V409 > V) were also detected in patients with suspected MPN and *JAK2*-mutated MPN in another study albeit with a lower frequency.[15] These *CALR* alterations were also found to co-occur with *MPL, CSF3R*, *ASXL1* and *ZRSR2*. Currently, the role of these *CALR* point mutations and inframe deletions in the molecular pathogenesis of MPN is not yet clear. Because they frequently co-occurred with mutations involving the JAK-STAT pathway and affected disease phenotype in *JAK2*-mutated ET patients, these non-classic *CALR* mutant proteins are suspected to have a contributory role in the pathogenesis of MPN.[15] The frequency of these non-classic *CALR* mutations in PMF and other MPN requires further study.

In conclusion, we have detected a high frequency of both classic and non-classic *CALR* exon 9 alterations in *JAK2*-mutated ET patients by HRMA. The presence of *CALR* alterations in *JAK2*-mutated ET defines a specific subgroup of patients requiring careful follow-up and management for their increased risk of thrombotic events. Because our study is limited by small patient number, larger study is warranted to confirm our observation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DISCLAIMER

The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

K-HL, CG-SC, Y-YK and W-CC conceived of the study, participated in its design and/or coordination, and edited the manuscript. K-HL, H-CL, CG-SC, Y-CC, Y-HC, H-IC, N-WS, JL, Y-FC, M-CC and R-KH enrolled patients into the study. K-HL and W-TW carried out experiments and data analysis. K-HL and W-TW drafted the manuscript. All authors approved the manuscript.

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Research Paper

Increased B cell activation is present in *JAK2*V617F-mutated, *CALR*-mutated and triple-negative essential thrombocythemia

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ABSTRACT

Essential thrombocythemia (ET) is a *BCL-ABL1*-negative myeloproliferative neoplasm. We have reported that increased activated B cells can facilitate platelet production mediated by cytokines regardless *JAK2* mutational status in ET. Recently, *calreticulin* (*CALR*) mutations were discovered in ~30% *JAK2*/*MPL*-unmutated ET and primary myelofibrosis. Here we sought to screen for *CALR* mutations and to evaluate B cell immune profiles in a cohort of adult Taiwanese ET patients. B cell populations, granulocytes/monocytes membrane-bound B cell-activating factor (mBAFF) levels, B cells toll-like receptor 4 (TLR4) expression and intracellular levels of interleukin (IL)-1β/IL-6 and the expression of CD69, CD80, and CD86 were quantified by flow cytometry. Serum BAFF concentration was measured by ELISA. 48 healthy adults were used for comparison. 19 (35.2%) of 54 ET patients harbored 8 types of *CALR* exon 9 mutations including 4 (7.4%) patients with concomitant *JAK2*V617F mutations. Compared to *JAK2*V617F mutation, *CALR* mutations correlated with younger age at diagnosis (p=0.04), higher platelet count (p=0.004), lower hemoglobin level (p=0.013) and lower leukocyte count (p=0.013). Multivariate analysis adjusted for age, sex, follow-up period and hematological parameters confirmed that increased activated B cells were universally present in *JAK2*-mutated, *CALR*-mutated and triple-negative ET patients when compared to healthy adults. *JAK2*- and *CALR*-mutated ET have significantly higher fraction of B cells with TLR4 expression when compared to triple-negative ET (p=0.019 and 0.02, respectively). *CALR*-mutated ET had significantly higher number of CD69-positive activated B cells when compared to triple-negative ET (p=0.035). In conclusion, increased B cell activation is present in ET patients across different mutational subgroups.

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INTRODUCTION

Essential thrombocythemia (ET) is a *BCL-ABL1*-negative myeloproliferative neoplasm (MPN), and is characterized by increased number of mature megakaryocytes (MKs) in the bone marrow and sustained thrombocytosis in the peripheral blood [1]. ET is associated with an increased risk of hemorrhagic and thrombotic complications and leukemic transformation [1]. Most ET patients can have a normal life expectancy but some may encounter serious events during their disease course. In 2005, the *JAK2*V617F mutation was discovered in MPNs including 50-60% patients with ET and primary myelofibrosis (PMF) [2-5]. *JAK2*V617F mutation plays an important role in cytokine-independent hematopoietic stem cells (HSCs) proliferation in MPNs. Also, hypersensitivity of hematopoietic cells to cytokines stimulation is noted in MPNs through the interaction between *JAK2*V617F mutation and various cytokine receptors [6]. Recently, a high frequency of *calreticulin* (*CALR*) mutations was discovered in *JAK2*/*MPL*-unmutated ET and PMF [7-9]. We and others have reported that *CALR* mutations are associated with distinct clinical characteristics including higher platelet counts, lower leukocyte counts and hemoglobin levels, and a lower thrombosis risk when compared to *JAK2*-mutated ET patients [7-11]. Using *in vitro* and/or *in vivo* models, we and others have recently reported that mutant *CALR* can activate JAK-STAT signaling pathway through an MPL-dependent mechanism to mediate pathogenic thrombopoiesis [12-18].

CALR is a 46-kDa Ca2+ binding chaperone protein located in the endoplasmic reticulum, but it can also localize to cell surface and accumulate in extracellular compartments [19]. In addition to ensuring proper protein and glycoprotein folding within the lumen of endoplasmic reticulum, CALR was also found to involve the immune response to pre-apoptotic cancer cells, and early cell surface exposure of CALR was followed by expression and release of heat-shock proteins (e.g. HSP70), and high-mobility group I (HMGB1) protein [20]. Recombinant *CALR* fragment was shown to exhibit potent stimulatory activities against B cells [21, 22]. Recently, we reported that activated B cells are increased in ET patients, and can facilitate platelet production mediated by cytokines, such as interleukin (IL)-1β and IL-6 regardless *JAK2*V617F mutational status [23]. We found that increased production of B cell-activating factor (BAFF) by granulocytes and monocytes up-regulates toll-like receptor 4 (TLR4) expression on B cells and promotes B cell activation in ET patients. Consequently, these activated B cells play a pathogenic role in augmenting thrombocytosis by producing IL-1β and IL-6 in ET patients through cytokine-dependent thrombopoiesis in the bone marrow. However, ET with *CALR* mutations was not included in our previous study because *CALR* mutations have not yet been discovered in MPNs when we conducted our study in 2013. The discovery of *CALR* mutations in *JAK2*/*MPL*-unmutated ET patients in December 2013 have prompted us to ask the question that whether increased B cell activation can also be found in ET with *CALR* mutations similar to that in *JAK2*V617F-mutated ET [7-9]. Hence, we sought to screen for *CALR* mutations in a cohort of adult Taiwanese ET patients and to evaluate B cell immune profiles in *JAK2*V617F-mutated, *CALR*-mutated and triple-negative ET in this study.

RESULTS

Mutational analysis

Among 54 ET patients (median age at diagnosis 54.5 years; 54% females; median follow-up 4.4 years), 27 (50%) patients harbored the *JAK2*V617F mutation and one (1.9%) patient harbored the *MPL* W515K mutation. By nucleotide sequencing and HRMA, 19 (35.2% overall and 68.2% in *JAK2*/*MPL*-unmutated cases) patients harbored 8 types of *CALR* exon 9 mutations: 2 type 1 (p.L367fs*46), 10 type 2 (p.K385fs*47), 2 type 3 (p.L367fs*48), 1 type 34 (p.K385fs*47), and 4 other types (one each of p.L367fs*43, p.E370fs*60, p.E371fs*59 and p.E381del). Except p.E381del which is a 3 base-pair inframe deletion, all other *CALR* exon 9 mutations are indels causing +1 base-pair reading frameshift, with type 2 (10/19, 52.6%) being the most prevalent mutational type. One patient with *JAK2*V617F mutation harbored a single nucleotide polymorphism in *CALR* exon 9 (c.1142 A > C, rs143880510). Four (21%) of the 19 *CALR*-mutated patients had simultaneous *JAK2*V617F mutation; one each of type 3, p.E370fs*60, p.E371fs*59 and p.E381del, and the latter 3 *CALR* mutations were only detected by HRMA and required TA-cloning to confirm the presence of mutations indicating that they were low allelic burden mutants. Seven patients (13%) were triple-negative (TN) for *JAK2*, *CALR* and *MPL* mutations. No *DNMT3A* exon 23 or *IDH1*/*2* exon 4 mutation was detected in this cohort of ET patients. The only one *MPL*-mutated and the 4 *CALR*/*JAK2*V617F co-mutated ET patients were excluded from further clinical and molecular correlation analysis to avoid statistical bias.

Clinical and molecular correlates

In 49 ET patients used for analysis, there was no significant difference in gender among the three major mutational groups. In this cohort, ET patients with *CALR* mutations had statistically significant longer follow-up (median 6.2 year, $p = 0.031$, Table 1), highest platelet count at the time of diagnosis ($p = 0.01$), and lower hemoglobin level at the time of diagnosis ($p =$

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Table 1: Clinical and laboratory characteristics in healthy adults and patients with essential thrombocythemia.

Variables	HA (n=48)	*JAK2* mutation (n= 27)	*CALR* mutations (n=15)	Triple-negative (n=7)	*CALR* mutations vs *JAK2* mutation vs Triple-negative *p* value	*CALR* mutations vs *JAK2* mutation *p* value	*CALR* mutations vs Triple-negative *p* value	*JAK2* mutation vs Triple-negative *p* value	*CALR* mutations vs HA *p* value	*JAK2* mutation vs HA *p* value	Triple-negative vs HA *p* value
Male/Female gender, n (%)	15/33 (31/69)	11/16 (41/59)	7/8 (47/53)	3/4 (43/57)	NS	NS	NS	NS	-	-	-
Age at diagnosis (y), median (range)	-	55 (25-89)	45 (21-76)	52 (20-79)	NS	0.04	NS	NS	-	-	-
Follow-up (y), median (range)	-	3.6 (0.1-20.8)	6.2 (0.8-13.4)	3.3 (0.1-10.3)	0.031	0.019	0.039	NS	-	-	-
Hemoglobin at diagnosis (g/dL), median (range)	-	13.5 (8.6-17.1)	11.9 (8.5-15.2)	12.9 (10.1-15.2)	0.037	0.013	NS	NS	-	-	-
WBC at diagnosis ($x10^9$/L), median (range)	-	12.3 (5.7-27.7)	8.7 (4.3-17.5)	7.8 (5.3-10.2)	0.002	0.013	NS	0.002	-	-	-
Platelet at diagnosis ($x10^9$/L), median (range)	-	948 (335-1437)	1275 (759-2606)	900 (608-1374)	0.01	0.004	0.039	NS	-	-	-
Hemoglobin at testing (g/dL), median (range)	12.9 (10.3-16.7)	13.4 (7.2-15.9)	12.5 (9.1-15)	13.3 (10.1-15.6)	NS	NS	NS	NS	NS	0.039	NS
WBC at testing ($x10^9$/L), median (range), n=56	5.5 (3.9-7)	12.0 (6.8-21.8)	8.3 (4-13.8)	7.8 (4.6-8.6)	< 0.001	0.001	NS	0.001	0.005	< 0.001	0.034
Platelet at testing ($x10^9$/L), median (range)	241.5 (118-366)	842 (449-1227)	734(247-2215)	824 (551-1127)	NS	NS	NS	NS	< 0.001	< 0.001	< 0.001
Cytoreductive therapy with hydroxyurea, n (%)	-	20 (74.1)	11 (73.3)	3 (42.9)	NS	NS	NS	NS	-	-	-

Abbreviations: HA, healthy adults; No. and n, number; NS, not significant; WBC, white blood cell; y, year.

0.037). When compared with *JAK2*V617F-mutated ET patients, *CALR* mutations also correlated with younger age at diagnosis (p = 0.04) and lower leukocyte count (p = 0.013). *JAK2*V617F mutation was associated with leukocytosis (p = 0.002) and white blood cell count was lowest in TN ET patients.

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Table 2: Univariate analysis of B cell immune profiles in healthy adults and patients with essential thrombocythemia.

Variables	HA (*n*=48)	*JAK2* mutation (*n*= 27)	*CALR* mutations (*n*=15)	Triple-negative (*n*=7)	*CALR* mutations vs *JAK2* mutation vs Triple-negative *p* value	*CALR* mutations vs *JAK2* mutation *p* value	*CALR* mutations vs Triple-negative *p* value	*JAK2* mutation vs Triple-negative *p* value	*CALR* mutations vs HA *p* value	*JAK2* mutation vs HA *p* value	Triple-negative vs HA *p* value
CD19+ B cells (/μL), median (range), n=71	230.5 (143-455)	129.0 (25.8-358)	121.8 (17.8-318)	97.2 (38.1-219.5)	NS	NS	NS	NS	0.001	< 0.001	0.001
Early transitional B cells (T1) (/μL), median (range), n=61	3 (1-11)	1.1 (0.0-15.5)	2 (0.0-19.4)	2.5 (1-10)	NS	NS	NS	NS	NS	NS	NS
Late transitional B cells (T2) (/μL), median (range), n=61	7 (3-15)	15 (1-46)	14 (0.4-29.5)	9.2 (2-46)	NS	NS	NS	NS	NS	NS	NS
Pre-germinal center B cells (/μL), median (range), n=61	4 (1-24)	7 (2-17.6)	6.6 (2-19)	4.0 (3-25)	NS	NS	NS	NS	NS	NS	NS
Memory B cells (/μL), median (range), n=61	75.5 (32-181)	34 (6.8-108)	34 (3.4-145)	28.5 (20-165)	NS	NS	NS	NS	< 0.001	< 0.001	NS
Plasmablast (/μL), median (range), n=61	0.0 (0.0-1)	0.4 (0.0-4)	0.3 (0.0-3)	0.5 (0.0-2)	NS	NS	NS	NS	< 0.001	< 0.001	0.025
Naive B cells (/μL), median (range), n=61	134.5 (75-238)	65.6 (5.8-293)	45.8(5.8-223)	57.5 (16-591)	NS	NS	NS	NS	0.003	0.01	0.014
MFI of mBAFF on granulocytes, n=63	6.7 (4.6-8.3)	25.4 (7.8-75.2)	34.2 (10.2-67.7)	33.7 (4.5-65.4)	NS	NS	NS	NS	< 0.001	< 0.001	0.007
MFI of mBAFF on monocytes, n=62	7.6 (2.8-8.0)	14.2 (3.5-54.6)	28.9 (4.4-49.4)	14.3 (3.2-40.7)	NS	NS	NS	NS	< 0.001	< 0.001	0.005
Serum BAFF level (ng/mL), n=66	1.1 (0.6-2.4)	2.3 (0.8-4.9)	1.6 (0.9-3.9)	1.8 (1.4-3.8)	0.049	0.02	NS	NS	0.015	< 0.001	0.001
IL-6 in B cells (%), n=39	1.0 (0.2-1.9)	6.7 (2.6-9.6)	6.8 (4.0-13.7)	8.2 (4.5-10.3)	NS	NS	NS	NS	< 0.001	< 0.001	< 0.001
IL-1β in B cells (%), n=39	4.8 (0.9-13.4)	11.5 (1.4-32.5)	15.4 (4.6-32.6)	12.9 (3.6-41.7)	NS	NS	NS	NS	0.002	0.002	0.012
TLR4 in B cells (%), n=54	2.3 (0.3-3.0)	4.5 (0.4-24.2)	11.3 (2.3-22.8)	3.4 (1.0-5.4)	0.021	NS	0.001	NS	< 0.001	0.001	0.02
CD69+ B cells (/μL), median (range), n=48	2.2 (1.0-4.6)	12.5 (0.6-39.1)	20.8 (2.5-51.4)	7.6 (1.8-18.6)	NS	NS	0.048	NS	< 0.001	0.002	NS
CD80+ B cells (/μL), median (range), n=46	9.2 (0.8-12.5)	9.0 (1.9-79.1)	13.8 (1.0-72.6)	10.0 (1.6-44.7)	NS	NS	NS	NS	0.036	NS	NS
CD86+ B cells (/μL), median (range), n=46	10.9 (3.0-41.9)	26.3 (4.3-101.6)	18.3 (5.9-63.4)	24.2 (8.0-82.3)	NS	NS	NS	NS	0.041	0.012	NS

Abbreviations: BAFF, B cell-activating factor; HA, healthy adults; IL, interleukin; mBAFF, membrane-bound B cell-activating factor; MFI, mean fluorescence intensity; No. and n, number; NS, not significant; TLR4, toll-like receptor 4; WBC, white blood cell; y, year.

Distribution of B cells and B cell subsets

Among 49 ET patients in the three major mutational groups, there were no significant differences in the number of total B cells and all the B cell subset populations (Table 2). When compared to healthy adults, ET patients had significantly lower numbers of total B cells and naïve B cells, but had significantly higher number of plasmablast in all three mutational groups. The number of memory B cells was statistically lower in *CALR*- and *JAK2* mutated-ET patients when compared with healthy adults. There were no statistically significant differences in the numbers of early and late transitional B cells and pre-germinal

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Table 3: Correlation of platelet count at testing, serum BAFF levels, and B cell immune profiles in this study.

Variables	Platelet at testing		MFI of mBAFF on granulocytes		MFI of mBAFF on monocytes		Serum BAFF levels	
	Spearman's rho	*p* value	Spearman's rho	*p* value	Spearman's rho	*p* value	Spearman's rho	*p* value
MFI of mBAFF on granulocytes	0.377	0.002	-	-	0.786	<0.001	0.29	0.021
MFI of mBAFF on monocytes	0.312	0.011	0.786	<0.001	-	-	0.304	0.016
Serum BAFF levels	0.486	<0.001	0.29	0.021	0.304	0.016	-	-
IL-6 in B cells	0.61	<0.001	0.545	<0.001	0.398	0.009	0.572	<0.001
IL-1β in B cells	0.543	<0.001	0.55	<0.001	0.472	0.002	0.293	NS
CD69+ B cells	0.325	0.021	0.388	0.005	0.161	NS	0.225	NS
CD86+ B cells	0.26	NS	0.42	0.003	0.252	NS	0.141	NS

Abbreviations: BAFF, B cell-activating factor; IL, interleukin; mBAFF, membrane-bound B cell-activating factor; MFI, mean fluorescence intensity; NS, not significant.

center B cells between ET patients and healthy adults.

B cell immune profiles

Among 49 ET patients in the three major mutational groups, the B cell immune profiles in 34 (69.4%; 19 *JAK2*V617F-mutated, 9 *CALR*-mutated and 6 TN) patients had been previously described [23]. When compared with *JAK2*V617F-mutated and TN ET patients, *CALR* mutations correlated with significantly lower serum BAFF level (median 1.6 ng/mL, $p = 0.049$) (Figure 1A) and higher fraction of B cells with TLR4 expression (median 11.3%, $p = 0.021$) (Figure 2A). Besides, ET patients with *CALR* mutations had statistically higher number of CD69-positive activated B cells when compared to TN group (median: 20.8/μL *vs* 7.6/μL, $p = 0.048$) (Figure 3A). There were no significant differences in mean fluorescence intensity (MFI) of mBAFF on both granulocytes and monocytes (Figure 1B and 1C, respectively), in the fraction of B cells with intracellular IL-1β or IL-6 expression (Figure 2B and 2C, respectively), and the numbers of CD80-positive and CD86-positive activated B cells among the three mutational groups of ET patients (Figure 3B and 3C, respectively).

When compared to healthy adults, patients with ET had statistically significant higher serum BAFF level and higher MFI of mBAFF on both granulocytes and monocytes (Figure 1), and higher fraction of B cells with TLR4 expression and higher fractions of B cells with intracellular IL-1β and IL-6 expression irrespective of their genotypes (Figure 2) (Table 2). Although ET patients had significantly lower numbers of CD19-positive B cells and naïve B cells when compared to healthy adults, ET patients with *CALR* and *JAK2* mutations had statistically higher numbers of CD69-positive and CD86-positive activated B cells (Figure 3A and 3C, respectively). 38 (70.4%) of 54 ET patients were treated with hydroxyurea to lower their blood counts in this cohort. There were no significant differences in all the B cells immune profiles in ET patients with or without hydroxyurea treatment, except lower IL-1β expression level in B cells (median 6.9% *vs* 16.4%, $p = 0.014$) was found in ET patients being treated with hydroxyurea (Table 1 and Supplementary Table S1).

In this study, platelet count at testing had moderately positive correlation with the fractions of B cells with intracellular IL-1β and IL-6 expression (Table 3). MFI of mBAFF on granulocytes had strong positive correlation with MFI of mBAFF on monocytes, and had moderately positive correlation with the fractions of B cells with intracellular IL-1β and IL-6 expression. In addition, serum BAFF levels had moderately positive correlation with the fraction of B cells with intracellular IL-6 expression. Interestingly, only MFI of mBAFF on granulocytes, but not MFI of mBAFF on monocytes or the serum BAFF levels, had weak positive correlation with the numbers of CD69-positive and CD86-positive activated B cells in our cohort. We also analyzed the correlation between platelet count, serum BAFF levels, and B cell immune profiles in the group of healthy controls. Platelet count of healthy controls only had moderately negative correlation with the MFI of mBAFF on monocytes (Spearman's rho = -0.625,

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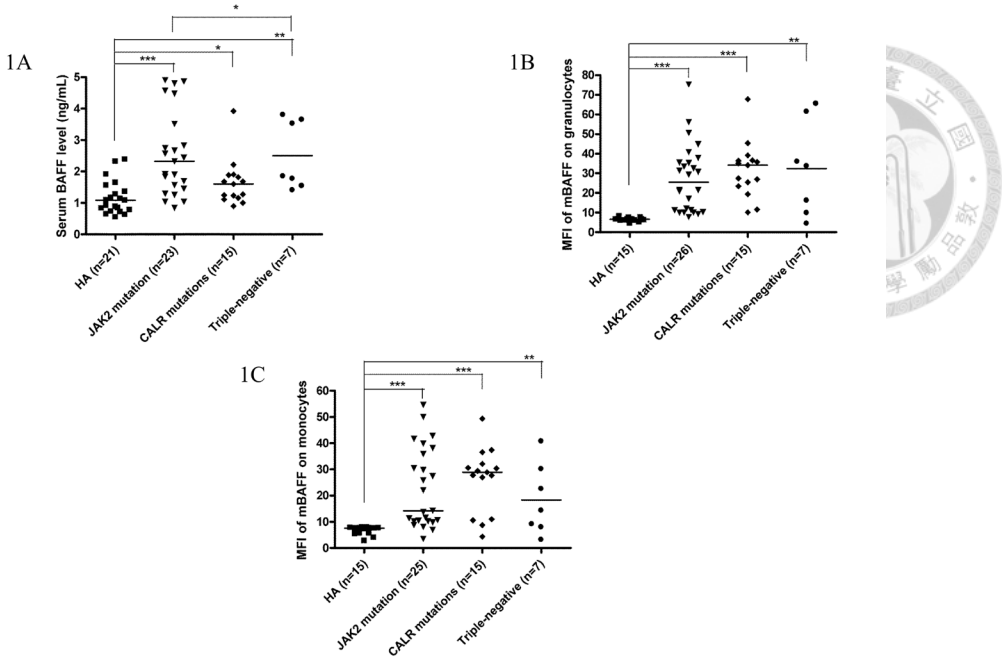

Figure 1: Elevated serum BAFF levels and higher membrane-bound BAFF expression in peripheral granulocytes and monocytes of ET patients. 1A, Elevated serum BAFF levels were found in ET patients, regardless of genotypes compared to healthy adults. *CALR*-mutated ET patients had lowest serum BAFF levels compared to *JAK2*-mutated and triple-negative ET patients in univariate analysis. **1B** and **1C**, Membrane-bound BAFF expression in peripheral granulocytes and monocytes was higher in ET patients, regardless of genotypes compared to healthy adults, respectively. Median values are indicated by the short horizontal bars. Asterisks represent significant differences between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

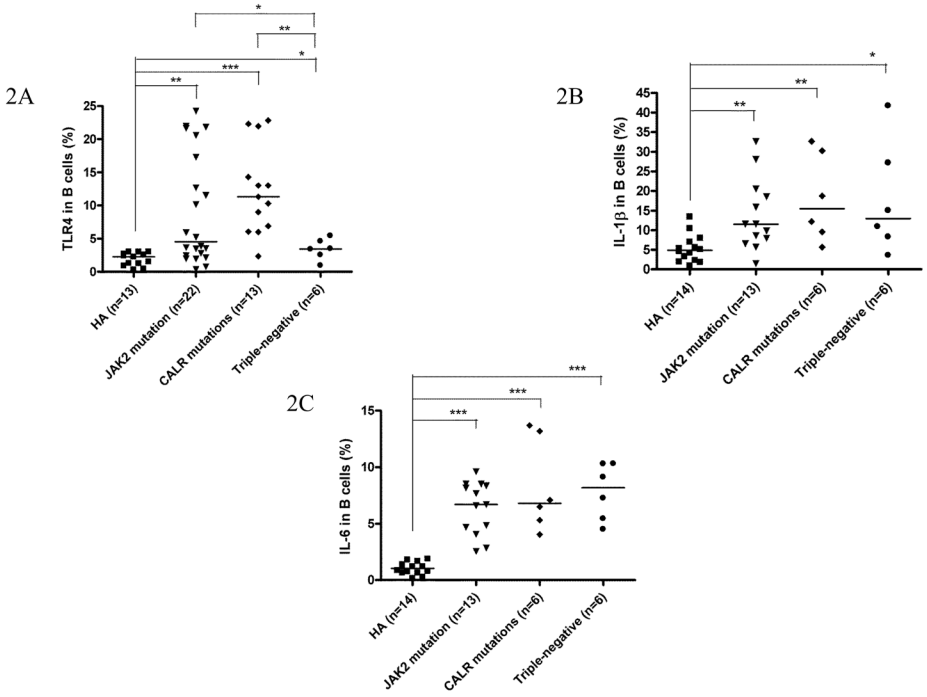

Figure 2: Fractions of activated B cells with TLR4 expression, and IL-1β and IL-6 production were higher in ET patients. 2A, B cells from patients with ET, regardless of genotypes expressed significantly higher levels of TLR4 compared to healthy adults. *CALR*-mutated ET patients had highest TLR4 expression compared to *JAK2*-mutated and triple-negative ET patients. **2B** and **2C**, B cells from patients with ET, regardless of genotypes expressed significantly higher levels of IL-1β and IL-6 compared to healthy adults, respectively. Median values are indicated by the short horizontal bars. Asterisks represent significant differences between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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Table 4: Comparison of B cell immune profiles between healthy adults and patients with essential thrombocythemia using linear regression model adjusted for hematological parameters.

Variables	ET subgroups	Unstandardized Coefficients		*p* value	95% Confidence Interval for B	
		B	Std. Error		Lower Bound	Upper Bound
CD19+ B cells (/μL)	*JAK2*-mutated	-109.918	42.713	0.013	-195.846	-23.990
	CALR-mutated	-102.613	40.102	0.014	-183.289	-21.938
	TN	-148.166	46.096	0.002	-240.899	-55.433
Neutrophil mBAFF MFI	*JAK2*-mutated	25.969	9.115	0.006	7.642	44.297
	CALR-mutated	33.242	8.565	<0.001	16.020	50.463
	TN	34.018	9.410	0.001	15.099	52.938
Monocyte mBAFF MFI	*JAK2*-mutated	14.897	7.287	0.047	0.237	29.556
	CALR-mutated	23.159	6.779	0.001	9.522	36.796
	TN	16.591	7.446	0.031	1.612	31.571
Serum BAFF levels (ng/ml)	*JAK2*-mutated	0.915	0.625	NS	-0.343	2.173
	CALR-mutated	0.194	0.586	NS	-0.986	1.373
	TN	1.102	0.643	NS	-0.194	2.397
Total B IL-6 %	*JAK2*-mutated	4.488	1.912	0.027	0.551	8.425
	CALR-mutated	4.906	2.337	0.046	0.093	9.719
	TN	5.463	1.724	0.004	1.913	9.014
Total B IL-1β%	*JAK2*-mutated	11.566	7.807	NS	-4.513	27.644
	CALR-mutated	16.529	9.544	NS	-3.128	36.186
	TN	14.697	7.040	0.047	0.199	29.196
Total B TLR4 %	*JAK2*-mutated	14.090	4.281	0.002	5.439	22.742
	CALR-mutated	14.262	3.746	<0.001	6.692	21.832
	TN	5.888	4.378	NS	-2.961	14.737
CD69+ B cells (/μL)	*JAK2*-mutated	35.629	9.431	0.001	16.440	54.817
	CALR-mutated	34.581	7.583	<0.001	19.153	50.008
	TN	19.206	9.302	0.047	0.282	38.130
CD80+ B cells (/μL)	*JAK2*-mutated	18.875	15.117	NS	-11.956	49.705
	CALR-mutated	15.519	12.409	NS	-9.790	40.828
	TN	16.876	14.963	NS	-13.640	47.393
CD86+ B cells (/μL)	*JAK2*-mutated	38.875	17.586	0.035	3.007	74.742
	CALR-mutated	27.556	14.437	NS	-1.888	57.000
	TN	33.042	17.407	NS	-2.460	68.545

Abbreviations: mBAFF, membrane-bound B cell-activating factor; IL: interleukin; MFI, mean fluorescence intensity; NS, not significant; Std., standard; TLR4: toll-like receptor 4; TN, triple-negative.

$p = 0.013$) suggesting that the platelet count of healthy controls did not have obvious correlation with their B cell immune profiles.

Multivariate analysis of B cell immune profiles in ET

The results of multivariate analysis using linear regression model adjusted for multiple parameters

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Table 5: Comparison of B cell immune profiles among patients with essential thrombocythemia using linear regression model adjusted for age, sex, follow-up period and hematological parameters.

Variables	ET subgroups	*CALR*-mutated group as control					*JAK2*-mutated group as control				
		Unstandardized Coefficients		*p* value	95% Confidence Interval for B		Unstandardized Coefficients		*p* value	95% Confidence Interval for B	
		B	Std. Error		Lower Bound	Upper Bound	B	Std. Error		Lower Bound	Upper Bound
Serum BAFF levels (ng/ml)	*JAK2*-mutated	0.436	0.479	NS	-0.536	1.408	–	–	–	–	–
	CALR-mutated	–	–	–	–	–	-0.436	0.479	NS	-1.408	0.536
	TN	0.571	0.578	NS	-0.601	1.743	0.135	0.577	NS	-1.035	1.304
Total B TLR4 %	*JAK2*-mutated	0.437	2.759	NS	-5.183	6.057	–	–	–	–	–
	CALR-mutated	–	–	–	–	–	-0.437	2.759	NS	-6.057	5.183
	TN	-8.169	3.329	0.02	-14.951	-1.388	-8.606	3.467	0.019	-15.669	-1.543
CD69+ B cells (/μL)	*JAK2*-mutated	-1.303	7.025	NS	-15.801	13.196	–	–	–	–	–
	CALR-mutated	–	–	–	–	–	1.303	7.025	NS	-13.196	15.801
	TN	-18.480	8.265	0.035	-35.539	-1.421	-17.177	8.605	NS	-34.936	0.582
CD80+ B cells (/μL)	*JAK2*-mutated	6.135	11.382	NS	-17.470	29.740	–	–	–	–	–
	CALR-mutated	–	–	–	–	–	-6.135	11.382	NS	-29.740	17.470
	TN	3.273	13.175	NS	-24.051	30.597	-2.862	13.175	NS	-30.185	24.462
CD86+ B cells (/μL)	*JAK2*-mutated	16.243	13.696	NS	-12.161	44.647	–	–	–	–	–
	CALR-mutated	–	–	–	–	–	-16.243	13.696	NS	-44.647	12.161
	TN	11.584	15.854	NS	-21.294	44.463	-4.658	15.854	NS	-37.537	28.221

Abbreviations: BAFF, B cell-activating factor; NS, not significant; Std., standard; TLR4: toll-like receptor 4; TN, triple-negative.

confirmed that increased activated CD69+ B cells were universally present in *JAK2*-mutated, *CALR*-mutated and triple-negative ET patients when compared to healthy adults, although the number of total B- cells was significantly lower in ET patients (Table 4). Activated B cells were characterized by the expression of CD69 and CD86, increased intracellular IL-6 and IL-1β levels, and higher expression of TLR4. Interestingly, peripheral granulocytes and monocytes mBAFF expression was significantly higher in ET patients compared to healthy controls. *JAK2*-mutated and *CALR*-mutated ET patients had significantly higher number of B-cells expressing TLR4 and IL-6, and TN ET patients had significantly higher number of B-cells expressing IL-6 and IL-1β (Table 4). TN ET patients had significantly lower number of B-cells expressing TLR4 when compared to *CALR*-mutated and *JAK2*-mutated ET patients (Table 5). TN ET patients also had significantly lower number of CD69+ B-cells when compared to *CALR*-mutated ET patients.

DISCUSSION

CALR mutations have been found to have phenotypic and prognostic significances in patients with ET from both Caucasian and Chinese populations [7, 8, 11, 24-27]. In this cohort of adult Taiwanese ET patients, *CALR* mutations were found to have a similar phenotypic correlation with higher platelet count, lower hemoglobin level and younger age at diagnosis. However, we detected a higher frequency of type 2 *CALR* mutation (10 of 19 patients) in this study while there was only 2 type 1 *CALR* mutation detected. These results are contradictory to the

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Table 6: Characteristics and the frequency of CALR and JAK2V617F co-mutations in patients with essential thrombocythemia.

Author	Population	Method to detect *CALR* mutations	*CALR* mutation	Frequency of *CALR* and *JAK2*V617F co-mutations		
				In ET no. (%)	In *CALR*-mutated ET no. (%)	In *JAK2*V617F-mutated ET no. (%)
Lundberg *et al.* [28]	Caucasian	Allele-specific PCR	p.K385fs*47	1/69 (1.4)	1/17 (5.9)	1/41 (2.4)
Fu *et al.* [29]	Chinese	Sanger sequencing	L367fs*46 c.997 C>T (arginine>tryptophan)	2/436 (0.5)	2/99 (2)	2/240 (0.8)
Shirane *et al.* [30]	Japanese	Fragment analysis and deep sequencing	p.E378fs*45	1/111 (0.9)	1/22 (4.5)	1/60 (1.7)
Ha and Kim [31]	Korean	Sanger sequencing	p.L367fs*46	1/114 (0.9)	1/25 (4)	1/68 (1.5)
Al Assaf *et al.*[32]	Caucasian	Sanger sequencing	p.K385fs*47	1/160 (0.6)	1/59 (1.7)	1/57 (1.8)
Lin *et al.*[33]	Chinese	Sanger sequencing	2 p.L367fs*46 2 p.K385fs*47	4/428 (0.9)	4/101 (4.0)	4/254 (1.6)
Lim *et al.* [34]	Taiwanese	HRMA and Sanger sequencing	p.L367fs*48 p.E381A p.K385fs*47 p.E370fs*60 p.E371fs*59 p.E371del p.E378del p.E396del p.E374X p.E380X p.K391X p.E372G p.E380G	13/92 (14.1)	13/34 (38)	13/59 (22)
Usseglio *et al.* [35]	Caucasian	HRMA	2 p.K385fs*47 p.L367fs*48 c.1125_1147del	4/103 (3.9)	4/48 (8.3)	4/56 (7.1)
Lim *et al.* (this study)	Taiwanese	HRMA and Sanger sequencing	p.L367fs*48 p.E370fs*60 p.E371fs*59 p.E381del	4/54 (7.4)	4/19 (21.1)	1/31 (3.2)

Abbreviations: ET, essential thrombocythemia; HRMA, high-resolution melting analysis; no., number.

vast majority of the reports in the literature. The possible explanations for this discrepancy in our results might be related to small sample size and selection bias cannot be excluded completely in this study.

In accordance with our previous report, a relatively high frequency of *CALR* and *JAK2*V617F co-mutations (21% in 19 *CALR*-mutated ET) was still found in this study. Several papers have reported the co-occurrence of *CALR* and *JAK2*V617F mutations in ET across different ethnic groups including one of our previous publication (Table 6). The frequency of *CALR* and *JAK2*V617F co-mutations ranges from 0.5 to 14.1%, 1.7 to 38%, and 0.8 to 22%, in ET, *CALR*-mutated ET, and *JAK2*V617F-mutated ET, respectively [28-35]. The cause of the difference in the frequency of *CALR* and *JAK2*V617F co-mutations in these studies might be related to the different methods used to

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detect *CALR* mutations. Higher frequency of *CALR* and *JAK2*V617F co-mutations was detected by using HRMA, whereas Sanger sequencing will likely miss to detect low allelic burden (< 10%) *CALR* mutants. On the other hand, Usseglio *et al*. found that *CALR* mutations could be detected in low allelic burden (< 4%) *JAK2*V617F-mutated ET suggesting that the frequency of *CALR* and *JAK2*V617F co-mutations might be further increased if a highly sensitive test was employed to detect *JAK2*V617F mutation in *CALR*-mutated ET [35]. Since both of our studies used a sensitive in-house developed HRMA followed by TA-cloning to detected *CALR* mutations, we were able to identify many low allelic burden *CALR* mutants resulting in the higher frequency of *CALR* and *JAK2*V617F co-mutations in our series. However, because our study was limited by small patient size, larger study using sensitive screening methods for the detection of both *CALR* and *JAK2*V617F mutations will be warranted to confirm our results.

Recently, we have shown that ET patients have quantitative and qualitative changes in their B cell immune profiles regardless of *JAK2*V617F mutational status [23]. In our previous report, we found that the number of CD19+ B cells did not differ between ET patients and age-matched healthy adults using univariate analysis. However, we found that ET patients had significantly lower numbers of total CD19+ B cells in univariate analysis (Table 2) and also in multivariate analysis adjusted for age, sex, follow-up period and hematological parameters (Table 4) in this study. We believe that the results reported in this study are more accurate because *CALR*-mutated ET patients were not included in our previous report and the results from multivariate analysis are more reliable. In the present study, we found that ET patients with *CALR* mutations also had similar quantitative and qualitative changes in most of the B cell immune profiles when compared to healthy adults using univariate and multivariate analyses (Tables 2 and 4, respectively). Although the number of

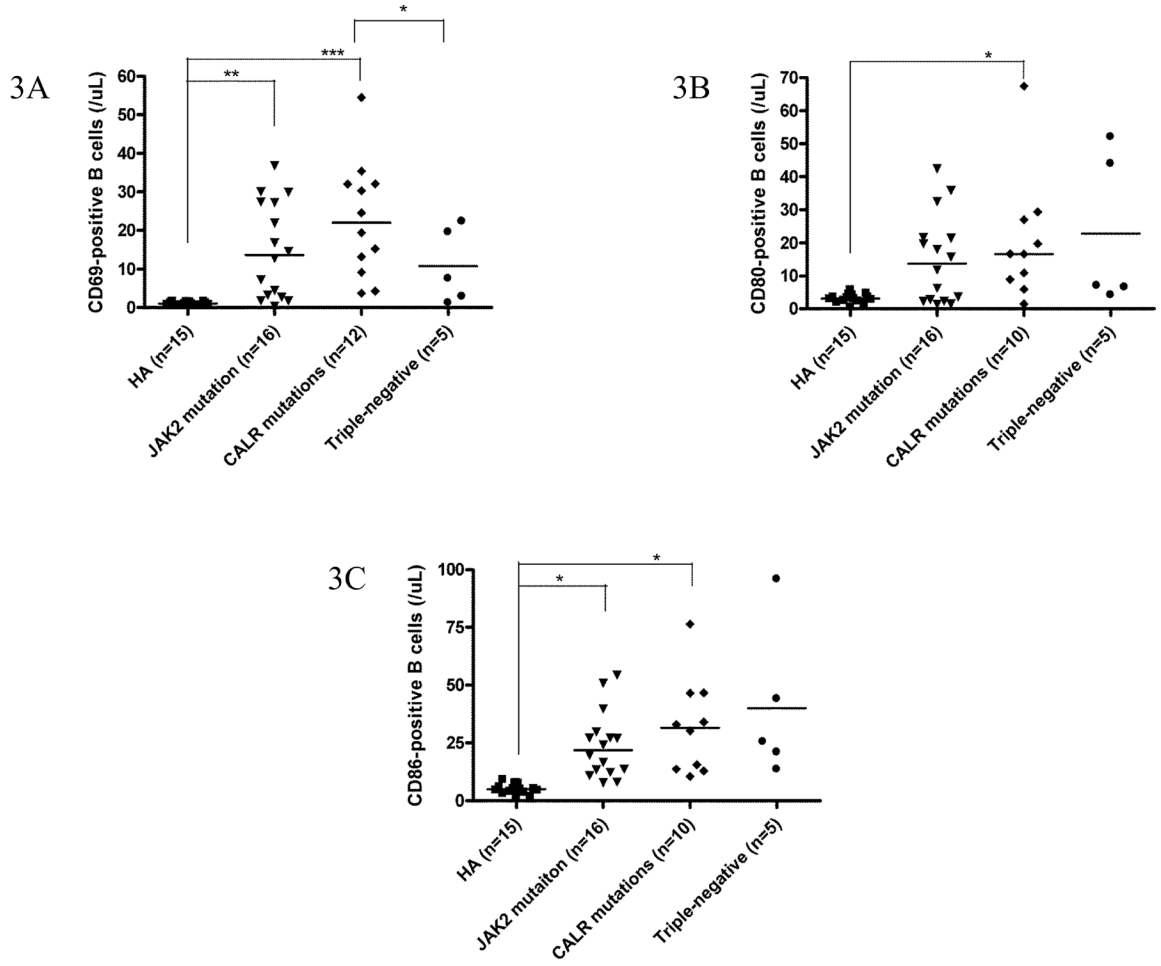

Figure 3: *CALR* mutations were associated with activated B cells in patients with ET. 3A, 3B and **3C**, The number of activated B cells was higher in *CALR*-mutated ET patients, as evidenced by expression of CD69, CD80 and CD86, respectively. Data are presented as the number of B cells expressing these markers. Median values are indicated by the short horizontal bars. Asterisks represent significant differences between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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total B cells was lower in ET patients including those with *CALR* mutations when compared with healthy controls, the number of activated B cells was significantly increased in ET patients across all 3 genotypes that characterized by the expression of CD69 and CD86, increased intracellular IL-6 and IL-1β levels, and higher expression of TLR4.

Regarding to the mechanism of B cell activation in ET patients, it has been well documented that elevated serum levels of inflammatory cytokines are frequently detected in patients of MPN, especially PMF, and may correlate to their constitutional symptoms which could be effectively ameliorated by the use of JAK inhibitor [36]. Previous study has reported that cytokine levels were also significantly increased in ET and PV patients [37]. Therefore, it is reasonable to argue that B cell activation could only be an epiphenomenon in ET rather than a cause of thrombopoiesis. However, we found that increased B cell activation was only present in ET patients but not in PV patients when compared to healthy controls or patients with reactive thrombocytosis (Supplementary Table S2). Although we did not evaluate B cell immune profiles in PMF patients due to difficulty in patient enrollment, our findings provided evidence to illustrate that increased B cell activation in ET patients could not be solely explained by the increased cytokine levels in MPN patients, and therefore might not be an epiphenomenon in these patients. Nevertheless, we had previously reported that some humoral factors such as endogenous toll-like receptor 4 (TLR4) ligands HSP70 and HMGB1 or other inflammatory cytokines, might participate in the activation of B cells in ET patients because peripheral B cells of ET patients could be stimulated by ET patients' sera to cause IL-1beta and IL-6 production [23]. In addition, we had also demonstrated that increased production of BAFF by granulocytes and monocytes up-regulates TLR4 expression on B cells and promotes B cell activation in ET patients. Consequently, these activated B cells play a pathogenic role in augmenting thrombocytosis by producing IL-1β and IL-6 in ET patients through cytokine-dependent thrombopoiesis in the bone marrow. Altogether, our data suggested that increased B cell activation in ET might be caused by the stimulation of specific humoral factors on B cells and the interaction of B cells with BAFF on granulocytes and monocytes. Importantly, our studies suggested that activated B cells in ET could play a role in mediating pathogenic thrombopoiesis in the bone marrow.

Besides, we had previously reported that TLR4 expression is upregulated in both naïve and memory B cell subsets, and BAFF receptor signaling has reciprocal effects on TLR interaction [23]. B cells are characterized by the expression of a clonally rearranged, antigen-specific B cell receptor (BCR) in combination with the expression of one or more members of the TLRs [38]. This dual expression feature allows B cells to integrate both antigen-specific signals and environmental danger signals *via* these key receptor systems. Since we did not measure or characterize the expression level of BCR on B cells, whether dual BCR and TLRs engagement may also play a role in the activation and/or affect the function of B cells in ET patients remains to be elucidated in future study.

Furthermore, we did not favor the paracrine effect of serum BAFF secreted by peripheral granulocytes and monocytes because its level was not different between ET patients and healthy controls in multivariate analysis. Rather, we hypothesized that the direct interaction between peripheral granulocytes and monocytes and B-cells might play a role in the activation of B-cells in ET patients since mBAFF expression was significantly higher in ET patients compared to healthy controls. Recently, mBAFF has been found to be a more potent stimulus for B cells than soluble BAFF thus supporting our view [39]. Our observation was also supported by the finding that mBAFF expression on peripheral granulocytes significantly correlated with higher number of IL-1β/IL-6-producing B cells and activated B-cells in ET patients (Table 3). In addition, higher number of TLR4-producing B cells in *JAK2*-mutated and *CALR*-mutated ET patients might also augment the production of IL-1β/IL-6 in B cells in these patients. We had previously shown that IL-1β and IL-6 play an important role in thrombopoiesis in ET patients, and hematopoietic stem cells of ET patients differentiated towards a megakaryocytic lineage after incubation with their own B cells [23]. Therefore, our data suggested that activated B-cells in ET patients might link to the pathogenic thrombopoiesis in these patients through the production of IL-1β/IL-6 in activated B cells regardless of their genotypes.

It is possible that the use cytoreductive therapy might affect B cell immune profiles in ET patients. However, most B cell immune profiles in ET patients were not affected by the treatment of hydroxyurea in this study (Supplementary Table S1). Therefore, we believed that the changes in B cell immune profiles may be more closely related to the underlying pathogenic mechanisms that could not be altered by non-specific cytoreductive therapy such as hydroxyurea.

Currently, the exact molecular mechanism of B cell activation in ET patients has not yet been fully elucidated. However, most of the changes in B cell immune profiles are independent of the three genotypes in ET patients, and the activation of JAK-STAT signaling pathway can be seen in most ET patients regardless of their molecular profiles [40]. *JAK2*V617F is a gain-of-function mutation resulting in the cytokine-independent growth of hematopoietic progenitors [41]. However, *JAK2*V617F mutation requires the presence of cytokine receptors (especially MPL) to be constitutively active [42]. *JAK2*V617F mutation can activate erythropoietin receptor, thrombopoietin receptor or granulocyte colony-stimulating factor receptor on progenitor cells to promote erythropoiesis, megakaryopoiesis, or granulopoiesis. Interestingly, *CALR* mutations are recently found to activate the JAK-STAT

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signaling through a MPL-dependent mechanism, and cause thrombocytosis both *in vitro* and *in vivo*. Hence, both *JAK2*V617F and *CALR* mutations can activate the JAK-STAT signaling in megakaryocytes. Although *CALR* mutations can be detected in hematopoietic stem/progenitor cells, it largely promotes the growth and the differentiation of megakaryocytic precursors resulting in the phenotype of ET and/or PMF. Therefore, *CALR* mutations are exclusively detected in around 25 % of ET or PMF, but not in PV. On the other hand, *JAK2*V617F mutation can be identified in about 95% of PV and in around 60 % of ET or PMF. Several observations have suggested that megakaryocytes play a major role in the pathogenesis of MPNs [43]. There is evidence suggesting that MPN associated mutations could alter megakaryocyte differentiation, migratory ability, and proplatelet formation, leading to increased platelet production [44]. *JAK2*V617F mutation was also found to lead to intrinsic changes in both megakaryocyte and platelet biology in a mouse model of ET [45]. Recently, CALR mutations have been shown to activate essential MAPK signaling through MPL-dependent mechanism and facilitate megakaryocyte differentiation [46]. Current evidences suggest that both *JAK2*V617F and *CALR* mutations intrinsically play a major role in the pathogenesis of ET through the promotion of megakaryopoiesis and thrombopoiesis. Based on our findings, increased platelet production in ET patients may be resulted from activating mutations synergistic with bystander thrombopoietic cytokines produced by activated B cells. We believe that these results would help advance our understanding of the pathogenesis of ET.

Our study is limited by a total number of 54 ET patients. However, the distribution and the percentage of the 3 driver mutations in these 54 ET patients were comparable with most studies: 27 (50%) patients harbored the *JAK2*V617F mutation, 1 (1.9%) patient with the *MPL*W515K mutation, 19 (35.2% overall and 68.2% in *JAK2*/*MPL*-unmutated cases) patients with *CALR* exon 9 mutations, and 7 (13%) TN patients. In this study, we detected a higher percentage of *CALR*/*JAK2*V617F co-mutations in 4 (7.4%) ET patients due to the use of a sensitive HRMA followed by TA-cloning to detect low allelic burden *CALR* mutants. To avoid statistic bias on the results, we excluded these 4 *CALR*/*JAK2*V617F co-mutated ET patients and the only one *MPL*-mutated ET patient from further analysis. We have also consulted our bio-statistician for help with the analysis of our data. Our results showed that increased B cell activation is present in *JAK2*V617F-mutated, *CALR*-mutated and triple-negative ET, and these findings are consistent with our previous report. Although we believe that there was no statistical bias on the results, larger study is still warranted to confirm our findings. In conclusion, increased B cell activation is present in ET patients across different mutational subgroups.

PATIENTS AND METHODS

Patient enrollment

The screening for mutations in patients with hematologic neoplasms was approved by the Institutional Review Board of MacKay Memorial Hospital (09MMHIS157 and 12MMHIS034). 54 adult Taiwanese ET patients were enrolled and written informed consent was obtained. The clinical and laboratory characteristics at the time of diagnosis/referral and at testing were determined retrospectively by chart review. Parts of the clinical data of 48 patients in this cohort have been described in our recent publication [10].

Mutation screening

Genomic DNA derived from bone marrow granulocytes, peripheral blood leukocytes, peripheral blood granulocytes or peripheral blood mononuclear cells were used for the screening of *CALR* exon 9 mutations spanning codons 352-417 [GenBank: NM_004343]. Oligonucleotide primers targeting *CALR* exon 9 were used to amplify a 285 bp product: (*CALR* Forward 5'-CCTGCAGGCAGCAGAGAAAC-3') (*CALR* Reverse 5'-ACAGAGACATTATTTGGCGCG-3'). The PCR were amplified using GoTaq Green Master Mix (Promega, CA, USA) on a Thermal Cycler® PCR System 2720 (Applied Biosystems, CA, USA). The final concentrations were as follows: 3 mM MgCl2 and 0.4 mM deoxyribo-nucleotide triphosphate, 2.5 μM each of forward and reverse oligo primer, 50 ng of DNA template and water to a final reaction volume of 20μl. Cycling parameters consisted of an initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30s, and extension at 72°C for 45s; and final extension at 72°C for 10 min. The EXO-SAP reagent (USB, CA, USA) was used to clean up the PCR product prior to sequencing. Direct DNA sequencing was conducted using the same primers for amplification and a BigDye terminator v3.1 Cycle sequencing kit (Applied Biosystems, CA, USA) on an ABI 3730 sequencer. Mutations were identified using DNA Dynamo sequence analysis software (Blue Tractor Software Ltd, Conwy, UK). All identified sequence variants were subjected to repeated bidirectional sequencing for confirmation. *CALR* exon 9 mutations were also independently screened by high-resolution melting analysis (HRMA) and TA-cloning was used to detected low allelic burden mutants in selected samples as previously described [47]. *JAK2*V617F mutation was determined by allele-specific PCR as previously described and/or mutation-enrich high sensitive PCR method over *JAK2* exon 14 mutation hot spot area [48, 49]. *MPL* exon 10 mutation was screened by nucleotide sequencing

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as previously described [50]. In order to exclude the influence of other possible mutations on B cell immune profiles, *DNMT3A* exon 23 and *IDH1/2* exon 4 mutations were also screened as previously described [50].

B cell immune profiles

The quantification of B cell populations and various B cell subsets including T1, T2, pre-germinal center, memory, and plasmablast/plasma cells, based upon the surface expression of CD19, CD24, CD27, CD38, and IgD was assessed by flow cytometric analysis as previously described [23]. Granulocytes and monocytes membrane-bound BAFF (mBAFF) levels, TLR4 expression and intracellular levels of IL-1β/IL-6 and the expression of CD69, CD80, and CD86 on B cells were quantified by flow cytometry using appropriated antibodies [23]. Serum BAFF concentration was measured by ELISA kit from R&D Systems according to the manufacturer's instructions. The B cell immune profiles of 38 patients in this cohort had been described in our previous publication [23]. B cell immune profiles from 48 healthy adults were used for comparison.

Statistical analysis

The correlation between *CALR* mutational status and clinical characteristics was calculated by the chi-square test or Fisher's exact test. Kolmogorov-Smirnov test was used to test normality of numerical variables. The independent *t*-test and the one-way analysis of variance (ANOVA) were used to compare differences between two and three independent groups when the dependent variables were normally distributed, respectively. When the dependent variables were not normally distributed, non-parametric Mann-Whitney U test and Kruskal-Wallis H test were used to compare differences between two and three independent groups, respectively. Spearman's rank correlation coefficient was used to evaluate the relationship between two variables. Multivariate analysis was performed using linear regression model adjusted for age, sex, follow-up period and hematological parameters. Statistical significance was defined as a two-sided p value < 0.05 and SPSS version 22.0 (IBM, New York, USA) was used for analyses.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Expression of CALR mutants causes mpl-dependent thrombocytosis in zebrafish

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CALR mutations are identified in about 30% of *JAK2*/*MPL*-unmutated myeloproliferative neoplasms (MPNs) including essential thrombocythemia (ET) and primary myelofibrosis. Although the molecular pathogenesis of *CALR* mutations leading to MPNs has been studied using *in vitro* cell lines models, how mutant CALR may affect developmental hematopoiesis remains unknown. Here we took advantage of the zebrafish model to examine the effects of mutant CALR on early hematopoiesis and model human *CALR*-mutated MPNs. We identified three zebrafish genes orthologous to human *CALR*, referred to as *calr*, *calr3a* and *calr3b*. The expression of CALR-del52 and CALR-ins5 mutants caused an increase in the hematopoietic stem/progenitor cells followed by thrombocytosis without affecting normal angiogenesis. The expression of CALR mutants also perturbed early developmental hematopoiesis in zebrafish. Importantly, morpholino knockdown of *mpl* but not *epor* or *csf3r* could significantly attenuate the effects of mutant CALR. Furthermore, the expression of mutant CALR caused jak-stat signaling activation in zebrafish that could be blocked by JAK inhibitors (ruxolitinib and fedratinib). These findings showed that mutant CALR activates jak-stat signaling through an mpl-dependent mechanism to mediate pathogenic thrombopoiesis in zebrafish, and illustrated that the signaling machinery related to mutant CALR tumorigenesis are conserved between human and zebrafish.

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INTRODUCTION

The *BCR-ABL*-negative classic myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders including polycythemia vera, essential thrombocythemia (ET) and primary myelofibrosis (PMF).[1] The *JAK2*V617F and *MPL* exon 10 mutations are two important driver mutations in MPNs and cause the activation of the JAK-signal transducer and activator of transcription (STAT) signaling that is central to the pathogenesis of MPNs.[2] Calreticulin (CALR) is a 46-kDa highly conserved, multicompartmental and multifunctional protein.[3] CALR has its role as a Ca^{2+}-binding chaperone protein and acts in concert with calnexin to ensure proper protein and glycoprotein folding in the endoplasmic reticulum (ER).[4] Recently, two research groups discovered *CALR* mutations in about 30% of *JAK2* and *MPL*-unmutated ET and PMF patients.[5,6] All *CALR* mutations are indels mutations in exon 9 and cause +1 base frameshift generating a novel C-terminus characterized by the loss of the ER retention signal KDEL and the change from acidic to basic amino-acid sequence. Although there are >50 *CALR* mutants identified in MPNs, the most prevalent types of *CALR* mutations are a 52 bp deletion (L367fs*46, type 1 mutation, CALR-del52) and a 5 bp insertion of TTGTC (K385fs*47, type 2 mutation, CALR-ins5) accounting for $>80\%$ of all patients with mutant *CALR*.[5,6] Most *CALR* mutations are mutually exclusive with the *JAK2* and *MPL* mutations, but some patients were found to have *JAK2* and *CALR* co-mutations.[7] ET and PMF patients with *CALR* mutations have been found to have different clinical characteristics such as younger age and higher platelet count and to carry a better prognosis than those patients with *JAK2*V617F mutation.[7–10]

Recent studies have focused on the underlying mechanism of *CALR* mutations in the pathophysiology of MPNs. With the use of *in vitro* cell lines and retroviral mouse models, *CALR* mutants were found to activate the JAK-STAT signaling in an MPL-dependent manner.[11–15] Although the expression of *CALR* mutants resulted in pathogenic thrombocytosis in adult mice, whether *CALR* mutants may disrupt normal hematopoiesis during early development remains unknown. The zebrafish is a useful disease model system and has been successfully utilized in studying hematopoiesis and leukemogenesis.[16–20] The early hematopoietic system in zebrafish involves two distinct primitive and definitive waves of development that is rapidly established within a few days after fertilization.[18] The developmental hematopoiesis of zebrafish also shows broad conservation with mammalian species and is regulated by conserved molecular pathways.[18] The transparency of zebrafish at the embryonic and larval stages has made it suitable for direct observation of the hematopoietic process. In addition, zebrafish can be used for *in vivo* high throughput screening due to its good permeability to chemical added to water during early developmental stages.[21–23] Here we aimed to evaluate the pathophysiologic effects of mutant CALR during embryonic hematopoietic development and to test the

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therapeutic effects of JAK inhibitors on mutant CALR using the *in vivo* zebrafish model.

MATERIALS AND METHODS

Zebrafish husbandry

Wild-type AB strain of zebrafish (*Danio rerio*) and the transgenic lines Tg (*cd41*:GFP)[24] and Tg(*fli1*:EGFP)[25] were maintained and manipulated with standard measure as previously described.[26] The stages of embryonic development were determined based on Kimmel *et al*.[27] Pigmentation was blocked by using 0.003% 1-phenyl-2-thiourea in some experiments. For pharmacologic inhibition, embryos were incubated with ruxolitinib (Abmole Bioscience, Houston, TX, USA) or fedratinib (Abmole Bioscience) from 1–2 cells stage to 5 days post fertilization (d.p.f.) with or without microinjection of *CALR* mRNA. The zebrafish experiments were approved by the MacKay Memorial Hospital Animal Care and Use Committee.

Identification of zebrafish ortholog of human CALR

Human genes located in 19p13.11-13.2 were identified using the National Center for Biotechnology Information (NCBI) Map Viewer.[28] Genes surrounding the three zebrafish *calr* genomic regions were identified using Ensembl[29] and Synteny database.[30] Human CALR protein sequence was used to BLASTP against zebrafish GRCz10 using the Ensembl platform (Ensembl release 82).[29] Alignment and comparative analysis between protein sequences was performed using the Clustal Omega algorithm[31] and edited by GeneDoc.[32]

Human and zebrafish CALR cDNAs cloning and mRNA synthesis

Full-length CALR cDNAs were subcloned in the pCS2^{+} vector and into a bicistronic pSYC-102 T2A vector (a gift from Dr Seok-Yong Choi) replacing the mCherry-CAAX reporter gene using the In-Fusion Cloning Kit (Clontech, Mountain View, CA, USA) (Supplementary Figure S1).[33] All vector sequences were verified by sequencing. The mMessage mMachine SP6 kit (Ambion, Austin, TX, USA) was used for *in vitro* transcription of capped mRNAs from vectors according to the manufacturer's protocol. mRNAs from the bicistronic pSYC-102-CALR vectors were only used to express EGFP and CALR concurrently in wild-type zebrafish embryos and only embryos expressing green fluorescence were collected under fluorescence microscope for use in the reverse transcription and real-time PCR.

Morpholinos and microinjection

Morpholinos (MOs) blocking splicing of *mpl* and *epor*, and translation (ATG/5′UTR) of *csf3r* were purchased from Gene Tools (Philomath, OR, USA; MO sequences are listed in Table 1).[24,34,35] Standard control MO was used as negative control. Embryos at the 1–2 cells stage were injected with MO (1 ng) or mRNAs (100 pg). Co-injection of each MO and *CALR* mutant mRNA was performed in a subset of embryos.

Reverse transcription and real-time PCR

Total RNA was extracted from embryos using miTotal Miniprep System (Viogene, New Taipei City, Taiwan) and reverse transcribed using a High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Primer sequences are listed in Supplementary Table 1. Fast SYBR Green Master Mix (Applied Biosystems) was used for real-time quantitative PCR according to the manufacturer's instructions.

Western blotting

Total proteins were extracted from zebrafish embryos at 24 h post fertilization (h.p.f.). Equal amounts of protein were denatured and electrophoresed. Membranes were immunoblotted with the following primary antibodies: CALR (Abcam, Cambridge, UK; recognizing N-terminal sequences of both human and zebrafish wild-type CALR proteins), gapdh and customized mutant CALR (GeneTex, Hsinchu City, Taiwan; specifically recognizing human CALR exon 9 indel mutant protein sequence), STAT5 (Santa Cruz, Dallas, TX, USA) and phospho-STAT5 (Cell Signaling, MA, USA).

Imaging

Live embryos were imaged using a Leica MSV269 fluorescence stereomicroscope and photographed using a Leica DFC425 C digital camera and Leica Application Suite software (Leica Microsystems, Wetzlar, Germany). GraphPad Prism 7 software and ImageJ (National Institutes of Health) were used to process images.

Statistical analysis

The Student *t*-test or analysis of variance test were used. Data were expressed as mean ± s.e.m. Significance was determined at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

RESULTS

Zebrafish ortholog of human CALR

To search for the zebrafish ortholog of human *CALR* gene, human CALR protein sequence was used to BLASTP against zebrafish GRCz10 (Ensembl release 82). We identified three annotated zebrafish orthologs of the human *CALR* gene (ENSG000001792), *calr* (ENSDARG00000076290 at chromosome 8), *calr3a* (ENSDARG00000103979 at chromosome 22) and *calr3b* (ENSDARG00000102808 at chromosome 2). After comparative analysis using the Clustal Omega algorithm, the amino-acid sequence of zebrafish calr, calr3a and calr3b proteins shares an overall 75%, 71% and 70% identity to human CALR protein sequence, respectively. The three functional domains in CALR are conserved in all three zebrafish calr proteins, including the KDEL ER retention signal at the C-terminus (Figure 1a). In addition, the genomic loci surrounding human chromosome 19p13.2 containing the *CALR* gene are syntenic with the regions of zebrafish *calr* on chromosome 8, *calr3a* on chromosome 22 and *calr3b* on chromosome 2 based on the search in NCBI Map Viewer, Ensembl database and Synteny database (Figure 1b). These results indicated that the three zebrafish *calr* genes are likely true orthologs of human *CALR*.

Effects of mutant CALR expression on thrombopoiesis and angiogenesis in zebrafish

For the expression of mRNA in zebrafish embryo, we first performed a dose-finding study ranging from 50 to 200 pg *CALR* mRNA. Phenotype could be observed at dose of 100 pg mRNA per embryo which was compatible with normal development for most embryos. All CALR proteins were adequately expressed at comparable amount at dose of 100 pg (Figure 2a, middle panel). The expression of CALR-del52 and CALR-ins5 mutant proteins was

Table 1. Morpholino sequences for *mpl*, *epor* and *csf3r* knockdown

Gene	*Target*	*ZFIN identity*	*MO sequence*
mpl	Intron1/exon2 boundary of exon 2	ZDB-MRPHLNO-060421-1	5′-CAGAACTCTCACCCTTCAATTATAT-3′
epor	Intron1/exon2 boundary of exon 2	ZDB-MRPHLNO-080325-2	5′-AACTGGGCCACTGAACAATCAAATT-3′
csf3r	ATG/5′UTR	ZDB-MRPHLNO-111213-1	5′-GAAGCACAAGCGAGACGGATGCCAT-3′
Standard control	Human beta-globin intron mutation	NA	5′-CCTCTTACCTCAGTTACAATTTATA-3′

Abbreviations: NA, not applicable; ZFIN, Zebrafish International Resource Center.

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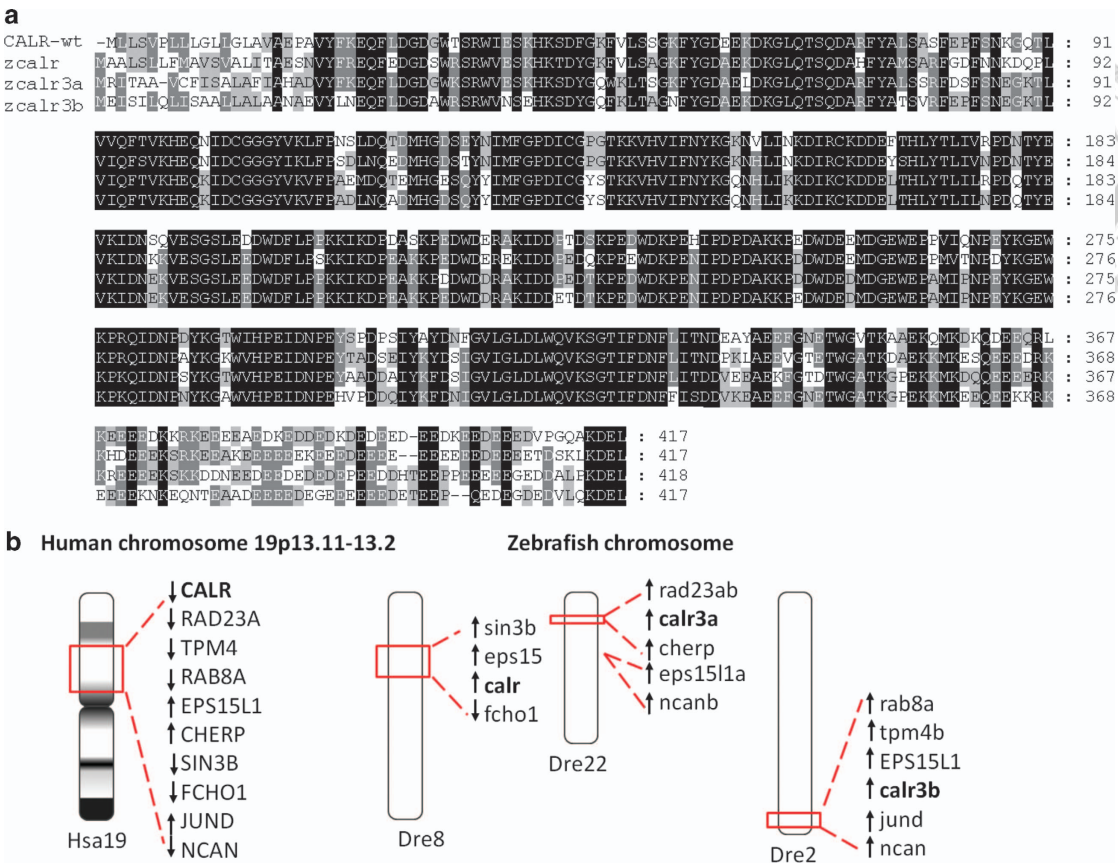

Figure 1. Identification of three zebrafish *calr* genes. (**a**) Alignment of human CALR (top row), zebrafish calr (second row), calr3a (third row) and calr3b (bottom row) protein sequences. The regions of sequence identity in the four proteins are shaded. (**b**) The genomic loci surrounding human *CALR* on chromosome 19p13.2 (Hsa19) are syntenic with the regions where zebrafish *calr* (on chromosome 8, Dre8), *calr3a* (on chromosome 22, Dre22) and *calr3b* (on chromosome 2, Dre2) are located in the zebrafish genome, respectively.

also confirmed by mutant CALR-specific antibody (Figure 2a, top panel). Therefore, 100 pg mRNA was injected throughout the study. To determine whether mutant CALR had an effect on hematopoietic stem and progenitor cells (HSPCs) in zebrafish, we injected the three mRNAs encoding *CALR* wild-type (CALR-wt), *CALR*-del52, and *CALR*-ins5 into 1–2 cells stage embryos of the *cd41*:GFP line, and the numbers of CD41$^+$ cells in the caudal hematopoietic tissue (CHT) at 3 d.p.f. indicating the HSPCs were counted.[24] Expression of both *CALR*-del52 and *CALR*-ins5 mutant mRNA significantly increased the numbers of HSPCs in the CHT when compared with *CALR*-wt mRNA (Figure 2b). However, the numbers of HSPCs did not have statistically significant difference between *CALR*-del52 and *CALR*-ins5 mutant groups at this developmental stage. To ascertain that the increase of HSPCs was not affected by the change in angiogenesis during early development, mRNAs encoding *CALR*-wt, *CALR*-del52, and *CALR*-ins5 were injected into 1–2 cells stage embryos of the *fli1*:EGFP line. No obvious changes in the angiogenesis were visualized in CALR-wt and mutant CALR expressing embryos at 3 d.p.f. when compared with uninjected control (Figure 2c). To determine whether CALR had an effect on mature thrombocyte, the numbers of CD41$^+$ thrombocytes in the *cd41*:GFP line were counted at 5 d.p.f. after injection. Mutant *CALR*-del52 significantly increased the number of CD41$^+$ thrombocytes (mean 162.5 ± 4.1 per embryo) when compared with *CALR*-wt (mean 117.1 ± 3.1 per embryo, $P < 0.001$), mutant *CALR*-ins5 (mean 128.3 ± 6.1 per embryo, $P < 0.001$) and uninjected control (mean 136.7 ± 3.0 per embryo, $P < 0.001$; Figure 2d). Although mutant *CALR*-ins5 slightly increased the number of CD41$^+$ thrombocytes when compared with *CALR*-wt, there was no statistically significant difference. Altogether, our data demonstrated that the effect of mutant CALR on thrombopoiesis in zebrafish is dependent on the presence of the novel C-terminus and is also related to specific CALR mutant protein sequences.

Mutant CALR requires mpl to cause thrombocytosis in zebrafish

To test whether cytokine receptors are involved in the pathogenesis of thrombocytosis caused by mutant CALR in zebrafish, *mpl*, *epor* and *csf3r* MOs (each with 1 ng) were injected in 1–2 cells stage embryos of *cd41*:GFP line and assayed for their effects on the number of CD41$^+$ thrombocytes at 5 d.p.f. Co-injection of *CALR*-del52 mutant mRNA (100 pg) with each MO was also performed in a subset of embryos. At 5 d.p.f., the number of CD41$^+$ thrombocytes significantly decreased upon *mpl* knockdown (mean 43.6 ± 4.9 per embryo) when compared with the control MO group (mean 123.5 ± 5.9 per embryo, $P < 0.001$) and the mutant *CALR*-del52 group ($P < 0.001$; Figure 3). Importantly, co-injection of *CALR*-del52 mutant mRNA (mean 73.7 ± 5.1 per embryo) can only partially reverse the knockdown effect of *mpl* MO. In contrast, the numbers of CD41$^+$ thrombocytes did not decrease significantly upon *epor* MO (mean 110.6 ± 5.5 per embryo) or *csf3r* MO (mean 116.6 ± 5.6 per embryo) knocked down compared with the control MO group. When *CALR*-del52 mutant mRNA was co-injected with *epor* (mean 151.7 ± 9.2 per

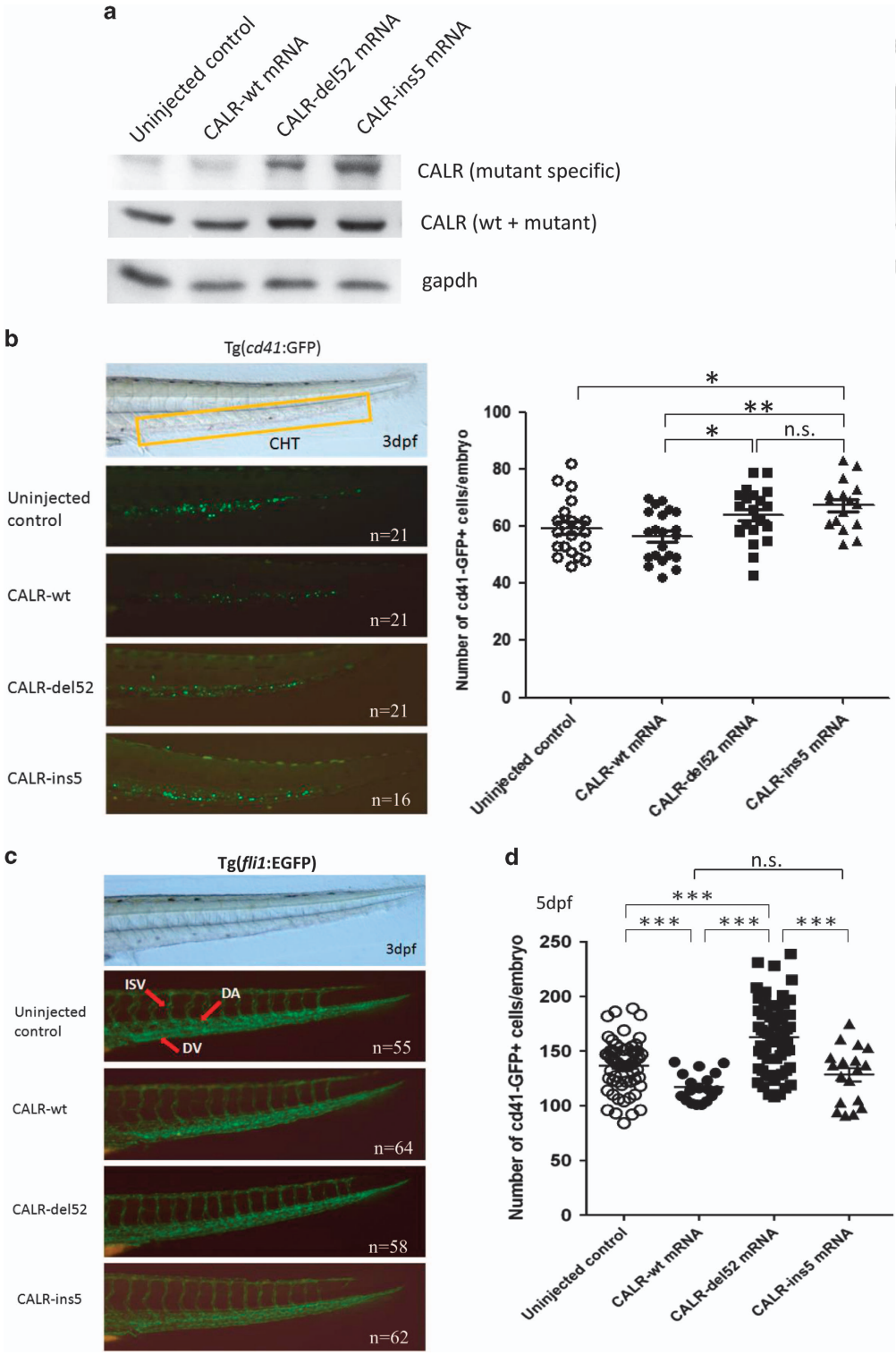

Figure 2. Effects of the expression of mutant CALR on the number of hematopoietic stem/progenitor cells and angiogenesis. (**a**) CALR protein expression in uninjected, *CALR*-wt-, *CALR*-del52- or *CALR*-ins5-injected embryos at 24 h.p.f. gapdh was used to normalize the total amount of protein in each sample. The expression of human CALR mutant proteins was confirmed in *CALR*-del52- and *CALR*-ins5-injected embryos by a customized human CALR mutant-specific polyclonal antibody (top panel). (**b**) Brightfield image (left panel) of a 3 d.p.f. embryo with a box area indicating caudal hematopoietic tissue where $CD41^+$ cells were counted. Green cells in the darkfield images (left panel) indicated expression of GFP under the control of the *cd41* promoter, and were counted and showed on the right panel. (**c**) The development of dorsal aorta (DA), dorsal vein (DV) and intersegmental vessel (ISV) as indicated by the red arrows (top panel) in uninjected, *CALR*-wt-, *CALR*-del52- or *CALR*-ins5-injected embryos of the Tg(*fli1*:EGFP) line at 3 d.p.f. (**d**) The total numbers of $CD41^+$ thrombocytes were counted in uninjected, *CALR*-wt-, *CALR*-del52- or *CALR*-ins5-injected embryos of the Tg(*cd41*:GFP) line at 5 d.p.f. (the direction of embryos was anterior to the left, dorsal upwards, lateral view; n.s., not significant; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Student *t*-test). The number of embryos used in each experiment is indicated by 'n' in figures.

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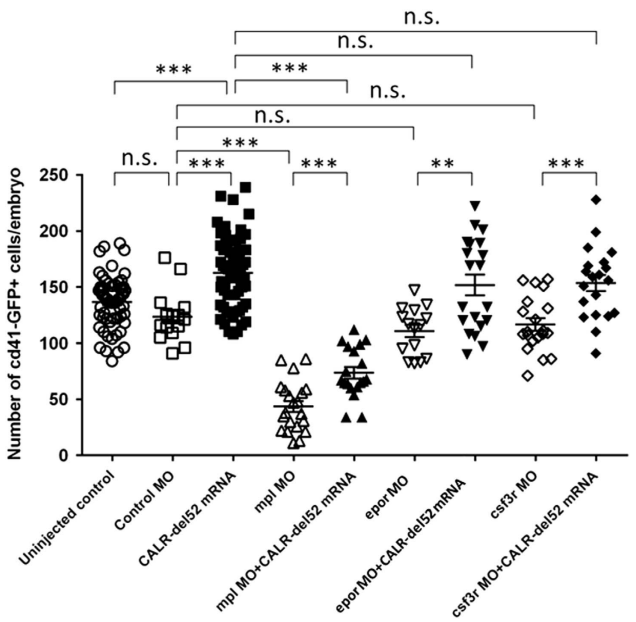

Figure 3. Mutant CALR requires mpl to cause thrombocytosis in zebrafish. Morpholinos (MOs) targeting *mpl*, *epor* or *csf3r* (1 ng per embryo) were injected into 1–2 cells stage embryos of the Tg(*cd41*:GFP) line with or without co-injection of *CLAR*-del52 mRNA (100 pg). Standard control MO was used as negative control. The total numbers of $CD41^+$ thrombocytes were counted at 5 d.p.f. and compared as indicated. The number of $CD41^+$ thrombocytes significantly decreases upon *mpl* knockdown when compared with the control MO group as well as the *CALR*-del52 group. Co-injection of *CALR*-del52 mRNA can only partially reverse the knockdown effect of *mpl* MO. When *CALR*-del52 mRNA was co-injected with *epor* MO or *csf3r* MO, the numbers of $CD41^+$ thrombocytes were comparable to those of *CALR*-del52-injected embryos (n.s., not significant; $^{**}P < 0.01$, $^{***}P < 0.001$; Student *t*-test).

embryo) or *csf3r* (mean 153.6 ± 7.2 per embryo) MOs, the numbers of $CD41^+$ thrombocytes were comparable to those of *CALR*-del52–injected embryos (both $P = 0.3$). Collectively, these findings indicated that the expression of mutant CALR causes thrombocytosis through an mpl-dependent mechanism in zebrafish.

Effects of CALR mutants on lineage-specific and cytokine gene expression

The increase in thrombopoiesis upon expression of mutant CALR prompted us to evaluate their effects on hematopoietic lineage-specific, thrombopoiesis,[36] cytokine and cytokine receptor gene expression in zebrafish embryos at 3 d.p.f. The expression of HSC gene *runx1* was significantly upregulated in *CALR*-ins5 group but was modestly downregulated in *CALR*-del52 group (Table 2). Also, the expression of *c-myb* and *scl* was only downregulated in *CALR*-del52 group. Although *gata1* was modestly downregulated in mutant CALR groups, the expression of *α-eHb* and *β-eHb* was not affected by both CALR mutants. The expression of early (*spi1b)* and late myeloid (*mpo*: granulocytic; *l-plastin*: macrophage) lineage genes, *epo* and *epor* showed no significant changes. However, the expression of lymphoid lineage genes (*rag1*, *rag2* and *lck*) was modestly downregulated in mutant CALR groups. Although the expression of *mpl* was significantly downregulated in both mutant CALR groups, both *tpo* and *csf3r* expressions were only downregulated in *CALR*-del52 group. In the group of genes related to thrombopoiesis, only the expression of *nbeal2* was significantly downregulated in *CALR*-del52 group.

Effects of mutant CALR on jak-stat signaling in zebrafish

We then investigated whether the expression of mutant CALR can activate the jak-stat signaling in zebrafish. The expression of *CALR*-del52 mRNA significantly increased stat5 phosphorylation (Figure 4a, lane 2). Furthermore, treatment with ruxolitinib and fedratinib significantly ameliorated the enhanced stat5 phosphorylation induced by *CALR*-del52 mRNA (Figure 4a, lane 3 and 4). In addition, treatment with ruxolitinib significantly decreased the numbers of $CD41^+$ thrombocytes in uninjected control as well as *CALR*-del52-injected embryos in a dose-dependent manner (Figure 4b). Whereas treatment with fedratinib only had minimal inhibitory effect on the number of $CD41^+$ thrombocytes in uninjected control embryos, and had a modest and significant dose-independent inhibitory effect on mutant CALR-induced thrombocytosis (Figure 4c). Our results demonstrated that mutant CALR-mediated pathogenic thrombopoiesis involves jak-stat activation that can be blocked by JAK inhibitors.

DISCUSSION

In this study, we have used the zebrafish animal model to examine the pathogenesis of mutant CALR in MPNs. We first identified three zebrafish orthologs for human *CALR* gene. We have shown that expression of the CALR-del52 mutant disturbs thrombopoiesis and increases the number of HSPCs in the CHT followed by significant thrombocytosis in the zebrafish embryo. These findings are consistent with the myeloproliferative phenotype in retroviral mouse bone marrow transplantation models elicited by mutant CALR expression characterized by thrombocytosis and megakaryocytic hyperplasia recapitulating those seen in patients with ET and myelofibrosis.[12,14]

The highly conserve protein sequences between human *CALR* and the three zebrafish *calr* genes suggested functional conservation between human and zebrafish *CALR*. Ma *et al.*[37] recently reported that MO knockdown of *calr* perturbs myeloid and HSCs lineages during zebrafish embryonic development including a decrease in the expression of genes associated with myeloid lineages at 24 h.p.f. and an increase in the expression of *cmyb* at 48, 72 and 96 h.p.f. We have also shown that the expression of genes involved in lineage-specific hematopoiesis, thrombopoiesis, cytokines and cytokine receptors could be perturbed by the expression of mutant CALR in zebrafish during early development. These data suggested that zebrafish *calr* genes have an important role in the regulation of vertebrae hematopoiesis. In addition, our data suggested that mutant CALR does not promote thrombopoiesis through the upregulation of *mpl* and *tpo* levels. Rather, the downregulation of *mpl* and *tpo* might represent a negative-feedback mechanism related to increased thrombopoiesis due to mutant CALR expression.

On the basis of the data from the murine and zebrafish animal models, the causative relationship between CALR mutations and thrombocytosis can be confirmed, and *CALR* mutations have been established as one of the driver mutations in MPNs. Furthermore, we demonstrated that expression of *CALR*-del52 (type 1 mutation) causes higher thrombocyte count than *CALR*-ins5 (type 2 mutation) at 5 d.p.f. in zebrafish embryo. Similar finding has been reported in murine model that marked thrombocytosis was rapidly induced in *CALR*-del52-expressing mice and then progressed to myelofibrosis, and *CALR*-ins5-expressing mice only developed modest thrombocytosis resembling mild ET phenotype.[12] This is consistent with the clinical finding that *CALR*-del52 mutation is more frequently detected in PMF than in ET,[5] and also confirms the differential effects of *CALR* variants on thrombopoiesis and clinical phenotypes.[10,38,39]

To further elucidate the molecular pathogenesis of mutant *CALR* in our zebrafish model, we have used MO knockdown experiments to show that only the *mpl* MO can significantly

Table 2. Effects of *CALR* mutant mRNA injection on the expression of genes in zebrafish embryo genes involved in lineage-specific hematopoiesis, thrombopoiesis, cytokines and cytokine receptors were examined based on real-time quantitative PCR of zebrafish embryos at 3 days post fertilization, with reference to that of *CALR* wild-type mRNA injection

Category	*Gene*	*CALR-wt*[a]	*CALR-del52* Mean ± s.e.m.	*CALR-ins5* Mean ± s.e.m.	*CALR-wt* vs *CALR-del52* vs *CALR-ins5* P-*value*[b]	*CALR-wt* vs *CALR-del52* P-*value*[c]	*CALR-wt* vs *CALR-ins5* P-*value*[c]	*CALR-del52* vs *CALR-ins5* P-*value*[c]
Hematopoietic stem cells	*cmyb*	1.00	0.73 ± 0.04	1.07 ± 0.02	< 0.001	0.02	NS	0.001
	runx1	1.00	0.78 ± 0.06	1.20 ± 0.06	0.003	NS	0.029	0.008
Hemangioblast	*scl*	1.00	0.75 ± 0.08	0.91 ± 0.05	0.043	NS	NS	NS
	lmo2	1.00	0.85 ± 0.06	0.99 ± 0.09	NS	NS	NS	NS
Erythropoiesis	*gata1*	1.00	0.87 ± 0.01	0.89 ± 0.02	0.002	0.012	0.036	NS
	α eHb	1.00	0.82 ± 0.11	0.89 ± 0.11	NS	NS	NS	NS
	β eHb	1.00	0.73 ± 0.12	0.96 ± 0.22	NS	NS	NS	NS
Vasculature	*fli1*	1.00	0.76 ± 0.06	0.84 ± 0.08	NS	0.017	NS	NS
Early myelomonocytic lineage	*spi1b*	1.00	1.13 ± 0.10	1.01 ± 0.10	NS	NS	NS	NS
Late myelomonocytic lineage	*L-plastin*	1.00	0.99 ± 0.17	0.94 ± 0.09	NS	NS	NS	NS
Lymphoid lineage	*rag1*	1.00	0.56 ± 0.07	1.01 ± 0.12	0.012	0.003	NS	0.033
	rag2	1.00	0.96 ± 0.07	0.84 ± 0.03	NS	NS	0.01	NS
	lck	1.00	0.50 ± 0.06	1.08 ± 0.13	0.006	0.015	NS	0.017
Thrombopoiesis	*arhgef3*	1.00	0.80 ± 0.09	0.90 ± 0.07	NS	NS	NS	NS
	emilin1a	1.00	0.90 ± 0.03	0.91 ± 0.04	NS	0.036	NS	NS
	nbeal2	1.00	0.64 ± 0.10	1.08 ± 0.06	0.007	NS	NS	0.019
	max	1.00	0.88 ± 0.03	0.87 ± 0.06	NS	0.021	NS	NS
Cytokines and cytokine receptors	*tpo*	1.00	0.88 ± 0.05	1.12 ± 0.05	0.016	NS	NS	0.026
	mpl	1.00	0.57 ± 0.05	0.52 ± 0.06	0.001	0.014	0.002	NS
	epo	1.00	0.99 ± 0.10	1.12 ± 0.23	NS	NS	NS	NS
	epor	1.00	0.80 ± 0.10	0.96 ± 0.09	NS	NS	NS	NS
	mpo	1.00	0.85 ± 0.09	0.84 ± 0.06	NS	NS	NS	NS
	csf3r	1.00	0.62 ± 0.04	1.15 ± 0.14	0.01	0.001	NS	0.021

Abbreviations: ANOVA, analysis of variance; NS, not significant; wt, wild-type. Data are from triplicate results. [a]Data of *CALR*-wt was arbitrarily set to 1.00 in all cases. [b]ANOVA test. [c]Student *t*-test.

attenuate the effect of mutant CALR on thrombopoiesis. Both *epor* and *csf3r* MOs were not able to inhibit the effect of mutant CALR. These findings indicated that mpl has an essential and specific role required by mutant CALR to cause thrombocytosis in zebrafish. Because CALR is physiologically functioning as a chaperone for MPL, it is reasonable to speculate that mutant CALR may interact directly with mpl to cause thrombocytosis in zebrafish. Our data are consistent with those recently reported by several groups of researchers using *in vitro* cell line models.[11–15] In these studies, both the novel C-terminus of CALR mutants and the direct interaction of mutant CALR with MPL receptor are required to activate MPL and the downstream JAK-STAT signaling, which in turn is responsible for cytokine-independent growth of Ba/F3-MPL and UT-7/TPO cell lines. Chachoua *et al.*[13] reported that the specific activation of MPL receptor by mutant CALR required both the presence of extracellular *N*-glycosylation residues of MPL and the glycan-binding site at the novel C-terminus of mutant CALR. In addition, Elf *et al.*[14] reported that the physical interaction between mutant CALR and MPL is dependent on the positive electrostatic charge of the C-terminus of the mutant CALR but not dependent on specific novel C-terminal sequence. Recently, Balligand *et al.*[40] reported similar finding that highly similar but not identical murine *Calr* exon 9 frameshift mutants also require Mpl interaction to activate the JAK-STAT signaling. Moreover, the positive charge predominant novel C-terminus of the mutant CALR results in different calcium-binding capacity, which may alter calcium homeostasis and signaling processes in mutant cells. All these structural differences and changes will likely contribute to the different clinical phenotypes seen in different *CALR* variants.[10,38,39,41]

We have also demonstrated that the expression of human CALR mutant is able to activate jak-stat signaling in zebrafish. In addition, jak-stat signaling in zebrafish can also be inhibited by JAK2 inhibitors used in clinical trials illustrating that the conserved signaling machinery in human and zebrafish.[42–44] Our data showed that ruxolitinib treatment results in a dose-dependent inhibitory effect on both normal thrombopoiesis and thrombocytosis caused by mutant CALR in zebrafish. By contrast, JAK2-selective inhibitor fedratinib has only minimal inhibitory effects on normal thrombopoiesis but has modest and dose-independent inhibitory effect on thrombocytosis caused by mutant CALR. Our data suggested that fedratinib can normalize the thrombocytosis caused by the expression of mutant CALR and does not cause significant thrombocytopenia in zebrafish model. These observations are comparable to the findings that both ruxolitinib and fedratinib have been demonstrated to have clinical responses in MPN patients harboring *CALR* mutations.[45–47] However, fedratinib has less hematological toxicities than ruxolitinib especially thrombocytopenia, which is a dose-limiting toxicity of ruxolitinib.[42–44] Despite both JAK inhibitors are effective in the reduction of splenomegaly and the relief of clinical symptoms, they are not likely to substantially modify the natural history of the *BCR-ABL*-negative classic MPNs including *CALR*-mutated PMF. Importantly, these JAK inhibitors are not specifically designed for *JAK2*V617F mutation. However, the unique pathogenic mechanism of mutant CALR in MPNs has led to the possibility

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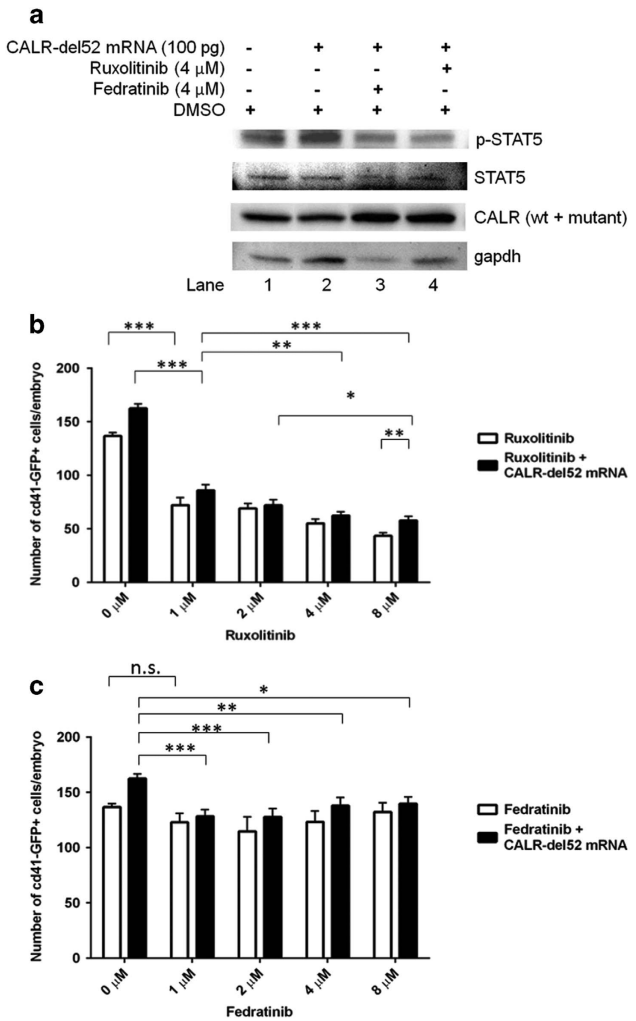

Figure 4. The expression of mutant CALR activates jak-stat signaling in zebrafish. (**a**) Western blotting showing embryos of wild-type zebrafish injected with *CALR*-del52 mRNA (100 pg) have significantly increased signal transducer and activation of transcription (stat) 5 phosphorylation (lane 2) as compared with uninjected control embryos from the same batch at 24 h.p.f. (lane 1). Pharmacologic treatment of embryos with a JAK2-selective inhibitor (fedratinib) and a dual JAK1/JAK2 inhibitor (ruxolitinib) significantly attenuated the enhanced stat5 phosphorylation induced by *CALR*-del52 mRNA stat5 phosphorylation (lane 3 and 4, respectively). Total amount of stat5 protein was not affected in all experiments. (**b** and **c**) Effects of the pharmacologic treatment with ruxolitinib and fedratinib (from 1 μM to 8 μM) on the numbers of $CD41^+$ thrombocytes at 5 d.p.f. with or without the injection of *CALR*-del52 mRNA. Treatment with ruxolitinib significantly decreases the numbers of $CD41^+$ thrombocytes in uninjected control as well as *CALR*-del52-injected embryos in a dose-dependent manner. Whereas treatment with fedratinib has minimal and insignificant inhibitory effect on the number of $CD41^+$ thrombocyte in uninjected control embryos, and has a modest and significant dose-independent inhibitory effect on mutant CALR-induced thrombocytosis (n.s., not significant; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Student *t*-test).

of new therapeutic approach targeting the interaction and binding between mutant CALR and MPL. In this regard, our results highlight the advantage and support the use of zebrafish as a relevant *in vivo* whole-organism model for the testing and screening of therapeutic compounds targeting mutant CALR.[22]

In conclusion, we have used the zebrafish model to show that mutant CALR promotes the activation of jak-stat signaling through an mpl-dependent mechanism to mediate pathogenic thrombopoiesis during zebrafish early hematopoiesis. These findings are consistent with those observed in *in vitro* cell line and mouse models and illustrated that the signaling machinery related to mutant CALR tumorigenesis are conserved between human and zebrafish. Zebrafish has also been shown to be a relevant *in vivo* model for the development of novel therapeutic compounds targeting mutant CALR. Future studies using stable mutant CALR transgenic or knock-in zebrafish models for this purpose will be warranted.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DISCLAIMER

All the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

KHL and YYK conceived the project. KHL, YCC, YHC, HCL, CGC, YYK and WCC participated in its design, results discussion and edited the manuscript. KHL, CYC, CSL, LH, WTW and TYL performed the experiments and analyzed the data. KHL and LH drafted the manuscript. All authors approved the manuscript.

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