Experimental Hematology

Experimental Hematology 2016;44:817–825

Calreticulin mutation does not contribute to disease progression in essential thrombocythemia by inhibiting phagocytosis

Shinya Daitoku[a], Katsuto Takenaka[a], Takuji Yamauchi[a], Ayano Yurino[a], Fumiaki Jinnouchi[a], Takuya Nunomura[a], Tetsuya Eto[b], Tomohiko Kamimura[c], Masakazu Higuchi[d], Naoki Harada[e], Noriyuki Saito[b], Toshihiro Miyamoto[a], Hiromi Iwasaki[f], and Koichi Akashi[a,f]

[a]*Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan;* [b]*Department of Hematology, Hamanomachi Hospital, Fukuoka, Japan;* [c]*Department of Hematology, Hara-Sanshin Hospital, Fukuoka, Japan;* [d]*Department of Hematology, Japan Community Health Care Organization Kyushu Hospital, Fukuoka, Japan;* [e]*Department of Hematology, National Hospital Organization Kyushu Medical Center, Fukuoka, Japan;* [f]*Center for Cellular and Molecular Medicine, Kyushu University Hospital, Fukuoka, Japan*

(Received 3 March 2016; revised 29 April 2016; accepted 4 May 2016)

Somatic mutations of calreticulin (CALR) have been observed in many cases of essential thrombocythemia (ET) or primary myelofibrosis that harbor non-mutated Janus kinase 2 (JAK2). CALR mainly localizes within the endoplasmic reticulum lumen, but a small fraction of the total CALR pool is distributed over the cell surface. Cell surface CALR is known to transduce prophagocytic "eat me" signals to macrophages and acts as one of the important regulators for macrophage engulfment. In this study, we attempted to clarify whether mutant CALR may affect the threshold for macrophage engulfment and play an integral role in the pathogenesis of CALR-mutated ET. First, we compared the surface expression levels of CALR on hematopoietic stem and progenitor cells (HSPCs) and mature blood cells in patients with myeloproliferative neoplasms and found that the surface expression of mutant CALR did not change. Next, we compared the threshold for macrophage phagocytosis of each HSPC fraction and mature blood cells and found no significant change in the efficiency of macrophage engulfment. Our data suggest that CALR mutation does not affect sensitivity to phagocytosis by macrophages. Finally, we analyzed the phosphorylation statuses of molecules downstream of JAK2 at each HSPC level in patients with ET and found that CALR mutations activated the JAK-STAT pathway in a manner similar to that associated with JAK2 mutations. These results indicate that mutant CALR causes myeloproliferation because of the activation of JAK-STAT pathway and not by the inhibition of phagocytosis, which is similar to the myeloproliferation caused by JAK2 V617F mutation.

Myeloproliferative neoplasms (MPNs) are chronic hematopoietic stem cell (HSC) disorders characterized by an excess of mature myeloid lineage cells (i.e. granulocytes, erythrocytes, and/or platelets) [1]. The *Janus kinase 2* (*JAK2*) gain-of-function mutation is the gene mutation most frequently involved in the pathogenesis of *breakpoint cluster region* (*BCR*)–*Abelson leukemia virus* (*ABL*)-negative MPNs such as polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) [2–6]. Previous reports suggested that *JAK2* V617F definitely induces MPNs and that phosphorylated signal transducers and activators of transcription 5 (STAT5s) play a crucial role in MPN pathogenesis. However, although a *JAK2* mutation is detected in most PV cases, approximately 50% of all ET and PMF cases harbor the wild-type *JAK2*; other mutations associated with these cases remain unclear.

Using whole-exome sequencing, a somatic mutation in *calreticulin* (*CALR*) exon 9 was recently detected in a considerable number of ET or PMF cases harboring a non-mutated *JAK2* or non-mutated *myeloproliferative leukemia virus oncogene* (*MPL*) [7,8]. CALR mainly localizes within the endoplasmic reticulum (ER) lumen and is involved in

---

Offprint requests to: Koichi Akashi, M.D., Ph.D., Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan; E-mail: akashi@med.kyushu-u.ac.jp

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exphem.2016.05.001.

http://dx.doi.org/10.1016/j.exphem.2016.05.001

lectin-like chaperoning of proteins and glycoproteins [9,10]. The vast majority of *CALR* exon 9 mutations found in MPN cases can be divided into two types, a 52-base pair (bp) deletion or type 1 mutation, which is the most common type, and a 5-bp insertion or type 2 mutation. Regardless of the type, all *CALR* mutations cause a common frame shift and result in an abnormal peptide sequence in the C-domain of CALR. This mutant CALR loses its calcium-ion-binding capacity and the sequence necessary for retention and retrieval into the ER lumen and is known to cause a change in the intracellular distribution [7,8].

Klampfl et al. showed that type I *CALR* mutations contribute to hypersensitivity to interleukin-3 (IL-3), IL-3-independent proliferation, and constitutive STAT5 phosphorylation when transduced into an IL-3-dependent cell line [7], a situation that closely resembles the *JAK2* V617F mutation [3,6]. Moreover, an earlier gene expression study of peripheral blood (PB) granulocytes from patients with MPN showed universally activated JAK2 signaling, regardless of the *JAK2* or *CALR* mutational status and a significantly enriched gene expression signature in patients with *CALR*-mutated MPN compared with those with JAK2-mutated MPN [11]. These observations indicated that the extent of JAK-STAT pathway activation by *CALR* mutations was similar to that by *JAK2* mutations in the pathogenesis of MPNs. Recently, several studies revealed the direct interaction between CALR and MPL by using cell lines transfected with mutant CALR [12–15]. Mutant CALR binds to the extracellular domain of MPL and induces the dimerization of MPL and the phosphorylation of JAK2. These interactions activate the JAK-STAT pathway and result in enhanced megakaryopoiesis. These findings indicate that both the *CALR* and the *JAK2* mutation cause JAK-STAT pathway activation and contribute to the pathogenesis of MPNs; however, several studies have described differences between the clinical features of MPNs harboring mutant *CALR* and those harboring mutant *JAK2*. Patients with the *CALR* mutation (which is more frequently observed in males) tended to be younger at the time of diagnosis and have lower white blood cell counts, a lower hemoglobin concentration, higher platelet counts, and a lower thrombosis occurrence rate [16–30]. These findings indicate that mutant *JAK2* and *CALR* may induce myeloproliferation via different mechanisms.

A small fraction of the total CALR pool is distributed over the cell surface [31]. These molecules have a function distinct from that of ER-residing CALR and are known to transduce prophagocytic "eat me" signals to phagocytes by binding to low-density lipoprotein receptor-related protein 1 (LRP1) on phagocytes [31–39]. On apoptotic cells, surface CALR acts as a receptor when conjugated with LRP1 and stimulates phagocytes to promote efficient apoptotic cell removal. Therefore, we hypothesized that the *CALR* mutation changes the cell surface expression level of CALR and affects intracellular signaling, which mediates phagocytic signals, resulting in the inhibition of phagocytic removal of myeloid cells and facilitating excess myeloproliferation.

Herein, we have attempted to elucidate the pathogenesis of ET cases harboring *CALR* mutations relative to those harboring *JAK2* mutations from a standpoint of phagocytic regulation.

Methods

Patients and samples

We obtained bone marrow (BM) or PB samples from patients diagnosed with *BCR-ABL*-negative MPNs, including PV, ET, and PMF, at institutions affiliated with the Fukuoka Blood and Marrow Transplantation Group and from healthy volunteers after obtaining their written informed consent. This study was approved by the ethics committee of each institution. Diagnoses of MPNs were made in accordance with the categories specified in 2008 by the World Health Organization. For DNA extraction, granulocytes were isolated from PB by dextran sedimentation, hypotonic lysis of contaminating erythrocytes, and centrifugation with lymphocyte separation medium (MP Biomedicals, Irvine, CA), as described previously [40].

Mutation analysis and DNA sequencing of JAK2, CALR, and MPL

Genomic DNA was obtained from PB cells with a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Genomic regions of interest were amplified by polymerase chain reaction (PCR) using pfu turbo DNA polymerase (Agilent Technologies, Santa Clara, CA). The primers used are listed in Supplementary Table E1 (online only, available at www.exphem.org). For the *JAK2* V617F mutation, PCR was performed as follows: 95°C for 2 min; 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min; 72°C for 10 min; and then maintained at 4°C. To detect *JAK2* exon 12 mutations, PCR was performed as follows: 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; 72°C for 7 min; and then maintained at 4°C. To detect *CALR* exon 9 mutations, PCR was performed as follows: 95°C for 2 min; 35 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 1 min; 72°C for 2 min; and then maintained at 4°C. To detect *MPL* exon 10 mutations, PCR was performed as follows: 95°C for 2 min; 35 cycles of 95°C for 30 s, 68°C for 30 s, and 72°C for 1 min; 72°C for 2 min; and then maintained at 4°C. The PCR products were purified and subjected to sequencing with a BigDye Terminator Cycle Sequencing Kit, version 1.1 (Life Technologies, Waltham, MA). Sequencing products were analyzed using an ABI3130XL DNA sequencer (Applied Biosystems, Foster City, CA).

Antibodies, cell staining, and sorting

To sort hematopoietic progenitor cells (HSPCs), BM mononuclear cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (581), a PerCP-Cy5.5-conjugated lineage mixture including anti-CD3 (SK7), anti-CD4 (OKT4), anti-CD8 (HIT8a), anti-CD19 (HIB19), anti-CD20 (2H7), anti-CD235a/b (HIR2), APC-Cy7-conjugated anti-CD38 (HIT2), brilliant violet 421-conjugated anti-CD45RA (HI100), and biotinylated anti-CD123 (6H6) monoclonal antibodies, as well as phycoerythrin (PE)-Cy7-conjugated