論文の内容の要旨

論文題目　Elucidation of functional roles of CAMK2G in myelofibrosis.
（骨髄線維症における CAMK2G の機能的役割の解明）

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[Background]

Philadelphia chromosome-negative Myeloproliferative neoplasms (MPNs), comprising polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (MF), are a heterogeneous group of diseases. MPNs are caused by the acquisition of somatic mutations in the hematopoietic stem cells, including *JAK2*V617F, *CALR*, and *MPL* mutations. Recurrent somatic mutation in *JAK2* is found in approximately 90-95 % of patients with PV and in approximately 50-60 % with ET and MF. Analysis of a large number of patients revealed that somatic mutations of *MPL* are present in approximately 3% of patients with ET and 8% of patients with MF. *CALR* mutations were most recently found and are present in approximately 25% of patients with ET and 35% of patients with MF. While patients with PV and ET show often a relatively mild clinical course and only sometimes require chemotherapeutic intervention, they can progress to secondary myelofibrosis. MF is a life-threatening condition characterized by megakaryocytic atypia, fibrosis in the bone marrow (BM), and extramedullary hematopoiesis. MF is a pre-leukemic disease with a poor prognosis and it can progress to acute myeloid leukemia in 10 to 20% of patients. The median life expectancy of MF patients ranges from 27 to 135 months. Currently the only curative treatment of MF is allogeneic stem cell transplantation. However, it is associated with high treatment-related mortality and morbidity. The 100-day treatment-related mortality was reported to be 18% for HLA identical sibling transplants, and 35% for unrelated transplants. Five-year overall survival rates were 37% and 40% respectively. Furthermore, most patients are not candidates for transplantation because of their age and comorbidity. The JAK1/JAK2 inhibitor ruxolitinib can reduce clinical symptoms and splenomegaly, whereas it fails to induce molecular remission and inhibits wild-type JAK2. Fedratinib, pacritinib and other drugs are JAK1/JAK2 inhibitors and have been used in clinical trials. Clinical trials demonstrated these new JAK1/JAK2 inhibitors reduced spleen volume, improved the symptoms related to MF and prolonged the overall survival (OS). Although the benefits associated with JAK1/JAK2 inhibitors are well established, not all patients respond to these inhibitors, and long-term exposure to these inhibitors results in the emergence of resistant clones based on the reactivation of JAK-STAT pathway. Therefore, new therapeutic strategy targeting other molecules can provide additional benefits to patients with MF.

[**Method**]

Compound Screening

MF-iPSCs previously established were used for the compound screening. Screening Committee of Anticancer Drugs (SCADS) inhibitor kit1(ver3.1) and kit2(ver1.4) were provided from SCADS.

Cell lines

Ba/F3 cells were purchased from RIKEN BRC and 32D cells were purchased from JCRB Cell Bank. The mouse C3H10T1/2 cells were cultured as previously descried.

Cell culture

Ba/F3 cells and 32D cells were cultured in RPMI supplemented with 10% fatal bovine serum (FBS) and 1% penicillin (PS).

Proliferation Assay

In each experiment, Ba/F3 and 32D cells were cultured in the RPMI containing 10% FCS supplemented with or without a 10% WEHI-3B supernatant. Each assay was performed in duplicate.

Colony Forming Assay

In each experiment, five hundred CD34+ cells from peripheral blood of MF patients or five hundred purified c-kit+ murine cells transduced with vectors were plated onto a MethoCult GF M4434 and M3434 medium (STEMCELL Technologies), respectively. The number of colonies in each experiment was scored on day seven following manufacturer's instructions.

Retrovirus production and transduction of cells

To obtain retroviral supernatants, Plat-E packaging cells were transiently transfected with pGCDNsam-huMPLwt-IRES-GFP and pGCDNsam-huMPLW515L-IRES-GFP. C-Kit+ BM cells of BALB/cCrSlc mice were transduced with retroviruses in the presence of RetroNectin (Takara Bio Inc.). The infected cells were collected 48 hours after retroviral infection, and vector-transduced cells were injected (2.5×10^5 cells per animal) into lethally irradiated (9.5 Gy) recipient mice.

[Results]

CAMK2 inhibitor was identified by compound screening using MF-iPSCs

In our laboratory, Miyauchi, et al established MF-iPSCs with integration-free episomal vectors from three different MF patients harboring JAK2 V617F, CALR type 1, and CALR type 2 mutations. After three rounds of screening using MF-HPCs, we identified KN-93 as a compound which inhibited the viability of MF-HPCs from all three patients, compared to normal-HPCs.

CAMK2 inhibition is effective in JAK2 inhibitor-sensitive cells and overcomes the resistance against JAK2 inhibitor

Based on the screening results, we focused on CAMK2 for further analysis. To address the efficacy of CAMK2 inhibition in other models of MF, I used BaF/3 cells expressing MPL W515L. Ba/F3 were stably transduced with pGCDNsam vectors of wildtype MPL and MPLW515L.

CAMK2 inhibitors alone or the combination of CAMK2 inhibitors (KN93, TFP) and ruxolitinib dramatically decreased cell growth and induced apoptosis of Ba/F3 cells overexpressing wild type MPL and MLW515L.

It is known long-term exposure to JAK2 inhibitors results in drug resistance. I next sought to test the therapeutic efficacy of CAMK2G inhibitors in MF cells resistant against ruxolitinib (INCB18424). I cultured Ba/F3 MPLmu_S with ruxolitinib (250 nM) for four weeks and established ruxolitinib-resistant Ba/F3_MPLmu. CAMK2 inhibitors decreased cell growth and induced apoptosis of Ba/F3 cells overexpressing wild type MPL and MLW515L resistant against ruxolitinib.

CAMK2 gamma plays a critical role in MF and efficacy of CAMK2G inhibition is shown in vitro and in vivo

We used 32D cells ectopically expressing wild type MPL or MPL W515L (32D_MPLwt, 32D_MPLmu) and Ba/F3 cells ectopically expressing wild type MPL or MPL W515L (Ba/F3_MPLwt, Ba/F3_MPLmu). In these cells CAMK2 gamma subtype (CAMK2G) was exclusively expressed among all CAMK2 subtypes by qPCR. Stable knock down of CAMK2G by short-hairpin RNA decreased IL3-independent growth of Ba/F3_MPLmu. We also used a specific CAMK2G inhibitor called berbamine. To test the efficacy of CAMK2G inhibition in vivo, we transplanted GFP+ c-kit+ cells form the bone marrow ectopically expressing MPL W515L into irradiated Balb/c mice. Berbamine was effective in treating MPL W515L-induced disease as there was a statistically significant prolongation of survival ($P < 0.05$). Mice receiving berbamine had significant reduction in WBC counts compared with mice treated with PBS. Hemoglobin levels and platelet counts were not significantly different between groups on day 24. Mice receiving berbamine 100 mg/kg had a significant reduction in spleen length.

CAMK2G is phosphorylated by MPL signal via JAK2.

We next sought to elucidate the molecular mechanism of CAMK2G in the pathogenesis of MF. We investigated the potential interaction between MPL-JAK2 pathway and CAMK2G. To elucidate the

signaling pathway related to CAMK2G, we made 32D cells expressing wild type or mutant MPL together with CAMK2G. The phosphorylation of CAMK2G was enhanced when cells were stimulated by thrombopoietin (TPO 10 ng/mL) both in the presence and absence of IL3, indicating that CAMK2G is located downstream of MPL. Ruxolitinib (250 nM) suppressed the phosphorylation of CAMK2G in 32D cells expressing both CAMK2G and wild type MPL or W515L in the presence of IL3. These results suggest that both wild type and mutant MPL signals phosphorylate CAMK2G protein via JAK2. We also assessed signaling pathway using Ba/F3_MPLmu treated with berbamine. Berbamine promptly suppressed CAMK2G and STAT5 and did not affect phosphorylation of JAK2 (**Fig. 18**), indicating STAT5 is one of the downstream targets of CAMK2G. Taken together, CAMK2G is an effector down stream of MPL-JAK2 signal and STAT5 is one of the effectors downstream of CAMK2G.

[Conclusion]

Two CAMK2 inhibitors, KN93 and TFP, markedly attenuated proliferation as well as increased cell apoptosis specifically in 32D and Ba/F3 cells overexpressing MPL W515L. These inhibitors also attenuated proliferation as well as increased cell apoptosis specifically in 32D and Ba/F3 cells overexpressing MPL W515L which become resistant against JAK2 inhibitor. I further identified the critical subtype of CAMK2 in MF. Stable knockdown of CAMK2G by short hairpin RNA decreased cell growth of Ba/F3 cells overexpressing MPLW515L. CAMK2G inhibition significantly prolonged survival of MF mice induced by MPLW515L and ameliorated disease phenotypes such as splenomegaly and leukocytosis. This study also showed CAMK2G is activated by MPL signal in MF model cells and is an effector in MPL-JAK2 signaling pathway in these cells. These results indicate CAMK2G plays an important role in MF, and CAMK2G inhibition can be a novel therapeutic strategy that overcomes resistance to JAK1/2 inhibition.