Leukemia Research 83 (2019) 106166

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Research paper

The upregulation of Pim kinases is essential in coordinating the survival, proliferation, and migration of KIT D816V-mutated neoplastic mast cells

Hyejoo Park[a], Dongchan Kim[a], Youngil Koh[b], Sung-Soo Yoon[a,b,*]

[a] Cancer Research Institute, Seoul National University College of Medicine, Seoul, 03080, Republic of Korea
[b] Department of Internal Medicine, Seoul National University College of Medicine, Seoul National University Hospital, Seoul, 03080, Republic of Korea

ARTICLE INFO

Keywords:
*KIT*D816V
Pim kinase
Mast cell neoplasm
Cell survival
Cell migration

ABSTRACT

About ~80% of mast cell neoplasm patients harbor the c-Kit activating mutation D816 V, which is associated with c-Kit inhibitor resistance and poor prognosis. However, the molecular basis for these effects is not fully known. To address this issue, in this study we screened molecules whose expression is altered by *KIT* D816 V mutation and found that Pim kinases were overexpressed in D816V-mutant neoplastic mast cells. This was accompanied by upregulation of signal transducer and activator of transcription (STAT) and mammalian target of rapamycin (mTOR) and downregulation of Akt and extracellular signal-regulated kinase (ERK1/2). Activated Pim kinases promoted the survival of D816 V cells by maintaining mTOR and p70S6K activation even under nutrient starvation. Conversely, cell proliferation was suppressed by inhibiting Pim kinases. The mRNA level of *C-X-C chemokine receptor type 4 (CXCR4)* was about 2-fold higher in D816 V cells; this was associated with a 2-fold increase in migratory capacity, which was modulated by Pim kinases. We also confirmed that upregulation of Pim kinases is a feature specific to cells with the D816 V mutation and is not observed in cells with the c-Kit activating N822 K mutation. These data suggest Pim kinases as a promising therapeutic target for the treatment of mast cell neoplasms with *KIT* D816 V mutation.

1. Introduction

c-Kit is a type III receptor tyrosine kinase (TK) expressed in hematopoietic stem cells and normally developing melanocytes, germ cells, and interstitial cells [1]. *KIT* gene alterations are detected in various cancers including acute myeloid leukemia (AML), mast cell leukemia, systemic mastocytosis, melanoma, germ cell tumors, and gastrointestinal stromal tumors, with a high frequency of mutation found in mast cell neoplasms and core binding factor (CBF)-AML. In particular, the D816 V (Asp816Val) point mutation in the exon 17 TK2 region is present in up to 30% of CBF-AML cases [2,3] and up to 80% of mast cell neoplasms [4], and is linked to c-Kit inhibitor resistance and poor prognosis [5,6]. N822 K (Asn822Lys), another high-frequency mutation in the TK2 domain of exon 17, is also a c-Kit activating mutation but shows better prognosis and drug response than D816 V [7]. A third c-Kit activating mutation, V560 G (Val560Gly), occurs not in TK2 but in the juxtamembrane domain and confers responsiveness to c-Kit inhibitors such as imatinib [8]. These difference in therapeutic response may be attributable to the major signaling pathways that are activated by each mutation [9]. For instance, mammalian target of rapamycin (mTOR) [10], SRC kinase [11], phosphoinositide 3-kinase (PI3K) p85 [12], and signal transducer and activator of transcription (STAT) [13] have been implicated in the survival and drug response resistance of D816V-mutant cells. However, the molecular basis for these properties is not well understood; clarifying the underlying mechanisms can provide a basis for developing novel and more effective therapeutic strategies.

Pim serine/threonine protein kinases are highly expressed in various hematologic malignancies as well as in solid cancers, and are considered promising therapeutic targets owing to their critical roles in cancer cells [14]. Overexpression of Pim kinases promotes cancer cell survival by regulating the cell cycle, apoptosis, and metabolism via modulation of signal transduction molecules such as mTOR [15] as well as C-X-C chemokine receptor type (CXCR) 4, which regulates the cell migration that is critical for drug response and disease relapse. Pim kinase expression is regulated by Janus kinase (JAK)/STAT, PI3K/AKT, mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK), and nuclear factor (NF)-κB signaling [16]. In particular, STAT directly regulates Pim kinases expression by binding the transcription site [17] and plays a key role in the survival of cells harboring *KIT* D816 V mutation (Fig. 1). Additionally, although the mechanism by

* Corresponding author at: Department of Internal Medicine, Seoul National University Hospital, Seoul National University College of Medicine, 101, Daehak-ro, Jongno-gu, Seoul, 03080, Republic of Korea.
E-mail address: ssysmc@snu.ac.kr (S.-S. Yoon).

https://doi.org/10.1016/j.leukres.2019.106166
Received 29 March 2019; Received in revised form 19 May 2019; Accepted 6 June 2019
Available online 12 June 2019

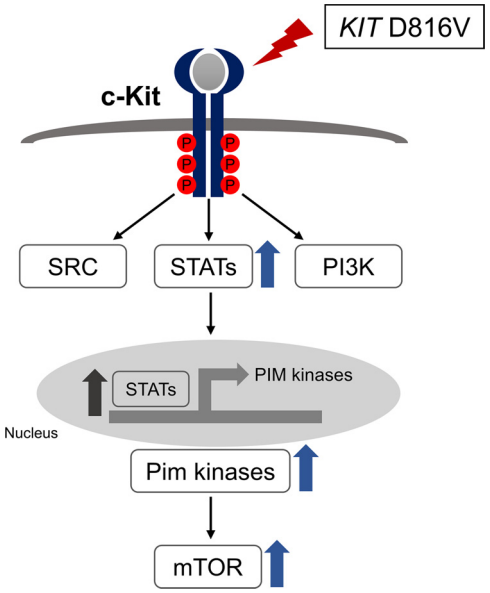

Fig. 1. Connection between Pim kinases and upregulated signaling molecules of *KIT* D816 V cells.

which aberrantly activated c-Kit affects cancer cells remains unclear, it was recently reported that Pim-1 regulates *KIT* translation [18].

The present study investigated the functional significance of *KIT* D816 V mutation and Pim kinase overexpression in relation to various characteristics of mast cell neoplasms including cell survival, proliferation, and migration. We also compared the signal transduction pathways activated by *KIT* mutations to determine whether Pim kinase overexpression is specifically associated with D816 V. Our findings indicate that Pim kinases are a promising therapeutic target in patients harboring the D816 V mutation.

2. Materials and methods

2.1. Cell cultures

The HMC-1.1 and HMC-1.2 leukemic human mast cell lines were provided by Dr. Joseph H. Butterfield of Mayo Clinic (Rochester, MN, USA). The Kasumi-1 human AML cell line (American Type Culture Collection, Manassas, VA, USA) and the MV4-11, MOLM14, NB4, HL-60, HEL, and KG-1 leukemic cell lines were purchased from DSMZ (Braunschweig, Germany). HMC-1 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific, Waltham, MA, USA; 12440053). Kasumi-1 cells were cultured in Roswell Park Memorial Institute (RPMI)1640 medium (Welgene, Gyeongsan, South Korea; LM011-51), as were the other cell lines (LM011-01). All culture media were supplemented with 10%–20% heat-inactivated fetal bovine serum (Thermo Fisher Scientific; 16000044) and 1% PenStrep (Thermo Fisher Scientific; 15140122). Cells were passaged every 2–4 days.

2.2. Western blotting

Cells were lysed using Kinexus lysis buffer (Kinexus Bioinformatics, Vancouver, Canada). Total protein was quantified with the Micro BCA Protein Assay kit (Thermo Fisher Scientific), separated by 8%–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride (PVDF) membrane (MilliporeSigma, Burlington, MA, USA) that was blocked with 5% skim milk (GeorgiaChem, Norcross, GA, USA) in Tris-buffered saline with 5% Tween-20 for 1 h at room temperature. The membrane was probed overnight at 4 °C with primary antibodies followed by secondary antibodies diluted 1:10,000 in 1% skim milk solution. Protein bands were detected by enhanced chemiluminescence. The antibodies used are listed in Supplementary Table 1.

2.3. Small interfering (si)RNA transfection

Pre-designed siRNAs targeting Pim-1, -2, and -3 were purchased from Genolution (Seoul, South Korea). The sequences are shown in Supplementary Table 2. Transfection was performed using RNAiMAX (Thermo Scientific Scientific) and an electroporation kit (Amaxa Cell Line Nucleofector Kit V [VCA-1003] and program O-17; Lonza, Basel, Switzerland). The final concentration of siRNAs was 2 μM.

2.4. Transwell migration assay

Cell migration was evaluated using a commercial kit (BioVision, Milpitas, CA, USA; K909-12) in a 24-well plate with inserts (8-μm pore size) according to the manufacturer's protocol. Cells were starved in serum-free IMDM medium for 24 h before experiments.

2.5. Adhesion assay

To evaluate cell adhesion, 2.5 μg/ml of human plasma fibronectin (MilliporeSigma; 2549971) was dispensed into a 96-well plate (Corning Inc., Corning, NY, USA) and allowed to stand for 1 h at room temperature until it coated. Cells (3×10^5) were then seeded in the wells, followed by incubation for 2 h at 37 °C. The wells were washed three times with 300 μl of serum-free IMDM and then incubated in conditioned IMDM for 30 min at 37 °C. After adding 10 μl of EZ-Cytox solution (DoGenBio, Seoul, Korea) to each well and incubating for 2 h, the absorbance at 450 nm was measured.

2.6. Cell proliferation assay

siRNA-transfected cells were seeded in 96-well plates (5000 cells/well), and cell proliferation was measured on days 1, 4, and 6 by adding EZ-Cytox solution to each well and incubating for 2 h, and measuring the absorbance at 450 nm.

2.7. Cell viability assay

Cells (3000/well) were seeded in a 96-well plate and were treated 1 day later with the drug at eight different concentrations (1/10 serial dilution from 10 μM). After 72 h, EZ-Cytox solution was used to evaluate cell viability. The drugs used are listed in Supplementary Table 1.

2.8. Outgrowth assay

HMC-1.1 and HMC-1.2 cells were cultured in T-25 flasks (Corning Inc.) up to 120% and 150% confluence in a conditioned medium as described in Section 2.1.

2.9. Generation of KIT knock-in cells by genome editing

KIT knock-in cells were generated by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). Recombinant Cas9 protein, single guide (sg)RNA (5′-CAAGAA TGATTCTAATTATGTGG-3′), and single-stranded DNA donor (ssDonor) were purchased from ToolGen (Seoul, Korea). A mixture of 1 μg sgRNA, 1 μg Cas9 protein, and 100 pM ssDonor was transfected into Kasumi-1 cells using an Amaxa II electroporator (program V-001; Lonza).

2.10. Total RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated with TRIzol reagent (Thermo Fisher

Scientific; 15596018) and the concentration was measured on a NanoDrop spectrophotometer (Thermo Fisher Scientific; ND-1000). About 1–5 μg of RNA was synthesized into cDNA using RNA to cDNA EcoDry Premix (Random Hexamers) kit (Takara Bio, Otsu, Japan; 639546). Real-time PCR was performed with SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio; RR820A) on an Applied Biosystems 7500 PCR system (Thermo Fisher Scientific). The target mRNA level was normalized to that of 18S rRNA. Primer sequences are listed in Supplementary Table 3.

2.11. DNA extraction and single nucleotide polymorphism (SNP) sequencing

DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. SNP sequencing was performed by Macrogen (Seoul, Korea) and data were analyzed using Chromas v.2.6.6 software (Technelysium, Brisbane, Australia).

2.12. Statistical analysis

Group means were compared with the Student's *t*-test and by two-way analysis of variance with Bonferroni correction using Prism 5 software (GraphPad, San Diego, CA, USA). $P < 0.05$ was considered significant (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

3. Results

3.1. Pim kinases are upregulated in KIT D816 V cells and promote cell survival under nutrient deprivation and correlate with cell viability

HMC-1.1 cells harbor the *KIT* V560 G mutation (in the juxtamembrane region), whereas HMC-1.2 cells have both the V560 G and D816 V mutations. We examined changes in intracellular signaling molecules—including c-Kit, PI3K p85/p55, Akt, mTOR, STAT3, STAT5, Erk1/2, and Pim kinases, which are known to influence cancer cell survival—induced by *KIT* D816 V by western blotting (Fig. 2A). c-Kit phosphorylation was increased in *KIT* D816 V cells relative to wild-type cells, indicating that c-Kit signaling is activated by D816 V. Phosphorylated PI3K p85/p55, mTOR, STAT3 (S727), and STAT5 (Y694) levels were increased in HMC-1.2 cells, as previously reported [19]. Phospho-Akt (S473 and T308) and Erk1/2 expression was downregulated in D816V-mutant cells. Pim kinases (Pim-1, -2, and -3) were upregulated in *KIT* D816 V cells.

To confirm whether upregulation of Pim kinases is a feature unique to *KIT* D816 V cells, we examined Pim kinase expression in nine human leukemic cell lines (Fig. 2B) and detected phospho-c-Kit in three *KIT* mutant cell lines—i.e., HMC-1.1, HMC-1.2, and Kasumi-1 (harboring the *KIT* N822 K mutation). HMC-1.2 cells showed the highest expression levels of phospho-c-Kit and Pim kinases. Pim-1 was expressed only in HMC-1.1 and HMC-1.2 cells, with higher levels in the latter. Pim-2 and -3 were expressed in all nine cell lines, but the levels were highest in cells with D816 V mutation. These data indicate that the expression of intracellular signaling molecules varies according to D816 V mutation status and that all Pim kinases are highly expressed in *KIT* D816 V cells.

To determine how upregulation of Pim kinases affects cell survival, we evaluated Pim kinase expression under conditions of nutrient deprivation caused by cell outgrowth (Fig. 2C). Phospho-c-Kit expression increased in HMC-1.2 cells and decreased in HMC-1.1 cells as outgrowth progressed. STAT expression was unchanged or strongly increased in HMC-1.2 cells but decreased or only slightly increased in HMC-1.1 cells. Pim-1 level was downregulated in association with HMC-1.1 cell outgrowth, but the opposite trend was observed in HMC-1.2 cells. Pim-2 has three isoforms (34, 38, and 40 kDa), one (34 kDa) of which induces apoptosis when phosphorylated at Ser112 [20] as well as cell cycle arrest at the G1 phase [21]. Expression of the 34-kDa isoform of Pim-2 in HMC-1.1 cells increased with outgrowth, implying that apoptotic signaling was activated under these conditions. In contrast, the 40- and 34-kDa isoforms were up- and downregulated, respectively, in HMC-1.2 cells, suggesting that anti-apoptotic signaling pathways were instead activated. Pim-3 was upregulated in both cell lines but the level was higher in HMC-1.2 cells in the early phase of outgrowth. The expression of mTOR—which acts downstream of Pim kinases and is involved in cell metabolism—was increased along with that of its effector p70S6K, but only in HMC-1.2 cells. Expression of these molecules was reduced by treatment with the Pim kinase inhibitor AZD1208 (Fig. 2D). HMC-1.1 cells showed increased expression of mTOR (S2448) at the final stage of outgrowth.

To clarify the roles of Pim kinases and STATs in the viability of *KIT* D816 V cells, we examined their expression following treatment with the c-Kit inhibitors imatinib, dasatinib, and BLU-285. As in previous studies [22–24], HMC-1.2 cells responded only to dasatinib and BLU-285 (Fig. 2E), with the latter affecting cell viability at a lower concentration than the former. Imatinib had no effect on the expression of Pim kinases and STATs or on cell viability, but these all declined upon dasatinib or BLU-285 treatment (Fig. 2F), with the latter having a more potent effect and Pim-3 being the least affected by the drugs. These results indicate that *KIT* D816V-mutant cells have a survival advantage under adverse conditions such as nutrient deprivation that is conferred by upregulation of phospho-STAT5 and Pim kinases and activation of mTOR and p70S6K signaling.

3.2. Pim kinases regulate the proliferation and migration of KIT D816 V cells

We explored the effects of Pim kinases on the proliferation of *KIT* D816 V cells by downregulation of their expression using siRNAs and measuring optical density 1, 4, and 6 days later (Fig. 3A). HMC-1.2 cells transfected with scrambled siRNA served as the siControl. Although there were no observable changes on day 1, cell growth was markedly reduced on days 4 and 6. Cell proliferation was most strongly inhibited in siPIM all (*PIM1*, *2*, and *3* were all knocked down). The suppression of Pim kinase expression was confirmed by western blotting (Fig. 3B).

Cell migration and adhesion are important features of malignant cancers and are associated with CXCR4 activation by Pim kinases [25,26]. We examined whether *CXCR4* expression changes according to *KIT* D816 V mutation status (Fig. 3C) and found that relative *CXCR4* mRNA level was almost 2-fold higher in *KIT* D816 V cells than in wild-type cells. To determine whether overexpression of Pim kinases affects the migration and adhesion of D816 V cells, we carried out transwell migration and fibronectin adhesion assays. The results showed that *KIT* D816 V cells had a greater capacity for migration than HMC-1.1 cells (Fig. 3D). Moreover, the number of migrating cells was decreased upon knockdown of Pim kinases (Fig. 3E). Consistent with these observations, the motility of D816 V cells was reduced 2-fold by treatment with the Pim kinase inhibitor PIM447 for 24 h (Fig. 3F). However, cell adhesion was unaffected by *KIT* D816 V mutation and by Pim kinase knockdown (Fig. 3G). These data demonstrate that increased proliferation and migration of the mutant cells—which are closely related to poor prognosis—are regulated by Pim kinases.

3.3. Upregulation of Pim kinases is a specific feature of KIT D816 V cells

To confirm whether the D816 V mutation having a worse impact on prognosis than the c-Kit TK2 domain activating mutation N822 K, is related to Pim kinase upregulation, we examined the expression of signal transduction proteins in N822 K and wild-type (N822) cells. The *KIT* exon 17 wild-type (N822) clone #8 was established by replacing the mutated A (Lys [K]: AAA) of N822 K with T (Asn [N]: AAT) by CRISPR-Cas9 genome editing (Fig. 4A), and the knock-in was confirmed by SNP sequencing (Fig. 4B).

We evaluated the effect of *KIT* N822 K on cell proliferation and

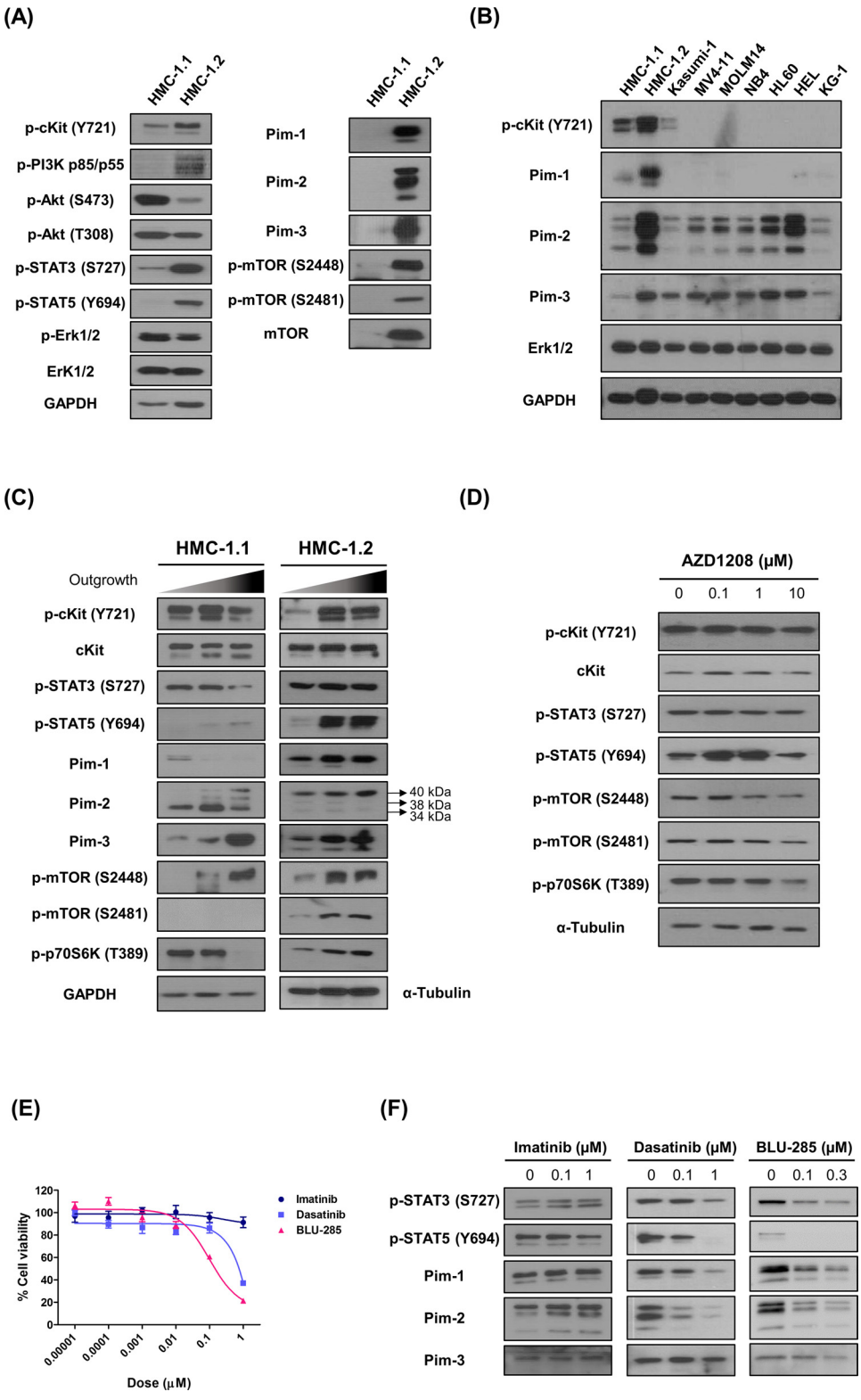

Fig. 2. Expression of signaling molecules and viability of *KIT* D816 V cells. (A) Western blot analysis of signaling molecules. Phospho-c-Kit, mTOR, STATs, and Pim kinases were upregulated in *KIT* D816 V cells. (B) Expression of Pim kinases in nine human leukemic cell lines. Phospho-c-Kit was detected only in *KIT* mutant cell lines. Erk1/2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as loading controls. (C) Signal transduction in HMC-1.1 and HMC-1.2 cells during outgrowth. Phosphorylation of c-Kit, STAT5, Pim kinases, and mTOR was increased in *KIT* D816 V cells. (D) Expression levels of molecules up- and downstream of Pim kinases in D816 V cells treated with AZD1208. (E) Cytotoxicity evaluation of c-Kit inhibitors in *KIT* D816 V cells. Dasatinib and BLU-285 reduced cell viability. (F) Decreased expression of Pim kinases, STAT3, and STAT5 following dasatinib or BLU-285 treatment.

found that compared to N822 K cells, clone #8 had reduced proliferative capacity (Fig. 4C). This result shows that the N822 K mutation also alters cell growth and could confer a survival advantage to cancer cells in a manner similar to D816 V.

To determine whether Pim kinase overexpression is specific to cells with the D816 V mutation, we examined the levels of signaling molecules by western blotting (Fig. 4D). Clone #8 showed decreased expression of phospho-c-Kit and total c-Kit, while PI3K, Erk1/2, and mTOR levels showed the same trend as in D816 V cells. However, in contrast to D816 V cells, Akt T308 and S473 expression were increased in N822 K cells. Furthermore, expression of Pim-3 and STAT3, which was elevated in D816 V cells, were unaffected by *KIT* N822 K mutation (phospho-STAT5 was not detected).

Thus, PI3K p85/p55 and mTOR signaling were induced by N822 K mutation as by D816 V mutation. However, the expression of Pim and STAT was unaffected by N822 K mutation even though it enhanced cell

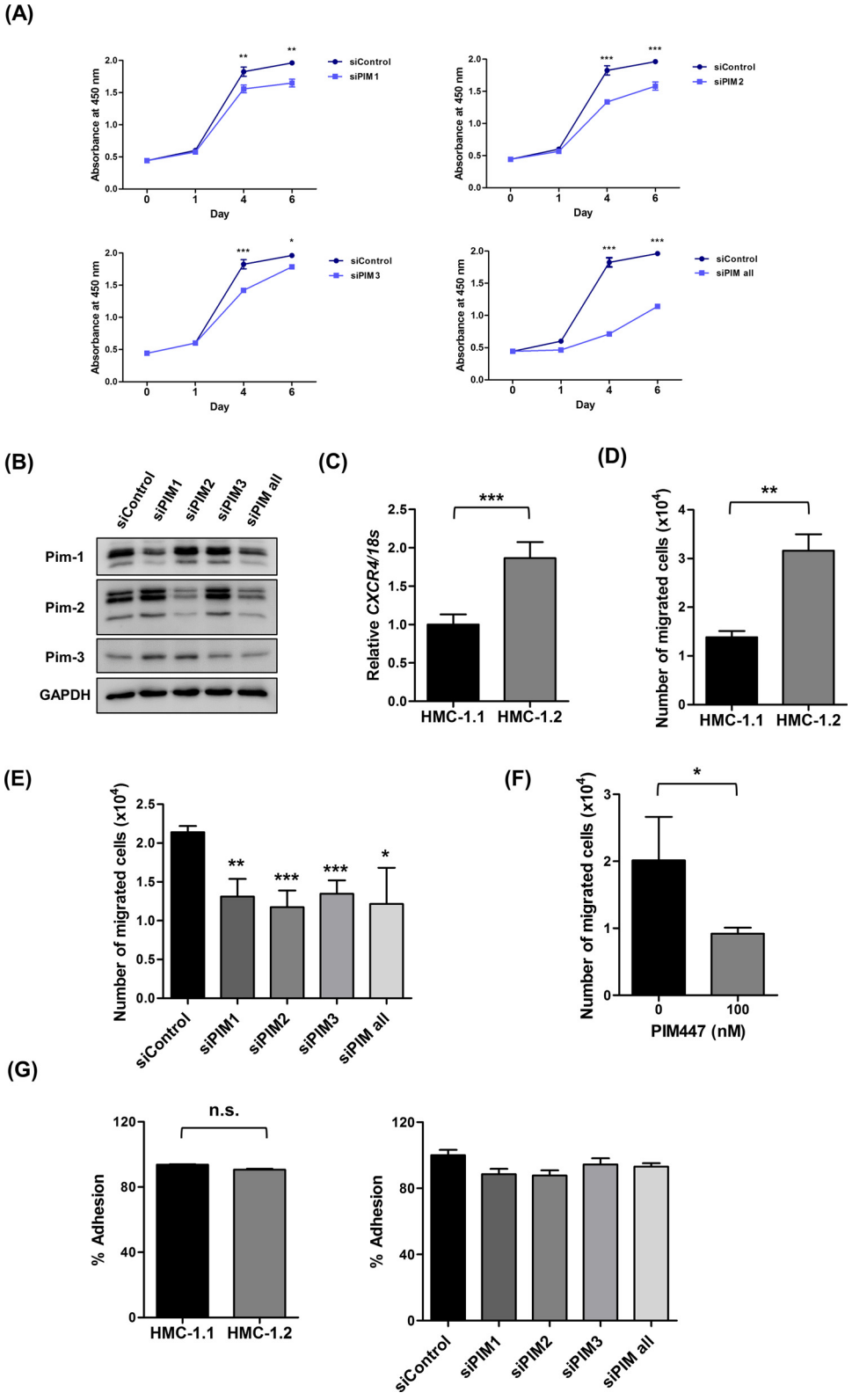

Fig. 3. Evaluation of *KIT* D816 V cell proliferation, migration, and adhesion according to Pim kinase expression level. (A) Proliferation assay after knockdown of Pim kinases. Cell proliferation assessed by measuring optical density (OD) on days 1, 4, and 6. (B) Knockdown of Pim kinases was confirmed by western blotting, with GAPDH serving as a loading control. (C) Relative mRNA expression of *CXCR4* according to D816 V mutation status. (D–F) Effect of D816 V and Pim kinase knockdown on cell motility was assessed with the transwell migration assay. Nutrient-starved cells were seeded in the top chamber and migrated cells were counted after 24 h. (G) Fibronectin adhesion assay for evaluating cell adhesion according to D816 V mutation status and Pim kinase depletion.

proliferation like D816 V. Thus, although both D816 V and N822 K promote the survival of cancer cells, only D816 V exerts its effects via Pim kinase-mediated signaling. These data demonstrate that upregulation of Pim kinases is a specific feature of D816 V cells and may explain the worse prognosis of patients harboring D816 V than those having N822 K mutation.

4. Discussion

The *KIT* D816 V mutation occurs at a high rate (> 80%) in indolent systemic mastocytosis (SM) and smoldering SM and is detected in > 70% of aggressive SM cases [27,28]. The mutation significantly impacts patient prognosis depending on overall mutation burden [6]. In mast cell leukemia, a rare disease characterized by mast cell neoplasms,

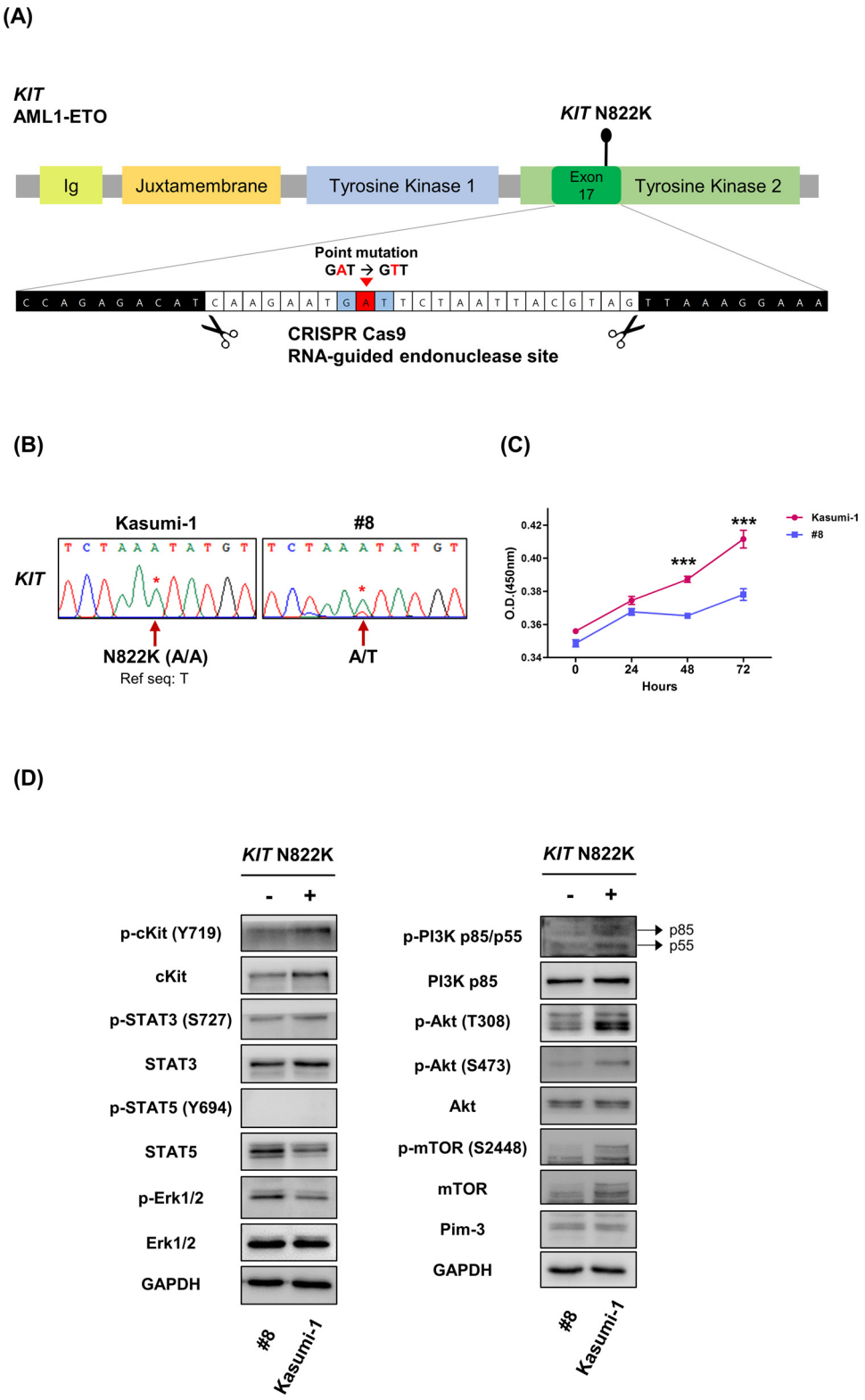

Fig. 4. Effects of *KIT* N822 K mutation on leukemic cells. (A) Schematic representation of *KIT* N822 K knock-in by CRISPR-Cas9 genome editing to replace the mutated sequence with a reference sequence. (B) *KIT* N822 knock-in clone #8 was confirmed by SNP sequencing (knock-in site is marked with a red star; T is the reference sequence). (C) Proliferation assay of N822 K and N822 cells. Proliferation was determined by measuring OD values. (D) Expression of signal transduction molecules in the presence of N822 K mutation. Phospho-c-Kit, PI3K p85/p55, Akt, and mTOR levels were increased by N822 K mutation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

KIT D816 V is present in about 46% of patients and is linked to poor prognosis [29]. Thus, therapeutic strategies that target D816 V cells have potential clinical benefits and are currently being explored in clinical trials.

Drugs targeting D816 V cells are classified as agents that regulate c-Kit receptor activation or as multi-TK inhibitors (TKIs) that act on a broad spectrum of TKs including c-Kit. Cells harboring D816 V are inherently resistant to the c-Kit inhibitor imatinib. Masitinib, which also targets c-Kit, has recently been tested in clinical trials, and BLU-285 trials are actively ongoing. Ponatinib [22] and dasatinib [23] are multi-TKIs that act on signaling molecules other than c-Kit (i.e., STAT5 and Src) to regulate D816 V cell survival. Thus, targeting different factors that are upregulated in this context is likely to be more effective than c-Kit receptor blockade alone.

A study of SM patients with D816 V mutation showed that a high proportion shown upregulation of *PIM1* [30], implying that Pim kinases

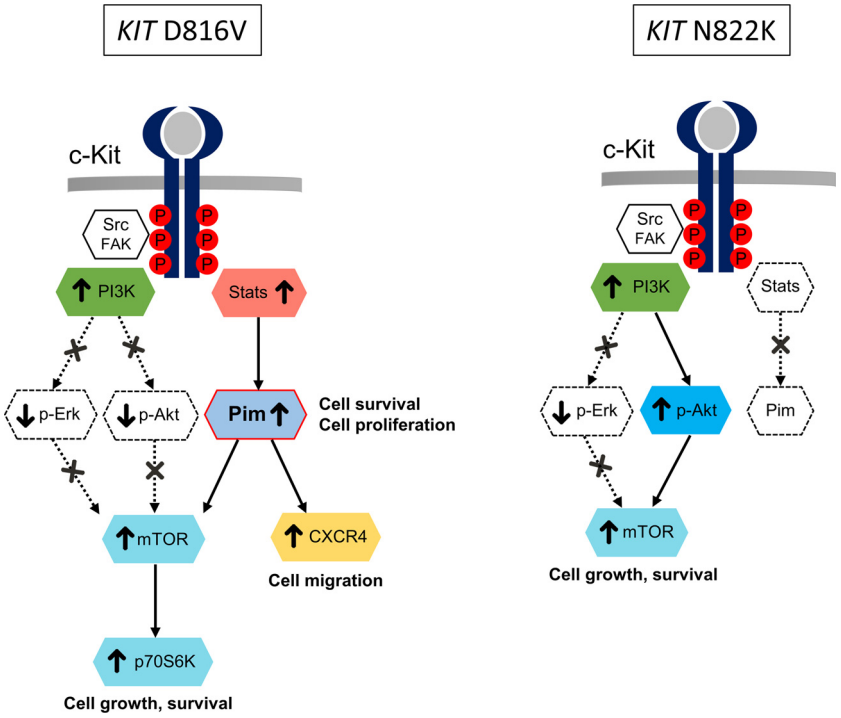

Fig. 5. Summary of signal transduction cascades associated with *KIT* D816 V and N822 K mutations.

play a biologically important role in this disease. Our results demonstrate that Pim kinases may also contribute to mast cell neoplasms harboring D816 V mutation through as modulation of STATs and Src.

Given their important roles in the progression of hematologic and solid cancers, drugs targeting Pim kinases and clinical trials are currently being developed. Most of these are pan-Pim kinase inhibitors that act on Pim-1, -2, and -3 and are predicted to have potent anti-cancer effects. Indeed, in our proliferation assay, the greatest degree of inhibition was observed by suppressing all three proteins. In a study of the pan-Pim kinase inhibitor AZD1208 on AML cell lines showed that drug response correlates with Pim-1 and STAT5 expression and AZD1208 induces cell death by decreasing 4EBP1 and p70S6K activation [31]. A phase I trial for AZD1208 has been completed in advanced solid tumors and malignant lymphomas and a clinical trial for another pan-Pim kinase inhibitor, PIM447, combined with the JAK1/2 inhibitor ruxolitinib and cyclin-dependent kinase 4/6 inhibitor LEE011 is ongoing. Combination treatment with an inhibitor of JAK1/2, which associated with the STAT activity, is especially promising as demonstrated by the results of the present study.

Precision medicine improves treatment outcome by targeting the specific factors promoting cancer cell survival in a patient. It is therefore important to identify the main signaling molecules involved in cancer cell survival and clarify their functions. We determined that Pim kinases are overexpressed in *KIT* D816 V cells and described their functions in neoplastic mast cells. Pim-2 was the most highly expressed, followed by Pim-1 and -3; it was also the first molecule to be downregulated by a low concentration of inhibitor, which had the greatest impact on cell proliferation on day 6. In contrast, Pim-3 showed only a slight change in expression level upon drug treatment and was associated with the lowest degree of growth inhibition on day 6. Thus, the function of each Pim molecule in *KIT* D816 V cells is related to its expression level, with Pim-2 being the most important in terms of the survival of *KIT* D816 V cells.

Various non-canonical pathways are known to be involved in cell survival [32] and tumorigenesis [33]. For instance, we found that D816 V cells use Pim kinases rather than Akt or Erk1/2 to activate downstream effectors such as mTOR (PI3K/STAT/Pim kinase/mTOR), unlike N822 K cells that use a canonical signaling axis (PI3K/Akt/mTOR) (Fig. 5). This could explain why the D816 V mutation is associated with worse prognosis compared to other c-Kit activating mutations.

5. Conclusions

The results of this study demonstrate that Pim kinase overexpression is associated with *KIT* D816 V mutation, which is frequently detected in mast cell neoplasms and results in poor prognosis. Given their critical role in promoting cancer cell survival and migration, Pim kinases are promising therapeutic targets in patients with *KIT* D816 V.

Author contributions

HP performed all of the experiments and wrote the manuscript. DK supported experimental discussion for CRISPR-Cas9 genome editing. YK suggested the concept of the study. SSY revised the manuscript and supervised the overall study process.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. NRF-2016R1A5A1011974 and NRF-2014M3A6A4074727).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2019.106166.

References

[1] M. Miettinen, J. Lasota, KIT (CD117): a review on expression in normal and neoplastic tissues, and mutations and their clinicopathologic correlation, Appl. Immunohistochem. Mol. Morphol. 13 (3) (2005) 205–220.
[2] H. Ayatollahi, A. Shajiei, M.H. Sadeghian, M. Sheikhi, E. Yazdandoust, M. Ghazanfarpour, et al., Prognostic importance of C-KIT mutations in core binding

factor acute myeloid leukemia: a systematic review, Hematol. Stem Cell Ther. 10 (1) (2017) 1–7.
[3] J.M. Ziai, A.J. Siddon, Education Committee of the Academy of Clinical Laboratory P, Scientists, Pathology consultation on gene mutations in acute myeloid leukemia, Am. J. Clin. Pathol. 144 (4) (2015) 539–554.
[4] P. Valent, C. Akin, W.R. Sperr, M. Mayerhofer, M. Fodinger, R. Fritsche-Polanz, et al., Mastocytosis: pathology, genetics, and current options for therapy, Leuk. Lymphoma 46 (1) (2005) 35–48.
[5] J.D. Growney, J.J. Clark, J. Adelsperger, R. Stone, D. Fabbro, J.D. Griffin, et al., Activation mutations of human c-KIT resistant to imatinib mesylate are sensitive to the tyrosine kinase inhibitor PKC412, Blood 106 (2) (2005) 721–724.
[6] G. Hoermann, K.V. Gleixner, G.E. Dinu, M. Kundi, G. Greiner, F. Wimazal, et al., The KIT D816V allele burden predicts survival in patients with mastocytosis and correlates with the WHO type of the disease, Allergy 69 (6) (2014) 810–813.
[7] S. Wakita, H. Yamaguchi, K. Miyake, Y. Mitamura, F. Kosaka, K. Dan, et al., Importance of c-kit mutation detection method sensitivity in prognostic analyses of t(8;21)(q22;q22) acute myeloid leukemia, Leukemia 25 (9) (2011) 1423–1432.
[8] Y. Ma, S. Zeng, D.D. Metcalfe, C. Akin, S. Dimitrijevic, J.H. Butterfield, et al., The c-KIT mutation causing human mastocytosis is resistant to STI571 and other KIT kinase inhibitors; kinases with enzymatic site mutations show different inhibitor sensitivity profiles than wild-type kinases and those with regulatory-type mutations, Blood 99 (5) (2002) 1741–1744.
[9] I.J. Chan, S. Kasprowicz, M.D. Tharp, Distinct signalling pathways for mutated KIT(V560G) and KIT(D816V) in mastocytosis, Clin. Exp. Dermatol. 38 (5) (2013) 538–544.
[10] M. Gabillot-Carre, Y. Lepelletier, M. Humbert, P. de Sepulvelda, N.B. Hamouda, J.P. Zappulla, et al., Rapamycin inhibits growth and survival of D816V-mutated c-kit mast cells, Blood 108 (3) (2006) 1065–1072.
[11] J. Sun, M. Pedersen, L. Ronnstrand, The D816V mutation of c-Kit circumvents a requirement for Src family kinases in c-Kit signal transduction, J. Biol. Chem. 284 (17) (2009) 11039–11047.
[12] R. Chian, S. Young, A. Danilkovitch-Miagkova, L. Ronnstrand, E. Leonard, P. Ferrao, et al., Phosphatidylinositol 3 kinase contributes to the transformation of hematopoietic cells by the D816V c-Kit mutant, Blood 98 (5) (2001) 1365–1373.
[13] A. Chaix, S. Lopez, E. Voisset, L. Gros, P. Dubreuil, P. De Sepulveda, Mechanisms of STAT protein activation by oncogenic KIT mutants in neoplastic mast cells, J. Biol. Chem. 286 (8) (2011) 5956–5966.
[14] R. Swords, K. Kelly, J. Carew, S. Nawrocki, D. Mahalingam, J. Sarantopoulos, et al., The Pim kinases: new targets for drug development, Curr. Drug Targets 12 (14) (2011) 2059–2066.
[15] A.U.R. Aziz, S. Farid, K. Qin, H. Wang, B. Liu, PIM kinases and their relevance to the PI3K/AKT/mTOR pathway in the regulation of ovarian cancer, Biomolecules 8 (1) (2018).
[16] P. Mondello, S. Cuzzocrea, M. Mian, Pim kinases in hematological malignancies: where are we now and where are we going? J. Hematol. Oncol. 7 (2014) 95.
[17] X. Zhang, M. Song, J.K. Kundu, M.H. Lee, Z.Z. Liu, PIM kinase as an executional target in cancer, J. Cancer Prev. 23 (3) (2018) 109–116.
[18] N. An, B. Cen, H. Cai, J.H. Song, A. Kraft, Y. Kang, Pim1 kinase regulates c-Kit gene translation, Exp. Hematol. Oncol. 5 (2016) 31.
[19] I. Omori, H. Yamaguchi, K. Miyake, N. Miyake, T. Kitano, K. Inokuchi, D816V mutation in the KIT gene activation loop has greater cell-proliferative and anti-apoptotic ability than N822K mutation in core-binding factor acute myeloid leukemia, Exp. Hematol. 52 (2017) 56–64 e4.
[20] B. Yan, M. Zemskova, S. Holder, V. Chin, A. Kraft, P.J. Koskinen, et al., The PIM-2 kinase phosphorylates BAD on serine 112 and reverses BAD-induced cell death, J. Biol. Chem. 278 (46) (2003) 45358–45367.
[21] D. Levy, A. Davidovich, S. Zirkin, Y. Frug, A.M. Cohen, S. Shalom, et al., Activation of cell cycle arrest and apoptosis by the proto-oncogene Pim-2, PLoS One 7 (4) (2012) e34736.
[22] B. Jin, K. Ding, J. Pan, Ponatinib induces apoptosis in imatinib-resistant human mast cells by dephosphorylating mutant D816V KIT and silencing beta-catenin signaling, Mol. Cancer Ther. 13 (5) (2014) 1217–1230.
[23] N.P. Shah, F.Y. Lee, R. Luo, Y. Jiang, M. Donker, C. Akin, Dasatinib (BMS-354825) inhibits KITD816V, an imatinib-resistant activating mutation that triggers neoplastic growth in most patients with systemic mastocytosis, Blood 108 (1) (2006) 286–291.
[24] E. Evans, A. Gardino, B. Hodous, A. Davis, J.L. Zhu, N.E. Kohl, et al., Blu-285, a potent and selective inhibitor for hematologic malignancies with KIT exon 17 mutations, Blood 126 (23) (2015).
[25] R. Grundler, L. Brault, C. Gasser, A.N. Bullock, T. Dechow, S. Woetzel, et al., Dissection of PIM serine/threonine kinases in FLT3-ITD-induced leukemogenesis reveals PIM1 as regulator of CXCL12-CXCR4-mediated homing and migration, J. Exp. Med. 206 (9) (2009) 1957–1970.
[26] L. Brault, T. Menter, E.C. Obermann, S. Knapp, S. Thommen, J. Schwaller, et al., PIM kinases are progression markers and emerging therapeutic targets in diffuse large B-cell lymphoma, Br. J. Cancer 107 (3) (2012) 491–500.
[27] M. Arock, P. Valent, Pathogenesis, classification and treatment of mastocytosis: state of the art in 2010 and future perspectives, Expert Rev. Hematol. 3 (4) (2010) 497–516.
[28] S. Bibi, F. Langenfeld, S. Jeanningros, F. Brenet, E. Soucie, O. Hermine, et al., Molecular defects in mastocytosis: KIT and beyond KIT, Immunol. Allergy Clin. North Am. 34 (2) (2014) 239–262.
[29] S. Georgin-Lavialle, L. Lhermitte, P. Dubreuil, M.O. Chandesris, O. Hermine, G. Damaj, Mast cell leukemia, Blood 121 (8) (2013) 1285–1295.
[30] C. Teodosio, A.C. Garcia-Montero, M. Jara-Acevedo, L. Sanchez-Munoz, C.E. Pedreira, I. Alvarez-Twose, et al., Gene expression profile of highly purified bone marrow mast cells in systemic mastocytosis, J. Allergy Clin. Immunol. 131 (4) (2013) 1213–1224 24 e1-4.
[31] E.K. Keeton, K. McEachern, K.S. Dillman, S. Palakurthi, Y. Cao, M.R. Grondine, et al., AZD1208, a potent and selective pan-Pim kinase inhibitor, demonstrates efficacy in preclinical models of acute myeloid leukemia, Blood 123 (6) (2014) 905–913.
[32] G. Corda, A. Sala, Non-canonical WNT/PCP signalling in cancer: Fzd6 takes centre stage, Oncogenesis 6 (7) (2017) e364.
[33] R. Raman, V. Kotapalli, R. Adduri, S. Gowrishankar, L. Bashyam, A. Chaudhary, et al., Evidence for possible non-canonical pathway(s) driven early-onset colorectal cancer in India, Mol. Carcinog. 53 (Suppl. 1) (2014) E181–6.