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Genetic disruption of the scaffolding protein, Kinase Suppressor of Ras 1 (*KSR1*), differentially regulates GM-CSF-stimulated hyperproliferation in hematopoietic progenitors expressing activating *PTPN11* mutants D61Y and E76K

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Abstract

Activating *PTPN11* mutants promote hematopoietic progenitor hyperactivation of Erk and hypersensitivity to GM-CSF. We hypothesized that Kinase Suppressor of Ras 1 (*KSR1*) contributes to activating *PTPN11*-induced GM-CSF hypersensitivity. Bone marrow progenitors from WT and *KSR1*^{−/−} mice expressing WT Shp2, Shp2E76K, or Shp2D61Y were evaluated functionally and biochemically. *KSR1* activation and interaction with phospho-Erk was enhanced in Shp2D61Y- and Shp2E76K-expressing cells. Genetic disruption of *KSR1* partially normalized Shp2E76K-induced GM-CSF hypersensitivity, but failed to correct Shp2D61Y-induced GM-CSF hypersensitivity. Collectively, these studies suggest that cells expressing Shp2E76K have a greater dependence on *KSR1* for GM-CSF hypersensitivity than cells expressing Shp2D61Y.

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Authors' Contributions

ZY – designed/performed research, wrote paper; MC, SAS, TS, and FY – designed and performed research; MY – performed statistical analysis; FCY and RJC designed research and wrote paper.

Conflict of Interest

The authors have no conflicting financial interests.

Keywords

juvenile myelomonocytic leukemia; *PTPN11*; *KSR1*; GM-CSF hypersensitivity; Shp2

Introduction

Juvenile myelomonocytic leukemia (JMML) is a lethal childhood disease clinically characterized by overproduction of myelomonocytic cells and by the *in vitro* phenotype of hematopoietic progenitor hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF). At the molecular level, Ras hyperactivation is implicated based on the majority of JMML patients bearing either loss-of-function *NF1* mutations or gain-of-function *RAS* or *PTPN11* mutations [1]. We demonstrated previously that the *PTPN11* gain-of-function mutants Shp2E76K and Shp2D61Y induce constitutively elevated and sustained activation of Erk [2], verifying the relevance the Ras-mitogen activated protein kinase (Raf1/MEK/Erk) cascade in this disease.

Signal transduction among Raf1/MEK/Erk kinases is mediated through direct phosphorylation, but scaffolding proteins also play an important role in regulating the location, strength, and duration of Raf1/MEK/Erk signaling. One of the best-defined scaffolding proteins that positively facilitates the Raf1/MEK/Erk cascade is kinase suppressor of Ras (KSR1) [3]. In quiescent cells, KSR1 is phosphorylated and constitutively associated with MEK. In response to growth factor stimulation or Ras activation, KSR1 is dephosphorylated (serine 392), translocates to the cell membrane, co-localizes MEK with Raf1 and Erk, and promotes Erk activation [4]. Inhibition and/or genetic deletion of Ksr1 have been demonstrated to reduce the capacity of gain-of-function Ras-induced tumorigenesis [5, 6]. Based on our previous observation of constitutively activated Erk in Shp2E76K- and Shp2D61Y-expressing cells, we hypothesized that KSR1 may contribute to the hyperproliferation and GM-CSF hypersensitivity of mutant Shp2-expressing cells.

Methods and Materials

Mice and cells

Generation of mice bearing genetic disruption of *KSR1* has been previously described [6]. *KSR1*^{+/-} mice were crossed to generate *KSR1*^{-/-} progeny and WT (*KSR1*^{+/+}) littermate controls. Progeny from these crosses were genotyped by polymerase chain reaction (PCR), as previously described [6]. All animal studies were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. Bone marrow low density mononuclear cells (LDMNCs) were either plated into progenitor assays, as previously described [7], or were retrovirally transduced with empty vector (MIEG3), WT Shp2, or the Shp2 mutants Shp2D61Y or Shp2E76K [2] and subjected to ³H-thymidine incorporation assays [7] or differentiated to macrophage progenitors for biochemical and mRNA expression studies [2], as previously described.

Immunoblot and co-immunoprecipitation analyses

Transduced and sorted cells were differentiated into macrophage progenitors and protein extracts were prepared, as described previously [2]. To examine Erk and Akt activation, cells were either starved (baseline) or stimulated with GM-CSF (50 ng/mL) and probed with anti-phospho-Erk, anti-Erk, anti-phospho-Akt, or anti-Akt (Cell Signaling Technology, Beverly, MA). To examine total KSR1 levels, protein extracts were immunoprecipitated with anti-KSR1 (#611576, BD Biosciences) and protein A sepharose beads (CL-4B, GE Healthcare) and blotted with the same antibody. To examine phospho-KSR1 levels and

interaction between KSR1 and phospho-Erk, protein extracts were immunoprecipitated with anti-KSR1 (clone C3H7, Upstate Biotechnology) and probed with anti-phospho-KSR1 (ser392, #4951, Cell Signaling Technology) or anti-phospho-Erk, respectively.

Semi-Quantitative RT-PCR

Total cellular RNA was prepared from macrophage progenitors using RNeasy (Qiagen, Valencia, CA) and RNA preparations were treated with RNase-free DNase I (Ambion, Austin, TX). First strand synthesis was performed using Omniscript RT kit (Qiagen). The following primer sets were used for PCR: *KSR1*, 5'-GCA GAG CGG GTG CCA GCA-3' and 5'-CCC AGC TGG GAC TCG TGG G-3', product length 303 bp; *Shp2*, 5'-GAT GGT TTC ACC CCA ACA TC-3' and 5'-GAC GTG GGT CAC TTT GGA CT-3', product length 497 bp; and *β-actin*, 5'-CGA GCA CAG CTT CTT TGC AG-3' and 5'-TTC TGA CCC ATT CCC ACC AT-3', product length 202 bp.

Results

KSR1 activation and interaction with phospho-Erk is increased in Shp2D61Y- and Shp2E76K-expressing macrophage progenitors

Previously, we demonstrated hyperactivation of GM-CSF-stimulated Erk activation in macrophage progenitors expressing the Shp2 gain-of-function mutants, D61Y and E76K [2]. Following GM-CSF stimulation, decreased levels of phospho-KSR1 were found in cells expressing Shp2 gain-of-function mutants compared to cells transduced with empty vector or WT Shp2, demonstrating enhanced KSR1 activation in the mutant Shp2-expressing cells (Figure 1). Consistently, significantly higher levels of phospho-Erk were associated with KSR1 after GM-CSF stimulation (Figure 1). These results provide biochemical evidence suggesting that KSR1 contributes to the Erk hyperactivation observed in mutant Shp2-expressing cells, leading us to hypothesize that KSR1 may contribute to Shp2 gain-of-function-induced hyperproliferation.

Genetic disruption of KSR1 reduces GM-CSF hypersensitivity of Shp2E76K-expressing hematopoietic progenitors, but not of Shp2D61Y-expressing hematopoietic progenitors

To examine the functional role of KSR1 in Shp2D61Y- and Shp2E76K-induced hypersensitivity to GM-CSF, we utilized mice bearing genetic disruption of KSR1 [6]. *KSR1*^{-/-} mice demonstrated normal development and no residual KSR1 protein expression persisted in splenocytes from the *KSR1*^{-/-} animals (Figure 2A). Pilot experiments demonstrated no overt abnormalities in spleen size or hematopoietic progenitor numbers within the bone marrow compartment of the *KSR1*^{-/-} mice (Figure 2B, 2C, and 2D).

Bone marrow LDMNCs from *KSR1*^{-/-} and WT littermate controls were isolated, retrovirally transduced with WT Shp2, Shp2D61Y, or Shp2E76K and subjected to ³H-thymidine assays. The GM-CSF-stimulated proliferation of *KSR1*^{-/-} cells expressing Shp2E76K was significantly reduced, but not entirely normalized to the level of WT Shp2-expressing cells (Figure 2F). In contrast, the proliferation of *KSR1*^{-/-} cells expressing Shp2D61Y was essentially unchanged compared to WT cells expressing Shp2D61Y (Figure 2E).

Genetic disruption of KSR1 reduces GM-CSF-induced Erk activation in Shp2E76K- and Shp2D61Y-expressing hematopoietic progenitors, but augments Akt activation

Given the differential effect of reduced KSR1 expression on GM-CSF-stimulated proliferation in the Shp2E76K v. the Shp2D61Y-expressing progenitors, we examined the effect of *KSR1* disruption on GM-CSF-stimulated Erk and Akt activation. Phospho-Erk levels were reduced by approximately 50% upon genetic disruption of *KSR1*, both at

baseline (Figure 3, compare phospho-Erk baseline, *KSR1*^{-/-} lanes 3 and 4 to baseline, WT lanes 3 and 4) and in response to GM-CSF (Figure 3, compare phospho-Erk 60 minutes, *KSR1*^{-/-} lanes 3 and 4 to 60 minutes, WT lanes 3 and 4). Strikingly, however, GM-CSF-stimulated Akt activation was increased, rather than decreased, in both Shp2E76K- and Shp2D61Y-expressing *KSR1*^{-/-} cells (Figure 3, compare phospho-Akt 60 minutes, *KSR1*^{-/-} lanes 3 and 4 to 60 minutes, WT lanes 3 and 4), suggesting that the gain-of-function Shp2 mutants induce a compensatory mechanism in the absence of KSR1 leading to enhanced signaling through the phospho-inositol-3-kinase (PI3K) pathway.

Given the differential effect of *KSR1* disruption on Shp2E76K- and Shp2D61Y-induced hypersensitivity to GM-CSF (Figure 2E and 2F) and the unexpected enhanced GM-CSF-stimulated phospho-Akt levels in the absence of KSR1 (Figure 3), we examined *KSR1* and Shp2 mRNA expression in each of the transduced cell populations at baseline and in response to GM-CSF stimulation. Using semi-quantitative RT-PCR, *KSR1* mRNA expression was similar in MIEG3-, WT Shp2, Shp2D61Y-, and Shp2E76K-transduced WT cells and was not altered by GM-CSF stimulation (Figure 3). For Shp2 mRNA expression, an approximately 2-fold higher level was found in the WT Shp2-, Shp2D61Y-, and Shp2E76K-transduced cells compared to MIEG3-transduced cells (Figure 3), as previously observed [2]. In *KSR1*^{-/-} cells, Shp2 expression was unaltered at baseline and in response to GM-CSF compared to that in WT cells. Additionally, no significant difference in expression was observed between Shp2D61Y and Shp2E76K, suggesting that the differential response of these mutants to loss of *KSR1* is not due to differential Shp2 expression.

Discussion

By regulating Raf1/MEK/Erk signaling, KSR1 is involved in many fundamental cellular processes including inhibition of tumor development [5, 6, 8]; thus, we predicted that KSR1 contributes to the regulation of GM-CSF-induced hyperactivation of Erk and hyperproliferation in mutant Shp2-expressing progenitors. This prediction was supported by our biochemical findings demonstrating increased KSR1 activation and association with phospho-Erk in Shp2D61Y- and Shp2E76K-expressing macrophage progenitors. However, while ablation of *KSR1* reduced proliferation of Shp2E76K-expressing cells, we observed no correction of GM-CSF-stimulated hyperproliferation of Shp2D61Y-expressing cells. Additionally, Akt activation was unexpectedly enhanced in mutant Shp2-expressing cells in the absence of KSR1. This effect was specific for the mutant Shp2-expressing cells, as the *KSR1*^{-/-} cells transduced with empty vector or WT Shp2 demonstrated reduced GM-CSF-stimulated phospho-Akt levels compared to the WT cells (Figure 3, compare phospho-Akt 60 minutes, *KSR1*^{-/-} lanes 1 and 2 to 60 minutes, WT lanes 1 and 2). We also found that disruption of *KSR1* did not aberrantly increase Shp2E76K or Shp2D61Y mRNA expression. Collectively, these findings suggest that compensatory upregulation of the PI3K pathway in the absence of KSR1 is not a general phenomenon observed in a normal cellular environment and is not due to aberrantly increased Shp2 expression, but is induced by gain-of-function Shp2 mutants. This observation highlights the need to simultaneously block multiple pathways to successfully normalize the oncogene-induced cellular proliferation and transformation.

The absence of GM-CSF hypersensitivity correction in the Shp2D61Y-expressing cells lacking KSR1 suggests that the Shp2D61Y-expressing cells are more responsive to the compensatory upregulation of Akt activation and are less dependent on KSR1 facilitating the Ras-MAPK pathway. This is the first report demonstrating a discrepancy between the functional effect of Shp2E76K and Shp2D61Y in hematopoietic progenitors. The mechanism of how hematopoietic progenitors expressing Shp2D61Y are more reliant on Akt

hyperactivation compared to that of cells expressing Shp2E76K will require additional work to ferret out. However, this observation provides a potentially important clue that the various *PTPN11* mutants exert their functional effects utilizing molecular mechanisms beyond the mere hyperactivation of Ras and implies that various Shp2 mutants function differentially downstream of Ras to promote GM-CSF hypersensitivity.

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Figure 1. KSR1 activation and interaction with phospho-Erk is increased in Shp2D61Y- and Shp2E76K-expressing macrophage progenitors

Total cellular protein extracts from baseline (serum- and growth factor-deprived) and GM-CSF-stimulated macrophage progenitors transduced with empty vector, WT Shp2, Shp2D61Y, or Shp2E76K were immunoprecipitated (IP) with anti-KSR1 and immunoblotted (IB) with anti-phospho-KSR1 or anti-phospho-Erk.

Figure 2. Genetic disruption of *KSR1* reduces GM-CSF hypersensitivity of Shp2E76K-expressing hematopoietic progenitors, but not of Shp2D61Y-expressing hematopoietic progenitors

(A) Immunoblot analysis to examine KSR1 expression in spleen LDMNCs from *KSR1*^{-/-} mouse and WT littermate control; (B) Spleen size was compared between *KSR1*^{-/-} mice and WT controls, spleen weight (mg) was normalized to body weight (gm); (C) and (D) Bone marrow LDMNCs from *KSR1*^{-/-} mice and WT littermate controls were plated into progenitor assays and analyzed for total colonies (CFU-C) per femur and per 25,000 cells and granulocyte-macrophage colonies (CFU-GM) per femur and per 25,000 cells; (E) and (F) ³H-thymidine incorporation assay of transduced, sorted bone marrow progenitors in increasing concentrations of GM-CSF. One of four independent experiments shown with similar results, each experiment performed in duplicate, **p*<0.05, for WT cells + Shp2E76K v. *KSR1*^{-/-} cells + Shp2E76K, statistics performed using two-tailed, unpaired student's *t* test.

Figure 3. Genetic disruption of *KSR1* reduces GM-CSF-induced Erk activation in Shp2E76K- and Shp2-D61Y-expressing hematopoietic progenitors, but augments Akt activation

Representative immunoblots showing baseline and GM-CSF-stimulated Erk and Akt activation and ethidium bromide-stained agarose gels showing baseline and GM-CSF-stimulated *KSR1*, *Shp2*, and β -actin mRNA expression in macrophage progenitor cultures derived from WT and *KSR1*^{-/-} bone marrow LDMNCs transduced with MIEG3 (empty vector), WT *Shp2*, *Shp2*D61Y, or *Shp2*E76K.