Colony-stimulating factor 3 receptor (CSF3R) M696T mutation does not impact on clinical outcomes of a Ph+ acute lymphoblastic leukemia patient

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Abstract

Colony-stimulating factor 3 receptor (CSF3R) mutations have been identified in a variety of myeloid disorders. Although CSF3R point mutations (eg, T618I) are emerging as key players in chronic neutrophilic leukemia/atypical chronic myelogenous leukemia , the significance of rarer CSF3R mutations is unknown. Here, we report a 32-year-old female who was diagnosed as Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph^+ ALL) with the CSF3R M696T mutation and was undergone unrelated donor hematopoietic stem cell transplantation. The patient achieved complete remission with chemotherapy in combination with tyrosine kinase inhibitor (TKI) and long-term survival by unrelated donor transplantation. Meanwhile, we performed a series of experiments using murine interleukin 3 (IL-3)-dependent Ba/F3 cell line to evaluate the transforming capacity of the CSF3R M696T mutation. We confirmed the presence of a CSF3R M696T germline mutation in this patient which was inherited from her mother. The in vitro experiment results showed that the CSF3R M696T mutation contributes marginally to the tumor transformation of Ba/F3 cells, indicating that CSF3R M696T mutation was neutral in tumor transformation ability. We concluded that TKI is effective in patients with the CSF3R M696T mutation in Ph^+ ALL and donors with CSF3R M696T mutation might still be selected as the candidate for transplantation.

Keywords: CSF3R M696T mutation, Familial inheritance, Pathogenicity, Ph+ acute lymphoblastic leukemia

1. INTRODUCTION

The colony-stimulating factor 3 receptor (CSF3R) gene is located on chromosome band 1p34 and encodes the transmembrane receptor for colony-stimulating factor 3, which plays a prominent role in the growth and differentiation of granulocytes.[1,2] CSF3R belongs to the cytokine receptor type I superfamily, which engages the canonical Janus kinase (JAK)/signal transducer and activator of transcription (STAT), RAS/RAF/MAP kinase, and PKB/AKT pathways.[1] There is a number of myeloid disorders that have been related to mutations in CSF3R, including severe congenital neutropenia (SCN), chronic neutrophilic leukemia (CNL), myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and atypical chronic myelogenous leukemia (aCML).[3–7] However, there is no report of Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL) associated with it. We report a new case of Ph+ ALL

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with the M696T mutation in the CSF3R gene and analysis showed that the gene mutated into familial inheritance.

2. RESULTS

2.1. Identification of the CSF3R M696T mutation in patient and her family members

The patient was diagnosed as Ph+ ALL with the CSF3R M696T mutation. This patient was treated and achieved complete remission with chemotherapy in combination with tyrosine kinase inhibitor (TKI) (Figure 1).

The patient intended to undergo sibling-matched hematopoietic stem cell transplantation (HSCT). On routine bone marrow gene workup, the sibling donor (sister) was also found to have the M696T mutation in the CSF3R gene (nucleotide changes c.2087 T > C, amino acid change p.M696T, mutation frequency 50.6%).

The over 50% allele fraction of the M696T mutation prompted us to determine whether the mutation was germline or somatic. We confirmed with sequencing the presence of a heterozygous M696T mutation in the oral mucosa sample of the patient and her sibling donor. We also sequenced the blood samples of the patient's parents. The results showed that the mutation was present in her mother (nucleotide changes c.2087 T > C, amino acid change p.M696T), but not the father. We conclude that CSF3R M696T mutation is a germline mutation in this patient, which was inherited from her mother, and BCR-ABL is the second mutation.

To exclude the possibility that CSF3R M696T mutation may have an adverse effect on hematopoiesis and/or transplantation, we subsequently chose a 28 years old male HLA 10/10 point matched unrelated donor instead of her sister. This patient received unrelated donor transplantation on April 15, 2019. She received conditioning based on etoposide 20 mg/kg/day × 2 days, busulfan 3.2 mg/kg/day × 3days, cyclophosphamide 40 mg/kg/day × 2 days, rabbit antithymocyte globulin 2 mg/kg/day × 4 days. Graft versus host disease prophylaxis regimen consisted of short-course methotrexate at a dose of 15 mg/m^2 intravenously on day 1 followed by 10 mg/m^2 intravenously on days 3, 6, and 11, tacrolimus 0.03 mg/kg/day intravenously beginning day -5, and mycophenolate mofetil 1 g/day beginning day -8. She received peripheral blood stem cell transplantation, the median numbers of MNC and CD34$^+$ cells were 18.17×10^8/kg, 1.64×10^6/kg, respectively. So far, there is no discomfort, the blood routine is recovered well, and the bone marrow minimal residual disease (MRD), BCR-ABL P210, and CSF3R gene mutation have been continuously negative.

2.2. CSF3R M696T mutation was neutral in tumor transformation ability

To ascertain CSF3R M696T mutation has any effect on normal or malignant hematopoiesis, we evaluate the transforming capacity of the CSF3R M696T mutation, we performed a series of experiments using murine interleukin 3 (IL-3)-dependent Ba/F3 cell line. We detected the relative mRNA level of *Csf3r* in the Ba/F3 cell line and found that, in comparison with whole bone marrow cells (WBM) and granulocytes (Gran), the expression level of *Csf3r* was barely detected (Figure 2A), indicating that Ba/F3 is a suitable cell line to investigate mutate CSF3R. We then used lentivirus-mediated infection to establish several stable cell lines expressing CSF3R or its mutations, including wild-type CSF3R, M696T from our patients, T618I and S783fs mutations both were previously reported as transformative mutations.[11] Empty vector was used as a negative control. The mRNA and protein levels of CSF3R in transduced cell lines were confirmed by RT-qPCR and Western blotting, respectively (Figure 2B and C). Under normal culture conditions (containing sufficient IL-3), there were no significant differences in cell proliferation among these groups (Figure 2D). However, with IL-3 withdrawal, M696T expression cell lines showed obvious growth retardation, while T618I and S783fs mutations expression cell lines could maintain proliferate (Figure 2E).

It was reported that various mutations of CSF3R acted through different pathways to induce oncogenesis, such as the truncated mutation S783fs preferentially activated SRC family kinases, while the membrane-proximal mutation T618I could mediate ligand-independent pathway through activation of the JAK-STAT pathway.[11] We first conducted G-CSF dependence assay and found that M696T expressing Ba/F3 cells were dose-dependent on G-CSF as well as wild-type CSF3R, which suggested that CSF3R M696T mutation did not affect the G-CSF response of G-CSF receptor (Figure 2F). We further performed immunoblot analysis for Stat and Src phosphorylation in Ba/F3 cells containing CSF3R mutations after 5-days culturing with or without IL-3. Consistent with previous studies,[11] Ba/F3 cells harboring T618I

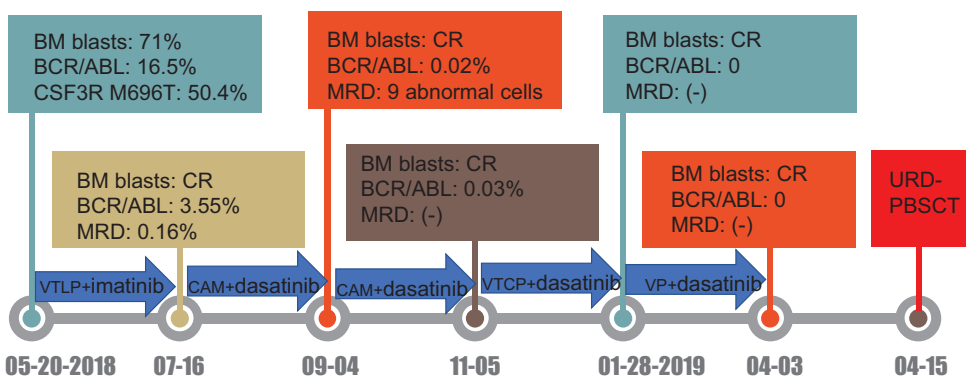

Figure 1. Clinical treatment and pedigree analysis of CSF3R gene mutation in this patient. (A) Clinical treatment of this patient. (B) Pedigree analysis of CSF3R gene mutation in this patient. BM = bone marrow morphology, CAM = cyclophosphamide + cytarabine + mercaptopurine, CR = complete response, MRD = minimal residual disease, PBSCT = peripheral blood hematopoietic stem cell transplantation, URD = unrelated donor, VP = vindesine + prednisone), VTLP = vinorelbine + pirarubicin + Pegaspargase + prednisone, VTCP = vindesine + pirarubicin + cyclophosphamide + prednisone.

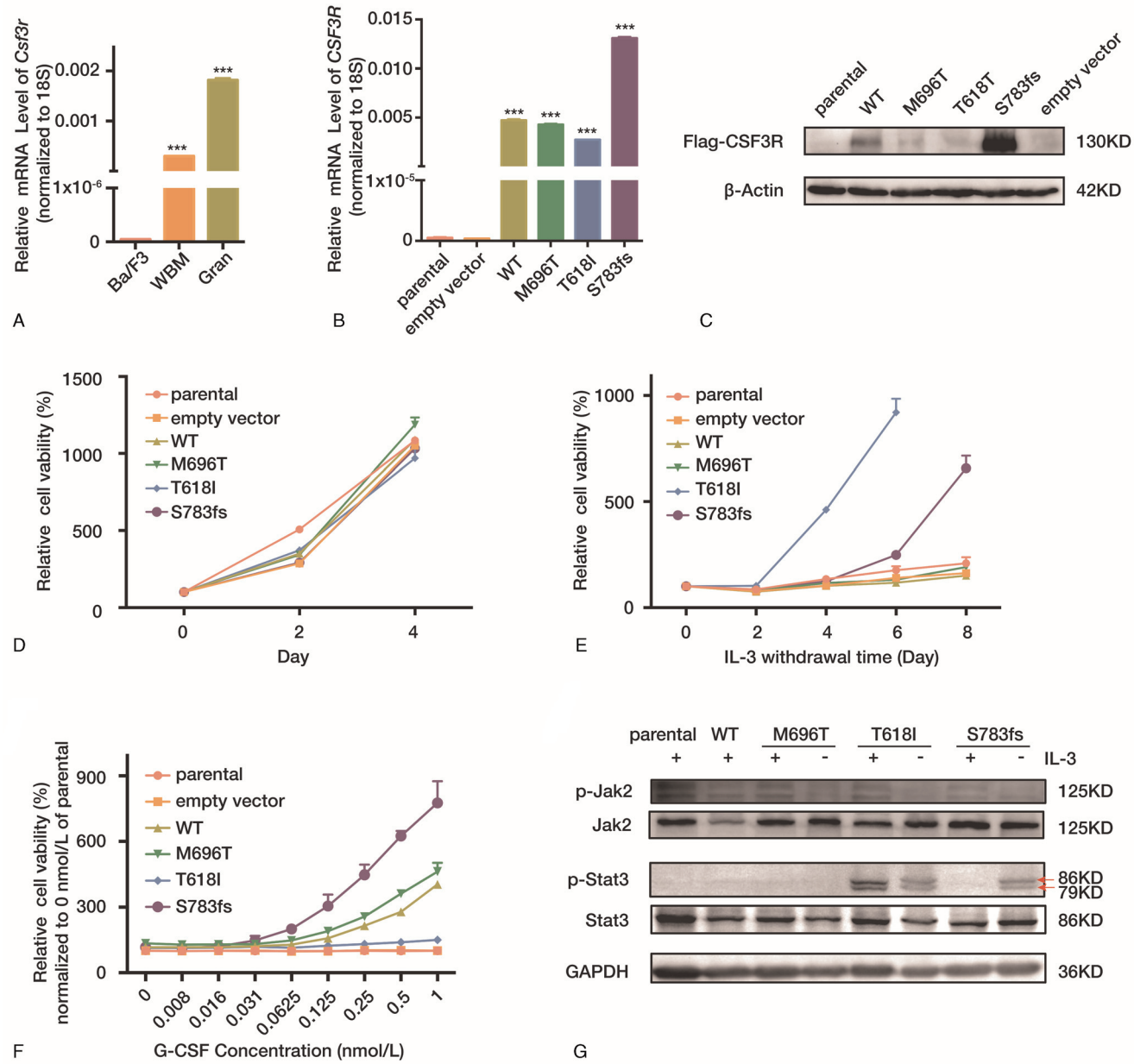

Figure 2. Tumor transformation ability and G-CSF dependency for various CSF3R mutations in Ba/F3 cells. (A) Csf3r mRNA levels quantified by RT-qPCR (n = 3). WBM, whole bone marrow cells of mice; Gran, granulocytes from bone marrow cells of mice, Gr-1^{+}Mac-1^{+}. Both two types of cells above were positive control due to their high expression of Csf3r. Data were normalized to 18S expression. $^{***}P < .001$. (B and C) Relative RNA and protein expression of CSF3R in Ba/F3 cell lines expressing wild-type CSF3R and its three mutations (n = 3). Parental Ba/F3 cells and empty vector (EV) infected Ba/F3 cells were used as negative controls. $^{***}P < .001$. (D and E) Relative cell viabilities of transduced Ba/F3 cells were measured in the presence (D) or absence (E) of IL-3. (F) Transduced Ba/F3 cells were cultured with a concentration gradient of G-CSF following IL-3 withdrawal and assessed by CCK8 assay at 72 hours. Data were normalized to parental Ba/F3 cells without G-CSF stimulation. (G) Western blotting analysis of the phosphorylation levels and total protein levels of Stat3 and Src in Ba/F3 cells expressing CSF3R mutations after 5 days culture in the presence and absence of IL-3. Parental Ba/F3 cells and Ba/F3 cells expressing wild-type CSF3R were used as controls. β-Actin and GAPDH as the internal controls. EV = empty vector.

mutation showed an abnormally upregulated phosphorylation level of Stat3 and Src in the presence of IL-3, and when IL-3 was withdrawn, both T618I and S783fs mutant cells displayed elevated phosphorylation level of Stat3 than those of parental and CSF3R WT groups. No obvious changes of Stat3 phosphorylation were observed in Ba/F3 cells expressing CSF3R M696T with or without IL-3 (Figure 2G). Motivated by these observations, the CSF3R M696T mutation harbors marginal contribution to the tumor transformation ability of Ba/F3 cells.

3. DISCUSSION

Granulocyte colony-stimulating factor (G-CSF), also known as colony-stimulating factor 3 (CSF3), is the main growth factor that controls both the proliferation and differentiation of myeloid progenitor cells into neutrophils.[12] It is a transmembrane protein consisting of 813 amino acids: 604 as an extracellular domain, 26 in the transmembrane domain, and 184 in the intracellular cytoplasmic domain. The extracellular domain of the receptor is composed of an immunoglobulin-like (Ig-like) domain, a

cytokine receptor homology domain (CRH domains), and three fibronectin type III (FN III) domains.[13] G-CSF is the primary ligand for granulocyte colony-stimulating factor receptor (G-CSFR). G-CSFR is encoded by the CSF3R gene.[13] A variety of CSF3R mutations have been identified from patient-derived samples. These various mutations may provide the key to understanding the divergence of disease phenotypes and can be divided into three groups: extracellular domain, transmembrane proximal, and intracellular truncation mutations.[13]

There is a number of myeloid disorders that have been related to mutations in CSF3R. Extracellular domain mutations in the CSF3R gene affect the extracellular domain of G-CSFR and are commonly associated with SCN and chronic idiopathic neutropenia (CIN). The extracellular mutant P229H was the first reported extracellular CSF3R mutation. Another series of extracellular domain mutations in CSF3R cause truncations at the WSXWS motif of the receptor.[14,15] These mutations result in amino acid substitutions at S296G, S319G, or S299G, causing truncations of the extracellular regions of the receptor and affecting ligand binding efficiency.[14–16] Another extracellular domain mutation resulting in S624R causes a frameshift truncation after the fibronectin domain of the receptor and has been reported in CIN patients.[15] These extracellular CSF3R mutations do not typically lead to myeloid malignancy but result in a neutropenic condition.[13] Transmembrane proximal point mutations in CSF3R affect the transmembrane domain and adjacent residues of the G-CSFR. The most prevalent proximal mutation is T618I.[17] In recent studies, around 83% of CNL patients have this mutation.[18] This mutation has also been reported to have a predisposition to myeloid malignancies in neutropenic patient.[19,20] Furthermore, T618I is observed in aCML, chronic myelomonocytic leukemia (CMML), de novo AML, and early T-cell precursor acute lymphoblastic leukemia (ETP-ALL), but with lower frequencies.[11,21,22] Other less frequent mutations of this class are N630K and R631del, leading to prolonged activation of STATs similar to G-CSFR-T618I after stimulation with G-CSF.[21,22] Intracellular truncation mutations consist of nonsense or frameshift somatic mutations in CSF3R that cause a truncation between 82 and 98 amino acids from the carboxyl terminus of the G-CSFR receptor.[15,23–25] These mutations are the most common type of mutation in the MDS/AML patient.[26,27] Certain CSF3R variants that have been reported at low frequencies in hematologic malignancies or found through sequencings, such as the P706C, P733T, E808K, and M696T, and R698C mutations, have not exhibited transformative capacity in the Ba/F3 cytokine independent model.[28] So far there is no report of Ph+ ALL associated with the gene mutation. Our case had a short medical history and without splenomegaly. The diagnosis of Ph+ ALL was confirmed based on bone marrow morphology, flow cytometry, and fusion gene. She was detected a mutation in the CSF3R gene (nucleotide change c.2087 T>C, amino acid change p.M696T, mutation frequency 50.4%). In the early stage of treatment, BCR-ABL fusion gene did not turn negative after imatinib combined with chemotherapy. Gerlach et al[29] found that primary myelofibrosis patients frequently show additional somatic mutations, the cumulative number of which seems to determine a worse prognosis. Although these mutations alone cannot cause a myeloproliferative neoplasms-phenotype and are frequently found in other hematologic malignancies, they seem to generally increase the genetic instability of the affected cells, thereby potentially giving them additional pro-oncogenic advantages and are probably able to modify disease progression. Wu et al[30] demonstrated high expression levels of GM-CSFR and G-CSFR, as well as their promotable role for viability in Ph+ ALL cells. They further found that recombinant human G-CSF (rhG-CSF) influenced the sensitivity of SUP-B15 (a Ph+ ALL cell line) cells to TKIs. Is it possible that our patient's poor response to imatinib is due to G-CSFR affecting TKI sensitivity due to the combination of CSF3R mutations? However, the BCR-ABL fusion gene gradually turned negative and continued to be negative after using dasatinib. Therefore, the M696T mutation of the CSF3R gene in the Ph+ ALL patient does not affect the therapeutic effect of TKI on the BCR-ABL fusion gene.

The patient's risk was stratified into high-risk groups and was ready for HSCT. She had an HLA 10/10 point matched sibling donor. Normally, we should choose this sibling donor. But on routine bone marrow gene workup, the sibling donor was also found to have the M696T mutation in the CSF3R gene (nucleotide changes c.2087 T>C, amino acid change p.M696T, mutation frequency 50.6%). Kosmider et al[31] found that the M696T variant observed in three CMML patients was a somatic mutation. The significance of this mutation in a normal person is not clear. However, from the in vitro and in vivo data and from the fact that somatic CSF3R mutations are frequently detected prior to morphologic evidence of transformation, we have hypothesized that they represent an important step in the progression of SCN to MDS/AML.[26,32] Germeshausen et al[33] presented data on a total of 218 patients with chronic neutropenia, including 148 patients with SCN (23/148 with secondary malignancies). Of 23 patients with SCN with signs of malignant transformation, 18 (78%) were shown to harbor a CSF3R mutation, indicating that these mutations, although not a necessary condition, are highly predictive for malignant transformation even if detected in a low percentage of transcripts. Their results strongly suggested that acquisition of a CSF3R mutation is an early event in leukemogenesis that has to be accompanied by cooperating molecular events, which remain to be defined. Since the over 50% allele fraction of the M696T mutation prompted us to determine whether the mutation was germline or somatic. Our sequencing confirmed the presence of a heterozygous M696T mutation in the oral mucosa sample of the patient and her sibling donor. We tested the blood samples of the patient's parents. The results showed that the mutation was present in the mother, but not in the father. We confirmed the presence of the mutation in this patient was germline, and the mutation was inherited from her mother. No one knows its pathogenicity. Finally, we chose a 28 years old male HLA 10/10 point matched unrelated donor. This patient received unrelated donor transplantation on April 15, 2019. So far, there is no discomfort, the blood routine is recovered well, and the bone marrow MRD, BCR-ABL P210, and CSF3R gene mutation have been continuously negative.

Meanwhile, to evaluate the transforming capacity of the CSF3R M696T mutation, we performed a series of experiments in vitro. The results were similar to Kosmider et al[31] study, which showed that the CSF3R M696T mutation harbors marginal contribution to the tumor transformation ability of Ba/F3 cells. CSF3R M696T mutation was neutral in tumor transformation ability. Although in our current case, donors with CSF3R M696T mutations showed neutral effects, long-term tracing and further investigations with more cases and animal model is needed.

4. CONCLUSION

Our case has a typical presentation of Ph+ ALL with the CSF3R M696T mutation. Clinical treatment process showed that the

M696T mutation of the CSF3R gene in the Ph+ ALL patient does not affect the therapeutic effect of TKI on the BCR-ABL fusion gene. We confirmed the presence of the mutation in this patient was germline, and the mutation was inherited from her mother. The in vitro experiment result showed the CSF3R M696T mutation harbors marginal contribution to the tumor transformation ability of Ba/F3 cells. CSF3R M696T mutation was neutral in tumor transformation ability. Therefore, we believe that TKI is still effective in patients with the CSF3R M696T mutation in Ph+ ALL. Donor with CSF3R M696T mutation might still be selected.

5. MATERIALS AND METHODS

5.1. Clinical specimens

All clinical samples were obtained with informed consent by the patient and her family members. Here, we report on a 32-year-old female who presented with asthenia in May 2018. Medical history was 20 days. The physical examination was pale without splenomegaly. The initial hematological workup revealed a white blood cell (WBC) count of 97×10^9/L (normal range 4–10×10^9/L), hemoglobin 54 g/L (normal 110–150 g/L), and the platelet count was 94×10^9/L (normal range of 100–300×10^9/L). There was 84% prolymphocyte in the bone marrow. The immunophenotype of the blasts as judged from flow cytometry was in accordance with a B-ALL: CD19 (87%), cTdT (67%), CD79a (43%), CD10 (23%), CD33 (34%), CD24 (24%), and CD22 (14%). The fusion gene for BCR-ABL P210 was positive. Hot mutation closely related to diseases was: CSF3R (nucleotide change c.2087 T > C, amino acid change p. M696T, mutation frequency 50.4%). Mutation that may be disease-related was: WT1, ARID1A, KMT2D, and RELN. Cytogenetic analysis showed 46, XX, t(9;22) (q34;q11).

5.2. Cell culture

HEK293T (ATCC) cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Biological Industries), and penicillin/streptomycin (Invitrogen). Ba/F3 cells (National Infrastructure of Cell Line Resource) were maintained in IMDM basic media (Gibco) supplemented with 10% FBS (Biological Industries), penicillin/streptomycin (Invitrogen), normocin (InvivoGen), and 10 ng/ml rmIL-3 (Peprotech).

5.3. Retroviral vector production and transduction

The gene sequence of wild-type CSF3R was from NCBI (NM_156039.3) and CSF3R mutations were all synthesized by YouBio Co., Ltd. The plasmid backbone of CSF3R and its mutations was pCDH-MSCV-EF1-mcherry, with a packaging system of pMD2G and psPAX2. Virus was produced by transfecting HEK293T cells with polyetherimide and transduced into Ba/F3 cells. Transduced Ba/F3 cells with mCherry positive were sorted by flow cytometry (FACS, Aria III, BD Biosciences).

5.4. RT-qPCR

Total RNA from Ba/F3 cells was extracted using RNeasy Mini Kit (QIAGEN). Then a reverse transcription system (TAKARA) was used to reverse-transcribe mRNA to cDNA. The cDNA of different samples was amplified by QuanStudio 5 Real-Time PCR Instrument with FastStart Universal SYBR Green Master (Roche). The pairs of primer for detecting mRNA expression of Csf3r and CSF3R were as follows: Csf3r-CAAGAAGCTTG CACCCGATG, GGCATGTTCTGCTGGTCTCT; CSF3R-GA CCCAAGGGGACTCCATCC, GGGGCTCCAGTTTCACAA-CAT. All the data were normalized to 18S expression. Relative expression changes were calculated with $2-\Delta Ct$ method.

5.5. Western blotting analysis

The cells were washed 3 times with pre-chilled PBS, discarded the supernatant followed by lysing with an appropriate amount of 1× loading buffer (500 μl/1×10^7 cells) at room temperature for 30 minutes, and stored at −20°C. The mixed samples were boiled for 10 minutes in a 100°C-heat block before loading on a 10% SDS-PAGE gel. The following primary antibodies and reagents were used: anti-FLAG (F1804, Sigma), anti-Src (#2109, Cell Signaling Technology), anti-Phospho-Src (#6943, Cell Signaling Technology), anti-Stat3(#9139, Cell Signaling Technology), anti-Phospho-Stat3 (#9145, Cell Signaling Technology), anti-β-Actin (#MA5-15739, Invitrogen), and anti-GAPDH (#8884, Cell Signaling Technology). All primary antibodies were used at a 1:1000 dilution, and affinipure donkey anti-mouse or anti-rabbit IgG (H+L) secondary antibodies (Jackson ImmunoResearch) were used at a 1:10000 dilution. Finally, the bands were detected by Bio-Rad ChemiDoc (Bio-Rad).

5.6. Ba/F3 IL-3 withdrawal assay

The IL-3 withdrawal assay was performed as described[8,9] with some modifications. Briefly, parental Ba/F3 cells or those stably expressing wild-type CSF3R or its mutations were washed 3 times and grown in 96-wells plates at a density of 5×10^4 cells per mL with cytokine-free media (IMDM with 10% FBS, penicillin/streptomycin, and normocin). Cell viability was measured using CCK8 assay and read at 450 nm after 4 hours using a Synergy 4 plate reader (BioTek) every 1 to 2 days. All results are normalized with the absorbance value of each cell line on day 0.

5.7. G-CSF dependence assay

Ba/F3 cells expressing wild-type CSF3R and mutations were plated with a concentration gradient of G-CSF after removing IL-3 and cultured for 72 hours and subjected to CCK8 assay.[10]

5.8. Statistical analysis

The GraphPad Prism 6.0 software was used to measure statistical analyses. The data were presented as the mean (±SEM). Statistical significance was determined using Student *t* tests and expressed as *P* value ($^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$).

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