Hematol Oncol Stem Cell Ther (2019) 12, 146–154

Available at www.sciencedirect.com

ScienceDirect

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

ORIGINAL RESEARCH REPORT

Phenotypic characterization of malignant progenitor cells in patients with idiopathic myelofibrosis

Yonghua Bao, Wenyang Hu *, Yongchen Guo, Wancai Yang

Department of Pathology, Institute of Precision Medicine, Jining Medical University, Jining, China

Received 16 August 2018; received in revised form 7 January 2019; accepted 21 January 2019
Available online 20 February 2019

KEYWORDS
Chromosome abnormality;
Idiopathic myelofibrosis;
IL3-Rα;
JAK2V617F

Abstract
Objective/Background: Idiopathic myelofibrosis (IM) is a clonal hematological malignancy originating from pluripotent hematopoietic stem cells (HSC). HSC are very rare potent cells that reside in the bone marrow (BM) and at a lower level in peripheral blood (PB). Previous studies showed that IM PB $CD34^+$ cells contain not only BM repopulating cells belonging to the malignant clone but also residual normal HSC.
Methods: In the current study, we separated the subpopulations of IM PB $CD34^+$ cells using IL-3Rα/CD123 labeling and further characterized them by genetic and functional analyses.
Results: We differentiated IM PB $CD34^+$ cells into three subpopulations (IL-$3R\alpha^{high}$, IL-$3R\alpha^{low}$, and IL-$3R\alpha^{negative}$). IL-$3R\alpha^{high}$ $CD34^+$ cell subgroup represents a small population in IM PB $CD34^+$ cells which was not seen in normal G-CSF mobilized $CD34^+$ cells. IM IL-$3R\alpha^{high}$ $CD34^+$ cells contained significant higher percentage of cells bearing marker chromosome detected by fluorescence in situ hybridization (FISH) analysis. In the absence of growth factors, IM IL-$3R\alpha^{high}$ $CD34^+$ cells exhibited abnormal colony forming ability and carried greater percentage of JAK2V617F mutant allele compared with IL-$3R\alpha^{low}$ and IL-$3R\alpha^{negative}$ $CD34^+$ cells.
Conclusion: These data indicate that IL-$3R\alpha^{high}$ $CD34^+$ cells from IM enriched for the malignant progenitor cells and IL-3Rα/CD123 may be a potential biomarker and therapeutic target for IM. Our findings will be further validated in future studies with a larger sample size and serial transplant in murine models.

* Corresponding author at: Department of Pathology, Institute of Precision Medicine, Jining Medical University, 16 Hehua Road, Jining 272067, China.
E-mail addresses: baoyonghua2005@126.com (Y. Bao), wyhu02@yahoo.com (W. Hu).

https://doi.org/10.1016/j.hemonc.2019.01.001

Introduction

Idiopathic myelofibrosis (IM) is a clonal hematological malignancy which originates at the level of the pluripotent hematopoietic stem cell (HSC) [1—4] HSC are multipotent cells which are capable of self renewal that reside in the bone marrow (BM) and at lower levels in peripheral blood (PB) [5]. The HSC defect results in a profound hyperplasia of morphologically abnormal megakaryocytes (MKs) and clonal populations of monocytes, which have been shown to locally release fibrogenic growth factors, leading to BM fibrosis [6—10]. Mobilized PB $CD34^+$ cells provide a source of stem cells for use as grafts for transplantation. Barosi et al. [11] have demonstrated that the PB of IM patients contained 360 times greater numbers of $CD34^+$ cells than normal controls. IM represents a unique situation in which the numbers of PB $CD34^+$ cells are frequently increased in the absence of extrinsic stimuli. Previous studies showed that IM PB $CD34^+$ stem cells contain not only BM repopulating cells belonging to a malignant clone but also residual normal HSC [12,13]. The malignant phenotype in IM likely results from a combination of genetic abnormalities leading to dysregulation of critical genes that contribute to cell proliferation, differentiation, and cell death [14].

Molecular markers for IM progenitor cells have long been searched for, but little progress has been made although recently recurrent chromosomal abnormalities have been documented in 30% of cases. A single point mutation (V617) has been identified in JAK2, a cytoplasmic tyrosine kinase, enables constitutive tyrosine kinase activity without the need for ligand binding to hematopoietic receptors. The number of V617F JAK2 copies likely contributes to the phenotypic heterogeneity of the JAK2V617F positive myeloproliferative disorders (MPD) [15—17]. This mutation has been observed in 50% of patients with IM, making it unlikely that it accounts entirely for the origin of IM [17]. Studies had been performed for the characterization of IM PB $CD34^+$ progenitor cells using various molecular markers [12—14]. However, a suitable phenotypic marker for IM tumor progenitor cells has yet to be identified. Interleukin 3 (IL-3) plays an important role in the promotion of hematopoietic progenitor cell survival, proliferation, and differentiation [18—23]. IL-3 receptor is composed of an α- and β-subunit [18,19]. IL-3Rα, also known as CD123, has been found to be widely expressed in cells belonging to a variety of hematological malignancies [23—28]. Human acute myeloid leukemia (AML) stem cells express elevated levels of IL-3Rα/CD123 and it serves as a unique marker for AML stem cells [26—29]. Elevated expression of IL-3Rα in AML is associated with enhanced blast proliferation, increased cellularity, and a poor prognosis [27]. Therefore, IL-3Rα/CD123 has diagnostic, prognostic as well as therapeutic value in AML [30,31]. Ouyang et al. [32] showed that $CD34^+$ myeloblasts in myeloproliferative neoplasms (MPN) have frequently increased CD123 expression level. In addition, IL-3Rα/CD123 is also highly expressed in blastic plasmacytoid dendritic cell neoplasm (1 of the immunohistochemical criteria in BPDCN) and minimally expressed on normal cells, suggesting it is an appropriate target for therapy [33,34].

In this study, we are the first to characterize IM tumor progenitor cells by using IL-3Rα/CD123 as a phenotypic marker. We have been able to separate IM PB $CD34^+$ cells into three subgroups according to the differential expression of IL-3Rα: IL-3Rα^{high}, IL-3Rα^{low}, and IL-3R$\alpha^{negative}$. We found that there was a near 40-fold increase in the average percentage of IL-3Rα^{high} $CD34^+$ cells over total $CD34^+$ cells in IM patients as compared with that in G-CSF mobilized normal volunteers. We utilized four IM patients with chromosome abnormalities and evaluated the percentages of chromosome abnormalities in each IL-3Rα subpopulation by FISH analysis. We found that there was a significant increase in the percentage of cells containing marker chromosomes in IL-3Rα^{high} $CD34^+$ cells compared with IL-3Rα^{low} and IL-3R$\alpha^{negative}$ $CD34^+$ cells. In vitro colony forming assays in the presence of six growth factors demonstrated that three subpopulations of IM PB $CD34^+$ cells were capable of forming hematopoietic colonies in vitro. However, in the absence of growth factors, IL-3Rα^{low} and IL-3R$\alpha^{negative}$ cells formed significantly reduced or no colonies in vitro whereas IL-3Rα^{high} cells retained the similar colony forming ability as seen in the presence of growth factors. Finally, we evaluated the JAK2V617F allele status of colonies cloned from IM PB $CD34^+$ cells of IL-3Rα^{high}, IL-3Rα^{low}, and IL-3R$\alpha^{negative}$ subpopulations, we found that colonies cloned from IL-3Rα^{high} $CD34^+$ cells exhibit increased percentage of JAK2V617F mutant allele which result an increased tumor burden.

We have characterized a small subpopulation of IM PB $CD34^+$ cells highly expressed the IL-3Rα/CD123 which was not observed in normal G-CSF mobilized PB $CD34^+$ cells, this small subgroup of IL-3Rα^{high} $CD34^+$ cells may be enriched for the IM progenitor cells.

Materials and methods

Reagents

Human recombinant granulocyte colony-stimulating factor (G-CSF), IL-3, IL-6, stem cell factor (SCF), erythropoietin (EPO), and thrombopoietin (TPO) were kindly provided by Amgen Biologicals (Thousand Oaks, CA, USA). Purified recombinant forms of fluorescein isothiocyanate (FITC)-conjugated antihuman $CD34^+$ antibody and phycoerythrin (PE)-conjugated antihuman IL-3Rα antibody were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Patients and healthy control individuals

All human tissue samples were obtained after informed consent following the guidelines of the Institutional Review Board of Jining Medical University, Jining, China. PB samples were obtained from: (a) healthy donors mobilized receiving G-CSF at 5 μg/kg/d subcutaneously; and (b) patients with IM who meet the World Health Organization (WHO) diagnostic criteria for IM. Patients receiving cytotoxic agents at the time of study or patients having evidence of transformation to acute leukemia were excluded.

Purification of human PB $CD34^+$ cells

The PB samples were layered onto Ficoll-Hypaque (1.077 g/mL; Amersham Biosciences, Piscataway, NJ,

USA), and low-density mononuclear cells (MNCs) were separated after density centrifugation. A $CD34^+$ cell population was isolated using a Magnetic Activated Cell Sorting $CD34^+$ Isolation Kit (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. The purity of the $CD34^+$ cell population was analyzed using a FACSCaliber flow cytometer (Becton Dickinson, Mountain View, CA, USA). Cell fractions showing a $CD34^+$ cell purity of $\geq$90% will be used for subsequent experiments.

Phenotypic analysis of IM PB $CD34^+$ cells and cell sorting selection

We performed the phenotypic analysis and sorted the PB $CD34^+$ cells using anti-$CD34^+$ and anti-IL-3Rα antibodies. PB $CD34^+$ cells (1—5 million) were used for cell sorting. Separate aliquots of isolated PB $CD34^+$ were double stained with FITC-conjugated anti-$CD34^+$ and PE-conjugated anti-IL-3Rα (Becton Dickinson, San Jose, CA, USA) antibodies. The percentage of $CD34^+$ cells expressing IL-3Rα was determined by flow cytometric analysis. Cell viability analysis of PB $CD34^+$ cells was performed using propidium iodide (PI) staining. Cell populations expressing different levels of IL-3Rα (IL-$3R\alpha^{high}$, IL-$3R\alpha^{low}$, IL-$3R\alpha^{negative}$) were collected by means of a high-speed cell sorter (Dako, Carpinteria, CA, USA). Sorted cells with different expression levels of IL-3Rα were used for subsequent experiments. In each cell group, 500 cells were used for colony assays. In case the patient has a known marker chromosomal abnormality, glass slides with 1×10^3 cells were prepared by cytospin and were used for FISH analysis.

Chromosome abnormality detected by FISH analysis

$CD34^+$ cells from four patients with marker cytogenetic abnormalities (monosomy 7, isochromosome 17, and 13q deletion) were studied in an identical fashion. Cytospin preparations of $CD34^+$ cell subpopulations of IL-$3R\alpha^{high}$, IL-$3R\alpha^{low}$, and IL-$3R\alpha^{negative}$ were stained with chromosome enumeration probes (CEP) (VYSIS, Abbott, Laboratories, Des Plaines, IL, USA) for monosomy 7, isochromosome 17, and 13q deletion, respectively, using interphase FISH analysis according to the standard methods. An attempt was made to analyze at least 100 metaphases. The description of karyotypes followed the recommendations of the International System for Human Cytogenetic Nomenclature and was performed by pathologists at Jining Medical University.

Functional analysis of $CD34^+$ cells by colony forming assay

$CD34^+$ cell subpopulations were plated at a concentration of 500 cells/mL in semisolid medium. One milliliter of culture mixture containing 500 $CD34^+$ cells, 0.9% methylcellulose, 30% fetal bovine serum (FBS), 1% bovine serum albumin (BSA), 0.05 mM 2-mercaptoethanol (2-ME; StemCell Technologies, Vancouver, BC, Canada) and with or without a cytokine cocktail containing SCF (100 ng/mL), IL-3 (100 ng/mL), IL-6 (100 ng/mL), G-CSF (20 ng/mL), EPO (4 U/mL), and TPO (100 ng/mL) (all from Amgen) were placed in 35-mm nontissue culture dishes and incubated at 37 °C in 5% CO_2. All cultures were performed in triplicate and various colony types enumerated using an inverted microscope at Days 12—14 of culture, according to previously described criteria.

JAK2V617F mutation in colonies analyzed by nested allele-specific Polymerase Chain Reaction (PCR)

Individual hematopoietic colonies were randomly plucked from the semisolid media and genomic DNA (gDNA) was isolated by using the Extract-N-Amp™ Blood PCR Kits (Sigma St. Louis, MO, USA). The JAK2V617F mutation was detected by using nested allele-specific PCR as previous described. Briefly, a 521 bp DNA fragment containing the V617F mutation site was amplified from the gDNA using forward primer P1 5′-GATCTCCATATTCAGGCTTACACA-3C and reverse primer P1r 5′-TATTGTTTCCTTTTTCACAAGA-3′. PCR was carried out by 35 cycles consisting of 30 s at 90 °C, 30 s at 60 °C, and 30 s at 72 °C. A sample of 0.5 μL PCR products were further amplified by nested and allele-specific PCR using an outer primer set of P2 5′-CCTCAGAACGTT GATGGCA-3′, P2r 5′-ATTGCTTTCCTTTTTCACAAGA-3′ for amplifying a 453-base pair (bp) DNA fragment and two inner primers of Pnf 5′-AGCATTTGGTTTAAATTATGGAGTATATG-3′, Pmr 5′-GTTTTACTTACTCTCGTCTCCACAAAA-3′ for detecting the wild type (229 bp) and mutant type (279 bp) sequences respectively. Second round PCR cycle was carried out by 35 cycles of 90 °C for 30 s, 59 °C for 25 s, and 72 °C for 25 s. Colonies classified as homozygous for JAK2V617F mutant contained only the 279 bp band and homozygous wild type contained only the 229 bp band, whereas heterozygous colonies were identified based upon the presence of both the 279 bp and 229 bp bands.

Results

Phenotypic characterization of IM PB $CD34^+$ cells by Fluorescence Assisted Cell Sorting (FACS) analysis using IL-3Rα

Using IL-3Rα/CD123 as a phenotypic marker, we were able to separate IM PB $CD34^+$ cells into three subpopulations: IL-$3R\alpha^{high}$, IL-$3R\alpha^{low}$, and IL-$3R\alpha^{negative}$. Fig. 1 shows a representative dot plot of flow cytometric analysis of IL-3Rα expression on PB $CD34^+$ cells from (A) a G-CSF mobilized volunteer and (B) a patient with IM. The majority of the $CD34^+$ cells from both G-CSF mobilized controls and patients with IM express IL-3Rα at low level. There were ~4% of the IM PB $CD34^+$ cells which express IL-3Rα at high level, by contrast, only 0.1% of the normal G-CSF mobilized PB $CD34^+$ cells express IL-3Rα at high level ($p < .01$, IM vs. G-CSF mobilized control, Table 1). The significant increase of cell number in IL-$3R\alpha^{high}$ $CD34^+$ cells in patients with IM indicates that IL-3Rα could be a useful phenotypic marker for IM $CD34^+$ cells.

IM PB IL-$3R\alpha^{high}$ $CD34^+$ cells contain higher percentage of marker chromosome abnormalities

Because the percentage of IM PB IL-$3R\alpha^{high}$ $CD34^+$ cells was much higher than that in normal G-CSF mobilized PB $CD34^+$ cells we therefore hypothesized that the IM PB IL-$3R\alpha^{high}$

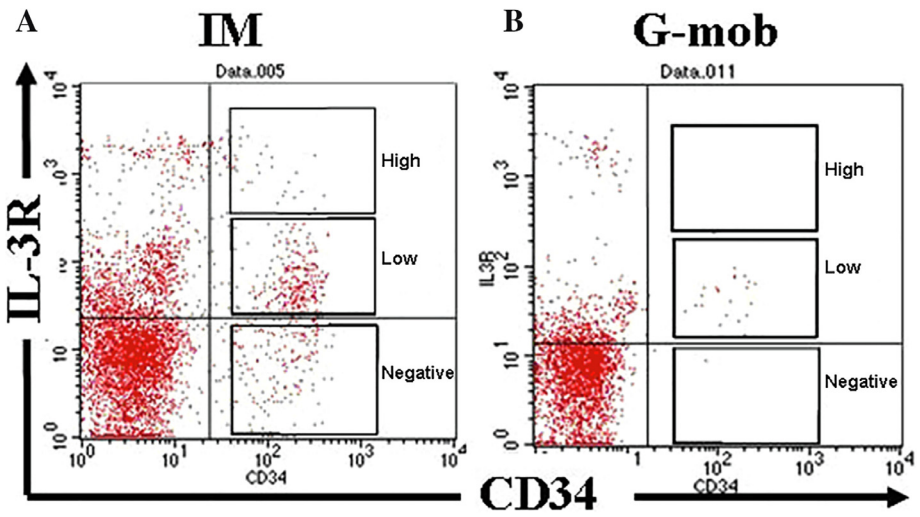

Fig. 1 Flow cytometric analysis of IL-3Rα expression on PB CD34⁺ cells from G-CSF mobilized volunteers and patients with IM. Representative dot plots of IL-3Rα labeling in cells from (A) a G-CSF mobilized volunteer; (B) a patient with IM are shown. *Note*. G-CSF = granulocyte colony-stimulating factor; IM = idiopathic myelofibrosis.

Table 1 Percentage distributions of CD34⁺ cells from G-CSF mobilized volunteers and patients with IM in IL-3Rα^{high}, IL-3Rα^{low}, and IL-3Rα^{negative} subpopulations.

CD34⁺ cells	IL3Rα^{high}, %	IL3Rα^{low}, %	IL3Rα^{negative}, %
G-CSF mobilized velunteers (G-Mob) (n = 4)	0.1 ± 0.1	81.1 ± 10.2	18.2 ± 6.4
IM (n = 7)	3.9 ± 2.1*	62.6 ± 8.3	33.5 ± 7.1

Note. G-CSF = granulocyte colony-stimulating factor; IM = idiopathic myelofibrosis.
* p < .05 versus G-CSF mobilized volunteers.

CD34⁺ cells may be enriched for the malignant progenitor cell. We then selected four patients with IM (IM2, IM13, IM40, and IM44) with marker chromosome abnormalities detectable by FISH (2 patients with monosomy 7, 1 with isochromosome 17, and 1 with 13q deletion). We did the selection of IL-3Rα^{high}, IL-3Rα^{low}, and IL-3Rα^{negative} CD34⁺ cell subpopulations using cell sorting and the percentages of chromosome abnormalities in each subpopulation were evaluated by FISH analysis. The average percentage of cells containing marker chromosome from four patients with IM are shown in Fig. 2, and the detail marker chromosome abnormalities for each patient with IM in IL-3Rα^{high}, IL-3Rα^{low}, and IL-3Rα^{negative} subpopulations are listed in Table 2. There was a significant increase in the average percentage of cells containing marker chromosome abnormalities in IL-3Rα^{high} CD34⁺ cells compared with IL-3Rα^{low} and IL-3Rα^{negative} CD34⁺ cells (51 ± 8% vs. 24 ± 9%, and 16 ± 9%, p < .05). Actual value varied in these four patients, three out of four patients had greater different value between cell groups, one patient had less difference. These results strongly suggest that IM PB IL-3Rα^{high} CD34⁺ cells maybe enriched for malignant progenitor cells.

IM PB IL-3Rα^{high} CD34⁺ cells exhibit abnormal colony forming ability

The next question we asked was if this small group of IM PB IL-3Rα^{high} CD34⁺ cells has increase in vitro cloning efficiency. We performed the colony-forming assays under two different conditions: with and without growth factors. In the presence of six growth factors, cells from all three CD34⁺ cell subpopulations formed hematopoietic colonies

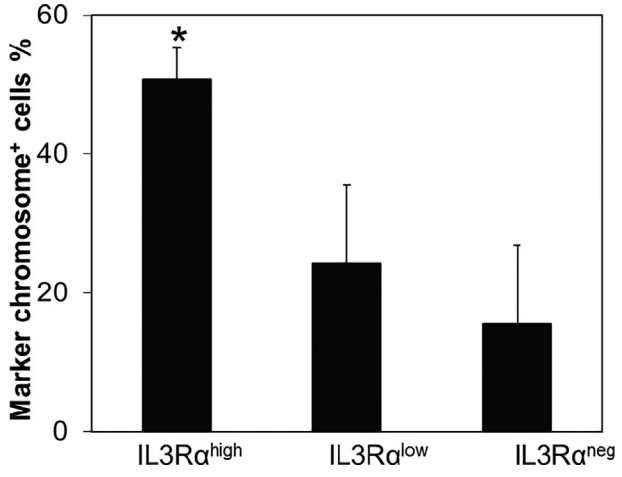

Fig. 2 Percentages of chromosome abnormalities in three populations of CD34⁺ enriched cells from IM PB: IL-3Rα^{high}, IL-3Rα^{low}, and IL-3Rα^{negative} cells. There was a significant increase in the percentage of cells containing marker chromosome abnormalities in IL-3Rα^{high} CD34⁺ cells as compared with IL-3Rα^{low} and IL-3Rα^{negative} CD34⁺ cells (57% vs 24% and 17%). N = 4, p < .05 vs IL-3Rα^{low} and IL-3Rα^{negative} cells. *Note*. IM = idiopathic myelofibrosis; PB = peripheral blood.

Table 2 Percentage of marker chromosome abnormalities[a] in patients with IM in IL-3Rα^{high}, IL-3Rα^{low} and IL-3Rα^{negative} subpopulations.

Patients	IL3Rα^{high}	IL3Rα^{low}	IL3Rα^{negative}
IM1	47	3	0
IM2	61	21	9
IM3	64	48	43
IM4	31	25	10
Mean	51[a]	24	16

Note. IM = idiopathic myelofibrosis.
[a] Chromosome abnormalities include monosomy 7, isochromosome 17, 20q-.

in vitro with the highest efficiency occurring in the IL-3Rα^{low} subpopulation (Fig. 3A). However, in the absence of growth factors, cells in IL-3Rα^{negative} and IL-3Rα^{low} groups formed significantly reduced or no colony; cells in IL-3Rα^{high} retained this colony forming ability (Fig. 3B), indicating that the colony forming ability of IL-3Rα^{high} CD34^{+} cells is relatively independent of the addition of exogenous growth factors.

IM PB IL-3Rα^{high} CD34^{+} cells have increased JAK2V617F mutation frequency

The JAK2V617F allele status of granulocytes isolated from patients with IM was determined. Three patients (IM17, IM35, and IM41 with JAK2V617F mutation levels in granulocytes at 70%, 10%, and 5% respectively) were used for the colony JAK2V617F status analysis. JAK2V617F allele status in colonies cloned from three subpopulations of IL-3Rα^{high}, IL-3Rα^{low}, and IL-3Rα^{negative} of IM PB CD34^{+} cells was determined by nested allele-specific PCR.

In IM35 and IM41, colonies from three subpopulations contained mutant homozygote, heterozygote, and wild type status for JAK2V617F in the presence of growth factors (Fig. 4A), whereas in the absence of growth factors, colonies from three subpopulations only contained mutant homozygote and heterozygote but not wild type for JAK2V617F (Fig. 4B), suggesting that colony forming ability of colonies burden JAK2V617F mutant allele are relatively independent of the addition of exogenous growth factors whereas colonies with JAK2 wild type allele are relatively dependent of exogenous growth factors. In the presence of growth factors, colonies from IL-3Rα^{high} and IL-3Rα^{low} CD34^{+} cells have higher percentage of JAK2V617F mutant homozygote and heterozygote allele (Table 3), whereas in the absence of growth factors, colonies from IL-3Rα^{high} CD34^{+} cells have significant higher percentage of JAK2V617F mutant homozygote allele compared with colonies from IL-3Rα^{low} and IL-3Rα^{negative} CD34^{+} cells (Table 2). These data suggest that colonies from IL-3Rα^{high} CD34^{+} cells exhibit increased percentage of JAK2V617F mutant allele especially in the absence of growth factors. In IM17, colonies from all three subpopulations of cells were heterozygote for JAK2V617F, suggesting that JAK2V617F mutation allele varied in patients with IM and it does not account entirely for the origin of IM.

Discussion

IM, one of the Philadelphia chromosome (Ph) negative MPD, is a clonal HSC disorder of unknown etiology [1—4]. A growing set of molecular and genetic markers, some possibly contributing to disease development, some more likely

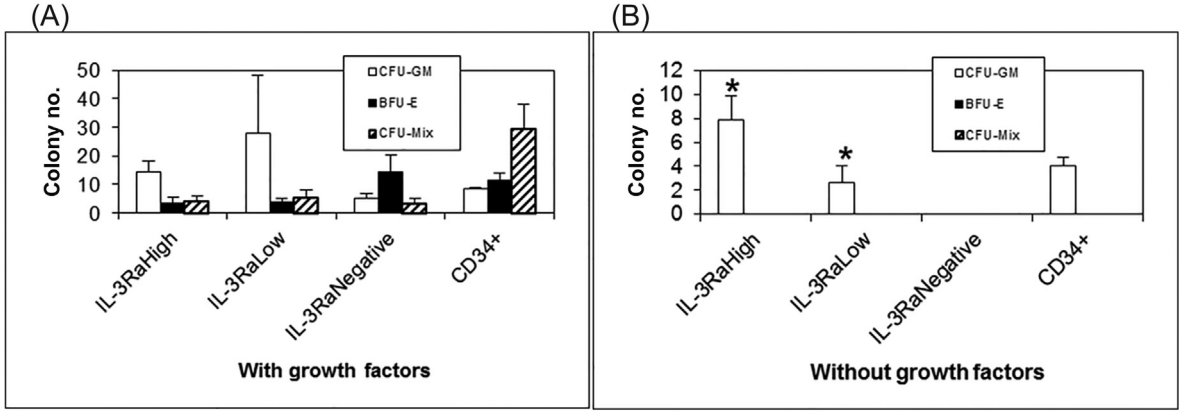

Fig. 3 Colony forming abilities of IL-3Rα^{high}, IL-3Rα^{low} and IL-3Rα^{negative} cells of IM PB CD34^{+} cells. (A) In the presence of growth factors, cells from all three CD34^{+} cell subpopulations were capable of forming hematopoietic colonies in vitro with the highest efficiency occurring in the IL-3Rα^{low} group; (B) by contrast, in the absence of growth factors, cells in IL-3Rα^{low} and IL-3Rα^{negative} groups formed significant reduced or no colony; whereas cells in IL-3Rα^{high} group retained this colony forming ability. $N = 4$, $p < .05$ vs IL-3Rα^{low} and IL-3Rα^{negative} cells. *Note*. IM = idiopathic myelofibrosis; PB = peripheral blood.

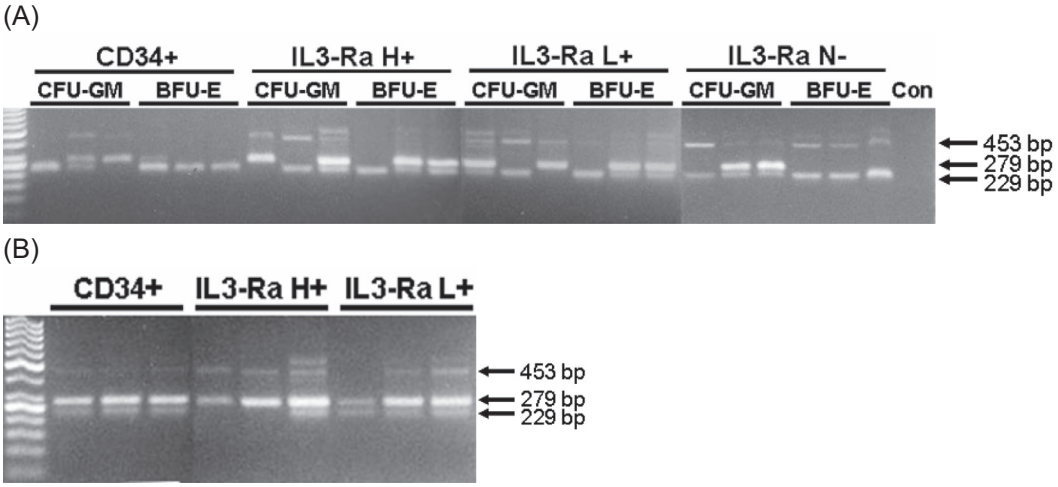

Fig. 4 JAK2V617F allele status in colonies cloned from three populations of IL-3Rα^{high}, IL-3Rα^{low}, and IL-3Rα^{negative} of IM PB CD34^{+} cells. (A) In IM35 and IM41, colonies from three subpopulations contained mutant homozygote, heterozygote, and wild type status for JAK2V617F in the presence of growth factors; (B) in the absence of growth factors, colonies from three subpopulations only contained mutant homozygote and heterozygote but not wild type for JAK2V617F, suggesting that colony forming ability of colonies burden JAK2V617F mutant allele are relatively independent of the addition of exogenous growth factors whereas colonies with JAK2 wild type allele are relatively dependent of exogenous growth factors. *Note*. BP = base pair; BFU-E = burst-forming unit-erythroid; CFU-GM = colony-forming unit-granulocyte-macrophage; CFU-Mix = colony-forming unit-multipotent progenitormixed colonies; IM = idiopathic myelofibrosis; PB = peripheral blood.

Table 3 Colony formation and JAK2V617F status in IL-3Rα^{high}, IL-3Rα^{low}, and IL-3Rα^{negative} subpopulations of CD34^{+} cells from patients with IM.[a]

	With growth factors			Without growth factors		
CD34^{+} cells	IL3-Rα High	IL3-Rα Low	IL3-Rα Negative	IL3-Rα High	IL3-Rα Low	IL3-Rα Negative
Colony no.	23	15	21	15	13	0
JAK2 wild type, %	30	33	67	0	0	0
JAK2V617F heterozygote, %	61	60	29	60	100	0
JAK2V617F Homozygote, %	9	7	5	40	0	0

Note. IM = idiopathic myelofibrosis.

[a] In the presence of growth factors, colonies from IL-3Rαhigh and IL-3Rαlow CD34^{+} cells contained significant higher percentages of JAK2V617F mutant homozygote and heterozygote compared with IL-3Rαnegative CD34^{+} cells. In the absence of growth factors, a significant increased percentage of JAK2V617F mutant homozygous colonies were observed in IL-3Rαhigh CD34^{+} cell population compared with IL-3Rαlow and IL-3Rαnegative CD34^{+} cells.

epiphenomena, have been characterized in these patients over the past few years [14]. Compiling and synthesizing the increasing knowledge on the genetic changes observed in patients with IM will allow us to generate testable hypotheses on the molecular etiology of disease development [14].

IL-3 plays an important role in the promotion of hematopoietic progenitor cell survival, proliferation, and differentiation [18—23]. The IL-3Rα/CD123 was shown to be necessary for the activation of STAT5, this region was also found to mediate transcriptional activation of c-*fos*, c-*jun*, and c-*Raf* [18,35—39]. In the presence of IL-3, heterodimerization of α- and β-subunit activates receptor and receptor associated JAK2 kinase and phosphorylates the β-subunit cytoplasmic tail, which is sufficient for IL-3 induced mitogenesis [20,21]. IL-3Rα has been found to be widely expressed in hematological malignancies [24—29]. Studies from Jordan et al. [25] and others had demonstrated that human AML stem cells expressed elevated levels of IL-3Rα, >99% of CD34^{+} cells from primary AML peripheral blood expressed IL-3Rα, by contrast, only 12% of CD34^{+} cells from normal BM expressed IL-3Rα, indicating that IL-3Rα is a unique marker for AML stem cells [24—29]. Testa et al. [27] also showed that deregulated expression of IL-3Rα in AML may contribute to the proliferative advantage of the leukemic blasts and hence, to a poor prognosis. Riccioni et al. [26] further characterized AML stem cells using IL-3Rα as a phenotypic marker. They divided AML stem cells into three subgroups: IL-3Rα^{high}, IL-3Rα^{middle}, and IL-3Rα^{low}, and found that AML IL-3Rα^{high} cell population represents a subgroup of cells with a peculiar immunophenotype mainly consisting in the elevated expression of CD34 and

several receptor membrane tyrosine kinases, such as c-kit and Flt3, indicating the AML IL-3Rα^{high} cells are blocked at an early stage of differentiation and express at elevated levels several growth factor receptors [26]. However, it has not yet been documented if IL-3Rα is a phenotypic marker for IM stem cells. In this study, we have characterized an IL-3Rα^{high} cell population from IM PB CD34$^+$ cells which is not seen in G-CSF mobilized normal volunteers, implying that IL-3Rα could be a useful phenotypic marker IM PB CD34$^+$ cells and that IL-3Rα^{high} CD34$^+$ cells may be enriched for the malignant clone.

The malignant phenotype in IM likely results from a combination of genetic abnormalities leading to dysregulation of critical genes that contribute to cell proliferation, differentiation, and cell death [14]. Baxter et al. studied the chromosome abnormalities (including trisomy 1q, 13q deletion, 20q deletion, and trisomy 8) in 51 patients with IM, they found that 21 patients had an abnormal clone, whereas 30 patients had a normal karyotype. Although the series of patients studied displayed chromosomal aberrations that are frequently observed in IM, they also found some new abnormalities (balanced translocations and polyploidy) that are rarely observed in IM [40]. In this report, we studied four IM patients with chromosome abnormalities (including monosomy 7, isochromosome 17, and 13q deletion) and analyzed the chromosome abnormalities in three subpopulations of CD34$^+$ cells by FISH analysis. We found a significant increase in the averaged percentage of cells containing marker chromosome abnormalities in IL-3Rα^{high} CD34$^+$ cell group as compared with IL-3Rα^{low} and IL-3Rα^{negative} CD34$^+$ cells, indicating that IL-3Rα^{high} CD34$^+$ cells in IM may be enriched for the malignant progenitor cells. Although three out of four patients had greater different value between cell groups, one patient had less difference between cell groups, suggesting that marker chromosome abnormalities may not account for the entire origin of IM.

IM is characterized by the constitutive mobilization of CD34$^+$ cells. We have previously reported that IM PB CD34$^+$ cells had a reduced cloning efficiency and a lower frequency of cobblestone areas compared with normal G-CSF-mobilized PB CD34$^+$ cells [13]. Because MPD hematopoietic progenitor cells have been shown to be able to form colonies even in the absence of growth factors [16], implying that stem cells derived from malignant clones from MPD may have abnormal colony forming ability, we therefore performed the colony-forming assays under two different conditions: with and without growth factors. Cells from all three subpopulations of IM PB CD34$^+$ cells form colonies in the presence of six growth factors although the colony forming abilities vary, the depletion of growth factors caused significant decrease or loss of colony forming ability of cells in IL-3Rα^{low} and IL-3Rα^{negative} groups. Surprisingly, cells in IL-3Rα^{high} group still maintained similar colony forming ability as seen in the presence of growth factors. Because cells which formed colonies in the absence of growth factors may derive from malignant clones, suggesting that IM PB IL-3Rα^{high} CD34$^+$ cells may include a higher percentage of tumor progenitor cells, and the abnormal colony forming ability of IM PB IL-3Rα^{high} CD34$^+$ cells may result from the increase of autocrine function.

JAK2V617F and MPLW515K/L are recently discovered somatic activating mutations that confer increased sensitivity of hematopoietic cells to cytokines [40—43]. The JAK2V617F mutation has been documented in 50% of patients with IM [18]. JAK2V617F is a subtle mutation that induces a limited gain of function in JAK2. There is evidence that its activation requires the presence of cytokine receptors to induce signaling [15,41,42]. The JAK2V617F mutation has become the biological marker and has spurred the development of a specific therapy to neutralize its effects. The number of V617F JAK2 copies likely contributes to the phenotypic heterogeneity of the JAK2V617F positive MPD [15]. The discovery of JAK2V617F mutation is leading to rapid advancements in understanding the pathophysiology and in treatment of the diseases. We further characterized the IL-3Rα^{high} CD34$^+$ cells by evaluating the JAK2V617F allele status in colonies cloned from IM PB CD34$^+$ cells three subpopulations of IL-3Rα^{high}, IL-3Rα^{low}, and IL-3Rα^{negative}. In two out of three patients, colonies from all three subpopulations contained mutant homozygote, heterozygote, or wild type status for JAK2V617F in the presence of growth factors. Colonies from three subpopulations only show mutant homozygote or heterozygote but not wild type for JAK2V617F status in the absence of growth factors, suggesting that colony forming ability of colonies burden JAK2V617F mutant allele are relatively independent of the addition of exogenous growth factors. Colonies with JAK2 wild type allele are relatively dependent of exogenous growth factors. Moreover, in the absence of growth factors, colonies from IL-3Rα^{high} CD34$^+$ cells had a significantly higher percentage of mutant homozygote type compared with colonies from IL-3Rα^{low} and IL-3Rα^{negative} CD34$^+$ cells, whereas in the presence of growth factors, colonies from IL-3Rα^{high} and IL-3Rα^{low} CD34$^+$ cells had a higher percentage of mutant homozygote and heterozygote. These data suggest that colonies cloned from IL-3Rα^{high} CD34$^+$ cells had an increased percentage of JAK2V617F mutant allele and may have increased tumor burden. In one patient with higher JAK2V617F mutant allele in granulocytes, colonies cloned from all three cell subgroups were all JAK2V617F heterozygous, suggesting that JAK2V617F mutation allele varied in patients with IM and it does not account entirely for the origin of IM.

Currently there are five potential clinical trials targeting IL-3Rα/CD123 for BPDCN therapies. SL-401 is one of the novel biologic targeted therapies directed to CD123. SL-401 is a recombinant protein consisting of human IL-3 (the natural ligand of IL-3Rα/CD123) fused to a truncated diphtheria toxin (DT). The IL-3 domain of SL-401 directs the cytotoxic DT to cells expressing CD123 (its natural receptor). Upon internalization, SL-401 inhibits protein synthesis and induces apoptosis of the target cells [44—46]. These results demonstrate preclinical proof of principle of high anti-BPDCN activity by targeting IL-3Rα/CD123 and warrant further clinical testing of this approach in human clinical trials in IM/PMF.

In conclusion, we have characterized a small subpopulation of IM PB CD34$^+$ cells highly expressed the IL-3Rα/CD123 which was not observed in normal G-CSF mobilized PB CD34$^+$ cells. This small subgroup of IL-3Rα^{high} CD34$^+$ cells have an increased percentage of chromosome abnormalities, abnormal colony-forming ability, and increased JAK2V617F mutant allele burden, and may be enriched for the IM tumor progenitor cells. Additional phenotypic markers for IM

tumor progenitor cells will be clarified and can be used by combination with IL-3Rα/CD123 for further characterization of IM/PMF tumor progenitor cells. We will further validate our findings with a larger sample size in the future study and establish the stemness properties of IL-3Rα^{high} CD34+ cells by serial transplant in murine models.

Conflict of interest

All authors have no conflicts of interest to declare.

Acknowledgments

The authors acknowledge funding ZR2016HL35 received from Nature Science Foundation of Shandong, China.

References

[1] Shantzer L, Berger K, Pu JJ. Primary myelofibrosis and its targeted therapy. Ann Hematol 2016:1—5.
[2] Savona MR. Are we altering the natural history of primary myelofibrosis. Leuk Res 2014;38:1004—12.
[3] Tefferi A. Myelofibrosis with myeloid metaplasia. N Engl J Med 2000;342:1255—65.
[4] Barosi G. Myelofibrosis with myeloid metaplasia: diagnostic definition and prognostic classification for clinical studies and treatment guidelines. J Clin Oncol 1999;17:2954—70.
[5] Adams GB, Scadden DT. The hematopoietic stem cell in its place. Nat Immunol 2006;7:333—7.
[6] Spangrude GJ, Lewandowski D, Martelli F, Marra M, Zingariello M, Sancillo L, et al. P-selectin sustains extramedullary hematopoiesis in the gata1 low model of myelofibrosis. Stem Cells 2016;34:67—82.
[7] LeBousse-Kerdiles MC, Martyré MC. Dual implication of fibrogenic cytokines in the pathogenesis of fibrosis and myeloproliferation in myeloid metaplasia with myelofibrosis. Ann Hematol 1999;78:437—44.
[8] Martyré MC, Romquin N, LeBousse-Verdiles MC, et al. Transforming growth factor-β and megakaryocytes in the pathogenesis of idiopathic myelofibrosis. Br J Haematol 1994;88:9—16.
[9] Reilly JT. Pathogenesis of idiopathic myelofibrosis: present states and future directions. Br J Haematol 1994;88:1—8.
[10] Kimura A, Katoh O, Hyodo H, Kuramoto A. Transforming growth factor-β regulates growth as well as collagen and fibronectin synthesis of human marrow fibroblasts. Br J Haematol 1989;72:486—91.
[11] Barosi G, Viarengo G, Pecci A, Rost V, Piaggio G, Marchetti M, et al. Diagnostic and clinical relevance of the number of circulating CD34$^+$ cells in myelofibrosis with myeloid metaplasia. Blood 2001;98:3249—55.
[12] Xu M, Bruno E, Chao J, Ni H, Lindgren V, Nunez R, et al. The constitutive mobilization of bone marrow repopulating cells into the peripheral blood in idiopathic myelofibrosis. Blood 2005;105:1669—705.
[13] Xu M, Bruno E, Chao J, Huang S, Finazzi G, Fruchtman SM, et al. The constitutive mobilization of CD34$^+$ cells into the peripheral blood in idiopathic myelofibrosis may be due to the action of a number of proteases. Blood 2005;105:4508—15.
[14] Bench AJ, Pahl HL. Chromosomal abnormalities and molecular markers in myeloproliferative disorders. Semin Hematol 2005;42:196—205.
[15] Angona A, Alvarez-Larrán A, Bellosillo B, Longarón R, Fernández-Rodríguez C, Besses C. Dynamics of JAK2 V617F allele burden of CD34$^+$ haematopoietic progenitor cells in patients treated with ruxolitinib. Br J Haematol 2016;172:639—42.
[16] Lu X, Levine R, Tong W, Wernig G, Pikman Y, Zarnegar S, et al. Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation. Proc Natl Acad Sci USA 2005;102:18962—7.
[17] Pomicter AD, Eiring AM, Senina AV, Zabriskie MS, Marvin JE, Prchal JT, et al. Limited efficacy of BMS-911543 in a murine model of Janus kinase 2 V617F myeloproliferative neoplasm. Exp Hematol 2015;43:537—45.
[18] Reddy EP, Korapati A, Chaturvedi P, Rane S. IL-3 signaling and the role of Src kinases, JAKs, and STATs: a covert liaison unveiled. Oncogene 2000;19:2532—47.
[19] Stomski FC, Sun Q, Bagley CJ, Woodcock J, Goodall G, Andrews RK, et al. Human interleukin-3 (IL-3) induces disulfide-linked IL-3 receptor alpha- and beta-chain heterodimerization, which is required for receptor activation but not high-affinity binding. Mol Cell Biol 1996;16:3035—46.
[20] Orban PC, Levings MK, Schrader JW. Heterodimerization of the alpha and beta chains of the interleukin-3 (IL-3) receptor is necessary and sufficient for IL-3-induced mitogenesis. Blood 1999;94:1614—22.
[21] Evans CA, Pierce A, Winter SA, Spooncer E, Heyworth CM, Whetton AD. Activation of granulocyte-macrophage colony-stimulating factor and interleukin-3 receptor subunits in a multipotential hematopoietic progenitor cell line leads to differential effects on development. Blood 1999;94:1504—14.
[22] Blalock WL, Weinstein-Oppenheimer C, Chang F, Hoyle PE, Wang XY, Algate PA, et al. Signal transduction, cell cycle regulatory, and anti-apoptotic pathways regulated by IL-3 in hematopoietic cells: possible sites for intervention with anti-neoplastic drugs. Leukemia 1999;13:1109—66.
[23] Agger K, Miyagi S, Pedersen MT, Kooistra SM, Johansen JV, Helin K. Jmjd2/Kdm4 demethylases are required for expression of Il3ra and survival of acute myeloid leukemia cells. Genes Dev 2016;30:1278—88.
[24] Munoz L, Nomdedeu JF, Lopez O, Carnicer MJ, Bellido M, Aventin A, et al. Interleukin-3 receptor alpha chain (CD123) is widely expressed in hematologic malignancies. Haematologica 2001;86:1261—9.
[25] Jordan CT, Upchurch D, Szilvassy SJ, Guzman ML, Howard DS, Pettigrew AL, et al. The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. Leukemia 2000;14:1777—84.
[26] Riccioni R, Rossini A, Calabro L, Diverio D, Pasquini L, Lococo F, et al. Immunophenotypic features of acute myeloid leukemias overexpressing the interleukin 3 receptor alpha chain. Leuk Lymphoma 2004;45:1511—7.
[27] Testa U, Riccioni R, Militi S, Coccia E, Stellacci E, Samoggia P, et al. Elevated expression of IL-3Ralpha in acute myelogenous leukemia is associated with enhanced blast proliferation, increased cellularity, and poor prognosis. Blood 2002;100:2980—8.
[28] Huang S, Chen Z, Yu JF, Young D, Bashey A, Ho AD, et al. Correlation between IL-3 receptor expression and growth potential of human CD34$^+$ hematopoietic cells from different tissues. Stem Cells 1999;17:265—72.
[29] Matsuda T, Hirano T. Association of p72 tyrosine kinase with Stat factors and its activation by interleukin-3, interleukin-6, and granulocyte colony-stimulating factor. Blood 1994;83:3457—61.
[30] Kovtun Y, Jones GE, Adams S, Harvey L, Audette CA, Wilhelm A, et al. A CD123-targeting antibody-drug conjugate, IMGN632, designed to eradicate AML while sparing normal bone marrow cells. Blood Adv 2018;2:848—58.
[31] Eldin TK, Abdelsalam EMN, Razek GA, Nafady H, Saleh MFM. Diagnostic and prognostic value of CD123 in acute myeloid leukemia (AML). Clin Lymphoma Myeloma Leuk 2017;17:S272—3.

[32] Ouyang J, Zheng W, Shen Q, Goswami M, Jorgensen JL, Medeiros LJ, et al. Flow cytometry immunophenotypic analysis of Philadelphia-negative myeloproliferative neoplasms: correlation with histopathologic features. Cytometry B Clin Cytom 2015;88B:236—43.
[33] Sullivan JM, Rizzieri DA. Treatment of blastic plasmacytoid dendritic cell neoplasm (BPDCN). Hematol Am Soc Hematol Educ Program 2016;2016:16—23.
[34] Falcone U, Sibai H, Deotare U. A critical review of treatment modalities for blastic plasmacytoid dendritic cell neoplasm (BPDCN). Crit Rev Oncol Hematol 2016;107:156—62.
[35] Doyle SE, Gasson JC. Characterization of the role of the human granulocyte-macrophage colony-stimulating factor receptor alpha subunit in the activation of JAK2 and STAT5. Blood 1998;92:867—76.
[36] Lee J, Seong S, Kim JH, Kim K, Kim I, Jeong BC, et al. STAT5 is a key transcription factor for IL-3-mediated inhibition of RANKL-induced osteoclastogenesis. Sci Rep 2016;6:30977.
[37] Itoh T, Muto A, Watanabe S, Miyajima A, Yokota T, Arai K. Granulocyte-macrophage colony-stimulating factor provokes RAS activation and transcription of c-fos through different modes of signaling. J Biol Chem 1996;271:7587—92.
[38] Tian SS, Tapley P, Sincich C, Stein RB, Rosen J, Lamb P. Multiple signaling pathways induced by granulocyte colony-stimulating factor involving activation of JAKs, STAT5, and/or STAT3 are required for regulation of three distinct classes of immediate early genes. Blood 1996;88:4435—44.
[39] Watanabe S, Ishida S, Koike K, Arai K. Characterization of cis-regulatory elements of the c-myc promoter responding to human GM-CSF or mouse interleukin 3 in mouse proB cell line BA/F3 cells expressing the human GM-CSF receptor. Mol Biol Cell 1995;6:627—36.
[40] Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 2005;365:1054—61.
[41] Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 2005;352:1779—90.
[42] Pardanani AD, Levine RL, Lasho T, Pikman Y, Mesa RA, Wadleigh M, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood 2006;108:3472—6.
[43] Hu W-Y, Zhao Y, Ishii T, Sozer S, Shi J, Zhang W, et al. Haematopoietic cell lineage distribution of MPLW515L/K mutations in patients with idiopathic myelofibrosis. Br J Haematol 2007;137:378—9.
[44] Frankel AE, Woo JH, Ahn C, Pemmaraju N, Medeiros BC, Carraway HE, et al. Activity of SL-401, a targeted therapy directed to interleukin-3 receptor, in blastic plasmactyoid dendritic cell neoplasm patients. Blood 2014;124:385—92.
[45] Sun W, Liu H, Kim Y, Karras N, Pawlowska A, Toomey D, et al. First pediatric experience of SL-401, a CD123-targeted therapy, in patients with blastic plasmacytoid dendritic cell neoplasm: report of three cases. J Hematol Oncol 2018;11:61.
[46] Cai T, Galetto R, Gouble A, Smith J, Cavazos A, Konoplev A, et al. Preclinical studies of anti-CD123 CAR-T cells for the treatment of blastic plasmacytoid dendritic cell neoplasm (BPDCN). Blood 2016;128:4039.