CD45dimCD34+CD38-CD133+ cells function as leukemic stem cells in acute myeloid leukemia

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Abstract

Leukemia stem cells (LSCs) in acute myeloid leukemia (AML) played important roles in development of leukemia, chemotherapeutic drug resistance, and disease relapse and progression. The identification of LSCs and targeting therapies for them has been under investigation. We examined the CD45 dim CD34 + CD38 - CD133 + cells on bone marrow samples of hematologic malignancies and healthy controls using four-color flow cytometry experiments. Interestingly, the CD45 dim CD34 + CD38 - CD133 + cells were highly expressed in bone marrow of patients with AML compared to that of healthy controls (HC). Moreover, the proportions of CD45 dim CD34 + CD38 - CD133 + cells were also examined in diverse hematology malignancies including AML, CML, DLBCL, MM, MDS, HL, ALL and CLL. These cells were prominently detected in BMCs of AML and CML, but rarely in DLBCL, MM, MDS, ALL, CLL and HL. Additionally, the high levels of the CD45 dim CD34 + CD38 - CD133 + cells in AML patients were an independently significant poor risk factor for overall survival and event free survivals. Therefore, our results suggest that CD45 dim CD34 + CD38 - CD133 + cells in AML might have the potential of leukemia stem cells. In addition, this cell population might be a novel therapeutic target for AML.

Background

Acute myeloid leukemia (AML) is generally regarded as a stem cell disease, and it originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity [1, 2]. Also, AML is a very heterogeneous disease, with abundant pathogenetic mutations defined, numerous mutations present, and significant differences in clinical outcome seen in individual patients [3]. Generally, it is rapidly lethal, and survival outcomes for adults with AML are very poor despite intensive chemotherapy and/or targeted therapies together with supportive care [4].

The leukemia stem cells (LSCs) in AML play important roles in development of leukemia, chemotherapeutic drug resistance, and disease relapse and progression [5]. Actually, the available evidence has recently been suggested that LSCs, which are capable of developing identical daughter cells as well as differentiated cells and maintaining AML [6, 7]. For examples, Rhenen et al showed that a high percentage of CD34⁺CD38⁻ stem cells at diagnosis significantly correlated with a high minimal residual disease frequency and subsequently to relapse especially after the third course of chemotherapy in AML patients. Also, these cell populations directly correlated with poor survival [2, 5].

Searching for the LSCs population is one of the best ways to find treatment strategies, or improve treatment outcomes in AML and other malignant diseases [3]. Therefore, basic research on identification and targeting LSCs is actively under way. Many scientists have been interesting in this area and made efforts to find the appropriated biological markers of LSCs population in AML including CD34⁺CD38⁻ cells [8, 9], CD34⁺lin⁻ cells [10], CD34⁺Thy1⁺CD38^{low} cells [11], CD34⁺CD117⁺ cells [12], CD34⁺CD38⁻CD123⁺ cells [13–15], CD34⁺CD38⁻CD123⁺CD33⁺ cells [16], CD34⁺CD38⁻C-type lectin-like molecule-1 (CLL-1)⁺ cells [17], CD34⁺CD38⁻CD96⁺ cells [18], CD34⁺CD38⁻CD45^{-/low} cells [19], CD34/CD123/CD25/CD99⁺ [5] and so on. Moreover, the CD34⁺CD38⁻ progenitor cells expressed variable amounts of the target receptor

CD33, CD133 and c-kit (CD117) [20]. But all the researches have been still very confused. Therefore, we analyzed various existing information and created new combinations that could become LSCs. Furthermore, it was developed using the basic platform of the CD34⁺CD38⁻ markers, but applying more advanced antigens such as CD45^{dim} and CD133. In this study, we focused on measuring LSCs easily in the bone marrow cells from AML patients by developing a four-color flow cytometric analysis. Our studies suggest that CD45^{dim}CD34⁺CD38⁻CD133⁺ cells have the potential of leukemic stem cells in AML patients.

Methods

Reagents

Mouse anti-human CD45-FITC (Clone 2D1, Cat No. 347463), Mouse Anti-human CD34-PE [Clone 8G12 (also known as HPCA2), Cat No. 348057], Mouse anti-human CD38-PE-Cy[™]5 (Clone HIT2, Cat No. 555461) and appropriated isotype control antibodies were purchased from BD Biosciences (San Diego, CA, USA). Mouse anti-human CD133-APC (Clone CD133, Cat No. 130-090-826) was obtained from Miltenyi Biotec (San Diego, CA, USA).

Patient samples

We analyzed bone marrow samples collected from 87 patients who were newly diagnosed with AML (n = 40), chronic myeloid leukemia (CML, n = 6), diffuse large B-cell lymphoma (DLBCL, n = 19), multiple myeloma (MM, n = 10), myelodysplastic syndrome (MDS, n = 5), Hodgkin lymphoma (HL, n = 4), acute lymphocytic leukemia (ALL, n = 3) or chronic lymphocytic leukemia (CLL, n = 2) and from 27 healthy controls at Ulsan University Hospital, Ulsan, South Korea. Baseline clinical characteristics of 40 patients with AML were summarized in Supplementary Table 1. And the other patients' characteristics (except AML) were summarized in Supplementary Table 2.

Isolation of bone marrow cells

The bone marrow cells (BMCs) were isolated by the density gradient method, as previously described [21]. In brief, BMCs were isolated via density gradient centrifugation at 400 × *g* using Lymphoprep (Axis-Shield, Oslo, Norway; density, 1.077 g/mL). They were washed with phosphate-buffered saline (PBS).

Flow cytometric phenotypic analysis

The BMCs were collected and washed twice with FACS buffer (PBS containing 0.3% BSA and 0.1% NaN₃). Cells were incubated with four antibodies against each cell surface antigens including CD45, CD34, CD38, and CD133 on ice for 30 min. First of all, the live cells of BMCs were collected, and SSC^{low}

and CD45^{dim} cells were gated, as shown in Fig. 1A and 1B. The BMCs were incubated with three types of combinations of monoclonal antibodies (mAbs) on ice for 30 min such as isotype control 1 (Mouse anti-human CD45-FITC, Mouse IgG-PE, Mouse IgG-PE CY5, and Mouse IgG-APC), isotype control 2 (Mouse anti-human CD45-FITC, Mouse anti-human CD34-PE, Mouse anti-human CD38-PE CY5, and Mouse IgG-APC), and sample (Mouse anti-human CD45-FITC, Mouse anti-human CD34-PE, Mouse anti-human CD38-PE CY5, and Mouse human CD133-APC), as shown in Fig. 1C and Fig. 1D. Cells were then washed twice with FACS buffer and analyzed using the FACSCalibur flow cytometer and CellQuest Pro software (BD Bioscience) as shown Fig. 1. Finally, CD45^{dim}CD34⁺CD38⁻CD133⁺ cells, CD133 positive cells of the R1, R2, R3-gated cells were measured, and the results were expressed as percentage changes from the base conditions including isotype control 2.

ELISA for cytokine measurements

Cell-free plasmas from bone marrow samples from patients with AML were collected and frozen at -80°C. Plasma levels of interleukin (IL)-1 β , IL-6, IL-17 and IL-23 were measured using ELISA kits according to manufacturer's introductions (R&D Systems).

Statistics

The data presented here represent the means $\pm$ standard error of mean (SEM) of at least three independent experiments. All values were evaluated by one-way analysis of variance followed by Turkey range tests implemented in GraphPad Prism 7.0. Differences were considered significant at $P < 0.05$. For patients with AML, continuous variables were compared using Student's t-test, whereas categorical variables were analyzed using the Pearson chi-square test or Fisher's exact test. Overall survival (OS) was calculated from the date of HCT to the date of death or last follow-up. Event-free survival (EFS) was defined from the date of HCT to the date of relapse or death from any cause. Survival probabilities were estimated by the Kaplan-Meier method. Univariate and multivariate analyses for OS, EFS, and relapse probability were performed using the log rank test and Cox proportional hazards model, respectively. The following variables were included for univariate analyses: CD45^{dim}CD34⁺CD38⁻CD133⁺ cell proportion, age, white blood cell (WBC) count, platelet count, bone marrow blast percentage, cytogenetic risk groups, chemotherapeutic regimens and immunophenotypings including CD7, CD33, CD34, and HLA-DR. Variables with a P -value < 0.1 in the univariate analyses were included in the multivariate analyses. The statistical analyses were performed with SPSS version 21.0 software (IBM Corp., Armonk, NY). For all analyses, the P -values were two-sided; a P -value of < 0.05 was considered statistically significant.

Results

The CD45^{dim}CD34⁺CD38⁻CD133⁺ cells are highly expressed in bone marrow of patients with acute myeloid leukemia, not healthy controls.

The process of four-color flow cytometry experiments using monoclonal antibodies (mAbs) are shown in Fig. 1. As shown in Fig. 1A and 1B, the live cells of BMCs were collected, and $SSC^{\text{low}}/CD45^{\text{dim}}$ cells were obtained. The BMCs were stained with various combinations of mAbs on ice for 30 min such as isotype 1, isotype 2, and sample (Fig. 1C). Lastly, the CD133 positive cells of the R1, R2, R3-gated cells were measured by flow cytometer, and the results were expressed as percentage changes from the isotype 2 (Fig. 1D). A total of 40 AML patients were examined for expression of the target antigens, $CD45^{\text{dim}}CD34^+CD38^-CD133^+$ cells on BMCs. These cells were highly expressed only in bone marrow samples of patients with AML, not in those of healthy controls (Fig. 2). These results indicated that $CD45^{\text{dim}}CD34^+CD38^-CD133^+$ cells in bone marrow have the potential of AML stem cells.

Elevated IL-1 β , IL-6, IL-17 and IL-23 cytokine production of plasma in patients with AML

We examined the IL-1 β , IL-6, IL-17 and IL-23 cytokine production in BM plasma samples, which they were matched BMCs in AML patients. Then plasma samples of the AML patients were shown higher levels of IL-1 β , IL-6, IL-17 and IL-23 cytokine production than those of healthy controls, as shown in Fig. 3.

The $CD45^{\text{dim}}CD34^+CD38^-CD133^+$ cells are prominently detected in BMCs of patients with AML and CML

As shown in Fig. 1, the $CD45^{\text{dim}}CD34^+CD38^-CD133^+$ cells were examined by four-color flow cytometry experiments in diverse hematology malignancy including AML (n = 40), CML (n = 6), DLBCL (n = 19), MM (n = 10), MDS, (n = 5), HL (n = 4), ALL (n = 3) and CLL (n = 2). These cells are significantly detected in BMCs of patients with AML and CML, not DLBCL, MM, MDS, ALL, CLL and HL. Once again, these results indicated that $CD45^{\text{dim}}CD34^+CD38^-CD133^+$ cells in bone marrow have the potential of acute myeloid leukemia stem cells. In addition, these cells might be feasible for AML stem cells marker.

Clinical characteristics according to levels of the $CD45^{\text{dim}}CD34^+CD38^-CD133^+$ cells

FLT3-ITD mutation in AML patients with higher $CD45^{\text{dim}}CD34^+CD38^-CD133^+$ cells ($\geq 10\%$) was significantly rarely found compared to those with lower $CD45^{\text{dim}}CD34^+CD38^-CD133^+$ cells ($< 10\%$) (0% vs. 23.1%, respectively, $P = 0.031$). In addition, higher $CD45^{\text{dim}}CD34^+CD38^-CD133^+$ cells ($\geq 20\%$) were

significantly associated with lower levels of IL-17 expression, comparing to lower CD45^{dim}CD34⁺CD38⁻CD133⁺ cells (< 20%) (118.0 vs. 35.0 pg/ml, respectively, $P = 0.028$). However, there was no significant difference in IL-1 β , L-6, and IL-23 levels according CD45^{dim}CD34⁺CD38⁻CD133⁺ cells proportion.

High proportion of the CD45^{dim}CD34⁺CD38⁻CD133⁺ cells predicts poor survival in AML patients

When we divided AML patients into three groups according to CD45^{dim}CD34⁺CD38⁻CD133⁺ cell proportions (<10% vs. 10–40% vs. $\geq 40\%$), the 2-year OS rate was 64.3% vs. 57.9% vs. 0%, respectively ($P < 0.001$) and the 2-year EFS was 62.3% vs. 37.2% vs. 0% ($P = 0.002$) on univariate analysis (Supplementary Table 3). Among three groups (CD45^{dim}CD34⁺CD38⁻CD133⁺ cell proportions <10%, 10–40% and $\geq 40\%$), however, there were no significant differences in baseline clinical factors including age ($P = 0.085$), white blood cell count ($P = 0.397$), platelet count ($P = 0.737$), chemotherapy intensity ($P = 0.158$). On univariate analyses for OS and EFS in patients with AML, older age (> 60 years) was significantly associated with worse OS compared to younger age (32.8% vs. 75% at 2-year, respectively, $P = 0.041$) (Supplementary Table 3). In addition, patients with higher marrow blast % ($\geq 60\%$) showed significantly lower OS rates than those with lower marrow blast % (< 60%) (36.7% vs. 66.7%, $P = 0.038$) (Supplementary Table 3). Patients who were treated with intensive chemotherapy showed significantly better OS than those with hypomethylating agents (57.8% vs. 30.0%, $P = 0.012$). When we consider together other clinical parameters in univariate and multivariate analysis, higher proportion of CD45^{dim}CD34⁺CD38⁻CD133⁺ cells ($\geq 40\%$) was an independently significantly prognostic factor for OS (hazard ratio [HR], 6.810, $P = 0.003$) and EFS (HR, 9.028, $P = 0.002$) (Fig. 5. and Table 1).

Discussion

The conception that cancer stem cells including LSCs are responsible for initiation, drug resistance, and relapse of cancers has excited this area of research, and the importance of cancer stem cells has been demonstrated in a variety of tumors [6, 22–26]. Especially, LSCs have potential of unlimited self-renewal and are responsible for the maintenance of leukemia. Because selective eradication of LSCs could propose considerable therapeutic benefit, there has been interest in identifying the characterization of LSCs population that controls their development [27, 28]. Therefore, the research related to prognostically relevant and potentially reliable molecular targets are needed.

AML is a hematopoietic disease that is characterized by clonal growth and the accumulation of myelopoietic progenitor cells [28]. It still remains a devastating and mostly incurable disease [4]. Moreover, therapy for AML involves intense cytotoxic treatment and yet approximately 70% of AML are refractory to initial therapy or eventually relapse [2]. This is at least partially driven by the chemo-resistant nature of the LSCs that maintain the disease. Therefore, novel anti-LSC therapies could decrease relapses and improve survival.

The first LSC compartment that was described had the CD34⁺CD38⁻ immunophenotype [1, 9]. The CD34⁺CD38⁻ compartment was shown to contain both CD34⁺CD38⁻ LSCs and normal hematopoietic stem cells (HSCs) [11]. Mawali et al. have proposed CD34⁺CD38⁻CD123⁺ cells as AML LSCs [15]. In the present study, the CD45^{dim}CD34⁺CD38⁻CD133⁺ cells are examined by four-color flow cytometer experiments to define more specific and prognostically significant LSC population (Fig. 1).

The CD133 has been reported as a cancer stem cell marker in solid tumors [12, 29–31]. Several studies have shown that CD133positive cells have self-renewal properties, capacity for differentiation, high proliferation, and capacity for forming tumors in xenografts [30, 31]. Although the precise function of CD133 remained unknown, it is associated with aggressive cancers and poor prognosis. CD133 has been known to be required for tumor growth and survival [12, 26, 29]. In hematologic malignancies including AML, however, clinical implications of CD133 expression have not been well known. Interestingly, CD45^{dim}CD34⁺CD38⁻CD133⁺ cells are highly expressed in bone marrow of patients with AML, not healthy controls (Fig. 2).

We also found raised IL–1 β , IL–6, IL–17 and IL–23 cytokine production in BM microenvironment of AML patients at diagnosis (Fig. 3). Carey et al. also reported that IL–1 and IL– β might be associated with AML cell growth [32]. IL–3 has known to play a key role within the network of cytokines involved in the regulation of hematopoiesis and leukemic blast formation, although IL–3 has no prognostic significance [18]. Higher serum IL–17 levels have been known as an adverse prognostic factor in AML [22]. Otherwise, our results showed that CD45^{dim}CD34⁺CD38⁻CD133⁺ LSC proportions in our study inversely correlated with IL–17 levels in BM microenvironments. There has been little data for cytokine IL–23 levels at AML diagnosis, although IL–23 have been reported to be associated with AML leukemogenesis and disease susceptibility in a previous study [33].

In addition, the CD45^{dim}CD34⁺CD38⁻CD133⁺ cells were prominently detected in BMCs of patients with AML and CML, not in DLBCL, MM, MDS, ALL, CLL and HL (Fig. 4). Moreover, the prognostic significance of LSC has been reported in previous studies [1, 15]. Tervinjin et al. showed higher CD34⁺CD45⁻LAP⁺ cell proportions were related to poor survivals [1]. But, our study demonstrated that higher expression of the CD45^{dim}CD34⁺CD38⁻CD133⁺ cells predict poor OS and EFS in AML (Fig. 5). These results also indicated that CD45^{dim}CD34⁺CD38⁻CD133⁺ cells compartment in bone marrow could more discriminate LSC and normal hematopoietic stem cell and be a strong prognostic marker. Therefore, targeting CD45^{dim}CD34⁺CD38⁻CD133⁺ cells could be a novel therapeutic direction in AML. Future studies will focus on the search about diminishment or elimination of the CD45^{dim}CD34⁺CD38⁻CD133⁺ cells in patients with AML. Most importantly, high levels of the population are correlated with poor survival in AML patients. Therefore, our results indicate that CD45^{dim}CD34⁺CD38⁻CD133⁺ cells function as LSCs in AML.

Conclusions

We found that CD45^{dim}CD34⁺CD38⁺CD133⁺ cells have the potential of AML stem cells. Moreover, high levels of the CD45^{dim}CD34⁺CD38⁺CD133⁺ cells are correlated with poor survival in AML patients. Therefore, our results indicate that CD45^{dim}CD34⁺CD38⁺CD133⁺ cells function as LSCs in AML.

Additional Files

Additional file 1: Supplementary Table 1. Baseline characteristics of AML patients.

Additional file 2: Supplementary Table 2. Patient characteristics.

Additional file 3: Supplementary Table 3. Univariate analysis for AML patients.

Abbreviations

BM, bone marrow; BMCs, bone marrow cells; HC, healthy controls; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; DLBCL, Diffuse large B-cell lymphoma; MM, multiple myeloma; MDS, myelodysplastic syndrome; HL, Hodgkin lymphoma; ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

SKH, EKN, YC, and JCJ designed the study. SKH, EKN, and YKJ performed the experiments. SKH, EKN, YKJ, JC, SK, and YJM analyzed and interpreted the experimental data. SKH, EKN, YKJ, JC, SK, and YJM provided the discussion and suggestions to the experiments. SKH, EKN, YC, and JCJ wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files. Please contact the author Jae-Cheol Jo (jcjo97@hanmail.net) upon reasonable requests.

Ethics approval and consent to participate

All experiments were performed in accordance with the relevant guidelines and regulations. All patients provided written informed consent before the commencement of the study. The study protocol and patient informed consent form were approved by the Ulsan University Hospital Ethics Committee and Institutional Review Board (UUH-IRB–2016–07–026). The informed written consent was obtained from all included subjects before collecting the bone marrow samples.

Consent for publication

Not applicable.

Competing interests

The authors declare that no competing interests exist.

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Tables

Table 1. Multivariate analysis for AML patients.

	HR for OS	P-value	HR for EFS	P-value
CD45^{dim}CD34⁺CD38⁻CD133⁺				
<10%	1		1	
10-<40%	2.460	0.110	2.731	0.089
≥40%	6.810	0.003	9.028	0.002
Platelet count, /mm³				
< 40, x 10 ³	3.196	0.076	-	-
≥ 40, x 10 ³	1			
Chemotherapy				
Intensive chemotherapy	1		1	
Hypomethylating agent	3.845	0.014	4.829	0.010

Figures

Fig. 1. Heo et al.

Figure 1

The process of four-color staining flow cytometry experiments using monoclonal antibodies. The BMCs were collected and washed twice with FACS buffer. Cells were incubated with four antibodies against each cell surface antigens including CD45, CD34, CD38, and CD133 on ice for 30 min. (A, B) The live cells of BMCs were collected, and SSClow and CD45dim cells were gated. (C, D) The BMCs were incubated with three types of combinations of monoclonal antibodies (mAbs) on ice for 30 min such as isotype

control 1 (Mouse anti-human CD45-FITC, Mouse IgG-PE, Mouse IgG-PE CY5, and Mouse IgG-APC), isotype control 2 (Mouse anti-human CD45-FITC, Mouse anti-human CD34-PE, Mouse anti-human CD38-PE CY5, and Mouse IgG-APC), and sample (Mouse anti-human CD45-FITC, Mouse anti-human CD34-PE, Mouse anti-human CD38-PE CY5, and Mouse human CD133-APC). Cells were then washed twice with FACS buffer and analyzed using the FACSCalibur flow cytometer and CellQuest Pro software (BD Bioscience). Finally, CD45dimCD34+CD38-CD133+ cells, CD133 positive cells of the R1, R2, R3-gated cells were measured, and the results were expressed as percentage changes from the base conditions including isotype control 2. The filled histogram represents the isotype control 2, and the open histogram represents CD45dimCD34+CD38-CD133+ cells.

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Figure 2

The CD45^{dim}CD34⁺CD38⁻CD133⁺ cells are highly expressed in bone marrow of patients with AML, not healthy controls. Bone marrow cells from healthy controls and AML patients were examined for expression of the target antigens, CD45^{dim}CD34⁺CD38⁻CD133⁺ cells. Data represent mean $\pm$ SEM representing three independent experiments from different AML patients. Significantly different from the control (*); ***, $P < 0.001$. HC, healthy controls; AML, Acute myeloid leukemia patients.

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Figure 3

Measure the production of cytokines in plasma from healthy controls and AML patients. Cell-free plasmas from bone marrow samples from patients with AML were collected and frozen at -80°C. Plasma levels of interleukin (IL)-1 β , IL-6, IL-17 and IL-23 were measured using ELISA kits according to manufacturer's introductions (R&D Systems). Data represent mean $\pm$ SEM representing three independent

experiments from different AML patients. Significantly different from the control (*); ***, P < 0.001. HC, healthy controls; AML, Acute myeloid leukemia patients; BM, Bone marrow.

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Figure 4

. The CD45^{dim}CD34⁺CD38⁻CD133⁺ cells are prominently detected in BMCs of patients with AML and CML. As shown in Fig. 1, the CD45^{dim}CD34⁺CD38⁻CD133⁺ cells were examined by four-color flow cytometry experiments in diverse hematology malignancy including AML (n = 40), CML (n = 6), DLBCL (n = 19), MM (n = 10), MDS, (n = 5), HL (n = 4), ALL (n = 3) and CLL (n = 2). Data represent mean ± SEM representing three independent experiments from different AML patients. Significantly different from the control (*) or AML (#); #: P < 0.05.; ***, ###: P < 0.001. HC, healthy controls; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; DLBCL, Diffuse large B-cell lymphoma; MM, multiple myeloma; MDS, myelodysplastic syndrome; HL, Hodgkin lymphoma; ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia.

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Figure 5

High proportion of the CD45^{dim}CD34⁺CD38⁻CD133⁺ cells predicts poor survival in AML patients. (A) Higher CD45^{dim}CD34⁺CD38⁻CD133⁺ cell proportion was significantly associated with worse OS ($P < 0.001$). (B) Poorer EFS was significantly associated with higher CD45^{dim}CD34⁺CD38⁻CD133⁺ cell proportion ($P = 0.002$).

Supplementary Files

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- [LSCSupplementaryTablesHeoetal0717.docx](#)