CD9 is a leukemic stem cell-specific marker in human acute myeloid leukemia

Yongliang Liu
Center of Biological Therapy, Southwest Hospital, Army Medical University

Guiqin Wang
Center of Biological Therapy, Southwest Hospital, Army Medical University

Jiasi Zhang
Department of Hematology, Southwest Hospital, Army Medical University

Xue Chen
Department of Hematology, Southwest Hospital, Army Medical University

Huailong Xu
Center of Biological Therapy, Southwest Hospital, Army Medical University

Gang Heng
Center of Biological Therapy, Southwest Hospital, Army Medical University

Jun Chen
Chongqing Institute of Precision Medical and Biotechnology Co., Ltd.

Yongchun Zhao
Chongqing Institute of Precision Medicine and Biotechnology Co., Ltd.

Jiatao Li
Center for Precision Medical of Cancer, Chongqing Key Laboratory of Translational Research for Cancer Metastasis and Individualized Treatment, Chongqing University Cancer Hospital

Yuanli Ni
Center for Precision Medical of Cancer, Chongqing Key Laboratory of Translational Research for Cancer Metastasis and Individualized Treatment, Chongqing University Cancer Hospital

Yingzi Zhang
Center for Precision Medicine of Cancer, Chongqing Key Laboratory of Translational Research for Cancer Metastasis and Individualized Treatment, Chongqing University Cancer Hospital

Juanjuan Shan (juanjuansh@gmail.com)
Chongqing University cancer hospital

Cheng Qian (cqian8634@gmail.com)
Center of Biological Therapy, Southwest Hospital, Army Medical University

Research
Keywords: Acute myeloid leukemia (AML), Leukemia stem cells (LSCs), CD9, Alpha-2-macroglobulin (A2M), Biomarker

DOI: https://doi.org/10.21203/rs.3.rs-55355/v2

License: ☑️  This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background:** Leukemia stem cells (LSCs) are responsible for the initiation, progressing and relapse of acute myeloid leukemia (AML). Therefore, the therapy strategy of targeting LSCs is hopeful to eradicate AML. In this study, we aimed to identify LSCs-specific surface markers and uncover the underlying mechanism of AML LSCs.

**Methods:** Microarray gene expression data were used to investigate the candidate AML-LSCs specific markers. CD9 expression was evaluated by flow cytometry (FC) in AML cell lines, patients with AML and normal donors. The biological characteristics of CD9-positive (CD9+) cells were analyzed by in vitro proliferation, chemotherapeutic drug resistance, migration and *in vivo* xenotransplantation assays. The molecular mechanism involved in CD9+ cell function was investigated by gene expression profiling. Effects of alpha-2-macroglobulin (A2M) on CD9+ cells were analyzed in the aspects of proliferation, drug resistance and migration.

**Results:** CD9, as a cell surface protein, is specifically expressed on AML LSCs, but barely can be detected on normal hematopoietic stem cells (HSCs). CD9+ cells exhibits more resistance to chemotherapy drugs and higher migration potential than CD9-negative (CD9-) cells. More importantly, CD9+ cells possess the ability to reconstitute human AML in immunocompromised mice and promote leukemia growth, suggesting CD9+ cells define the LSCs population. Furthermore, we identified A2M as an important role in CD9+ LSCs stemness maintenance. Knock down of A2M impairs drug-resistance and migration of CD9+ cells.

**Conclusion:** Our findings suggested that CD9 is a new biomarker of AML LSCs and may serve as a promising therapeutic target.

Background

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, accounting for approximately 80% of cases in this group that is characterized by infiltration of the bone marrow, blood, and other tissues by proliferative, clonal, abnormally differentiated, and occasionally poorly differentiated cells of the hematopoietic system [1,2]. AML is caused by the disorders of the hematopoietic system which is commonly treated by chemotherapeutic and hematopoietic stem cell transplantation [3]. However, 43% of patients eventually end up with relapse after complete remission in young adult patients [4]. Residual rare leukemia stem cells (LSCs) are the major cause of recurrence of AML, which possess chemoresistant and the ability to self-renew and differentiate thus reconstitute AML. Therefore, the LSCs concept has inspired the design of innovative treatment strategies for AML aiming at targeting LSCs hidden in cancers.

To identify the potential therapeutic targets, many groups have reported cell surface proteins preferentially expressed on AML LSCs, including CD47 [5], CD44 [6], CD96 [7], CD123 [8,9] CD99 [10] and
TIM-3 [11,12]. During the past few years, some strategies for targeting LSCs markers antibody or immune cells have already been tested in patients, but still face the problems of toxicity and LSCs resistance. Therefore, more specific LSCs markers still need to be explored.

Here we identified a potential AML LSCs specific molecule CD9 by analyzing three microarray data of AML LSCs and a minimal residual disease (MRD) expression profiling. As one member of the tetraspanins family, CD9 is the third most abundant protein on the platelet surface and is required for the release of microparticles from coated-platelets [13,14]. Furthermore, it was reported that CD9 plays an important role in cell adhesion, movement, differentiation, proliferation, apoptosis and resistance to chemotherapy [15-19]. Although CD9 has been reported to identify cancer stem cells in several types of cancers including pancreatic cancer, glioblastoma and B-acute lymphoblastic leukemia, and is related to the prognosis of AML [15,20-22]. However, biological characteristic and regulatory mechanism of CD9+ AML LSCs remains to be elucidated.

In this study, we found the high-expression of CD9 in AML patient LSCs and extremely-low-expression in normal hematopoietic stem cells (HSCs). CD9+ cells exhibited stem cell characteristics, including drug resistance, migration ability and remodel human AML in immuneocompromised mice. Mechanically, we identified that A2M plays a crucial role in CD9+ LSCs maintenance by transcription profiling analysis. Down-regulation of A2M impairs drug-resistance and migration ability of CD9+ cells. In summary, our data suggested CD9 is a potential new target for AML therapy and A2M controls stemness characteristics of CD9+ AML LSCs.

Materials And Methods

Data sources

Three AML LSCs sequencing chips and one AML minimal disease residue (MDR) sequencing chip were sourced from the publicly available database. Microarray 1: (GSE24006) https://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-24006/?query=GSE24006; Microarray 2: (GSE24395) https://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-24395/?query=GSE24395; Microarray 3: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3005290/; MRD: A group of Minimal Residual Disease was from https://insight.jci.org/articles/view/98561/sd/3.

Cell lines and leukemic cells from patients

Leukemia cells including THP-1 and KG-1α were obtained from America Type Culture Collection (ATCC); U937 was obtained from the Chinese Academy of Sciences, Shanghai, China; MV-4-11 was obtained from the Query Network for Microbial Species of China; MOLM-13 was obtained from the COBIOER, Nanjing, China; HL-60 was obtained from the JOINN Labs, Suzhou, China.

Primary AML cells were obtained from bone marrows of patients with AML who have all signed the informed consent according to the protocols approved by the Institutional Review Board of the Southwest...
Hospital, Army Medical University. All the patients’ information was in supplementary table S1.

The primary AML cells were cultured in endotoxin-free RPMI 1640 (Gibco) medium supplemented with 20% FBS, 1% penicillin-streptomycin, 10ng/ml human cytokines IL-3, Flt-3 ligand, TPO and SCF (Pepro Tech). The normal bone marrow samples were obtained from volunteers.

**Antibodies, Cell Staining, and Sorting**

All the antibodies for FC were purchased from BioLegend. For analyses of CD9 expression in AML cell lines, cells were stained with PE anti-human CD9 Antibody (312106). For primary AML cells, cells were stained with FITC anti-human CD3/CD19 antibody (300306, 392508), PerCP anti-human CD45 antibody (368506), APC/Cyanine7 anti-human CD34 antibody (343614), APC anti-human CD38 antibody (356606) and PE anti-human CD9 antibody (312106). Briefly, cells were harvested and suspended with 50µl staining/washing buffer (PBS including 1% FBS), then stained with antibodies and incubated for 30 minutes at 4°C. Cells were washed with staining/washing buffer and suspended in buffer for flow cytometry or cell sorting.

**Migration assay**

The migration AML cells were tested by Falcon® Permeable Support for 24-well Plate with 8.0 µm Transparent PET Membrane (Corning). 2x10^5 CD9^+ and CD9^- cells were suspended in 200µl RPMI 1640 medium (without FBS) and seeded in the upper chambers, respectively. 900µl medium with 20% FBS was added to bottom chamber of each well. After the 6-hour incubation, migrated cells were counted by trypan blue at the indicated time points [23].

**Drug resistance assay**

The drug resistances of AML cells were assessed by MTS assay. 1x10^5 of AML cells were seeded in 96-well microtiter plates (NEST) with different concentrations of cytarabine (10, 100µg/ml) in 100µl medium [24]. After 24-hour incubation, 10µl MTS (Promega) was added to each well. After 2 hours’ incubation, the plate was measured at wavelength of 490nm with microplate reader (Thermo Fisher Scientific).

**Cell proliferation assay**

The AML cells were seeded in 96 well plates with 5x10^3 cells/well. At the indicated time, 10µl MTS (Promega) was added to cells and then detected the absorbance at 490 nm after 2 hours by the microplate reader (Thermo Fisher Scientific). The medium without cells was used as a negative control [25].

**Transplantation of AML cells into immunodeficient mice**

The animal study was performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Southwest Hospital, Army Medical University. NOG mice (Vital River
Laboratories) at age of 6 to 8 weeks were used for xenogeneic transplantation assays. THP-1 cells were infected with lentivirus co-expressing luciferase and GFP according to the previous method[26]. Sorted CD9+ and CD9− THP-1 cells were transplanted into NOG mice via tail vein injection. The progression of leukemia was monitored by bioluminescence imaging with *In Vivo* Imaging System (IVIS) Spectrum (Perkin Elmer, USA) and Living Image Software for IVIS (Perkin Elmer).

**Microarray analysis**

Three pairs of sorted CD9+ and CD9− primary AML cells (patient 6, patient 7, and patient 8) were investigated by the BGISEU-500 platform for gene expression. In brief, total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions from sorted CD9+ and CD9− primary AML cells, respectively. Then all samples are submitted to the BGISEQ-500 platform for RNA sequencing (RNA-seq).

**Real time PCR**

Total RNA was extracted from CD9+ and CD9− THP-1 cells using RNAiso Plus (Takara), and then Single-stranded cDNA was synthesized with the PrimeScript RT Reagent Kit (Takara) according to the manufacturer's instruction. Quantitative-PCR (Q-PCR) analysis was performed using SYBR premix Ex Taq (Takara). The sequence of the primers and the housekeeping gene GAPDH were all from Primer Bank (https://pga.mgh.harvard.edu/primerbank/).

**Western blotting**

Cells were washed by ice PBS and lysed with RIPA buffer added with protease and phosphatase inhibitor cocktail (Roche). Primary antibody for GAPDH (2118), A2M (4929) were purchased from Abcam and EGR1 (100899-T32) from Sino Biological.

**shRNA-mediated A2M knockdown**

THP-1 and HL-60 cells were transduced with lentiviruses expressing shRNAs (sh-A2M 1, 5'-CCTAACATCTATGTACTGG-3'; sh-A2M 2, 5'-ATAGTGAAAGTCTATGATT-3'; and sh-Control, 5'-TTCTCCGAACGTGTCACGT-3'). Briefly, the concentrated virus or the viral supernatant (50 MOI) were directly added into cells in the presence of 8µg/ml polybrene then spinoculation was conducted at 32°C, 800g for 60 min.

**A2M network with CD9 utilizing the GeneMANIA database**

Datasets, including physical interactions, pathway, and genetic interactions, were collected from the public domain GeneMANIA database. The dataset relevant to A2M and CD9 network was produced from the GeneMANIA database (http://www.genemania.org).

**Chromatin immunoprecipitation**
Chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer's instruction (Cell Signaling Technology, 9005S). Anti-EGR1 antibody was purchased from Cell Signaling Technology (4154S). PCR primers for the CD9 promoter were listed in Supplementary table S2.

Statistical analysis

All experiments were repeated at least in triplicate. Collected data were analyzed with GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA) and estimated variation was taken into account for each group of data and indicated as SEM or SD in each figure legend. Comparison between two groups was carried out with unpaired Student's t test (two tailed), and differences among more than two groups were determined by a one-way ANOVA followed by Newman-Keuls test. Difference with p < 0.05 was considered statistically significant.

Results

1. **CD9 is highly expressed in CD34^+CD38^- cell population of AML patients and almost not expressed in normal HSCs**

To identify cell surface markers selectively expressed on AML LSCs, we surveyed three sets of AML LSCs sequencing chips data [11,27,28]. Fifty-five commonly up-regulated genes were detected by comparing the three datasets (Fig. 1A). MRD (Minimal Residual Disease) in AML patients refers to residual cancer cells after treatment, and it was reported to be a powerful and independent prognostic factor in treatment outcome [29-38]. Whether the expression level of the fifty-five LSCs up-regulated genes was also up-regulated in the MRD microarray has lead us to a further investigation. By analyzing the MRD microarray data, we found that among these fifty-five genes, twenty-three genes were significantly up-regulated in the MRD microarray, including eight cell surface proteins CD33, CD96, HCK, C3AR1, TYROBP, FCER1G, LPXN and CD9 (Fig. 1A, 1B, supplementary table S3). Interestingly, CD96 has already been reported as a LSCs-specific marker in human AML [7], which strongly confirmed the reliability of our guess.

Among these membrane proteins, CD9 was the most intensively up-regulated gene. We firstly analyzed the CD9 expression level in AML cell lines by flow cytometry (FC). The results showed that CD9^+ cells account for 11.14% (1.9%-42.3%, Fig. 1C, S1). Then, we examined CD9 expression in the bone marrow of AML patients and normal donors. With the previously described gating strategy in flow cytometry, we firstly gated away CD3 and CD19 positive T cells and B cells, then focused on CD45^lowSSC^low population, and analyzed CD34^+CD38^- cells within CD45^lowSSC^low population, then further investigated CD9 expression in CD3^-CD19^-CD45^lowCD34^+CD38^- [10] (Fig. 1D). Our data revealed that the average percentage of CD9^+ cells was 12.9% (5.84%-36%) in AML blasts. It has been shown that the AML LSCs mainly reside within the CD34^+CD38^- fraction of leukemic cells. CD9^+ cells account for 62.76% (37.2%-87.1%) in CD34^+CD38^- cells (Fig. 1E), suggesting that CD9^+ cells were enriched in AML LSCs. To determine whether CD9 expression could distinguish LSCs from normal HSCs, we examined CD9 expression in the bone marrow of normal donors. The results showed that CD9 expression was very low in normal bone marrow cells (1.7%,
1.1%-2.4%) and normal HSCs (0.9%, 0.3%-1.3%) (Fig.1F). These data together demonstrate that the cell surface protein CD9 could be a promising marker for targeting AML LSCs.

2. CD9\(^+\) cells exhibited LSCs characteristics

To investigate the biological function of CD9\(^+\) cells, we isolated CD9\(^+\) cells and CD9\(^-\) cells from THP-1 and AML patients by fluorescence-activated cell sorting. Cell proliferative assay showed that there was no significant difference in the proliferative capacity of CD9\(^+\) cells and CD9\(^-\) cells either from THP-1 cells or AML patients (Fig. 2A, 2B, \(p=0.9669, p=0.9005\)), which was consistent with previous reports that stem cells do not exhibit superior proliferation capacity in the normal condition \(39-41\). In addition, cell cycles were analyzed by FC, and the results demonstrated that there are no differences between CD9\(^+\) and CD9\(^-\) cells (Fig. S2). To assess the ability of drug resistance, we treated the sorted cells with different doses of Ara-C (10\(\mu\)g/ml and 100\(\mu\)g/ml) which is a commonly used as leukemia chemotherapy drug, and checked cell survival rate after 24 hours. The results showed that CD9\(^+\) cells were more resistant to Ara-C than CD9\(^-\) cells (Fig. 2C, 2D). Furthermore, the transwell migration assay showed CD9\(^+\) cells exhibited higher migration potential than CD9\(^-\) cells (Fig. 2E, 2F).

To study the function of CD9\(^+\) AML cells \textit{in vivo}, THP-1 cells were stably infected with lentivirus co-expressing luciferase and GFP to facilitate subsequent observation of leukemia growth \textit{in vivo}. 1\(\times\)10\(^6\) CD9\(^+\) cells and CD9\(^-\) cells from THP-1 were respectively injected into NOG mice via tail vein. Due to the severe development of leukemia, mice were sacrificed on day 50 and the results showed that CD9\(^+\) cells exhibited superior proliferation capacity than CD9\(^-\) cells \textit{in vivo} (Fig. 3A, 3B). The proportion of CD9\(^+\) cells in the bone marrow of mice were tested by flow cytometry and the results showed that CD9\(^+\) cells injected mice bone marrow contained a large number of infiltrating CD9\(^+\) cells (50.9%, 17.5%-73.5%). Unexpectedly, varied degrees of CD9\(^+\) cells were also contained in CD9\(^-\) cells-injected mice bone marrow (21.02%, 4.27%-33.9%) (Fig. 3C), which may explain why CD9\(^-\) mice can also form leukemia. In addition, the existence of the same phenomenon in the peripheral blood of mice was observed (Fig. 3D). Previous studies have shown that cancer stem cells and differentiated tumor cells can be transformed into each other in tumor microenvironment \(42,43\). Furthermore, survival research showed that mice survived for a shorter period of time after being transplanted with THP-1 CD9\(^+\) cells compared with mice transplanted with THP-1 CD9\(^-\) cells (Fig. S3). In conclusion, CD9\(^+\) cells display LSCs characteristics, drug resistance, increased capacity of migration and promoting cancer progression.

3. A2M is expressed in CD9\(^+\) cells at high levels

To investigate the molecular mechanisms involved in CD9\(^+\) LSCs maintenance, we performed global gene expression profiles in CD9\(^+\) cells and CD9\(^-\) cells from three AML patients by cDNA microarray. The Venn diagram was used to analyze the differential genes that were up-regulated in these 3 gene sets, and 52 differential genes that were commonly up-regulated in the CD9\(^+\) population were detected (Fig. 4A, 4B).
We further performed gene ontology analysis of the 52 genes, among which the top cluster was genes involved in the extracellular matrix organization, including A2M, SULF2, TGFB1, LRP1, MMP9, SERPINE1 and CRISPLD2 (Fig. 4C). We then confirmed the expression levels of these extracellular matrix-associated genes by real-time PCR in THP-1 (Fig. 4D). We focused on A2M not only because its expression level in CD9+ cells was at high level, but also because activation of A2M signals was reported to promote proliferation and survival of cancer cells [44]. We also confirmed the high expression of A2M protein in CD9+ cells of THP-1 and HL-60 by Western blotting (Fig. 4E).

4. **A2M regulates CD9+ LSCs maintenance**

To further investigate the connection between CD9 and A2M, the GeneMANIA webserver were applied to predict their interactions in the network with the parameters limited to physical interactions, genetic interactions, and pathways to score nodes and source organism Homo sapiens as additional parameters (Fig. 5A). From the Gene MANIA network, we found that A2M has networked with CD9. To test whether A2M regulates CD9 expression, the expression of A2M in CD9+ cells was knocked down by short hairpin RNAs (shRNAs). The results showed that A2M knockdown significantly reduced the expression levels of EGR1 and CD9 (Fig. 5B, 5C). EGR1, as an important transcription factor, is a node in the network of A2M and CD9 (Fig. 5A). The results revealed that A2M possibly regulates CD9 expression by regulating its downstream protein EGR1 and this conclusion was confirmed by ChIP (Fig. S4). Functionally, even though knockdown of A2M had no effect on the proliferative of CD9+ cells (Fig. 5D), but significantly increased the sensitivity of CD9+ cells to Ara-C treatment and attenuate CD9+ cells migration, compared with control groups (Fig. 5E, 5F). Therefore, we concluded that A2M is an upstream gene that regulates CD9 gene expression through EGR1 and controls AML LSCs characteristics (Fig. 5G).

**Discussion**

Cancer stem cells (CSCs) drive tumor initiation, progression and metastasis. AML is a clonal malignant disorder derived from a small number of LSCs. LSCs could be the ultimate cellular target to cure human AML. Scientists are dedicated to searching specific LSCs markers, which can effectively distinguish between LSCs and normal HSCs. Many molecules were reported to be differently expressed on AML LSCs, such as CD47, CD44, CD96, TIM3, CD99 and CD123 [5-7,9-11], however, some of these markers are not specific for AML LSCs. For example, targeting CD123 antibody impairs cytokine signaling and is toxic to common myeloid precursors (CMPs) [9], and targeting CD44 antibody disrupts blast-niche interactions [6].

To find a more specific marker of AML LSCs, three RNA-sequencing data of LSCs and an AML MRD microarray data were analyzed. CD9, the most intensively upregulated membrane molecule, was selected as a candidate marker for AML LSCs, and has been reported to be involved in several types of CSCs, including pancreatic cancer stem cells, breast cancer stem cells, ovarian cancer stem cells, glioblastoma stem cells, LSCs in B-acute lymphoblastic leukemia [15,20,22,45,46].
As a member of the tetraspanin superfamily, CD9 was first identified by Kersey et al as the human hematopoietic progenitor cell surface antigen p24 using a monoclonal antibody that bound to acute lymphoblastic leukemia cells [47]. CD9 has been reported to be expressed in 40% of human AML samples and associated with clinical outcomes in AML [21]. In this study, we demonstrate that CD9 is highly expressed in the CD34+CD38- AML LSCs, and extremely low or no expression in normal HSCs, which could serve as a hopeful therapeutic target in AML. Nevertheless, the evaluation of targeting-CD9 therapy still requires further study.

Understanding the underlying mechanisms of CSCs maintenance also provides potential for patient care and improved prognosis. For example, Hedgehog (Hh), Notch and Wnt signaling exhibit significant crosstalk during embryogenesis. Inhibitors of Hh and Notch pathways have achieved considerable progress in early phase clinical trials [48]. To identify the mechanisms that regulate the characteristics of CD9+ LSCs, we performed RNA-sequencing of CD9+ and CD9- cells from three AML patients. We verified A2M was involved in regulating stemness characteristics of CD9+ cells. Importantly, it has been reported that activated A2M signals promote proliferation and survival of cancer cells predominantly through cell surface GRP78 (CS-GRP78) [49]. {Misra, 2005 #2}Therefore, we believe that A2M signal play a crucial role in AML and the treatment of targeting-A2M signaling pathway will bring new hope to AML patients.

**Conclusion**

Our study demonstrated that CD9 was highly expressed in AML LSCs, but almost not expressed in normal HSCs, which allowed it to serve as a potential LSCs marker. CD9-positive cells possess CSCs characteristics, including drug-resistance, migration ability and promoting leukemia progression. Importantly, we found that A2M signal plays a crucial role in the stemness maintenance of CD9-positive cells in AML. Overall, our results found CD9 a new target for AML therapy.

**Abbreviations**

LSCs: Leukemia stem cells; AML: Acute myelogenous leukemia; A2M: Alpha-2-macroglobulin; HSCs: Hematopoietic stem cells; CD9+: CD9-positive; CD9-: CD9-negative; Minimal residual disease: MRD; CSCs: Cancer stem cells; CMPs: Common myeloid precursors; Flow cytometry: FC; Cytarabine: Ara-C.

**Declarations**

**Acknowledgements**

None.

**Authors’ contributions**

Yongliang Liu, Guiqin Wang and Juanjuan Shan: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; Jiasi Zhang, Xue
Chen, Gang Heng and Guiqin Wang: Acquisition of patient specimens, collection and assembly of data, final approval of manuscript; Jun Chen and Yongchun Zhao: Collection and assembly of data, final approval of manuscript; Huailong Xu and Yuanli Ni: Data analysis and interpretation, final approval of manuscript; Jiatao Li and Yingzi Zhang: Data analysis and interpretation, final approval of manuscript; Cheng Qian: Conception and design, data analysis and interpretation, financial support, final approval of manuscript;

**Funding**

This work was supported by National Key Research and Development Program (2016YFC1303405), National Science Foundation of Chongqing (cstc2016shms-ztzx10006), National Natural Science Foundation of China (No. 81572464;81602590), Fundamental Research Funds for the Central Universities (No. 1061120206, 2019CDYGZD008).

**Availability of data and materials**

For data requests, please contact the authors.

**Ethics approval and consent to participate**

This study was approved by the Ethical/Scientific Committee of Southwest Hospital.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Yamamoto JF, Goodman MT. Patterns of leukemia incidence in the United States by subtype and demographic characteristics, 1997-2002 [in eng]. Cancer causes & control : CCC 2008;19(4):379-390.

2. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia [in eng]. The New England journal of medicine 2015;373(12):1136-1152.

3. Ding Y, Gao H, Zhang Q. The biomarkers of leukemia stem cells in acute myeloid leukemia [in eng]. Stem cell investigation 2017;4:19.

4. Mardiros A, Forman SJ, Budde LE. T cells expressing CD123 chimeric antigen receptors for treatment of acute myeloid leukemia [in eng]. Current opinion in hematology 2015;22(6):484-488.
5 Majeti R, Chao MP, Alizadeh AA et al. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells [in eng]. Cell 2009;138(2):286-299.

6 Jin L, Hope KJ, Zhai Q et al. Targeting of CD44 eradicates human acute myeloid leukemic stem cells [in eng]. Nature medicine 2006;12(10):1167-1174.

7 Hosen N, Park CY, Tatsumi N et al. CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia [in eng]. Proceedings of the National Academy of Sciences of the United States of America 2007;104(26):11008-11013.

8 Mardiros A, Dos Santos C, McDonald T et al. T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia [in eng]. Blood 2013;122(18):3138-3148.

9 Jin L, Lee EM, Ramshaw HS et al. Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells [in eng]. Cell stem cell 2009;5(1):31-42.

10 Chung SS, Eng WS, Hu W et al. CD99 is a therapeutic target on disease stem cells in myeloid malignancies [in eng]. Science translational medicine 2017;9(374).

11 Kikushige Y, Shima T, Takayanagi S et al. TIM-3 is a promising target to selectively kill acute myeloid leukemia stem cells [in eng]. Cell stem cell 2010;7(6):708-717.

12 Li C, Chen X, Yu X et al. Tim-3 is highly expressed in T cells in acute myeloid leukemia and associated with clinicopathological prognostic stratification [in eng]. International journal of clinical and experimental pathology 2014;7(10):6880-6888.

13 Herr MJ, Longhurst CM, Baker B et al. Tetraspanin CD9 modulates human lymphoma cellular proliferation via histone deacetylase activity [in eng]. Biochemical and biophysical research communications 2014;447(4):616-620.

14 Reyes R, Cardeñes B, Machado-Pineda Y et al. Tetraspanin CD9: A Key Regulator of Cell Adhesion in the Immune System [in eng]. Frontiers in immunology 2018;9:863.

15 Podergajs N, Motaln H, Rajčević U et al. Transmembrane protein CD9 is glioblastoma biomarker, relevant for maintenance of glioblastoma stem cells [in eng]. Oncotarget 2016;7(1):593-609.

16 Levy S, Shoham T. The tetraspanin web modulates immune-signalling complexes [in eng]. Nature reviews Immunology 2005;5(2):136-148.

17 Leung KT, Chan KY, Ng PC et al. The tetraspanin CD9 regulates migration, adhesion, and homing of human cord blood CD34+ hematopoietic stem and progenitor cells [in eng]. Blood 2011;117(6):1840-1850.
18 Hemler ME. Specific tetraspanin functions [in eng]. The Journal of cell biology 2001;155(7):1103-1107.

19 Hemler ME. Tetraspanin proteins promote multiple cancer stages [in eng]. Nature reviews Cancer 2014;14(1):49-60.

20 Wang VM, Ferreira RMM, Almagro J. CD9 identifies pancreatic cancer stem cells and modulates glutamine metabolism to fuel tumour growth. 2019;21(11):1425-1435.

21 Touzet L, Dumezy F, Roumier C et al. CD9 in acute myeloid leukemia: Prognostic role and usefulness to target leukemic stem cells. 2019;8(3):1279-1288.

22 Nishida H, Yamazaki H, Yamada T et al. CD9 correlates with cancer stem cell potentials in human B-acute lymphoblastic leukemia cells [in eng]. Biochemical and biophysical research communications 2009;382(1):57-62.

23 Li H, Guo L, Jie S et al. Berberine inhibits SDF-1-induced AML cells and leukemic stem cells migration via regulation of SDF-1 level in bone marrow stromal cells [in eng]. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 2008;62(9):573-578.

24 Harrison JS, Wang X, Studzinski GP. The role of VDR and BIM in potentiation of cytarabine-induced cell death in human AML blasts [in eng]. Oncotarget 2016;7(24):36447-36460.

25 Wobus M, Bornhäuser M, Jacobi A et al. Association of the EGF-TM7 receptor CD97 expression with FLT3-ITD in acute myeloid leukemia [in eng]. Oncotarget 2015;6(36):38804-38815.

26 Shan J, Shen J, Wu M et al. Tcf7l1 Acts as a Suppressor for the Self-Renewal of Liver Cancer Stem Cells and Is Regulated by IGF/MEK/ERK Signaling Independent of β-Catenin. 2019;37(11):1389-1400.

27 Gentles AJ, Plevritis SK, Majeti R et al. Association of a leukemic stem cell gene expression signature with clinical outcomes in acute myeloid leukemia [in eng]. Jama 2010;304(24):2706-2715.

28 Saito Y, Kitamura H, Hijikata A et al. Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells [in eng]. Science translational medicine 2010;2(17):17ra19.

29 Ivey A, Hills RK, Simpson MA et al. Assessment of Minimal Residual Disease in Standard-Risk AML [in eng]. The New England journal of medicine 2016;374(5):422-433.

30 Krönke J, Schlenk RF, Jensen KO et al. Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: a study from the German-Austrian acute myeloid leukemia study group [in eng]. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2011;29(19):2709-2716.
Inaba H, Coustan-Smith E, Cao X et al. Comparative analysis of different approaches to measure treatment response in acute myeloid leukemia [in eng]. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2012;30(29):3625-3632.

Buccisano F, Maurillo L, Del Principe MI et al. Prognostic and therapeutic implications of minimal residual disease detection in acute myeloid leukemia [in eng]. Blood 2012;119(2):332-341.

Terwijn M, van Putten WL, Kelder A et al. High prognostic impact of flow cytometric minimal residual disease detection in acute myeloid leukemia: data from the HOVON/SAKK AML 42A study [in eng]. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2013;31(31):3889-3897.

Walter RB, Buckley SA, Pagel JM et al. Significance of minimal residual disease before myeloablative allogeneic hematopoietic cell transplantation for AML in first and second complete remission [in eng]. Blood 2013;122(10):1813-1821.

Othus M, Estey E, Gale RP. Assessment of Minimal Residual Disease in Standard-Risk AML [in eng]. The New England journal of medicine 2016;375(6):e9.

Taub JW, Berman JN, Hitzler JK et al. Improved outcomes for myeloid leukemia of Down syndrome: a report from the Children's Oncology Group AAML0431 trial [in eng]. Blood 2017;129(25):3304-3313.

Hourigan CS, Gale RP, Gormley NJ et al. Measurable residual disease testing in acute myeloid leukaemia [in eng]. Leukemia 2017;31(7):1482-1490.

Buldini B, Rizzati F, Masetti R et al. Prognostic significance of flow-cytometry evaluation of minimal residual disease in children with acute myeloid leukaemia treated according to the AIEOP-AML 2002/01 study protocol [in eng]. British journal of haematology 2017;177(1):116-126.

Fuchs E, Horsley V. Ferreting out stem cells from their niches [in eng]. Nature cell biology 2011;13(5):513-518.

Medema JP, Vermeulen L. Microenvironmental regulation of stem cells in intestinal homeostasis and cancer [in eng]. Nature 2011;474(7351):318-326.

Simons BD, Clevers H. Strategies for homeostatic stem cell self-renewal in adult tissues [in eng]. Cell 2011;145(6):851-862.

Cabrera MC, Hollingsworth RE, Hurt EM. Cancer stem cell plasticity and tumor hierarchy [in eng]. World journal of stem cells 2015;7(1):27-36.

Gupta PB, Pastushenko I, Skibinski A et al. Phenotypic Plasticity: Driver of Cancer Initiation, Progression, and Therapy Resistance [in eng]. Cell stem cell 2019;24(1):65-78.
44 Gopal U, Gonzalez-Gronow M, Pizzo SV. Activated α2-Macroglobulin Regulates Transcriptional Activation of c-MYC Target Genes through Cell Surface GRP78 Protein [in eng]. The Journal of biological chemistry 2016;291(20):10904-10915.

45 Nagare RP, Sneha S, Krishnapriya S et al. ALDH1A1+ ovarian cancer stem cells co-expressing surface markers CD24, EPHA1 and CD9 form tumours in vivo [in eng]. Experimental cell research 2020;392(1):112009.

46 Ullah M, Akbar A, Thakor AS. An emerging role of CD9 in stemness and chemoresistance [in eng]. Oncotarget 2019;10(40):4000-4001.

47 Kersey JH, LeBien TW, Abramson CS et al. P-24: a human leukemia-associated and lymphohemopoietic progenitor cell surface structure identified with monoclonal antibody [in eng]. The Journal of experimental medicine 1981;153(3):726-731.

48 Saygin C, Matei D, Majeti R et al. Targeting Cancer Stemness in the Clinic: From Hype to Hope [in eng]. Cell stem cell 2019;24(1):25-40.

49 Misra UK, Deedwania R, Pizzo SV. Binding of activated alpha2-macroglobulin to its cell surface receptor GRP78 in 1-LN prostate cancer cells regulates PAK-2-dependent activation of LIMK [in eng]. The Journal of biological chemistry 2005;280(28):26278-26286.

Figures
Figure 1

CD9 is enriched in AML-LSC population. (A) The Venn diagram compares the number of specifically expressed and shared differentially expressed genes among the three AML LSCs sequencing chips. Fifty-five common differentially expressed genes were analyzed in the MRD microarray by the Venn diagram. (B) The expression levels of 8 membrane protein-related genes. (C) Flow cytometry was used to detect the expression of CD9 in AML cell lines THP-1 and HL-60. (D) Gating strategy for comparing the expression
of CD9 on AML blasts (CD3-CD19-CD45[low]SSC[low]) and LSC-enriched CD34+CD38- populations (CD3-CD19-CD45[low]SSC[low]CD34+CD38-). (E) The expression of CD9 on AML blasts and LSCs-enriched CD34+CD38- populations from patients bone marrow (n=5) with AML. Error bars show the ±SEM. (F) CD9 expression in normal bone marrow. Error bars show the ±SEM.
CD9+ cells exhibit stemness characteristics. (A, B) Proliferation ability of CD9+ cells and CD9- cells. Error bars represent ±SD of triplicates. (C, D) Arc-c resistance of CD9+ cells and CD9- cells. Error bars represent ±SD of triplicates. (E, F) Migration ability of CD9+ cells and CD9- cells. Error bars represent ±SD of triplicates. * P < 0.05, **p<0.01, ***p<0.001.

Figure 3
CD9+ cells promote leukemia growth. (A) Bioluminescent images showed that the total flux of the leukemia derived from CD9+ THP-1 cells (left) and CD9- THP-1 cells (right) after tail vein injected in NOG mice. Signal intensity is represented as p/s/cm²/sr. (B) Quantification showed that the total flux of the leukemia derived from CD9+ THP-1 cells and CD9- THP-1 cells after tail vein injected in NOG mice. (n = 5–6 mice/time point). (C) The proportion of CD9+ cells in mice bone marrow. Error bars show the ±SD. (D) The proportion of CD9+ cells in mice peripheral blood. Error bars show the ±SD.

Figure 4
High expression of A2M in CD9+ AML-LSCs (A) Venn diagram analysis of RNA-seq results from AML-LSCs (CD9+) and non-LSCs (CD9-) of three AML patients. (B) Heatmap showing of 52 common differentially expressed genes in three AML patients. (C) Gene Ontology analysis of genes in the heatmap. (D) The expression levels of 7 extracellular matrix organization-associated genes are detected by RT-PCR. Error bars show the ±SD. (E) The expression levels of A2M in CD9+ cells than CD9- cells are detected by Western blotting.
A2M regulates the stem cell characteristics of CD9+ cells (A) The interactions of CD9 and A2M in the network were analyzed by GeneMANIA using the parameters limited to physical interactions, genetic interactions, and pathways to score nodes and source organism Homo sapiens as additional parameters. (B) Protein expression of A2M and EGR-1 in CD9+ cells after A2M knockdown. (C) Flow cytometry to detect CD9 expression after A2M knockdown. (THP-1: sh-Control=78.66%, sh-A2M 1=53.2%, sh-A2M 2=51.89%; HL-60: sh-Control=58.62, sh-A2M 1=34.3%, sh-A2M 2=31.6%. (D) The proliferation ability of CD9+ cells after A2M knockdown. Error bars represent ±SD of triplicates. (E, F) The drug-resistant ability and migration ability of CD9+ cells after A2M knockdown. Error bars represent ±SD of triplicates. (G) Schematic summary of the role of A2M in regulating the stemness of CD9+ AML-LSCs. *p<0.05, **p<0.01, ***p<0.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable.xlsx
- SupplementaryFigure.pptx