Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Resveratrol drives ferroptosis of acute myeloid leukemia cells through Hsa-miR-335-5p/ NFS1/ GPX4 pathway in a ROS-dependent manner

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ARTICLE INFO

Original paper

Article history:
Received: April 07, 2023
Accepted: July 12, 2023
Published: July 31, 2023

Keywords:

Leukemia, acute myelogenous leukemia, survival analysis, ferroptosis, NFS1

ABSTRACT

To explore the potential target to induce ferroptosis for treating acute myeloid leukemia (AML) as well as its mechanism and latent drugs. Using the keyword "acute myelogenous leukemia", the related dataset in TCGA and GEO were used for searching differentially expressed genes. After the filtrate by ROC curve, AUC values, and survival analysis, RT-qPCR as well as Western-blot analysis were performed to verify the high expression level of NFS1 in AML-193 and OCI-AML-3 cells. After CCK-8 detection with and without various cell death inhibitors, ferroptosis were further detected by the expression level of GPX4. After taking the intersection in Starbase and TargetScan, the upstream regulatory miRNA of NFS1 was found. Then the relation of hsa-miR-335-5p, NFS1, as well as GPX4, was ascertained by knockdown and overexpression study in AML-193 and OCI-AML-3 cells. In addition, cellular ROS was detected by DCFH-DA. Finally, resveratrol was used to intensify ferroptosis of AML-193 and OCI-AML-3 cells. NFS1 was highly expressed in AML cells, positively associated with AML-related mortality, and can be used to diagnose AML. Knockout of NFS1 facilitated ROS accumulation and ferroptosis-associated labile iron pool increase. si-NFS1 can inhibit the expression level of GPX4, facilitate ROS accumulation and induce ferroptosis-associated labile iron pool increase. Besides, overexpressed GPX4 can lead to down-regulated cell death after si-NFS1 treatment. Hsa-miR-335-5p was found as the upstream regulator of NFS1. The expression of NFS1 can be up-regulated by sh-hsa-miR-335-5p transfection and can be inhibited by hsa-miR-335-5p transfection. Resveratrol was found can increase the expression level of hsa-miR-335-5p and decrease the expression of NFS1 and GPX4. Resveratrol can intensify ferroptosis of AML cells via Hsa-miR-335-5p/NFS1/ GPX4 pathway through a ROS-dependent manner.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.7.21

Introduction

Leukemia, also known as blood cancer, is a kind of hematopoietic stem cell malignant clonal disease (1). As one of the most common cancers, its incidence rate ranks 11th among all cancers worldwide (2,3). Leukemia includes a series of subtypes, which can be divided into acute leukemia and chronic leukemia on the basis of its advanced speed (4,5). Thereinto, acute leukemia could be further divided into acute lymphoblastic leukemia and acute myelocytic leukemia (AML) (5,6). Among them, AML is a highly heterogeneous malignancy in the blood system, accounting for about 85% of leukemia and is most commonly found in patients over 45 years of age (7). The incidence of AML has increased by 40 percent in developed countries over the past 40 years (8).

The prognosis of relapsed AML patients is generally poor, which overall survival less than 30%[9]. High incidence and low survival rate make AML bring about a global serious medical burden (8,9). Current therapies include supportive therapy, chemotherapy, targeted drug therapy, hematopoietic stem cell transplantation, and cellular immunotherapy (10). However, the above treatments did not significantly improve clinical outcomes (10,11). Therefore, it is necessary to further discover the molecular basis of AML, new diagnostic and prognostic biomarkers of AML, and search for better therapeutic targets to promote the treatment and prognosis of AML.

Ferroptosis, an iron-dependent regulatory cell death, is deemed as an imbalance of redox balance instigated by lipid peroxidation or decreased antioxidant capacity (12). It is different from autophagy, apoptosis, necrosis, necrosis, pro-death and other forms of cell death (12). The occurrence of ferroptosis is a complex and continuous process that relies on signals generated by a series of organelles to activate cell membrane oxidative damage, in which mitochondria, peroxisome, endoplasmic reticulum and lysosome play an important role in producing reactive oxygen species (ROS) and lipid peroxidation and causing to lipid membrane rupture (13,14). The accumulation of iron is also a key factor in the occurrence of iron death (14). Small molecule activators including erastin as well as RSL3 can resist the antioxidant system by boosting the amount of iron in the system (15). This accretion of iron lead to the Fenton reaction, which can further produce ROS and promote oxidative damage to lipids (16).

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There is increasing evidence suggesting ferroptosis participates in the occurrence as well as the development of various diseases, such as cancer, tissue reperfusion injury, and neurodegenerative diseases (17). Ferroptosis inducers can induce cell growth inhibition and change AML cell resistance to chemotherapy drugs through ferritin and necrosis (18,19). These findings may suggest that ferroptosis inducer is a new antitumor treatment strategy for AML. Therefore, this study aimed to discover the latent target to induce ferroptosis for the treatment of AML as well as its mechanism and latent drugs.

Materials and Methods

Public data collection

By gathering omics data associated with cancer, the cancer genome atlas (TCGA) provides a huge reference library free for cancer research through the website https://cancergenome.nih.gov. Using the keyword "acute myelogenous leukemia", we downloaded the TCGA-LAML RNA-Seq gene expression data, clinical data and GTEx from the UCSC Xena database. In addition, we downloaded GSE12417 (n=79, GPL570) from the GEO database as a verification set.

Cell culture

Normal human bone marrow monocytes and AML cell lines (AML-193 and OCI-AML-3) were purchased from American Type Culture Collection and seeded in RPMI-1640 medium containing 10% fetal bovine serum, and 100 g/ml penicillin and streptomycin. The incubated cell was placed into the environment with 5% CO_2 at 37°C. The short tandem repeat analysis was used to verify the authenticity of all human cell lines, and all of the cells used in each experiment were mycoplasma free.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cell lines, and a Moloney leukemia virus reverse transcriptase kit was used for reverse transcriptase reaction. 1 μg total RNA was used as a template to generate complementary deoxyribose nucleic acid (cDNA). To evaluate mRNA expression, cDNA was amplified by qRT-PCR using Green Mix SYBR and their expression was analyzed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. $2^{-\Delta\Delta CT}$ was used to calculate the relative expression level of mRNA and each sample was tested three times. Primers are as follows: β-actin: 5′-CGC CTC AGG CAC AAG GCG-3′and 5′-GCT GGCGTG TTG TAG GT-3′. Hsa-miR-335-5p: 5′-GTG CAA ACC TGTATC GCC TG-3′and 5′-GAA CAG TAT GAT GCC CGAAG-3. NFS1: 5′-TGT GAG GAG ACC ACT CTA GAC-3′and 5′-TGT GCA TCT TCT GGC TAC AGC-3′

Western blot

The cell line was first lysed with a RIPA with protein inhibitor, then the concentration of protein was detected by the Pierce protein assay kit. Primary antibodies were purchased from Abcam (Cambridge, MA, USA) and were used to recognize the protein after it has been transferred to a polyvinylidene fluoride (PVDF) membrane by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins are measured using a chemiluminescence method (Bio-Rad, Hercules, CA, USA).

Cellular proliferation experiment

Cell viability was measured using the cell count Kit 8 (CCK-8) on the basis of the manufacturer's protocol. In short, the cells were inoculated in 96-well plates. Different amounts of TMZ or dimethyl sulfoxide controls were then incubated with the cells. After that, the CCK-8 solution is dropped into the wells. Cells were subsequently detected at an absorbance of 450nm in a microplate reader.

In vitro transfection

In 6-well plates, normal human bone marrow monocytes, AML-193 and OCI-AML-3 cells were seeded at 5×10^4cells/well, respectively, the day prior to transfection. For subsequent transfection, the culture medium was substituted by the serum-free medium. Then, cells were transfected via Lipofectamine (Invitrogen, Carlsbad, CA, USA) complexed with 2 ug si-RNA or sh-RNA.

Statistical analysis

Statistical analysis was achieved with the aid of GraphPad Prism 7 (La Jolla, CA, USA) or Statistical Product and Service Solutions (SPSS) 23.0 (IBM, Armonk, NY, USA). Three independent experiments were involved in each study and expressed as average with SD. Student t-test as well as analysis of variance were respectively used to compare two or three groups. ROC curve analysis was performed to assess the sensitivity as well as specificity of AML diagnostic genes and to calculate the area under the curve (AUC). Starbase and TargetScan were performed to find the upstream molecule of NFS1. $P<0.05$ is considered to present statistically significant.

Results

Highly expressed NFS1 is the key gene to diagnose AML

We downloaded the gene expression level data of TCGA-LAML as well as GTEx to study the expression pattern of NFS1 in AML. Various expression analyses showed that NFS1 was highly expressed in AML ($P<0.001$, Figure 1A). To determine the sensitivity and specificity of NFS1 for the diagnosis of AML, we then performed an ROC curve analysis and compared AUC values. The results showed that NFS1 had a high diagnostic significance for AML (AUC=0.980, Figure 1B). As shown in Figure 1C, D, the mRNA as well as the protein expression levels of NFS1 were higher in AML cell lines (AML-193 and OCI-AML-3) than in normal bone marrow mononuclear cells.

Survival analysis of NFS1 in AML

In order to discover the relationship between NFS1 and the prognosis of AML, a survival analysis based on the TCGA database was executed. The results determined that enlarged levels of NFS1 were negatively connected to the prognosis of AML patients. That is NFS1 was positively associated with cancer-related mortality ($P = 0.0038$, Figure 2A-B). In the GSE12417 validation dataset, patients with high expression levels of NFS1 had significantly worse overall survival outcomes than those in the low expression level group ($P = 0.042$; Figure 2C, D).

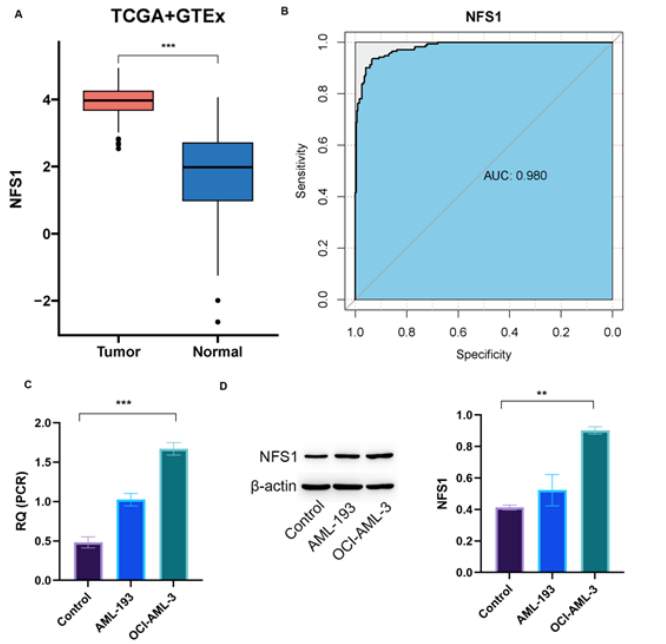

Figure 1. Highly expressed NFS1 is the key gene to diagnose AML. (A) Expression analyses in the TCGA database showed that NFS1 was highly expressed in AML. (B) ROC curve analysis revealed NFS1 had a high diagnostic significance for AML (AUC=0.980). (C-D) RT-qPCR and Western-Blot suggested NFS1 were higher in AML cell lines (AML-193 and OCI-AML-3) than in normal bone marrow mononuclear cells. **: P<0.01; *** P<0.001.

Knockout of NFS1 promotes cell ferroptosis and inhibits cell proliferation in vitro in a ROS-dependent manner

We used small interferon RNAs (siRNAs) to ascertain the function of NFS1 in AML-193 and OCI-AML-3 cells. The effectiveness of gene knockout was validated by RT-qPCR as well as Western blotting (Figure 3A-B). For these two AML cells, CCK-8 detection showed that si-NFS1 significantly restrained cell proliferation as well as promoted cell death (Figure 3C-D).

In order to ascertain the effect of si-NFS1 caused cell death, normal human bone marrow monocytes, AML-193 and OCI-AML-3 cells were transfected with si-NFS1 in the absence as well as the presence of a series of inhibitors related to cell death. The treatment with the aid of ferrostatin-1 (a potent inhibitor of ferroptosis), and deferoxamine (an iron-chelating agent), but not with chloroquine (a potent inhibitor of autophagy), necrostatin-1 (a potent inhibitor of necroptosis), or Z-VAD-FMK (a general caspase inhibitor), thwarted si-NFS1 induced growth inhibition in these cells (Figure 4A-C). Therefore, the above data suggested that ferroptosis could participate in si-NFS1-induced growth inhibition in those cells.

Iron as an indispensable reactive element can participate in many biological processes such as ferroptosis as well as ROS generation. The labile iron pool, which is a crossroad of the transport for cellular iron, was previously deemed as relating to ferroptosis via directly facilitating ROS generation (20,21). At first, we verified the cellular labile iron pool. si-NFS1 treatment aroused a remarkable up-regulation of the cellular labile iron pool (Figure 5A). Then, we tested whether an inhibitor of ferroptosis can prevent si-NFS1-induced labile iron pool increase. As shown in Figure 5A, liproxstatin-1 can block ferroptosis-associated labile iron pool up-regulation.

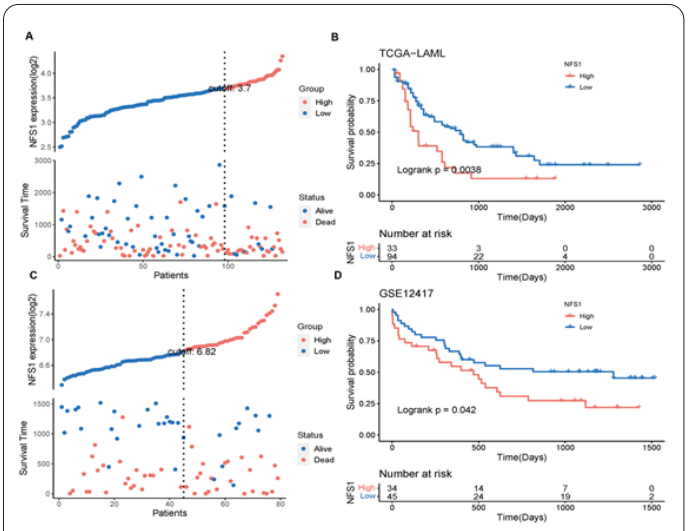

Figure 2. Survival analysis of NFS1 in AML. (A-B) TCGA data revealed that increased levels of NFS1 were negatively associated with the prognosis of AML patients. (C-D) The GSE12417 dataset suggested patients with high expression of NFS1 had significantly worse overall survival outcomes.

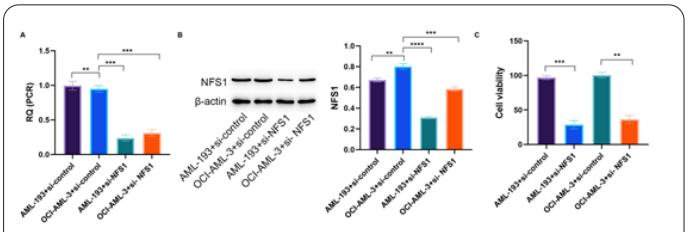

Figure 3. si-NFS1 significantly restrain cell proliferation and promoted cell death. (A) The effectiveness of NFS1 knockout was verified by RT-qPCR. (B) The effectiveness of NFS1 knockout was validated by Western blotting. (C) CCK-8 detection of AML-193 and OCI-AML-3 cells showed that si-NFS1 significantly restrain cell proliferation and promoted cell death. **: P<0.01; *** P<0.001;****P<0.0001.

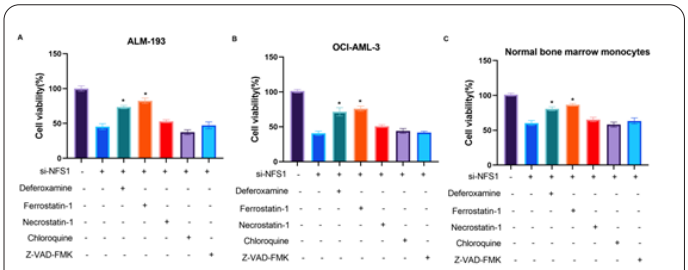

Figure 4. Ferroptosis may participate in si-NFS1-related growth inhibition in those cells. The effect of si-NFS1-induced cell death for normal human bone marrow monocytes (A), AML-193 (B) and OCI-AML-3 cells (C) were transfected with si-NFS1 in the absence or presence of a series of cell death inhibitors. *: P<0.05; +: did not used for the cell line; -: added to the cell line.

ROS accumulation is deemed a remarkable characteristic of ferroptosis. Increasing data suggest that a series of ROS inhibitors and ferroptosis scavengers could completely suppress ferroptosis and cellular ROS accumulation (20). In order to ascertain whether ROS participated in si-NFS1-induced cell death, we detected cellular ROS on the basis of DCFH-DA, a fluorescent probe sensitive to oxidation and can be oxidized to DCF under the circumstances of ROS. Our results suggested si-NFS1 elevated cellular ROS levels. (Figure 5B). In addition, these up-regulated ROS levels can be restrained by the addition

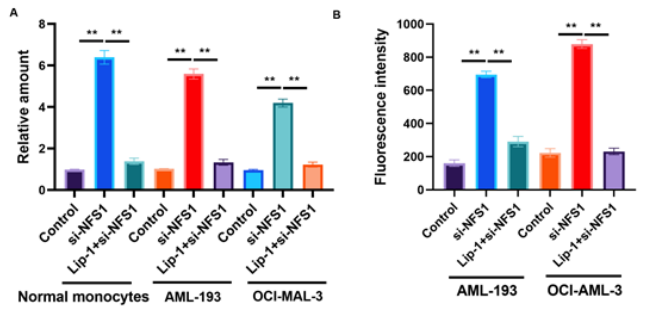

Figure 5. Si-NFS1 promotes ferroptosis-associated labile iron pool increase and ROS accumulation. (A) ferroptosis-associated labile iron pool. (B) ROS accumulation. Lip-1: liproxstatin-1. **: $P<0.01$

of liproxstatin-1 (Figure 5B). That is, si-NFS1 facilitated ROS accumulation and ferroptosis-associated labile iron pool increase.

GPX4 as a crucial antioxidant enzyme is a vital controller of ferroptosis for cancer cells (22). Previous studies have revealed the activation of GPX4 could restrain ferroptosis (22-24). In accordance with previous studies, our study suggested si-NFS1 can inhibit the expression level of GPX4 and this effect could subsequently take part in si-NFS1-induced ferroptosis (Figure 6A). Besides, overexpressed GPX4 can lead to down-regulated cell death after si-NFS1 treatment (Figure 6B). Therefore, GPX4 inhibition could serve as a crucial process in si-NFS1-related ferroptosis.

As is well-known, the ROS generation-induced labile iron pool rise may be ascribed to either the decrease of cellular iron storage protein ferritin or the amassment of iron uptake (25). The iron transport mediated by transferrin is the most familiar iron uptake pathway, in which transferrin serves as a crucial controller for ferroptosis (25,26). In order to ascertain if iron regulatory proteins including transferrin and ferritin are altered after si-NFS1 transfected, Western blotting analysis was implemented to confirm the expression levels of those iron regulatory proteins. After si-NFS1 treatment, the expression level of transferrin was significantly up-regulated (Figure 6A). In addition, to verify this effect was not caused by the change of iron transport regulatory proteins, the expression level of transferrin receptor ferritin was ascertained. The results suggested that, after si-NFS1 treatment, transferrin receptor ferritin did not significantly change (Figure 6A). That si-NFS1 treatment led to ferroptosis by changing iron transport.

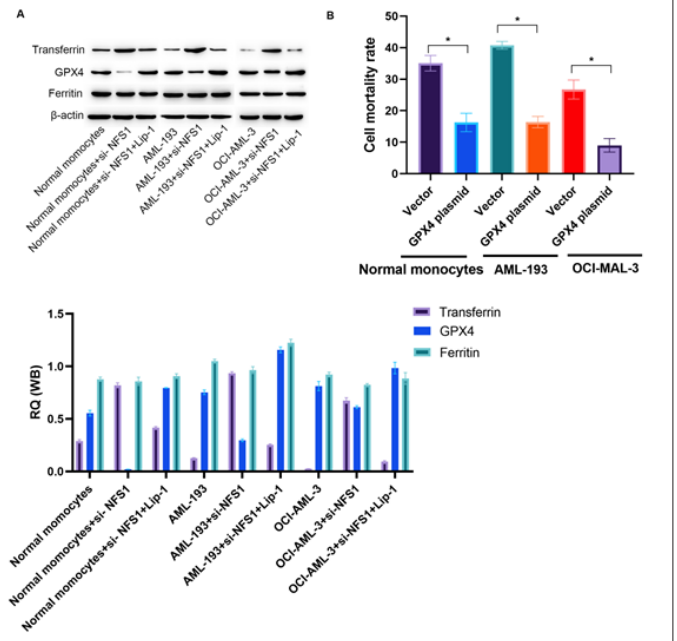

Figure 6. Si-NFS1 treatment led to ferroptosis by changing iron transport. (A) Expression of iron regulatory proteins. (B) The effect of GPX4 on si-NFS1 induced cell death through ferroptosis.*: $P<0.05$.

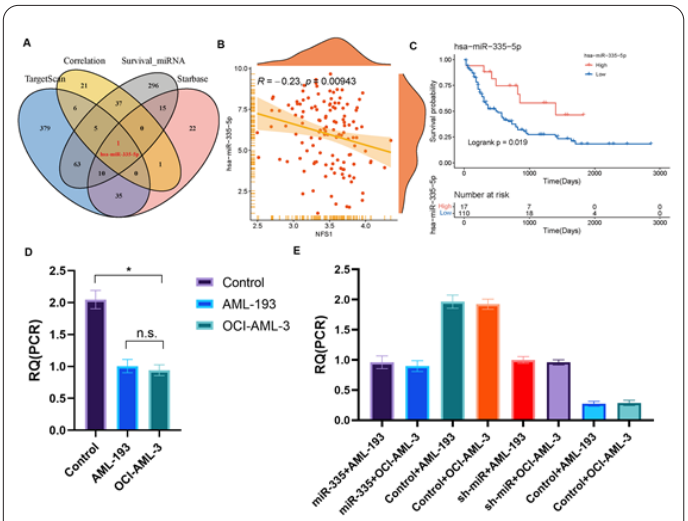

Figure 7. Hsa-miR-335-5p is the upstream regulatory miRNA of NFS1. (A) Taking intersection, hsa-miR-335-5p was found to be the upstream miRNA of NFS1. (B) Correlation analysis of hsa-miR-335-5p and NFS1. (C) Survival analysis of hsa-miR-335-5p for the prognosis of AML patients. (D) RT-qPCR analysis of the expression level of hsa-miR-335-5p in AML and control cells. (E) RT-qPCR analysis of NFS1 in AMLs transfected by hsa-miR-335-5p and sh-hsa-miR-335-5p. *: $P<0.05$; n.s.: no significant difference.

Hsa-miR-335-5p is the upstream regulatory miRNA of NFS1

110 miRNAs were identified by Starbase as the upstream miRNAs of NFS1. 587 miRNAs were identified as upstream miRNAs of NFS1 by TargetScan, including 71 miRNAs that were identified to negatively correlate with NFS1 expression and 427 miRNAs that had prognostic significance (P<0.05). Finally, after taking the intersection, we found that hsa-miR-335-5p was the upstream miRNA of NFS1 (Figure 7A), and Hsa-miR-335-5p was negatively correlated with NFS1 (Cor = -0.23, P=0.00943; Figure 7B). Survival analysis determined that increased levels of hsa-miR-335-5p were positively correlated with the prognosis of AML patients and negatively correlated with their mortality (P = 0.019; Figure 7C).

RT-qPCR results showed that the expression level of hsa-miR-335-5p in AML cell lines was lower than that in normal bone marrow monocytes (Figure 7D). After being transfected by hsa-miR-335-5p, AML-193 cells showed inhibited expression of NFS1, while the expression of NFS1 can be up-regulated by sh- hsa-miR-335-5p (Figure 7E).

Resveratrol can inhibit the proliferation of AML cells through the hsa-miR-335-5p/NFS1-dependent approach.

Resveratrol is a multiple kinases inhibitor and is known for its anti-cancer effect (27,28). Furthermore, resveratrol

can regulate cell viability by restraining cell proliferation and facilitating apoptosis (27). In recent years, increasing studies suggested resveratrol is a ferroptotic inducer and can cause various ferroptosis events in diverse types of cells (27). Ferroptosis has been detected in resveratrol-treated hemopoietic stem cells as well (27,29). Yet, the latent mechanism is still unclear.

In this study, resveratrol could decrease the expression of NFS1 and GPX4 and lead to ferroptosis of AML cell lines (Figure 8A). In addition, NFS1 and GPX4 proteins were significantly upregulated in hsa-miR-335-5p down-regulated AML cell lines (Figure 8A). Besides, resveratrol can increase the expression of hsa-miR-335-5p, however, resveratrol-induced ferroptosis can be restrained by NFS1 overexpression (Figure 8B-C). Taken together, resveratrol can induce ferroptosis of AML cell lines through the hsa-miR-335-5p/NFS1 dependent pattern.

Discussion

With the development of sequencing and information technology, increasing public repositories are established to accelerate medical research (30). Thereinto, TCGA is a publicly funded project to catalogue and reveal major tumor-causing genomic changes to build a comprehensive "atlas" of tumor genomic database (31,32). TCGA has included large cohorts of more than 30 human cancers on the basis of large-scale genome sequencing as well as integrated multi-dimensional analyses. A primary aim of the TCGA was to offer publicly accessible datasets to help facilitate diagnostic approaches, and treatment standards, and ultimately to prevent tumors (33). GEO is also a global public repository for both high-throughput microarray and next-generation sequencing data sets (34). The dataset in GEO was submitted by the global research community (34).

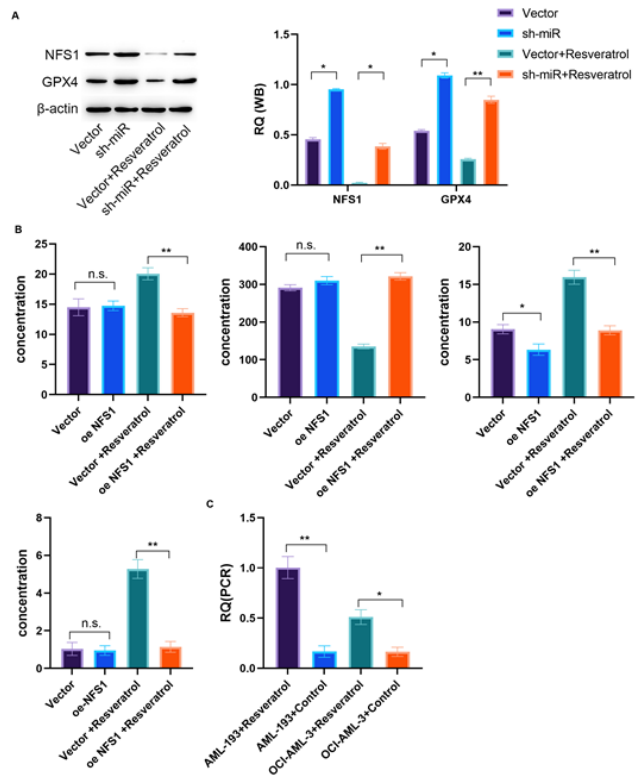

Figure 8. Resveratrol can inhibit the proliferation of AML cells through the hsa-miR-335-5p/NFS1-dependent approach. (A) Western-blot analysis of the expression of NFS1 and GPX4 in sh-hsa-miR-335-5p transfected AML cell line with or without the appearance of resveratrol. (B) The contents of iron, MDA, GSH, and ROS generation in NFS1 overexpressed AML cell lines with or without the appearance of resveratrol. (C) RT-qPCR analysis of hsa-miR-335-5p in AML cell line with or without the appearance of resveratrol. *: $P<0.05$; **: $P<0.01$; n.s.: no significant difference.

After downloading the gene expression data from TCGA and GEO, we found that NFS1 was highly expressed in AML. Subsequent detection for mRNA, as well as protein expression levels of NFS1 in AML cells, verified this tendency. Since the AUC value for diagnosing AML is 0.980, NFS1 could be deemed as the biomarker of AML. Survival analysis is a method of analyzing and inferring the occurrence time of a given event to study the relationship between survival time, outcome and biomarker. To discover the connection between NFS1 and the prognosis of AML, survival analysis based on TCGA and GSE12417 was performed. The results determined that patients with high expression levels of NFS1 had significantly worse overall survival outcomes than those who possess low NFS1 expression. That is, NFS1 may serve as a latent important role in the occurrence and progress of AML.

We used si-NFS1 transfection to ascertain the function of NFS1 in AML-193 and OCI-AML-3 cells. For two types of AML cells, CCK-8 detection showed that si-NFS1 significantly restrain cell proliferation and promoted cell death. In order to further ascertain which kind of cell death was caused by si-NFS1, a series of cell death inhibitors were involved in this study. Thereinto, necrostatin-1, chloroquine, and Z-VAD-FMK failed to rescue si-NFS1 transfected AML cells, which suggested necroptosis, autophagy, and apoptosis did not participate in the si-NFS1 induced cell death. By contrast, deferoxamine and ferrostatin-1 succeeded to preserve the si-NFS1 transfected AML cells. Thus, the knockout of NFS1 can promote the ferroptosis of AML cells.

The labile iron pool, which is a crossroad of the transport for cellular iron, was previously deemed as relating to ferroptosis via directly facilitating ROS generation (4). In order to reveal the mechanism of si-NFS1-induced ferroptosis of AML cells, we detected the cellular labile iron pool and found that si-NFS1 treatment can arouse a remarkable up-regulation of the cellular labile iron pool and this influence can be blocked by liproxstatin-1, inhibitor of ferroptosis. Since ROS inhibitors can completely suppress ferroptosis and cellular ROS accumulation, we detected cellular ROS and found that si-NFS1 can elevate cellular ROS levels and these up-regulated ROS levels can be restrained by the addition of liproxstatin-1. That is the up-regulated labile iron pool involved in the si-NFS1-induced ferroptosis of AML cells in a ROS-dependent approach.

Since the ROS generation-induced labile iron pool rise could be ascribed to either the degradation of cellular iron storage protein ferritin or the amassment of iron uptake (22), we further revealed the expression level of transferrin and transferrin receptor ferritin. We discovered that transferrin significantly up-regulated in AML cells after si-NFS1 treatment, while transferrin receptor ferritin did not significantly change. That si-NFS1 treatment impaired the iron transport and led to ROS accumulation which eventually caused ferroptosis.

GPX4 as a crucial antioxidant enzyme is a vital controller of the ferroptosis for cancer cells. Previous studies have revealed the activation of GPX4 could restrain ferroptosis. This study suggested si-NFS1 can inhibit the expression level of GPX4 and this effect could subsequently take part in si-NFS1-induced ferroptosis. Therefore, GPX4 inhibition could serve as a crucial process in si-NFS1-related ferroptosis.

MiRNAs are kinds of single-stranded and noncoding RNA molecules, which are endogenously generated. It generally regulates posttranscriptional protein synthesis by base pairing to partially complementary sequences in the 3′UTRs of target mRNAs. Then, miRNAs mediate mRNA repression by recruiting the miRNA-induced silencing complex, a ribonucleoprotein complex, to target mRNAs. After taking the intersection in Starbase and TargetScan, the upstream regulatory miRNA of NFS1 was discovered to be hsa-miR-335-5p. Subsequent in vitro study suggested the expression of NFS1 can be up-regulated by sh-hsa-miR-335-5p transfection and can be inhibited by hsa-miR-335-5p transfection. Maryam Kay et al. (35) found that has-miR-335 can regulate cardiac mesoderm as well as progenitor cell differentiation. Since the cell differentiation is exactly related to the prognosis of AML (36), the treatment targets to hsa-miR-335-5p for AML may conduce to a better prognosis.

This study remains a few flaws. First, in vivo study was not involved. Second, the side effect of resveratrol for normal human bone marrow monocytes was not ascertained. However, this study still suggested that AML cells can restrain the expression level of hsa-miR-335-5p to up-regulated the expression of NFS1. Then, up-regulated NFS1 can elevate the iron transport, stabilize the labile iron pool, restrain ROS accumulation, and finally protect AML cells from ferroptosis. To intercept this self-preserving mechanism, in this study. Resveratrol was found can intensify ferroptosis of AML cells via Hsa-miR-335-5p/ NFS1/ GPX4 pathway in a ROS-dependent manner.

This study found that AML cells can restrain the expression level of hsa-miR-335-5p to up-regulated the expression of NFS1, which could elevate the iron transport, stabilize labile iron pool, restrain ROS accumulation, and finally protect AML cells from ferroptosis. Besides, resveratrol was found can intercept this self-preserving mechanism and promote the ferroptosis of AML cells.

Conflict of Interests

The authors declared no conflict of interest.

Funding Acknowledgements

This work was supported by the Qiqihar Science and Technology Bureau (LHYD-2021087) [Effects of resveratrol on proliferation and apoptosis of leukemia HL-60 cells through PI3K/AKT pathway].

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