NADH dehydrogenase subunit 1/4/5 promotes survival of acute myeloid leukemia by mediating specific oxidative phosphorylation

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Received November 1, 2021; Accepted March 16, 2022

DOI: 10.3892/mmr.2022.12711

Abstract. Acute myeloid leukemia (AML) is a type of hematological malignancy caused by uncontrolled clonal proliferation of hematopoietic stem cells. The special energy metabolism mode of AML relying on oxidative phosphorylation is different from the traditional 'Warburg effect'. However, its mechanism is not clear. In the present study, it was demonstrated that the mRNA expression levels of NADH dehydrogenase subunit 1, 4 and 5 (ND1, ND4 and ND5) were upregulated in AML samples from The Cancer Genome Atlas database using the *limma* package in the R programming language. Reverse transcription-quantitative PCR and ELISA were used to verify the upregulation of ND1, ND4 and ND5 in clinical samples. Pan-cancer analysis revealed that the expression of ND1 was upregulated only in AML, ND2 was upregulated only in AML and thymoma, and ND4 was upregulated only in AML and kidney chromophobe. In the present study, it was demonstrated that silencing of ND1/4/5 could inhibit the proliferation of AML cells in transplanted tumor of nude mice. Additionally, it was found that oxidative phosphorylation and energy metabolism of AML cells were decreased after silencing of ND1/4/5. In conclusion, the present study suggested that ND1/4/5 may be involved in the regulation of oxidative phosphorylation metabolism in AML as a potential cancer-promoting factor.

Introduction

Acute myeloid leukemia (AML) is a type of hematological malignancy caused by uncontrolled clonal proliferation of hematopoietic stem cells (1,2). The World Health Organization identifies primordial cells ≥20% as the diagnostic standard of AML (3). In order to improve the accuracy of acute leukemia diagnosis, morphology, immunology, cytogenetics and molecular biology typing is commonly used, including cytomorphology, immunology, cytogenetics and molecular biology (4). The detection of specific immunophenotypic markers (such as CD13, CD33, CD34 and CD117) is important for the classification of AML (5,6). Among them, the antigens associated with good prognosis include myeloperoxidase, CD38 and CD19, and the antigens associated with poor prognosis include CD56, CD7, CD123, CD34 and CD11b. Common cytogenetic abnormalities in AML include t (15;17), 8-trisomy, t (8;21), inv (16) or t (16;16) and 11q23 rearrangements (7,8). Different karyotypes affect the clinical prognosis and have independent prognostic value (9). Molecular genetic abnormalities are also important in improving the prognosis and treatment of AML. Mutations in CCAAT enhancer binding protein α, isocitrate dehydrogenase NADP+1 and nucleophosmin 1 indicate a good prognosis, while mutations in FMS-related receptor tyrosine kinase 3, mixed lineage leukemia, ASXL transcriptional regulator 1 and PHD finger protein 6 indicate a poor prognosis (10,11). Finally, the abnormal expression of oncogenes (N-ras, K-ras and Bcl-2) and suppressor genes (RB transcriptional corepressor 1, p53 and lactate dehydrogenase) in the serum is closely associated with the occurrence of AML (12,13). In conclusion, the identification and detection of pathogenic genes is an important basis for the study of the pathogenesis, clinical treatment and prognosis of AML.

Glycolysis is the main energy supply mode for most tumor cells to maintain the high metabolic demand for proliferation, which is called the 'Warburg effect' (14-16). Proteins related to oxidative phosphorylation are highly enriched in the protein expression profile of AML cells (17). Simultaneously, the survival of AML cells is highly dependent on mitochondrial function (18). Inhibition of mitochondrial protein synthesis and mitochondrial DNA replication in AML cells can effectively inhibit AML cell proliferation (19). Compared with most tumor cells, AML cells have their own unique metabolic mode and their energy metabolism depends more on oxidative phosphorylation, which is different from the traditional Warburg effect. However, to the to the best of the authors' knowledge, its mechanism is not clear.

The electron transport chain, also known as the respiratory chain, is a system composed of a series of electron carriers that transfer electrons from NADH or flavin adenine dinucleotide

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Key words: acute myeloid leukemia, NADH dehydrogenase subunit 1/4/5, oxidative phosphorylation, proliferation

to oxygen (20-22). In the process of electron transfer, free energy is gradually released. Simultaneously, most of the energy is stored in ATP molecules through oxidative phosphorylation. NADH dehydrogenase is the main entrance of the electron transport chain, catalyzing the transfer of electrons from NADH to coenzyme Q. This reaction is the first step in the electron transport chain and serves a vital role in energy metabolism (23,24). NADH dehydrogenase subunits 1/2/3/4/4 L/5/6 (ND 1/2/3/4/4 L/5/6) are involved in encoding NADH dehydrogenase. Previous studies on ND1/2/3/4/4 L/5/6 have focused more on genetic diseases, such as Leber hereditary optic neuropathy, Leigh syndrome and myocardial mitochondrial disease (25,26). However, the relationship between NADH dehydrogenase subunit (ND1/2/3/4/4L/5/6) and AML metabolism has yet to be reported.

In the present study, analysis of The Cancer Genome Atlas (TCGA) database revealed that the expression levels of ND1/4/5 in AML were higher compared with those in normal samples. Notably, a pan-cancer analysis (27) demonstrated that the expression of ND1 was upregulated only in AML, ND2 was upregulated only in AML and thymoma, and ND4 was upregulated only in AML and kidney chromophobe). It was demonstrated that silencing of ND1/4/5 could inhibit the proliferation of AML cells in cell and animal models. Furthermore, it was revealed that oxidative phosphorylation and energy metabolism of AML cells were decreased after silencing of ND1/4/5. In conclusion, the present study suggested that ND1/4/5 may be involved in the regulation of oxidative phosphorylation metabolism in AML as a potential cancer-promoting factor.

Materials and methods

Data collection. The gene expression RNA sequencing data and clinical information of 132 patients with AML from TCGA (28) were obtained from UCSC Xena (https://xenabrowser.net/datapages/?dat-aset=TCGA-LAML.htseq_counts.tsv). The dataset ID in UCSC Xena was TCGA-LAML.htseq_counts.tsv. The RNA expression data were processed by $\log_2$ (count+1) standardization and the genes with >20% missing values (value=0) were removed. Additionally, the clinical information of the patients with AML was collected from TCGA.

Clinical sample collection. Between December 2019 and December 2020, the peripheral blood of 20 healthy individuals and 20 patients with AML was obtained from Kunming Yan'An Hospital (Kunming, China). There were 10 males and 10 females, with a median age of 41 years (range, 8-79 years). The diagnostic criteria of AML patients followed FAB classification (29) and NCCN (National Comprehensive Cancer Network) AML diagnosis and treatment guidelines (30). The present study was approved by the ethics committee of Kunming Yan'An Hospital (Kunming, China; approval no. 2021-03-01). All participants provided written informed consent. The clinical information of the patients with AML was also collected (Table I).

Cell lines. The human acute promyelocytic leukemia cell line (HL-60) was purchased from Cobioer Biosciences Co., Ltd. HL-60 cells were cultured in Iscove's modified Dulbecco's medium (cat. no. 12440053; Thermo Fisher Scientific, Inc.) containing 20% fetal bovine serum (cat. no. 10099141; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin solution (cat. no. C0222; Beyotime Institute of Biotechnology). Cells were grown with 5% CO_2 at 37˚C. The HL-60 cells were cultured in hypoxia under 95% N_2 and 5% CO_2. The HL-60 cells were treated with 0.5 μM rotenone (cat. no. R8875; MilliporeSigma) or 1 μM oligomycin (cat. no. SC0366; Beyotime Biotechnology Inc.) at 37˚C for 24 h. The endoribonuclease-prepared small interfering RNAs (esiRNAs) targeting human mitochondrion (MT)-ND1, MT-ND4 or MT-ND5 (cat. nos. EHU100901, EHU101821 and EHU101561; MilliporeSigma) were used to silence ND1, ND4 or ND5 in HL-60 cells. esiRNA targeting enhanced green fluorescent protein (cat. no. EHUEGFP; MilliporeSigma) was used as a control. pCMV3-C-GFPSpark (cat. no. CV026; Sino Biological, Inc.) was used as an overexpression vector to construct the pCMV3-ND1/4/5 recombinant plasmid, and pCMV3-untagged (cat. no. CV011; Sino Biological, Inc.) was used as the negative control vector. siRNA/plasmids (20 pmol) were added to 50 μl opti-MEM (cat. no. 11058021; Thermo Fisher Scientific, Inc.) medium without serum. 1 μl Lipofectamine (cat. no. A12621; Thermo Fisher Scientific, Inc.) was also added to 50 μl opti-MEM serum-free medium. The two were mixed and placed at room temperature for 20 min to form the complex. The mixture was added to the cell suspension which was cultured at 37˚C and 5% CO_2. After 48 h, other experimental steps were carried out. HL-60 cells stably transfected with siRNA negative control (siNC) or siRNA ND1/4/5 (siND1/4/5) were constructed. siRNA ND1: TGATCAGGGTGAGCATCAAACTCAAACTACGCCCTGATCGGCGCACTGCGAGCAGTAGCCCAAACAATCTCATATGAAGTCACCCTAGCCATCATTCTACTATCAACATTACTAATAAGTGGCTCCTTTAACCTCTCCACCCTTATCACAACACAAGAACACCTCTGATTACTCCTGCCATCATGACCCTTGGCCATAATATGATTTATCTCCACACTAGCAGAGACCAACCGAACC. siRNA ND4: CAGCCACATAGCCCTCGTAGTAACAGCCATTCTCATCCAAACCCCCTGAAGCTTCACCGGCGCAGTCATTCTCATAATCGCCCACGGACTCACATCCTCATTACTATTCTGCCTAGCAAACTCAAACTACGAACGCACTCACAGTCGCATCATAATCCTCTCTCAAGGACTTCAAACTCTACTCCCACTAATAGCTTTTTGATGACTTCTAGCAAGCCTCGCTAACCTCGCCTTACCCCCCACTATTAACCTACTGGGAGAACTCTCTGTGCTAGTAACCACGTTCTCCTGATCAAAsiRNA ND5: ACATCTGTACCCACGCCTTCTTCAAAGCCATACTATTTATGTGCTCCGGGTCCATCATCCACAACCTTAACAATGAACAAGATATTCGAAAAATAGGAGGACTACTCAAAACCATACCTCTCACTTCAACCTCCCTCACCATTGGCAGCCTAGCATTAGCAGGAATACCTTTCCTCACAGGTTTCTACTCCAAAGACCACATCATCGAAACCGCAAACATATCATACACAAACGCCTGAGCCCTATCTATTACTCTCATCGCTACCTCCCTG.

Tumor xenograft model. A total of 20 BALB/c-nu mice (male, 6 weeks old, 18-20 g) were purchased from SipeiFu Biotechnology Co., Ltd. Nude mice were adaptively fed in a specific pathogen-free environment for 7 days. The study protocol was ethically approved by the Kunming Yan'An Hospital Experimental Animal Ethics Committee (Kunming,

Table I. Clinical features of collected tissue samples from AML patients

Clinical characteristics	Value (n=20)
Age (years)	
Median	41
Mean	41.8
Range	8-79
Sex (n)	
Female	10
Male	10
FAB systems (n)	
M0	1
M1	2
M2	3
M3	4
M4	4
M5	3
M6	2
M7	1
White blood cell ($x10^9$/l)	
Median	38.3
Mean	50.4
Range	1-230
Hemoglobin (g/l, n)	
<80	14
≥80	6
Platelet ($x10^9$/l, n)	
<50	16
≥50	4
Ratio of bone marrow blasts (n)	
<60%	9
≥60%	11

FAB classification, French-American-British classification.

China; approval no. 2021016). The whole animal experiment process also followed the Animal Research: Reporting *In Vivo* Experiments guidelines (31). Mice were randomly divided into a control group (siNC) and an experimental group (siND1/4/5) with 10 mice in each group. The cell suspension (HL-60-siNC or HL-60-siND1/4/5) with $2x10^6$ cells per mouse was injected into the right axillary skin of mice after inhalation anesthesia with 2% isoflurane (cat. no. 26675-46-7; MilliporeSigma). The induction concentration of isoflurane in mice was 4% and the maintenance concentration was 2%. The whole experimental period was 23 days starting with the receipt of nude mice. Mice were raised in a specific pathogen-free level environment with suitable temperature (26-28˚C), humidity (40-60%), ventilation (10-15 times per hour) and light (10 h of light, 14 h of no light). Each mouse had an independent sterile cage to ensure sufficient activity space. The feed and drinking water of mice were also pathogen-free and was used for ad libitum feeding. The health and behavior of the mice were observed and recorded every day. Weight loss, loss of appetite, weakness, infection of body organs and excessive tumor volume in mice were regarded as the humane end points. A total of 20 mice were euthanized at the terminal point of the experiment and no mice died during the experiment. All mice were sacrificed using intraperitoneal injection of 200 mg/kg pelltobarbitalum solution (cat. no. P-010; MilliporeSigma) on the 18th day after injection. Before euthanasia, the mice were given orally administrated ibuprofen (40 mg/kg; cat. no. 14883; MilliporeSigma) with water to relieve pain. The criteria for euthanasia were respiratory and cardiac arrest and disappearance of nerve reflex. The long diameter (A) and short diameter (B) of the tumor were measured every 2 days and tumor volume was calculated according to the following formula: $V = AB^2/2$. The tumors were removed surgically and images were captured using a camera (Alpha 7S III; Sony Corporation).

Differentially expressed mRNA (DEmRNA) screening. Isolation of protein-coding genes from the RNA expression matrix from the TCGA database was performed using the *dplyr* and *tidyr* packages (32,33) in the R programming language (version 3.6.2; https://www.r-project.org/). DEmRNAs were screened using the *limma* (version 3.50.1) package (34) in R3.6.2 (35) according to the threshold defined as $P<0.01$ and $|\log_2^{foldchange}|>4$ (AML group vs. normal group). DEmRNAs were visualized by a heatmap using the *pheatmap* (version 1.0.12) package (36) in R3.6.2.

Pan-cancer analysis. The differential expression of ND1, ND4 and ND5 across cancers was analyzed using the Gene Expression Profiling Interactive Analysis (http://gepia2.cancer-pku.cn/#index) online website (37).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the cell lines or tumor tissues using a Micro Scale RNA Isolation Kit (cat. no. AM1931; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The total RNA concentration was measured by a micro ultraviolet-visible spectrophotometer (NanoDrop™ One; Thermo Fisher Scientific, Inc.). Equal amounts of RNA (2 μg) in each group were reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (cat. no. 4374967; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The reverse transcription conditions were as follows: 25˚C for 10 min; 37˚C for 120 min; and 85˚C for 4 min. qPCR assays were performed using a DyNAmo ColorFlash SYBR Green qPCR Kit (cat. no. F416S; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The qPCR conditions were as follows: Pre-denaturation (95˚C for 7 min, 1 cycle); denaturation and annealing (95˚C for 10 sec, 60˚C for 20 sec, 40 cycles); extension (95˚C for 15 sec, 60˚C for 15 sec, 1 cycle). The following primer sequences were used: ND1 forward, 5'-CAACATCGAATACGCCGCAG-3' and reverse, 5'-AATCGGGGGTATGCTGTTCG-3'; ND4 forward, 5'-ACAAGCTCCATCTGCCTACG-3' and reverse, 5'-GAAGCTTCAGGGGGTTTGGA-3'; ND5 forward, 5'-CACATCTGTACCCACGCCTT-3' and reverse, 5'-TGCTATAGGCGCTTGTCAGG-3'; and GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'.

GAPDH was the internal reference for the RT-qPCR assay. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (38).

Reactive oxygen species (ROS) accumulation. ROS accumulation was detected using a Reactive Oxygen Species Assay Kit (cat. no. CA1410; Beijing Solarbio Science & Technology Co., Ltd.). Briefly, dichlorofluorescin diacetate (DCFH-DA) was diluted with serum-free medium at a ratio of 1:1,000 to make the final concentration 10 μmol/l. The cells were fixed at room temperature with 95% ethanol for 30 min. After fixation, the cells (10^6 cells/ml) were suspended in diluted DCFH-DA and incubated in a cell incubator at 37˚C for 20 min. The cells were washed three times with serum-free cell culture medium. The staining state of cells was directly observed under a fluorescence microscope (BX53; Olympus Corporation). Quantitative analysis was performed by flow cytometry (Model: BD Accuri C6; BD Biosciences Inc.) with an excitation wavelength of 525 nm and an emission wavelength of 488 nm. The result of the flow cytometry was analyzed using BD Accuri C6 software (version: 1.0.264.21).

ELISA. ELISA was used to detect the contents of ND1, ND4 and ND5 in peripheral blood of AML patients and healthy people according to the manufacturer's instructions of Human NADH Dehydrogenase Subunit 1, 4 and 5 ELISA kit (cat. nos. abx576646, abx536145 and abx152448; Abbexa, Ltd.). The optical density was determined at OD_{450} nm using an microplate photometer (Multiskan FC; Thermo Fisher Scientific, Inc.).

ATP content detection. ATP in cells and tissues was detected using an ATP content detection kit (cat. no. BC0300; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. ATP content was detected at a wavelength of 340 nm by an enzyme-labeled instrument (Multiskan FC; Thermo Fisher Scientific, Inc.).

Extracellular oxygen consumption assay. Extracellular oxygen consumption in cells was detected using an Extracellular Oxygen Consumption Assay kit (cat. no. ab197243; Abcam) according to the manufacturer's instructions. The extracellular O_2 consumption signal was measured at 1.5 min intervals for 90 min by an enzyme-labeled instrument (Multiskan FC; Thermo Fisher Scientific, Inc.). The excitation wavelength was 380 nm and the emission wavelength was 650 nm.

Human NADH dehydrogenase (complex I) content detection. Human NADH dehydrogenase (complex I) content in cells and mouse tumor tissues was detected using a Human NADH Dehydrogenase SimpleStep ELISA® Kit (cat. no. ab178011; Abcam) according to the manufacturer's instructions. The absorbance value was recorded at 450 nm and substituted into the standard curve to calculate the content of complex I.

Cell Counting Kit-8 (CCK-8) assay. Cell suspension was cultured in a 96-well plate (100 μl; 5,000 cells per well). The cells were cultured at 37˚C with 5% CO_2, and 10 μl CCK-8 solution (cat. no. CA1210; Beijing Solarbio Science & Technology Co., Ltd.) was added to each well. The cells were cultured in the incubator for 4 h. The absorbance at 450 nm was measured by an enzyme-labeled instrument (Multiskan FC; Thermo Fisher Scientific, Inc.).

Table II. Clinical and demographic data from the 132 patients with acute myeloid leukemia in The Cancer Genome Atlas database.

Clinical characteristics	Value (n=132)
Mean age ± SD, years	53.27±16.36
Age, ≥60 years (n)	55
Sex, n (%)	
Female	61 (46.21)
Male	71 (53.79)
Vital status, n (%)	
Dead	80 (60.61
Alive	52 (39.39
Mean days to death ± SD	418.94±387.92
Cytogenetics risk category, n (%)	
Poor	27 (20.45)
Intermediate/normal	73 (55.30)
Favorable	30 (22.73)
Unknown	2 (1.52)
FAB classification, n (%)	
M0	12 (9.09)
M1	32 (24.24)
M2	32 (24.24)
M3	14 (10.61)
M4	27 (20.45)
M5	12 (9.09)
M6	2 (1.52)
M7	1 (0.76)

FAB classification, French-American-British classification.

Statistical analysis. The R programming language (version 3.6.2; https://www.r-project.org/) and SPSS 15.0 software (SPSS, Inc.) were used for statistical analysis. All experiments were repeated at least three times and data are presented as the mean ± SD. A two-tailed unpaired Student's t-test was used to determine the statistical significance of the experimental results. One-way ANOVA with the Least Significant Difference post hoc test was used for the comparison of multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical features of patients with AML from TCGA. RNA sequencing data from a total of 132 patients with AML were included in the present study. The average age of the patients with AML was 53 years and only 55 patients were over 60 years old. There were 61 female patients and 71 male patients. Among them, 80 patients succumbed to AML disease and the average survival time was 418 days. The

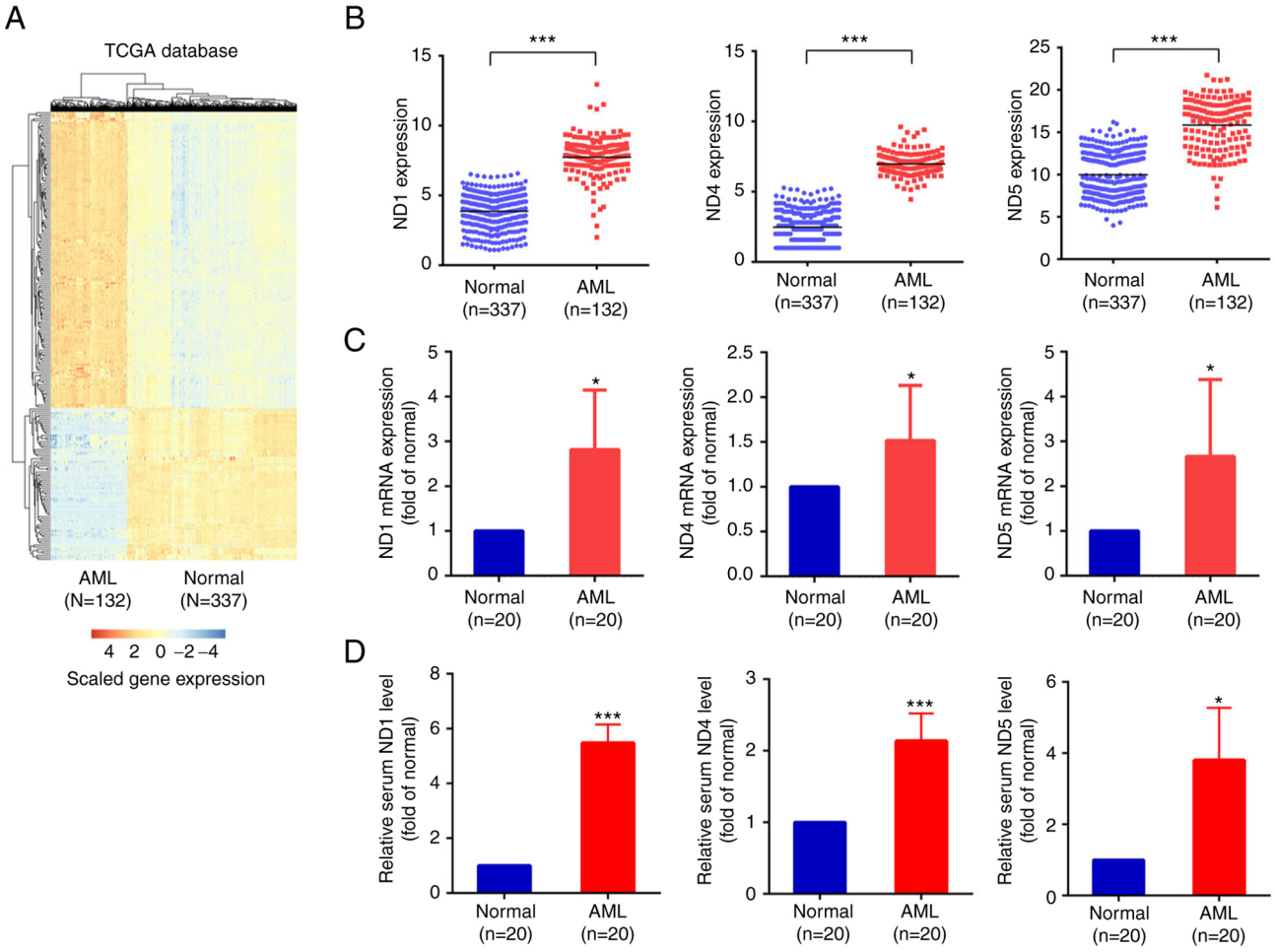

Figure 1. Expression levels of ND1, ND4 and ND5 are upregulated in AML. (A) Differentially expressed mRNAs in AML based on TCGA data were visualized using a heatmap. Blue indicates downregulated genes and red indicates upregulated genes. (B) Scatter diagram indicating the expression levels of ND1, ND4 or ND5 in TCGA. Each scatter represents an independent sample. Normal group vs. AML group, ***P<0.001. (C) Reverse transcription-quantitative PCR assay indicating the mRNA expression levels of ND1, ND4 or ND5 in normal and AML clinical samples. Normal groups vs. AML groups, *P<0.05. (D) ELISAs indicated the secretion level of ND1, ND4 or ND5 in serum in normal and AML clinical samples. Normal group vs. AML group, *P<0.05, ***P<0.001. AML, acute myeloid leukemia; ND1, NADH dehydrogenase subunit 1; ND4, NADH dehydrogenase subunit 4; ND5, NADH dehydrogenase subunit 5; TCGA, The Cancer Genome Atlas.

French-American-British classification of patients was as follows: M0, 12 (9.09%); M1, 32 (24.24%); M2, 32 (24.24%); M3, 14 (10.61%); M4, 27 (20.45%); M5, 12 (9.09%); M6, 2 (1.52%); and M7, 1 (0.76%). Detailed clinical information is presented in Table II.

DEmRNAs. A total of 284 mRNAs were differentially expressed between the normal and AML groups. Among them, 245 (86.27%) mRNAs were upregulated in the AML samples, while 39 (13.73%) were downregulated in the AML samples from TCGA database (Table SI). A heatmap was used to visualize differentially expressed genes (Fig. 1A). The mRNA expression levels of ND1, ND4 and ND5 were upregulated in the AML samples compared with the normal samples (Fig. 1B; P<0.001). Peripheral blood samples were collected from 20 healthy individuals and 20 patients with AML to verify the differential expression of ND1, ND4 and ND5. RT-qPCR results demonstrated that the mRNA expression levels of ND1, ND4 and ND5 were upregulated in the AML samples compared with in the normal samples (Fig. 1C; P<0.05). ELISA results revealed that the secretion levels of ND1, ND4 and ND5 in the serum of patients with AML were higher than those in the normal group (Fig. 1D; ***P<0.001, *P<0.05).

Differential expression analysis of ND1/4/5 in pan-cancer. The differential expression of ND1, ND4 and ND5 across cancers was analyzed using the Gene Expression Profiling Interactive Analysis online website. Compared with in normal samples, ND1 was significantly upregulated only in AML samples. ND4 was significantly upregulated in AML and thymoma samples. ND5 was significantly upregulated in AML and kidney chromophobe samples (Fig. 2; P<0.001). Thus, the upregulation of ND1, ND4 and ND5 expression is almost a unique phenomenon in AML.

Carcinogenic effect of ND1, ND4 and ND5. siRNA-mediated silencing of ND1, ND4, ND5 or ND1/4/5 in HL-60 cells resulted in decreased mRNA expression levels of these genes (Fig. 3A). Additionally, ND1, ND4 and ND5-silenced HL-60 cells showed decreased cell viability, with the triple-KD (siND1/4/5) group showing the largest decrease to 24.22±7.12%

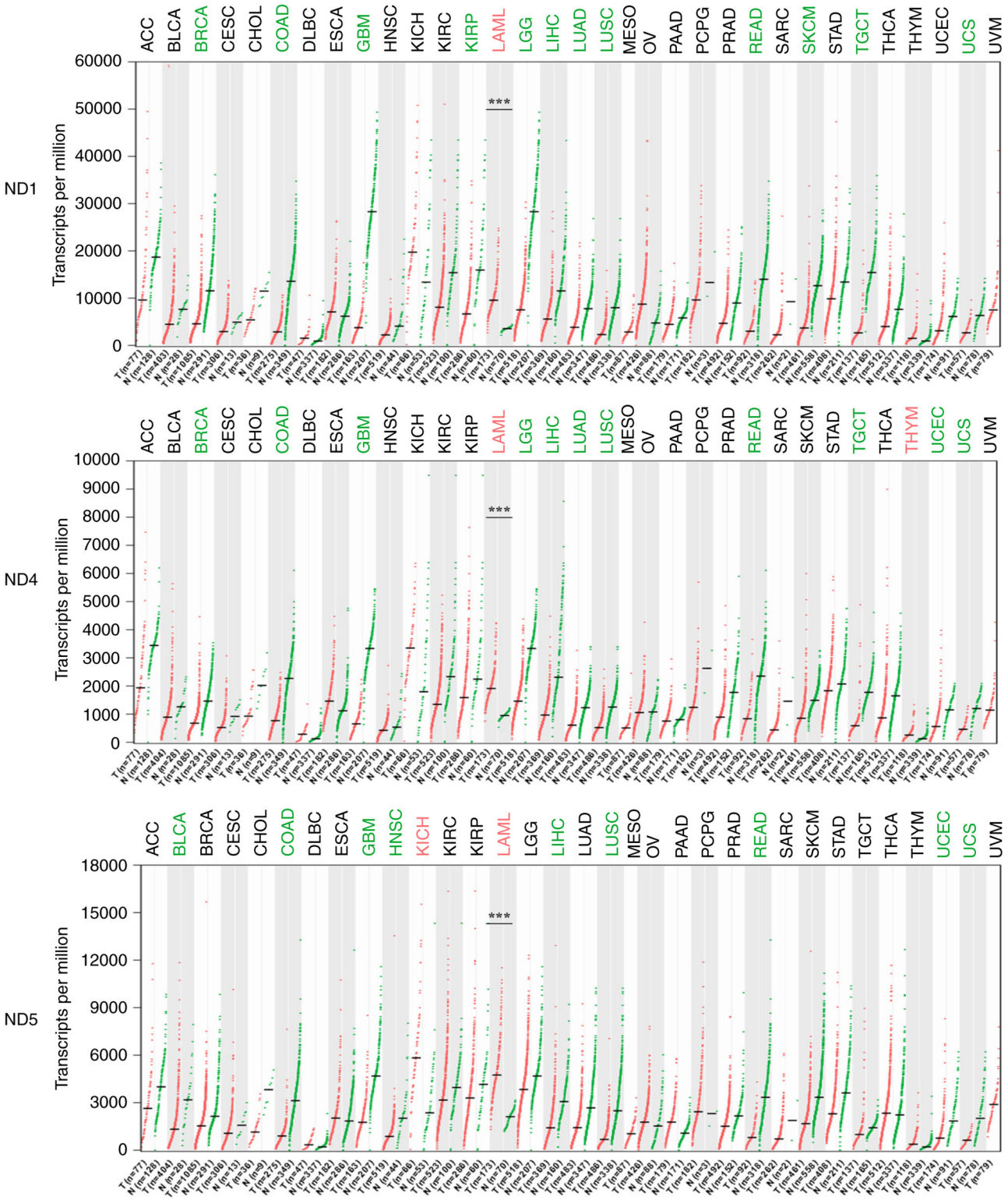

Figure 2. Pan-cancer expression analysis of ND1, ND4 and ND5. Red lines indicate the genes in tumor tissues and green lines indicate the genes in normal tissues. The labels at the top indicate the type of cancer, and red labels indicate upregulation, green labels indicate downregulation and black labels indicate no significant change in the expression of the gene in the specific type of cancer. Normal group vs. AML group ***P<0.001. ND1, NADH dehydrogenase subunit 1; ND4, NADH dehydrogenase subunit 4; ND5, NADH dehydrogenase subunit 5.

compared with the control (siNC) group (Fig. 3B). A xenograft model was used to identify the effect of ND1/4/5 on AML growth *in vivo*. As shown in Fig. 3C, tumors in the siND1/4/5 group grew slower than those in the negative control group. RT-qPCR was performed on all tumor tissues. RT-qPCR showed that the mRNA expression levels of ND1, ND4 and ND5 were significantly downregulated in the siND1/4/5 group compared with the siNC group (P<0.001; Fig. 3D). The volume of tumors in the siNC was larger than that of siND1/4/5 groups over time (Fig. 3E). The average volume of tumors in the

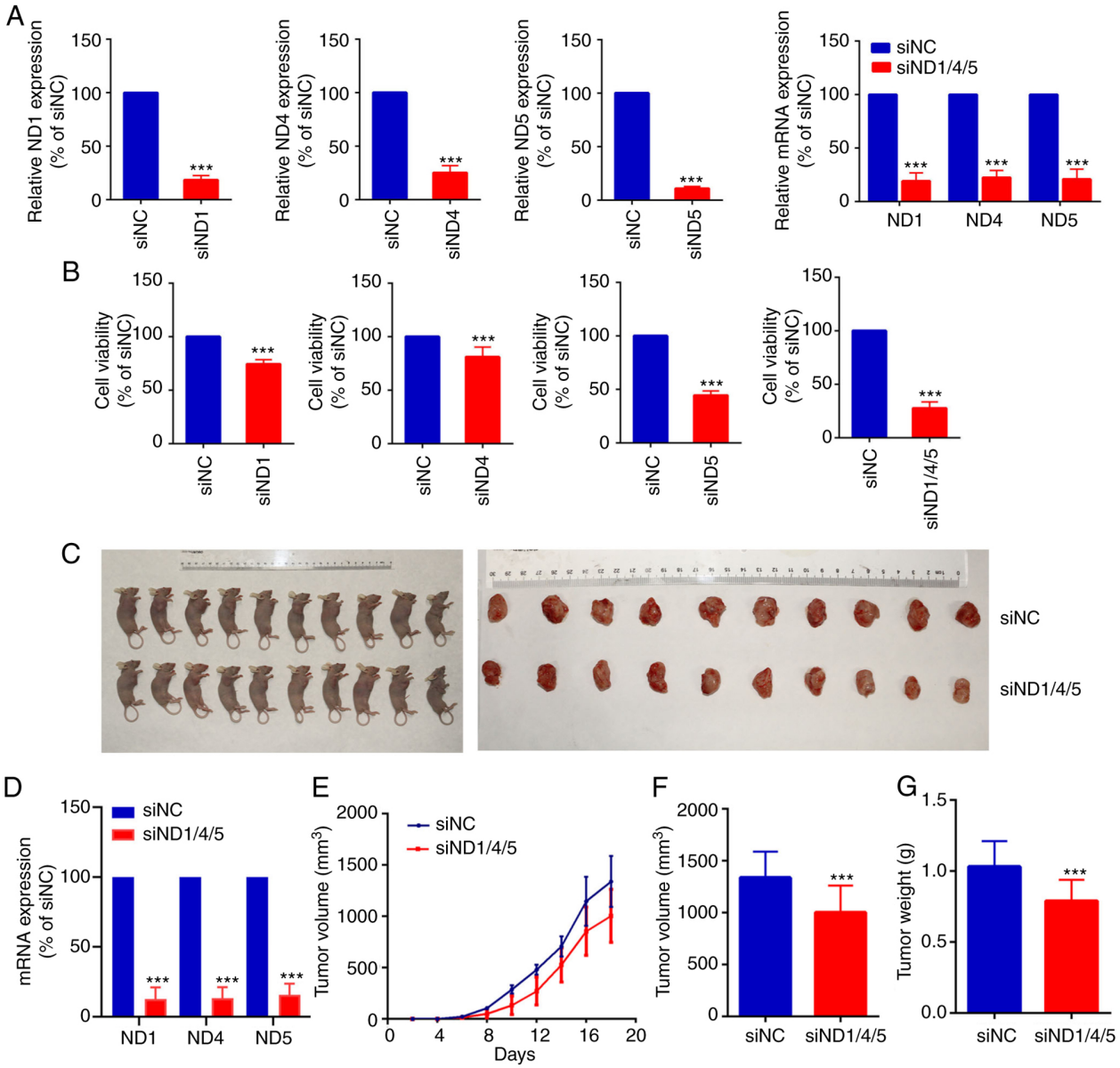

Figure 3. ND1, ND4 and ND5 promote the proliferation of AML cells. (A) Silencing of ND1, ND4, ND5 and ND1/4/5 in the HL-60 cell line. RT-qPCR assays indicated the gene silencing effect in the HL-60 cell line. siND1, siND4, siND5 or siND1/4/5 group vs. siNC group, ***P<0.001. (B) Cell Counting Kit-8 assays demonstrated that HL-60 cells had decreased viability after silencing of ND1, ND4, ND5 or ND1/4/5. siND1, siND4, siND5 or siND1/4/5 group vs. siNC group, ***P<0.001. (C) A xenograft model was used to identify the effect of ND1/4/5 on AML growth *in vivo*. (D) RT-qPCR demonstrated that the mRNA expression levels of ND1, ND4 and ND5 were downregulated in the siND1/4/5 group compared with the siNC group. siND1/4/5 group vs. siNC group, ***P<0.001. (E) Volume of tumors in the siNC and siND1/4/5 groups over time (4, 6, 8, 10, 12, 14, 16 and 18 days). (F) The volume of tumors in the siNC group and siND1/4/5 group on the 18th day. siND1/4/5 group vs. siNC group, ***P<0.001. (G) Weight of tumors in the siNC group and siND1/4/5 group. siND1/4/5 group vs. siNC group, ***P<0.001. AML, acute myeloid leukemia; ND1, NADH dehydrogenase subunit 1; ND4, NADH dehydrogenase subunit 4; ND5, NADH dehydrogenase subunit 5; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering RNA; siNC, small interfering RNA negative control.

siNC or siND1/4/5 group at day 18 was 1,339.33±247.53 mm^3 or 1,004.91±257.25 mm^3. Compared with siNC group, the average volume in siND1/4/5 group was decreased (P<0.001; Fig. 3F). The average weight of tumors in the siND1/4/5 group at 18th days was 77.21±16.72% of that in the control (siNC) group (P<0.001; Fig. 3G).

ND1/4/5 promotes oxidative phosphorylation in AML cells. Most tumors can survive and proliferate rapidly under hypoxia (39). The present study demonstrated that the viability of HL-60 cells was decreased under hypoxia; this was 81.89±3.80% of that under normoxia (Fig. 4A). Furthermore, when HL-60 cells were treated with 0.5 μM rotenone or 1 μM oligomycin (respiratory chain inhibitors) for 24 h, cell viability was decreased to 63.56±7.38% and 75.12±2.13%, respectively, compared with that in the control group (Fig. 4B). The present study explored whether ND1/4/5 overexpression could alleviate the negative effects of rotenone. After verification of the overexpression of ND1/4/5 in HL-60 cells transfected with the PCMV3-ND1/4/5 recombinant plasmid, it was found that it could not alleviate the inhibition of cell viability in AML cells caused by rotenone, an inhibitor of respiratory chain complex I (Fig. 4C). However, silencing of ND1/4/5 downregulated the expression levels of complex I

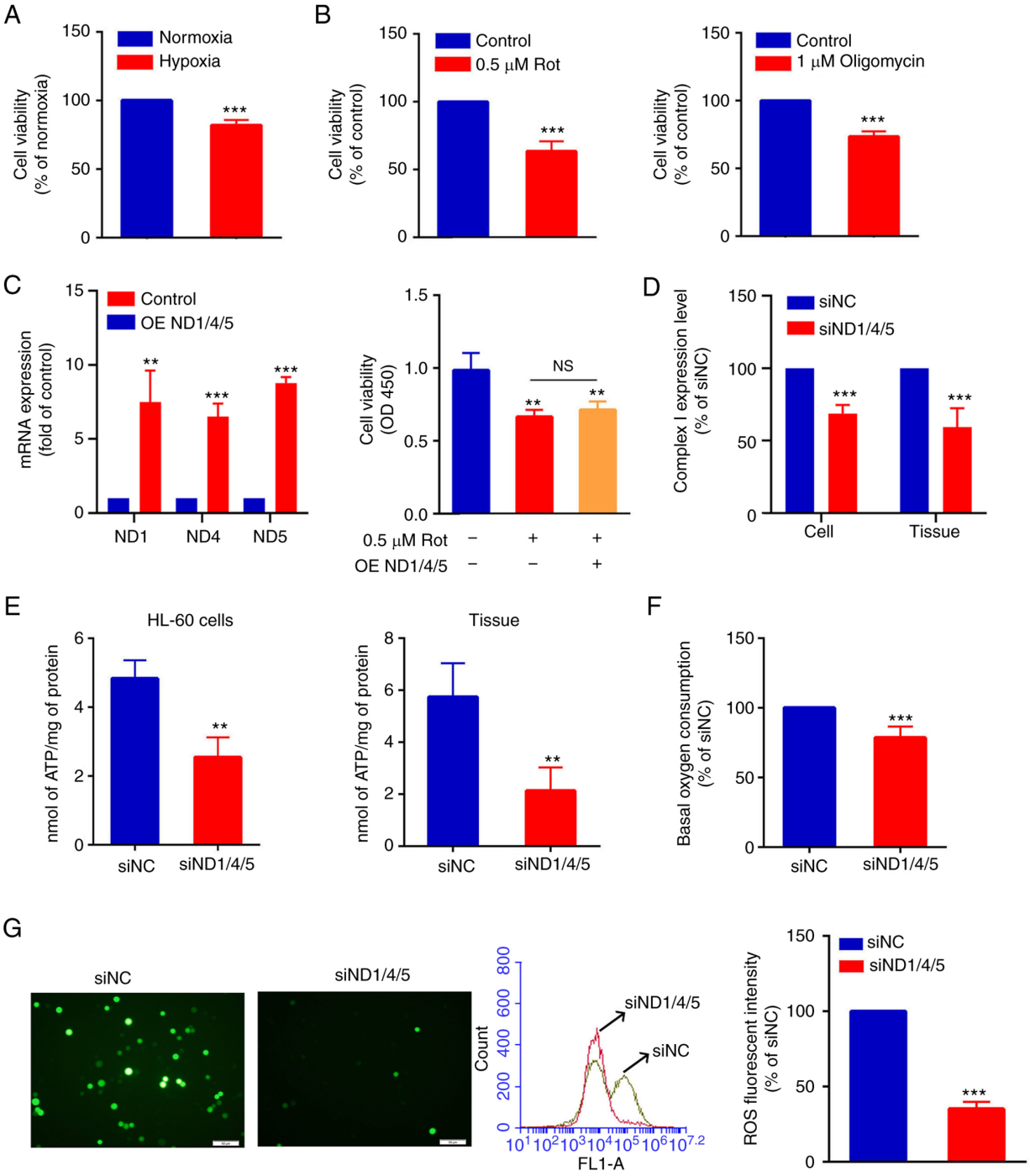

Figure 4. ND1/4/5 promotes oxidative phosphorylation of acute myeloid leukemia cells. (A) CCK-8 assays showed that HL-60 cells had decreased viability under hypoxia conditions. Normoxia group vs. hypoxia group, ***P<0.001. (B) CCK-8 assays showed that the cell viability of HL-60 cells treated with 0.5 μM rotenone or 1 μM oligomycin was decreased, compared with that in the control group. Rotenone or oligomycin groups vs. control groups, ***P<0.001. (C) Reverse transcription-quantitative PCR assay (left) showing that the mRNA expression levels of ND1, ND4 and ND5 were increased in the HL-60 cells transfected with the ND1/4/5 overexpression vector compared with the negative control cells. OE ND1/4/5 group vs. control group, **P<0.01, ***P<0.001. The CCK-8 assay (right) showed that overexpression of ND1/4/5 could not alleviate the inhibition of HL-60 cell viability by rotenone. Rotenone group or rotenone + OE ND1/4/5 group vs. control group, **P<0.01. (D) ELISAs indicated the differences in the expression levels of complex I between the siNC and siND1/4/5 groups in HL-60 cells and tumor tissues. siNC group vs. siND1/4/5 group, ***P<0.001. (E) ATP production in the siNC group and siND1/4/5 group in HL-60 cells and tumor tissues. siNC group vs. siND1/4/5 group, **P<0.01. (F) Basal oxygen consumption in the siNC group and siND1/4/5 group in HL-60 cells. siNC group vs. siND1/4/5 group, ***P<0.001. (G) ROS production was detected using DCFH-DA probes by fluorescence microscopy (magnification, x200, left). Flow cytometry histogram showing ROS levels in the siNC group and the siND1/4/5 group (middle). The fluorescence intensity of ROS was measured by flow cytometry (right). siNC group vs. siND1/4/5 group, ***P<0.001. CCK-8, Cell Counting Kit-8; DCFH-DA, dichlorofluorescin diacetate; ND1, NADH dehydrogenase subunit 1; ND4, NADH dehydrogenase subunit 4; ND5, NADH dehydrogenase subunit 5; OD, optical density; OE ND1/4/5, overexpression of ND1/4/5; ROS, reactive oxygen species; Rot, rotenone; si, small interfering RNA; siNC, small interfering RNA negative control.

in HL-60 cells and mouse tumor tissues (Fig. 4D). Next, the effect of ND1/4/5 on the energy metabolism of AML cells was assessed in terms of ATP production in HL-60 cells or tumor tissues. After silencing of ND1/4/5, ATP production in

HL-60 cells or tumor tissue was decreased to 50.48±7.56% or 40.48±21.68% of the control (siNC), respectively (Fig. 4E). After silencing of ND1/4/5, the oxygen consumption of HL-60 cells was decreased significantly to 78.56±8.34% compared with that in the control (siNC) group (Fig. 4F). Similarly, after silencing of ND 1/4/5, the ROS production of HL-60 cells was decreased significantly to 38.26±2.13% of that of the control (siNC) group (Fig. 4G). In conclusion, these data suggest that the silencing of ND1/4/5 can inhibit the proliferation of AML cells and reduce the oxidative phosphorylation.

Discussion

AML is one of the most common types of leukemia, with a 5-year overall survival rate of 40-45% and <10% in young and elderly patients, respectively (40). In the present study, it was found and verified that the expression levels of ND1/4/5 in AML were upregulated using a pan-cancer analysis of the TCGA database. A number of studies have reported the association between the ND1/4 and the development of other tumors. For example, Jiang *et al* (41) analyzed the ND1, ND4 and GAPDH levels in plasma and blood cells from 75 patients with thyroid cancer, which indicated that the ratio of ND1/ND4 in thyroid cancer was abnormally elevated. Liu *et al* (42) found a total of 39 gene mutations in human esophageal cancer cells (EC9706, TE-1 and ECA109). Among them, the mutation frequency of the mitochondria encoded cytochrome B, ND5 and ND4 genes was the highest. Therefore, the aforementioned data together with the abnormal upregulation of ND1/4/5 in AML suggest that ND1/4/5 could be considered as an important factor in the occurrence and development of AML.

The present study demonstrated that the viability of AML cell lines *in vitro* was decreased under respiratory chain inhibitor treatment and hypoxia. Most tumors can survive under hypoxia (43). Therefore, it is suggested that the survival of AML cells depends on oxidative phosphorylation. The present study further demonstrated that ND1/4/5 silencing inhibited the proliferation of AML cells *in vitro* and in a nude mouse model. Human mitochondrial DNA is involved in encoding NADH dehydrogenase (ND1/2/3/4/4L/5/6), the expression of which can affect oxidative phosphorylation (44,45). It is suggested that the special oxidative phosphorylation metabolism of AML is related to the upregulation of ND1/4/5, which maintains the survival of AML cells. Our subsequent experiments confirmed our hypothesis. ND1/4/5 silencing of AML cells directly resulted in decreased expression of complex I. After silencing ND1/4/5, ATP production, oxygen consumption and ROS production in AML cells were decreased. Combined with the aforementioned results, we hypothesized that the silencing of ND1/4/5 can inhibit the proliferation of AML cells and reduce the oxidative phosphorylation. ND1/4/5 may be a potential oncogene.

In recent years, there have been a number of scientific reports on the treatment of AML by inhibiting oxidative phosphorylation. For example, Zhang *et al* (46) reported that a near-infrared fluorescent dye, IR-26, exerted targeted therapeutic effects on AML cells although impaired oxidative phosphorylation. Baccelli *et al* (17) reported that mubritinib, a known Erb-b2 receptor tyrosine kinase 2 inhibitor, had anti-carcinogenic effects through ubiquinone-dependent inhibition of complex I activity. Carter *et al* (47) reported that the small molecule compounds IACS-010759 and ME-344 inhibited oxidative phosphorylation by targeting the electron transfer chain, thus inhibiting the proliferation of AML cells. In the present study, the potential oncogene ND1/4/5 was identified, which preliminarily confirmed that upregulation of ND1/4/5 was associated with the oxidative phosphorylation in AML. Similar to the reported oxidative phosphorylation inhibitors, ND1/4/5 is expected to become a novel target for the treatment of AML.

Acknowledgements

Not applicable.

Funding

This work was supported by The Key Laboratory of Tumor Immunological Prevention and Treatment of Yunnan Province (grant no. 2017DG004), The Joint Special Program of Applied Basic Research of Kunming Medical University Young Doctor Program (grant no. 202001AY070001-153) and The Kunming Health Research Project (grant no. 2020-21-01-111).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YK and YW designed the experiment and wrote the manuscript. CP and HG contributed to the cell experiments. YD and XY performed the statistical and bioinformatics analysis. JW and FK performed the transplanted tumor experiment in nude mice. YK, HG and YW confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of The Kunming Yan'An Hospital (approval no. 2021-03-01; Kunming, China), and all patients signed informed consent forms. The study protocol was ethically approved by the Kunming Yan'An Hospital Experimental Animal Ethics Committee (approval no. 2021016; Kunming, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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