



## Research Paper

# High Expression of *CPT1A* Predicts Adverse Outcomes: A Potential Therapeutic Target for Acute Myeloid Leukemia



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## ABSTRACT

*Carnitine palmitoyl transferase 1A (CPT1A)* protein catalyzes the rate-limiting step of *Fatty-acid oxidation (FAO)* pathway, which can promote cell proliferation and suppress apoptosis. Targeting *CPT1A* has shown remarkable anti-leukemia activity. But, its prognostic value remains unclear in Acute Myeloid Leukemia (AML). In two independent cohorts of cytogenetically normal AML (CN-AML) patients, compared to low expression of *CPT1A* (*CPT1A<sup>low</sup>*), high expression of *CPT1A* (*CPT1A<sup>high</sup>*) was significantly associated with adverse outcomes, which was also shown in European Leukemia Network (ELN) Intermediate-I category. Multivariable analyses adjusting for known factors confirmed *CPT1A<sup>high</sup>* as a high risk factor. Significant associations between *CPT1A<sup>high</sup>* and adverse outcomes were further validated whether for all AML patients (OS:  $P = 0.008$ ; EFS:  $P = 0.002$ ,  $n = 334$ , no M3) or for National Comprehensive Cancer Network (NCCN) Intermediate-Risk subgroup (OS:  $P = 0.021$ , EFS:  $P = 0.024$ ,  $n = 173$ ). Multiple omics analysis revealed aberrant alterations of genomics and epigenetics were significantly associated with *CPT1A* expression, including up- and down-regulation of oncogenes and tumor suppressor, activation and inhibition of leukemic (AML, CML) and immune activation pathways, hypermethylation enrichments on CpG island and gene promoter regions. Combined with the previously reported anti-leukemia activity of *CPT1A*'s inhibitor, our results proved *CPT1A* as a potential prognosticator and therapeutic target for AML.

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## 1. Introduction

Acute myeloid leukemia (AML) represents a group of myeloid malignancies with remarkably heterogeneous outcomes. Finding effective prognostic biomarkers has been being one of the most urgent clinical needs and research hotspots. Cytogenetically normal acute myeloid leukemia (CN-AML) accounts for about one-half of total AML and constitutes the main body of intermediate-risk AML (Dohner et al., 2010). Since there are no microscopically detectable chromosome abnormalities in leukemic

blasts, CN-AML shows a particular uniformity of cytogenetics and provides a perfect platform of biomarker recognition for AML. Many kinds of molecular signals (such as DNA mutations, aberrant expression of mRNA and microRNAs) have been identified independently as favorable or adverse prognostic biomarkers. Recent studies showed that metabolic signatures were involved in leukemogenesis and could inspire novel therapeutic regimens for AML (Cheong et al., 2012).

Aggressive metabolic changes are key hallmarks of cancer (Hanahan and Weinberg, 2000), Warburg effect of aerobic glycolysis has been regarded as an important bioenergetic source for rapid cell proliferation (Hsu and Sabatini, 2008). Beyond the Warburg effect, other effects, especially fatty acid oxidation (FAO) (Carracedo et al., 2013), also show important roles in the cancer pathogenesis. Series reports of therapeutic applications for metabolic signatures have appeared for AML, including a distinct glucose metabolism signature proved as a prognostic biomarker (Chen et al., 2014), glutamine uptake (Willems et al., 2013) and arginine dependence (Mussai et al., 2015) reported as novel therapeutic targets. Particular concerns were paid on *FAO*, an original article and editorial discussed function blocking of *FAO* with *CPT1A*'s inhibitor (ST1326) and its inherent mechanisms for anti-leukemia treatment

**Abbreviations:** AML, Acute myeloid leukemia; CN-AML, cytogenetically normal AML; *CPT1A*, Carnitine palmitoyl transferase 1A; *CPT1A<sup>high</sup>*, high expression of *CPT1A*; *CPT1A<sup>low</sup>*, low expression of *CPT1A*; *FAO*, fatty-acid oxidation; OS, overall survival; EFS, event-free survival; ELN, European Leukemia Net; NCCN, National Comprehensive Cancer Network.

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(Ricciardi et al., 2015, Samudio and Konopleva, 2015). But, up to now, the prognostic value of *CPT1A* expression remains unclear for AML patients.

Here, we presented *CPT1A*<sup>high</sup> as an adverse prognostic biomarker for AML with concrete data, and also explored the distinctive genomic and epigenomic patterns associated with *CPT1A* expression. Compared to the existing basic experiments of *CPT1A*, our work offered more direct evidences for using *CPT1A* expression as a prognostic biomarker in risk stratification or as a potential therapeutic target for AML patients.

## 2. Materials & Methods

### 2.1. Patients and Treatment

The first cohort was derived from a whole AML cohort ( $n = 334$ , no M3), which included 156 *de novo* CN-AML patients (median age, 50 years, range: 16–77 years), which were all diagnosed and collected at Erasmus University Medical Center (Rotterdam) between 1990 and 2008. 83% of patients ( $n = 129$ ) were aged under 60 years (younger patients) and 17% patients were  $\geq 60$  years (older patients). The study was approved by the institutional review boards at Weill Cornell Medical College and Erasmus University Center, and all subjects provided written informed consent in accordance with the Declaration of Helsinki. The methods were carried out in accordance with the approved guidelines. All patient were uniformly treated under the study protocols of Dutch-Belgian Cooperative Trial Group for Hematology Oncology (HOVON, details of therapeutic protocol available at <http://www.hovon.nl>). All clinical, cytogenetic and molecular information as well as microarray data of these patients can be publicly downloaded at the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>, GSE6891 (Verhaak et al., 2009)). All samples contained 80–100% blast cells after thawing. Conventional cytogenetic examination of at least 20 metaphases from bone marrow (BM) was used to determine normal karyotype. RT-PCR assays were used to assess the mutations of *NPM1*, *CEBPA*, *N-RAS*, *K-RAS*, *IDH1* and *IDH2*, the presence of *FLT3-ITD* and *FLT3-TKD* [D835].

Another independent cohort included 162 previously untreated CN-AML patients (median age: 57.5, range: 17–83 years), which also received uniform intensive double-induction and consolidation chemotherapy provided by the multicenter AMLCG-1999 trial of German AML Cooperative Group between 1999 and 2003. The AMLCG-1999 clinical trial was approved by the local institutional review boards, and written informed consent for each patient were obtained in accordance with the Declaration of Helsinki. The methods were carried out in accordance with the approved guidelines. Microarray data as well as patient information were also publicly available (GEO accession number: GSE12417 (Metzeler et al., 2008)).

### 2.2. Microarray and Sequencing Data Analyses

Several previously published dataset were used for gene expression profiles, including GSE6891 (Verhaak et al., 2009), GSE12417 (Metzeler et al., 2008), GSE1159 (Valk et al., 2004), GSE9476 (Stirewalt et al., 2008) and GSE30029 (de Jonge et al., 2011), all of which can be obtained from GEO. Microarray expression profiles were obtained by Affymetrix Human Genome 133 plus 2.0 and U133A Gene Chips. All experiments' design, quality control and data normalization were in line with the standard Affymetrix protocols. Expression profiles of mRNA and microRNA were obtained by high throughput sequencing (RNA-Seq), and genome-wide methylation data were obtained by Illumina Infinium 450K beadchips, derived from the Cancer Genome Atlas (TCGA), which provided 73 CN-AML patients with all mRNA and microRNA expression and methylation profiles (Cancer Genome Atlas Research, 2013). For microarray data, expression levels of a gene were computed as the mean value of all probe sets annotated to it, while for RNA-Seq data, expression levels of mRNA and microRNA were computed as RPKM and

RPM (Reads Per Kilo-base per Million reads). To choose the appropriate cut-off value for subdivision, we accessed the distribution of *CPT1A* expression level and compared survivals for the 156 CN-AML patients dividing into 4 quartiles, results represented a normal distribution (Fig. S1A) and evident distinction along the median value (Fig. S1B and C). Thus, median value of *CPT1A* expression was used to divide patients into *CPT1A*<sup>high</sup> and *CPT1A*<sup>low</sup> groups. Other dividing of patients according to a gene's expression (such as *ERG*, *WT1* and *DNMT3A* etc.) were dealt using the same strategy.

### 2.3. Statistical Analyses

The definition of overall survival (OS) was the time from date of diagnosis to death due to any causes. Event free survival (EFS) was defined as the time from date of diagnosis to removal from the study due to the absence of complete remission (CR), relapse or death. The Kaplan-Meier method and log-rank test were used to estimate the association between *CPT1A* expression and OS, EFS. The Fisher exact and Wilcoxon rank-sum tests were used, respectively for categorical and continuous variables, to assess the association between expression levels and clinical, molecular characteristics. Multivariable hazards models were used to evaluate the impacts of *CPT1A* expression to OS and EFS in the presence of other known risk factors. Student's *t*-test and multiple hypothesis correction (False Discovery Rate, FDR) were used to identify differences in genome-wide gene, microRNA and methylation profiles between *CPT1A*<sup>high</sup> and *CPT1A*<sup>low</sup> groups. All analyses were performed using the R 3.2.3 software packages.

## 3. Results

### 3.1. Overexpression of *CPT1A* in AML Patients

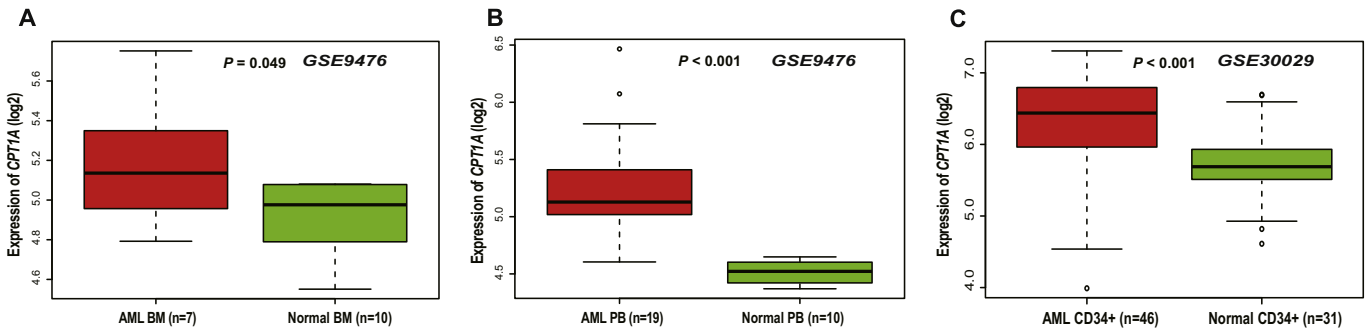
Three public microarray datasets were used to compare *CPT1A* expression between AML patients and healthy donors using bone marrow (BM), peripheral blood (PB) and CD34+ cells. For the comparison of BM samples, *CPT1A* was highly expressed in AML (Fig. 1A,  $P = 0.049$ , 7 AML vs 10 NB, GSE9476), which was validated in PB samples (Fig. 1B,  $P < 0.001$ , 19 AML vs 10 normal, GSE9476). Additionally, CD34+ cells derived from AML patients and healthy donors were used to further verify *CPT1A*'s overexpression in AML patients (Fig. 1C,  $P < 0.001$ , 46 AML CD34+ vs 31 normal CD34+, GSE30029). Besides, higher expression levels of *CPT1A* was shown in various different AML-subtypes than normal BM, including 19 CBFβ-MYH11, 115 CN-AML, 10 Complex, 17 *MLL*-translocation, 86 Others, 22 *RUXN1-RUNX1T1* and 5 normal BM (Fig. S2, GSE1159). All these results showed significant overexpression of *CPT1A* in AML patients.

### 3.2. Different Molecular Characteristics Between *CPT1A*<sup>high</sup> and *CPT1A*<sup>low</sup> Groups

In the cohort of 156 CN-AML derived from GSE6891 (334 AML, no M3), patients in *CPT1A*<sup>high</sup> group tended to be younger ( $P = 0.03$ ) and belong more to FAB M1 ( $P = 0.03$ ) than that in *CPT1A*<sup>low</sup>. *CPT1A*<sup>high</sup> group carried more *FLT3-ITD* than *CPT1A*<sup>low</sup> ( $P = 0.05$ ), while no additional significant associations between *CPT1A* expression and other mutations were found. In addition, *CPT1A*<sup>high</sup> was more likely to accompany with higher expression of many known adverse prognostic biomarkers, such as *ERG* ( $P < 0.001$ ), *BAALC* ( $P < 0.04$ ), *WT1* ( $P < 0.001$ ), *DNMT3A* ( $P < 0.001$ ), *DNMT3B* ( $P = 0.006$ ), *MAPKBP1* ( $P = 0.04$ ), *ITPR2* ( $P < 0.001$ ), *ATP1B1* ( $P < 0.001$ ), *RUNX1* ( $P < 0.001$ ), *TCF4* ( $P = 0.002$ ) and *CXXC5* ( $P < 0.001$ ). (See Table 1 and Fig. S3A)

### 3.3. *CPT1A*<sup>high</sup> Is Associated With Adverse Outcomes in AML

Survival analyses were carried out in the whole cohort of 156 CN-AML patients and European Leukemia Net (ELN) Intermediate-I



**Fig. 1.** Differential expression of *CPT1A*. (A) AML-BM ( $n = 7$ ) vs NBM ( $n = 10$ ). (B) AML-PB ( $n = 19$ ) vs NPM ( $n = 10$ ). (C) AML CD34+ cells ( $n = 46$ ) vs NBM CD34+ cells ( $n = 31$ ).

category ( $n = 121$ ). Compared to *CPT1A*<sup>low</sup> groups, the former results showed that *CPT1A*<sup>high</sup> group was significantly associated with shorter OS and EFS ( $P = 0.042$  and  $P = 0.037$ , respectively, Fig. 2A), and the latter results also showed significant associations between *CPT1A*<sup>high</sup> and shorter OS and EFS ( $P = 0.013$  and  $P = 0.008$ , respectively, Fig. 2B). Further, in order to confirm the prognostic significance of *CPT1A* expression, multivariable analyses were performed in the whole CN-AML and ELN Intermediate-I category. After adjusting for the impacts of known risk factors, multivariable models of OS and EFS were built respectively. For the whole CN-AML, *CPT1A*<sup>high</sup> had 1.68 times higher risks on OS ( $P = 0.014$ ) and 1.64 times higher risks on EFS ( $P = 0.014$ ). Other negative factors included older age ( $P = 0.031$  for OS), wild type of *NPM1* (OS:  $P = 0.007$ , EFS:  $P = 0.003$ ) and the presence of *FLT3-ITD* (OS:  $P = 0.002$ , EFS:  $P = 0.003$ ). For the ELN Intermediate-I category, *CPT1A*<sup>high</sup> still showed adverse impacts on OS and EFS ( $P = 0.006$  and  $P = 0.004$ , respectively) (See Table 2). To further investigate the prognostic significance of *CPT1A* expression, same analyses were executed for the whole cohort of 334 AML (No M3) and National Comprehensive Cancer Network (NCCN) Intermediate-Risk category ( $n = 173$ ). Compared to *CPT1A*<sup>low</sup> groups, *CPT1A*<sup>high</sup> groups still had remarkable shorter OS and EFS in the total AML ( $P = 0.008$  and  $P = 0.002$ , respectively, Fig. 2C) and in the NCCN Intermediate-Risk category ( $P = 0.021$  and  $P = 0.024$ , respectively, Fig. 2D).

**Table 1**  
Patients' characteristics in the primary cohort of 156 CN-AML patients according to *CPT1A* expression.

Variable	<i>CPT1A</i> <sup>high</sup> , $n = 78$	<i>CPT1A</i> <sup>low</sup> , $n = 78$	<i>P</i>
Median age, y (range)	47.5 (16–77)	53 (18–77)	0.03
FAB subtype, no.			
M0	0	3	0.25
M1	29	16	0.03
M2	20	12	0.16
M4	10	14	0.51
M5	16	23	0.62
M6	1	0	1
Other	2	10	0.03
Double <i>CEBPA</i> (No <i>FLT3-ITD</i> ), no	6	8	0.78
<i>NPM1</i> (No <i>FLT3-ITD</i> ), no	17	18	1
<i>FLT3-ITD</i> , no.	39	26	0.05
High <i>ERG</i> , no.	52	26	<0.001
High <i>BAALC</i> , no.	46	32	0.04
High <i>WT1</i> , no.	51	27	<0.001
High <i>DNMT3A</i> , no	50	28	<0.001
High <i>DNMT3B</i> , no	48	30	0.006
High <i>MAPKBP1</i> , no	46	32	0.04
High <i>IIPR2</i> , no	51	27	<0.001
High <i>ATP1B1</i> , no	51	27	<0.001
High <i>RUNX1</i> , no	55	23	<0.001
High <i>TCF4</i> , no	49	29	0.002

**FAB**, French-American-British classification; **ITD**, internal tandem duplication; **TKD**, tyrosine kinase domain; **ELN**, European Leukemia Net.

High *ERG*, *BAALC*, *WT1*, *DNMT3A*, *DNMT3B*, *MAPKBP1*, *IIPR2*, *ATP1B1*, *RUNX1* and *TCF4* expression were defined as an expression level above the median of all samples, respectively.

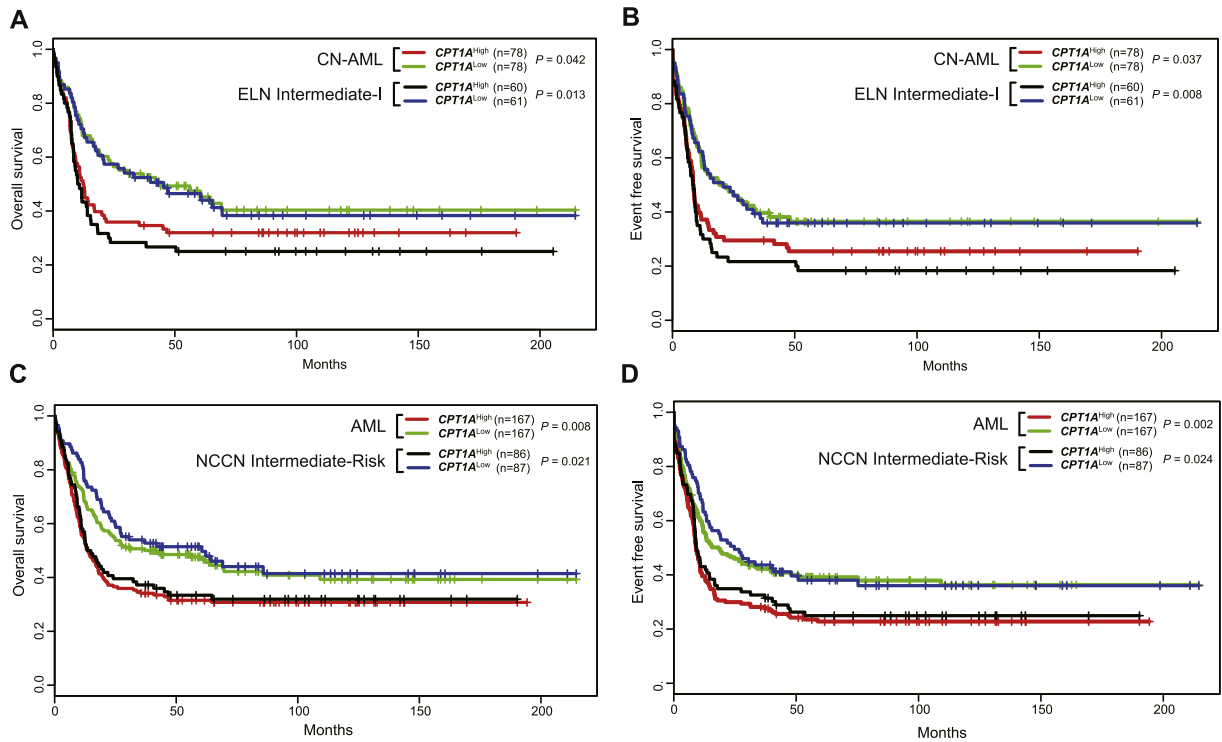
### 3.4. *CPT1A*<sup>high</sup> Is Associated With Adverse Outcomes in Another Independent Cohort of 162 CN-AML Patients

Another independent patient cohort of 162 *de novo* CN-AML was further studied (*GSE12417*), results showed that *CPT1A*<sup>high</sup> was significantly associated with shorter OS ( $P = 0.01$ , Fig. S4). In addition, *CPT1A*<sup>high</sup> patients were more likely to have higher expression of *ERG*, *WT1*, *DNMT3B*, *TCF4*, *IIPR2*, *MAPKBP1*, *ATP1B1* and *RUNX1* (All  $P < 0.001$ , see Table S1 and Fig. S3B).

### 3.5. Associations Between Genome-wide Expression Profiles and *CPT1A* Expression

To further investigate the biological role of *CPT1A* in leukemogenesis, gene expression profiles associated with *CPT1A* were derived based on genome-wide microarray analysis. 131 up-regulated and 271 down-regulated genes were identified as to be significantly associated with *CPT1A* expression (*fdr-adjusted P* < 0.05 and *FC* > 1.5 or *FC* < 1/1.5, Fig. 3A). Further, these aberrant genes were presented as an expression heatmap (Fig. 3B). The up-regulated genes included: 1) genes involving in leukemogenesis (such as *HHEX* (Shields et al., 2016), *NET1* (Ahmad et al., 2014)), tumorigenesis promoters (such as *CDK6*, *HOX* family genes (Shah and Sukumar, 2010)), tyrosine kinase genes (*c-KIT*, *GRB10* (Kazi and Ronnstrand, 2013)); 2) Independent adverse prognostic factors in AML including *WT1*, *CXCS5* (Kuhnl et al., 2015), *MSI2* (Byers et al., 2011), etc.; 3) *CD34*, which acts as a marker for hematopoietic progenitor cells; 4) genes correlating with drug-resistant in AML (such as *IGFBP2* (Kuhnl et al., 2011) and *ABCC1* (Xu et al., 2013a)). The down-regulated genes included: 1) immune system activators such as *ICAM1* and *CD86*; 2) Hematopoietic tumor suppressor *ID2* (Ishiguro et al., 1996) and *KLF4* (Seipel et al., 2016); 3) *CEBPB*, a gene negatively regulated by BCR/ABL, inhibits proliferation and promotes differentiation of BCR/ABL-expressing cells (Guerzoni et al., 2006).

Cell signal pathways, acting as functional units of gene groups, play important biological roles in major cellular processes, including cancer development and progression. Thus, we assessed the associations between *CPT1A* expression and cell signal pathways based on MSigDB database (Subramanian et al., 2005). Using the mean expression of all genes in a pathway to quantize its expression level, 14 down-regulated and 38 up-regulated pathways were found to be significantly associated with *CPT1A*<sup>high</sup> ( $P < 0.05$ ). The up-regulated included: 1) Leukemia-related pathways, such as AML and CML; 2) Metabolic pathways of nucleic acid, amino acid and protein, such as pyrimidine metabolism, basal transcription factors and arginine and proline metabolism; 3) Pathways of lipid metabolism, such as biosynthesis of unsaturated fatty acids, fatty acid metabolisms; 4) Other molecule-related pathways, such as RNA polymerase, DNA replication and Cell-cycle. However, the down-regulated were mainly immune-activation pathways, such as Natural Killer Cell-Mediated-Cytotoxicity, Toll-like



**Fig. 2.** The prognostic value of *CPT1A* expression in CN-AML and AML patients. (A) OS and (B) EFS of the entire 156 CN-AML and 121 patients of ELN Intermediate-I category. (C) OS and (D) EFS of the entire 334 AML and 173 patients of NCCN Intermediate-Risk.

Receptor, Nod-like Receptor and Antigen Processing-Presentation. These dysregulated genes and pathways were consistent with their known understanding about leukemogenesis, which might explain the

involvement between *CPT1A* and the prognosis of CN-AML. (See Figs. 3C and S5)

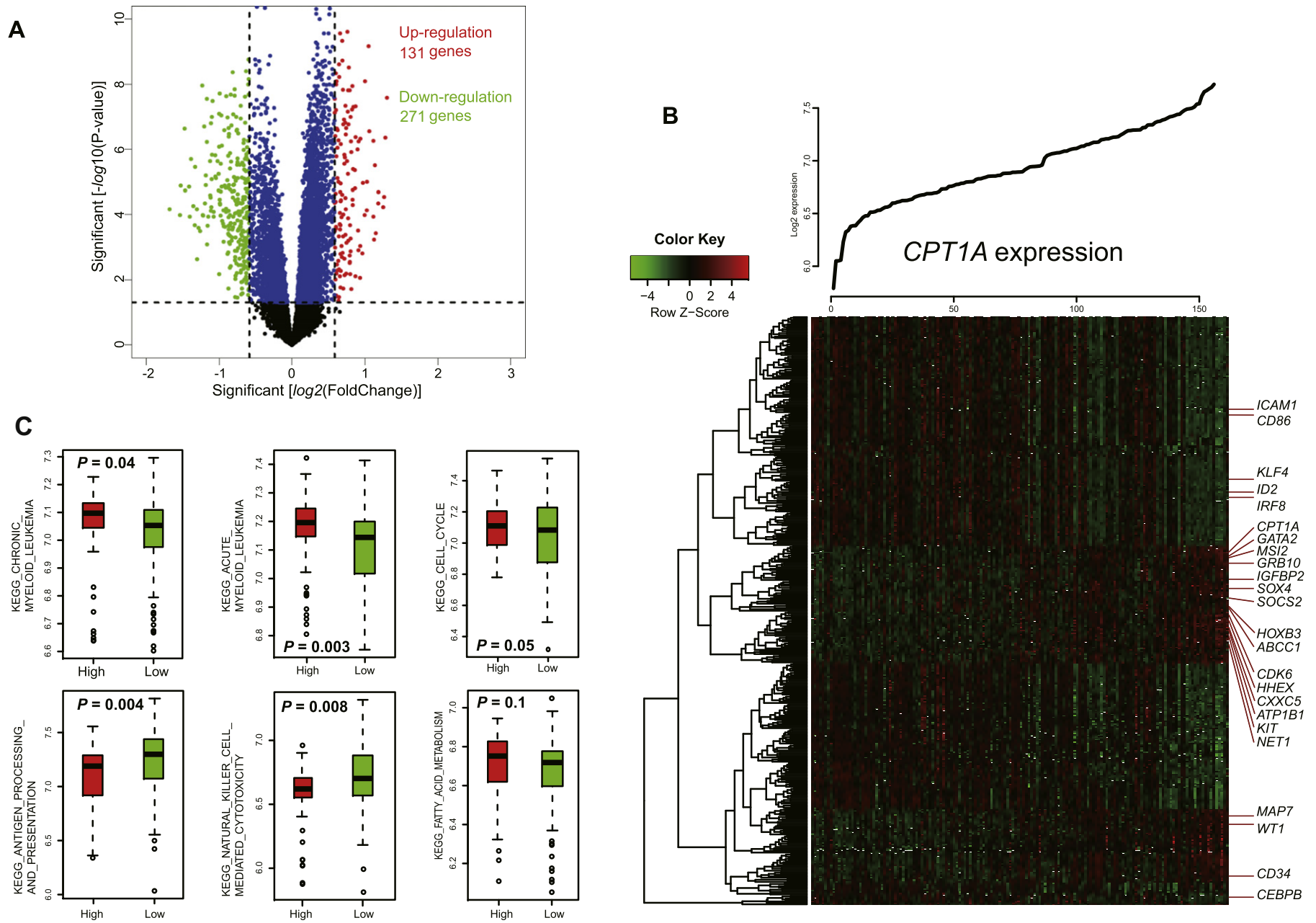
### 3.6. Associations Between Genome-wide MicroRNA Profiles and *CPT1A* Expression

A genome-wide analysis of microRNA-sequencing data were carried out to identify microRNA profiles showing significant correlation to *CPT1A* expression. 109 microRNAs were found, including 89 positive and 20 negative ( $P < 0.05$ , Fig. 4A). Via filtering profiles with missing values, expression profiles of 76 microRNAs were presented as a heatmap (Fig. 4B). Firstly, positively correlated microRNAs included *miR-222*, *miR-221*, *miR-20a*, *miR-17*, *miR-155*, *miR-26a*, *miR-335* and so on. All of those microRNAs have been found to have important tumor-promoting values in previous studies. *miR-222/221* can enhance proliferation and differentiation blockade of melanoma cells (Felicetti et al., 2008). *miR-20a* and *miR-17* can participate in the acquisition of apoptotic resistance in HL50, K562 and K562/ADR cells, inhibition of which helps to decrease the chemoresistance in leukemia therapy (Weng et al., 2014). Up-regulation of *miR-155* is significantly associated with lower complete remission (CR), shorter disease-free survival (DFS) and OS, and can independently identify high-risk patients of CN-AML (Marcucci et al., 2013). *miR-26a* can target *E2F7*, and significantly sustains cell cycle progression and inhibits monocytic differentiation of AML cells (Salvatori et al., 2012). High bone marrow *miR-335* level was significantly associated with a poor treatment response and shorter relapse-free survival and OS in AML patients (Yingchun et al., 2015). Secondly, negatively correlated microRNAs included *miR-193a*, *miR-708*, *miR-152*, *miR-148a*, *miR-326*, *miR-22* and *miR-27a*. Previous study reported that *miR-193a* could repress *c-Kit* expression, and silencing of *miR-193a* contributed to leukemogenesis in AML (Li et al., 2013). Its down-regulation in *CPT1A*<sup>high</sup> hints higher expression of *c-Kit*, which further aggravates worse outcome, which is consistent to *CPT1A*'s prognostic role. *miR-152* is crucial for anti-tumor effect of nature

**Table 2**

Multivariable analysis with OS and EFS in the primary cohort of 156 CN-AML patients.

Variables in final model by end point	HR	95% CI	P-value
OS (All CN-AML, n = 156)			
<i>CPT1A</i> expression, high vs low	1.68	1.11–2.55	0.014
Age, per 10-y increase	1.19	1.02–1.40	0.031
Single <i>CEBPA</i> mutation vs wild type	0.95	0.40–2.26	0.915
Double <i>CEBPA</i> mutation vs wild type	0.49	0.21–1.13	0.095
<i>NPM1</i> , mutated vs wild type	0.54	0.34–0.84	0.007
<i>FLT3-ITD</i> , presented vs others	2.03	1.29–3.17	0.002
EFS (All CN-AML, n = 156)			
<i>CPT1A</i> expression, high vs low	1.64	1.11–2.42	0.014
Age, per 10-y increase	1.08	0.93–1.26	0.305
Single <i>CEBPA</i> mutation vs wild type	1.03	0.43–2.45	0.952
Double <i>CEBPA</i> mutation vs wild type	0.49	0.23–1.04	0.064
<i>NPM1</i> , mutated vs wild type	0.52	0.34–0.80	0.003
<i>FLT3-ITD</i> , presented vs others	1.92	1.25–2.95	0.003
OS (ELN Intermediate-I, n = 121)			
<i>CPT1A</i> expression, high vs low	1.90	1.12–3.01	0.006
Age, per 10-y increase	1.18	1.00–1.40	0.050
Single <i>CEBPA</i> mutation vs wild type	0.94	0.30–2.24	0.891
Double <i>CEBPA</i> mutation vs wild type	0.47	0.20–1.11	0.085
<i>NPM1</i> , mutated vs wild type	0.57	0.30–1.11	0.098
<i>FLT3-ITD</i> , presented vs others	1.86	0.96–3.61	0.065
EFS (ELN Intermediate-I, n = 121)			
<i>CPT1A</i> expression, high vs low	1.90	1.23–2.95	0.004
Age, per 10-y increase	1.11	0.94–1.30	0.213
Single <i>CEBPA</i> mutation vs wild type	1.0	0.41–2.41	0.999
Double <i>CEBPA</i> mutation vs wild type	0.49	0.23–1.05	0.068
<i>NPM1</i> , mutated vs wild type	0.50	0.27–0.94	0.032
<i>FLT3-ITD</i> , presented vs others	2.01	1.07–3.76	0.030



**Fig. 3.** Genome-wide genes and cell signaling pathways associated with *CPT1A* expression. (A) Volcano plot of differential gene profiles between *CPT1A*<sup>high</sup> and *CPT1A*<sup>low</sup>. (B) Expression heatmap of *CPT1A*-associated genes. The top curve shows *CPT1A*'s expression distribution of 156 CN-AML samples. (C) Boxplot of direct-related signaling pathways.



killer-cells by upregulating HLA-G (Bian et al., 2015). Down-regulation of *miR-326* promotes cell proliferation in CML CD34+ cells (Babashah et al., 2013). Silencing of *miR-708* could promote *NF- $\kappa$ B* signaling in CLL (Baer et al., 2015). *miR-27a* functions as a tumor suppressor in acute leukemia cells via regulation of apoptosis (Scheibner et al., 2012). All these aberrant regulations of microRNAs would contribute to the adverse outcome of *CPT1A*<sup>high</sup> group.

To further clarify the biological functions of microRNAs, interaction-network was constructed based on the overlapping of results derived from microRNA-target predicting algorithm (Garcia et al., 2011) and correlation analysis (Fig. 4C). Up- and down-regulation of microRNAs and mRNAs were represented separately as red and green rectangles and ellipses, separately. Results showed some tumor suppressors were repressed by the up-regulated microRNAs, thus aggravated worse outcome. For example, *CEBPB*, targeted by *miR-155*, could inhibit proliferation and promote differentiation of BCR/ABL-expressing cells (Guerzoni et al., 2006). Meanwhile, targets of the down-regulated microRNAs included many oncogenes, such as *ABI2*, targeted by *miR-193a*, participated in the MLL translocation in AML (Coenen et al., 2012). *RICTOR* was targeted by *miR-152*. *HHEX* (Shields et al., 2016), *PBXIP1* (Xu et al., 2013b) and *EZH1* (Xu et al., 2015) were all targets of *miR-148a*, *NET1* (Ahmad et al., 2014) and *HOXA4* (Zangenberg et al., 2009) were targeted by *miR-22*; *MAP3K4*, *ERG*, *ABL2*, *CDK6* and *CDK8* were targeted by *miR-27a*; *JAK1*, *MAP3K3*, *SRSF6* (Cohen-Eliav et al., 2013) and *SRSF1* (Zou et al., 2012) were targeted by *miR-708*. These discoveries perhaps helped to understand why *CPT1A*<sup>high</sup> could act as an adverse prognosticator.

In addition, the dynamic microRNA-mRNA pairs (MMR) were also investigated between *CPT1A*<sup>high</sup> and *CPT1A*<sup>low</sup> groups. Distinct MMR pairs were screened out for *CPT1A*<sup>high</sup> and *CPT1A*<sup>low</sup> separately, with significant negative correlation ( $P < 0.01$ ) in *CPT1A*<sup>high</sup> or *CPT1A*<sup>low</sup> and without significant negative correlation ( $P > 0.1$ ) in *CPT1A*<sup>low</sup> or *CPT1A*<sup>high</sup> groups, additionally with significant changes in absolute correlation coefficients between these two groups (Fig. S6A for *CPT1A*<sup>high</sup>, Fig. S6B for *CPT1A*<sup>low</sup>). 14 most significant MMR pairs were filtered out in *CPT1A*<sup>high</sup>, including *miR-196a* and *PIP4K2C* (a tyrosine kinase), *miR-32* and *AKIRIN2*, which promotes tumorigenicity and metastasis of Lewis lung carcinoma cells (Komiya et al., 2014), *miR-7* and *TRIM11*, which is overexpressed in high-grade gliomas (Di et al., 2013). Our discoveries were consistent to all those known studies. 13 MMR pairs were found to be significantly correlated in *CPT1A*<sup>low</sup>, including *miR-374b* and *ZMAT3*, the gene can promote cell cycle rather than apoptosis via directing the p53 stress response (Bersani et al., 2014). Taking together, those observations possibly provided rational interpretations for the prognostic role of *CPT1A* expression.

### 3.7. Abnormal Genome-wide Methylation Alterations Associated With *CPT1A*<sup>high</sup>

DNA methylation is an important epigenetic mechanism, which can regulate gene expression through 3 DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*) and affect the behaviors of cancer cells. Considering the fact that *CPT1A*<sup>high</sup> remarkably accompanied with higher expression of *DNMT3A* and *DNMT3B*, differential methylated regions (DMR) were derived to see the different methylation patterns between *CPT1A*<sup>high</sup> and *CPT1A*<sup>low</sup> groups of CN-AML. Firstly, compared to *CPT1A*<sup>low</sup>, *DNMT3A* and *DNMT3B* showed significantly higher expression in *CPT1A*<sup>high</sup> group ( $P = 0.001$  and  $P < 0.001$ , respectively), and *DNMT1* also showed a trend of higher expression ( $P = 0.182$ ) (Fig. 5A). Secondly, 1201 hypermethylation and 455 hypomethylation DMRs were derived from the comparison between *CPT1A*<sup>high</sup> and *CPT1A*<sup>low</sup> ( $P < 0.05$ ,  $FC > 2$  or  $FC < 1/2$ , Fig. 5B). Thirdly, position distribution around CpG islands for these aberrant DMRs were compared. More hypermethylated DMRs were around the CpG islands (Island:  $P < 0.001$ , S\_Shore:  $P = 0.03$  and N\_Shore:  $P < 0.001$ ), while more hypomethylated DMRs lied in open sea regions ( $P = 0.02$ ). (See upper

subfigure of Fig. 5C). Finally, relative position distributions of different structural fragments for genes were presented. More hypomethylated DMRs lied on UTR regions (5' UTR:  $P < 0.001$  and 3' UTR:  $P = 0.01$ ), while more hypermethylation DMRs fell onto other regions (TSS200, TSS1500, Body and Others, all  $P < 0.001$ , lower subfigure of Fig. 5C). Further, *CPT1A*-associated DMRs were presented as heatmaps for those enriched on island and gene promoter regions (Fig. 5D and E).

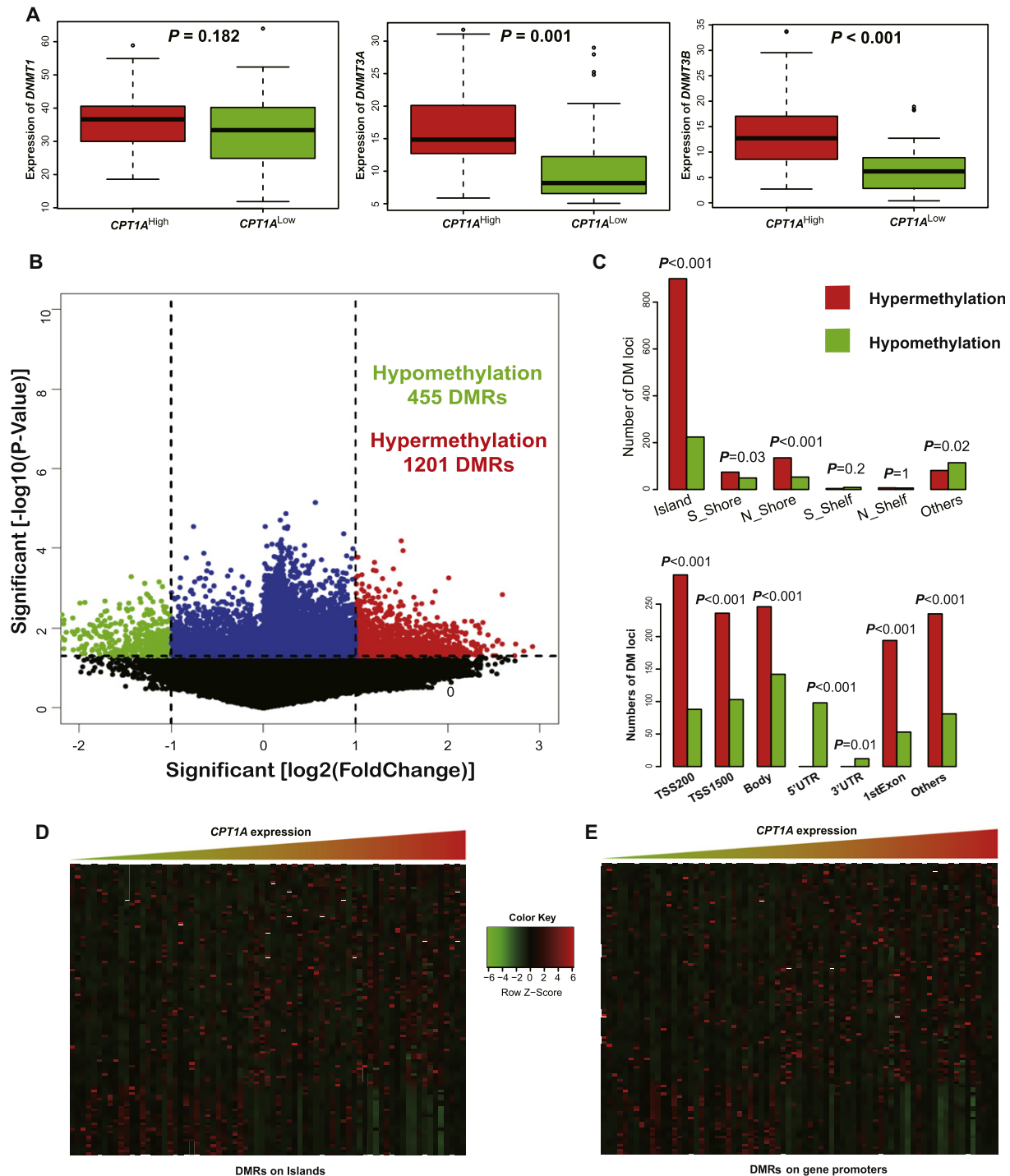
## 4. Discussion

*CPT1A* protein can catalyze the rate-limiting step of *FAO* pathway, which may represent an alternative carbon source for anabolic processes of dNTP (Schoors et al., 2015). Targeting *CPT1A* has shown positive results for impairing cancer cell survival and inhibiting tumor cell proliferation in *in vitro* and *in vivo* models of Burkitt lymphoma (Pacilli et al., 2013). Recent study showed that *CPT1A* was widely expressed in all leukemia cell lines, and targeting *CPT1A* had a strong anti-leukemia activity in *in vitro* on human leukemia cell lines and primary cells of different hematologic diseases (Ricciardi et al., 2015). Furthermore, an editorial illustrated molecular mechanisms of *FAO*'s support to mitochondrial function, and confirmed the therapeutic effectiveness of targeting *CPT1A* for hematological malignancies, alone or in combination with cytarabine (AraC) (Samudio and Konopleva, 2015).

Our study provided direct evidences that *CPT1A*<sup>high</sup> predicted adverse outcomes for AML. Firstly, overexpression of *CPT1A* was shown in AML than normal BM and PB, and also in AML CD34+ and normal BM CD34+ cells (Fig. 1A, B and C), which facilitated the clinical use by qPCR detection. Secondly, based on two relatively larger, independent CN-AML cohorts, *CPT1A*<sup>high</sup> was proved to have significant associations with adverse outcomes, which was also shown in ELN Intermediate-I subgroup and multivariable analyses (Fig. 2A and B, Table 2). Further, the prognostic value of *CPT1A*<sup>high</sup> was confirmed in the whole AML patients and NCCN Intermediate Risk group (Fig. 2C and D), both of which contained a variety of karyotypes of AML samples. Combined the anti-leukemia activity of *CPT1A*'s inhibitors (Ricciardi et al., 2015, Pacilli et al., 2013), all these results proved *CPT1A* as a potential prognosticator and therapeutic target of AML, which could promote further fine stratification of ELN Intermediate-I category and NCCN Intermediate-Risk group.

The pathogenesis of AML remains unclear, but previous studies have reported many aberrant molecular signatures, which could be used independently as favorable or adverse prognosticators and promote the understanding of pathogenesis for AML. The former includes *NPM1* and double *CEBPA* mutation. The latter includes the presence of *FLT3-ITD*, higher expression of *ERG* and *BAALC*, *WT1*, *DNMT3B*, *ITPR2* (Shi et al., 2015), *TCF4* (In 'T Hout et al., 2014), *ATP1B1* (Shi et al., 2016), *MAPKBPI* (Fu et al., 2015) and *RUNX1* (Fu et al., 2016). Associations between *CPT1A* expression and these known prognosticators were assessed, including some pretreatment molecular characteristics. Compared to the *CPT1A*<sup>low</sup> group in the 156 CN-AML cohort, *CPT1A*<sup>high</sup> group contained significantly more patients with M1 ( $P = 0.03$ ) FAB subtype, suggesting that *CPT1A*<sup>high</sup> expressers carried more immature cells, which might indicate adverse malignancy. *CPT1A*<sup>high</sup> expressers presented significantly more *FLT3-ITD* and only slight trends of fewer *NPM1* and double *CEBPA* mutations. In addition, *CPT1A*<sup>high</sup> remarkably accompanied with high expression of *ERG*, *BAALC*, *WT1*, *DNMT3A*, *DNMT3B*, *MAPKBPI*, *ITPR2*, *ATP1B1*, *RUNX1*, *TCF4* and *CXCC5* in both cohorts of CN-AML (Fig. S3A and B), suggesting that *CPT1A* might be another independent biomarker of adverse outcomes for CN-AML.

Recent studies shows both genetics and epigenetics mechanisms play important roles in tumorigenesis, the former focuses primarily on aberrant gene expression, the latter mainly included microRNA's post-transcriptional regulation and DNA methylation. Thus, we further investigated possible mechanisms by which *CPT1A*<sup>high</sup> affects outcomes from these three aspects. Firstly, we derived *CPT1A*-associated gene and microRNA expression profiles, including related cell signaling pathways.



**Fig. 5.** Genome-wide methylation patterns associated with *CPT1A* expression. (A) Differential expression of three DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*). (B) Volcano plot of differential methylated regions. (C) Distribution of DMRs around the islands and on gene's different structural regions. (D) Methylation heatmap of DMRs on genome-wide CpG island regions. (E) Methylation heatmap of DMRs on genome-wide promoter regions.

Many genes involved in tumorigenesis were up-regulated such as *c-Kit*, *CDK6* and *HOX* family genes, including some known independent adverse prognosticators (*WT1*, *ATP1B1*, *MSI2*, etc.), down-regulated genes included many tumor suppressors and immune factors, such as *ID2* (Ishiguro et al., 1996) and *CD86* (Fig. 3B). The consistency to *CPT1A*'s role of adverse prognosticator could also be seen in aberrant expression of cell signaling pathways, including up-regulation of AML, CML and cell cycle pathways, and down-regulation of immune-activation pathways such as Natural Killer pathway and Toll-like or Nod-like receptor pathways (Figs. 3C and S5).

More interesting results were the aberrant changes related to metabolisms. Many pathways of glycometabolism and lipid metabolisms were significantly associated with *CPT1A* expression, including many metabolic pathways of nucleic acid, amino acid and protein (Fig. S5). Especially, fatty acid pathway also showed a trend of up-regulation, which was reported previously as involved by *CPT1A* and promote tumor cell proliferation.

Secondly, genome-wide analysis also revealed some microRNA-involved mechanisms consist with *CPT1A*'s prognostic role (Fig. 4B and C). Except those *CPT1A*-positive or -negative correlated microRNA



individuals (such as *miR-155* and *miR-193a*), which were known involved in tumor-promotion or progression or tumor suppression, many pairs of microRNA-mRNA were also shown aberrantly synchronized changes along with *CPT1A* expression, such as positive correlation of *miR-155* and down-regulation of *CEBPB*, negative correlation of *miR-193a* and up-regulation of *ABI2*, etc. (Fig. 4C). Those synchronized changes of microRNAs and target genes may aggravate the malignancy of AML. Further, aberrant regulations of dynamic MMR pairs were also explored, which also showed some consistency to *CPT1A*'s prognostic value, such as *miR-196a* and *PIP4K2C*, the significantly negative regulation of *miR-196a* to *PIP4K2C* could contribute to adverse outcome for *CPT1A*<sup>high</sup> patients (Fig. S6A and B). Those aberrant genes, pathways, microRNAs and MMR pairs may contribute to the unfavorable outcomes for CN-AML.

Many microRNAs associated with *CPT1A* also participated in metabolic regulations, especially fatty acid metabolisms. *miR-22* could repress fatty acid synthesis and was an important regulator of lipid and folate metabolism in breast cancer cells (Koufaris et al., 2016). *miR-27a* was reported to accelerate adipolysis releasing significantly more glycerol and free fatty acids (Wang et al., 2011). *miR-155* could driver the metabolic reprogramming in ER+ breast cancer cells (Bacci et al., 2016), and its overexpression could promote the aberrant expression of hepatic genes associated with lipid metabolism in a mice model of liver (Lin et al., 2015). All those results might illustrate the metabolic alterations derived from expression changes of *CPT1A*. Metabolic alterations have been proposed to support extra bioenergy and special microenvironment needed by tumor cell proliferations (Cairns et al., 2011). Our work and previous studies about *FAO* further showed catabolic signatures could participate in controlling cell growth, survival and chemoresistance and might be used as a potential target for leukemia treatment (Ricciardi et al., 2015, Samudio and Konopleva, 2015).

Finally, inspired by the aberrant expression of those three DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*, Fig. 5A), associations between *CPT1A* expression and genome-wide methylation were exploited. Result showed that the nearer to the CpG islands lied more hypermethylation DMRs (Fig. 5C). Also, more DMRs related to genes' different functional fragments were in hypermethylation, especially the gene promoter regions, while hypomethylation DMRs fell more on the 3' UTR or 5' UTR regions (Fig. 5C). Hypermethylation on gene promoters and CpG islands were consistent to known understanding of the methylation influences to gene expression, which might contribute to the down-regulation of tumor suppressors in *CPT1A*<sup>high</sup> expressers. Possibly, those abnormal methylation might be accompanying phenomenon to the adverse prognosis, and indicated extra understanding to leukemogenesis.

In summary, the involvement of metabolic alterations in tumorigenesis has obtained more and more acceptance, and attracted great attentions. In recent publications, *CPT1A* has been proved to have clear anti-leukemia efficacy in basic experiments. Combined with existing results of *CPT1A* in AML, our results in clinical further confirmed that high expression of *CPT1A* was an adverse biomarker for AML, and *CPT1A*<sup>high</sup> patients carried various kinds of molecular adverse events. Taken together, *CPT1A* plays an important role in leukemogenesis and will be potentially a therapeutic target for AML.

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## Conflict of Interest Disclosures

The authors declare no competing financial interests.

## Authorship Contributions

J.L. Shi, L. Fu and H.P. Fu designed and performed the study and wrote the manuscript; Z.L. Jia participated in analyzing data and revising manuscript; L. Fu, W.D. Wang and K.L. He coordinated the study over the entire time. All authors reviewed the final manuscript.

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