Leukemia Research 85 (2019) 106198

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

CircPAN3 contributes to drug resistance in acute myeloid leukemia through regulation of autophagy

Jin Shang[a], Wei-Min Chen[a,*], Shan Liu[a], Zhi-Hong Wang[a], Tian-Nan Wei[a], Zhi-Zhong Chen[b], Wen-Bing Wu[b]

[a] *Provincial Clinical Medical College of Fujian Medical University, Department of Hematology, Fujian Provincial Hospital, Fuzhou, Fujian 350001, China*
[b] *Provincial Clinical Medical College of Fujian Medical University, Department of Pathology, Fujian Provincial Hospital, Fuzhou, Fujian 350001, China*

ARTICLE INFO

Keywords:
Acute myeloid leukemia
Drug resistance
Circular RNA
Autophagy
RNA sequencing

ABSTRACT

The aim of this study was to investigate the role and underlying mechanism of circular RNA (circRNA) circPAN3 in mediating drug resistance in acute myeloid leukemia (AML). We first established two doxorubicin (ADM)-resistant AML cell lines and then utilized high-throughput RNA sequencing (RNA-seq) to compare their circRNA expression profiles with those of the parental cell lines. With bioinformatic analysis, we identified key circRNA molecules involved in drug resistance and validated our findings in clinical specimens. The target microRNAs (miRNAs) and downstream mRNAs were also explored bioinformatically. Using RNA interference technique, the potential mechanism was further investigated. Twenty-nine circRNAs were identified to be differentially expressed between ADM-resistant and sensitive cells. We found that circPAN3 is most likely a key mediator in the development of AML drug resistance, evidenced by the increased expression in ADM-resistant cell lines and BM samples from relapsed patients. Additionally, downregulation of circPAN3 by small interfering RNA (siRNA) significantly restored drug sensitivity to ADM in the two ADM-resistant cell lines, but lentivirus-mediated circPAN3 overexpression had the opposite effect. Subsequent bioinformatic analysis and mechanistic experiments revealed that circPAN3 may facilitate AML drug resistance through regulating autophagy and influencing expression of apoptosis-related proteins, while AMPK/mTOR signaling plays a key role in the regulation of circPAN3 on autophagy. These findings may provide new important insights into the role of circRNAs in mediating AML drug resistance, and suggest that circPAN3 might be a potential target for treatment of drug-resistance AML, which merits further investigation and validation.

1. Introduction

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by proliferation of malignant precursors of the myeloid lineage [1]. Despite progress in AML treatment and emerging new therapies such as autologous/allogeneic hematopoietic stem cell transplantation, the combinations of cytarabine (Ara-C) and anthracycline antibiotics (idarubicin, daunorubicin, and doxorubicin (ADM)) remain the first-line regimen for the disease, which can achieve around 70% initial complete remission (CR) rate in patients under the age of 60 [2]. However, the projected 5-year survival rate for AML is around 55% for patients with favorable cytogenetics, but only 24% and 5% for those with intermediate and poor cytogenetics, respectively [3]. Particularly, the poor outcome is closely related to a high proportion of cases who will eventually relapse after an initial response, which is mainly due to acquired resistance of AML cells to chemotherapeutic drugs.

Macroautophagy (here referred to simply as autophagy) is an evolutionarily-conserved lysosomal pathway, which is characterized by formation of a closed double-membrane vacuole, autophagosome [4]. Autophagosome matures in a stepwise process involving engulfing

---

Abbreviations: circPAN3, circular RNA PAN3; AML, acute myeloid leukemia; miRNAs, microRNAs; siRNA, small interfering RNA; Ara-C, cytarabine; CR, complete remission; ncRNA, non-coding RNA; RBP, RNA-binding protein; ADM, doxorubicin; AMPK, AMP-activated protein kinase; p-AMPK, phosphorylated AMPK; p-mTOR, phosphorylated mTOR; CCK-8, Cell Counting Kit-8; 3-MA, 3-methyladenine; BM, bone marrow; IC_{50}, half maximal inhibitory concentration; TMM, trimmed mean of M-value; mRFP, monomeric red fluorescent protein; GFP, green fluorescent protein; qRT-PCR, quantitative real-time PCR; SD, standard deviation; MRE, miRNA response element

[*] Corresponding author at: Department of Hematology, Fujian Provincial Hospital, Provincial Clinical Medical College of Fujian Medical University, 134 Dong Jie Road, Fuzhou, Fujian 350001, China.

E-mail address: chenwm1962@163.com (W.-M. Chen).

https://doi.org/10.1016/j.leukres.2019.106198
Received 18 September 2018; Received in revised form 7 July 2019; Accepted 1 August 2019

events and fusion with endolysosomal vesicles [5]; then lysosomal hydrolases digest the contents of autolysosome to recyclable breakdown products, generating energy to confer stress tolerance [6]. Recently, an increasing number of studies show that the imbalance of autophagy is involved in cancer development and apoptosis [7,8]. Increased autophagic activity has been observed in a variety of drug-resistant tumor cells [9], while inhibition of autophagy significantly enhances cell death induced by chemotherapeutic agents in certain cancer cells [10]. These findings indicate that autophagy plays a critical role in drug resistance of cancer. Nevertheless, the underlying molecular mechanisms are largely unknown and still need to be clarified.

Circular RNAs (circRNAs), a novel type of endogenous non-coding RNA (ncRNA), have drawn intense interest to researchers in recent years and been recognized to function in gene regulation and contribute to pathogenesis of various human cancers [11–13]. They are considered to act as miRNA sponge to prevent mRNA translation. Additionally, circRNAs have been found to influence gene expression by regulating splicing or transcription, and by interacting with RNA-binding proteins (RBPs) [14]. Interestingly, according to the latest literature, circRNAs have been implicated in regulating autophagy in some certain diseases [15]. Given these findings, we hypothesized that circRNAs might be related to autophagy and drug resistance in AML cells. In this study, we established two AML cell models with acquired ADM-resistance and characterized their circRNA expression profile using high-throughput RNA sequencing (RNA-seq). With bioinformatic analysis, we identified a key circRNA molecule involved in drug resistance and validated our findings with the established cell models and clinical specimens from AML patients.

2. Materials and methods

2.1. Cell lines and reagents

THP-1 and K562 human AML cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Hieff Trans™ Liposomal Transfection Kit and the related transfection reagents were purchased from Yeasen Bio (Shanghai, China). The primary monoclonal antibodies against microtubule-associated protein 1A/1B-light chain 3 (LC3)-I, LC3-II, Beclin-l, P62, Bax, Bcl-2, Caspase-3 (Cas-3), cleaved Cas-3 (CL-Cas-3), Caspase-9 (Cas-9), cleaved Cas-9 (CL-Cas-9), AMP-activated protein kinase (AMPK), phosphorylated AMPK (p-AMPK), mTOR, phosphorylated mTOR (p-mTOR), and transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) were purchased from Affinity Biosciences (Cincinnati, OH, USA). Cell Counting Kit-8 (CCK-8) reagent kit was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Autophagy inhibitor 3-methyladenine (3-MA) and AMPK inhibitor Compound C were purchased from MedChemExpress (Monmouth Junction, NJ, USA).

2.2. Establishment of ADM-resistant cell lines and collection of human samples

ADM-resistant cell lines (THP-1/ADM and K562/ADM) were established by exposing THP-1 and K562 cells to gradually increasing concentrations (0.2–2.0 μg/mL) of ADM for six months. The untreated parental cell lines were used as controls. Bone marrow (BM) samples were collected from 42 AML patients who were hospitalized at Fujian Provincial Hospital (Fuzhou, China) between January 2015 and May 2018. The patient population consisted of 25 males and 17 females with a median age of 28. Of these patients, 22 (52.4%) had newly diagnosed AML; while the rest ($n = 20$, 47.6%) had relapse AML. Pathological diagnosis and confirmation of relapse of AML were defined according to the published criteria [16]. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Fujian Provincial Hospital. All patients provided written informed consent before participation.

2.3. Assessment of cell sensitivity to ADM

CCK-8 was used to assess cell sensitivity to ADM. The two ADM-resistant cell lines (THP-1/ADM and K562/ADM) and their parental cell lines (THP-1 and K562) were plated in 96-well plates at a density of 1×10^4 cells/mL and then treated with 200 μL of medium containing various concentrations of ADM (0.01–2.4 μg/mL) for 48 h (h). Afterwards, 10 μL of CCK-8 reagent was added to cultured cells, and incubated in a humidified incubator containing 5% CO_2 at 37 °C for 2 h. The absorbance was detected at a wavelength of 490 nm. The half maximal inhibitory concentration (IC_{50}) and inhibitory ratio values were calculated from the concentration-response curve generated for each cell line.

2.4. Expression profile analysis of circRNAs

The expression profile of circRNAs was analyzed using a high-throughput sequencing technology. Briefly, total RNA was isolated from cells using a Hipure Total RNA Mini Kit (Magen, Guangzhou, China) according to the protocol. Afterwards, ribosomal RNA was removed from total RNA samples, followed by an incubation with 10-unit RNase R (Epicentre Technologies, Madison, WI, USA) for 30 min at 37 °C to remove linear RNAs. The resulting total RNA samples were purified with VAHTS RNA Clean Beads (Vazyme Biotech, Nanjing, China). RNA-seq library was prepared with approximately 2 μg of total RNA using a KAPA RNA HyperPrep Kit with RiboErase (HMR) for Illumina (Kapa Biosystems, Inc., Woburn, MA). Each library was diluted to a final concentration of 10 nM and pooled equimolar prior to clustering. Paired-end sequencing (PE150) was performed on all samples with an Illumina HiSeq X10 system (Illumina Inc. San Diego, CA, USA). The amount of sequencing data was 10 gigabytes (GB). The differentially expressed circRNAs were screened by Bioconductor software packages (https://www.bioconductor.org/). For circRNA expression analysis, the reads were mapped to the genome using the Spliced Transcripts Alignment to a Reference (STAR) software (https://github.com/alexdobin/STAR) [17]. The DCC software (https://github.com/dieterich-lab/DCC/tree/master/DCC) was used to identify circRNA candidates and estimate their expression [18]. Differentially expressed genes were identified using the edgeR program ((https://www.bioconductor.org/) [19]. Trimmed mean of M-values (TMM) were used to normalize gene expression. Genes with altered expression ($P < 0.05$ and more than 2-fold change) were considered differentially expressed.

2.5. Autophagy assay using fluorescence microscope

To analyze autophagic flux, we monitored the formation of autophagic vesicles using the monomeric red fluorescent protein (mRFP)-green fluorescent protein (GFP)-LC3 method, because GFP quenching occurs in acidic lysosomal environment, which allows distinguishing autophagosome from autolysosome by detecting the mRFP and GFP signals [20]. In brief, cells were transfected with mRFP-GFP-LC3-expressing pLenti6 lentivirus (Nanjing Mergene Life Science, Nanjing, China). Autophagic flux was determined by detecting punctate signals of GFP and mRFP (puncta/cell). Fluorescence was analyzed using an Olympus Cell-imaging Station (Olympus, Shinjuku, Japan) and Cell M software (Olympus).

2.6. Quantitative real-time PCR

Total RNA was extracted from the tested cell lines and BM tissue specimens using TRIzol reagent. Particularly, the total RNA samples from the tested cell lines were aliquoted. With the purpose to examine circPAN3, one-half of the aliquots were treated with RNase R to remove linear RNAs before reverse transcription into cDNA. The other half was directly subjected to reverse transcription for a purpose to detect PAN3

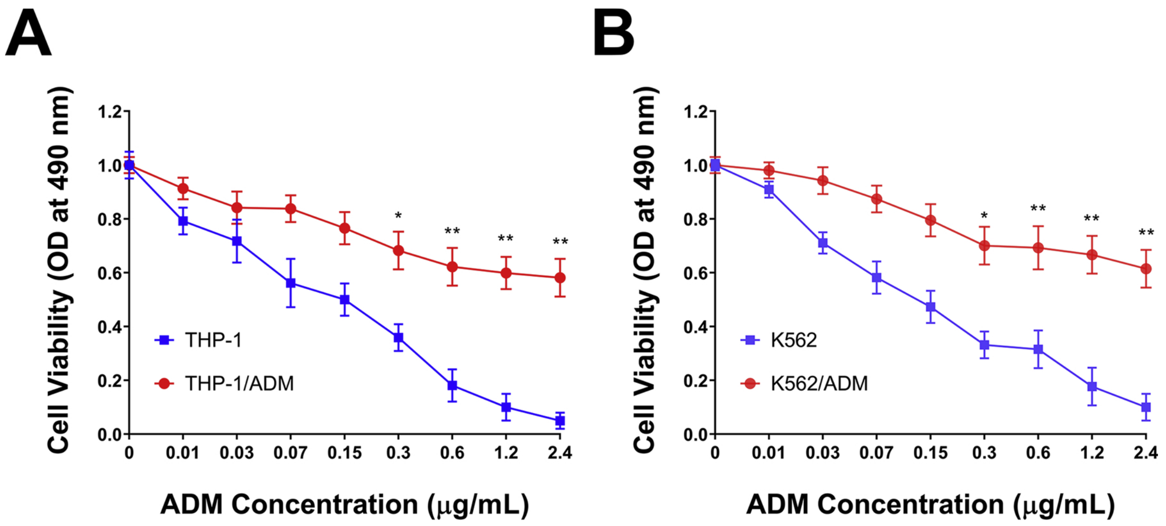


Fig. 1. Drug resistance of THP-1/ADM (**A**) and K562/ADM cells (**B**) to ADM. THP-1, THP-1/ADM, K562 and K562/ADM cells were exposed to increasing concentrations of ADM for 48 h, and cell viability was detected by CCK-8 assay. Data are presented as means ± SD of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the control. ADM: doxorubicin; OD: optical density.

mRNA and 10 target miRNAs of circPAN3. The reverse transcription was performed using Hifair™ II First Strand cDNA Synthesis Kit (Yeasen, Shanghai, China). Quantitative real-time PCR (qRT-PCR) was conducted with a LightCycler 480II RT-PCR system (Roche Applied Science, Indianapolis, IN, USA) in accordance to the manufacturer's instruction. The primer sequences used for qRT-PCR were listed in Supplementary Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 served as the internal references. The reaction condition was 45 cycles of denaturation at 95 °C for 15 s (sec), annealing at 60 °C for 30 s. and extension at 72 °C for 10 s. The delta-delta cycle threshold (ΔΔCt) method was used for quantification [21].

2.7. *Small interfering RNA transfection*

Small interfering RNAs (siRNAs) targeting the junction region of circPAN3 sequence (5′-GAGAAAGTGGGGAATGTCGTT-3′) and negative control siRNA (NC) (sequence: 5′- TTCTCCGAACGTGTCACGT -3′) were provided by Geneseed Bio-Tech (Guangzhou, China). Before transfection, THP-1/ADM and K562/ADM cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) to a log phase of growth. Then, cells were plated in 6-well culture plates at a density of 1×10^6 cells/mL (medium containing 1% FBS) and transfected with 5 μL 20 μM siRNAs or NC for 6 h using the Hieff Trans™ Liposomal Transfection Reagent (Promega, Madison, WI, USA) according to the manufacturer's instruction. Afterwards, the transfected cells were cultured in fresh complete medium for additional 48 h. Knock-down of circPAN3 was confirmed by RT-PCR.

2.8. *Lentiviral overexpression of circPAN3*

The plasmid pLC5-ciR (Geneseed Bio-Tech) was used to construct a recombinant lentiviral vector for circPAN3. In brief, the human circPAN3 (circBase: hsa_circ_0100181) gene were amplified from a cDNA library. The PCR primers were designed as follows: CGGAATTC GAATG (forward) and CGGGATCCCCCACTTTCTC (reverse). The amplified fragments and plasmid were doubly digested with restriction endonucleases EcoR I and BamHI (Promega), purified, and ligated using T4 DNA ligase. The ligation mixture was then transformed into competent cells for generating a lentiviral overexpression vector: pLC5-ciR/circPAN3. The vectors were harvested and used to transfect target cells (cell density: 1×10^5 cells/well in 24-well culture plates) in log phase growth at a multiplicity of infection (MOI) of 300. Transfection was performed for 24 h at 37 °C; then the transfected cells were cultured in fresh complete medium for an additional 72 h. Transfection efficiency was evaluated by monitoring GFP expression with flow cytometry. The overexpression of circPAN3 was confirmed by RT-PCR.

2.9. *Western blot analysis*

Protein lysates from cells and BM tissue samples were subjected to Western blot analysis using the standard protocol as previously described [22].

2.10. *Statistical analysis*

The Wilcoxon signed-rank test was performed to analyze significant differences in the expression levels of circRNAs and miRNAs between samples. The Pearson's analysis was applied to determine correlation coefficients for different variables. Other data are presented as means ± standard deviations (SD). The Student's *t*-test (two-tailed) or one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison test was performed for data analysis. $P < 0.05$ was considered significant.

3. Results

3.1. *Characterization of drug resistance in THP-1/ADM and K562/ADM cells*

We first characterized ADM resistance in the two established drug-resistant AML cell lines. After exposure to increasing concentrations of ADM for 48 h, the IC_{50} values were 2.62 ± 0.83 μg/mL and 5.31 ± 1.13 μg/mL for THP-1/ADM cells and K562/ADM cells, respectively, showing a 17.5- and 20.4-fold increase as compared to those for their parental cell lines (0.15 ± 0.05 μg/mL for THP-1 cells and 0.26 ± 0.06 μg/mL for K562 cells) (Fig. 1A and 1B). These results verified ADM resistance in the two established cell lines. We also determined their IC_{50} values to Ara-C and etoposide (VP-16). Both ADM-resistant cell lines showed resistance to the two cytotoxic drugs compared with THP-1 and K562 cells (data not shown), indicating that multidrug resistance (MDR) was also developed.

3.2. *Differentiation in circRNA expression profile between ADM-sensitive and -resistant AML cells*

Next, the circRNA expression profiles of ADM-sensitive and

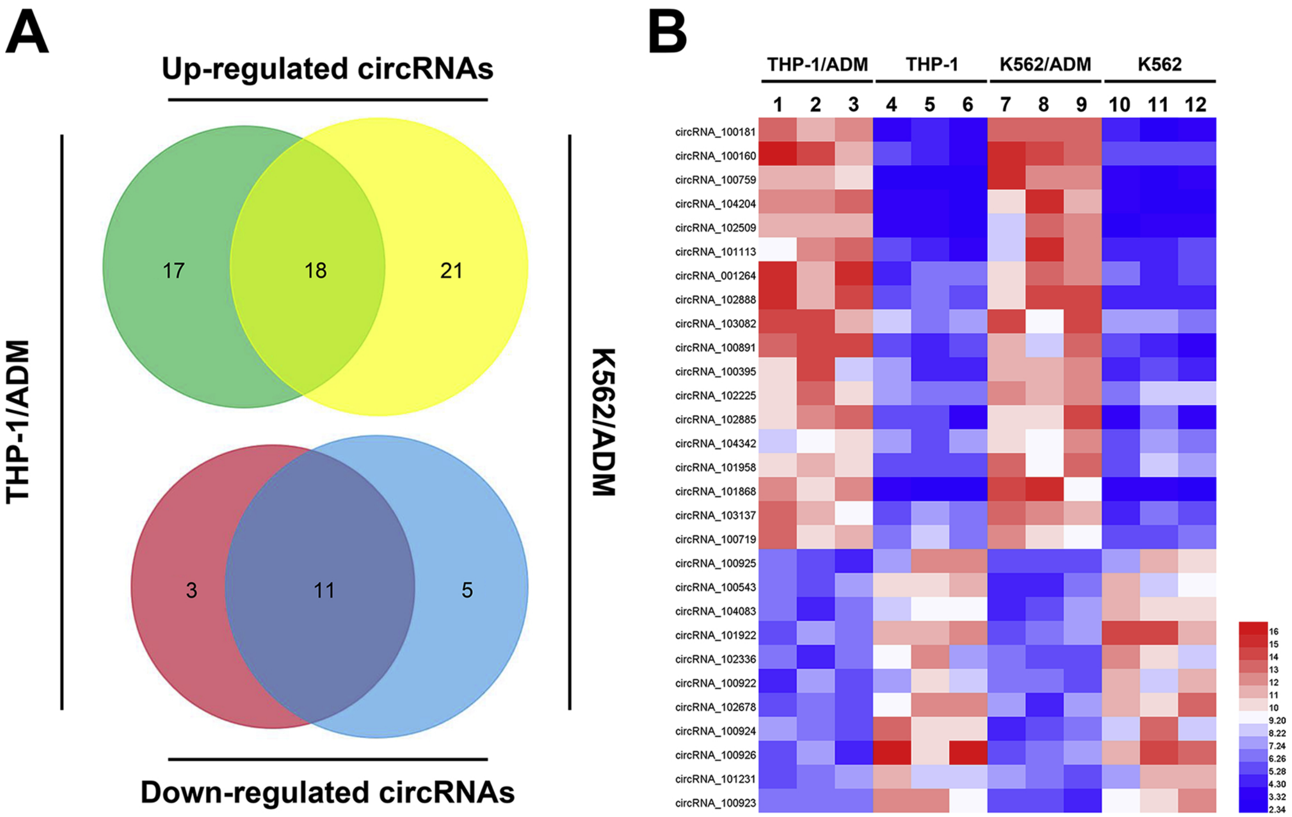


Fig. 2. Differentiation in circRNA expression profile between ADM-sensitive (THP-1 and K562) and –resistant (THP-1/ADM and K562/ADM) AML cells. (**A**) The Venn diagram revealed an intersection of differentially expressed circRNAs between the two cell groups. (**B**) Heat map of the intersection of 29 differentially expressed circRNAs between the two cell groups, of which circRNA_100181 refers to circPAN3. Color scale represents values for gene expression fold change, with red color indicating a high fold change while blue color indicating a low fold change. ADM: doxorubicin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

-resistant AML cells were analyzed using a high-throughput RNA-seq technology. After normalization, a total of 4573 circRNA targets were identified in three pairs of THP-1 and THP-1/ADM samples, and three pairs of K562 and K562/ADM samples. As a result, 49 distinct circRNAs were differentially expressed between THP-1 and THP-1/ADM cell lines by a fold change > 2.0 and a P value < 0.05, of which 35 were up-regulated and 14 were down-regulated. Between K562 and K562/ADM cells, 55 distinct circRNAs were differentially expressed, of which 39 were up-regulated and 16 were down-regulated. The Venn diagram revealed an intersection of 29 differentially expressed circRNAs between the two cell groups, of which 18 were up-regulated and 11 down-regulated (Fig. 2A). These up- and down-regulated circRNAs were presented in Fig. 2B (heat map).

3.3. *Increased basal autophagy level in drug-resistant AML cells*

The relationship between autophagy and drug resistance has been demonstrated in multiple solid tumors [23], but rarely reported in AML cells. We evaluated basal autophagy level in the two drug-resistant AML cells by examining the expression of three autophagy markers, the ratio of LC3-II/LC3-I, Beclin-1, and P62 [24], with Western blot. It has been documented that conversion of LC3-I to LC3-II displays autophagy initiation and that greater LC3-II/LC3-I ratio indicates stronger autophagy [25]. Beclin-1 is involved in both the formation and maturation of autophagosomes, while P62 is a selective substrate for degradation by autophagy [26,27]. Therefore, increased Beclin-1 expression and decreased P62 level are also relevant to enhanced autophagy.

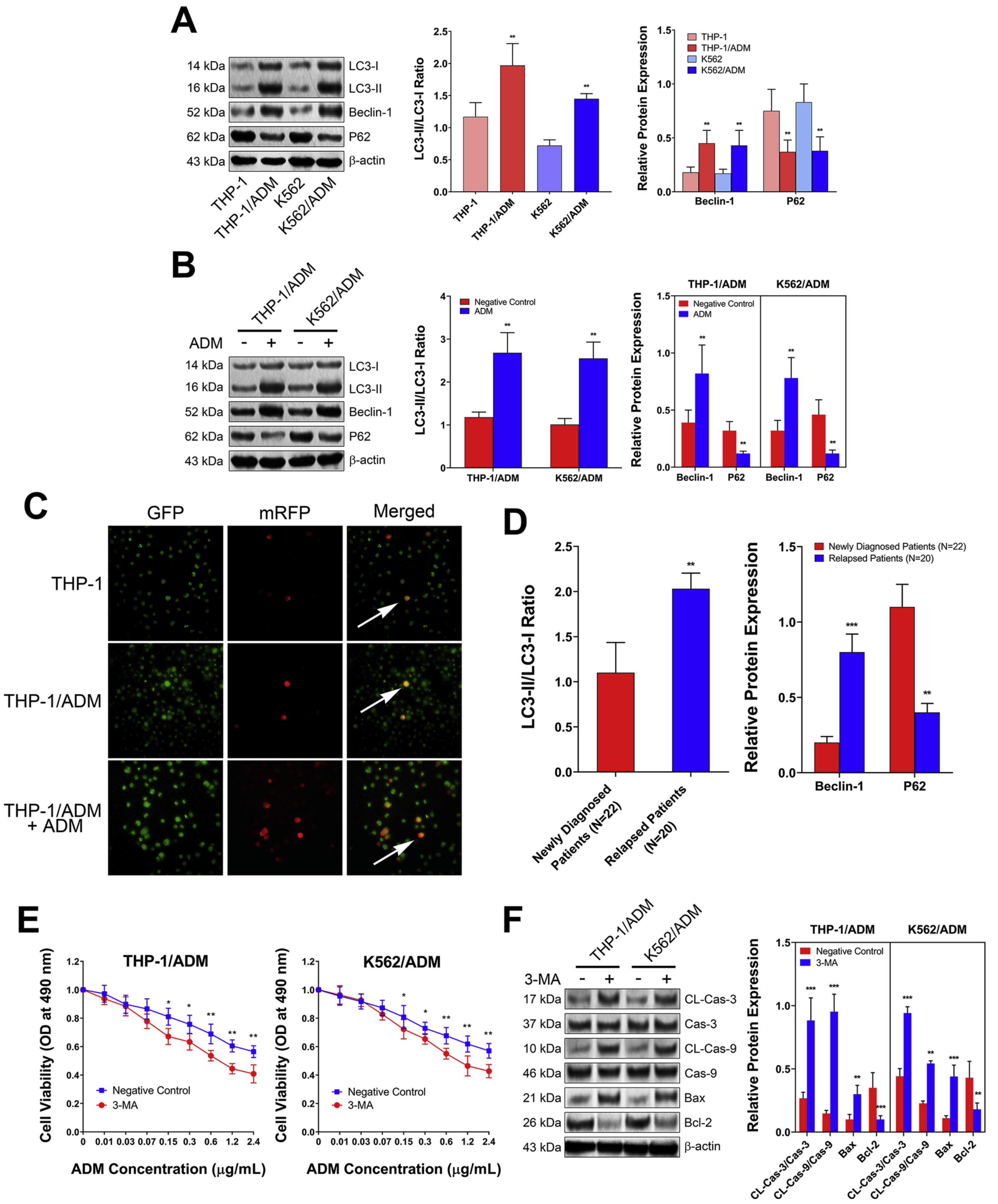
The results showed that the ratio of LC3-II/LC3-I and the expression of Beclin-1 significantly increased, while the expression of P62 dramatically decreased in THP-1/ADM and K562/ADM cells, as compared with their parental cells (Fig. 3A), indicating that basal autophagic activity is most likely enhanced in drug-resistant AML cells. Moreover, treatment with 2 μg/mL ADM for 24 h resulted in more prominent changes in the expression of these autophagy markers, thereby suggesting a further increased autophagic activity in response to ADM in THP-1/ADM and K562/ADM cells (Fig. 3B). The images of fluorescence microscope also confirmed these findings, manifested by enhanced signals of mRFP-GFP-LC3 in autolysosomes in ADM-treated (2 μg/mL for 24 h) THP-1/ADM cells (Fig. 3C). BM samples from 20 relapsed patients showed considerably higher LC3-II/LC3-I ratio and Beclin-1 expression, as well as significantly lower P62 expression than those with 22 newly diagnosed AML, as shown in Fig. 3D, which revealed an elevated autophagic activity in BM leukemic cells of the relapsed disease.

Recent studies have demonstrated that autophagy is an underlying mechanism for resistance to multiple cytotoxic drugs in a range of solid tumors [28]. Therefore, we further analyzed the relationship between autophagic activity and drug resistance in AML cells using 3-methyladenine (3-MA), an autophagy inhibitor, at a concentration of 5 mM that has no cytotoxicity [29]. Our results showed that treatment with 5 mM 3-MA for 24 h significantly restored drug sensitivity in THP-1/ADM and K562/ADM cells, evidenced by the decrease in IC_{50} value from 2.62 ± 0.83 μg /mL to 1.02 ± 0.25 μg /mL and from 5.31 ± 1.13 μg/mL to 1.25 ± 0.23 μg/mL, respectively (Fig. 3E). These results suggest that inhibition of autophagy can reverse drug resistance of AML cells.

To further explore the underlying mechanism behind drug resistance reversal upon autophagy inhibition, we then investigated the effect of autophagy inhibition on expression of apoptosis-related proteins, i.e. Cas-3, Cas-9, Bcl-2, and Bax, upon exposure to 2 μg/mL ADM for 24 h. As shown in Fig. 3F, as compared to negative control, pretreatment with autophagy inhibitor 3-MA (5 mM for 24 h) significantly increased the ratios of CL-Cas-3/Cas-3 and CL-Cas-9/Cas-9, as well as the expression of Bax, but dramatically decreased Bcl-2 expression in both THP-1/ADM and K562/ADM cells, suggesting that inhibition of autophagy may be associated with increased apoptotic cell death, which, at least in part, contributes to drug resistance reversal observed in Fig. 3E.

3.4. The effect of circPAN3 on drug resistance of AML cells is mediated by autophagy

Among the 18 up-regulated circRNAs, circPAN3 has been found to be associated with proliferation, apoptosis, and cell cycle of AML cells [30]. Therefore, we speculate that up-regulation of circPAN3 may be involved in the development of drug resistance in AML. To validate the



(caption on next page)

Fig. 3. The levels of autophagy markers in drug-sensitive and -resistant AML cells. (**A**) Autophagy related proteins LC3-II/LC3-I, Beclin-1, and P62 were detected and quantified by Western blot in THP-1, THP-1/ADM, K562 and K562/ADM cells. (**B**) THP-1/ADM and K562/ADM cells were exposed to 2 μg/mL ADM for 24 h. Autophagy related proteins were detected and quantified by Western blot. (**C**) THP-1 and THP-1/ADM cells were transfected with mRFP-GFP-LC3-expressing pLenti6 lentivirus. THP-1/ADM cells were exposed to 2 μg/mL ADM for 24 h. Autophagic flux was determined by fluorescence analysis. Green fluorescence represents GFP, and red fluorescence represents mRFP. White arrows indicate the cells with mRFP-GFP-LC3 signals in autolysosomes. Magnification is 100 × . (**D**) Relative expression of autophagy related proteins in AML cells of BM specimens from 22 newly diagnosed and 20 relapsed AML patients. The expression levels were detected and quantified by Western blot. (*E*) THP-1/ADM and K562/ADM cells were pre-treated with 5 mM 3-MA for 24 h before exposure to increasing concentrations of ADM for additional 48 h. Cell viability was examined by CCK-8 assay. (**F**) THP-1/ADM and K562/ADM cells were pre-incubated with 5 mM 3-MA for 24 h before exposure to 2 μg/mL of ADM for additional 24 h. Apoptosis-related proteins were detected and quantified by Western blot. All data are presented as means ± SD of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared to parental cell line (THP-1 or K562), negative control, or newly diagnosed patients. ADM: doxorubicin; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; OD: optical density. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

results of RNA-seq analysis, we performed qRT-PCR for circPAN3 and mRNA of its host gene *PAN3* in the two ADM-sensitive and -resistant AML cell sets. Although there was no significant difference in *PAN3* mRNA level and protein expression (by Western blot, data not shown), the expression of circPAN3 was increased by 36-fold in THP-1/ADM cells and by 45-fold in K562/ADM cells compared to their parental cell lines (Supplementary Fig. 2A and 2B). These results were similar to those of RNA-seq analysis (log2 fold changes are 5.5 and 6.5, respectively).

To confirm the role of circPAN3 in drug resistance of AML, we knocked down the expression of circPAN3 in THP-1/ADM cells using siRNA (70% reduction) and found that the ratio of LC3-II/LC3-I and the expression of Beclin-1 were significantly decreased upon silencing of circPAN3, but the expression of P62 was dramatically increased (Fig. 4A). These results suggest a decreased basal autophagy in circPAN3-silenced THP-1/ADM cells. Next, we treated the cells with various concentrations of ADM. As depicted in Fig. 4B, silencing circPAN3 expression did not enhance apoptosis itself, but caused a significant increase in apoptosis upon ADM exposure, evidenced by a considerably higher percentage of apoptotic cells detected by flow cytometry after treatment with 0.5, 1, or 2 μg/mL of ADM for 24 h. Collectively, these findings reveal that circPAN3 may induce ADM resistance in AML cells through enhancing autophagy activity, while silencing of circPAN3 could potentially reverse such drug-resistance.

For further validation, we used recombinant lentivirus to overexpress circPAN3 in THP-1 cells. The level of the overexpressed circPAN3 in transfected cells were about 5-fold higher than in non-transfected cells. With the transfected cells, we found that lentivirus-mediated overexpression of circPAN3 significantly increased the ratio of LC3-II/LC3-I (Fig. 4C), and considerably decreased cell apoptosis induced by ADM (2 μg/mL for 24 h) (Fig. 4D). However, incubation with autophagy inhibitor 3-MA (5 mM for 24 h) prior to ADM treatment could attenuate the anti-apoptotic effect of circPAN3 overexpression in THP-1 cells (Fig. 4D). Furthermore, we examined the relationship between circPAN3 and the three autophagy markers in BM samples from 42 AML patients. As shown in Figs. 4E to 4 G, the expression of circPAN3 was positively correlated with the ratio of LC3-II/LC3-I and the expression of Beclin-1 (Pearson's $r = 0.447$, $P = 0.003$ and Pearson's $r = 0.411$, $P = 0.007$, respectively), but had a negative correlation with the expression of P62 (Pearson's $r = -0.395$, $P = 0.01$). Taken together, these results demonstrate a close relationship between circPAN3 expression and autophagic activity in AML cells and indicate that overexpression of circPAN3 may, at least in part, contribute to development of AML drug resistance via up-regulation of autophagic activity.

3.5. *CircPAN3 regulates autophagy through the AMPK/mTOR pathway in AML cells*

We further explored the underlying molecular mechanism for circPAN3 to regulate autophagic activity in AML cells. Generally, it is believed that circRNAs serve as miRNA sponge by sequestering miRNAs through base pair complementarity, consequently regulating transcription of the mRNA targets [31]. With TargetScan and miRanda, we predicted target miRNAs and downstream mRNAs of circPAN3. The signaling pathways involved in the downstream genes were also analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG). The results showed that the target miRNAs of circPAN3 include miR-153-5p, miR-183-5p, miR-338-3p, miR-346, miR-545-3p, miR-574-5p, miR-599, miR-653-5p, miR-766-3p, and miR-767-3p. KEGG analysis showed that the downstream genes of these miRNAs were involved in several important signaling pathways associated with cancer development and progression, such as the TGF-β, mTOR, ErbB, and FoxO signaling pathways (Fig. 5A). We also applied qRT-PCR to examine the expression of these 10 target miRNAs in circPAN3-overexpressing THP-1 cells. We found that most of these miRNAs (8 of 10) showed a significantly decreased expression level compared to those in negative control cells (Supplementary Fig. 3), demonstrating the association between circPAN3 and its target miRNAs.

The AMPK/mTOR pathway has been proved to be involved in the regulation of autophagy [32]. Therefore, we examined activation of this pathway in THP-1/ADM cells. As we expected, the ratio of p-AMPK/AMPK was higher in THP-1/ADM cells than in THP-1 cells, while the ratio of p-mTOR/mTOR was lower (Fig. 5B), suggesting an activation of the AMPK/mTOR pathway in this ADM-resistant cell line. When THP-1/ADM cells were transfected with circPAN3 siRNA, this siRNA-induced downregulation of circPAN3 decreased the ratio of p-AMPK/AMPK but increased the ratio of p-mTOR/mTOR (Fig. 5C). We further verified the relationship among circPAN3 expression, AMPK/mTOR pathway activation, and autophagy activity in transfected THP-1 cells overexpressing circPAN3. The results showed that lentivirus-mediated overexpression of circPAN3 significantly enhanced the ratio of p-AMPK/AMPK but decreased the ratio of p-mTOR/mTOR (Fig. 5D). Together with the finding shown in Fig. 4A that transfection with circPAN3 siRNA caused a decrease in the ratio of LC3-II/LC3-I in THP-1/ADM cells, these results indicate that circPAN3 induces autophagy activity of THP-1 cells through activation of the AMPK/mTOR pathway.

For further validation, we pre-incubated circPAN3-overexpressing THP-1 cells with 10 μM of AMPK inhibitor Compound C for 12 h. We found that pre-treatment with Compound C could counteract the effects of circPAN3 overexpression on the ratios of LC3-II/LC3-I, p-AMPK/AMPK, and p-mTOR/mTOR (Fig. 5E and 5 F), suggesting that circPAN3 overexpression upregulates autophagy through activation of the AMPK/mTOR pathway. Taken together, these results provide concrete evidence that the impact of circPAN3 on autophagic activity may be mediated by AMPK/mTOR signaling.

4. Discussion

Impairment of apoptotic signaling is one of the leading mechanisms for development of drug resistance in tumor cells, while autophagy has been regarded a "double-edged sword" for tumor apoptosis [33]. Low level of autophagy can remove abnormal proteins and damaged organelles, and inhibit apoptosis of tumor cells. However, autophagy with high intensity inevitably disintegrates cell structure, leading to irreversible function loss and even apoptosis [34]. The different roles of

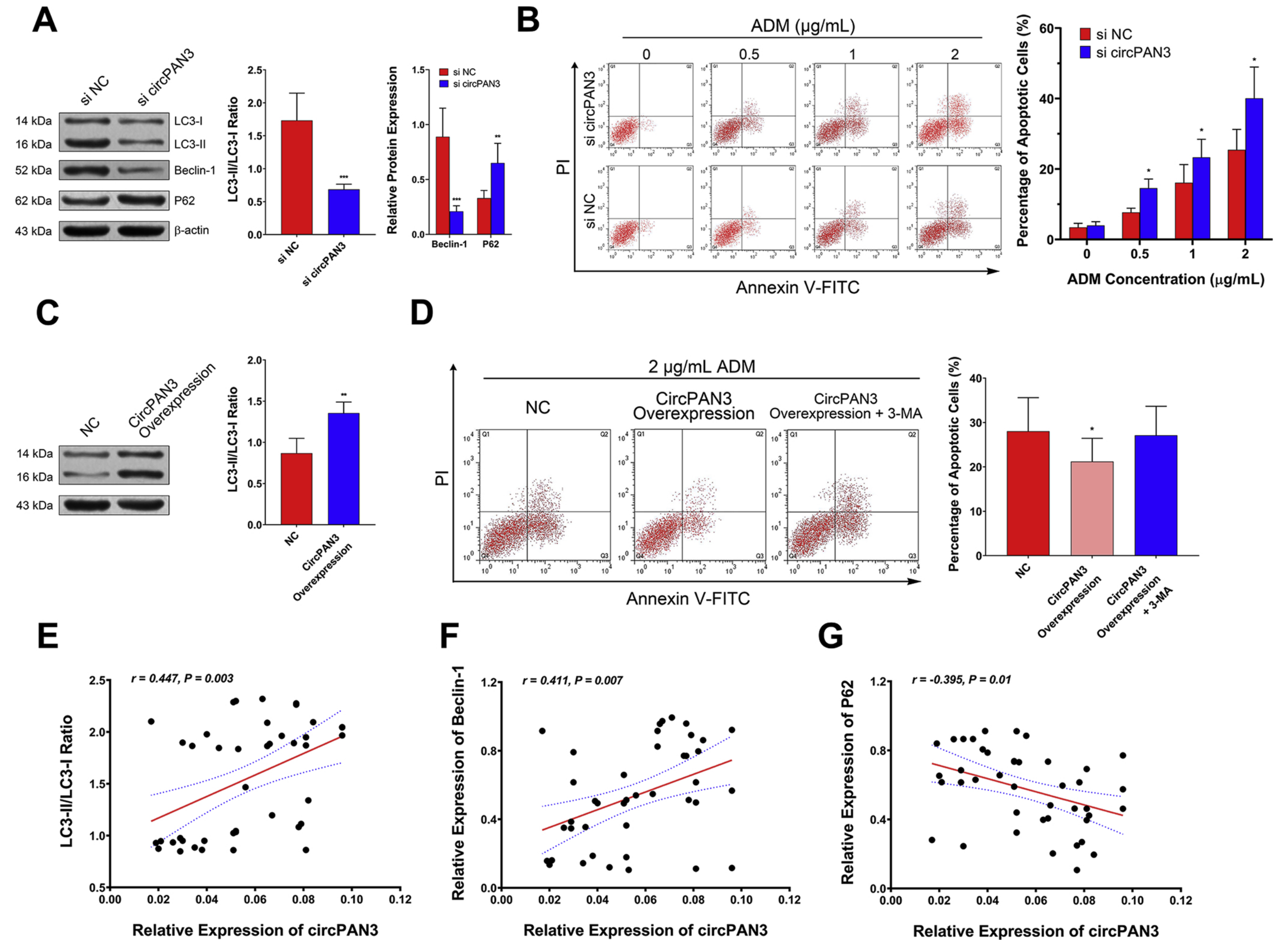


Fig. 4. CircPAN3 contributes to drug resistance in AML cells through mediating autophagy. (**A**) THP-1/ADM cells were transfected with circPAN3 siRNA. Expression of autophagy related proteins were detected and quantified by Western blot. (**B**) THP-1/ADM cells were transfected with circPAN3 siRNA, followed by exposure to 0, 0.5, 1, and 2 μg/mL ADM for 24 h. The apoptosis rates was examined and analyzed by flow cytometry. (**C**) THP-1 cells were transfected with lentiviral vector for overexpression of circPAN3. Autophagy related proteins of LC3-II/LC3-I were detected and quantified by Western blot. (**D**) THP-1 cells were transfected with lentiviral vector for overexpression of circPAN3. It should be noted that lentivirus-mediated overexpression of circPAN3 significantly decreased cell apoptosis induced by ADM (2 μg/mL for 24 h). However, incubation with autophagy inhibitor 3-MA (5 mM for 24 h) prior to ADM treatment could attenuate the anti-apoptotic effect of circPAN3 overexpression in THP-1 cells. The percentage of apoptotic cells were detected and analyzed by flow cytometry. All histogram data are presented as means ± SD of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared to negative control. (**E, F, and G**) The correlation between circPAN3 and autophagy related protein expression in BM specimens from 22 newly diagnosed and 20 relapsed AML patients was analyzed by Pearson's correlation analysis. Red line represents the fitted curve; two blue dotted lines represent 95% confidence intervals (CIs). NC: negative control; ADM: doxorubicin; OD: optical density. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

autophagy in cancer seem to depend on tumor type, stage, and genomic context and setting [4]. Nevertheless, the relationship between autophagy and circRNAs remains largely unknown. In this study, we established two AML cell lines with acquired drug resistance and analyzed differential expression of circRNAs between these two cell lines and their parental cell lines using RNA-seq. We identified circPAN3 as a key molecule involved in acquired resistance of AML, and found that this molecule was highly expressed in drug-resistant AML cells and promoted autophagy through the AMPK/mTOR pathway, consequently inducing occurrence of drug resistance. These findings widen understanding of the relationship between circRNAs and drug resistance in AML, and raise a new theoretical basis as well as a potential therapeutic target for clinical treatment of relapsed AML.

The relationship between autophagy and drug resistance has been confirmed in multiple solid tumor cells [35], but rarely reported in AML. In this study, one highlight was the establishment of two AML cell lines with acquired drug resistance by using concentration gradient exposure method, which served as useful in vitro models to investigate the underlying mechanisms of drug resistance in AML. We found that autophagic activity was elevated in the established drug-resistant AML cells compared with their parent drug-sensitive cells. This finding indicates that augmented autophagy occurs in response to ADM exposure in AML cells, which might be a self-protective and survival mechanism against apoptosis under the stress exerted by cytotoxic drugs [36]. On the other hand, we found that inhibition of autophagy by 3-MA could reverse drug resistance in our ADM-resistant AML cell lines. This result was in line with the findings observed in other solid tumor cells [10,37], suggesting that autophagy may be a potential therapeutic target for overcoming AML drug resistance. Nevertheless, it should be noted that the detailed mechanism for reversing drug resistance by autophagy inhibition is largely unknown. In our study, treatment with 3-MA not only inhibited autophagic activity, but up-regulated the expression of several pro-apoptotic proteins including CL-Cas-3, CL-Cas-9, and Bax, and down-regulated the expression of anti-apoptotic protein Bcl-2. Considering apoptotic inhibition has been regarded one of the leading causes of drug resistance in tumor cells [38], these results

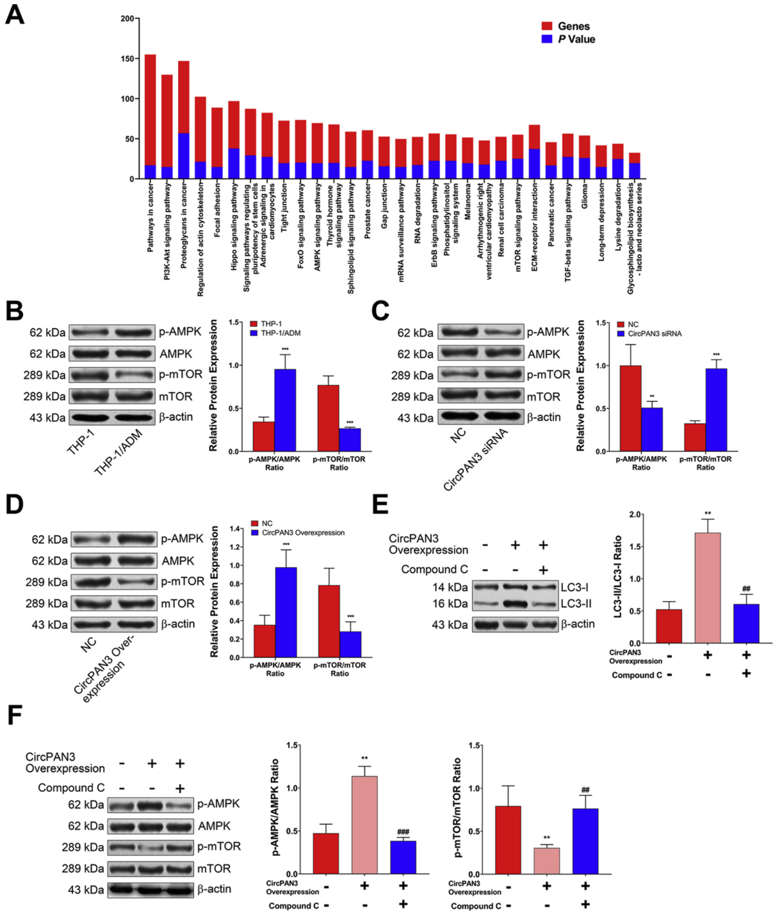


Fig. 5. CircPAN3 regulates autophagy through the AMPK/mTOR pathway in AML cells. (**A**) The signaling pathways involved in the downstream of circPAN3 were analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG). (**B**) Expression levels of key proteins in the AMPK/mTOR pathway in THP-1 and THP-1/ADM cells were detected and quantified by Western blot. (**C**) Expression of key proteins in the AMPK/mTOR pathway in THP-1/ADM cells transfected with circPAN3 siRNA. The protein levels were detected and quantified by Western blot. (**D**) THP-1 cells were transfected with lentiviral vector for overexpression of circPAN3. Expression of AMPK/mTOR pathway related proteins in these cells were detected and quantified by Western blot. (**E and F**) CircPAN3-overexpressing THP-1 cells were pre-treated with 10 μM of AMPK inhibitor Compound C for 12 h. Compound C could counteract the effects of circPAN3 overexpression on the ratios of LC3-II/LC3-I, p-AMPK/AMPK, and p-mTOR/mTOR. The protein levels were detected and quantified by Western blot. All data are presented as means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared to negative control. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared to circPAN3-overexpressing THP-1 cells without 3-MA pretreatment. NC: negative control.

suggested that autophagy may potentially contribute to AML resistance through regulation of apoptosis. It is worth noting that in in vitro experiments, the concentration of 3-MA showed no cytotoxicity but did significantly reverse drug resistance in AML cells. This result was consistent with previous studies [39], these findings suggest the great potential of autophagy inhibitors for clinical application. Therefore, preclinical study is needed to validate the activity of autophagy inhibitors against relapsed AML.

Another important finding of our study is that we identified circPAN3 as a key regulator of drug resistance in AML cells, which was highly expressed in drug-resistant AML cell lines and may induce drug resistance by enhancing autophagic activity. It is known that the host gene of circPAN3, *PAN3*, is located at chromosome 13q12 and its protein product serves as a regulatory subunit of the PAN2/PAN3 complex [40]. Recently, the PAN2/PAN3 complex has been found to be responsible for deadenylation process, which removes the poly(A) tail from RNA; while another study reports that *PAN3* rearrangement may be involved in the transition from MDS to AML, indicating that a potential association may exist between *PAN3* gene and AML development [41]. In this study, however, we find that there was no difference in *PAN3* mRNA and protein expression between ADM-sensitive and -resistant cells, although ADM-resistant cells showed significantly higher circPAN3 levels. These findings suggest that *PAN3* may not directly contribute to the development of drug resistance, but indirectly through circPAN3 by an alternative splicing regulation mechanism.

In our siRNA experiments, we found that down-regulation of circPAN3 by siRNA reduced autophagy activity and augmented ADM-induced apoptosis in THP-1/ADM cells. One possible explanation is that silencing circPAN3 expression by siRNA could inhibit basal autophagy. Although inhibition of basal autophagy may not directly induce apoptosis, but can mediates apoptosis sensitization to chemotherapy drugs. There have been a few of studies demonstrating this effect [42,43]. Moreover, lentivirus mediated circPAN3 overexpression results in opposite results in drug sensitive AML cells and this process could be interrupted by 3-MA treatment. Our findings confirmed that circPAN3 induces drug resistance in AML cells by increasing autophagy level.

To further study the mechanism of circPAN3 regulating autophagy, we first analyzed miRNA response elements (MREs) associated with circPAN3, and then predicted its potential target miRNAs and target genes regulated by these miRNAs through TargetScan. In siRNA experiments we found that down-regulation of circPAN3 by siRNA resulted in inactivation of the AMPK/mTOR pathway. On the other hand, Compound C, an inhibitor of AMPK, significantly attenuated the increase of autophagy activity induced by overexpression of circPAN3. In addition, based on TargetScan and miRanda, we hypothesized that circPAN3 might function as a miRNA sponge to interact with miR-545-3p, consequently regulating the expression of TAK1, a known activating AMPK kinase [44], and ultimately contributing to AMPK/mTOR signaling activation. In supporting this hypothesis, we found that in THP-1/ADM cells transfected with a specific siRNA for circPAN3, knockdown of circPAN3 resulted in a significant increase in miR-545-3p expression, while considerably decreased the protein level of TAK1 as compared to mock control (Supplementary Fig. 4A and 4B). Furthermore, luciferase assay showed that up-regulation of miR-545-3p by mimic transfection significantly reduced the luciferase activity of WT reporters, but this reduction was not observed in the reporters containing mutated miR-545-3p binding sites of circPAN3 or *TAK1* 3′-UTR with mutated binding sites for miR-545-3p (Supplementary Fig. 4C and 4D). These results indicate that there may some interactions between circPAN3, miR-545-3p, and AMPK activator TAK1 and therefore circPAN3 might lead to activation of AMPK/mTOR pathway in THP-1/ADM cells, at least partly, via the circPAN3-miR-545-3p-TAK1 axis.

However, considering circPAN3 interacts with multiple downstream miRNA molecules, other miRNA-mediated signaling axes may also contribute to AMPK/mTOR activation, which requires further studies to elaborate.

It is well known that AMPK/mTOR signaling is related to energy metabolism and can be activated under a variety of stressful states [45]. Particularly, activation of AMPK pathway has been demonstrated in drug-resistant tumors [46], and found to be closely associated with autophagy. Previous literature has reported that activated AMPK can phosphorylate ULK1 and TSC1/TSC2 complex and affect mTORC1 activation [47,48], while mTORC1 is an important component of the mTOR complex and suppression of mTORC1 activation inhibits ULK1 phosphorylation and up-regulates LC3-II expression, consequently promoting formation of autophagy bodies and inducing occurrence of autophagy [49]. These findings are in agreement with our study, confirm that the effect of circPAN3 on drug resistance of AML is, at least in part, mediated by autophagy via AMPK/mTOR signaling cascades.

In summary, the present study revealed that circPAN3 is most likely a key modulator for acquired drug resistance of AML, which may facilitate drug resistance in AML cells by regulating autophagy as an autophagy inducer via the AMPK/mTOR pathway.

Funding

This project was supported by Natural Science Foundation of Fujian Province (No.: 2018J01259), Fujian Provincial Health and Family Planning Commission Medical Innovation Fund (No.: 2018-CX-5), and High-level Hospital Foster Grants from Fujian Provincial Hospital, Fujian Province, China (No.: 2019HSJJ15).

Ethical approval and informed consent

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Fujian Provincial Hospital. All patients provided written informed consent before participation.

Declaration of Competing Interest

None.

Acknowledgments

This project was supported by Natural Science Foundation of Fujian Province (No.: 2018J01259), Fujian Provincial Health and Family Planning Commission Medical Innovation Fund (No.: 2018-CX-5), and High-level Hospital Foster Grants from Fujian Provincial Hospital, Fujian Province, China (No.: 2019HSJJ15).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2019.106198.

References

[1] J. Prada-Arismendy, J.C. Arroyave, S. Rothlisberger, Molecular biomarkers in acute myeloid leukemia, Blood Rev. 31 (1) (2017) 63–76.

[2] R.C. Lynch, B.C. Medeiros, Chemotherapy options for previously untreated acute myeloid leukemia, Expert Opin. Pharmacother. 16 (14) (2015) 2149–2162.

[3] J.C. Byrd, K. Mrozek, R.K. Dodge, A.J. Carroll, C.G. Edwards, D.C. Arthur, et al., Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461), Blood 100 (13) (2002) 4325–4336.

[4] Y. Kondo, T. Kanzawa, R. Sawaya, S. Kondo, The role of autophagy in cancer development and response to therapy, Nat. Rev. Cancer 5 (9) (2005) 726–734.

[5] M. Nadal, S.E. Gold, Assessment of autophagosome formation by transmission electron microscopy, Methods Mol. Biol. 835 (2012) 481–489.

[6] T. Yorimitsu, D.J. Klionsky, Autophagy: molecular machinery for self-eating, Cell Death Differ. 12 (Suppl 2) (2005) 1542–1552.

[7] S. Jin, E. White, Role of autophagy in cancer: management of metabolic stress, Autophagy 3 (1) (2007) 28–31.

[8] Y.J. Li, Y.H. Lei, N. Yao, C.R. Wang, N. Hu, W.C. Ye, et al., Autophagy and multidrug resistance in cancer, Chin. J. Cancer 36 (1) (2017) 52.

[9] M. Yang, P. Zeng, R. Kang, Y. Yu, L. Yang, D. Tang, et al., S100A8 contributes to drug resistance by promoting autophagy in leukemia cells, PLoS One 9 (5) (2014) e97242.

[10] W.L. Sun, J. Chen, Y.P. Wang, H. Zheng, Autophagy protects breast cancer cells from epirubicin-induced apoptosis and facilitates epirubicin-resistance development, Autophagy 7 (9) (2011) 1035–1044.

[11] E. Lasda, R. Parker, Circular RNAs: diversity of form and function, RNA 20 (12) (2014) 1829–1842.

[12] S. Qu, X. Yang, X. Li, J. Wang, Y. Gao, R. Shang, et al., Circular RNA: a new star of noncoding RNAs, Cancer Lett. 365 (2) (2015) 141–148.

[13] J. Zhou, Y. Ge, Y. Hu, D. Rong, K. Fu, H. Wang, et al., Circular RNAs as novel rising stars with huge potentials in development and disease, Cancer Biomark. 22 (4) (2018) 597–610.

[14] J. Greene, A.M. Baird, L. Brady, M. Lim, S.G. Gray, R. McDermott, et al., Circular RNAs: biogenesis, function and role in human diseases, Front. Mol. Biosci. 4 (2017) 38.

[15] J. Zhang, P. Wang, L. Wan, S. Xu, D. Pang, The emergence of noncoding RNAs as Heracles in autophagy, Autophagy 13 (6) (2017) 1004–1024.

[16] M.R. O'Donnell, M.S. Tallman, C.N. Abboud, J.K. Altman, F.R. Appelbaum, D.A. Arber, et al., Acute myeloid leukemia, version 3.2017, NCCN clinical practice guidelines in oncology, J. Compr. Canc. Netw. 15 (7) (2017) 926–957.

[17] M.D. Robinson, D.J. McCarthy, G.K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data, Bioinformatics 26 (1) (2010) 139–140.

[18] A. Dobin, C.A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, et al., STAR: ultrafast universal RNA-seq aligner, Bioinformatics 29 (1) (2013) 15–21.

[19] J. Cheng, F. Metge, C. Dieterich, Specific identification and quantification of circular RNAs from sequencing data, Bioinformatics 32 (7) (2016) 1094–1096.

[20] D.J. Klionsky, F.C. Abdalla, H. Abeliovich, R.T. Abraham, A. Acevedo-Arozena, K. Adeli, et al., Guidelines for the use and interpretation of assays for monitoring autophagy, Autophagy 8 (4) (2012) 445–544.

[21] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (6) (2008) 1101–1108.

[22] M. Mishra, S. Tiwari, A.V. Gomes, Protein purification and analysis: next generation Western blotting techniques, Expert Rev. Proteomics 14 (11) (2017) 1037–1053.

[23] A.S. Limpert, L.J. Lambert, N.A. Bakas, N. Bata, S.N. Brun, R.J. Shaw, et al., Autophagy in Cancer: regulation by small molecules, Trends Pharmacol. Sci. 39 (12) (2018) 1021–1032.

[24] I. Tanida, T. Ueno, E. Kominami, LC3 and autophagy, Methods Mol. Biol. 445 (2008) 77–88.

[25] I. Tanida, T. Ueno, E. Kominami, LC3 conjugation system in mammalian autophagy, Int. J. Biochem. Cell Biol. 36 (12) (2004) 2503–2518.

[26] R. Kang, H.J. Zeh, M.T. Lotze, D. Tang, The Beclin 1 network regulates autophagy and apoptosis, Cell Death Differ. 18 (4) (2011) 571–580.

[27] Y. Ichimura, M. Komatsu, Selective degradation of p62 by autophagy, Semin. Immunopathol. 32 (4) (2010) 431–436.

[28] V. Beljanski, C. Chiang, J. Hiscott, The intersection between viral oncolysis, drug resistance, and autophagy, Biol. Chem. 396 (12) (2015) 1269–1280.

[29] H. Liao, Y. Huang, B. Guo, B. Liang, X. Liu, H. Ou, et al., Dramatic antitumor effects of the dual mTORC1 and mTORC2 inhibitor AZD2014 in hepatocellular carcinoma, Am. J. Cancer Res. 5 (1) (2015) 125–139.

[30] W. Li, C. Zhong, J. Jiao, P. Li, B. Cui, C. Ji, et al., Characterization of hsa_circ_0004277 as a new biomarker for acute myeloid leukemia via circular RNA profile and bioinformatics analysis, Int. J. Mol. Sci. 18 (3) (2017) pii: E597..

[31] G. Militello, T. Weirick, D. John, C. Doring, S. Dimmeler, S. Uchida, Screening and validation of lncRNAs and circRNAs as miRNA sponges, Brief Bioinform 18 (5) (2017) 780–788.

[32] X. Fan, J. Wang, J. Hou, C. Lin, A. Bensoussan, D. Chang, et al., Berberine alleviates ox-LDL induced inflammatory factors by up-regulation of autophagy via AMPK/mTOR signaling pathway, J. Transl. Med. 13 (2015) 92.

[33] G. Marino, M. Niso-Santano, E.H. Baehrecke, G. Kroemer, Self-consumption: the interplay of autophagy and apoptosis, Nat. Rev. Mol. Cell Biol. 15 (2) (2014) 81–94.

[34] O. Oral, Y. Akkoc, O. Bayraktar, D. Gozuacik, Physiological and pathological significance of the molecular cross-talk between autophagy and apoptosis, Histol. Histopathol. 31 (5) (2016) 479–498.

[35] S. Chen, S.K. Rehman, W. Zhang, A. Wen, L. Yao, J. Zhang, Autophagy is a therapeutic target in anticancer drug resistance, Biochim. Biophys. Acta 1806 (2) (2010) 220–229.

[36] G. Kroemer, G. Marino, B. Levine, Autophagy and the integrated stress response, Mol. Cell 40 (2) (2010) 280–293.

[37] S. Chittaranjan, S. Bortnik, W.H. Dragowska, J. Xu, N. Abeysundara, A. Leung, et al., Autophagy inhibition augments the anticancer effects of epirubicin treatment in anthracycline-sensitive and -resistant triple-negative breast cancer, Clin. Cancer Res. 20 (12) (2014) 3159–3173.

[38] B. Mansoori, A. Mohammadi, S. Davudian, S. Shirjang, B. Baradaran, The different mechanisms of Cancer drug resistance: a brief review, Adv. Pharm. Bull. 7 (3) (2017) 339–348.

[39] Y. Pan, Y. Gao, L. Chen, G. Gao, H. Dong, Y. Yang, et al., Targeting autophagy augments in vitro and in vivo antimyeloma activity of DNA-damaging

chemotherapy, Clin. Cancer Res. 17 (10) (2011) 3248–3258.

[40] I.B. Schafer, M. Rode, F. Bonneau, S. Schussler, E. Conti, The structure of the Pan2-Pan3 core complex reveals cross-talk between deadenylase and pseudokinase, Nat. Struct. Mol. Biol. 21 (7) (2014) 591–598.

[41] I. Panagopoulos, L. Gorunova, H.K. Andersen, A. Bergrem, A. Dahm, K. Andersen, et al., PAN3-PSMA2 fusion resulting from a novel t(7;13)(p14;q12) chromosome translocation in a myelodysplastic syndrome that evolved into acute myeloid leukemia, Exp. Hematol. Oncol. 7 (2018) 7.

[42] B.E. Fitzwalter, C.G. Towers, K.D. Sullivan, Z. Andrysik, M. Hoh, M. Ludwig, et al., Autophagy inhibition mediates apoptosis sensitization in Cancer therapy by relieving FOXO3a turnover, Dev. Cell 44 (5) (2018) 555-565 e553.

[43] P. Bhat, J. Kriel, B. Shubha Priya, N.S. Basappa, B. Loos Shivananju, Modulating autophagy in cancer therapy: advancements and challenges for cancer cell death sensitization, Biochem. Pharmacol. 147 (2018) 170–182.

[44] Z. Chen, X. Shen, F. Shen, W. Zhong, H. Wu, S. Liu, et al., TAK1 activates AMPK-dependent cell death pathway in hydrogen peroxide-treated cardiomyocytes, inhibited by heat shock protein-70, Mol. Cell. Biochem. 377 (1-2) (2013) 35–44.

[45] Z. Luo, A.K. Saha, X. Xiang, N.B. Ruderman, AMPK, the metabolic syndrome and cancer, Trends Pharmacol. Sci. 26 (2) (2005) 69–76.

[46] H. Jin, S. Gao, H. Guo, S. Ren, F. Ji, Z. Liu, et al., Re-sensitization of radiation resistant colorectal cancer cells to radiation through inhibition of AMPK pathway, Oncol. Lett. 11 (5) (2016) 3197–3201.

[47] D.F. Egan, D.B. Shackelford, M.M. Mihaylova, S. Gelino, R.A. Kohnz, W. Mair, et al., Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy, Science 331 (6016) (2011) 456–461.

[48] M.M. Mihaylova, R.J. Shaw, The AMPK signalling pathway coordinates cell growth, autophagy and metabolism, Nat. Cell Biol. 13 (9) (2011) 1016–1023.

[49] J.K. Altman, A. Szilard, D.J. Goussetis, A. Sassano, M. Colamonici, E. Gounaris, et al., Autophagy is a survival mechanism of acute myelogenous leukemia precursors during dual mTORC2/mTORC1 targeting, Clin. Cancer Res. 20 (9) (2014) 2400–2409.