Original Article

Potential of circular RNA itchy E3 ubiquitin protein ligase as a biomarker and treatment target for multiple myeloma

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Background: This study aimed to investigate the association of circular RNA itchy E3 ubiquitin protein ligase (circ-ITCH) expression with disease risk, clinical characteristics, progression-free survival (PFS) and overall survival (OS) of multiple myeloma (MM), and to explore the influence of circ-ITCH overexpression on MM cell activities *in vitro*.

Methods: Bone marrow samples from 92 MM patients and 30 healthy controls were collected, and circ-ITCH expression was detected by quantitative polymerase chain reaction. PFS and OS in MM patients were calculated. Circ-ITCH in human MM cell lines and normal bone marrow mononuclear cells (BMMCs) were detected. Circ-ITCH overexpression and control overexpression plasmids were transfected to U226 cell line, and cell proliferation as well as apoptosis were assessed.

Results: Circ-ITCH expression was under-expressed in MM patients compared to healthy controls. And receiver operating characteristic curve displayed that circ-ITCH could distinguish MM patients from healthy controls [area under curve: 0.809 (95% CI: 0.722–0.895)]. Additionally, circ-ITCH high expression was associated with decreased International Staging System (ISS) stage in MM patients. Kaplan-Meier curves and Cox's regression analysis displayed that circ-ITCH expression was positively correlated with PFS and OS. *In vitro*, circ-ITCH expression was lower in MM cell lines (including RPMI8226, U226 and NCI-H929) compared to normal BMMCs. In U226 cells, cell proliferation was decreased but apoptosis was elevated by circ-ITCH overexpression.

Conclusions: Circ-ITCH might serve as a potential biomarker and treatment target for MM.

Keywords: Circular RNA itchy E3 ubiquitin protein ligase; multiple myeloma; survival; cell proliferation

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Introduction

Multiple myeloma (MM), as a B-cell malignancy that accounts for 1% of all cancers and around 13% of all hematologic malignancies, is characterized by clonal proliferation of malignant plasma cells in the bone marrow (BM) microenvironment and associated organ dysfunctions (1-5). With the introduction of novel therapies in the last decade, such as proteasome inhibitors, immunomodulatory agents, corticosteroids and alkylators, treatment outcomes of MM patients have been greatly improved, with 5-year median survival rate of over 50% (2). Whereas the

improvement is far away from uniform, with some patients living for more than 10 years whilst some others living for just 1–2 years, which may be related to the difference in patients' characteristics intrinsic to this disease (6). Thus, it is of great importance to explore novel biomarkers that identify patients at risk for severe disease progression and poor prognosis, which may further improve treatment outcomes of MM patients.

Circular RNA (circRNA), a type of non-coding RNA that consists of an unspecific length of nucleotides, is characterized by covalently closed-loop structures with neither 5' to 3' end nor poly-adenylated tail (3,7-11). Numerous studies have shown that circRNAs play key roles in the initiation, proliferation, migration and invasion of cancers (12). Notably, several studies have revealed that abnormal expressions of circRNAs are related to the disease risk or prognosis in hematological malignancies, such as the correlation of circ_0004277 low expression with increased AML risk and the association of circ_0000190 low expression with poor overall survival (OS) in MM (13,14). Circular RNA itchy E3 ubiquitin protein ligase (circ-ITCH), which is one of the recent research hotspots among circRNAs, is located on the chromosome 20q11.22 and derives from several exons of itchy E3 ubiquitin protein ligase (ITCH) (3,7,8). It has been revealed that circ-ITCH presents inhibitory effect on the initiation and progression of several cancers such as esophageal squamous cell carcinoma, hepatocellular carcinoma (HCC) and breast cancer, moreover, it has been identified as a biomarker for poor prognosis in several solid tumors (10,15,16). For hematological malignancies, ITCH, the linear RNA that circ-ITCH derived from via back-splicing, is reported to be closely implicated in hematological malignancies, including MM (17). Moreover, circ-ITCH is found to be expressed in human bone marrow samples, which is located on chromosome 20 from site 34413741 to 34445461 according to the Tissue-Specific CircRNA Database (http://gb.whu.edu.cn/TSCD/). Considering that circ-ITCH is identified as a tumor-suppressor in various solid tumors, meanwhile, the potential influence of ITCH in MM is reported and the expression of circ-ITCH is found in human bone marrow samples, we speculated that circ-ITCH might also participate in the pathogenesis of hematological malignancies, especially in MM, while little is known about the role of circ-ITCH in MM. Hence, this study aimed to investigate the association of circ-ITCH expression with disease risk, clinical characteristics, progression-free survival (PFS) and OS in MM, and to explore the influence of circ-ITCH overexpression on cell proliferation as well as apoptosis in MM cells *in vitro*.

Methods

Participants

Between January 2016 and December 2018, 92 symptomatic MM patients admitted to our hospital were consecutively recruited in this study. The inclusion criteria were as follows: (I) confirmed diagnosis of symptomatic MM in accordance with the updated criteria of International Myeloma Working Group (IMWG) (18); (II) age more than 18 years old; (III) absence of any plasma-cell disorder other than MM; (IV) absence of any immunoglobulin-related disorder other than MM; (V) able to be regularly followed up. The exclusion criteria were: (I) secondary MM or relapsed MM; (II) complicated with other malignancies; (III) infected with human immunodeficiency virus; (IV) history of tumors; (V) pregnant or lactating women. Besides, 30 healthy BM donors were recruited as healthy controls in the present study. Prior to the initiation, this study was approved by the Institutional Ethnic Committee of Xiangya Hospital (No. 201511298), and all participants provided the written informed consents.

Collection of baseline characteristics

After the diagnosis of MM was established, baseline clinical characteristics of all recruited MM patients were documented, which consisted of (I) demographic information including age and gender, (II) laboratory parameters including hemoglobin (Hb), calcium, serum creatinine (Scr), albumin (ALB), β2-microglobulin (β2-MG) and lactate dehydrogenase (LDH), (III) clinical features including immunophenotype, bone lesion, Durie-Salmon stage, International Staging System (ISS) stage, and cytogenetics abnormalities.

Collection and detection of BM samples

BM samples of MM patients were extracted before initiation of any therapy, and the BM samples of healthy controls were collected when they were undergoing BM donation. After collection, all BM samples were treated by density gradient centrifugation to isolate the mononuclear cells, then $CD138^+$ myeloma cells (plasma cells) were further separated and purified using the CD138-immunomagnetic beads (Miltenyi Biotec, Germany). The expression of circ-

ITCH in the myeloma cell was determined by quantitative polymerase chain reaction (qPCR).

Follow up and survival assessment

According to the MM patients' clinical status and individual willingness, treatment was administered as recommended by IMWG Clinical Practice Guideline (19), and the posttreatment response was evaluated in accordance with the criteria of the International Myeloma Workshop Consensus Panel 1 (20). Besides, all MM patients were followed up every 3 months or more frequently if clinically indicated. The last follow-up date was 2018/12/31, the longest follow-up duration was 36.0 months, and the median follow-up duration was 24.5 months. As for survival profiles, the progression-free survival (PFS) and overall survival (OS) of MM patients were calculated. PFS was defined as duration from the start of treatment date to the date of disease progression, death, or censored at the date of last contact. OS was defined as duration from the treatment date to the date of death or censored at the date of last contact.

Cell culture and circ-ITCH detection in cell lines

Human MM cell lines (RPMI8226, U226, NCI-H929 and OPM2) were purchased form Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, German) or kindly given by Fudan University (Shanghai, China), and cultured in 90% Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37 ℃ in a humidified atmosphere of 5% CO_2 in air. Then circ-ITCH expression in these MM cell lines was detected by qPCR assay. Also, plasma cells isolated from BM mononuclear cells of healthy donor was used as normal control to detect circ-ITCH expression by qPCR as well.

Transfection and detections

Circ-ITCH overexpression plasmid and control overexpression plasmid were established by Shanghai GenePharma Company (Shanghai, China) with pCD5-ciR vector (NTCC, China), then transfected into U226 cells. Post transfection, circ-ITCH expression was detected by qPCR at 24 h; cell viability was detected by Cell Counting kit-8 (CCK-8) (Beyotime, China) at 0, 24, 48 and 72 h according to the instructions; cell apoptosis rate was detected by Annexin V-FITC Apoptosis Detection Kit (Beyotime, China) at 48 h according to the instructions; then the samples were proposed to CytoFLEX system (Beckman Coulter, USA) and analyzed by the Flowjo Software 7.6 (FlowJo-LLC, USA).

qPCR

For the detection of circ-ITCH, total RNA was extracted by TRIzol™ Reagent (Thermo Fisher Scientific, Massachusetts, USA), and the linear RNA in total RNA was digested. Then, the reverse transcription to cDNA was conducted using PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara, Liaoning, China). qPCR was performed using KOD SYBR® qPCR Mix (Toyobo, Kansai, Japan), and GAPDH was applied as the internal reference. Primer sequences were as follows: circ-ITCH, forward: GTTCACCATCTGCCACTTCTGA, reverse: TCACAACTACTTCTTCAACCAGGAG; GAPDH, forward: GAGTCCACTGGCGTCTTCAC, reverse: ATCTTGAGGCTGTTGTCATACTTCT.

Statistical analysis

Statistical analysis was performed by the SPSS 22.0 (IBM, USA) and GraphPad Prism 7.02 (GraphPad Software Inc., USA). Variables were displayed as mean and standard deviation (SD), median and interquartile range (IQR), or count (percentage) as appropriate. Comparison was determined by the Chi-square test, Fisher's exact test, Student's t test, Wilcoxon rank sum test, or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. To assess the discrimination performance of variable, receiver operating characteristic (ROC) curve was constructed and the area under the curve (AUC) was calculated. PFS and OS were assessed by use of Kaplan-Meier curves, and the difference of PFS and OS between different subjects was determined by the Log-rank test. The variables related to the PFS and OS were evaluated by the univariable and forward-stepwise (conditional) multivariable Cox's proportional hazard regression model analyses. P value <0.05 was considered statistically significant.

Results

Baseline characteristics

Ninety-two MM patients with mean age of 56.7±8.3 years

Table 1 Baseline characteristics of MM patients

Items	MM patients (N=92)
Age (years), mean ± SD	56.7±8.3
Gender, No. (%)	
Male	59 (64.1)
Female	33 (35.9)
Immunophenotype, No. (%)	
IgG	51 (55.4)
IgA	21 (22.8)
IgM	1 (1.1)
IgD	1 (1.1)
Bence-Jones protein	18 (19.6)
Bone lesion, No. (%)	68 (73.9)
Biochemical indexes	
Hb (g/dL), mean ± SD	10.0±2.4
Calcium (mg/dL), mean ± SD	10.0±1.8
Scr (mg/dL), mean ± SD	1.6±0.5
Albumin (mg/dL), mean ± SD	3.9±0.7
β2-MG (mg/L), median (IQR)	4.4 (2.4–7.4)
LDH (U/L), median (IQR)	194.9 (170.9–229.5)
Durie-Salmon stage, No. (%)	
II	12 (13.0)
III	80 (87.0)
ISS stage, No. (%)	
I	23 (25.0)
II	32 (34.8)
III	37 (40.2)
Cytogenetics, No. (%)	
t(4;14)	10 (10.9)
t(14;16)	14 (15.2)
Del (17p)	9 (9.8)

MM, multiple myeloma; SD, standard deviation; Ig, immunoglobulin; Hb, hemoglobin; Scr, serum creatinine; β2-MG, β2-microglobulin; IQR, interquartile range; LDH, lactate dehydrogenase; ISS, international staging system.

were enrolled including 59 (64.1%) males and 33 (35.9%) females in this study (*Table 1*). The numbers of patients with immunophenotype IgG, IgA, IgM, IgD and Bence-Jones protein were 51 (55.4%), 21 (22.8%), 1 (1.1%), 1 (1.1%) and 18 (19.6%) respectively, and 68 (73.9%) patients presented bone lesion. For the biochemical indexes, levels of Hb, calcium, Scr, ALB, β2-MG and LDH were 10.0±2.4 g/dL, 10.0±1.8 mg/dL, 1.6±0.5 mg/dL, 3.9± 0.7 mg/dL, 4.4 (2.4–7.4) mg/L and 194.9 (170.9–229.5) U/L respectively. As to the disease stage, patients with Durie-Salmon stage II and III were 12 (13.0%) and 80 (87.0%) respectively, and the numbers of patients with ISS stage I, II and III were 23 (25.0%), 32 (34.8%) and 37 (40.2%) respectively. Additionally, there were 10 (10.9%), 14 (15.2%) and 9 (9.8%) patients presented cytogenetics t(4;14), t(14;16) and Del(17p) respectively.

Circ-ITCH expression in MM patients and healthy controls

Circ-ITCH expression was less expressed in BM samples from MM patients [0.465 (0.299–0.938)] than that from healthy controls [1.321 (0.719–1.876)] (P<0.001) (*Figure 1A*). Moreover, ROC curve displayed that circ-ITCH could distinguish MM patients from healthy controls, with AUC of 0.809 (95% CI: 0.722–0.895), and the sensitivity and specificity the median value of circ-ITCH expression (0.604) among MM patients and healthy controls were 59.8% and 80.0% respectively (*Figure 1B*).

Correlation of circ-ITCH expression with clinical characteristics in MM patients

Circ-ITCH high expression was associated with decreased ISS stage (P=0.036) in MM patients, and it was numerically associated with lower β2-MG level (P=0.056), but without statistical significance (*Table 2*). And no correlation of circ-ITCH expression with other characteristics including age (P=0.200), gender (P=0.828), immunophenotype (P=0.126), bone lesion (P=0.154), Hb (P=0.532), calcium (P=0.482), Scr (P=0.804), ALB (P=0.650), LDH (P=1.000), Durie-Salmon stage (P=1.000), t(4;14) (P=0.503), t(14;16) (P=0.562) and Del (17p) (P=0.158) in MM patients was observed.

Comparison of PFS and OS between circ-ITCH high expression patients and circ-ITCH low expression patients

Circ-ITCH high expression was positively correlated with PFS (P=0.017), meanwhile, circ-ITCH high expression was positively associated with OS (P=0.018) in MM patients (*Figure 2*).

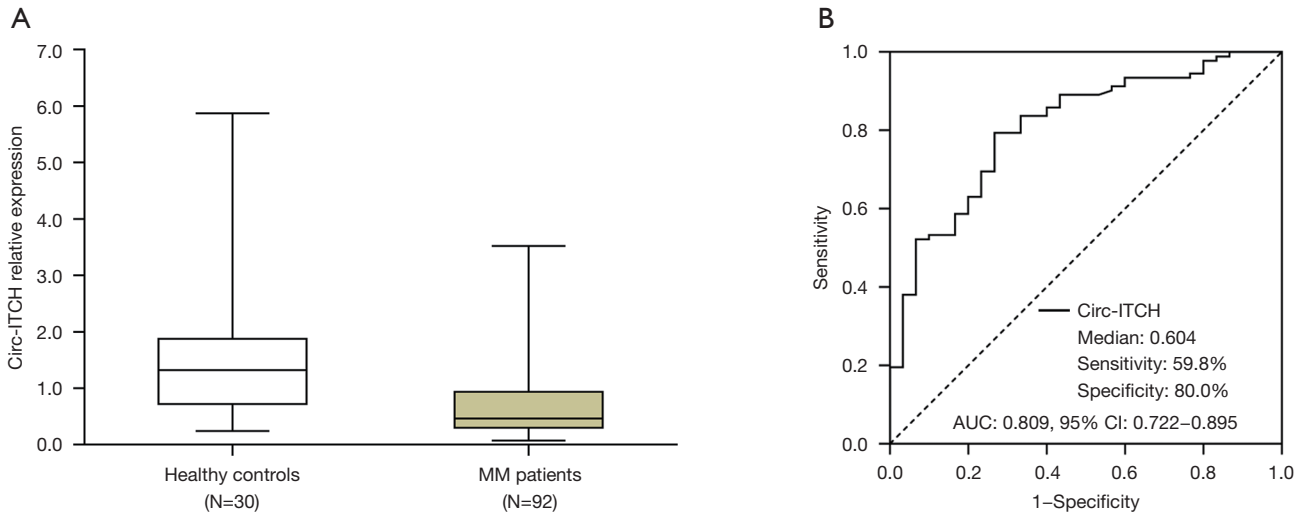

Figure 1 Comparison of circ-ITCH expression between MM patients and healthy controls. (A) Circ-ITCH expression in MM patients and healthy controls; (B) ROC curve of circ-ITCH expression in predicting MM risk. Comparison between groups was determined by Wilcoxon rank sum test. P<0.05 was considered significant. Circ-ITCH, circular RNA itchy E3 ubiquitin protein ligase; MM, multiple myeloma; ROC curve, receiver operating characteristic curve.

Analysis of factors affecting PFS and OS in MM patients

Univariate Cox's regression analysis showed that circ-ITCH high expression was associated with increased PFS (P=0.020) (*Table 3*). Whereas bone lesion (P=0.001), Scr (≥2.0 mg/dL) (P=0.012), β2-MG (≥5.5 mg/L) (P<0.001), higher ISS stage (P<0.001), t(4;14) (P=0.042), t(14;16) (P=0.043) and Del (17p) (P=0.012) were associated with worse PFS. Subsequently, the forward stepwise multivariate Cox's regression analysis displayed that circ-ITCH high expression was not an independent factor predicting PFS, while β2-MG (≥5.5 mg/L) (P<0.001) and t(14;16) (P=0.027) were independent predictive factors for reduced PFS in MM patients. Furthermore, analysis of factors affecting OS in MM patients was also performed, and univariate Cox's regression analysis showed that circ-ITCH high expression (P=0.022) was correlated with longer OS, while bone lesion (P=0.022), β2-MG (≥5.5 mg/L) (P<0.001), higher ISS stage (P<0.001), t(14;16) (P=0.011) and Del (17p) (P=0.002) were correlated with shorter OS in MM patients (*Table 4*). Additionally, multivariate Cox's regression analysis disclosed that circ-ITCH high expression was not an independent predictive factor for OS, while β2-MG (≥5.5 mg/L) (P<0.001), t(4;14) (P=0.019) and t(14;16) (P=0.006) were independent factors predicting worse OS in MM patients.

Comparison of circ-ITCH expression in MM cell lines and normal BM mononuclear cells

To further explore the circ-ITCH expression in MM cell lines and normal BM mononuclear cells, we detected circ-ITCH expression by qPCR assay, which showed that circ-ITCH expression was lower in RPMI8226 (P<0.01), U226 (P<0.001) and NCI-H929 (P<0.01) cell lines compared to that in normal BM mononuclear cells, while no difference of circ-ITCH expression was observed in OPM2 cell line compared to that in normal BM mononuclear cells (P>0.05) (*Figure 3*). These data indicated circ-ITCH was downregulated in MM cell lines compared to normal BM mononuclear cells.

Effect of circ-ITCH overexpression on cell proliferation and cell apoptosis in U226 cells

Circ-ITCH expression was elevated in circ-ITCH overexpression group than that in control overexpression group (P<0.001) (*Figure 4A*). Besides, cell proliferation was decreased after transfection at 72 h in circ-ITCH overexpression group than that in control overexpression group (P<0.05) (*Figure 4B*), and cell apoptosis rate was elevated at 48 h in circ-ITCH overexpression group than

Table 2 Correlations of circ-ITCH expression with clinical characteristics of MM patients

Items	Circ-ITCH expression*		P value
	Low	High	
Age, No. (%)			0.200
<60 years	31 (55.4)	25 (44.6)	
≥60 years	15 (41.7)	21 (58.3)	
Gender, No. (%)			0.828
Female	16 (48.5)	17 (51.5)	
Male	30 (50.8)	29 (49.2)	
Immunophenotype, No. (%)			0.126
IgG	21 (41.2)	30 (58.8)	
IgA	10 (47.6)	11 (52.4)	
IgM	1 (100.0)	0 (0.0)	
IgD	1 (100.0)	0 (0.0)	
Bence-Jones protein	13 (72.2)	5 (27.8)	
Bone lesion, No. (%)			0.154
No	9 (37.5)	15 (62.5)	
Yes	37 (54.4)	31 (45.6)	
Hb, No. (%)			0.532
<10.0 g/dL	24 (53.3)	21 (46.7)	
≥10.0 g/dL	22 (46.8)	25 (53.2)	
Calcium, No. (%)			0.482
<11.5 mg/dL	35 (52.2)	32 (47.8)	
≥11.5 mg/dL	11 (44.0)	14 (56.0)	
Scr, No. (%)			0.804
<2.0 mg/dL	36 (50.7)	35 (49.3)	
≥2.0 mg/dL	10 (47.6)	11 (52.4)	
Albumin, No. (%)			0.650
<3.5 mg/dL	15 (53.6)	13 (46.4)	
≥3.5 mg/dL	31 (48.4)	33 (51.6)	
β2-MG, No. (%)			0.056
<5.5 mg/L	23 (41.8)	32 (58.2)	
≥5.5 mg/L	23 (62.2)	14 (37.8)	
LDH, No. (%)			1.000
<220.0 U/L	31 (50.0)	31 (50.0)	
≥220.0 U/L	15 (50.0)	15 (50.0)	
Durie-Salmon stage, No. (%)			1.000
II	6 (50.0)	6 (50.0)	
III	40 (50.0)	40 (50.0)	
ISS stage, No. (%)			0.036
I	8 (34.8)	15 (65.2)	
II	15 (46.9)	17 (53.1)	
III	23 (62.2)	14 (37.8)	
t(4;14), No. (%)			0.503
No	40 (48.8)	42 (51.2)	
Yes	6 (60.0)	4 (40.0)	
t(14;16), No. (%)			0.562
No	38 (48.7)	40 (51.3)	
Yes	8 (57.1)	6 (42.9)	
Del(17p), No. (%)			0.158
No	39 (47.0)	44 (53.0)	
Yes	7 (77.8)	2 (22.2)	

Correlation was determined by Chi-square test, Fisher's exact test or Wilcoxon rank sum test. *, the high or low expression was classified according to the median value of circ-ITCH relative expression in MM patients; Ig, immunoglobulin; Hb, hemoglobin; Scr, serum creatinine; β2-MG, β2-microglobulin; LDH, lactate dehydrogenase; ISS, international staging system.

that in control overexpression group (P<0.01) (*Figure 4C,D*).

Discussion

Our results indicated that: (I) circ-ITCH expression was decreased in MM patients compared to that in heathy controls, which distinguished MM patients from healthy controls, and its high expression correlated with lower ISS stage in MM patients; (II) circ-ITCH high expression correlated with prolonged PFS and OS in MM patients; (III) circ-ITCH overexpression reduced cell proliferation but promoted cell apoptosis in MM cell lines.

Circ-ITCH, as the most frequently explored circRNAs, has been identified as a tumor suppressor through affecting cell activities (such as cell proliferation, apoptosis and invasion) in a number of cancers (12). For example, circ-

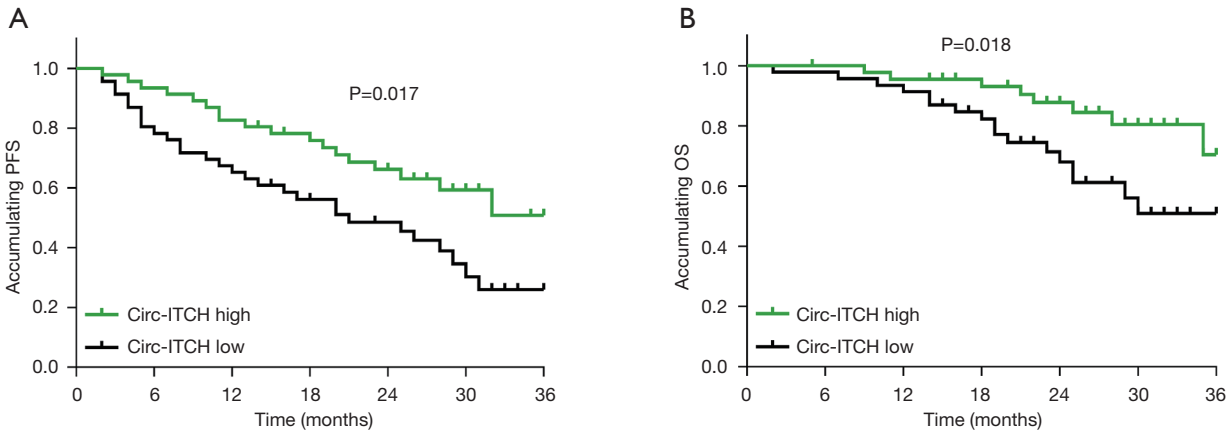

Figure 2 Survival profiles in circ-ITCH high expression patients and circ-ITCH low expression patients. (A) PFS in circ-ITCH high expression patients and circ-ITCH low expression patients; (B) OS in circ-ITCH high expression patients and circ-ITCH low expression patients. Comparison between groups was determined by log-rank test. P<0.05 was considered significant. Circ-ITCH, circular RNA itchy E3 ubiquitin protein ligase; PFS, progression-free survival; OS, overall survival.

Table 3 Univariate and multivariate Cox's proportional hazard regression model analyses of factors predicting PFS

Items	Cox's proportional hazard regression model			
	P value	HR	95% CI	
			Lower	Higher
Univariate Cox's regression				
Circ-ITCH high expression	0.020	0.498	0.276	0.898
Age (≥60 years)	0.345	1.320	0.742	2.347
Gender (male)	0.065	0.582	0.327	1.035
Immunophenotype (IgG *vs.* others)	0.776	0.920	0.518	1.634
Bone lesion	0.001	4.649	1.829	11.816
Hb (≥10.0 g/dL)	0.690	0.890	0.502	1.578
Calcium (≥11.5 mg/dL)	0.924	0.969	0.503	1.867
Scr (≥2.0 mg/dL)	0.012	2.217	1.193	4.122
Albumin (≥3.5 mg/dL)	0.220	1.527	0.777	3.002
β2-MG (≥5.5 mg/L)	<0.001	10.071	5.045	20.103
LDH (≥220.0 U/L)	0.168	1.528	0.837	2.791
Durie-Salmon stage (III *vs.* II)	0.082	2.493	0.889	6.986
Higher ISS stage	<0.001	5.166	3.050	8.749
t(4;14)	0.042	2.205	1.029	4.728
t(14;16)	0.043	2.013	1.023	3.964
Del(17p)	0.012	2.813	1.251	6.328
Forward stepwise multivariate Cox's regression				
β2-MG (≥5.5 mg/L)	<0.001	10.916	5.309	22.446
t(14;16)	0.027	2.180	1.093	4.351

PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; Ig, immunoglobulin; Hb, hemoglobin; Scr, serum creatinine; β2-MG, β2-microglobulin; LDH, lactate dehydrogenase; ISS, international staging system.

ITCH raises the level of ITCH, which further represses Wnt/β-catenin pathway and leads to suppression on cell proliferation in esophageal squamous cell carcinoma, HCC and breast cancer (10,15,16). Moreover, circ-ITCH inhibits cell proliferation via sponging miR-7 and miR-24 in bladder cancer, and it also represses cell proliferation but promotes cell apoptosis through sponging miR-124 in glioma (12,21). These previous studies disclose the tumor-suppressive influence of circ-ITCH in a variety of cancers. According to previous clinical studies, the dysregulation of circ-ITCH has been discovered in several cancers (21-23). For instance,

Table 4 Univariate and multivariate Cox's proportional hazard regression model analyses of factors predicting OS

Items	Cox's proportional hazard regression model			
	P value	HR	95%CI	
			Lower	Higher
Univariate Cox's regression				
Circ-ITCH high expression	0.022	0.367	0.156	0.865
Age (≥60 years)	0.688	0.846	0.373	1.918
Gender (male)	0.145	0.557	0.254	1.223
Immunophenotype (IgG *vs.* others)	0.571	1.260	0.566	2.808
Bone lesion	0.022	5.409	1.272	23.002
Hb (≥10.0 g/dL)	0.390	1.421	0.638	3.165
Calcium (≥11.5 mg/dL)	0.478	1.356	0.585	3.146
Scr (≥2.0 mg/dL)	0.771	1.158	0.431	3.109
Albumin (≥3.5 mg/dL)	0.615	1.266	0.504	3.182
β2-MG (≥5.5 mg/L)	<0.001	20.320	6.919	59.677
LDH (≥220.0 U/L)	0.381	1.443	0.635	3.279
Durie-Salmon stage (III *vs.* II)	0.115	4.998	0.674	37.070
Higher ISS stage	<0.001	10.598	4.264	26.343
t(4;14)	0.060	2.569	0.962	6.860
t(14;16)	0.011	2.997	1.292	6.954
Del (17p)	0.002	4.429	1.744	11.249
Forward stepwise multivariate Cox's regression				
β2-MG (≥5.5 mg/L)	<0.001	37.864	10.126	141.577
t(4;14)	0.019	3.430	1.225	9.604
t(14;16)	0.006	3.433	1.432	8.234

OS, overall survival; HR, hazard ratio; CI, confidence interval; Ig, immunoglobulin; Hb, hemoglobin; Scr, serum creatinine; β2-MG, β2-microglobulin; LDH, lactate dehydrogenase; ISS, international staging system.

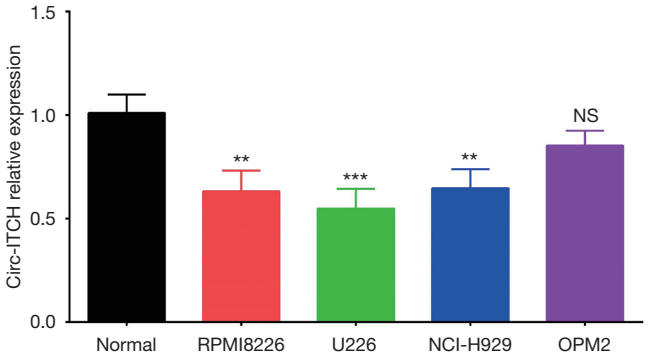

Figure 3 Circ-ITCH expression in various MM cell lines and normal BM mononuclear cells. Detection of circ-ITCH expression in RPMI8226, U226, NCI-H929, OPM2 and normal BM mononuclear cell lines by qPCR. Comparison between groups was determined by One-way ANOVA followed by Dunnett's multiple comparisons test. P<0.05 was considered significant. Circ-ITCH, circular RNA itchy E3 ubiquitin protein ligase; MM, multiple myeloma; BM, bone marrow; qPCR, quantitative polymerase chain reaction. ***P<0.001; **P<0.01; NS, no significance.

in HCC and esophageal squamous cell carcinoma, circ-ITCH is found downregulated in cancer tissues compared to normal tissues (22,23). As to the correlation of circ-ITCH expression with clinical characteristics, a study shows that circ-ITCH high expression is correlated with decreased histological grade in bladder cancer patients (21). These data suggest that circ-ITCH is downregulated in a variety of solid tumors, and its high expression correlates with attenuated clinical characteristics. For hematological malignancies, linear ITCH is reported to play a role in hematological malignancies, including MM (17), meanwhile, circ-ITCH exists in human bone marrow samples. Based on these indications, we speculated that circ-ITCH might also participate in the initiation and progression of MM, while related evidence was rarely reported. In our study, we enrolled 92 MM patients and 30 healthy controls to explore the association of circ-ITCH expression with disease risk, furthermore, we investigated the correlation of circ-ITCH with clinical characteristics in MM patients. We found that circ-ITCH expression was decreased in MM patients compared to healthy controls, and it presented good predictive value for MM risk. Meanwhile, circ-ITCH high expression correlated with lower ISS stage in MM patients. The possible reasons might be that circ-ITCH could sponge miRNAs (such as miR-7, miR-24 and miR-124) or regulate signaling pathways (such as Notch1 and Wnt/β-catenin signaling pathways) to repress cell proliferation and enhance cell apoptosis, which retarded initiation of MM, therefore, its low expression predicted increased MM risk (14,16,21,23). Additionally, circ-ITCH might repress cell invasion in MM patients and further decrease disease progression, such as reducing organ dysfunctions, thereby it resulted in a more normal macroglobulin level and

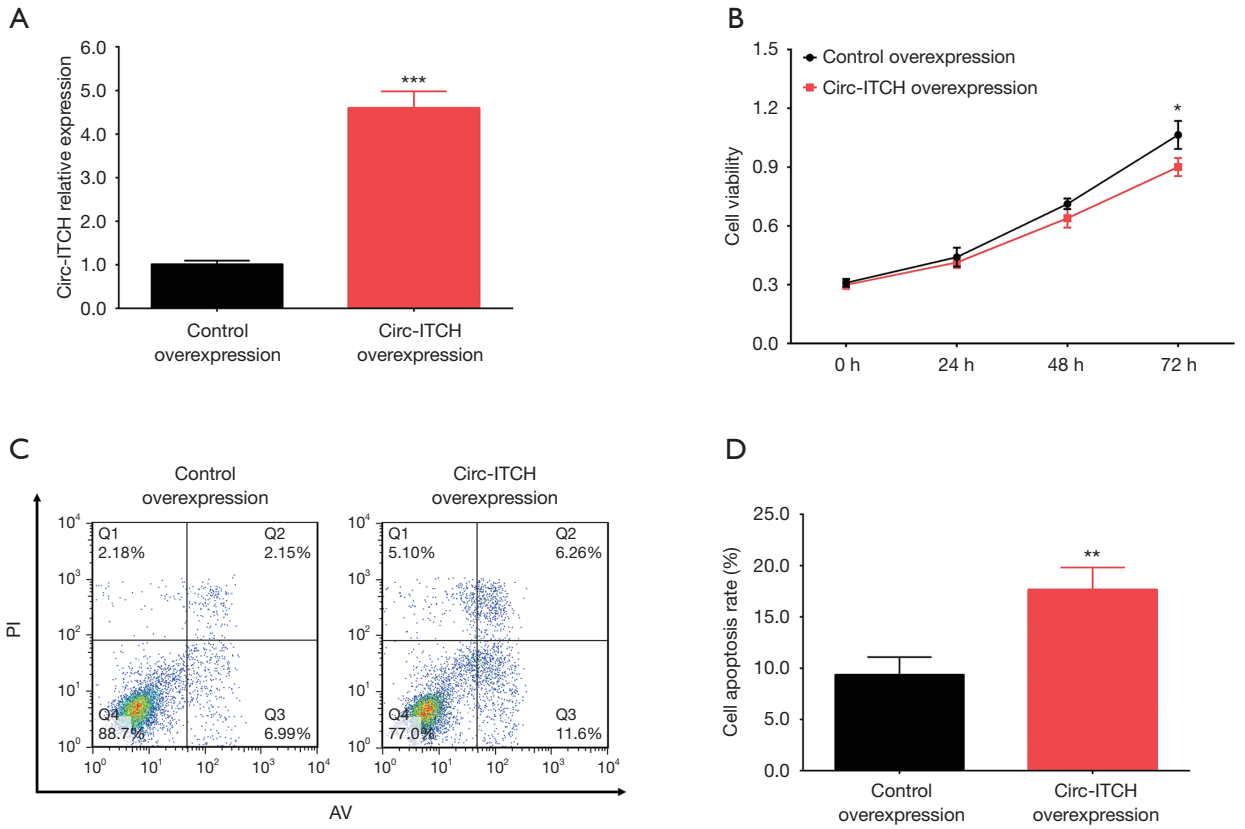

Figure 4 Cell proliferation and cell apoptosis in U226 cell line after plasmids transfection. (A) Circ-ITCH expression in circ-ITCH overexpression group and control overexpression group; (B) cell proliferation in circ-ITCH overexpression group and control overexpression group; (C,D) cell apoptosis in circ-ITCH overexpression group and control overexpression group. Comparison between groups was determined by independent samples *t*-test. P<0.05 was considered significant. Circ-ITCH, circular RNA itchy E3 ubiquitin protein ligase. ***P<0.001; **P<0.01; *P<0.05.

decreased ISS stage.

Regarding the predictive value of circ-ITCH for prognosis in cancers, several studies have shown that circ-ITCH has the potential to be a biomarker predicting cancer prognosis (12,21,22). For instance, a study discloses that circ-ITCH expression is positively associated with OS in bladder cancer patients (21). Also, a study shows that circ-ITCH expression is positively associated with OS in HCC patients (22). Besides, another study displays that circ-ITCH expression positively correlates with PFS as well as OS in glioma patients (12). Although these studies emphasize that circ-ITCH high expression is correlated with better survival profiles in several solid tumors and circRNAs have presented potential for predicting prognosis in various hematological malignancies, the related report about circ-ITCH in the prognosis of MM is rarely observed. In this present study, we found that circ-ITCH expression was positively associated with PFS and OS in MM patients, which might be due to: (I) circ-ITCH downregulated phosphorylated Dvl3 and inhibited the expression of oncogene c-myc to represses cell proliferation but promoted apoptosis, thereby it attenuated disease progression and further resulted in better PFS and OS in MM patients (10,15,16); (II) circ-ITCH might improve the sensitivity to treatment in MM patients and resulted in better treatment efficacy, therefore its high expression led to prolonged survival profiles.

Apart from these clinical practices about the role of circ-ITCH in MM patients, *in vitro* experiments were also performed in our study to further explore the effect of circ-ITCH overexpression on MM cell functions. And we observed that circ-ITCH expression was lower in several MM cell lines compared to normal BM mononuclear cells, besides, its overexpression inhibited cell proliferation but

promoted apoptosis in U226 cells, which were in line with the data from *in vitro* experiments of the previous studies (10,12,15,16,21). Our results contributed to deepening the understanding about the influence of circ-ITCH overexpression in MM cell lines, and expanded ideas for future studies that circ-ITCH might be a potential treatment strategy for MM.

In conclusion, circ-ITCH is under-expressed in MM patients, and its high expression correlates with lower ISS stage, longer PFS as well as better OS in MM, furthermore, circ-ITCH overexpression decreases cell proliferation but promotes apoptosis in MM.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.12.71). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Prior to the initiation, this study was approved by the Institutional Ethnic Committee of Xiangya Hospital (No. 201511298), and all participants provided the written informed consents.

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