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Original Article

CircCTNNA1 is Upregulated in Mantle Cell Lymphoma and Predicts Poor Survival by Sponging miR-34a to Increase Cell Proliferation

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Competing interests: The authors declare no conflict of Interest.

Abstract. ***Background*: It was reported that circular RNA (circRNA) circCTNNA1 plays an oncogenic role in colorectal cancer, while its role in mantle cell lymphoma (MCL) is unknown. This study aimed to explore the role of circCTNNA1 in MCL.**
***Methods*: Samples of B lymphocytes were collected from 56 MCL patients and 56 healthy controls. The expression of circCTNNA1 and miR-34a in these samples were determined by RT-qPCR. The direct interaction between circCTNNA1 and miR-34a in MCL cells was detected using RNA-RNA pulldown assay. Overexpression assays were performed to study the interactions between circCTNNA1 and miR-34a. Cell proliferation was assessed with BrdU assay.**
***Results*: The results showed that circCTNNA1 was upregulated in MCL and high expression levels of circCTNNA1 predicted the poor survival of MCL patients. MiR-34a was downregulated in MCL and inversely correlated with circCTNNA1. CircCTNNA1 was predicted to interact with miR-34a, and the interaction between them was confirmed by RNA pull-down assay. Interestingly, overexpression of circCTNNA1 and miR-34a did not affect the expression of each other. Cell proliferation analysis showed that overexpression of circCTNNA1 reversed the inhibitory effects of overexpression of miR-34a on cell proliferation.**
***Conclusion*: Upregulation of circCTNNA1 in MCL predicts poor survival of patients and it may sponge miR-34a to promote cancer cell proliferation.**

Keywords: Mantle cell lymphoma, circCTNNA1, miR-34a, Survival, Proliferation.

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Introduction. As a cancer develops in B-lymphocytes, mantle cell lymphoma (MCL) is a rare type of non-Hodgkin lymphoma that mainly affects patients older than 60 years old, and it is more common in men than that in women.[1-3] MCL affects about 1 case per 200, 000 people annually. In China, about 4.2 out of 100,000 people will develop MCL during their life time and more than half of these patients will die of MCL.[4] Although the clinical application of rituximab and novel agents, as well as the utilization of hematopoietic cell transplantation in the treatment of MCL have significantly increased the survival of patients, there is still no cure for MCL.[5-7] Actually, no more than 50% of MCL patients can survive more than 5 years after the initial diagnosis, and the 10-year overall survival rate is below 10%.[7] Therefore, more effective approaches are still needed to improve the outcomes of MCL treatment.

The development of novel anti-MCL approaches needs better elucidation of molecular factors involved in this malignancy.[7-11] Recent studies have identified several factors, such as the NF-κB signal pathway and CDK7, play critical roles in MCL and might serve as

potential therapeutic targets for the treatment of MCL.[9,11] Circular RNAs (circRNAs) are emerging novel players in human cancers that regulate cancer progression mainly by affecting gene expression.[12,13] Therefore, circRNAs are promising targets to treat cancers including MCL. CircRNA circCTNNA1 plays an oncogenic role in colorectal cancer,[14] while its role in MCL is unknown. We predicted that circCTNNA1 could interact with miR-34a, which plays critical roles in other types of B-cell lymphoma.[15] We therefore speculated that circCTNNA1 could interact with miR-34a to participate in MCL. This study was carried out to investigate the interaction between circCTNNA1 and miR-34a in MCL.

Materials and Methods.

MCL patients and survival analysis. This study enrolled a total of 56 MCL patients (34 cases of blastoid variant and 22 cases of pleomorphic variant; 63.4+/-6,6 years; 32 males and 24 females) and 56 healthy controls (63.5+/-6,5 years; 32 males and 24 females) at Zibo Central Hospital from May 2014 to May 2016. Ethics approval was obtained from the Ethics Committee of this hospital. Two groups of participants showed similar distributions of age and gender. The diagnosis of MCL was performed according to WHO classification of hematological neoplasms. Patients with leukemic phase MCL were excluded from this study. Fasting blood samples were collected from both groups of participants prior to the initiation of therapies. DETACHaBEAD CD19 Kit (Invitrogen) was used to separate B lymphocytes from all blood samples. Purity of higher than 90% was reached in all samples of B lymphocytes. All patients signed the informed consent. MCL patients were treated with chemoimmunotherapy R-CHOP, in which rituximab, cyclophosphamide, hydroxydaunomycin, vincristine sulfate and prednisone were included.

All 56 MCL patients were followed up for 5 years to evaluate the prognostic value of circCTNNA1 for MCL. Patients were visited through outpatient visit and telephone in some cases. All patients either died of MCL or survived until the end of follow-up. This study was conducted in accordance with the Declaration of Helsinki.

Cells and transfections. Two human MCL cell lines JVM-2 and Z138 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultivated in DMEM (GIBCO) containing FBS (10%), penicillin (50 units/mL) and streptomycin (50 mg/mL) in an incubator with temperature, humidity and CO_2 set to 37 °C, 95% and 5%, respectively.

Overexpression of circCTNNA1 and miR-34a was achieved in cells using Neon Electroporation Transfection (Thermo Fisher Scientific) to transfect circCTNNA1-pcDNA3.1 vector or miR-34a mimic. The dosages of vector and miRNA were 50 and 150 μM for 10^7 cells, respectively. The amount of vector and miRNA was blow 10% of the total volume.

RNA preparations. RNAstorm™ RNA Isolation Kit (Biotium) was used to extract total RNAs from both cultivated cells and tissue samples. DNase treatment was also included in this kit. All steps of RNA isolation and purification were carried out following the manufacturer's instructions. RNA concentrations were measured and RNA integrity was analyzed with Bioanalyzer. RNA samples with a RNA integrity number (RIN) higher than 8.5 were used in subsequent assays.

Reverse transcriptions and qPCR. Reverse transcriptions (RTs) were performed to prepare cDNA samples with 3 μg total RNAs as template using SSRT IV (Invitrogen). Briefly, a 13 μl mixture of RNA samples, primer (0.5 μl) and 10 mM dNTP (1 μl) were was incubated at 65 °C for 5 min, followed by incubation on ice for 2 min. After that, 1 μl RNaseout, 1 μl DTT, 1 μl reverse transcriptase and 4 μl 5x buffer were added to prepare a 20 μl mixture, which was then incubated at 23 °C for 5 min, 50 °C for 15 min and 80 °C for 10 min. cDNA samples were used as template to perform qPCRs (1 μl cDNA for 20 μl reaction system). The expression of circCTNNA1 and miR-34a were determined with 18S rRNA and U6 as the internal control, respectively. The $2^{-\Delta\Delta Ct}$ method was used to normalize Ct values.

RNA-RNA pulldown assay. A circCTNNA1 expression vector with T7 promoter was used to prepare circCTNNA1 and NC RNA *in vitro* transcripts using T7 reverse transcriptase (NEB). Both transcripts were labeled with biotin using Pierce™ RNA 3' End Biotinylation Kit (Thermo Fisher Scientific). The labeled RNAs were purified and named Bio- circCTNNA1 and Bio-NC, respectively. Neon Electroporation Transfection was used to transfect both labeled RNAs into cells, followed by cell culture for 48 h. After that, cell lysis was prepared to isolate RNA complex with magnetic beads. RNA samples were purified and quantified. RT-qPCR was then performed to quantify the expression levels of miR-34a.

BrdU incorporation cell proliferation assay. BrdU measurement was performed to analyze DNA synthesis, which directly reflects cell proliferation. Cell culture was performed in 96-well cell culture plates with 3 replicate wells set for each experiment. Cell culture was performed for 48 h, then 10 μM BrdU (Sigma-Aldrich) was added to culture medium, followed by incubation for 2 h. After incubation, peroxidase-coupled anti-BrdU-antibody (Sigma-Aldrich) was added, then peroxidase substrate (tetramethylbenzidine) was added at 60 min

later.

Statistical analysis. Mean ± standard deviation (SD) values of at least 3 biological replicates were presented. Shapiro-Wilk test was applied to test data distribution. Mann-Whitney test was used to explore differences with statistical significance between groups using GraphPad Prism software (GraphPad). The 56 patients were divided into high and low circCTNNA1 level groups (n = 28, median expression level of circCTNNA1 as the cutoff value). Survival curves were plotted and log-rank test was used to compare curves. Associations between patients' clinical features and the expression of circCTNNA1 were analyzed with Chi-squared test. Cox's proportional hazards regression was used for univariate and multivariate analysis. $p < 0.05$ was considered as statistically significant.

Results.

The expression of circCTNtNA1 in MCL and its prognostic value for this malignancy. The expression of circCTNNA1 in B lymphocytes samples of MCL patients and healthy controls were detected by performing RT-qPCRs. The results showed that the expression levels of circCTNNA1 were significantly increased in MCL patients (**Figure 1A**, $p < 0.01$). Survival analysis showed that the poor survival of MCL patients was closely correlated with patients' high expression levels of circCTNNA1 (**Figure 1B**). No obvious differences in treatment methods and drug dosages were observed between high and low level circCTNNA1 groups. It is worth noting that similar results were obtained using Youden index as cutoff value for grouping (data not shown). Chi-squared test showed that the expression levels of circCTNNA1 were not closely correlated with patients' age, gender, WBC, clinical stages, ECOG and serum LDH (**Table 1**). Univariate and multivariate analysis showed that circCTNNA1 was a significant prognostic factor for the overall survival of MCL patients (**Table 2**).

The expression of miR-34a in MCL and its correlations with circCTNNA1. The expression of miR-34a in B lymphocytes samples of MCL patients and healthy controls were also detected by performing RT-qPCRs. The results revealed significantly downregulated miR-34a in MCL compared to that in the Control group (**Figure 2A**, $p < 0.01$). Pearson's correlation coefficient showed that the expression of miR-34a was inversely correlated with circCTNNA1 across both MCL samples (**Figure 2B**) and the Control samples (**Figure 2C**).

CircCTNNA1 and miR-34a directly interacted with each other. The potential base pairing could be formed between circCTNNA1 and miR-34a was predicted using the online program IntaRNA 2.0. It was observed that circCTNNA1 and miR-34a could form strong base pairing (**Figure 3A**). RNA pull-down assay showed that compared to Bio-NC group, Bio-circCTNNA1 exhibited significantly higher expression levels of miR-34a (**Figure 3B**, $p < 0.01$). Therefore, circCTNNA1 and miR-34a could directly interact with each other.

The role of miR-34a and circCTNNA1 in regulating the expression of each other. Overexpression of miR-34a or circCTNNA1 were achieved in both JVM-2 and Z138 cells. It was observed that miR-34a and circCTNNA1 were significantly overexpressed from 24 h to 96 h (**Figure 4A**, $p < 0.05$). Interestingly, miR-34a showed no role in regulating the expression of circCTNNA1 (**Figure 4B**). Moreover, circCTNNA1 also did not alter the expression of miR-34a (**Figure 4C**).

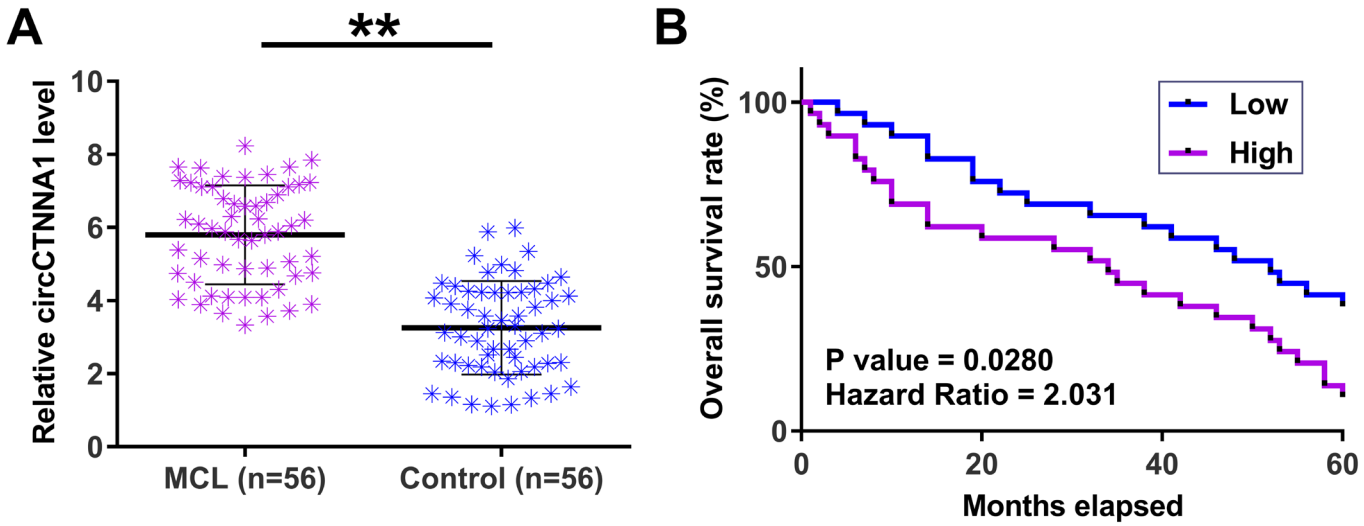

Figure 1 Analysis of the expression of circCTNNA1 in MCL and its prognostic value for this malignancy. Samples of B lymphocytes collected from MCL patients and healthy controls were subjected to RNA isolation, followed by performing RT-qPCRs to analyze the expression of circCTNNA1 (A). Prognostic value of circCTNNA1 for MCL was analyzed by performing survival analysis (B) **, $p < 0.01$.

Table 1. Associations between the expression of circCTNNA1 and patients' clinical features.

Clinical features	Cases	High (n = 28)	Low (n = 28)	Chi-square	*p*
Gender					
Male	32	15	17	0.29	0.59
Female	24	13	11		
Age (years)					
>= 60	40	18	22	1.4	0.24
< 60	16	10	6		
WBC					
>= 10 x 10^9/L	19	11	8	0.72	0.57
< 10 x 10^9/L	37	17	20		
Stage					
I or II	14	6	8	0.38	0.54
III or IV	42	22	20		
ECOG					
>= 2	36	19	17	0.31	0.58
< 2	20	9	11		
Serum LDH					
Normal	21	11	10	0.08	0.78
Elevated	35	17	18		

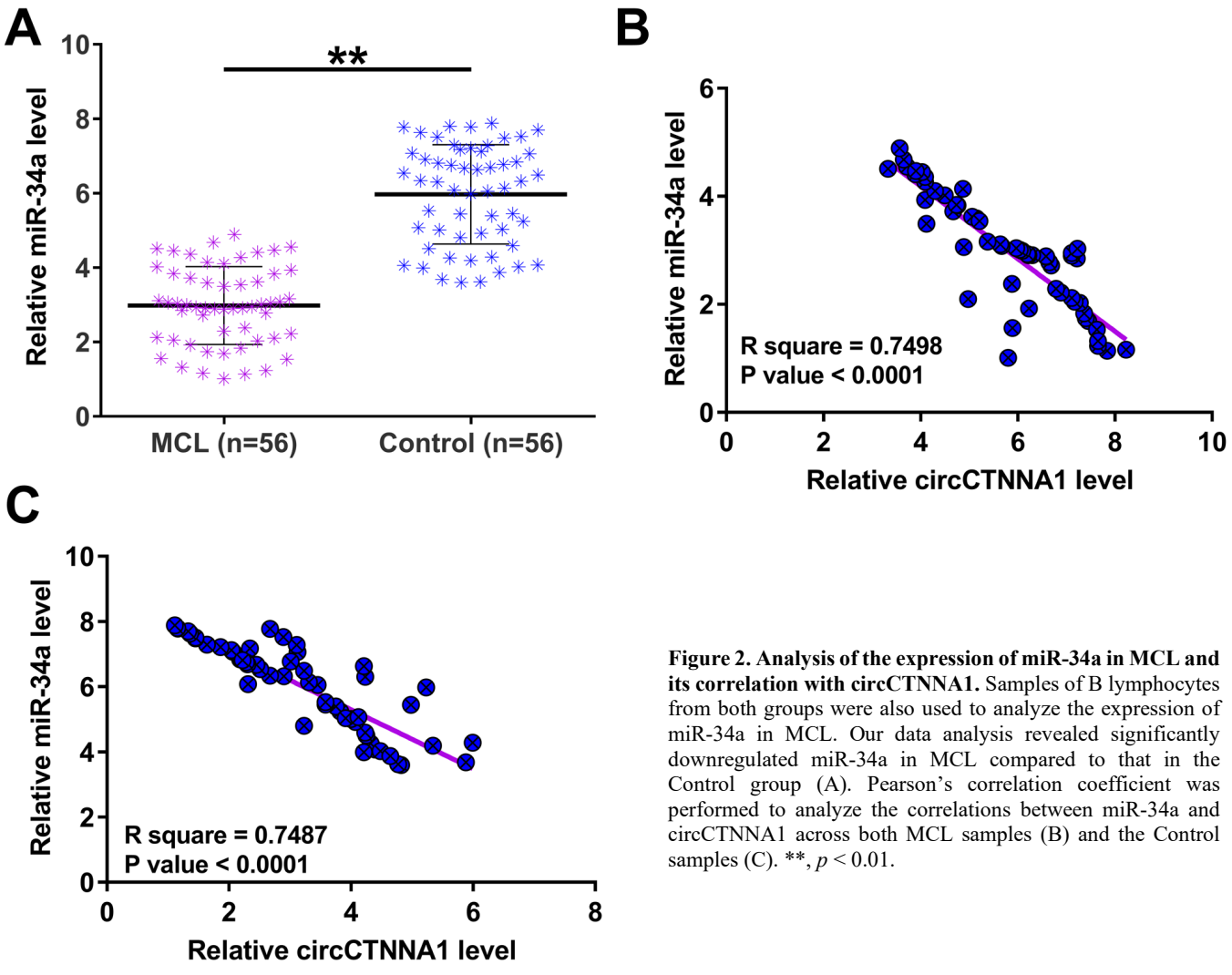

Figure 2. Analysis of the expression of miR-34a in MCL and its correlation with circCTNNA1. Samples of B lymphocytes from both groups were also used to analyze the expression of miR-34a in MCL. Our data analysis revealed significantly downregulated miR-34a in MCL compared to that in the Control group (A). Pearson's correlation coefficient was performed to analyze the correlations between miR-34a and circCTNNA1 across both MCL samples (B) and the Control samples (C). **, $p < 0.01$.

Table 2. Univariate and multivariate analysis of the MCL patients' overall survival.

Parameters	Univariate		Multivariate	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Gender, male vs. female	1.001 (0.701-1.788)	0.101		
Age (years), >= 60 vs.< 60	1.114 (0.866-1.601)	0.488		
WBC, > 10 x 10^9/l vs.<= 10 x 10^9/l	1.298 (0.801-1.872)	0.192		
Stages, III-IV vs. I-II	1.822 (1.601-2.204)	0.001	1.701 (1.399-2.121)	0.001
ECOG, ≥ 2 vs. < 2	0.882 (0.601-1.398)	0.103		
Serum LDH, elevated vs. normal	1.331 (0.782-1.768)	0.091		
circCTNNA1 expression, high vs. low	1.601 (1.234-1.891)	0.002	1.412 (1.102-1.781)	0.015

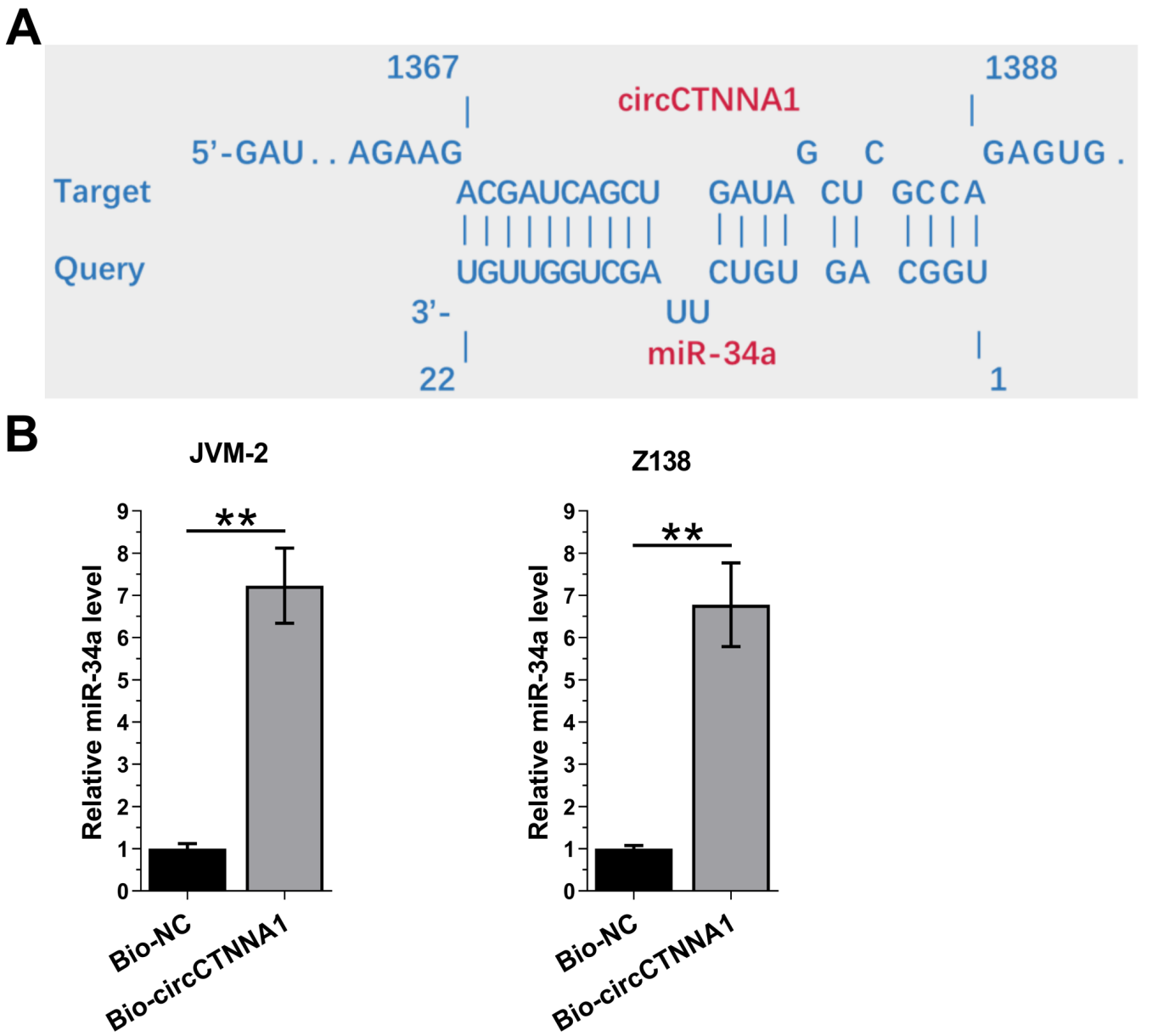

Figure 3. Analysis of the interaction between circCTNNA1 and miR-34a. The potential base pairing could be formed by circCTNNA1 and miR-34a was predicted with an online program IntaRNA 2.0 (A). RNA pull-down assay was performed to confirm the interaction (B). **, $p < 0.01$.

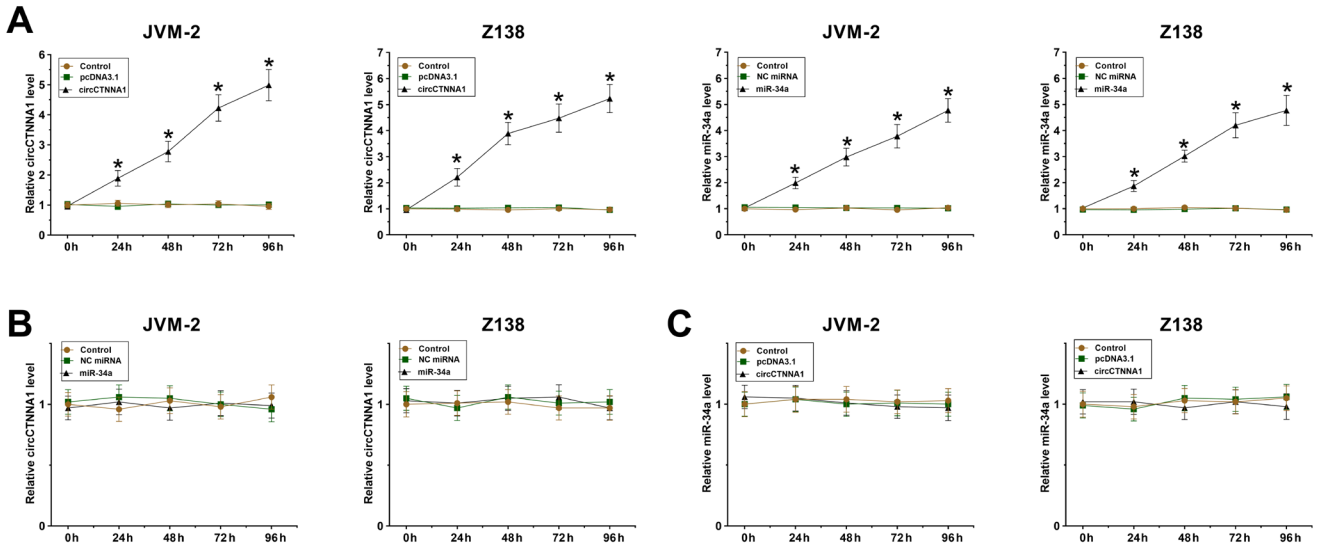

Figure 4. The role of miR-34a and circCTNNA1 in regulating the expression of each other. Overexpression of miR-34a or circCTNNA1 were achieved in both JVM-2 and Z138 cell lines. It was observed that miR-34a and circCTNNA1 were significantly overexpressed from 24 h to 96 h (A). The role of miR-34a in regulating the expression of circCTNNA1 (B) and the role of circCTNNA1 in regulating the expression of miR-34a (C) were analyzed with RT-qPCR. *, $p < 0.05$.

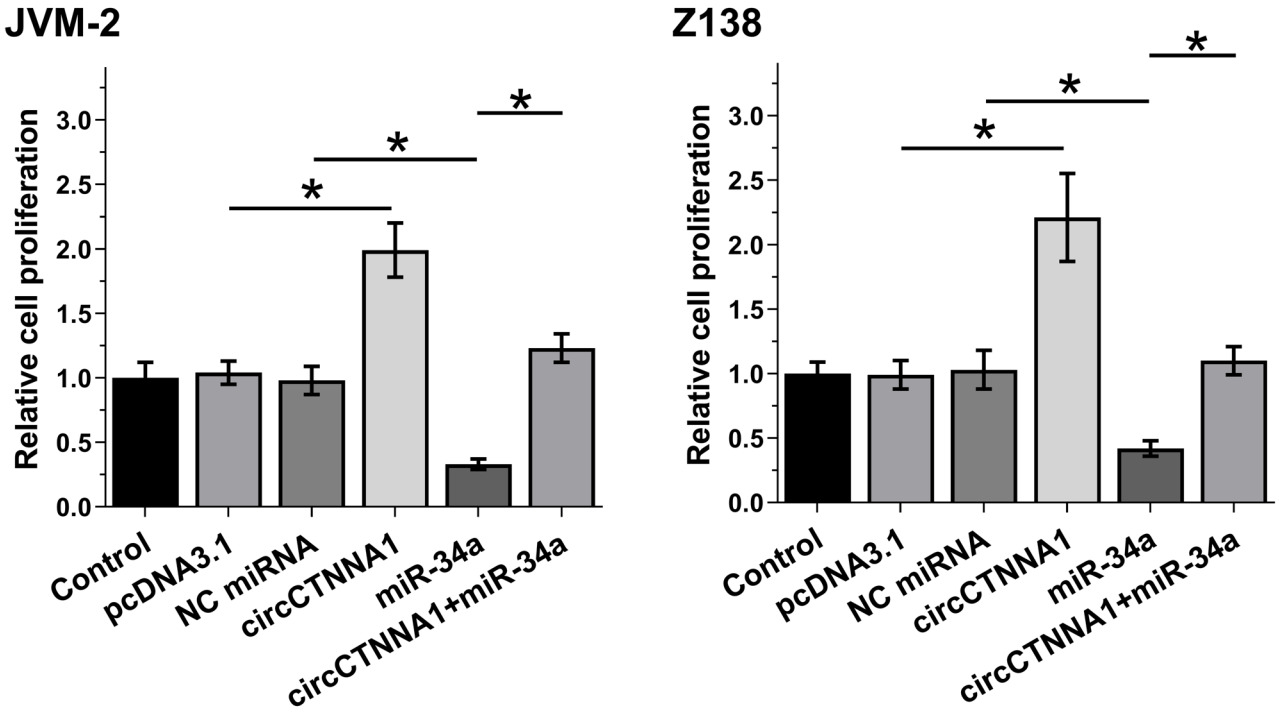

Figure 5. The role of circCTNNA1 and miR-34a in the proliferation of MCL cells. BrdU assay was performed to analyze the proliferation of MCL cells with overexpression of circCTNNA1 and/or miR-34a. *, $p < 0.05$.

The role of circCTNNA1 and miR-34a in the proliferation of MCL cells. BrdU assay was performed to evaluate the proliferation of MCL cells with overexpression of circCTNNA1 and/or miR-34a. It showed that miR-34a suppressed cell proliferation, while circCTNNA1 increased cell proliferation. Moreover, overexpression of circCTNNA1 totally reversed the inhibitory effects of overexpression of miR-34a on cell proliferation (**Figure 5**, $p < 0.05$).

Discussion. The present study explored the interaction between circCTNNA1 and miR-34a in MCL. We found that circCTNNA1 was highly expressed in MCL and it predicted the poor survival of MCL patients. Moreover, circCTNNA1 may sponge miR-34a to suppress its role in inhibiting MCL cell proliferation.

It has been well established that circCTNNA1 plays oncogenic roles in colorectal cancers.[14,16] Two previous studies showed that circCTNNA1 sponges miR-149-5p and miR-363-3p to upregulate FOXM1 and CXCL5, respectively, to promote the progression of MCL.[14,16] However, the prognostic value of circCTNNA1 for

human cancers remains unclear. The present study revealed upregulation of circCTNNA1 in MCL. In addition, overexpression of circCTNNA1 significantly increased the proliferation of MCL cells. Therefore, circCTNNA1 is likely an oncogenic circRNA in MCL. Moreover, our survival analysis showed that the high expression levels of circCTNNA1 were closely correlated with poor survival of MCL patients. Interestingly, the expression levels of circCTNNA1 in MCL patients were not correlated with their clinical features that may affect survival, such as clinical stages, age and WBC. Therefore, circCTNNA1 is likely an independent prognostic factor for MCL.

Although the involvement of miR-34a in MCL is unclear, its role in other types of B-cell lymphoma, such as diffuse large B-cell lymphoma, has been reported.[15] MiR-34a was downregulated in diffuse large B-cell lymphoma and overexpression of miR-34a increased the sensitivity of cancer cells to doxorubicin, thereby improving treatment outcomes. This study reported the decreased expression levels of miR-34a in MCL. In addition, decreased proliferation of MCL cells was observed after the overexpression of miR-34a. Therefore, miR-34a is a tumor suppressor in MCL. Interestingly, circCTNNA1 directly interacted with miR-34a, while they showed no role in regulating the expression of each other. Moreover, circCTNNA1 suppressed the role of miR-34a in inhibiting cancer cell proliferation. Therefore, circCTNNA1 may only sponge miR-34a, but not regulate its expression in MCL cells to promote cancer cell proliferation in MCL.

This study characterized a novel circCTNNA1/miR-34a pathway in MCL. However, expression of circCTNNA1 and the role of circCTNNA1/miR-34a pathway in other types of B-cell lymphoma is unclear. Future studies may focus on the involvement of circCTNNA1/miR-34a in other types of B-cell lymphoma.

Conclusions. In conclusion, circCTNNA1 is likely an oncogenic circRNA in MCL. It may promote MCL cell proliferation by sponging miR-34a. Moreover, measuring the expression levels of circCTNNA1 in MCL patients may assist the prognosis of MCL, thereby improving patients' survival.

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