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miR-330 regulates the proliferation of colorectal cancer cells by targeting Cdc42

Yuefeng Li [a,1], Xiaolan Zhu [b,1], Wenlin Xu [b], Dongqing Wang [a], Jinchuan Yan [a,*]

[a] The Affiliated Hospital of Jiangsu University, Zhenjiang, Jiangsu 212001, China
[b] The Fourth Affiliated Hospital of Jiangsu University, Zhenjiang, Jiangsu 212001, China

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ABSTRACT

MicroRNAs are small non-coding RNA molecules that play important roles in the multistep process of colorectal carcinoma (CRC) development. However, the miRNA–mRNA regulatory network is far from being fully understood. The objective of this study was to investigate the expression and the biological roles of miR-330 in colorectal cancer cells.

Cdc42, one of the best characterized members of the Rho GTPase family, was found to be up-regulated in several types of human tumors including CRC and has been implicated in cancer initiation and progression. In the present study, we identified miR-330, as a potential regulator of Cdc42, was found to be inversely correlated with Cdc42 expression in colorectal cancer cell lines. Ectopic expression of miR-330 down-regulated Cdc42 expression at both protein and mRNA level, mimicked the effect of Cdc42 knockdown in inhibiting proliferation, inducing G1 cell cycle arrest and apoptosis of the colorectal cancer cells, whereas restoration of Cdc42 could partially attenuate the effects of miR-330. In addition, elevated expression of miR-330 could suppress the immediate downstream effectors of Cdc42 and inhibit the growth of colorectal cancer cells in vivo.

To sum up, our results establish a role of miR-330 in negatively regulating Cdc42 expression and colorectal cancer cell proliferation. They suggest that manipulating the expression level of Cdc42 by miR-330 has the potential to influence colorectal cancer progression.

1. Introduction

MicroRNAs (miRNAs) are a class of small (~19–25nt), noncoding regulatory RNAs, transcribed from non-protein-coding genes or introns, which mediate translational suppression or cleavage of their target mRNAs by binding to complementary sites in their 3′UTR [1]. They play an essential role in cell cycle regulation, apoptosis and tumorigenesis. Aberrant miRNA expression profiles have been identified in tumors, as compared to normal tissues [2], thus establishing them as a relatively new and important class of oncogenes and tumor suppressors. One of these miRNAs, miR-330 was among the candidate miRNAs with potential tumor suppressor activity, evidence that miR-330 may suppress cell growth come from studies showing that miR-330 levels were much lower in tumor cell lines or primary tumors derived from prostate and lung tissue than in their respective normal tissues [3–5]. It also seemed to behave like a potential tumor suppressor in the central nervous system-derived tumor cells. Ruike et al. [6] performed a quantitative measurement of 155 types of mature human miRNAs in 16 human cell lines, they found that the expression of miR-330 was downregulated in many cancer cell lines. A recent study showed that miR-330 was significantly downregulated in colorectal carcinoma [5], suggesting a tumor suppressor role of miR-330 in colorectal carcinoma. It was reported level of miR-330 appeared to be inversely associated with the expression level of Cdc42 in breast cancer cells [7]. In this study, we aimed to determine the roles of miR-330 in the aggressiveness of colorectal cancer cells and study the regulatory mechanisms of miR-330 in colorectal cancer cells.

Cell division cycle 42 (Cdc42), as a member of Rho family of GTPases, belongs to the Ras superfamily [8]. It maps to 1p36.1 and encodes a 25-kDa protein [9]. Studies in primary gene targeted mammalian cells have shown that Cdc42 was critically involved in actin filopodia formation, cell motility, directional migration and cell growth [10]. Recently, Cdc42 has been shown to be up-regulated in several types of human cancers, including testicular cancer, colorectal cancer, breast cancer, head and neck carcinoma and melanoma [11–14]. The up-regulated Cdc42 activity may impair c-Cbl-mediated EGFR degradation, contribute to EGFR hyperactivity, and induce proteasomal degradation of p21CIP1, leading to an increase in cell proliferation and migration. These functional outcomes may be through regulation of PAK1, MLC, ERK1/2, and JNK pathways. In addition, suppression of Cdc42 signals can inhibit cell growth and induce apoptosis via the PI(3)K-Akt and Erk signaling cascades and the p53 tumor suppressor [15]. Given these di-

* Corresponding author.
E-mail address: jiangdalyf2009@126.com (J. Yan).
[1] These authors contributed equally to this work.

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verse functions and signaling events dependent on Cdc42, Cdc42 may contribute to multiple stages of tumorigenesis and tumor progression.

The current study was aimed at elucidating the regulatory mechanism of Cdc42 by miRNAs, and our data showed that miR-330 could authentically downregulate Cdc42 in colorectal cancer cells, moreover, the inhibition of cell proliferation and induction of G1 cell cycle arrest and apoptosis after re-expression of miR-330 was partially through suppression of Cdc42.

2. Materials and methods

2.1. Cell lines and cell culture

The colorectal cancer cells SW480, SW1116, WIDR, HT-29, Lovo were obtained from Shanghai Institute of Cell Biology, China Academy of Sciences (Shanghai, China). Cells were all cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, USA). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

2.2. Real-time quantification of miRNAs by stem–loop reverse transcription-polymerase chain reaction

Total RNA was extracted from the colorectal cancer cells using Trizol (Invitrogen), and the concentration of total RNA was quantitated by measuring the absorbance at 260 nm. The expression of mature miRNAs was assayed by stem–loop reverse transcription (RT) followed by real-time polymerase chain reaction (PCR). All reagents for stem–loop RT were obtained from Applied Biosystems (Foster City, USA). The relative amount of each miRNA was normalized to U6 snRNA. miR-330 RT primer: 5′-GTCGTATCC AGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCTCTGC-3′. miR-330 PCR primers are: forward:5′-CGGCAAAGCACA CGGCCTG-3′; reverse: 5′-TGCGTGTCGTGGAGTCGGC-3′. U6 RT primer is: 5′-TG GTGTCGTGGAGTCG-3′. U6 PCR primers are: forward: 5′-CTC GCTTCGGCAGCACA-3′; reverse: 5′-AACGCTTCACGAATTTGCGT-3′. The relative expression levels of each sample were measured using the $2^{-\Delta\Delta CT}$ method as described previously [16,17]. PCR was performed in triplicate.

2.3. miRNA transfection assay

The miR-330 mimic (miR-330), negative control miRNA mimic (NC:5′-UUCUCCGAACGUGUCACGUTT-3′) were chemically synthesized by Shanghai GenePharma Company (Shanghai, China). The colorectal cancer cells were plated in 6-well plates (5×10^5 cells/well) and transfected with 100 nM of the miR-330 mimic or negative control miRNA by Lipofectamine 2000 (Invitrogen, Long Island, USA) according to the manufacturer's protocol.

2.4. Cell cycle assay

After transfection for 72 h, cells were trypsinized. Cell pellets harvested by centrifugation were washed for twice with ice-cold PBS and fixed with ice-cold 70% ethanol for 48 h at 4 °C. The fixed cells were rehydrated in PBS and subjected to PI/RNase staining followed by fluorescence-activated cell sorter scan (FACS) analysis (Becton Dickinson, Mountain View, CA, USA). The percentage of cells in each phase of the cell cycle was estimated using ELITE software.

2.5. Apoptosis assay

Apoptosis was detected by flow cytometric (FCM) using Annexin V/FITC and PI apoptosis detection kit (Becton Dickinson, Franklin Lakes, USA). Briefly, adherent cells were harvested and suspended in the Annexin-binding buffer (1×10^6 cells/ml). Thereafter, cells were incubated with Annexin V-FITC and PI for 15 min at room temperature in the dark and immediately analyzed by FCM.

2.6. Cell growth assay

The colorectal cancer cells were seeded in 96-well plates 24 h before transfection. After transfection, the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was used to determine relative cell growth.

2.7. Western blot analysis

Recombinant human Cdc42 (PeproTech, Rocky Hill, USA) was added either alone or in combination with miR-330 mimic to colorectal cancer cells. Protein of treated cells was extracted by mammalian protein extraction reagent (Pierce, USA). Protein samples were resolved by 10% SDS–PAGE and then transferred to PVDF membranes. Protein levels were normalized to β-actin. The membranes were blocked and probed with antibodies against Cdc42, MLC, phospho-MLC (p-MLC), cyclin D1, Akt, phosphor-Akt (p-Akt) (Cell Signaling Technology, MA, USA), β-actin (Sigma). The secondary antibodies were purchased from Beyotime Ltd. (Haimen, China). Band detection via enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Pierce Biotechnology Inc., Rockford, USA).

2.8. Dual-luciferase activity assay

The Cdc42 3′UTR target site for miR-330 was amplified by PCR cloned into the XbaI site of pGL3 control (Promega, Madison, USA). This vector was sequenced and named Cdc42 3′UTR. Site-directed mutagenesis of the miR-330 target-site in the Cdc42 3′UTR was carried out using the Quick-change mutagenesis kit (Stratagene, Heidelberg, Germany) and named Mut-Cdc42 3′UTR. For reporter assays, the cells were transiently co-transfected with 800 ng Luciferase vector, including the Cdc42 3′UTR or Mut-Cdc42 3′UTR, and miR-330 mimic or mimic control at a final concentration of 50 nM by using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed by using the dual luciferase reporter assay system (Promega) 48 h after transfection.

2.9. siRNA knockdown

Cdc42 siRNAs (Si-Cdc42) were obtained from Qiagen Inc. (Valencia, CA), SW1116 cells were plated at a density of 3×10^4 -cells/well in 6-well plates. After 8 h, cells were transfected with 20 nM of validated siRNA specific to Cdc42. For controls, we transfected the cells with non-silencing control. The cells were prepared for next experiments 48 h after transfection. The transfection efficiency was evaluated by FCM by calculating the percentage of fluorescein-labeled cells.

2.10. In vivo tumor xenograft model

As transient miRNA expression system was simple and effective [18], Tumor growth studies using colorectal cancer xenografts were performed as previously described [19], to establish cancer model, SW1116 cells (9×10^7) were transfected with 200 nM miR-330 mimic or mimic negative control for 24 h before subcutaneous injection into the left and right flanks of 12 nude BALB/c

mice (SLAC Laboratory, Shanghai, China) respectively. 28 days after injection, all the mice were euthanized. The inhibitory effect of miR-330 on SW1116 tumors in nude mice was calculated using the following equation: inhibitory efficiency = [1 − tumor weight (experimental group)/tumor weight (control group)] × 100%.

2.11. Statistical analysis

Data were expressed as the mean ± SD from at least three independent experiments and compared using ANOVA by SPSS version 12.0 software (SPSS, Chicago, IL, USA). All *P* values were two-sided and a value of <0.05 was considered to be statistically significant.

3. Results

3.1. Expression of miR-330 was inversely correlated with Cdc42 levels in colorectal cancer cell lines

To establish functional association between Cdc42 and miR-330 in colorectal cancer, the expression levels of miR-330 and Cdc42 in five colorectal cancer cell lines were detected respectively. As showed in (Fig. 1A) the colorectal cancer cell lines (Lovo, WIDR, SW1116, HT-29) had low endogenous miR-330 levels, a relatively high level of Cdc42 protein was observed, whereas cell lines with relatively high miR-330 expression (SW480) showed significantly lower amounts of Cdc42 protein (Fig. 1A and B). These data from the available cell lines suggest a reciprocal relationship between levels of miR-330 and Cdc42 protein expression in human colorectal cancer cells, justifying a further examination of the role of miR-330.

3.2. Cdc42. is a direct target gene of miR-330

From the earlier observations, we examined whether miR-330 could functionally affect Cdc42 expression. Using the algorithms for target gene prediction, including PicTar, TargetScan, and miRanda, Cdc42 was identified as one of the potential targets of miR-330. To further confirm that Cdc42 was a direct target of miR-330, we constructed a luciferase reporter vector with the putative Cdc42 3′UTR target site for the miR-330 downstream of the luciferase gene. SW1116 cells transiently transfected with the Cdc42 3′UTR-reporter construct and miR-330 mimic led to a significant decrease of reporter activity when compared with the control (Fig. 2C) ($P < 0.05$). The activity of the reporter construct that car-

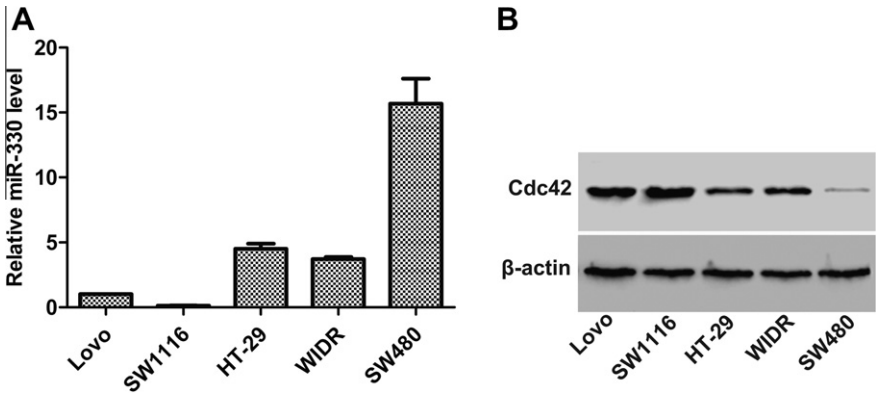

Fig. 1. miR-330 and Cdc42 expressions were inversely correlated in colorectal cancer cell lines. (A) qRT-PCR analysis for miR-330 expression in 5 colorectal cancer cell lines. (B) Expression levels of Cdc42 in 5 colorectal cancer cell lines analyzed by Western-blot accordingly.

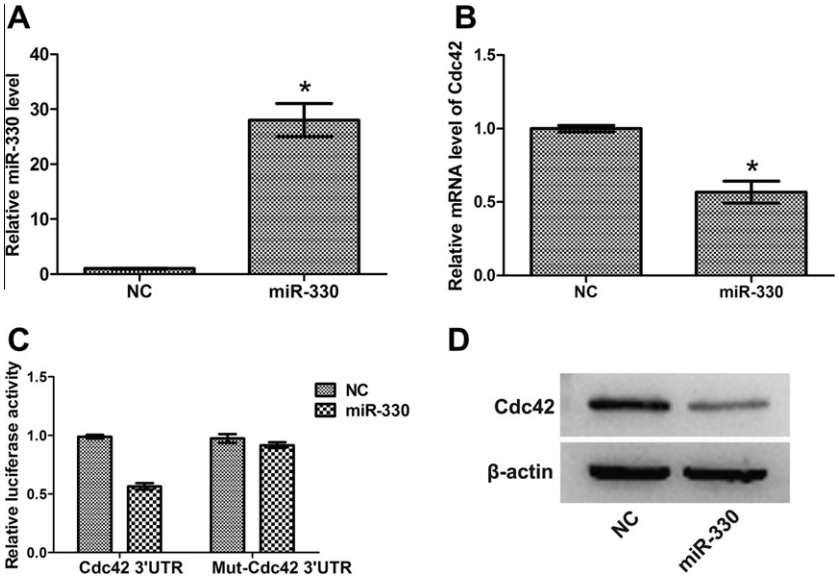

Fig. 2. Cdc42 is a putative target of miR-330. (A) qRT-PCR analysis of miR-330 in SW1116 cells transfected with miR-330 or control oligo. The miR-330 expression was normalized to U6 expression, the data were shown as a ratio of miR-330 cells to control oligo-transfected cells. (B) The mRNA expression of Cdc42. (C) The luciferase reporter assay showed a significant decrease in Cdc42 3′UTR relative luciferase activity in mimic-transfected SW1116 cells. (D) miR-330 regulated the protein level of Cdc42. *Indicates a significant difference from control oligo-transfected control cells ($P < 0.05$).

ried a mutated Cdc42 3′UTR was unaffected by a simultaneous transfection with miR-330. Further, as showed, the elevated miR-330 could significantly reduce both mRNA and protein expression levels of Cdc42 (Fig. 2B and D). This was similar to that caused by Si-Cdc42 transfection. The results suggest that there is a functional target site of miR-330 in the Cdc42 3′UTR.

3.3. Ectopic miR-330 expression alters colorectal cancer cells growth, cell cycle and apoptosis by down regulation of Cdc42

Cdc42 has been found up-regulated in colorectal cancer cells, and the overexpression of Cdc42 was associated with carcinogenesis and progression of colorectal carcinoma, and it was also known to play a key role in the regulation of cell cycle progression at the G1/S transition [20,21]. The frequent downregulation of miR-330 in colorectal carcinoma [5], and its inhibitory action on Cdc42 implied that miR-330 may have a role in colorectal carcinoma carcinogenesis by down regulation of Cdc42. To prove this, we tested whether the cell growth potential of colorectal cancer cells transfected with miR-330 was inhibited as a consequence of Cdc42 expression suppression. As showed in (Fig. 3A), the relative expression of Cdc42 was markedly decreased in miR-330 mimic transfected and Si-Cdc42 transfected cells compared with control cells, we also evaluated Cdc42 level by the addition of recombinant human Cdc42 to mimic-transfected SW1116 cells, the downregulation of Cdc42 by miR-330 was impaired by the Cdc42 restoration (Fig. 3B). Then, the effect of ectopic expression of miR-330 on cell growth was investigated in SW1116 cells. As shown in (Fig. 3C), the increased expression of miR-330 significantly inhibited the growth of SW1116 cells ($P < 0.05$). Analysis of cell cycle phase distribution by cytometry showed that compared with negative control group, the cell cycle progression of SW1116 cells transfected with miR-330 mimics or Si-Cdc42 were arrested at G1 phase with a significant decrease in S phase. (Fig. 3D) ($P < 0.05$). To further understand the role of miR-330 on colorectal cancer cells, we sought to evaluate whether the effect of cell growth was related to apoptosis. SW1116 cells were stained positively with Annexin V as determined by FCM, 31.1 ± 2.0% of miR-330 mimic transfected cells, 26.3 ± 2.9% of Si-Cdc42 and 7.4 ± 0.9% of miR-330 + Cdc42 transfected cells were stained positively with Annexin V as determined by FCM, and there were no significant changes in control cells (Fig. 3E) ($P < 0.05$). With the restoration of Cdc42 in mimic-transfected cells, the role of miR-330 overexpression in SW1116 cells was eliminated (Fig. 3C–E). These results indicate that miR-330 could functionally reduce cell proliferative potential by mimicking Cdc42 reduction, suggestting the role of miR-330 as a negative regulator of Cdc42 regulates cell growth in colorectal cancer cells.

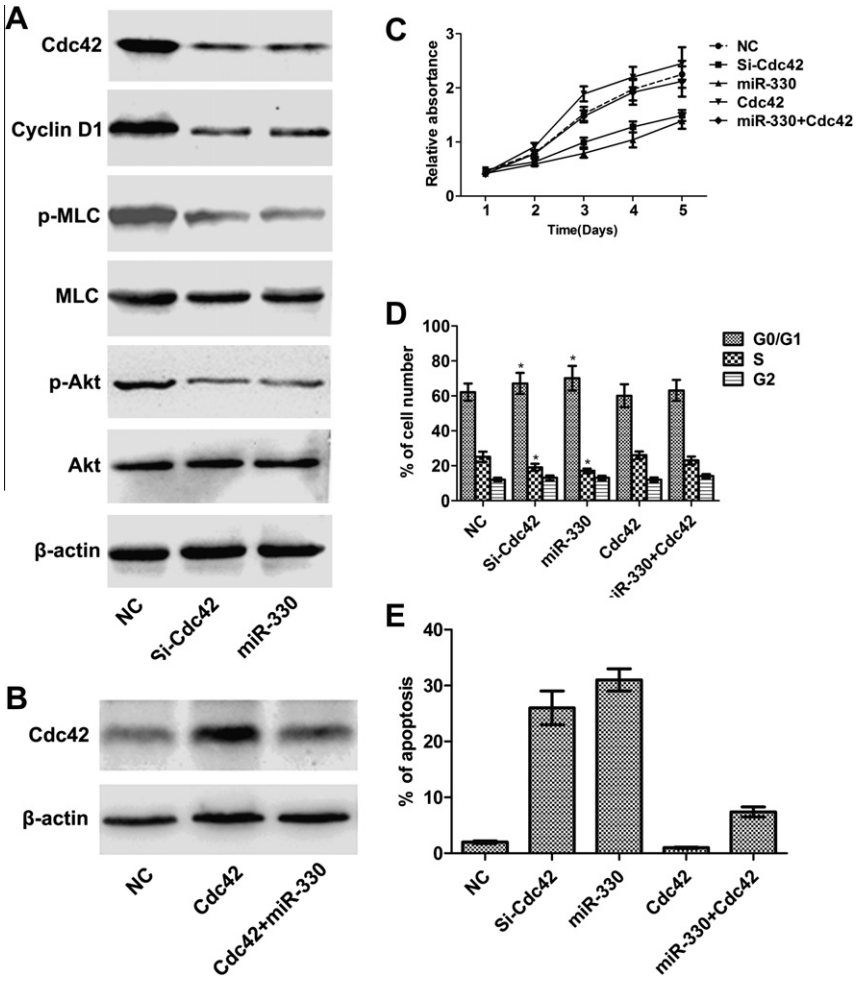

Fig. 3. Ectopic miR-330 expression alters colorectal cancer cells growth, cell cycle and apoptosis by downregulation of Cdc42. (A) Western-blot analysis of SW1116 cells transfected with miR-330, Si-Cdc42 or control oligo for Cdc42, Akt, MLC, cyclin D1, p-Akt, p-MLC. Overexpression of miR-330 suppressed downstream signals of Cdc42 similarly to that by Cdc42 knockdown. (B) The restoration of Cdc42 could rescue Cdc42 expression that was down-regulated by miR-330 mimic transfection. (C) Proliferation potential of SW1116 cells after transfection. (D) The cell cycle progression of SW1116 cells after transfection. (E) The apoptotic percentage of SW1116 cells after transfection. *Indicates a significant difference from control oligo-transfected control cells ($P < 0.05$).

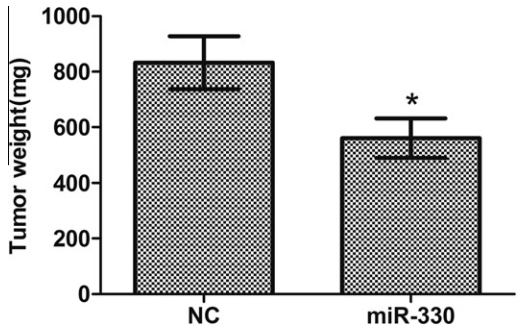

Fig. 4. Inhibitory effect of miR-330 on the growth of SW1116 cells in vivo. Nude mice were injected with SW1116 + miR-330 (miR-330) or SW1116 + miR-NC (NC) cells. The tumor was collected and weighted after 28 days. Data were presented as tumor weight (in milligrams) in the means ± SD ($n = 12$). $^{*}P < 0.05$.

3.4. Ectopic expression of miR-330 suppresses the downstream effectors of Cdc42

To determine whether the downstream signals of Cdc42 were affected by miR-330, SW1116 cells were transfected with miR-330, Si-Cdc42 or control oligo, the expression levels of Cyclin D1, phosphorylated and total Akt, MLC were analyzed. As showed in (Fig. 3A), the phosphorylation levels of PAK1, MLC, ERK1/2 and the expression of Cyclin D1, the known downstream signals of Cdc42, were markedly decreased by miR-330 and Si-Cdc42, suggesting that the biological function of miR-330 in the cancer cell proliferation suppression is likely, at least in part, through the downregulation of Cdc42.

3.5. Effect of miR-330 expression on SW1116 tumorigenicity

The average tumor weight of mice injected with SW1116 + mimic-NC cells at day 28 was 832.72 ± 95.51 mg, while that of the mice inoculated with SW1116 + miR-330 cells was 560.33 ± 71.89 mg, which were significantly lower than that of controls, with an inhibitory efficiency of 32.7% ($P < 0.05$) (Fig. 4).

4. Discussion

Colorectal cancer (CRC) is a significant health burden worldwide [22]. Despite the significant advancements in treatment options, improvements in CRC patient survival have been limited owing to lack of early detection and limited capacity for optimal therapeutic decision-making [23], CRC is still one of the most common causes of cancer-related mortality [24]. Biomarkers to improve CRC diagnosis, prognosis and prediction of treatment response therefore represent opportunities to improve patient outcome [25–27]. A recent surge in investigation of epigenetic biomarkers such as miRNA expression, have demonstrated that these alterations may be enticing translational biomarker candidates in CRC. The first report of dysregulated miRNAs in CRC was published in 2003, of which they discovered the down-regulation of miR-143 and miR-145 in CRC tumor tissues compared to the normal controls [28]. Subsequently, several miRNAs were found to be consistently deregulated in CRC specimens, such as overexpression of miR-20, miR-21, miR-31, and miR-99b, and the reduction of miR-143, miR-145, and miR-192 [29,30]. Recent studies showed that miR-330 decreased in colorectal tissue than in respective normal tissue [5], suggesting miR-330 a tumor suppressor gene, however, the expression level and downstream target genes of miR-330 as well as its biological roles in colorectal cancer are still unknown.

Cdc42, as a number of the Rho family of GTPases represents a class of Ras-related signaling molecules often deregulated in cancer. In its active form, the protein binds to a number of effectors molecules, activating signaling cascades which regulate a variety of cellular processes including cytoskeletal reorganization, cell cycle progression, cell polarity and transcription [9,31–33].

In this study, we found that miR-330 and Cdc42 expressions were inversely correlated in colorectal cancer cell lines. Of course, as the number of available colorectal cancer cell lines was limited and these cultured cell lines cannot stand for all subtypes of colorectal tumor, a more systemic study using clinical colorectal cancer samples is required to define the correlation between the endogenous expression level of Cdc42 and that of miR-330, however, it provided a possibility that the loss of miR-330 may be involved in the dysexpression of Cdc42 in colorectal tumorigenesis. Further studies confirmed that the restoration of miR-330 could efficiently reduce the expression level of Cdc42. By a luciferase-based reporter assay, we showed that one predicted miR-330 targeting site in the Cdc42 3′UTR was functional. By restoring miR-330 expression in colorectal cancer cells, we indeed showed that miR-330 suppressed cell growth, induced G1 cell cycle arrest and apoptosis, suggesting a tumor-suppressive role of miR-330 in colorectal cancer cells. Additionally, we found that inactivation of Cdc42 by siRNA was similar to the values obtained from ectopic expression of miR-330, and the restoration of Cdc42 can eliminate the effects of miR-330 overexpression. Furthermore, ectopic expression of miR-330 suppressed the downstream effectors of Cdc42: PAK1, MLC, ERK1/2 and Cyclin D1. The data suggest that miR-330 impacts on colorectal cancer cells partially by inactivation of Cdc42.

In conclusion, our data suggested that the oncogene Cdc42 was negatively regulated by miR-330 through a specific target motif at the Cdc42 3′UTR. Furthermore, elevated miR-330 induced apoptosis, G1 cell cycle arrest and antiproliferation in colorectal cancer cells. These data, together with our data from colorectal tumor xenograft model, may support a strategy for targeting with the miR-330/Cdc42 interaction or rescuing miR-330-expression as a new therapeutic application to treat colorectal cancer patients in the future.

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