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*Original Article*

# MicroRNA-335 targets the MEK/ERK pathway to regulate the proliferation and metastasis of colon cancer

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**Abstract:** Several studies have proved the tumor-suppressive effects of miR-335 but its role in colon cancer via regulation of the Raf/MEK/ERK signalling pathway is yet unknown. As such the main motive of conducting the present study was to elucidate the role of miR-335 in colon cancer via regulation of Raf/MEK/ERK signalling pathway and to explore its therapeutic potential. The results revealed significant ($P < 0.05$) downregulation of miR-335 in colon cancer and its overexpression led to a significant ($P < 0.05$) decline in viability of the HT-29 and SW948 cells. The TUNNEL assay showed miR-335 promotes apoptosis in the HT-29 and SW948 colon cancer cells and is also associated with increase in Bax and decrease in Bcl-2 expression. The results also revealed that miR-335 overexpression enhances the sensitivity of the HT-29 and SW948 cells to the apoptotic effects of cisplatin. From the transwell assays, it was found that the migration of the HT-29 and SW948 cells was decreased by 53% and 45% and while as invasion was decreased by 49% and 42% respectively ($P < 0.05$). Finally, western blot analysis showed that miR-335 blocks the Raf/MEK/ERK signalling pathway in HT-29 colon cancer cells. The results of in vivo study showed that miR-335 also exhibits tumor-suppressive effects on xenografted tumors. Taken together, it is concluded that miR-335 acts as tumor-suppressor in colon cancer and may exhibit therapeutic implications in its treatment.

**Keywords:** Colon cancer, microRNA-335, viability, invasion, apoptosis, TUNNEL assay, migration, expression

## Introduction

Colon cancer is responsible for around a million deaths annually across the world. Currently, it is considered as the 3rd prevalent cancer and 4th main reason for cancer associated deaths [1]. Annually, around two million new colon cancer patients are added to the list worldwide. Although a decline has been observed in colon cancer over the last few decades but owing to changing lifestyle and environmental factors, the colon cancer incidence is expected to increase significantly in the coming years [2]. According to reports, it is believed that the colon cancer incidence will increase by more than sixty percent [3]. Different treatment regimens are being used for the treatment of colon cancer, but late diagnosis and regular relapses adds to the hurdles faced in colon cancer treatment. It is believed that identification of biomarkers will enable the early diagnosis of the disease [4]. Additionally, the selection of the molecular therapeutic targets for drugs will enable efficient treatment of the disease with targeted therapy [5]. MicroRNAs (miRs) are non-coding RNA species consisting of about 20 nucleotides which regulate the expression of about 30% of protein coding gens in humans through post-transcriptional gene silencing or degradation of their mRNA transcripts [6, 7]. Crucial biological processes like cellular metabolism, differentiation and development, cell death, autophagy, cell cycle, metastasis etc are regulated by miRs [8]. For their profound regulatory potential in controlling the growth and tumorigenesis of human cancers, miRs are valued as therapeutic molecules of choice against many types of human cancers [9]. MicroRNA-335 (miR-335) has been shown to be involved in the regulation of different cancer related processes [10, 11]. However, the role of miR-335 in colon cancer development

via regulation of the Raf/MEK/ERK signalling has not been studied so far. Therefore, this study was undertaken to analyse the role of miR-335 in colon cancer growth and metastasis via modulation of Raf/MEK/ERK signalling. We strongly believe that study will form basis for the development of miR-335 as therapeutic target for colon cancer.

## Materials and methods

### *Cell lines and culture conditions*

The normal colon CCD-18Co and the colon cancer cell lines (HT-29, SW-948, RKO and SW480) were procured from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640; Gibco, Carlsbad, CA, USA) medium supplemented with 10% foetal bovine serum and 0.2% penicillin and streptomycin (Invitrogen, Carlsbad California, United States). All cells were cultured in a 5% $CO_2$ incubation chamber at 37°C.

### *Expression analysis*

The RNA was extracted from the transfected HT-29 and SW948 cells using a Trizol regent and then the RevertAid cDNA synthesis kit was employed to synthesize complementary DNA (cDNA). The relative expression was evaluated through quantitative real time polymerase chain reaction (qRT-PCR). The 20 µl PCR reaction mixture consisted of dNTPs (200 µM) $MgCl_2$ (1.5 mM), *Taq* DNA Polymerase (2.5 units), primers (0.2 µM each) and cDNA (0.5 µg). The parameters of PCR cycling were: 95°C for 30 sec, followed by 40 cycles of 95°C for 15 sec, and 58°C for 30 sec. U6 snRNA and GAPDH were used as internal controls and ΔΔCT methodology used for relative quantification as previously described [12].

### *Transfection*

The transfection of miR-NC and miR-335 mimics was carried out using Lipofectamine 2000 (Invitrogen) according to manufacturer instructions.

### *TUNEL assay*

TUNEL assay was carried out to detect apoptosis with the help of a commercial kit (Promega, Madison, WI) by following the user guidelines as described earlier [13].

### *Cell viability assay*

The transfected HT-29 and SW948 cells ($2.5 \times 10^3$) were cultured in each 96 well plates for 24 h at 37°C. Following an incubation of 3 days, the cells were subjected to treatment with 25 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The media was removed after about 4 h and addition of 170 µl Dimethyl sulfoxide (DMSO) was used for dissolving the formazan crystals. At the end, the absorbance readings were recorded at the wavelength of 570 nm for the quantitative estimation of HT-29 and SW948 cell viability.

### *Migration and invasion assay*

The Matrigel fitted transwell chambers were used for the quantitative assessment of the invasion of respective transfected cancer cells. In brief, the upper chamber of transwell was supplied with approximately 6000 transfected cells suspended in 100 µL while 750 µl of Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% FBS were poured into the lower chamber. After incubation of 48 h at 37°C, the cells from the upper surface of the intervening membrane were removed with care with the help of cotton swabs while the ones sticking to the membrane's lower side were fixed with 70% ethyl alcohol and 0.1% crystal violet stain was applied to them. Light microscopy (× 100) was used for visualizing the stained cells and photographs were obtained. The invasion was ascertained through manual counting from at least seven randomly selected microscopic fields. Cell migration was assessed by the same methodology except that Matrigel was not used.

### *Western blotting*

The protease inhibitor chilled hypotic buffer was used for the digestion of HT-29 and SW948 cells. The protein count of the CAMA-1 cell lysates was quantified by BCA assay. Exactly the same protein concentrations were resolved on SDS-PAGE from each cellular fraction. PAGE gels were blotted to nitrocellulose membranes and the latter were given an exposure of theprimary antibodies at 25°C for 55 min. Afterwards, the membranes were given

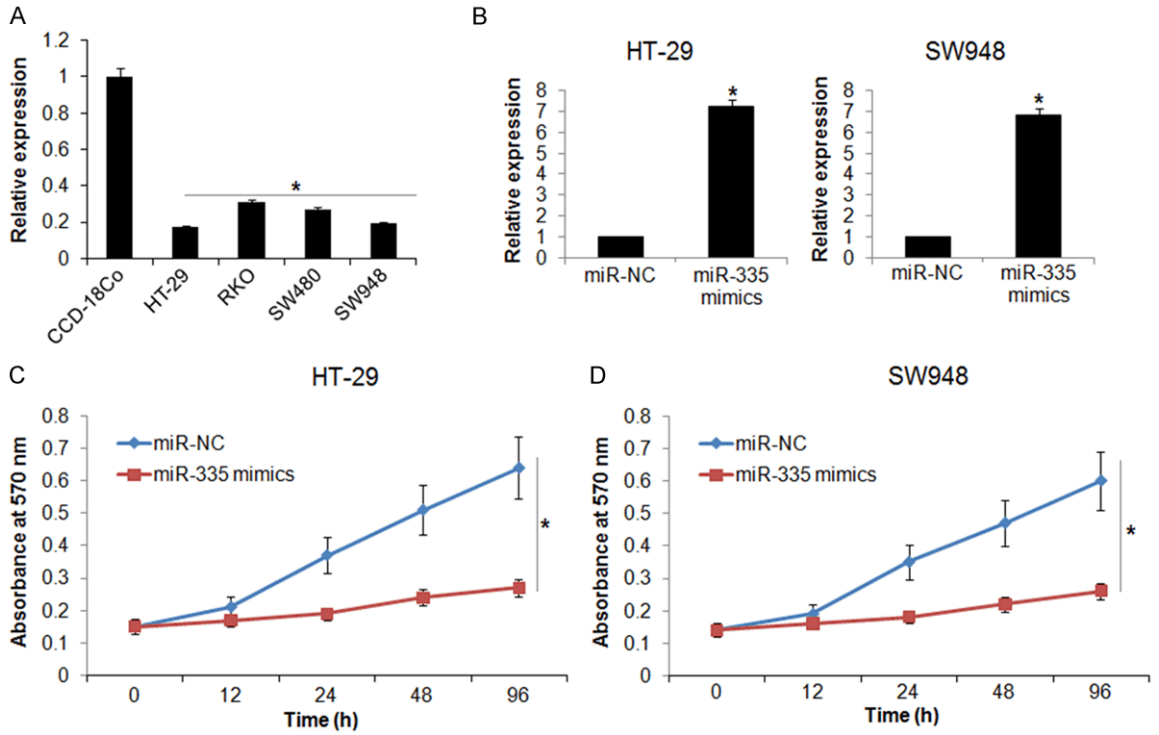


**Figure 1.** The miR-335 affects the colon cancer cell growth. A. Relative expression of miR-335 in normal and colon cancer cell lines. B. Expression of miR-335 in miR-NC and miR-335 mimics transfected HT-29 and SW948 cells. C. Viability assessment of miR-NC and miR-335 mimics transfected HT-29 cells. D. Viability assessment of miR-NC and miR-335 mimics transfected SW948 cells. Three replicas were used per experimental set up and expressed as mean ± SD (*P < 0.05 for miR-NC Vs miR-335 mimics).

the secondary antibody treatment. The visual detection of specific protein bands was made with the help of chemi-luminescence reagent.

*In vivo xenograft study*

The 18 ± 2 g nude mice (4-week-old) were procured from animal house of Harbin Medical University, Harbin China. For the maintenance of animals, well ventilated rooms were used, having an adjustable environment of a day: night, light/dark cycle with temperature of 28 ± 2°C. The mice were randomly divided into two groups ($n$ = 15/group). The animal study was approved by the animal ethics committee of the institute under approval number HMU/C55T/2019. The animals were randomly distributed into two experimental groups. The miR-NC group were injected with miR-NC transfected HT-29 cells and the miR-335 mimics group were injected with miR-335 mimics transfected HT-29 cells in the flanks. The tumor size of the mice was measured after every $4^{th}$ day using the formula, tumor volume ($mm^3$) = 0.5 × Length × $width^2$. On day 34, the tumor weight was determined after sacrificing the mice.

*Statistical analysis*

The present data was compiled by using three independent experimental replicates. The dat was presented as mean ± SD. Student's t-test was used for analysing the statistical difference between two data sets while the multi-set data groups were analysed through one way analysis of variance and Tukey's test The statistical tests were performed on GraphPad Prism software (version 7; GraphPad Software, Inc., La Jolla, CA, USA). A value of $P < 0.05$ was taken as statistically significant.

## Results

*Downregulation of miR-335 in colon cancer*

The study began with the analysis of gene expression of miR-335 in the colon cancer cell lines and normal cells by qRT-PCR. The qRT-PCR results were processed through ΔΔCT

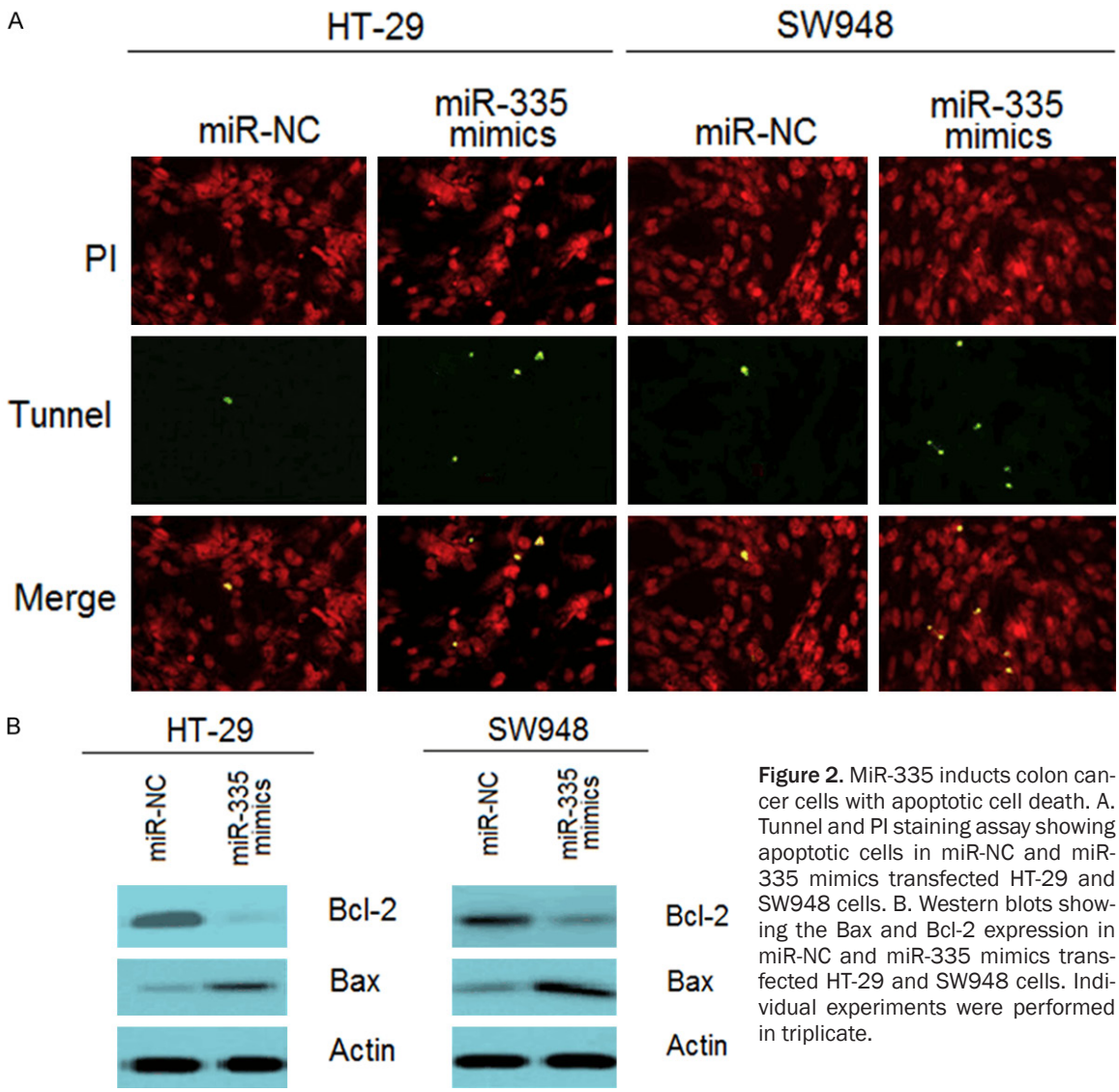


**Figure 2.** MiR-335 inducts colon cancer cells with apoptotic cell death. A. Tunnel and PI staining assay showing apoptotic cells in miR-NC and miR-335 mimics transfected HT-29 and SW948 cells. B. Western blots showing the Bax and Bcl-2 expression in miR-NC and miR-335 mimics transfected HT-29 and SW948 cells. Individual experiments were performed in triplicate.

methodology which revealed that all the cancer cell lines express significantly ($P < 0.05$) lower transcript count of miR-335 when compared with those of the normal cells (**Figure 1A**). The upregulation was upto more than 5.5 folds. Subsequently, the HT-29 and SW948 cells which showed lowest transcript levels of miR-335 were employed for further experimentation.

*Inhibition of cell viability of colon cancer cells by miR-335*

Next, the expression of miR-335 enhanced in HT-29 and SW948 cells via transfection with miR-335 mimics. The expression of miR-335 in HT-29 and SW948 cells increased by 7.2 and 6.8 folds upon miR-335 over-expression (**Figure 1B**). MTT assay showed that enhancement of the miR-335 transcription in the HT-29 and SW948 cells, caused a significant ($P < 0.05$) diminishment of cell viability (**Figure 1C** and **1D**). To ascertain the underlying mechanism, TUNNEL assay was performed. It was observed that enhancement of the miR-335 expression resulted in apoptosis of the HT-29 and SW948 cells (**Figure 2A**). The expression of the Bax and Bcl-2 was also examined in the HT-29 and SW948 cells. It was observed that Bax protein was increased in both HT-29 and SW948 cells while as Bcl-2 was decreased (**Figure 2B**).

*Enhancement of chemosensitivity of colon cancer cells by miR-335*

The effects of the enhancement of the miR-335 expression in HT-29 and SW948 cells

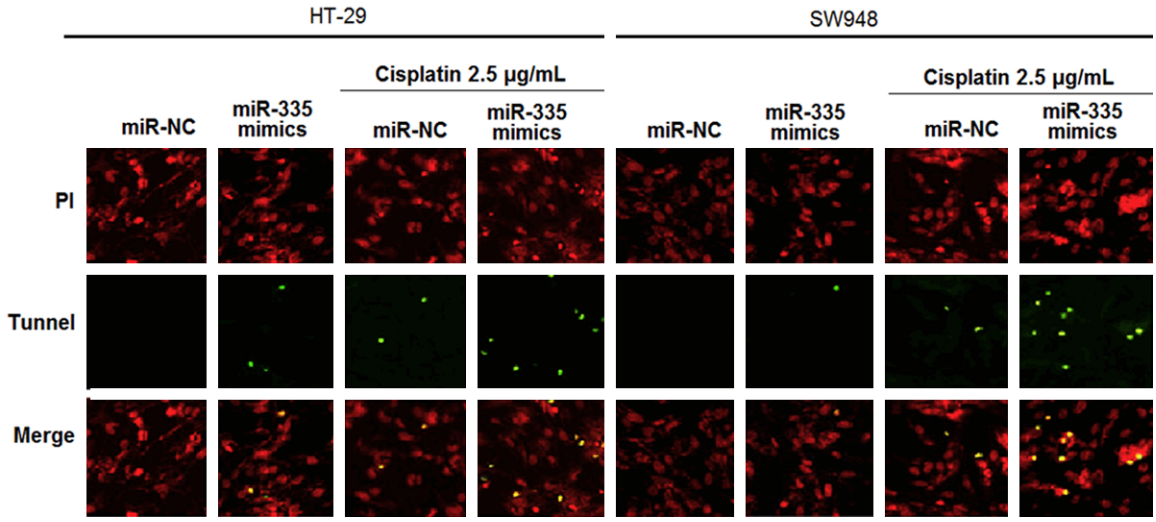


**Figure 3.** The miR-335 enhances the sensitivity of the colon cancer cells to cisplatin. Propidium Iodide (PI) and TUNNEL assay showing the effects of miR-335 on the cisplatin sensitivity of the HT-29 and SW948 cells. Individual experiments were performed in triplicate.

were examined on their sensitivity to cisplatin by using TUNNEL assay. The miR-NC and miR-335 mimics transfected colon cancer cells were treated with 2.5 μg/mL of cisplatin. The untreated miR-NC and miR-335 mimics transfected HT-29 and SW948 cells were kept as control. All these groups of cells were then subjected to MTT assay. The results showed that miR-335 over-expression remarkably increased the cisplatin triggered apoptosis 29 and SW948 cells (**Figure 3**).

*Suppression of colon cancer metastasis by miR-335*

The effects of the miR-335 to restrain the metastatic potential of colon cancer cells were also inspected through transwell assays. It was observed that the migration of the HT-29 and SW948 cells was decreased by 53% and 45% respectively (**Figure 4**). The invasion assay showed that the invasion of the HT-29 and SW948 cells decreased by 49% and 42% respectively (**Figure 5**). These observations suggest that miR-335 over-expression increases colon cancer metastasis.

*Inhibition of Raf/MEK/ERK pathway by miR-335 in colon cancer cells*

The effects of the enhancement of the miR-335 were examined on the Raf/MEK/ERK signalling pathway. It was found that the expression of Raf, p-Raf, p-MEK and p-ERK2 decreased remarkably upon over-expression of miR-335 in HT-29 cells. However, MEK and ERK1/2 remained unchanged (**Figure 6**).

*miR-335 inhibits xenografted tumor growth in vivo*

The effects of miR-335 over-expression were also examined in xenografted mice (**Figure 7A**). The results showed that miR-335 over-expression significantly suppressed the tumor volume in a time dependent manner (**Figure 7B**). Before concluding the animal study, the tumor weight was also examined and significant difference in the weight of the tumors of miR-NC and miR-335 mimics groups was observed. The average tumor weight weight under miR-335 over-expression was appreciably lower than that of the miR-NC tumors (**Figure 7C**).

## Discussion

Colon cancer results in remarkable human mortality and microRNAs have shown to exhibit great promise as therapeutic targets for the management of wide array of cancer types [14, 15]. The establishment of an efficient molecular therapeutic target for a disease enables targeted therapy for efficient treatment [16]. Herein, we explored the function of miR-335 and its implications in the treatment of colon. The gene expression studies showed that miR-335 was significantly suppressed in

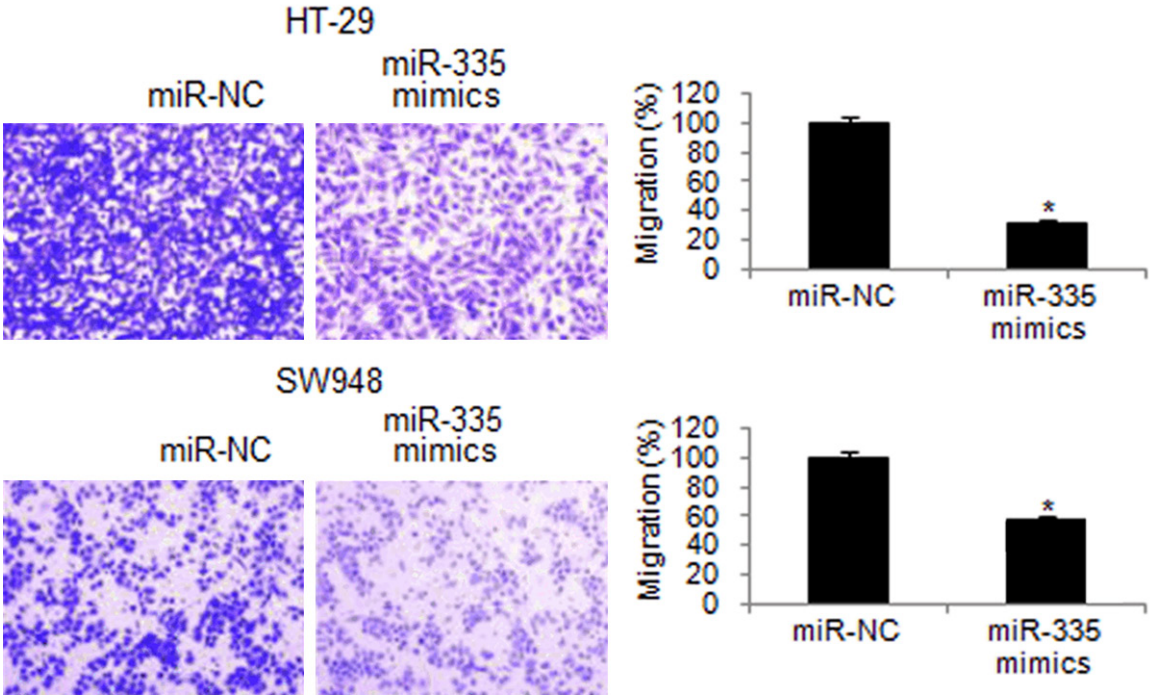


**Figure 4.** The miR-335 inhibits the migration of the colon cancer cells. The transwell assays showing the migration in the miR-NC and miR-335 mimics transfected HT-29 and SW948 cells. Individual experiments were performed in triplicate and data is presented as mean ± SD (*P < 0.05 for miR-NC Vs miR-335 mimics).

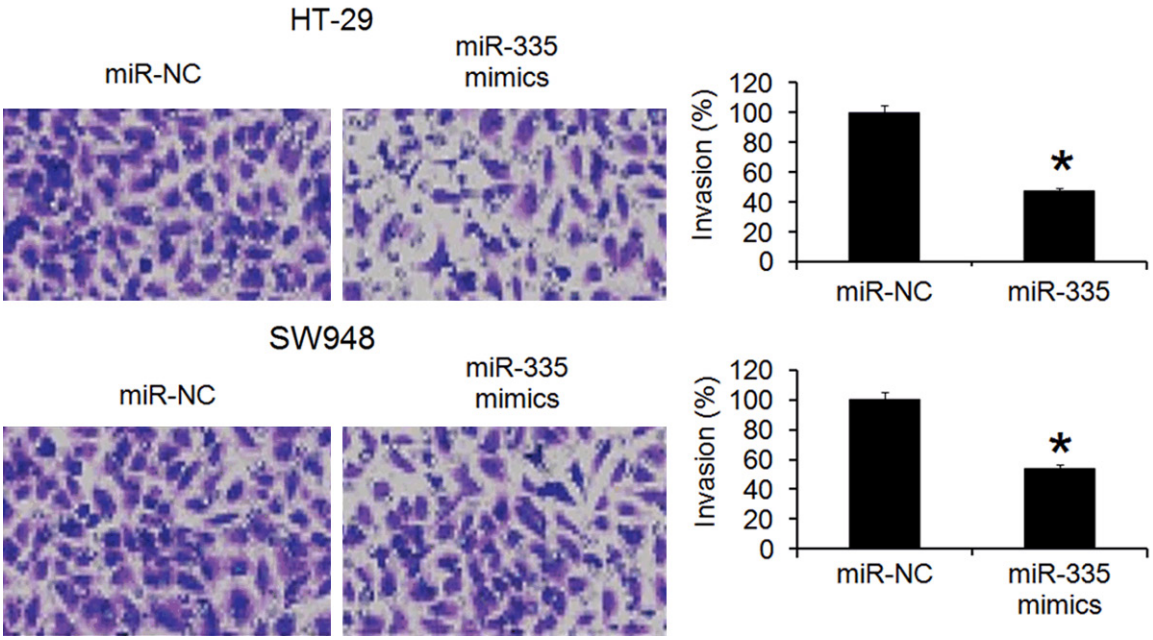


**Figure 5.** The miR-335 restrains the invasion of the colon cancer cells. The transwell assays showing the invasion in the miR-NC and miR-335 mimics transfected HT-29 and SW948 cells. Individual experiments were performed in triplicate and data is presented as mean ± SD (*P < 0.05 for miR-NC Vs miR-335 mimics).

colon cancer cells relative to the normal cells. This is consistent with several of the investigations carried out previously wherein miRs have been dysregulated in cancer cells, for instance,

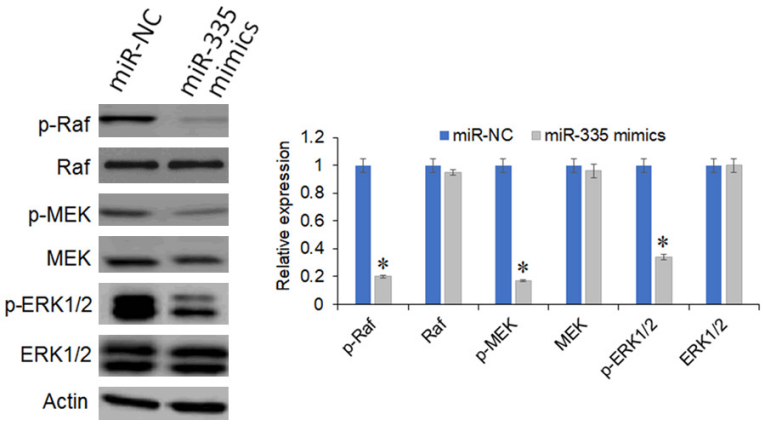


**Figure 6.** The miR-335 blocks the Raf/MEK/ERK signalling pathway. Western blot analysis showing the effects of miR-NC and miR-335 mimics transfection on the Raf/MEK/ERK signalling pathway in HT-29 cells. Individual experiments were performed thrice and data is presented as mean ± SD (*P < 0.05 for miR-NC Vs miR-335 mimics).

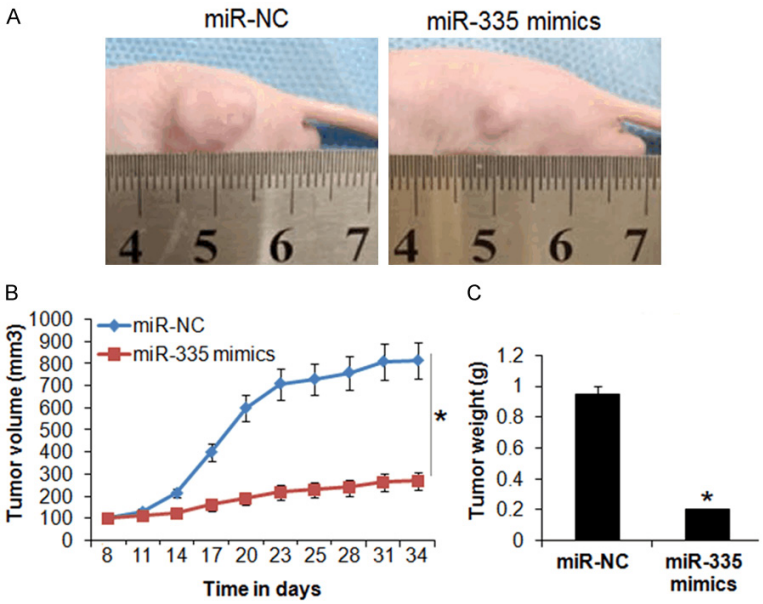


**Figure 7.** miR-335 over-expression inhibits the growth of the xenografted tumors. (A) Photographic illustration of the miR-NC and miR-335 mimics tumors (B) Tumor volume and (C) Tumor weight. Three replicas were used per experimental set up and expressed as mean ± SD (*P < 0.05 for miR-NC Vs miR-335 mimics).

miR-335 has been reported to be repressed in gastric cancer tissues [17]. The miRs have either been shown to suppresses the development and progression of cancer and referred as tumor suppressor miRs or promote the growth and development of cancer and referred as oncomiRs [18]. Herein, it was observed that miR-335 over-expression decreases the viability of the colon cancer cells and as such it acts as negative regulator of tumorigenesis of colon cancer. We also found that miR-335, by enhancing the apoptosis, negatively affected the growth of the colon cancer cells as revealed by the tunnel assay and western blot analysis which showed enhancement of Bax and suppression of Bcl-2 expression. This is in agreement with several previous studies wherein miR-335 has been reported to suppress the growth of the cancer cells, for instance, epigenetic silencing of miR-335 has been shown to correlate the tumorigenesis of the hepatocellular carcinoma [19]. We also observed that miR-335 enhances the sensitivity of the colon cancer cells to the apoptotic effects of cisplatin and inhibits their metastasis. This was in agreement with a study wherein miR-335 was previously found to inhibit the metastasis of the lung cancer to bone tissues by modulating the IGF-IR and RANKL signalling pathways [20]. There are several preclinical and clinical studies that have reported the aberration activation of Ras/Raf/MEK/ERK signaling in different human cancers including colon cancer [21]. Suppression of Raf/MEK/ERK signalling pathway has been shown to suppress the proliferation of colon cancer cells. For instance, the inhibition of colon cancer proliferation by tumor suppressor FOXD3 involves blocking of Raf/MEK/ERK signalling pathway [22]. Similarly, deactivation of the Raf/MEK/ERK pathway by HOXA3 has been reported to growth of colon cancer cells [23]. Additionally, several chemotherapeutic agents have been suppressing the growth of colon cancer cells via in activation of Ras/Raf/MEK/ERK signaling [24, 25].

In the present study we found that Raf/MEK/ERK signalling pathway is deactivated upon overexpressing miR-335 in colon cancer cells. These results indicate that miR-335 might be exerting its tumor suppressive via inactivation of Ras/Raf/MEK/ERK pathway. Nonetheless, how miR-335 regulates the Raf/MEK/ERK remains an important area of investigation. Additionally, identification of chemotherapeutic agents which can enhance the expression of miR-335 could open new avenues for the treatment of colon cancer.

## Conclusion

The findings of the present study revealed remarkable downregulation of miR-335 in colon cancer. The study was conclusive that miR-335 acts as tumor-suppressor in colon cancer and decreases the colon cancer viability via induction of apoptosis. The miR-335 also suppressed the metastasis of the colon cancer via inhibition of the MEK/ERK signalling pathway. Taken together, miR-335 may prove beneficent therapeutic target for the treatment of colon cancer and hence warrants further research endeavours.

## Disclosure of conflict of interest

None.

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