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miRNA-29a reverses P-glycoprotein-mediated drug resistance and inhibits proliferation via up-regulation of PTEN in colon cancer cells

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ABSTRACT

Colon cancer is a serious malignant type of cancer in the world. Acquisition of multi-drug resistance (MDR) during chemotherapy is still a controversial challenge during cancer treatment. Accordingly, detection of safe and impressive MDR-reversing targets such as microRNAs (miRNAs/miRs) can play critical role in cancer treatment. Here, the functional effects of miR-29a in chemo-resistant colon cancer cells is scrutinized. The effect of doxorubicin (DOX) on cell proliferation after miR-29a transfection has been evaluated using MTT assay in HT29 and HT29/DOX cells. Rhodamine123 (Rh123) assay is used to identify the activity of common drug efflux through membrane transporters P-glycoprotein (P-gp). P-gp and PTEN mRNA/protein expression levels were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot analyses. Flow cytometry was employed to the investigation of apoptosis. ANOVA followed by Bonferroni's and Sidak's tests were used to compare the data from different groups. Thus, it was shown that miRNA-29a overexpression considerably inhibited the HT29/DOX viability. miR-29a significantly down-regulated P-gp expression and activity in HT29/DOX cells and declined drug resistance through elevation of intracellular DOX. Furthermore, upon miRNA-29a transfection, PTEN expression could be restored in resistant cells. These results have indicated that miR-29a target PTEN ultimately P-gp, which is downstream of PTEN, inhibit drug resistance, proliferation, and apoptosis through PI3K/Akt pathway. As a result, miR-29a overexpression is led to enhance the sensitivity of HT29/DOX cells to DOX-treatment by targeting P-gp. MiR-29a might proffer a novel promising candidate for colon cancer therapeutics during chemotherapy.

1. Introduction

Colon cancer is one of the five most causing of cancer-related deaths worldwide in both sex (Coronel-Hernández et al., 2019). Whereby, the complex pathophysiologic mechanisms result in colon cancer, and despite improving surgical and adjuvant chemotherapy approaches, colon cancer is still a controversial challenge in public health (Liu et al., 2019). Despite signs of progress in chemotherapy against cancer, many of these chemotherapeutic agents developed resistance towards the cancer cells. This phenomenon, well-known as multidrug resistance (MDR), remains a big pending to positive treatment outcomes in most cancers (Nanayakkara et al., 2018). DOX is one of the most commonly used chemotherapeutic agents, which particularly in advanced or metastasis cancer patients. Mechanically, DOX represses topoisomerase II (Top II) and intercalates directly to DNA double-strand, finally, resulting in the intervention of gene transcription (Lage et al., 2006). In

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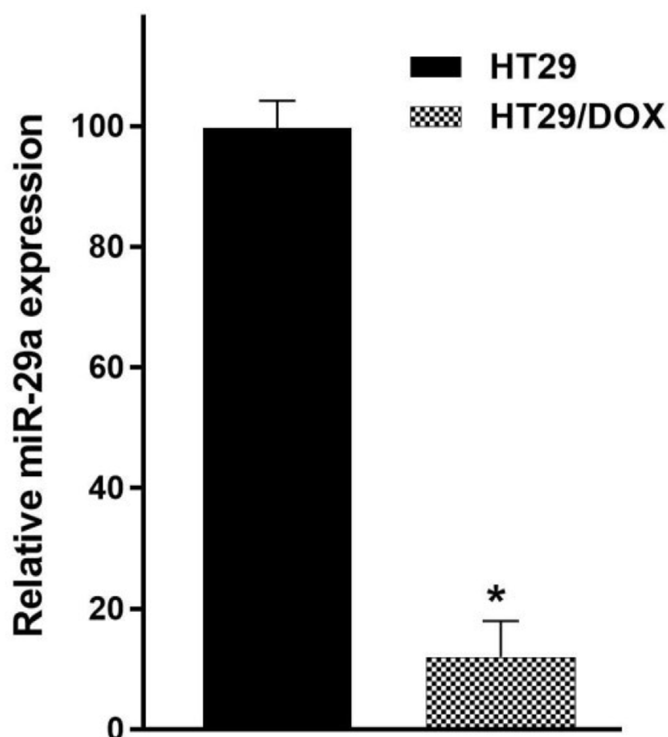


Fig. 1. The expression levels of miR-29a in HT29 and HT29/DOX cells (*$P < 0.05$). Each point represents the mean ± S.D. (n = 3 independent experiments).

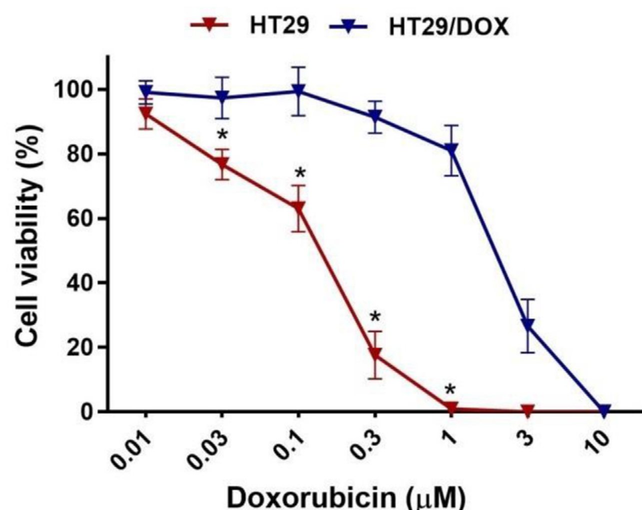
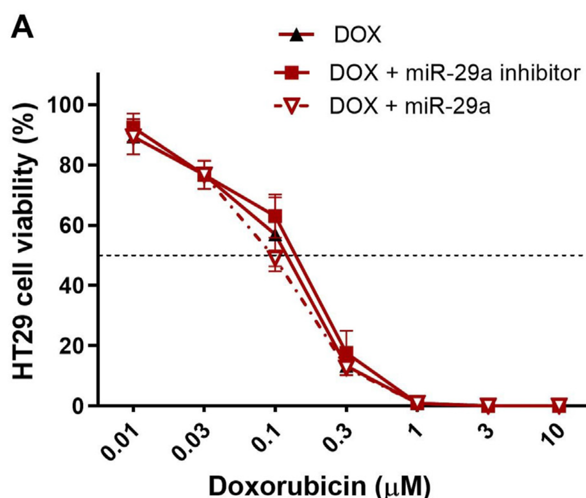


Fig. 2. The sensitivity of HT29 and HT29/DOX cells to DOX. Cells were treated with versatile concentrations of DOX. After 48 h of incubation, the viability rates of the cells were measured using the MTT assay. Each point represents the mean ± S.D. (n = 3 independent experiments; *$P < 0.05$ compared with HT29/DOX cells).

this regard, DOX-resistance is one of the pivotal leads of chemotherapy failure in the treatment of advanced colon cancer (Yang et al., 2015).

Many relevant mechanisms for MDR have been described and discussed, among which of the importance of these mechanisms is the overexpression of the ATP-binding cassette (ABC) transporter proteins, which regulate the intracellular concentrations of cytotoxic agents (Majidinia et al., 2017; Yousefi et al., 2017; Shafiei-Irannejad et al., 2018). These pumps troughs decrease the accumulation of anticancer drugs to sub-therapeutic levels in cells, therefore result in mitigating or



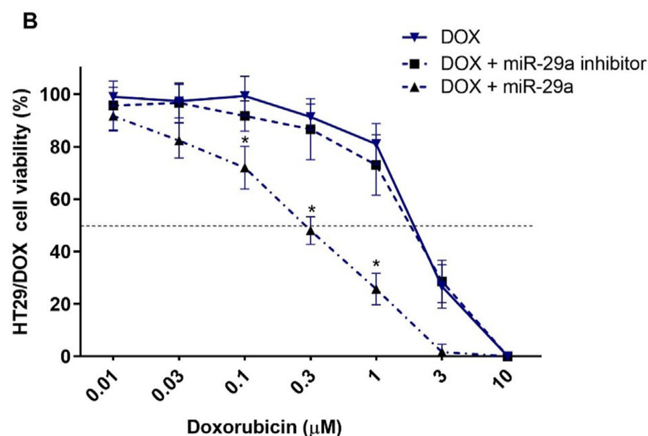


Fig. 3. Effects of transfection of mir-29a and its inhibitor on DOX cytotoxicity. Upon culture for 48 h after transfection with the miR-29a mimics, inhibitor or control, (A) HT29 cells and (B) HT29/DOX cells were incubated with various concentrations of DOX for 48 h; and the cell viability was defined by the MTT assay. Each point represents the mean ± S.D. (n = 3 independent experiments; *$P < 0.05$ compared with control).

abolishing chemotherapy efficacy (Katayama et al., 2014). In this regard, human P-170 glycoprotein (P-gp/ABCB1) is encoded by MDR1 gene, located on chromosome 7q2, which is dominant members of ABC transporters. In cancer cells, overexpression of P-gp is a common occurrence seen with resistant phenotype, which leads to pumps out various anticancer drugs, including anthracyclines (such as DOX), vinca alkaloids, epipodophyllotoxins, taxanes, and xenobiotics. Indeed, P-gp expression in tumor cells awards MDR to these anticancer drugs (Katayama et al., 2014; Yousefi et al., 2017, Teng and Chen, 2019).

For overcoming this scenario, recent cancer therapy approaches are focusing on targeting the mediators, which inhibit specific pathways involved of MDR in these cells (Yousefi et al., 2017). In this regard, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor gene, which is located at 10q23.31. PTEN is a multifunctional protein provided phosphatase activity on protein substrates including FAK, SHC, IRS1, Dvl2, PTK6, and PIP3 (Kotelevets et al., 2018). Deficiency or inactivation of a single PTEN allele is adequate to activation of the phosphoinositide 3-kinase (PI3K)/Akt/PKB

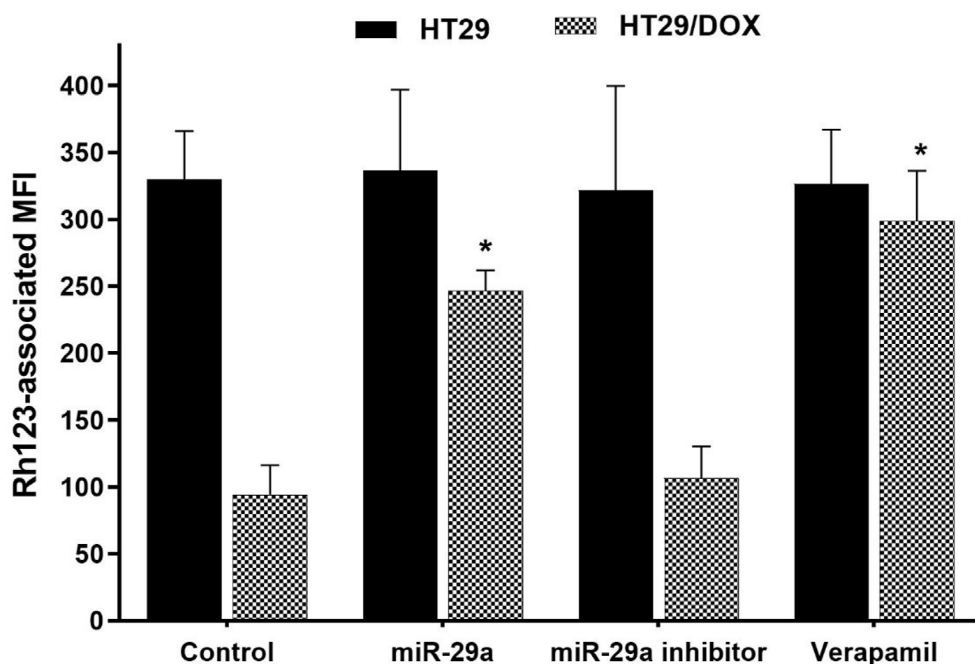


Fig. 4. Effect of miRNA-29a on the intracellular accumulation of Rh123. Upon culture for 48 h after transfection, the cells were incubated with 5 μM of Rh123 in the presence or absence of miRNA-29a, and its inhibitor and 10 μM of verapamil for 2 h and then, Rh123-associated MFI was measured. Each point depicts the mean ± S.D. from four tests (*$P < 0.05$ compared control).

signaling pathway, which may result in colon cancer (Colakoglu et al., 2008; Chen et al., 2019). PTEN inhibits the activation of PI3K/Akt cascade through dephosphorylation of PIP3 to produce PIP2; whereby, decrease cell proliferation, promote apoptosis, and revert invasiveness (Kotelevets et al., 2018). Inversely, when PKB activated by suppression PTEN leading to phosphorylation and activation in many proteins including mTOR, IKK, Bad, caspase 9, GSK3β, and P-gp (Shen et al., 2016; Chen et al., 2018a,b). Evidence illustrates that PTEN functions in a dosage-dependent manner during tumor development. Non-coding RNAs (ncRNAs) are critical regulators of PTEN, including microRNAs (miRNAs/miRs) and lncRNAs, which specifically modulate the PTEN expression (Li et al., 2018).

miRNAs are a family of RNAs with 18–22 nucleotides, which join to 3′ untranslated region (3′-UTR) of mRNAs, through Watson-Crick base pairing postulate (Taby and Issa, 2010; Zhou et al., 2017). It is worth mentioning that, miRNAs can function either as a promoter or a suppressor to regulate the MDR of gastric cancers (Berindan-Neagoe et al., 2014; Chen et al., 2018a,b). In gastric cancer, miRNAs including miR-508-5p miR-15b, miR-27a, miR-200c, miR-181b, miR-16, miR-106a, and miR-497, have been reported to be involved in MDR (Shang et al., 2014; Gong et al., 2019). The human miR-29 family includes three major members including miR-29a, b, and c. Transcriptional profiling studies showed that miR-29 is down-regulated in the majority of cancers and up-regulated in the minority, which is miR-29a up-regulation in colon cancer (Jiang et al., 2014). MiR-29a as a tumor suppressor gene in several cancers can affect tumor cell growth invasion, migration, and apoptosis (Liu et al., 2018). Despite the development of the biological function of miRNAs, the role of miRNAs in regulating drug resistance in colon cancer remains under review.

Overall, our understanding of the physiologic and pathophysiologic of colon cancer made possible by the elucidation of the signaling pathways and related molecular disorders. In conclusion, we evaluated effects of miR-29a on drug resistant in colon cancer cell with underlying molecular mechanisms involved in MDR. For this purpose, we investigated the key role of miR-29a on P-gp as a member of ABC transporters through PTEN/PI3K/Akt/MDR1/P-gp signaling pathway.

2. Materials and methods

2.1. Drugs and reagents

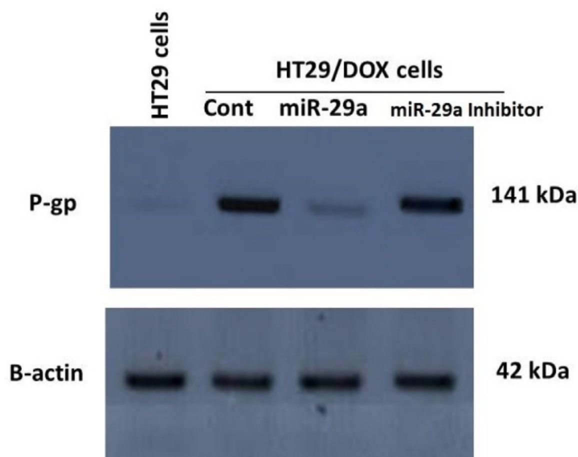
The human colon cancer cell line HT29 and DOX-resistant colon cancer HT29/DOX cells were purchased from Pasteur Institute Cell bank (Tehran, Iran); The miR-29a mimic and negative control were obtained from microsynth AG (Balgach, Switzerland). DOX, PD098059, LY294002, and verapamil were obtained from Cayman (USA). SF1670 was purchased from Echelon Biosciences Inc (USA). Lipofectamine 2000 purchased from Invitrogen. Rhodamine123 (Rh123), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and RNase were purchased from Sigma (USA). Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from Gibco (USA). Antibodies for P-gp, PTEN, and β-actin were obtained from Abcam (Cambridge, UK).

2.2. Cell culture and transfection

HT29 and HT29/DOX Cell lines were cultured in the RPMI 1640 medium, supplemented with 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin at 37 °C in a humidified in 95% O2 and 5% CO2 in a humidified incubator. HT29/DOX cells were seeded and cultured in the presence of 0.5 μM of DOX or DOX free medium for two weeks before being experiments. The sequence of the miR-29a mimic was 5′ UAGCACCAUCUGAAAUCGGUUA 3′. Cells (5×10^5 cells/2 ml/well) were seeded in six-well plates at 60% confluence. After 48 h, the miR-29a mimic or the negative control was transfected into the cells using Lipofectamine, at a final concentration of 50 nM, according to the manufacturer's instructions.

2.3. MTT assay

The effect of DOX on cell viability in colon cancer cells growth was performed with MTT assay. HT29 and TH29/DOX cells were plated onto 96-well plates (2×10^4 cells/200μl/well) and incubated overnight and transfected with miRNA-29a at 37 °C. Upon 24 h incubation, both cell lines were treated with diverse concentrations of DOX to 48 h. Then cells were treated with 10% MTT (5 mg/ml) for another 4 h. Following, 200 μl dimethyl sulfoxide (DMSO) was added to each well to dissolving



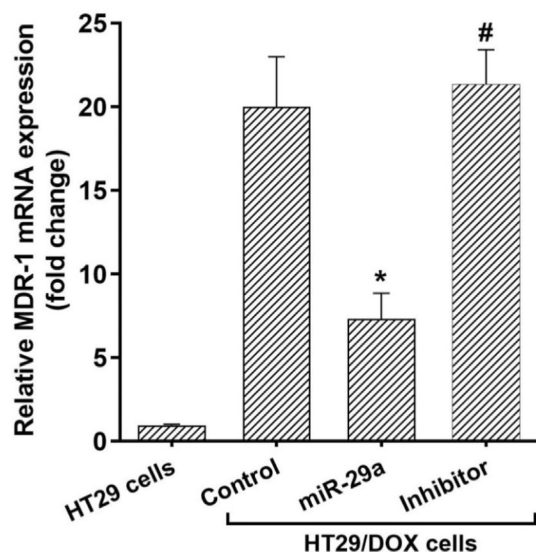


Fig. 5. Effect of miRNA-29a on the P-gp expression. (A) P-gp protein expression level (B) and MDR-1 mRNA expression in HT29 and HT29/DOX cells. Each point represents the mean ± S.D. from four experiments (n = 3; $*P < 0.05$ compared control; $^{\#}P > 0.05$ compared control).

formazan product. Then, absorbance values were measured at 450 nm with a micro plate reader (State Fax 2100; Awareness Technology Inc, USA) (Yan et al., 2019). The half maximal inhibitory concentration (IC_{50}) of each experiment was calculated. The fold reversal (FR) of MDR was determined by dividing IC_{50} values of cells treated with the indicated anticancer drug in the absence of a modulator by IC_{50} values of cells treated with the same anticancer drug in the presence of a modulator.

2.4. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from cells with AccuZolTM reagent, following the manufacturer's instructions (Bioneer; Daedeok-gu, Daejeon, Korea). Complementary DNA (cDNA) was synthesized using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI). Following qRT-PCR was performed using SYBR Premix Ex Taq (TaKaRa Bio, Otsu, Japan) and specific primers via the Rotor-Gene™ 6000 system (Corbett Life Science, Mortlake, Australia). PCR amplification was performed at 95 °C for 3 min prior to 35 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 60 s, followed by a final incubation at 72 °C for 5 min. All experiments were carried out in triplicates. Raw data were analyzed with the comparative Ct method using β-actin as a housekeeping gene.

2.5. Western blot analysis

Proteins were extracted from cells using a lysis buffer containing [20 mM Tris-HCl, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and pH 7.4,]. Then centrifuged at 14,000 ×g for 15 min at 4 °C. The protein concentration in the cell lysates was detected by Bradford reagent with bovine serum albumin (BSA) as standard (Bio-Rad, USA). For western blotting, protein samples boiled with SDS-PAGE loading dye and loaded at ~50 μg into each lane of PAGE and separated by a SDS-PAGE gel. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, and the membrane was then immersed in a blocking solution containing 5% BSA and 0.1% Tween-20 in PBS for 1 h at room temperature with constant shaking. Proteins were blotted with the primary antibodies at 4 °C overnight with constant shaking. Upon washed three times in Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature for 15 min, immunodetection was accomplished with incubation of secondary antibody conjugated with horseradish peroxidase (HRP) at room temperature for 2 h, followed by reaction with chemiluminescence HRP substrate. Bands were identified using the enhanced chemiluminescence system (ECL) Western blot analysis Kit (Amersham Pharmacia Biotech, Piscataway, NJ).

2.6. Assessment of P-gp activity

Rh123 operates as a good substrate for MDR-associated P-gp, and drugs that inhibit P-gp have been found to enhance the retention of Rh123 in MDR cells (Wang and Yang, 2008). To measure net Rh 123 uptake, 1 × 10^6 cells//2 ml/well were seed at 6-well plates. After 24 h of incubation, cells were treated with medium containing 5 μM Rh123 in the presence or absence of miR-29a and were incubated at 37 °C for 2 h. Verapamil was used for P-gp inhibition. The intracellular mean fluorescence intensity (MFI) associated with intracellular Rh 123 accumulation was measured using FP-6200 fluorometer (Jasco Co., Tokyo, Japan). Results were expressed in an arbitrary unit of the MFI. Excitation was achieved by an argon-ion laser operating at 488 nm and emitted at 530 nm.

2.7. Annexin V and propidium iodide (PI) staining

Cells were seeded at 1x106 cells/2 ml/well in 6-well plates, after 70% confluency incubated overnight and transfected with miRNA-29a at 37 °C. After 24 h of treatment, the cells were washed twice with PBS. Then incubated in the binding buffer (10 mM HEPES, 0.1% BSA, 140 mM NaCl, 2.5 mM CaCl2, and pH 7.4). In continue cell pellets were suspended in 10 μl of fluorescein isothiocyanate (FITC)-labeled Annexin V and PI solution for 10 min at dark in RT and then analyzed using a fluorescence-activated cell sorting (FACS) flowcytometer (BD LSR; Becton-Dickinson, USA) and Cell Quest software (Becton-Dickinson) (Yan et al., 2019).

2.8. Statistical analysis

Statistical analysis was performed using mean ± standard deviation (S.D.). Analysis of variance (ANOVA) followed by Bonferroni's and Sidak's tests was used to ascertain the statically significant differences

Table 1
Cytotoxicity effect of DOX and reversing MDR in HT29/DOX cells role of miR-29a and co-exposure with LY294002, PD098059, and SF1670 as selective inhibitors.

Treatment	HT29 / DOX	
	IC_{50} (μM)	FR
Control (DOX)	2.84 ± 0.37	–
miR-29a + DOX	0.32 ± 0.09[a]	8.87
miR-29a + LY294002 10 μM + DOX	0.29 ± 0.08[a,b]	9.71
miR-29a + PD098059 10 μM + DOX	0.38 ± 0.08[a,b]	7.47
miR-29a + SF1670 10 μM + DOX	1.99 ± 0.31[c]	1.42

MTT reduction activity was used to determine cell viability. Each value represents the mean ± S.D. of three independent tests. The fold reversal (FR) of MDR was distinguished by dividing the IC_{50} of control to IC50 of each test. Statically significant different from control at [a]$P < 0.05$; No significant difference from miR-29a + DOX group [b]$P > 0.05$; Significantly different from miR-29a + DOX at [c]$P < 0.05$.

between groups. *P* values lesser than 0.05 were considered to be statically significant. All statistical analyses were carried out with the GraphPad Prism software version 6.01 (GraphPad, San Diego, CA).

3. Results

3.1. Effects of miR-29a overexpression on the viability of HT29 and HT29/DOX cells

To investigate the possible role of miR-29a in the HT29/DOX colon cancer cell, first, RT-qPCR analysis was performed. Results illustrated that the expression level of miR-29a was higher in the HT29 cells compared with the HT29/DOX cells (Fig. 1), which suggested that miR-29a may be inversely associated with DOX-resistance in colon cancer cells. Next, we assessed the effect of DOX on cell viability was determined with the MTT assay. As shown in Fig. 2, DOX (concentration range from 0.01 to 10 μM) displayed lower cytotoxicity towards the MDR cells compared the corresponding parental cells, which caused enhancement in the viability of HT29/DOX cells. The IC_{50} values for DOX were 0.21 μM in HT29 and 2.84 μM in HT29/DOX cells, the latter showing 13.52-fold resistance to DOX, compared to the HT29 cells.

3.2. miR-29a reversed MDR in HT29/DOX cells

To ascertain the correlation between miR-29a and HT-29a/DOX cells, the effect of up-regulation and down-regulation of miR-29a on the DOX-resistance cells was analyzed. The HT29 cells, which were treated with the DOX, DOX + miR-29a mimic and DOX + miR-29 inhibitor exhibited equal cytotoxicity in these cells (Fig. 3A). In contrast, the HT29/DOX cells transfected with the DOX + miR-29a mimic and

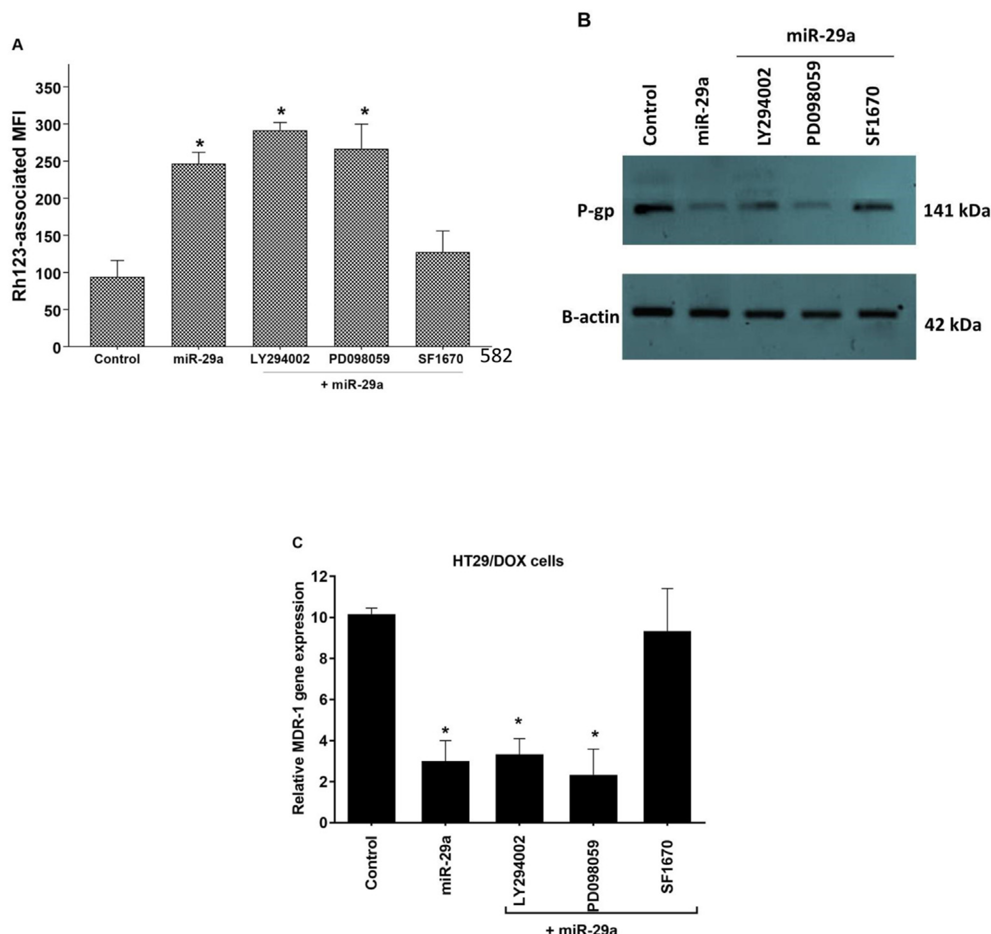


Fig. 6. PTEN inhibition abolished miR-29a effects on MDR. A) Intracellular Rh123 accumulation in HT29/DOX cells affected by miR-29a and specific inhibitors. Treatment with 10 μM of LY294002 or PD098059 increased Rh123-associated MFI and with 10 μM of SF1670 almost abolished the effect of the miR-29a overexpression. (B) P-gp protein expression and (**C**) MDR-1 mRNA in HT29/DOX cells 48 h after transfection. SF1670 suppress the effects of miR-29a. Data represent mean ± S.D. (n = 3; *$P < 0.05$ vs untreated HT29/DOX cells).

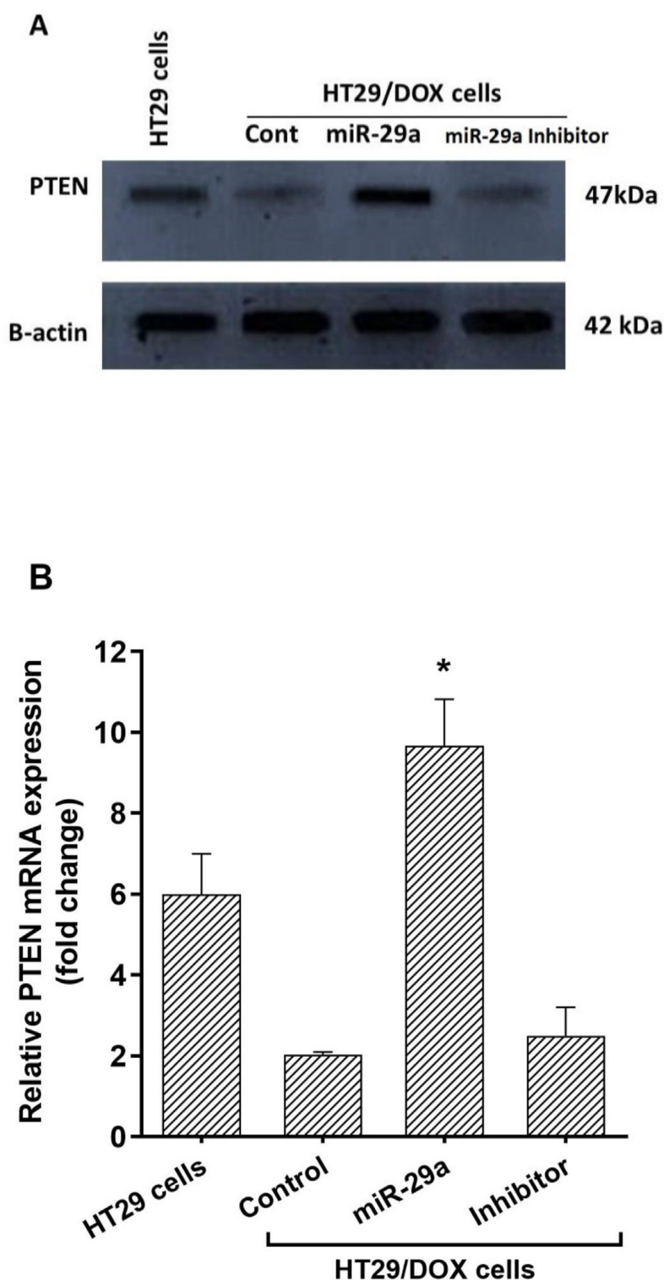


Fig. 7. PTEN is a direct target of miR-29a. Relative protein (A) and mRNA levels (B) in HT29/DOX cells. The data represent mean ± S.D. (n = 3, $^*P < 0.05$ vs untransfected HT29/DOX cells).

DOX + miR-29 inhibitor demonstrated statically significant lower and higher survival rates than DOX group, respectively (Fig. 3B). In other words, miR-29a overexpression can effectively reverse DOX resistance in HT29/DOX cells, while down-regulation of this miRNA may play critical role in the development of DOX resistance in HT29 cells.

3.3. miR-29a overexpression decreased P-gp activity

P-gp activity was determined using intracellular Rh123 accumulation assay. Verapamil used as a positive control. Fig. 4 clearly demonstrates that miR-29a enhances Rh123 accumulation in HT29/DOX cells after 2 h, while HT29 cells showed no such enhancement when treated with miR −29a. In addition, transfection of HT29/DOX cells with miR-29a inhibitor resulted in the significant decrease in the accumulation of Rh123 within these cells. Indeed, incubation of miR-29a-treated HT29/DOX cells with the Rh123 resulted in the increase in the MFI ($P < 0.05$). This effect comparable with that observed in HT29/DOX cells treated with verapamil. Nevertheless, no accumulation of substrate was recognized in parental HT29 cells over the same period. Therefore, it was proposed that the activity of P-gp was inhibited via miR-29a in HT29/DOX cells, and results from cells treatment with miR-29a inhibitor approved this finding.

3.4. miR-29a down-regulated P-gp expression in HT29/DOX cells

In the present study, we expanded our work to a HT29/DOX cell line to assess whether the observed effects were cancer cell type specific or whether they might be more generally applicable. Western blot analyses using a P-gp-specific primary antibody showed that while the HT29/DOX cells expressed significant amounts of P-gp, no P-gp was detectable in the parental HT29 cells. These results strongly suggest that the HT29/DOX cell line was phenotypically MDR and this MDR phenotype was correlated to overexpression of P-gp (Fig. 5). We found out that miR-29a down-regulated P-gp at the mRNA and protein levels in HT29/DOX cells that can describe the elevated intracellular accumulation of DOX and cytotoxicity in the miR-29a-treated HT29/DOX cells. Moreover, treatment of DOX resistant cells with miR-29a inhibitor led to significant overexpression of P-gp, as shown in Fig. 5.

3.5. PTEN inhibition abolished miR-29a effects on MDR

We speculated that miR-29a may play crucial role in the inhibition of MDR1/P-gp expression by down-regulating PI3K/Akt signaling pathway activity. Therefore, to further scrutinize the fundamental molecular mechanisms, we investigated the MDR-reversing effect of miR-29a in presence of special inhibitors of PI3K (LY294002), MAPK (PD098059), and PTEN (SF1670). As shown in Table 1, compared with the control group, DOX cytotoxicity was heightened in the miR-29a-treated group. The IC50 values (μM) were diminished from 2.84 ± 0.37 to 0.32 ± 0.09, 0.29 ± 0.08, 0.38 ± 0.08, and 1.99 ± 0.31 in Control (DOX), miR-29a + DOX, miR-29a + LY294002 10 μM + DOX, miR-29a + PD098059 10 μM + DOX, miR-29a + SF1670 10 μM + DOX, respectively. However, miR-29a effect was significantly attenuated by SF1670 treatment. Also, results reveal that FR in miR-29a + SF1670 10 μM + DOX significantly different from miR-29a + DOX. Following culture for 48 h after transfection with the miR-29a, the effect of LY294002, PD098059, and SF1670 on the miR-29a-induced intracellular Rh123 associated MFI accumulation was observed in HT29/DOX cells. Indeed, treatment with 10 μM of LY294002 or PD098059 enhanced Rh123-associated MFI and with 10 μM of SF1670 almost abolished the action of the miR-29a overexpression (Fig. 6A).

In this regard, our findings clearly demonstrated that miR-29a in HT29/DOX cells 48 h after transfection attenuated P-gp protein expression and MDR-1 mRNA. Besides, SF1670 suppress the effects of miR-29a (Fig. 6B and C). Moreover, further examinations showed that in the presence of 10 μM of SF1670 eliminated miR-29a effects on P-gp expression, while no similar effect with 10 μM ofLY294002 or 10 μM of PD098059 was noted. Overall, these data illustrated that miR-29a-induced down-regulation of the P-gp was associated with PTEN-dependent manner in HT29/DOX cells.

3.6. miR-29a down-regulated P-gp via up-regulation of PTEN

To investigate the correlation between miR-29a and PTEN, PTEN expression was evaluated in HT29/DOX cells treated with miR-29a and its inhibitor. We revealed that PTEN expression was significantly lower in HT29/DOX cells treated with miR-29a (Fig. 7A and B). More importnatly, miR-29a inhibitor resulted in the up-regulation of PTEN in HT29/DOX cells.

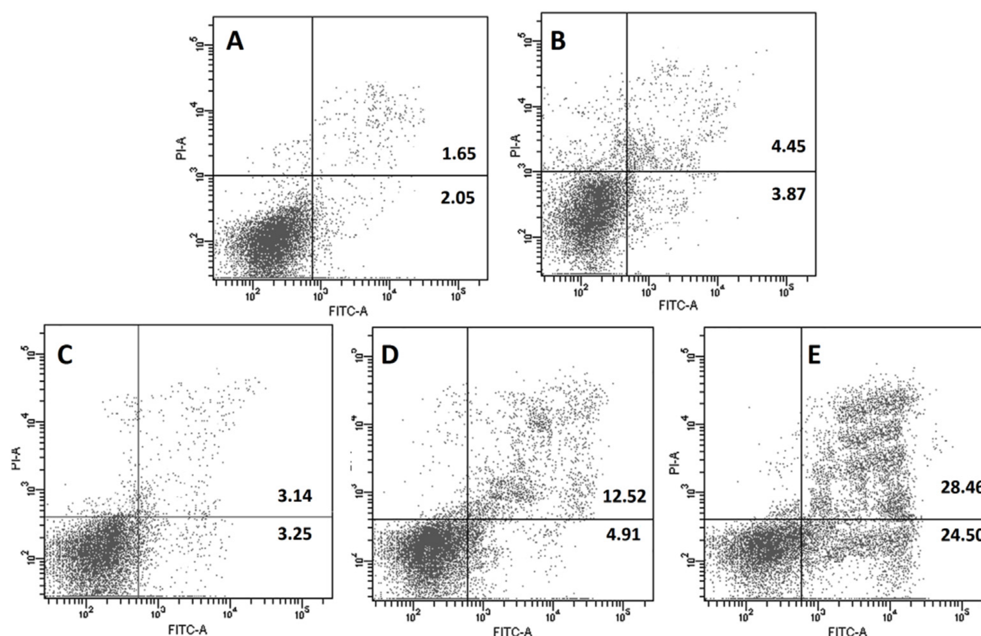


Fig. 8. Apoptotic analysis of HT29/DOX cells by flow cytometry. Cells were stained with Annexin V–FITC and PI 48 h after transfection with miR-29a. Control (A), miR-29a (B), miR-29a inhibitor (C) DOX, 0.5 μM (D), and miR-29a plus DOX, 0.5 μM (E).

3.7. miR-29a promoted DOX-induced apoptosis in HT29/DOX cells

Flow cytometric analysis was performed to evaluate the impact of HT29/DOX cells by Annexin V–FITC and PI staining, and apoptotic cells were detected 48 h after miR-29a transfection. The lower right quadrant indicates the pro-apoptotic cell rate. We reveal that combination of miR-29a with DOX enhanced the migration of HT29/DOX cells to apoptotic regions as compared with DOX (Fig. 8). In addition, cells treatment with miR-29a inhibitor could not exert significant effect in increasing apoptosis in resistant cells.

4. Discussion

Our results showed three major findings, which allow us to a better understanding of the role of miR-29a in colon cancer. 1) We have revealed that miR-29a overexpression considerably inhibited the HT29/DOX viability. 2) Our biological assays highlighted that miRNA-29a significantly down-regulated P-gp expression in HT29/DOX cells and alleviated drug resistance through elevation of intracellular DOX, which reduced colon cancer cell growth. 3) We indicated that miR-29a targets PTEN to inhibit drug resistance, proliferation, and apoptosis through PI3K/Akt pathway. These biological findings have demonstrated the critical role of miR-29a as a tumor suppressor in colon cancer.

It is pivotal to identify unique strategies to enhance the effectiveness of DOX for therapeutic purposes. Likewise, the enhancing popularity of circulating miRNAs as a prognostic agent in cancer offers that these molecules useful as non-invasive biomarkers to predict the patient response to chemotherapy, which provides new insights about the functional mechanism of MDR (Yang et al., 2015; Fruci et al., 2016). By analyzing the cell index (CI; reflects the number of cells in each well) identified more than 10 miRNAs that improve drug sensitivity when overexpressed, which one striking example is miR-508-5p reversed drug resistance most efficiently in gastric cancer. Shang et al. (Shang et al., 2014) demonstrated that by targeting ABCB1 and ZNRD1, miR-508-5p overexpression enhances the intracellular concentration of chemotherapeutics and promotes drug-induced apoptosis in MDR gastric cancer cells. In contrast, the results of the study on breast cancer by Zhong and colleagues (Zhong et al., 2013) revealed that miR-222 and -29a conferred DOX resistance in MCF-7 cells. The results of our study showed that miR-29a was significantly down-regulated in HT29/DOX cell line, compared with the parental HT29 colon cancer cell line. Indeed, Overexpression of miR-29a in HT29/DOX cells causes restored DOX-sensitivity and a significant decline in the viability of HT29/DOX cells. Indeed, we sought to clarify that combination of miR-29a with DOX enhanced the apoptosis of HT29/DOX cells compared with HT29 cells.

Ameliorating chemo-sensitivity by targeting P-gp has been extensively used as a strategy for cancer therapy (Bao et al., 2012; Majidinia et al., 2018; Majidinia et al. 2018a,b). The up-regulation of P-gp in carcinoma cells elevated drug resistance, whereas inhibiting the expression or function of P-gp was shown to reverse drug resistance. These indicate defined the classical MDR associated role of P-gp (Tsuji et al., 2012). In this regard, Zhao and colleagues (Bao et al., 2012) revealed that miR-302 cooperatively sensitizes breast cancer cells to DOX via repressing P-gp by targeting MAP/ERK kinase kinase1 (MEKK1) of ERK pathway. Also, Gao et al., (2016) showed that overexpression of miR-145 sensitized breast cancer cells to DOX through inducing intracellular DOX accumulation via inhibiting MRP1. PN and colleagues (Yu et al., 2014) showed that down-regulation of miR-29 enhances cisplatin resistance in ovarian cancer cells. In this regard, our results illustrate that miR-29a is capable of inhibiting the efflux of Rh123 from HT29/DOX cells. On the other hand, incubation of miR-29a-treated HT29/DOX cells with the Rho 123 resulted in the increase in the MFI (Fig. 4). In fact, our data show that exposing resistant cells with miR-29a can reverse MDR by increasing PTEN expression, inhibiting the P-gp activity, and enhancing the intracellular accumulation of DOX, in HT29/DOX cells. Additionally, verapamil, a known P-gp inhibitor, which enhances the intracellular retention of anticancer agents in MDR cells (Ledwitch et al., 2016), enhance the Rho 123 accumulation in HT29/DOX cells, which is a positive control for validation of our results. With this perspective, present study results showed that overexpression of miR-29a significantly decreased the activity as well as the mRNA/protein expression levels of P-gp in HT29/DOX cells. Moreover, inhibition of P-gp with specific inhibitor significantly enhanced the growth inhibition rate of the HT29/DOX cells, compared with the control group. As a result, miR-29a sensitized HT29/DOX cells to treatment with DOX.

Besides, overexpression of PTEN in tumor cells has been associated with the MDR-reversal effect and enhanced response of these cells to chemotherapeutic agents by suppression of PI3K/Akt signaling pathway (Chappell et al., 2011; Chen et al., 2018a,b; Mashayekhi et al., 2019; Chen et al., 2020). The previous studies results show that PTEN as a potential target of miR-29a. Indeed, PTEN as a tumor suppressor gene, which acts as a direct target of miR-29a in the endothelium cells (Wang et al., 2013). Du and colleagues (Du et al., 2016) revealed that miR-221 decreased gefitinib sensitivity via PI3K/Akt signaling pathway downstream of PTEN in human cervical cancer. Hernández et al. (Coronel-Hernández et al., 2019) revealed that in colorectal cancer miR-26a is significantly overexpressed, which means that miR-26a expression is inversely correlated with PTEN. Moreover, miR-26a could directly inhibit PTEN expression via 3′ UTR interaction. Furthermore, Xie et al., (2018) revealed that treatment with miR-132/212 result in diminished PTEN expression in MCF-7 cells in breast cancer. Inversely, we demonstrated that miR-29a a positive regulator of PTEN in improving drug resistance in colon cancer. Wu et al., (2018) revealed that overexpression of miR-503 leads to the sensitizer to cisplatin treatment in ovarian cancer by targeting PI3K/p85. Furthermore, Han and colleagues (Mirza-Aghazadeh-Attari et al., 2018) reported that the protective effects of miRNA-29a suppress the PTEN/Akt/GSK3β and Wnt/β-catenin signaling pathways in colon cancer. Also, Shen and colleagues (Shen et al., 2016) demonstrated that down-regulation of miR-29a expression in MCF-7/DOX cells in breast cancer enhanced PTEN expression levels, resulting in diminished p-Akt and p-GSK3β expression through PTEN/AKT/GSK3β signaling pathway. To our knowledge, we show for the first time a positive regulation of PTEN exerted by miR-29a in colon cancer cells. Surprisingly, we showed that PTEN expression was statically significant lower in HT29/DOX cells, in fact, miR-29a result in overexpression of PTEN in HT29/DOX cells. Up-regulation of PTEN in these cells results in suppression of the PI3K/Akt signaling pathway; in which finally, this mechanism results in the down-regulation of P-gp and enhances drug accumulation in cells.

Taken together, this study has provided insight into the role of the miR-29a/PTEN/PI3K/Akt/MDR1/P-gp axis in colon cancer drug resistance. In this study, we recognized miR-29a as a novel MDR suppressor through PTEN/PI3K/Akt/MDR1/P-gp pathway. These data show that interference of miR-29a expression could be potentially useful for the prediction of the clinical response to HT29/DOX, whereby providing us a promising target for the therapy on colon cancer. Maybe the results of this study can as an auxiliary bridge to future studies in curative improving colon cancer patients.

CRediT authorship contribution statement

Xiaoxin Shi: Supervision. **Amir Valizadeh:** Investigation. **Seyed Mostafa Mir:** Investigation. **Zatollah Asemi:** Writing - review & editing. **Ansar Karimian:** Investigation. **Maryam Majidina:** Conceptualization, Methodology. **Amin Safa:** Formal analysis, Writing - review & editing. **Bahman Yosefi:** Conceptualization, Methodology.

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