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Down-regulation of miR-135b in colon adenocarcinoma induced by a TGF-β receptor I kinase inhibitor (SD-208)

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ABSTRACT

Objective(s): Transforming growth factor-β (TGF-β) is involved in colorectal cancer (CRC). The SD-208 acts as an anti-cancer agent in different malignancies via TGF-β signaling. This work aims to show the effect of manipulation of TGF-β signaling on some miRNAs implicated in CRC.

Materials and Methods: We investigated the effects of SD-208 on SW-48, a colon adenocarcinoma cell line. The cell line was treated with 0.5, 1 and 2 μM concentrations of SD-208. Then, the xenograft model of colon cancer was established by subcutaneous inoculation of SW-48 cell line into the nude mice. The animals were treated with SD-208 for three weeks. A quantitative real-time PCR was carried out for expression level analysis of selected oncogenic (miR-21, 31, 20a and 135b) and suppressor-miRNAs (let7-g, miR-133b, 145 and 200c). Data were analyzed using the 2-ΔΔCT method through student's t-test via the GraphPad Prism software.

Results: Our results revealed that SD-208 could significantly down-regulate the expression of one key onco-miRNA, miR-135b, in either SW-48 colon cells (P=0.006) or tumors orthotopically implanted in nude mice (P=0.018). Our *in silico* study also predicted that SD-208 could modulate the expression of potential downstream tumor suppressor targets of the miR135b.

Conclusion: Our data provide novel evidence that anticancer effects of SD-208 (and likely other TGF-β inhibitors) may be owing to their ability to regulate miRNAs expression.

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer related death worldwide, making it a fine and attractive area for oncologists to study (1, 2). In numerous cancers including CRC, it has been documented that aberration of the growth factor signaling pathways specifically transforming growth factor-β (TGF-β) pathway play a vital role in tumor initiation, progression and metastasis (3-7). Therefore, it has become a proper target for cancer therapy (8-13). It is has been accepted that a major growth factor such as TGF-β, which has a wide range of effects on key physiological events, must be under extended regulation to control its expression and function. This regulation should include mechanisms that allow a variety of effects depending on special cellular and tissue contexts (14-16).

Recently, a new layer of cellular mechanisms as microRNA (miRNA)-mediated gene expression has been identified that is implicated in the post transcriptional negative regulation (17-20). The miRNAs were well-known to interact with signaling pathway components and to involve in multiple cellular processes as well as initiation, progression and metastasis of human cancers (21-25). Increasing evidences have revealed that cancer-associated miRNAs can function as oncogenes or tumor suppressors (18, 20). The miRNAs-mediated regulation networks can strictly influence the growth factors signaling pathways (24, 25). There are several studies showing relationship of TGF-β with diverse microRNAs (26-29). In addition, it has been shown that basic cell signaling pathways adjust the activity of the related components in miRNAs biogenesis pathway to achieve a fine miRNAs expression pattern (28).

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Recent emerging evidence suggests manipulation of growth factors signaling by special inhibitors can alter miRNAs expression in cancer cells *in vivo* and *in vitro* (30-35). Several strategies based on either restoration of silenced miRNAs or inhibition of overexpressed miRNAs has opened a new area of research in cancer therapy including CRC. It has been proposed that restoration of normal equilibrium for cancer-related miRNAs can inhibit colon tumor progression (36, 37). On the other hand, several reports imply that targeting of TGF-β signaling pathway at late stages of carcinogenesis could be a helpful tool for treatment of human cancers such as CRC, glioblastoma and breast cancer (4, 8). These studies show that a series of TGβRI kinase inhibitors such as SD-208 could be influential in treatment of a range of cancers (12). Our aim was to investigate the miRNAs (oncogene or tumor suppressor), whose expression might be altered due to inhibition of TGF-β signaling pathway.

Materials and Methods

Cell culture and treatment with SD-208

SW-48 cell line, a human colon adenocarcinoma cell line (Pasteur Institute, Tehran, Iran) was grown in 25 cm^2 flask (SPL Life Sciences; South Korea) containing RPMI-1640 medium (Gibco; Germany) supplemented with 5% fetal bovine serum (FBS) (Gibco; Germany) and 100 units/ml penicillin (Gibco; Germany). Drug treatment and cell viability assay were performed as previously explained (6). Briefly, the cells were trypsinized in exponential growth phase and were seeded in 6-well flat-bottom plates (SPL Life Sciences; South Korea) at a density of 5×10^5 cells/well (2000 µl media/well). 48 hr after treatment with 0.5, 1 and 2 µM concentrations of SD-208, the cells were harvested for total RNA extraction.

Animal model implanted with adenocarcinoma cell line (SW-48) and treatment protocol

The protocol for establishing the xenograft model of colon cancer was approved by the Committee on the Ethics of Animal Experiments of Tehran University of Medical Sciences (Ethical Code Number; ERC/S/277) as previously described (6). 6-week-old female athymic C56BL/6 nude mice (n= 8 per group) were obtained from Omid Institute for Advanced Biomodels (Tehran, Iran). After cell inoculation, xenograft tumors were allowed to achieve a size of 80 mm^3. Then, the animals were randomly divided into two groups of 8 to receive either SD-208 (50 mg/kg/d) or vehicle (DMSO-containing deionized water) orally for three weeks. Obtained tumors after isolating from animals, were fixed in formalin or frozen for histological staining and RNA extraction, respectively.

Histopathological diagnosis of colon tumors

To confirm colon adenocarcinoma, tumor tissues were excised and subjected to hematoxylin and eosin (H&E) staining (Dako, Denmark) as previously described (6).

Total RNA extraction from cultured and tumor tissues

Either SW-48 cells or tumor tissues at the mentioned time points after treatment with SD-208, were subjected for total RNA extraction using TRIzol reagent (Invitrogen; Germany) according to the manufacturer's instructions. Extracted total RNA was stored at -80 °C until use.

miRNA expression analysis by reverse-transcription (RT) real-time PCR

Four miRNAs as potential onco-miRs (miR-21, 31, 20a, 135b) and four miRNAs as potential suppressor-miRs (let7-g, miR-133b, 145, 200c) involved in colon cancer, were selected from the Sanger Center miRNA Registry at http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml. MicroRNA expression was analyzed by real-time quantitative polymerase chain reactions (qPCR) using the SYBR Green method (Parsgenom, Iran). After polyadenylation of total RNA and cDNA synthesis, miRNAs were expanded by the specific primers for mature forms according to the manufacturer's instructions. Real-time PCR was performed on a Bio Rad CFX96 Real-Time PCR System. RNU6B was used as an endogenous (internal) control, and the data were normalized compared to this housekeeping gene. All reactions were performed in triplicate and the absence of contamination was verified using non-template controls. PCR products also were visualized by electrophoresis on a 2% agarose gel.

Statistical analysis

Data analysis was performed using the 2-ΔΔcCT method. The standard error of means was computed and analysis of variance (ANOVA, Tukey's post tests) completed via GraphPad Prism 5.0 software. *P-values* less than 0.05 were considered to indicate statistically significant differences between data sets.

Results

SD-208 toxicity effects

To assess the potential toxicity effects of SD-208, the expression levels of miRNAs was examined by real time RT-PCR (Table 1). Then the appropriate numbers of SW-48 cells were injected into 8 mice to develop tumors. Following SD-208 treatment period, we could not observe any changes in animal behavior, body weight or lifespan compared to controls. Also all mice with an observed tumor growth survived with a balanced diet. These data suggest that SD-208 lacks toxic effects on animals (data not shown).

Table 1. miRNAs differentially expressed in SW-48 cells after 48 hr treatment with SD-208

miRNA	Fold change	*P*-value
miR-21	-0.923	0.401
miR-31	-0.938	0.467
miR-20a	-0.918	0.249
miR-135b	-0.519	0.006**
let7-g	+1.1	0.099
miR-133b	+1.07	0.163
miR-145	+1.09	0.15
miR-200c	+1.12	0.135

Colon adenocarcinoma confirmation

The H&E staining confirmed marked cellularity with significant hyperchromatism and pleomorphism (Figure 1). The pattern of adenocarcinoma was alike to the human origin (Figure 1 A, B).

Expression pattern of selected onco/suppressor miRNAs in colon adenocarcinoma SW-48 cell line

Real-time PCR analysis detected differentially expression of all selected miRNAs in SW-48 cell line (Table 2). Among the studied miRNAs, miRNA-135b and let7-g expressed at the highest and lowest levels, respectively (Figure 2).

Alteration of the miRNAs expression resulted by SD-208 treatment

Evaluation of miRNAs expression by q-PCR showed that the expression of miR-135b significantly down-regulated after treatment by SD-208 (*P*=0.006, Figure 3A). In the tumors treated with SD-208, miR-135b expression also was down-regulated significantly (*P*=0.018), compared to control tumors (Figure 3B). Our results showed that all the treated tumors express a lower number of miR-135b, but not other miRNAs, compared to the control tumors. However, there was no change in cell proliferation or tumor size (data not shown).

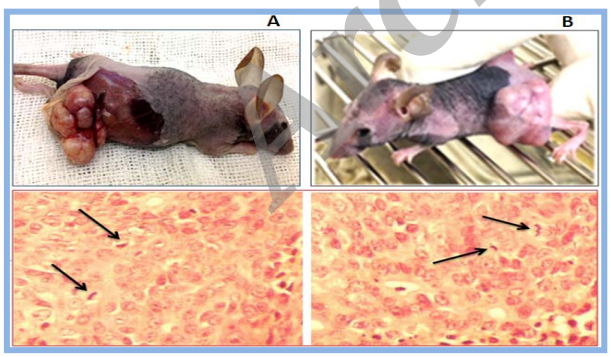

Figure 1. Representative results of orthotopic colon tumor in nude mice and pathological confirmation. Nude mice bearing developed SW-48 tumors were divided into two groups: SD-208 treatment (A) and vehicle (B). Hematoxylin and eosin staining of tumor tissues confirmed colon adenocarcinoma in both treated (A) and non-treated (B) tumor-bearing mice. The staining demonstrates marked cellularity with profound hyperchromatism and pleomorphism (arrows) and low differentiated tumor cells similar to human colon cancer

Table 2. miRNAs differentially expressed in SW-48-derived tumors after treatment with SD-208

miRNA	Fold change	*P*-value
miR-21	-0.923	0.401
miR-31	-0.938	0.467
miR-20a	-0.918	0.249
miR-135b	-0.519	0.006**
let7-g	+1.1	0.099
miR-133b	+1.07	0.163
miR-145	+1.09	0.15
miR-200c	+1.12	0.135

Prediction of the target genes of miR-135b using bioinformatics analysis

In order to determine the biological function of the down-regulated miRNA, miR-135b, we further predicted the putative downstream targets of this miRNA. We focused our attention on miR-135b because it was the only miRNA affected by SD-208 either *in vitro* or *in vivo*. *In silico* analysis using TargetScan (http://www.targetscan.org/) showed that miR-135b potentially targets transcripts encoding known tumor-suppressor factors, such as APC (adenomatosis polyposis coli), FOXO1 (forkhead transcription box1), RUNX1 (runt-related transcription factor 1) and ESRRA (estrogen-related receptor alpha).

Discussion

In this study, we evaluated the effect of a TGF-β receptor kinase inhibitor, SD-208, on some potential onco/suppressor-miRNAs expression in CRC. SD-208 is a TGF-β signaling pathway inhibitor that could exert anticancer effects on several tumor cells by reduction of growth rate or modification of other cell functions (6, 9, 12). Some investigators revealed SD-208 reduces tumor growth and metastasis in different cancers (9, 12), whereas, others showed this agent regulates the growth of tumors without changes in proliferation, apoptosis or angiogenesis and cannot reduce proliferation of cells (8). Another study reported that SD-093 (as an SD-208 analog) failed to alter morphology and growth rate of pancreatic carcinoma cells (38). Also it has been reported that SD-208 has no effect on the growth of

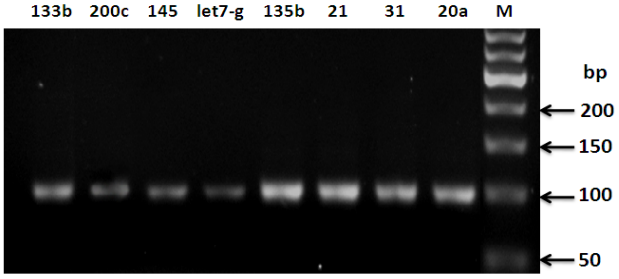

Figure 2. Electrophoresis of miRNAs genes pattern expressed in SW-48 cell line. As shown, all selected onco/suppressor miRNAs express differentially in SW-48 cell line

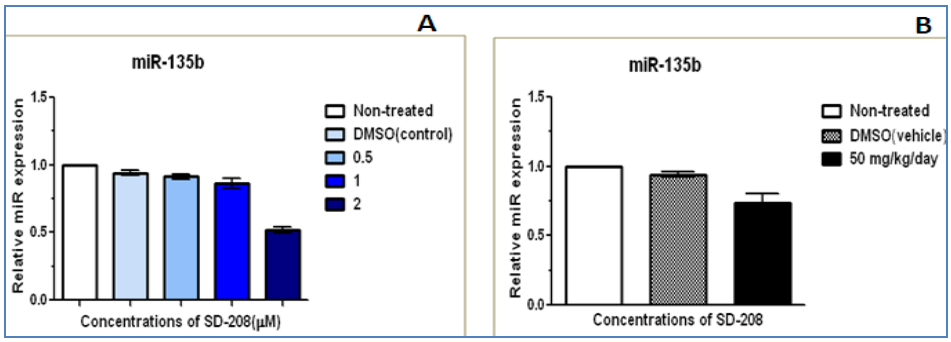

Figure 3. Modulation of miR-135b expression caused by SD-208 treatment. The expression of miR-135b significantly was down-regulated either in cell culture (A) or developed tumors (B) in SD-208 groups compared to controls

primary and metastatic R3T mammary tumors in athymic nude mice (9). These findings indicate that anti-cancer effect of SD-208 may be not due to suppression of cell growth and proliferation (36). Since efficacy of this inhibitor is a controversial issue in cancer treatment, we hypothesized the unknown mechanism(s) including alteration of cancer-related miRNAs might be involved.

Several chemopreventive agents have been shown to modulate the expression of numerous miRNAs in cancer cells that lead to sensitization of cancer cells to chemotherapeutic agents (39, 40), suggesting the potential of miRNAs as targets for anti-cancer drugs (18, 20). Hence, evaluation of the possible effects of chemotherapeutic drugs on the expression profile of miRNAs may have an important outcome for cancer therapy strategies (21, 33).

Although a number of studies have shown that chemotherapy drugs alter miRNAs expression in many cancer cells, there is no report on the effect of TGF-β receptor kinase inhibitors on miRNAs in human CRC. We selected some miRNAs that have already been confirmed to function as onco-miR (miR-21, miR-31, miR-20a, and miR-135b) or suppressor miR (miR-133b, miR-145, miR-200 and let7-g) in CRC (36-39). The present study focused on the alteration of these miRNAs in colon cancer treated by SD-208.

Expression analysis of miRNAs by a q-PCR revealed that expression of miR-135b significantly down-regulated after *in vitro* treatment with SD-208 (P=0.006). In the tumor tissues treated with SD-208, miR-135b expression significantly down-regulated, also. The results showed that all the treated tumors significantly expressed a lower number of miR-135b, but not other oncogenic-miRNAs, compared to controls (P=0.018). However, there was no change in cell proliferation or tumor size.

Interestingly, the miR-135b has been documented as a tumor promoting factor and to play a role in migration and metastasis in different cancers as well as CRC (37-39). In order to address the question of how can the possible molecular mechanisms of the miR-135b on CRC cell signaling pathways be defined, we performed an *in-silico* study. *In silico* analysis using TargetScan (http://www.targetscan.org/) showed that miR-135b potentially targets key tumor-suppressor genes involved in CRC: APC and FOXO1. APC and FOXO1 genes have also been validated as targets of miR-135b using luciferase reporter assay (41), hence, we would like to discuss these genes whose functions could potentially affect the cell signaling in CRC.

APC gene acts a tumor suppressor the inactivation of which is the key initiating event in colorectal carcinogenesis (42). Recently, one study demonstrated that up-regulation of miR-135b in CRC is associated with low APC mRNA levels (42). In the present study, we advocate a novel molecular mechanism: a kinase inhibitor molecule can reduce miR-135b expression, by which APC activation could be mediated.

Moreover, it has been reported that miR-135b affects FOXO1 as an endogenous target and suppresses protein expression. Several lines of evidence indicate that FOXO transcription factors might play an important role in tumor development (41, 43). On the other hand, FOXO1 alteration by miR-135b can affect sensitivity to chemotherapeutic drugs, such that tumor cells overexpressing miR-135b were more resistant to specific anticancer drugs. These results suggest the possibility that miR-135b may confer chemoresistance to tumor cells through FOXO1 modulation (41). Therefore, it is rational to hypothesize that the properties of anti-cancer drugs may be related to their alteration of miRNA profiles (43, 44). For example, suppression of miR-21 has been shown to sensitize MCF-7 cells to topotecan (44). As well, 5-FU was reported to be able to modify the expression of several miRNAs in human colon cancer cells (44). Similar studies exist for the drugs gemcitabine, doxorubicin and tamoxifen (38).

Overall, we primarily predicted that combination of SD-208 and one anticancer drug such as 5-FU, may show stronger inhibition of colon tumor cell growth by modification of onco-miRs. Additionally, as a novel approach in CRC therapy, pre-treatment by SD-

208 could improve chemosensitivity in resistant cancerous cells. However, the mechanism by which these agents alter miRNA expression may be dependent on genomic context.

Conclusion

The receptor kinase inhibitor SD-208 may partially inhibit colon tumorigenesis as well as chemoresistance by alteration of miRNAs. Hence, the TGβRI kinase inhibitor-based treatment may possibly uphold chemosensitivity in resistant colon cancer cells.

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Conflict of interests

The authors declare they have no conflict of interests.

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