

EXPERIMENTAL STUDY

Induction of miR-31 causes increased sensitivity to 5-FU and decreased migration and cell invasion in gastric adenocarcinoma

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ABSTRACT

Drug resistance is the main obstacle in the treatment of gastric cancer, the third most common cause of cancer-related death in the world. Due to their small size, easy entrance to cells and multiple targets, microRNAs (miRs) are considered novel and attractive targets. In the current study, parental MKN-45, MKN-45-control vector, and MKN-45-miR-31 populations were compared in terms of cell cycle transitions, migration, cell invasion, and proliferation. In addition, downstream targets of miR-31, including E2F6, and SMUG1 were examined using Real-time RT-PCR and western blotting. MKN-45-miR-31 showed an increased sensitivity to 5-FU, decreased migration and cell invasion compared to the control groups ($p = 0.0001$, $p = 0.01$ and $p = 0.01$, respectively). There was a significant increase in the percentage of cells in G1/pre-G1 phase in MKN-45-miR-31 relative to the control groups ($p = 0.001$). Induction of miR-31 expression in MKN-45 caused a significant reduction of E2F6 and SMUG1 genes. Our findings indicated that induction of miR-31 expression could increase drug sensitivity, and diminish tumor cell migration and invasion of gastric cancer cells. Therefore, miR-31 can be considered as a potential target molecule in the targeted therapy of gastric cancer (Fig. 2, Ref. 43). Text in PDF www.elis.sk. KEY WORDS: gastric cancer, miR-31, drug resistance, E2F6, SMUG1.

Introduction

Gastric cancer is the third most common cause of cancer-associated death, thus accounts for a considerable burden of global cancer-related mortality and morbidity (1, 2). The overall survival rate of gastric cancer has improved resulting from earlier surgical intervention, and chemo/radiotherapy regimens; however the vast majority of patients eventually develop drug resistance, recurrent disease and distant metastasis (2, 3). Therefore, finding new strategies for potentiation of the current therapeutic approaches or potential target molecules is an urgent clinical necessity.

MicroRNAs (miRNAs) are small non-coding RNA (20–22 nucleotides) involved in RNA silencing and post-transcriptional regulation of gene expression. In the last decade, miRNAs have been the focus of many research projects considering their role in tumorigenesis, drug resistance, invasion, metastasis and tumor relapse (4). Emerging evidence indicates that co-administration

of miRNAs with conventional chemotherapy drugs increases their anti-cancer effects (5–8). Co-administration of miR-200c and chemotherapeutic drugs enhances the therapeutic effects of these regimens on clear cell renal cell carcinoma (5). In addition, over-expression of miR-7 increases the sensitivity of cisplatin-resistant breast cancer cells to cetuximab and also non-small cell lung cancer cells (NSCLC) to paclitaxel (6, 9). It is also shown that restoration of miR-143 and miR-145 expression results in the re-sensitization colorectal cancer cells to cetuximab (10).

miR-31 may act as oncomiRs by targeting tumor suppressor genes or as tumor suppressor miRs by targeting oncogenes in a tissue-dependent manner (11). The preliminary studies indicated a strong association between decreased expression of miR-31 with advanced stage, lymph node metastasis and poor survival in gastric cancer (12, 13). A more recent experiment showed that induction of miR-31 by targeting of E2F2 can decrease the viability of gastric tumor cells, reduction of tumor cell invasion, and inhibition of in vivo tumorigenesis (13). Up-regulation of miR-31 by targeting integrin $\alpha 5$ (ITGA5) is shown to suppress the invasion and metastasis in SGC7901 gastric tumor cells (14). Additionally, Ruoming and co-workers demonstrated that increase in miR-31 through SGPP2, Smad4 and STAT3 molecules can result in the inhibition of cell proliferation and migration and increase in apoptosis (15).

Our recent immunohistochemical analysis showed the E2F6^{Low}/SMUG1^{High} expression pattern is associated with poorly differentiated tumors and with the omental involvement in gastric adenocarcinoma (16). In addition, the RhoA^{High}/SMUG1^{High} phenotype was more often found in poorly differentiated gastric adenocarcinoma

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clinical tumor samples. In the following study, induction of miR-31 caused an increased sensitivity to 5 fluorouracil (5-FU), inhibition of cell proliferation and invasion compared to the parental gastric adenocarcinoma cells, which are mediated by suppression of RhoA expression (17). E2F6, a member of E2F family, is targeted by Wnt signaling pathway and can act as a brake on the cell cycle progression (18). Up-regulation of E2F6 is more frequently found in gastric cancer compared to normal tissues (19). Base excision repair (BER) pathway is triggered by SMUG1, as DNA glycosylase, which eliminates non-bulky damaged bases after 5-FU chemotherapy treatment (22). Based on our previous study and new potential target molecules of miR-31, the current study was conducted to analyze the tumor-promoting effect of miR-31 on the malignant phenotype of gastric adenocarcinoma cells possibly through the E2F6 and SMUG1 downstream molecules.

Materials and methods

Cell lines and culture conditions

The gastric adenocarcinoma cell lines AGS (NCBI Code: C131) and MKN-45 (NCBI Code: C615) as well as human embryonic kidney cells (HEK 293) (NCBI Code: C497) were purchased from the Pasteur institute, Tehran, Iran. The cell lines were grown in DMEM medium (GIBCO) supplemented by 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin in 37 °C incubator.

RNA extraction, cDNA synthesis, and quantitative RT-PCR

Total RNA was extracted from MKN-45-miR-31 or the control groups using RNX-plus solution (Cat No. RN7713C, Cinnagen Inc., Tehran, Iran) using the manufacturer's instructions (23). cDNA synthesis was performed using a kit for mRNA synthesis (Cat No. K1641, Fermentas Life Sciences, Germany) and Expand™ Reverse Transcriptase (Cat No. 11785826001, Sigma-Aldrich, USA) for miRNA synthesis. The real-time PCR reactions were carried out using a Rotor-Gene 6000 system (Corbett, Concorde, NSW, Australia) and the data were normalized with the reference genes SNORD47 for miR-31 and β -actin for E2F6 and SMUG1 (24). In all experiments, we compared the MKN-45-miR-31 (termed "test"), MKN-45-control vector (termed "control") and parental MKN-45 (termed "control").

Retroviral transduction and GFP expression assay

For induction of miR-31, HEK293 cells were transduced with psPAX2, pMD2G, and pLEX-miR-31 or pLEX-control (Bon Yakhteh Cell Bank, Tehran, Iran). Purified lenti-miR-31 and lentiviruses containing control vector were used for transduction of MKN-45 cells. After that, transduced cells were exposed to puromycin for 24 h and the transduction efficiency was assessed by the visual analysis of GFP expression under a fluorescence microscope.

Viability and proliferation of MKN-45 miR-31-expressing cells

Effect of 5-FU on the viability and proliferation of the test (MKN-45-miR-31) and the control groups (MKN-45-control vector and parental MKN-45 cells) was evaluated using the MTT

assay. For this purpose, 1×10^4 cells from each population were seeded in 96-well plates and treated with a wide range of 5-FU (0.5, 1, 2, 4, 8 and 16 nanomolar) concentrations for 48 h.

Cell cycle analysis using flow cytometry

The test or the control group cells were harvested, washed with phosphate-buffered saline (PBS) and the single cell suspensions were fixed in 70 % ethanol. Subsequently, these cells were stained using propidium iodide (PI) staining solution, containing PI (50 mg/L), RNase A (1 g/L), and 0.1 % Triton X-100. Finally, the cell cycle analysis was performed using a fluorescence-activated cell sorting (FACS) flow cytometer (Partec, Germany) and the results were analyzed using FlowJo software (Tree Star, Ashland, OR) (25).

Cell migration and invasion assay

Examination of cell migration was undertaken using transwell inserts with a pore size of 8 μ m (SPL, cat number: CBA-100, Life Bioscience, Korea). For this purpose, 3×10^5 cells from each cell population were plated in the upper chamber and incubated for 24 h. Subsequently, the media in the lower chambers were collected and the cells in the chambers were harvested and counted.

For investigation of cell invasion, transwell inserts coated with Extracellular Matrigel Matrix (ECM, cat number: ECM550, Sigma-Aldrich, USA) were used. Briefly, 3×10^5 cells/well from the test or the control groups were plated and after incubation for 24 hr, the invaded cells at the bottom of the filters were counted.

Western blotting

The effect of miR-31 induction on E2F6 and SMUG1 protein expressions were assessed using western blotting. For this purpose, the protein was extracted from the three cell lines using RIPA buffer containing a protease inhibitor. Equal amounts of whole cell lysates from each group were separated by SDS-PAGE; the separated proteins were transferred to nitrocellulose membrane and incubated with the primary antibodies against E2F6 (ab152151, Abcam, Cambridge, UK), SMUG1 (ab11572, Abcam, Cambridge, UK), and β -actin. Primary antibodies were detected using secondary anti-rabbit or anti-mouse IgGs linked to horseradish peroxidase and then visualized using the ECL detection kit (Amersham, Life Science, USA).

Statistical analysis

Data were analyzed using the SPSS software version 20 using one-way ANOVA method (SPSS, Chicago, IL, USA). All the results are expressed as the mean \pm standard error. For all the analysis, a two-sided *p*-value of less than 0.05 was considered statistically significant. RT-qPCR results were analyzed using REST@2009 software.

Results

Low expression of miR-31 in MKN-45 cells compared to AGS cells

Expression levels of miR-31 in both cell lines, MKN-45 and AGS, were evaluated using quantitative real-time RT-PCR. The expression of miR-31 was significantly lower in MKN-45 cells

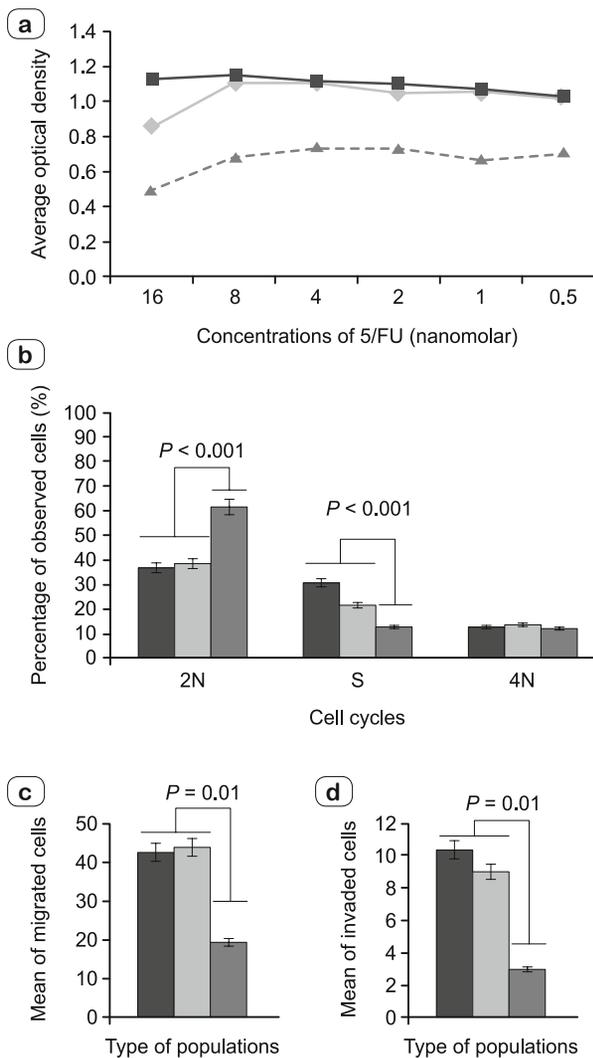


Fig. 1. The effect of miR-31 on the sensitivity of MKN-45 to 5-FU, cell proliferation, invasion, migration. (a) miR-31 increased the sensitivity of MKN-45 cells to 5-FU compared to control groups. (b) miR-31 decreased cell proliferation, (c) migration (d) invasion.

compared to AGS cells, so MKN-45 was used for transduction of miR-31 (Fig. 1a).

Stable miR-31 expression in MKN-45 cells

Stability of MKN-45 transduction with miR-31 was confirmed by the observation of green color in more than 85 % of cells under a fluorescence microscope. Validation of miR-31 expression using qRT-PCR assay showed a significant up-regulation of this transcript in MKN-45-miR-31 cell population compared to the MKN-45-control vector ($p = 0.01$) and parental MKN-45 cells ($p < 0.001$).

Expression of miR-31 increases sensitivity of MKN-45 cells to 5-FU

The effects of 5-FU on MKN-45 cells stably transduced by lenti-miR-31 or the control vector and the parental MKN-45 cells were

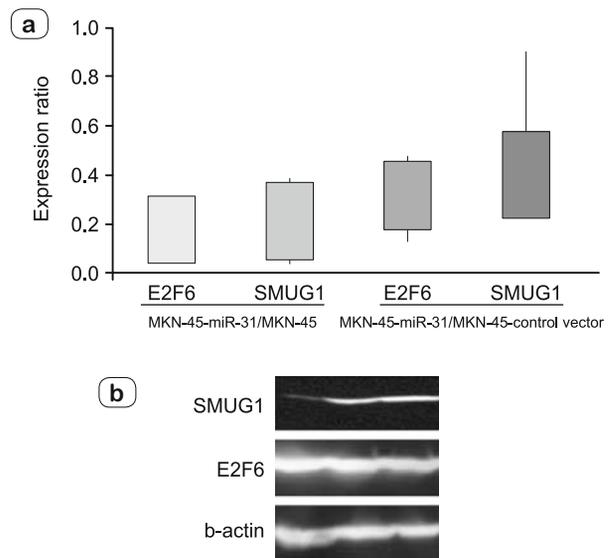


Fig. 2. The effect of miR-31 on E2F6 and SMUG1 expressions. Comparison of E2F6 and SMUG1 expressions in MKN-45-miR-31 compared to MKN-45-control vector and parental MKN-45 in terms of (a) genes using qRT-PCR and (b) protein using western blotting analysis (From left to right: MKN-45-miR-31, MKN-45-control vector and parental MKN-45).

determined using MTT assay. Stably transduced MKN-45 cells with pLEX-miR-31 displayed more obvious sensitivity to 5-FU than the control groups ($p = 0.0001$), whereas we did not find significant difference between the two control groups ($p = 0.098$) (Fig. 1a).

Overexpression of miR-31 decreased cell proliferation, migration, and invasion of MKN-45 cells

To understand the probable roles of miR-31 on cell cycle, we examined three populations. Percentage of cells in G1/pre-G1 and S phases in MKN-45 cells expressing miR-31 displayed a significant difference relative to the control groups ($p < 0.001$), while all the groups showed similar cell distribution in G2 phase ($p = 0.28$) (Fig. 1b).

Our analysis demonstrated a decreased migration ability in MKN-45 cells expressing miR-31 than the control groups ($p = 0.01$), whereas there was no significant difference between the control groups ($p = 0.99$) (Fig. 1c). In addition, cell invasion analysis revealed a significantly lower invasion capability in MKN-45-miR-31 cells ($p = 0.01$), but not between the control subgroups ($p = 0.75$) (Fig. 1d).

Decreased expression of SMUG1 and E2F6 genes in MKN-45-miR-31 cells

The effect of miR-31 induction on E2F6 and SMUG1 expressions was assessed using qRT-PCR. Our analysis showed a down-regulation of SMUG1 in MKN-45-miR-31 compared to the MKN-45-control vector and parental MKN-45 cells ($p < 0.001$ and $p < 0.001$, respectively). Furthermore, expression of E2F6 was significantly lower in the test group compared to the control groups (Fig. 2a).

Down-regulation of SMUG1 protein expressions in MKN-45-miR-31 cells

Expression of SMUG1 and E2F6 proteins were examined using western blotting. MKN-45-miR-31 cells showed a significant decrease in SMUG1 protein expressions compared to MKN-45-control vector and parental MKN-45 cells, whereas there was no significant difference in E2F6 protein expression between the test and the control groups (Fig. 2b).

Discussion

Gastric cancer ranks as the third leading cause cancer associated-death worldwide (1). The alarming trend of gastric cancer incidence rates is the most likely the result of the two facts: diagnosis of disease in advanced stages and drug resistance (26). Resistance to conventional chemo/radiotherapy regimens is a major obstacle in curing gastric cancer (27).

Alterations of signaling networks are evident in several malignant tumors. Importantly, elimination of cancer cells and cancer stem cells (CSCs) through aberrant specific pathways provides a new approach for targeted therapy. Wnt signaling pathway is a conserved network in CSCs, a subset of cancer cells, which exhibit tumor initiation capacity, metastasis, recurrence as well as drug resistance (28, 29). Base excision repair (BER) is major pathway in repair of DNA damages induced by cellular metabolism. Activation of Wnt signaling cascade recruit E2F6 and Ras homolog gene family, member A (RhoA), two main downstream molecules (29, 30). E2F6 encodes a transcriptional repressor that inhibits apoptosis and plays a key role in resistance to cisplatin (30, 31). Elevated expression of several members of the E2F family, including E2F6, has been demonstrated in gastric cancer samples using transcriptome and proteome analysis. (19). Furthermore, infection of normal gastric cells with *Helicobacter pylori* cause production of inflammation and subsequently ROS induces DNA damage, which leads to the activation of different DNA repair pathways, such as: base excision repair (BER) (28, 41, 42). SMUG1 is a key enzyme of BER pathway that encodes a single-stranded selective monofunctional uracil DNA glycosylase in the G1-S transition of the cell cycle (22). SMUG1 molecule can detach the 5-FU (a main component of many chemotherapeutic regimens in gastric adenocarcinoma patients) from DNA and confer drug resistance to gastric cancer cells (43). Immunohistochemical analysis of a panel of BER pathway molecules in gastric cancer patients showed high SMUG1 expression in specimens from patients with advanced-stage tumors and poor survival (41).

In a prior study, we examined the expression levels of E2F6, RhoA, and SMUG1 molecules and their correlations in gastric adenocarcinoma patients using tissue microarray-based immunohistochemistry (16). We showed E2F6^{Low}/SMUG1^{High} and RhoA^{High}/SMUG1^{High} indicative of aggressive tumor phenotype in gastric adenocarcinoma (16). In the current study, we examined the biological role of miR-31 in regulating E2F6 and SMUG1 molecules as well as cell proliferation, drug resistance, and invasion in gastric cancer cells. In a pioneer study, down-regulation of miR-31

was found in gastric cancer compared with adjacent normal tissues (12). In another study, Ruoming and co-workers showed low expression of miR-31 in gastric cancer specimens from patients with stages III/IV tumors and distant metastasis (15). In addition, the same authors showed that miR-31, by targeting Smad4 and SGPP2, diminished gastric cancer cell invasion and progression (15). A recent report indicated that down-regulation of miR-31 in gastric cancer tissues correlated with advanced tumor stage, lymph node metastasis and poor survival (13). The same group also showed induction of miR-31 expression in gastric cancer cells by inhibiting E2F2 (13). In yet another study, Zhang et al have demonstrated that miR-31 can suppress cell invasion and tumor progression through an integrin $\alpha 5$ mediated mechanism (14).

Conclusions

In summary, induction of miR-31 expression can result in increased drug sensitivity, decreased cell migration, and also in invasion of gastric cancer cells. Therefore, miR-31 may be considered as a potential target for the targeted therapy of gastric cancer.

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