

## RESEARCH ARTICLE

# MicroRNA-451 Inhibits Growth of Human Colorectal Carcinoma Cells via Downregulation of Pi3k/Akt Pathway

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### Abstract

MicroRNAs (MiRNAs) play important roles in coordinating a variety of cellular processes and abnormal expression has been linked to the occurrence of several cancers. The miRNA miR-451 is downregulated in colorectal carcinoma (CRC) cells, suggested by several research groups including our own. In this study, synthetic miR-451 mimics were transfected into the SW620 human CRC cell line using Lipofectamine 2000 and expression of miR-451 was analyzed by real time PCR, while expression of CAB39, LKB1, AMPK, AKT, PI3K and Bcl2 was analyzed by Western blot, and cell growth was detected by MTT assay. In comparison to the controls, a significant increase in the expression of miR-451 was associated with significantly decreased expression of CAB39, LKB1, AMPK, AKT, PI3K and Bcl2. The capacity of cell proliferation was significantly decreased by miR-451 expression, which also inhibited cell growth. Our study confirmed that miR-451 has a repressive role in CRC cells by inhibiting cell growth through down-regulating the PI3K/AKT pathway.

**Keywords:** Human colorectal carcinoma cells - SW620 - MiRNA-451 - PI3K/AKT pathway

*Asian Pacific J Cancer Prev*, **14** (6), 3631-3634

### Introduction

Colorectal carcinoma (CRC) is one common and frequently occurring malignant tumor. Even with the improved living standards and the changes in eating habits (e.g. smoking, insufficient physical activity, overweight and fatness, consumption of processed meat, and obesity which are risk factors for CRC), its total incidence is still on the rise. In 2008, 120,000 cases of CRC were newly diagnosed worldwide and more than 700 patients died (Jemal et al., 2011). The occurrence of CRC is a multifactor and multistep process, which involves genomic and epigenetic variation, activation of oncogene, inactivation of anti-oncogene, and occurrence of tumor (Eustace 2005; Ren et al., 2012; Dong et al., 2013). By probing into the pathogenic mechanism of CRC, we may discover the molecular markers that play key roles in its early diagnosis, individualized treatment, targeted therapy, and prognostic evaluation. Actually, microRNAs (miRNAs) show wide prospects in this area.

miRNA is a non-coding, single-strand and micromolecular RNA with 22 nucleotides and involved in posttranscriptional gene regulation. miRNA can specifically identify and join the 3' non-translated (3'UTR) region of mRNA, and induce the degradation or inhibited translation of mRNA, thereby regulating many important cell activities such as posttranscriptional gene expression (Chen et al., 2004). Studies on miRNA started in 1993

when (Lee et al., 1993) found the first miRNA (lin-4) in *C. elegans*. As studies go on, miRNA is known to be abnormally expressed in cells of several human tumors, and act as oncogene or anti-oncogene in the occurrence and development of tumors. So far nearly 1000 miRNAs have been identified, and they may be involved in regulation during differentiation, proliferation, migration, metabolism and death of cells.

Specific miRNAs that upregulate or downregulate the expression of CRC have been successively discovered. For instance, Bandres et al. (2006) examined 15 CRC cell strains by using real time PCR (RT-PCR), and detected the expressions of 156 types of mature miRNAs in 12 cases of paired tumor and non-tumor tissues. They found 13 types of miRNAs were expressed abnormally, where the expressions of miR-31, miR-96, miR-133b, miR-145, miR-183 and miR-135b were significantly disordered. The upregulation level of miR-31 was related to the pathological stage of CRC. CRC expression was significantly higher at stage IV than at stage II, indicating that miR-31 could induce tumors and render them with more invasive phenotype, and that miR-31 showed certain clinical effect in staging of CRC (Bandres et al., 2009) used qRT-PCR to examine the expressions of 250 types of miRNAs in the tissues of 21 gastric cancer patients, and the clinical pathological analysis showed that miR-451 was associated with the poor prognosis of gastric cancer patients. In-situ hybridization showed that the expressions

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of miR-451 decreased in epithelia of gastric cancer and colon cancer patients. Cell amplification decreased and radiation sensitivity increased in gastric cancer cell AGS and colon cancer epithelium DLD1 transfected by miR-451. These results support miR-451 as a regulator in cancer proliferation, and provide new vision for treatment of chemoradiotherapeutic resistance. Also the expressions of miR-451 in tissues of 12 CRC patients were examined by using qRT-PCR and found to be downregulated in 75% of CRC tissues. However, the association of miR-451 in the evolution of CRC, and the role of miR-451 in the emergence and development of CRC are unknown. In this study, the function of miR-451 in the inhibition tumor was discussed by using MTT assay, RT-PCR and Western blot.

## Materials and Methods

### Cell Culture and Transfection

Human CRC Cell Line SW620 was purchased from China General Microbiological Culture Collection Center (Beijing, China), and cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) in 5% CO<sub>2</sub> and 95% air at 37 °C. Transfections with hsa-miR-451 mimics were performed in serum-free medium for 24 h after plating, with Lipofectamine 2000 (Invitrogen). The oligonucleotide sequence of the hsa-miR-451 mimics was: 5'-AAACCGUUACCAUACUGAGUU-3'. A scrambled siRNA sequence (5'-TTCTCCGAACGTGTCACGT-3') was used as the negative control (Gima Biol Engineering Inc., Shanghai, China). Cells were cultured in complete medium after 6 h.

### Cell Growth Assays

Equal numbers of cells were plated in triplicate in 96-well culture plates and stained by 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) after transfection. Six replicates were prepared for transfection and culture until 24, 48 and 72 h. After the addition of 200 µl of dimethylsulfoxide (DMSO) in each well, the samples were incubated in dark for 30 min, and then swirled for mixing. Absorbance A at 490 nm was measured by using an enzymatic reader. Experiments were repeated three times.

### PCR Analysis

miRNA was isolated and then miRNA first-strand cDNA was synthesized using miRcute miRNA isolation kit and miRcute miRNA First-Strand cDNA synthesis kit (Tiangen, China) from SW620. Expressions of mature miR-451 were quantified by miR-qRT PCR using the SYBR PrimeScript™ miRNA RT-PCR Kit (Tiangen, China). U6 was used as the internal control. The forward primer of hsa-miR-451: 5'-UUUGGCAAUGGUA AUGACUCAA-3', U6: 5'-CTCGCTTCGGCAGCACAA-3'. Data are shown as fold change and analyzed initially using Opticon Monitor Analysis Software V2.02 (MJ Research, USA).

### Western Blot Analysis

Control cells, cells transfected with miR-451 mimics and scrambled oligonucleotides were lysed for total protein

extraction. The protein concentration was determined by the bicinchoninic acid (BCA) method (Beyotime, China), and 15 µg of protein lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresed proteins were transferred to 0.2 µm poly(vinylidene fluoride) (PVDF) membranes (MILLIPORE, USA), which were blocked in 5% non-fat milk and incubated overnight at 4 °C with diluted antibodies against Akt (1:800, Cell Signaling Technology, USA), Bcl-2 (1:200, Santa Cruz, USA), PI3K (1:200, Santa Cruz, USA), CAB39 (1:200, Santa Cruz, USA), and AMPK (1:200, Santa Cruz, USA). The membranes were then incubated with HRP-conjugated secondary antibody (1:500, Santa Cruz, USA). After 3 washings with phosphate buffer solution with Tween-20 (PBST), the membranes were probed using ultra-enhanced chemiluminescence Western blotting detection reagents. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

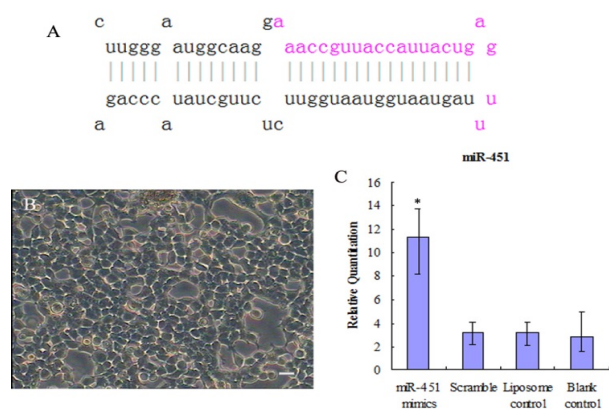
## Results

### miR-451 expression after transfection in Human CRC Cell Line, SW620

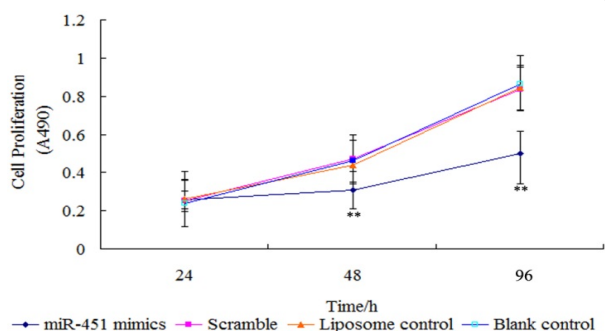
The expression of miR-451 was quantified by RT-PCR 48 h after transfection. As shown in Figure 1, miR-451 levels were significantly elevated by the miR-451 mimics.

### miR-451 Inhibited the Proliferation of Human CRC Cells In Vitro

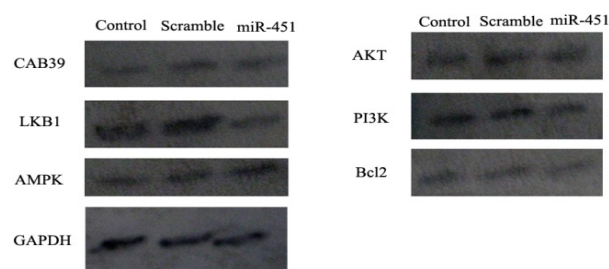
The MTT assay was used to measure the cell growth and viability of SW620 cells two days after transfection with miR-451 mimics oligonucleotides in comparison with black control and scrambled control. As shown in Figure 2, the growth inhibitory effect of the miR-451 mimics was time-dependent, with the maximum inhibition detected 3 days after transfection. These results suggest



**Figure 1. miR-451 mRNA Up-regulation in miR-451 Mimics Transfected Human Colorectal Carcinoma Cell Line SW620 Cells.** A Stem-loop of hsa-pre-miR-451, red color is the sequence of mature miR-451; B Human colorectal carcinoma cell line SW620 cells; C Cells were incubated with synthetic oligonucleotides as described in the materials and methods section, and miR-451 mRNA was quantified by real time PCR. Data represent mean ± SD of four independent experiments miR-451 mRNA was increased in miR-451 mimics-treated cells compared with the control and scramble groups ( $p < 0.05$ )



**Figure 2. Human Colorectal Carcinoma Cell Line SW620 Cells were Transfected with miR-451 Mimics and Cell Proliferation was Assessed Using the MTT assay.** Data are presented as the mean of triplicate experiments. The growth inhibitory effect of the miR-451 mimics was time dependent, with the maximum inhibition detected 3 days after transfection. Significant difference ( $p > 0.01$ )



**Figure 3. Effect of miR-451 Mimics on CAB 39, LKB1, AMPK, AKT, PI3K and Bcl-2 Expression.** Human colorectal carcinoma cell line SW620 cells were treated with synthetic oligonucleotides as described in the materials and methods section. miR-451 mimics down-regulated CAB 39, LKB1, AMPK, AKT, PI3K and Bcl-2 expression compared to the control, scrambled and oligonucleotides

that miR-451 might function as a tumor suppressor in human CRC cells in vitro.

#### Detection of miR-451 target genes

Bioinformatics analysis failed to predict and identify CAB39, LKB1 and AMPK as miR-451 target genes (GBandres et al., 2009; odlewski et al., 2010; Patrick et al., 2010; Li et al., 2011; Tian et al., 2012; Wang et al., 2013). The Western blot results showed that miR-451 over-expression decreased expression of miR-451 target genes, CAB39, LKB1 and AMPK (Figure3).

#### MiR-451 Over-Expression Decreased Akt, PI3K and Bcl-2 Levels

The expressions of Akt, PI3K and Bcl-2 were determined in SW620 cells treated with miR-451 mimics or scrambled oligonucleotides. Transfection with miR-451 mimics oligonucleotides downregulated Akt, PI3K and Bcl-2 expression compared to the blank, liposome and scramble groups (Figure 3). These data suggest that the tumor suppressor activity of miR-451 in human CRC cells may be associated with Akt, PI3K and Bcl-2.

## Discussion

MiRNAs are a type of non-coding single-strand RNA molecules with 20-25 nucleotides. MiRNAs, by complete or incomplete complementary pairing with target mRNAs,

can promote the target mRNA degradation, inhibit protein translation, and play important roles in cell proliferation, cell differentiation, cell apoptosis, gene regulation, and tumor occurrence (Chen et al., 2004, Huang et al., 2013). miRNA with diversified sequences, structure, abundance and expression ways can act as moderator in protein coding mRNA, and is important in gene expression, cell cycle regulation, and individual development. Recently, miRNA expression spectrum disorders were found in various tumors, including gastric cancer (Katada et al., 2009), nasopharyngeal carcinoma (Johnson et al., 2008), breast cancer (Hui et al., 2009), colon cancer (Chen et al., 2009) and lung cancer (Gao et al., 2011) indicating the important role of miRNA in regulation of tumor gene expressions. Because the gene regulation of miRNAs constitutes a powerful network, miRNAs show great potential: as a new treatment target and as a powerful intervention means. The overexpression of miRNA can be induced by using artificially synthesized miRNA imitation or chemically modified oligonucleotide induction. On the contrary, miRNA could be silenced by antisensing oligonucleotides and antagomirs. Regulation on miRNAs that inhibit tumor progression or on cancerogenic miRNAs may benefit the treatment of CRC and inhibit the progression of precancerous lesions (Tong and Nemunaitis 2008).

Cell proliferation and apoptosis play important roles in maintaining the balance of tissues. It is increasingly proved that miRNA can target at cancer genes or anti-oncogene, and participate in the occurrence and development of tumors by regulating proliferation and apoptosis (Akao et al., 2007). Transfected miR-143 and miR-145 precursors to CRC cell lines DLD-1 and SW480 respectively, and observed the growth inhibition and apoptosis promotion of dose-dependent cells, indicating that miR-143 and miR-145 can be used as anti-oncogenes in CRC (Chen et al., 2009). Transfected miR-143 mimic into Lovo cells, and consequently, the expression of K-RAS disappeared, while the K-RAS protein level was increased by the miR-143 inhibitor. Luciferase Reporter Gene Assay confirmed that miR-143 could directly identify the 3'-non-translated region of transcript K-RAS. Treatment on Lovo cells by miR-143 inhibitor could irritate cell proliferation, while the overexpression of miR-143 could restrain cell proliferation. This study proved for the first time that miR-143 could restrain the growth of colon cancer cells by restraining the translation of K-RAS (Gao et al., 2011). Used miRNA chips to examine the non small-cell lung cancer (NSCLC) tissues and found 40 differently expressed miRNAs, where the expression of miR-451 was the lowest and was related to tumor differentiation, pathologic staging, and lymph node metastasis. Also the low expression of miR-451 was related to the short total survival period of NSCLC patients. High ectopic expression of miR-451 could significantly restrain the proliferation of NSCLC cells, clone formation, and growth of nude mouse transplantation tumor. RAB14 was proved to be one target of miR-451, and miR-451 could regulate the survival of NSCLC cells by down-regulating the expression of RAB14. The serine-threonine protein kinase, AKT, is one major downstream target of PI3K.



Once activated, AKT moves to the cytoplasm and nucleus to phosphorylate, activate, or inhibit many downstream targets, regulating various cellular functions. PI3K regulates the single transmission of AKT phosphorylation. (Gallia et al., 2006, Kita et al., 2007) Several studies show that PI3K inhibition sensitizes glioma cells to radiation and chemical therapy (Opel et al., 2008; Prevo et al., 2008). Modulation of AKT signalling cascade using miRNAs in glioblastoma cell lines was described also in Nan et al. In this study, transfection of miR-451 mimicked reduced expression levels of Akt1, Cyclin D1, MMP-2, MMP-9 and Bcl-2. By contrast, miR-451 down-regulation led to increase in p27 levels. According to phenotypic experiments, miR-451 inhibited invasive ability, induced cell cycle arrest in the G0/G1 phase, delayed the progression of cell cycle, inhibited cell proliferation and induced apoptosis in glioblastoma cells in vitro. It seems that miR-451 affects glioblastoma cells via regulation of the PI3K/AKT signaling pathway (Gao et al., 2011). Akt plays a critical role in the control of diverse cellular functions, such as protein synthesis, cell cycle, cell survival and apoptosis (Yang et al., 2004; Song et al., 2005; Chen et al., 2012), and may represent an important link between miR-451 and Bcl-2. The regulation of cell proliferation, invasion and apoptosis, possibly via Akt, suggest that miR-451 may function as a tumor suppressor in human CRC. Future investigation will focus on the identification and validation of miR-451 targets for the development of novel strategies to treat CRC.

In summary, we showed for the first time that up-regulated expression of miR-451 suppressed cell proliferation in the CRC cell line SW620. In addition, the growth inhibitory mechanisms of miR-451 were examined in vitro using Western blot. The results suggested that miR-451 may function as a tumor suppressor. The potential application of miR-451 for the development of miRNA-based CRC therapy strategies should be further examined.

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