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Original Articles

# MicroRNA-195 desensitizes HCT116 human colon cancer cells to 5-fluorouracil

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ABSTRACT

Multidrug resistance is one major barrier to successful chemotherapy. Although several studies have attempted to overcome resistance of cancer cells to anti-cancer drugs, key determinants of resistance remain largely unknown. The objective of this study was to investigate whether microRNAs might play a role in the acquisition of resistance. Human colorectal cancer HCT-116 cell lines were transduced with a lentivirus library containing 578 precursor microRNAs (miRNAs) to establish cell lines resistant to 5-fluorouracil (5-FU). Specific miRNAs were identified from four different resistant clones and a miR-195-expressing resistant clone (HCT-116_lenti-miR-195) was further investigated. The HCT-116_lenti-miR-195 cells showed resistant phenotype. These cells grew faster after 5-FU treatment compared to control cells (HCT-116_lenti-control). Check point kinase 1 (CHK1) and G2 check point kinase WEE1 were found to be direct targets of miR-195. Downregulation of miR-195 sensitized HCT-116 cells after 5-FU treatment. Our results demonstrate that miR-195 can promote acquisition of drug resistance to 5-FU.



## Introduction

Chemotherapy is a useful way to treat cancer cells. However, acquisition of resistance to anti-cancer drugs is a major hindrance to effective chemotherapy. Cancer cells have multiple mechanisms to overcome toxic effects of anti-cancer drugs by altering drug transportation, inhibiting drug metabolism, increasing tolerance to genotoxic stresses, or decreasing cell death [1–3]. 5-fluorouracil (5-FU) is a widely used drug to treat a range of cancers, including colorectal, liver, and breast cancers. It interferes with DNA replication by inhibiting thymidylate synthase, thereby leading to cell cycle arrest or cell death [4]. High expression levels of thiamine synthase, Bcl-2, Bcl-XL, and Mcl-1 are related to 5-FU resistance [5–7]. Although diverse approaches have been made to manage chemo-resistance of cancer cells to 5-FU, core factors and key determinants of drug resistance are not fully elucidated yet.

MicroRNAs (miRNAs) are small noncoding RNAs that can negatively regulate gene expression by binding to the 3′ untranslated region (3′UTR) of target mRNAs [8,9]. MiRNAs play pivotal roles in diverse processes such as differentiation, proliferation, cell death, development, and pathogenesis of different diseases, including cancer [10,11]. Several studies have shown that miRNA expression profiles in drug-resistant cancer cells are significantly changed in comparison with those of parental drug-sensitive cancer cells [12,13]. Aberrant expressions of certain miRNAs can lead to drug resistance by abnormally affecting expression levels of genes involved in drug transport, metabolism, stress response, cell survival, and cell death [14,15]. For example, miR-19, -20, -21, and -204 can regulate 5-FU resistance in various types of cancer cells [16–21]. These findings indicate that miRNAs can function as active drivers in the regulation of resistance to anti-cancer drugs.

In this study, we performed functional screening of miRNAs involved in the acquisition of resistance to 5-FU in human colorectal cancer HCT-116 cells using a lenti-viral miRNA library and identified miR-195 as a regulatory miRNA governing the acquisition of resistance to 5-FU. We demonstrated that cell growth and viability were enhanced in resistant clone stably expressing miR-195 in response to various anti-cancer drugs. Inhibition of miR-195 sensitized resistant cells to 5-FU by downregulating WEE1 and CHK1. Our results suggest that miR-195 plays an essential role in the acquisition of drug resistance. Therefore, targeting miR-195 might be able to decrease resistance of colorectal cancer cells to anti-cancer drugs.

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## Materials and methods

### *Cell culture, transfection of plasmids, precursors and inhibitors of miRNA, and treatment*

Human colorectal cancer HCT-116 cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) and antibiotics. All transient transfections including precursor and inhibitor of miR-195 (Bioneer, Daejeon, Korea) and enhanced green fluorescent protein (EGFP) reporter plasmids were performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). EGFP reporters were cloned by inserting 3′UTR of *CHK1* or *WEE1* mRNA into pEGFP-C1 vector (BD Bioscience, Heidelberg, Germany).

### *Infection of lentivirus library into cells*

HCT-116 cells were seeded into a 6 well plate at a concentration of $1.0 \times 10^5$ cells/well one day prior to viral transduction. Lenti-miR™ Library (Cat. No. PMIRHPLVAHT-1, System Biosciences, Palo Alto, CA, USA) and polybrene (System Biosciences, Palo Alto, CA, USA) were diluted in complete medium to a final concentration of 5 μg/ml. Infections of Lenti-miR™ Library was performed with a multiplicity of infection of 10 according to the manufacturer's protocol. After incubation at 37 °C in a humidified atmosphere for 24 h, cell media were replaced with fresh complete medium. For phenotype selection cells were treated with 10 μM 5-FU until all transduced cells with lenti-miR™ virus control were dead. Survival cells were isolated as a single clone.

### *Identification of miRNA effector clone*

Genomic DNA was isolated from each clone using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. Precursors of miRNA integrated into gDNA of cells were amplified by polymerase chain reaction (PCR) with specific primers targeting lentivirus vectors (see Table 1). Effector miRNA amplicons with size ranging from 500 to 600 bp were recovered by gel purification, and verified by nucleotide sequencing (Cosmogentech, Seoul, Korea) using primers specific for lentiviral vectors as shown in Table 1.

### *MTT assay*

To assesses cell viability, a colorimetric assay was carried out using tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After cells ($1.0 \times 10^4$ cells) were inoculated into each well of a 96 well plate, samples were exposed to 5-FU, oxaliplatin, cisplatin, or doxorubicin for 72 h. MTT was then added to each well at 0.5 mg/ml followed by incubation for 2 h. After removing the medium, crystals formed by cells were solubilized by adding 100 μl of distilled water. Absorbance was then measured at wavelength of 570 nm using a Victor 3 microplate reader (Perkin Elmer, Turku, Finland).

### *Flow cytometric analysis of cell cycle*

Cell cycle was evaluated based on flow cytometric analysis of propidium-iodide-stained nuclei. Briefly, cells ($1 \times 10^6$ cells) were fixed in 75% ethanol at 4 °C overnight. After washing with PBS, cells were incubated at 37 °C in PBS containing 2 mM EDTA and RNase A (0.2 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Propidium iodide (0.02 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to cells and incubated at room temperature for 20 min. Cells were then subjected to flow cytometric analysis using FACSCanto II (Becton, Dickinson and Company, San Jose, CA, USA) and FACSCalibur™ cell analyzer. Results were analyzed with BD Cell Quest™ Pro version 5.2.1 (BD) and WinMDI version 2.9 (The Scripps Research institute, San Diego, Ca, USA). A total 10,000 events were calculated for each sample.

**Table 1**
Primer sequences used in this study.

| Primers for PCR | sequences |
|---|---|
| human GAPDH-F | 5′-TGCACCACCAACTGCTTAGC-3′ |
| human GAPDH-R | 5′-GGCATGGACTGTGGTCATGAG-3′ |
| human WEE1-F | 5′-AGGGAATTTGATGTGCGACAG-3′ |
| human WEE1-R | 5′-CTTCAAGCTCATAATCACTGGCT-3′ |
| human CHK1-F | 5′-ATATGAAGCGTGCCGTAGACT-3′ |
| human CHK1-R | 5′-TGCCTATGTCTGGCTCTATTCTG-3′ |
| miR-195-5p | 5′-TAGCAGCACAGAAATATTGGC-3′ |
| human U6 | 5′-CGCAAGGATGACACGCAAATTC-3′ |
| pCDH-5.1 (gDNA PCR-F) | 5′-GCCTGGAGACGCCATCCACGCTG-3′ |
| pCDH-3.1 (gDNA PCR-R) | 5′-GATGTGCGCTCTGCCCACTGAC-3′ |
| **Primers for EGFP-reporter** | **sequences** |
| human WEE1-3U-F | 5′-AAAAAGATCTTAACCCACTGGGAGCAC-3′ |
| human WEE1-3U-R | 5′-AAAAGGTACCCAGCATTCCAAAGGAGG-3′ |
| human WEE1-3UM-F | 5′-CTTGTGAATTTGGACGTATTTTAGTTTT-3′ |
| human WEE1-3UM-R | 5′-AAAACTAAAATACGTCCAAATTCACAAG-3′ |
| human CHK1-3U-F | 5′-AAAAAGATCTTAATCGGACCATCGGCTC-3′ |
| human CHK1-3U-R | 5′-AAAAGGTACCAATTCTATTCATCCTTTCC-3′ |
| human CHK1-3UM-F | 5′-GTGAATATAGTGGACGTATGTTGACATT-3′ |
| human CHK1-3UM-R | 5′-AATGTCAACATACGTCCACTATATTCAC-3′ |

### *Western blot analysis*

Whole cell lysates were prepared using RIPA buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, and 0.1% SDS], separated by SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Belfor, MA, USA). Incubations with primary antibodies to detect WEE1, CHK1, EGFP (Santa Cruz Biotechnology), CCNB1 (Cell Signaling Technology®), and β-actin (Abcam, Cambridge, MA, USA) were followed by incubations with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Protein bands were detected by using enhanced luminescence (Clarity™ Western ECL Substrate Kit, Bio-Rad, Hercules, CA, USA).

### *RNA and miRNA analysis*

Total RNA was prepared from whole cells using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA). After reverse transcription (RT) using ReverTra Ace® qPCR RT Kit (Toyobo, Japan), transcriptional levels of genes were assessed by real time quantitative (q) PCR analysis using KAPA SYBR® FAST qPCR Kits (Kapa Biosystems, Inc., Woburn, MA, USA) and gene-specific primer sets (Table 1). RT-qPCR analysis was performed on StepOnePlus™ (Applied Biosystems, Foster City, CA, US). Individual miRNAs were further quantified using QuantiMir™ cDNA Kit (System Biosciences Inc., Palo Alto, CA, USA). For QuantiMir™ cDNA analysis, forward primers were designed to be the exact sequences of miRNAs based on miRBase database, and a universal reverse primer provided by the kit was used.

### *Colony-forming assay*

Cells were seed into 6-well plates at density of $1 \times 10^3$ cells/well and incubated with or without 5-FU for three weeks. After fixation with 4% formaldehyde, cells were stained with 0.05% crystal violet for 10 min at room temperature, followed by washing with PBS and drying. Quantification of crystal violet stained area was performed using ImageJ software (Ver. 1.48).

## Results

### *Functional screening of cell lines resistant to 5-FU using Lenti-miRNA library*

To identify miRNAs involved in the regulation of drug resistance to 5-FU, we performed functional screening using Lenti-miRNA library. Human colorectal cancer HCT-116 cells were transduced with lenti-viral particles containing 578 precursor miRNAs (multiplicity of infection = 10) and sequentially incubated with 10 μM 5-FU until control cells were dead (Fig. 1A) (2017 EMM). Each GFP-positive HCT-116 clone survived after exposure to 5-FU was isolated and established as 5-FU resistant clone (Fig. 1B). Individual miRNA from each clone was identified by genomic DNA PCR using specific primer sets followed by sequencing of PCR amplicons (Fig. 1C). Four miRNA (miR-195, -133a-1, -616, and -671) expressing resistant clones were established and one lenti-miR-01 clone, lenti-miR-195, was further analyzed in this study. Relative levels of miR-195 in both lenti-Control (lenti-Ctrl) and the lenti-miR-195 cells were determined by RT-qPCR. As shown in Fig. 1D, mature forms of miR-195 were higher in lenti-miR-195 cells compared to those in lenti-Ctrl cells. To investigate sensitivity of lenti-Ctrl and lenti-miR-195 cells to 5-FU, cells were exposed to 5-FU and cell viability was assessed by MTT assay. Lenti-miR-195 cells were found to be more resistant to 5-FU than lenti-Ctrl cells (Fig. 1E). We further tested whether miR-195 was involved in the regulation of 5-FU resistance by assessing cell viability after ectopic expression of pre-miR-195. miR-195 overexpression resulted in increased viability of HCT-116 cells after exposure to 5-FU. These results indicated that miR-195 has potential to regulate resistance of HCT-116 cells to 5-FU.

### *Chemoresistance of lenti-miR-195 cells to several anti-cancer drugs*

Cell survival of lenti-miR-195 cells in response to 5-FU was increased compared to that of lenti-Ctrl cells (Fig. 1E). To evaluate