Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Prognostic potential of MNX1-AS1 in chemotherapy of colorectal carcinoma

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ARTICLE INFO

Original paper

Article history:
Received: September 30, 2023
Accepted: December 07, 2023
Published: December 20, 2023

Keywords:

CRC; MNX1-AS1; Chemotherapy resistance

ABSTRACT

This studt aimed to clarify the role of MNX1-AS1 in 5-FU resistance of Colorectal carcinoma (CRC). Relative levels of MNX1-AS1 in CRC and paracancerous tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Recruited CRC patients were treated with 5-FU-based FOLFOX chemotherapy, and they were divided into an effective group and a non-effective group according to the therapeutic efficacy, followed by comparison of their differences in clinical indicators. Influences of MNX1-AS1 on clinical features of CRC were analyzed. In addition, *in vitro* level of MNX1-AS1 in 5-FU-resistant HCT-8 cells and their parental cells was detected. After knockdown of MNX1-AS1 in HCT-8/5-FU cells, viability change was evaluated by cell counting kit-8 (CCK-8) assay. At last, regulatory effects of MNX1-AS1 on expression levels of ABC family genes were detected. MNX1-AS1 was upregulated in CRC tissues than paracancerous ones, and its level was higher in 5-FU-resistant CRC cases in comparison to 5-FU-sensitive cases. MNX1-AS1 level was linked to tumor size, tumor differentiation, depth of invasion, TNM staging and lymphatic metastasis in CRC. Notably, TNM staging, depth of invasion and lymphatic metastasis could affect the efficacy of FOLFOX chemotherapy in CRC patients. Knockdown of MNX1-AS1 reduced viability in HCT-8/5-FU cells, and downregulated ABCA1, ABCB1, ABCC1, ABCG1 and ABCG2. MNX1-AS1 triggers 5-FU resistance in CRC cells. Knockdown of MNXK1-AS1 is conducive to the good response to FOLFOX chemotherapy in CRC patients.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.14.41

Introduction

Colorectal carcinoma (CRC) is a common malignant tumor affecting human health. It is estimated by WHO that in 2018, CRC was the fourth leading cause of cancer death (1). Chemotherapy resistance is one of the most important reasons that limits the survival of CRC (2). AJCC data released that the 5-year survival of cTNP stage I CRC patients is up to 93.2%, which is only 8.1% in stage IV patients (3). Therefore, detection of CRC in the early phase contributes to timely health management.

Long non-coding RNAs (lncRNAs) are transcribed by RNA polymerase II, which are generally longer than 200 nt in transcripts (4). Recent studies have proven that dysfunctional lncRNAs are involved in tumor progression (5,6). They are considered as potential targets for diagnosing, treating and predicting the prognosis of malignant tumors (7,8). Multiple abnormally expressed lncRNAs have been discovered in CRC tissues, and their involvement in the CRC process and chemotherapy resistance remains to be comprehensively explored (9). LncRNA MNX1-AS1 is previously reported to be upregulated in epithelial ovarian cancer, which has a close relation to the poor prognosis (10). In addition, knockdown of MNX1-AS1 is able to block ovarian cancer cells to proliferate and migrate (11).

The high incidence of chemotherapy resistance leads to a high tumor recurrence rate and low survival rate, which limits the effectiveness of chemotherapy and poses a life-threatening to affected patients. Previous studies have elucidated various cytological mechanisms of chemotherapy resistance. Generally, changes in cancer cell behaviors, drug metabolism and cytotoxicity are attributed to the occurrence of chemotherapy resistance (12). Multiple lncRNAs have been discovered to regulate CRC cell phenotypes (13,14). It is reported that lncRNA snaR is remarkably downregulated in 5-FU-resistant cells (15). This study aims to elucidate the potential role of MNX1-AS1 in regulating 5-FU resistance of CRC. Our findings may provide references for clinical prevention and treatment of drug resistance of CRC.

Materials and Methods

Case collection

This study was approved by the Ethics Committee of our hospital. Signed written informed consent were obtained from all participants before the study. Sixty pairs of CRC and paracancerous tissues were collected from CRC patients treated with FOLFOX chemotherapy. Their clinical data were recorded in detail. Inclusion criteria: 1) Age > 18 years; 2) Subjects were preoperatively diagnosed as CRC; 3) CRC patients were treated with the palliative neoadjuvant 5-FU-based FOLFOX chemotherapy (> 3 cycles) after preoperative evaluation; 4) No surgery within 1 month of neoadjuvant chemotherapy; 5) Selective radical or palliative resection was conducted after neoadjuvant

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chemotherapy. Exclusion criteria: 1) Juveniles (age < 18 years); 2) Patients who did not meet the inclusion criteria; 3) Sudden diseases (i.e. sudden death, severe infection, multiple organ dysfunction); 4) Patients who asked to withdraw from the trial.

5-FU-based FOLFOX chemotherapy lasted for more than 3 cycles, every 2-3 weeks. The recommended dose schedule of FOLFOX chemotherapy was as follows:
Day 1: Oxaliplatin, 85 mg/m^2, IV infusion, 2 h;
Day 1 + 2: Calcium folinace (CF), 200 mg/m^2, IV infusion, 2 h;
Day 1 + 2: 5-FU, 400 mg/m^2, IV bolus, 2 min; 600 mg/m^2, IV infusion, 22-24 h.

Perioperative and postoperative evaluation

Patients were divided into a chemotherapy chemotherapy-effective group and a non-effective group according to the clinical evaluation of serum levels of tumor biomarkers, and perioperative and postoperative evaluation of tumor size, depth of invasion and lymphatic metastasis after FOLFOX chemotherapy. The effectiveness of chemotherapy was defined as an obvious contraction of primary tumor lesions and a decrease in malignant cavity effusion. Otherwise, enlargement of primary tumor lesions and infiltration, increased cavity effusion or new metastases within 6-12 months were considered as non-effectiveness of chemotherapy. CRC tissues used in the experiment were pathologically confirmed.

Cell culture

HCT-8 cells were cultivated in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 5% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and maintained at 37°C with 5% CO_2. After adherent cells were grown to 85-90% confluence, they were passaged using 0.25% trypsin. Second-generation cells were used in the following experiments.

Induction of 5-FU-resistant cells

HCT-8 cells were prepared to suspension with 1×10^6 cells/ml, which were cultured in the medium with 0.2 μM 5-FU. When cells were able to keep growing with the survival higher than 80%, the concentration of 5-FU in the medium was gradually enhanced from 0.2 μM, 2 μM, 20 μM, 40 μM, 60 μM, 80 μM, 100 μM, 120 μM, 140 μM, 160 μM, 180 μM to 200 μM within 5 months. HCT-8/5-FU cell line was generated once cells were able to normally grow in the medium containing 10% FBS and 200 μM 5-FU.

Cell transfection

Cells were seeded in a 6-well plate with 2×10^5 cells per well one day prior to transfection, with three replicates per sample. At about 60% confluence, the serum-free medium was replaced. Transfection plasmid was diluted in RNase-free water to prepare the working solution (20 mol/L) and stored at -20°C. 5 μL of transfection plasmid and 5 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was respectively diluted in 500 μL of serum-free Opti-MEM, and gently mixed 5 min later. The mixture was added per well for transfection, and the complete medium was replaced at 6 h. Sequences of si-MNX1-AS1 were: F:5'-CCGGGCTCTGCAGGTCGAACCTTATCTCGAGATAAGGTTCGACCTGCAGCTTTTTG-3', R:5'-AATTCAAAAAGCTCTGCAGGTCGAACCTTATCTCGAGATAAGGTTCGACCTGCAGAGC-3'.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs in tissues were collected using TRIzol (Invitrogen, Carlsbad, CA, USA), which were subjected to measurement of concentration and purity. Qualified RNAs were reversely transcribed to complementary deoxyribose nucleic acids (cDNAs) using the PrimeScript-RT kit. QRT-PCR was finally conducted using SYBR⑩Premix-Ex-Taq™ (TaKaRa, Tokyo, Japan) at 95°C for 10 min, and 45 cycles at 95°C for 10 s, 60°C for 30 s and 85°C for 20 s. Primer sequences were as follows: MNX1-AS1 F: 5'-CGCATTTTCAGATTCACGCAGG-3', R: 5'-TTGCAAGACTCACGTAGCACTGTG-3'; ABCA1 F: 5'-ACCCACCCTATGAACAACATGA-3', R: 5'-GAGTCGGGTAACGGAAACAGG-3'; ABCB1 F: 5'-ACCCATCATTGCAATAGCAGGAGTTGT-3', R: 5'-AGTTTGACTCACCTTCCCAGT-3'; ABCC1 F: 5'-TACCTCCTGTGGCTGAATCTGG-3', R: 5'- CCGATTGTCTTTGCTCTTCATG-3'; ABCG1 F: 5'-GGGGTCGCTCCATCATTTG-3', R: 5'-TTCCCCGGTACACACATTGTC-3'; ABCG2 F: 5'-GTGGCCTTGGCTTGTATGAT-3', R: 5'-GATGGCAAGGGAACAGAAAA-3'; GAPDH F: 5'-GCTGAGAACGGGAAGCTTGT-3', R: 5'-GCCAGGGGTGCTAAGCAGTT-3'.

Cell counting kit-8 (CCK-8)

Treated cells were inoculated in a 96-well plate with 1×10^5 cells/mL. At the indicated time points, the absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) was used for statistical analysis. Differences between groups were compared using the t-test. Enumeration data were analyzed by the Chi-square test. Data were expressed as mean ± standard deviation. $P<0.05$ was considered as statistically significant.

Results

Potential influences on the therapeutic efficacy of FOLFOX chemotherapy in CRC patients

After a comprehensive evaluation on the efficacy of FOLFOX chemotherapy, 60 recruited CRC patients were divided into chemotherapy effective group (n=35) and non-effective group (n=25). No significant differences in sex, age, location of tumor, tumor size and differentiation were found between groups ($P>0.05$). Nevertheless, significant differences in TNM staging, depth of invasion and lymphatic metastasis were identified, which were potential factors influencing the efficacy of FOLFOX chemotherapy ($P<0.05$) (Table 1).

MNX1-AS1 levels in CRC

In comparison to paracancerous tissues, MNX1-AS1 was highly expressed in CRC tissues (Figure 1A). In addition, its level remained higher in 5-FU-resistant CRC cases than that of 5-FU-sensitive ones (Figure 1B). It is

Table 1. Potential influences on the therapeutic efficacy of 5-FU-based FOLFOX chemotherapy in colorectal carcinoma patients.

Variable	n	Chemotherapy effective group (n=35)	Chemotherapy non-effective group (n=25)	χ^2	*p*
Sex					
Male	31	19	12	0.231	0.794
Female	29	16	13		
Age (years)					
<60	26	14	12	0.380	0.603
≥60	34	21	13		
Location					
Colon	30	20	10	1.714	0.295
Rectum	30	15	15		
Diameter (cm)					
<3	27	13	14	2.095	0.191
≥3	33	22	11		
Differentiation					
High	23	12	11	0.582	0.591
Poor/moderate	37	23	14		
TNM staging					
I/II	23	9	14	5.659	0.030
III/IV	37	26	11		
Depth of invasion					
T1/T2	29	11	18	9.613	0.004
T3/T4	31	24	7		
Lymphatic metastasis					
No	35	16	19	5.503	0.033
Yes	25	19	6		

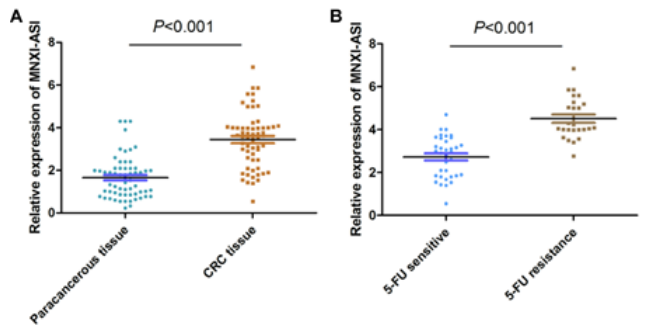

Figure 1. MNX1-AS1 levels in CRC. (A) MNX1-AS1 levels in CRC and paracancerous tissues; (B) MNX1-AS1 levels in 5-FU-sensitive and 5-FU-resistant CRC tissues. P<0.001.

indicated that MNX1-AS1 was unfavorable to 5-FU-based chemotherapy in CRC patients.

Influence of MNX1-AS1 on CRC pathology

To analyze the influence of MNX1-AS1 on CRC, we divided recruited patients to high-level (n=30) and low level group (n=30) according to the median level of MNX1-AS1. Significant differences were detected in tumor size, differentiation, TNM staging, depth of invasion and lymphatic metastasis between groups (Table 2). Hence, MNX1-AS1 was able to affect tumor size, differentiation, TNM staging, depth of invasion and lymphatic metastasis of CRC.

MNX1-AS1 levels in 5-FU-resistant CRC cells

Differential levels of MNX1-AS1 were detected in HCT-8/5-FU cells and the parental cells, which were highly expressed in the former (Figure 2A). Subsequently, the transfection efficacy of siMNX1-AS1 was tested in HCT-8/5-FU cells, showing an effective outcome (Figure 2B).

MNX1-AS1 upregulated drug-resistance genes

CCK-8 assay showed that cell viability was markedly declined after the knockdown of MNX1-AS1 in HCT-8/5-FU cells (Figure 3A). Furthermore, we detected relative levels of ABC family genes regulated by MNX1-AS1. Knockdown of MNX1-AS1 remarkably downregulated

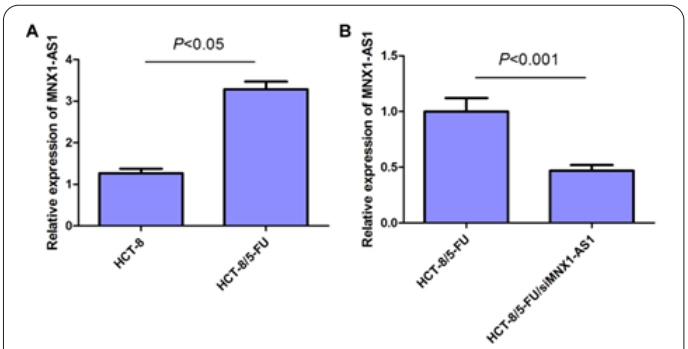

Figure 2. MNX1-AS1 levels in HCT-8/5-FU cells. (A) MNX1-AS1 levels in HCT-8/5-FU cells and the parental cells; (B) Transfection efficacy of siMNX1-AS1 in HCT-8/5-FU cells. P<0.001.

Table 2. The correlation between MNX1-AS1 expression and clinicopathological factors of colorectal carcinoma.

Variable	n	Low level (n=30)	High level (n=30)	χ^2	*p*
Sex					
male	31	16	15	0.067	1.000
female	29	14	15		
Age (years)					
<60	26	12	14	0.271	0.795
≥60	34	18	16		
Location					
Colon	30	14	16	0.267	0.797
Rectum	30	16	14		
Diameter (cm)					
<3	27	18	9	5.455	0.037
≥3	33	12	21		
Differentiation					
High	23	19	4	15.864	<0.001
Poor/moderate	37	11	26		
TNM staging					
I /II	23	18	5	11.915	0.001
III /IV	37	12	25		
Depth of invasion					
T1/T2	29	20	9	8.076	0.009
T3/T4	31	10	21		
Lymphatic metastasis					
No	35	23	12	8.297	0.008
Yes	25	7	18		

ABCA1, ABCB1, ABCC1, ABCG1 and ABCG2 in HCT-8/5-FU cells (Figure 3B).

Discussion

It is estimated that in 2015, there were 376,300 new onsets and 191,000 deaths of CRC in China (16). The incidence and mortality of CRC both rank fifth in malignant tumors in the Chinese population. The prognosis of early-stage CRC is relatively good. Nevertheless, the 5-year survival of metastatic CRC is only about 14% (17). At present, radiotherapy, chemotherapy and surgical resection are preferred to CRC patients, although their efficacies are often not ideal. It is of significance to explore novel biomarkers and therapeutic methods for CRC.

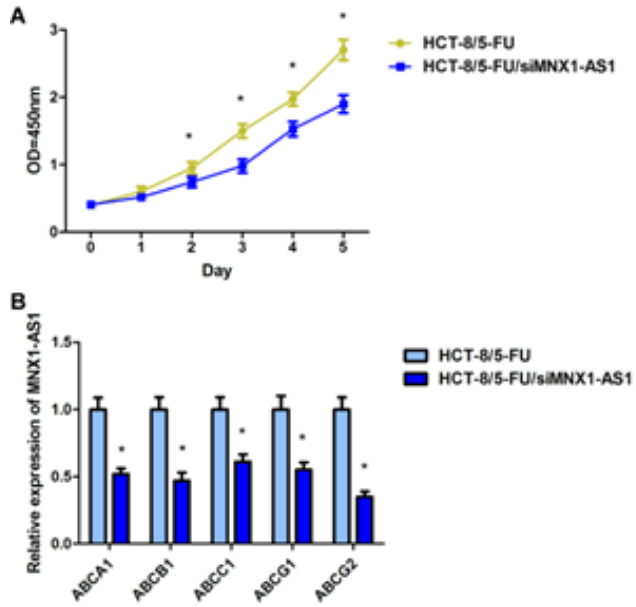

Figure 3. MNX1-AS1 upregulated drug-resistance genes. (A) Cell viability in HCT-8/5-FU cells with MNX1-AS1 knockdown; (B) Relative levels of ABC family genes in HCT-8/5-FU cells with MNX1-AS1 knockdown. P<0.05.

Great efforts have been made to prevent and reverse drug resistance during the past decades. LncRNAs have shown their promising applications in cancer treatment (18). Ma et al. (18) demonstrated that lncRNA CCAL is an oncogene leading to short survival and poor efficacy of chemotherapy in CRC patients, which induces multidrug resistance by activating the Wnt signaling via downregulating AP-2α. Knockdown of lncRNA snaR inhibits CRC apoptosis after 5-FU treatment, indicating that knockdown of snaR may lead to 5-FU resistance (15). Consistently, our findings showed that MNX1-AS1 was upregulated in CRC tissues, especially 5-FU-resistant ones. Besides, MNX1-AS1 was identically upregulated in HCT-8/5-FU cells, suggesting the involvement of MNX1-AS1 in 5-FU resistance of CRC.

ABC family genes on the cell membrane are drug pumps that pump the intracellular drugs to the outside, thus blocking the contact between drugs and cancer cells. They help cancer cells escape from the toxicity and killing effects of multiple chemotherapy drugs, resulting in a poor response to chemotherapy (19). ABC family members include ABCB1-encoded p-gp, ABCC1-encoded multidrug resistance genes and ABCG2-encoded BCRP (20). In $CD133^+$ CRC stem cells, ABCG2 and other ABC fa-

mily proteins mediate the membrane pump drug-resistant molecules on the cell membrane to clear the drugs in the cytoplasm, which display a strong resistance to chemotherapy drugs (21). Our results showed that knockdown of MNX1-AS1 remarkably downregulated ABCA1, ABCB1, ABCC1, ABCG1 and ABCG2 in HCT-8/5-FU cells. It is indicated that MNX1-AS1 could be a potential target for blocking drug resistance in CRC.

Conclusions

MNX1-AS1 triggers 5-FU resistance in CRC cells. Knockdown of MNXK1-AS1 is conducive to a good response to FOLFOX chemotherapy in CRC patients.

Conflict of Interest

The authors declared no conflict of interest.

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