

Original Article

Expression of IARS2 gene in colon cancer and effect of its knockdown on biological behavior of RKO cells

Ling Zhong^{1,2}, Yi Zhang³, Jing-Yu Yang⁴, Liang-Fa Xiong⁴, Tao Shen⁵, Ya-Lian Sa⁵, Yi-Ming O'Yang⁶, Si-Hui Zhao⁶, Jia-Yong Chen⁷

¹Kunming Medical University, Chenggong District, Kunming 650500, Yunnan Province, China; ²Department of Emergency Intensive Care Unit, The First People's Hospital of Yunnan Province, Kunming 650032, Yunnan Province, China; ³Department of Thyroid and Breast Surgery, The Second Affiliated Hospital of Kunming Medical University, Kunming 650101, Yunnan Province, China; ⁴Department of Thyroid and Breast Surgery, The First People's Hospital of Yunnan Province, Kunming 650032, Yunnan Province, China; ⁵Department of Research Laboratory, The First People's Hospital of Yunnan Province, Kunming 650032, Yunnan Province, China; ⁶Department of General Surgery, The First People's Hospital of Yunnan Province, Kunming 650032, Yunnan Province, China; ⁷Department of Emergency, The Second Affiliated Hospital of Kunming Medical University, Kunming 650101, Yunnan Province, China

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Abstract: Objective: To investigate the expression level of IARS2 gene in colon cancer tissues and various cell strains of the cancer; to explore cytologically the effect of IARS2 gene knockdown on proliferation, apoptosis and cell cycle of RKO cells in the cancer. Methods: Real-time, fluorescence-based quantitative PCR (qPCR) was used to detect the expression of IARS2 gene in human colon cancer and surrounding tissues and in various cell strains of the cancer; the RNA interference target of IARS2 gene was designed and the target was detected by Western blot; the IARS2-siRNA lentiviral vector was established and used to infect the RKO cells of colon cancer; qPCR was employed to determine the effect of gene knockdown; changes of the RKO cells in growth, apoptosis, cell cycle and clone formation were observed after IARS2 gene knockdown. Results: The expression of IARS2 gene was higher in human colon cancer tissues than in surrounding tissues; there was expression of IARS2 gene in colon cancer cells, and the expression level of IARS2 gene mRNA was higher in the RKO cells than in the SW480, HCT116, DLD1, HT-29 and SW620 cells. After infection of the RKO cells with IARS2-siRNA lentivirus, the expression of IARS2 gene was inhibited in the level of mRNA; proliferation rate of the RKO cells was significantly inhibited; the G1 phase arrest of the RKO cells was increased with less RKO cells in S phase; the apoptotic RKO cells increased significantly; and the number of colonies of the RKO cells reduced. Conclusion: The expression of IARS2 gene is different in human colon cancer and surrounding tissues; after knockdown of IARS2 gene, proliferation of the RKO cells is inhibited; there are more cells in G phase and fewer cells in S phase; apoptosis of cells is increased; and formation of colonies is reduced. IARS2 gene is probably a cancer-promoting gene.

Keywords: Colon cancer, IARS2, RNA interference, RKO cells

Introduction

Colorectal cancer (CRC) is one of the most commonly seen diseases at present and accounts for 10% of all tumors all over the world [1], and CRC is the world's fourth cancer-related cause of death [2]. Current studies have revealed that the occurrence and development of colon cancer may involve interreaction of multiple genes and multiple factors, including lifestyle, dietary habit, environmental factors, inflammatory bowel diseases, cancer gene mutation and inactivity of tumor suppressor genes [3-5]. At present, the primary therapeutic approach for

CRC is still surgery, new auxiliary radiation therapy (for patients with rectal cancer) and adjuvant chemotherapy (for patients with colon cancer at stage III or IV or with high risk colon cancer at stage II), but the total efficacy is poor and the prognosis is relatively poor [6, 7]. Therefore, to find out the pathogenesis of CRC, especially the biomarkers that can provide a clue for early diagnosis and to further deliver targeted prevention and treatment become the key in prevention and control of this disease.

IARS2 gene is a nuclear gene that encodes mitochondrial isoleucine-tRNA synthetase [8],

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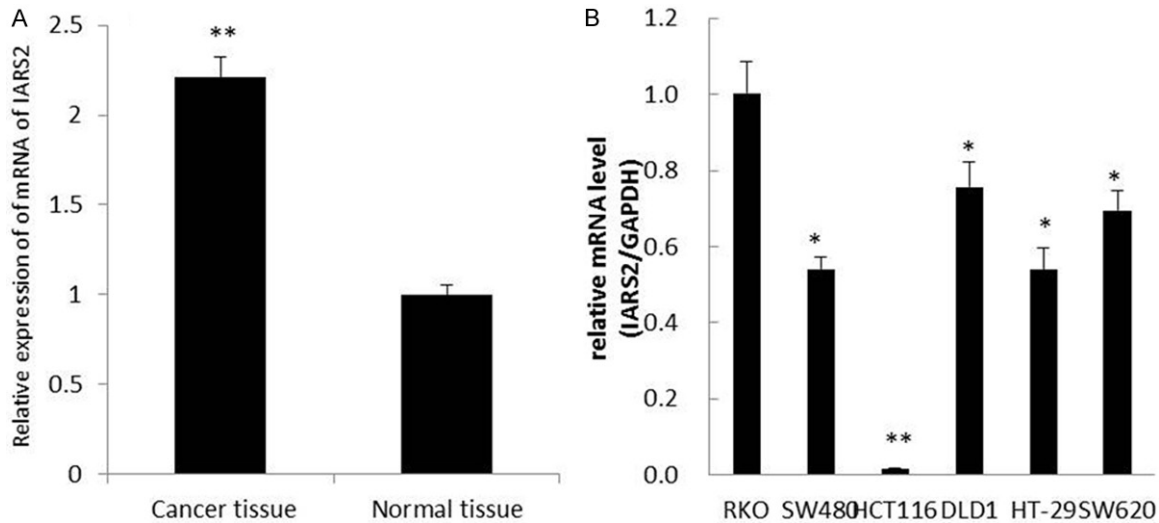


Figure 1. A. The mean expression level of IARS2 gene mRNA in colon cancer tissues relative to the surrounding tissues; B. The expression of IARS2 gene mRNA in different colon cancer cells relative to the RKO cells (*, $P < 0.05$, **, $P < 0.01$, $n = 6$).

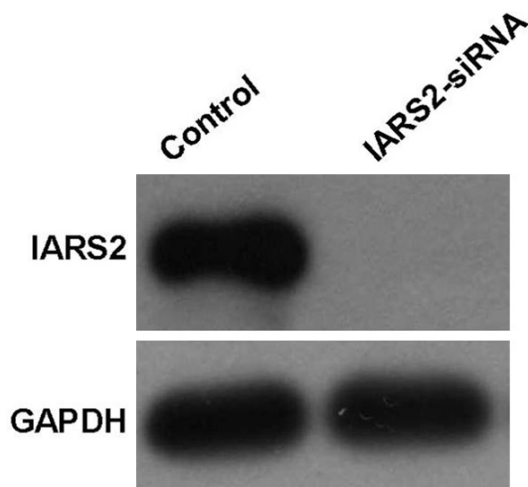


Figure 2. The changes as detected by Western blot at the protein level after IARS2-siRNA mediated knock-down of IARS2 gene.

and studies have indicated that mutation of the gene encoding the mitochondrial isoleucine-tRNA synthetase may cause diseases [9], but there has been no report of study on the relation between IARS2 gene and colon cancer. In the present study, the relation between IARS2 gene and colon cancer was preliminarily investigated by observing the difference in expression of IARS2 gene between tissues of human colon cancer confirmed pathologically and the surrounding tissues, establishing the IARS2-siRNA lentivirus vectors and infecting the RKO

cells of colon cancer with them, and detecting the changes in biological behavior of the RKO cells after knockdown of IARS2 gene.

Materials and methods

Clinical data

This experiment was approved by the Ethic Committee of the First People's Hospital of Yunnan Province. Tissue samples were collected from fresh cancer tissues and the surrounding colon tissues (tissues 10 cm away from the primary tumor) in 6 patients with colon cancer having surgery in the hospital and pathologically confirmed. Collection was performed within 30 min after separation of tumor, none of the patients received tumor treatment before surgery, and the pathological type was adenocarcinoma at stage II~III.

Reagents and instrument

Trizol was purchased from Invitrogen; extraction of total RNA was performed according to the Trizol operating instructions in Invitrogen; reverse transcription of RNA to obtain cDNA was performed according to the M-MLV operating instructions in Promega, and the required consumables were purchased from Axygen; the upstream primer sequence of GAPDH was TGACTTCAACAGCGACACCCA, the downstream primer sequence was CACCCTGTTGCTGTAGCCAAA, the upstream primer sequence of

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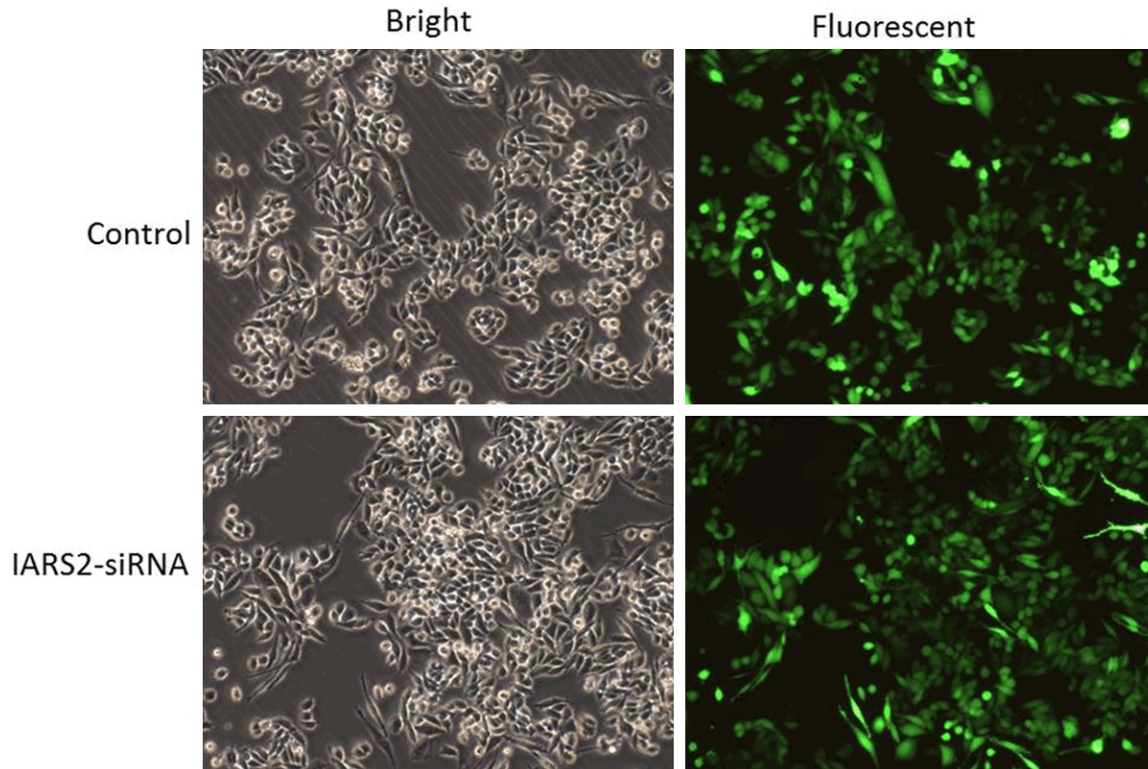


Figure 3. Infection of the RKO cells with lentivirus ($\times 100$).

IARS2 was TGGACCTCCTTATGCAAACGG, the downstream primer sequence was GGCAA-CCCATGACAATCCCA, and the IARS2-siRNA sequence was GCAGATGTAATCGAGCTTAAA, all of which were synthesized by Shanghai Genechem Co., Ltd.; the pGCSIL-GFP carrier and Age I/EcoRI enzyme digestion were purchased from Shanghai Genechem Co., Ltd.; the Cellomics instrument was purchased from Thermo; the PI was purchased from Sigma; the flow cytometer was purchased from Becton, Dickinson and Company; the fluorescence microscope was from Olympus; the Annexin V apoptosis kit was purchased from eBioscience (88-8007); the Giemsa staining solution was purchased from Chemicon, and paraformaldehyde was purchased from Sangon Biotech (Shanghai) Co., Ltd.

Extraction of RNA

Six pairs of colon cancer specimens were studied according to the principle of matching cancer tissues (C) with the surrounding tissues (N) of each patient, the total RNA was extracted after TRIZOL lysis of tissue samples and inversely transcribed into cDNA, and qPCR was utilized

to determine the expression level of mRNA of IARS2 gene in cancer and surrounding tissues. As an internal reference gene, GAPDH was analyzed using the $2^{-\Delta\Delta Ct}$ method, and the expression level of IARS2 gene mRNA in colon cancer tissues relative to the surrounding tissues was observed with the expression level of IARS2 gene mRNA in the surrounding tissues as the indicator.

Extraction of RNA from colon cancer cells

The RKO, SW480, HCT116, DLD1, HT-29 and SW620 cells of colon cancer were resuscitated, cultured and subcultured, RNA of each cell strain in good growth status was extracted and inversely transcribed into cDNA, and qPCR was used to detect the expression level of mRNA of IARS2 gene in different colon cancer cell strains. GAPDH was selected as an internal reference gene, the results were also analyzed using the $2^{-\Delta\Delta Ct}$ method [10-12], the expression level of mRNA of IARS2 gene in the RKO cells was selected as the indicator, and the expression level of IARS2 gene mRNA of the other 5 colon cancer cell strains relative to that in the RKO cell strains was observed.

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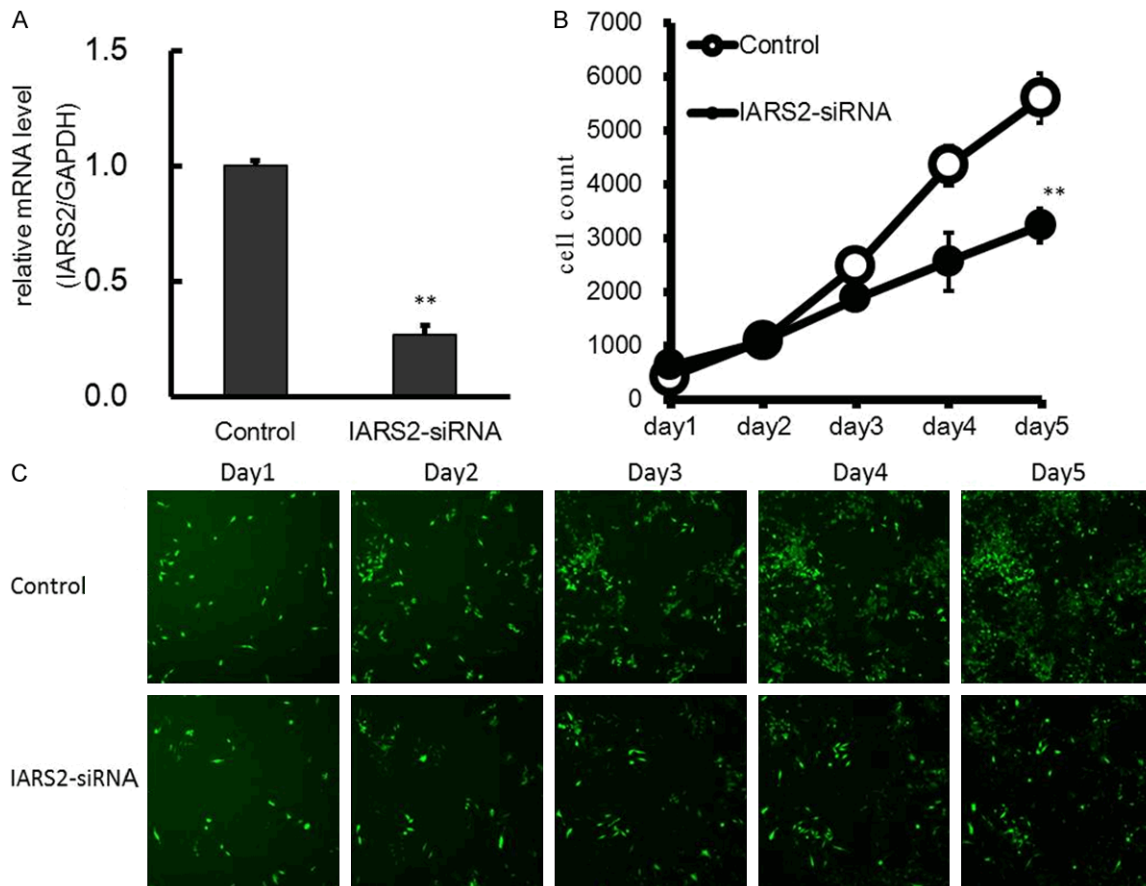


Figure 4. A. The expression level of IARS2 gene mRNA in IARS2-siRNA group relative to control group after the RKO cells were infected with lentivirus (*, $P < 0.05$, $n = 3$); B. The cell growth curve based on cell count for consecutive 5 days after the RKO cells were infected with lentivirus; C. The Cellomics scan images for consecutive 5 days after the RKO cells were infected with lentivirus ($\times 100$).

miRNA transfection

The IARS2-siRNA sequence was designed, the recombinant plasmid containing the IARS2-siRNA sequence was prepared to transfect *E. coli* to amplify the plasmid, and PCR identification and sequence comparison was performed on the positive clones. The method of RNAi exogenous screening targets was selected to verify the effectiveness of target interference due to the lack of antibody of IARS2 protein currently. The 293T cells cotransfected with plasmids containing the IARS2 sequence and having a flag tag and those containing IARS2-siRNA were designated as the experimental group, and in control group cotransfection was performed with the RNAi plasmids replaced with those containing the negative control sequence; cells in the experimental and control groups were collected 36 to 48 hours after the trans-

fection, protein was extracted and then Western blot was used to detect with the Flag antibody.

The 293T cells were cotransfected with the recombinant plasmids containing IARS2-siRNA and the two kinds of auxiliary incasing element plasmids, lentivirus incasing was performed in the 293T cells, and 48 hours later the cell supernatant rich in lentivirus granules was collected and concentrated to obtain the concentrated solution of lentivirus. The RKO cells in good growth status were assigned into two groups: control group (added with lentivirus containing negative control sequence) and experimental group (added with lentivirus containing IARS2-siRNA) to carry out the RNA interference experiment. At 3 days after the cells were infected with lentivirus, the expression of GFP was observed under a fluorescence microscope to find out the infection efficiency; at 5

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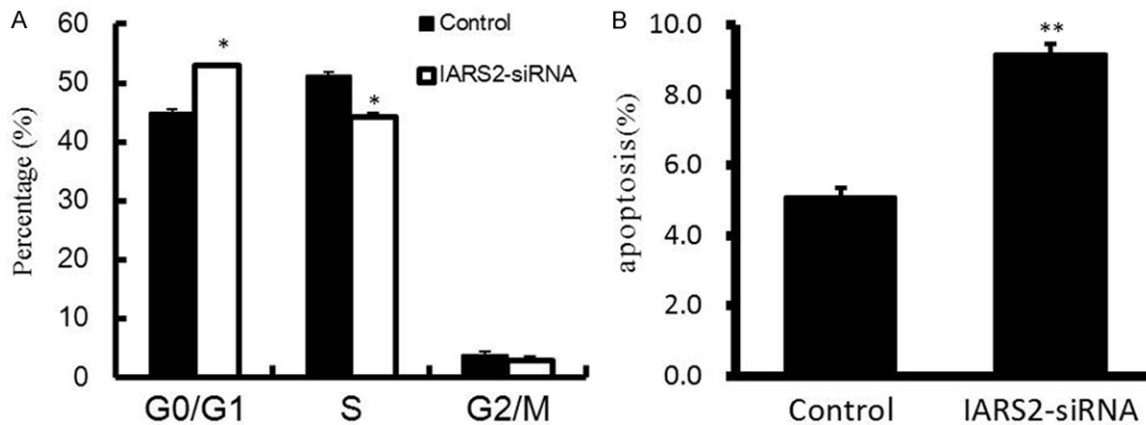


Figure 5. A. Effect of IARS2 gene knockdown on cell cycle of the RKO cells; B. Effect of IARS2 gene knockdown on apoptosis of the RKO cells (compared with control group, * $P < 0.05$, $n = 3$).

days after the cells were infected with lentivirus, the RKO cells were collected, and qPCR was used to determine the expression of IARS2 gene in the level of mRNA in cells of the two groups, and the $2^{-\Delta\Delta C_t}$ method was utilized to compare the difference in expression of mRNA of IARS2 gene between the experimental and control groups.

Analysis of biological characteristics of cells

The RKO cells in logarithmic growth phase in the two groups were re-suspended into cell suspension and inoculated into 96-well plates for continued culture. From day 2 after the initiation of inoculation, Cellomics detection was performed once every day to read the plates for consecutively 5 days, and proliferation of the cells was observed in the two groups. When coverage rate of the RKO cells was about 80% after growing on the 6 cm culture dishes, the cells were collected, 70% ethyl alcohol was used for fixation and PI staining was performed. The intracellular DNA content was determined by flow cytometry and PI staining, and the percentage of cells in G0/G1 phase, S phase or G2/M phase was calculated. When cell confluency of the RKO cells was up to 85% in the two groups, the cells were collected for Annexin V-APC staining, and flow cytometry was utilized to detect the apoptosis of cells in the two groups. The RKO cells in logarithmic growth phase in the two groups were re-suspended into cell suspension and inoculated into 6-well plates for continued culture for 7 days. The clonality of cells on the plates was observed and the number of clones was counted to com-

pare the two groups of cells in terms of tumor-formation ability.

Statistical analysis

Statistical analysis was performed using the software SPSS 11.0. For comparison of means between two groups, unpaired t test was employed for statistical analysis; for comparison of means among three or more groups, one-way ANOVA was used for statistical analysis and Dunnett t test was employed for comparison between two groups. For multiple measurements of a observational indicator of the same study subject at different time points, repeated measure ANOVA was used for statistical comparison, with a significance level of $\alpha = 0.05$.

Results

Expression of IARS2 gene

The results of qPCR detection of 6 pairs of human colon cancer tissues and surrounding tissues (over 10 cm away from the primary tumor) are shown in **Figure 1A**: the expression of IARS2 gene mRNA in tumor tissues was (2.213 ± 0.102) relative to the surrounding r tissues, and the difference was of statistical significance ($P < 0.05$).

There was expression of IARS2 gene in the 6 colon cancer cell strains; the expression level of IARS2 gene mRNA in the RKO cell was significantly higher than that in the SW480, HCT116, DLD1, HT-29 and SW620 cells, with statistically significant difference ($P < 0.05$) (**Figure 1B**).

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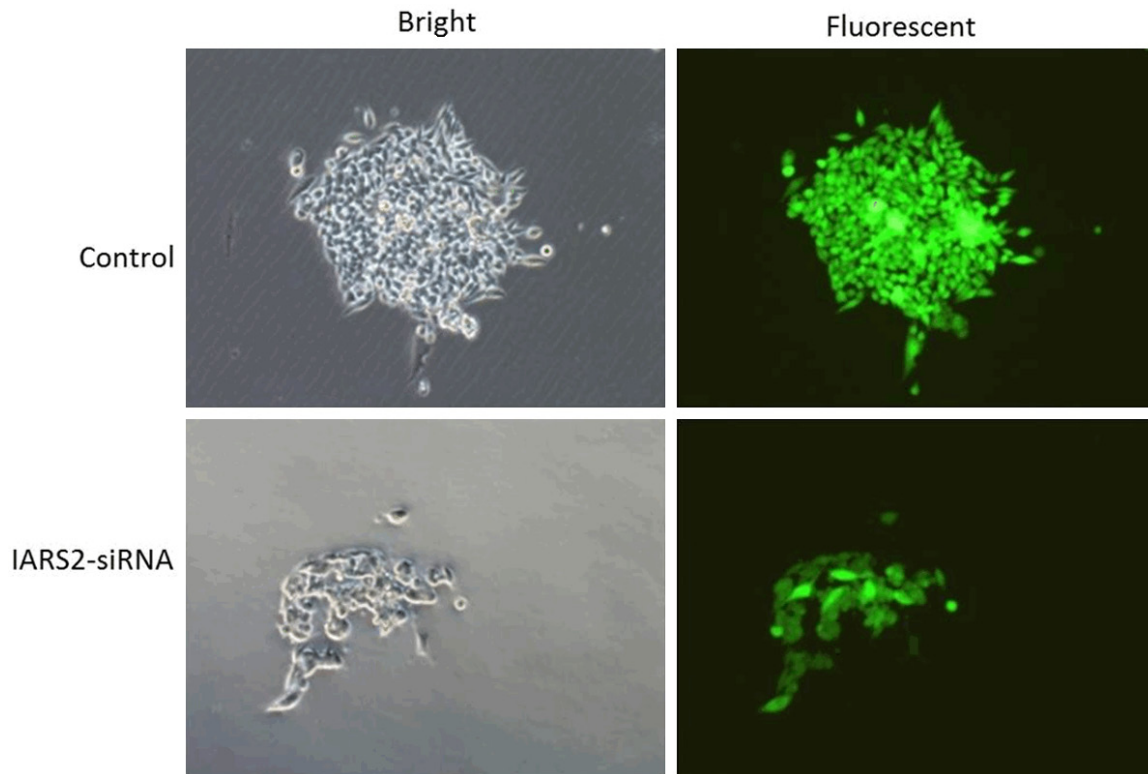


Figure 6. Clone formation of cells in IARS2-siRNA group ($\times 100$).

Changes in the level of IARS2 protein after transfection of the cells with IARS2-siRNA plasmids

After the cells in the experimental group were transfected with IARS2-siRNA plasmids, the level of IARS2 protein decreased significantly compared with the control group (**Figure 2**), indicating that the lentivirus plasmids containing IARS2-siRNA established in the experiment exerted a knockdown effect on the expression of IARS2 gene. In **Figure 3**, a large amount of green fluorescence was seen in the cells of IARS2-siRNA and control groups, indicating that the plasmids established in this study had very high transfection efficiency.

Effect of IARS2 gene knockdown on proliferation of the RKO cells

After infection with lentivirus, the expression level of mRNA of IARS2 gene was significantly lower in IARS2-siRNA group than in control group ($P < 0.05$), indicating that after infection with IARS2-siRNA lentivirus, the expression of IARS2 gene in the RKO cells at the mRNA level was inhibited (see **Figure 4A**). After infection of the RKO cells with lentivirus, the qualitative

observation under microscope (see **Figure 4C**) revealed that the number of cells in control group increased significantly over time, while the number of cells in IARS2-siRNA group increased slowly. Over the same culture time, the number of cells was significantly smaller in IARS2-siRNA group than in control group. In order to perform a quantitative study on the proliferation capability of cells after infection with IARS2-siRNA lentivirus, the cell growth curve (see **Figure 4B**) was plotted with the mean number of cells counted every day as the Y-axis and the days as the X-axis. It showed that from day 2, there was significant difference in the growth rate of cells between the groups, and the growth rate was faster in control group than in IARS2-siRNA group; this suggested that after infection with IARS2-siRNA lentivirus, the growth of RKO cells was slowed, which was consistent with the results obtained from the qualitative observation.

Effect of IARS2 gene knockdown on cell cycle and apoptosis of the RKO cells

The changes in cell cycle after the RKO cells were infected with lentivirus were plotted with cell cycle phase as the X-axis and the percent-

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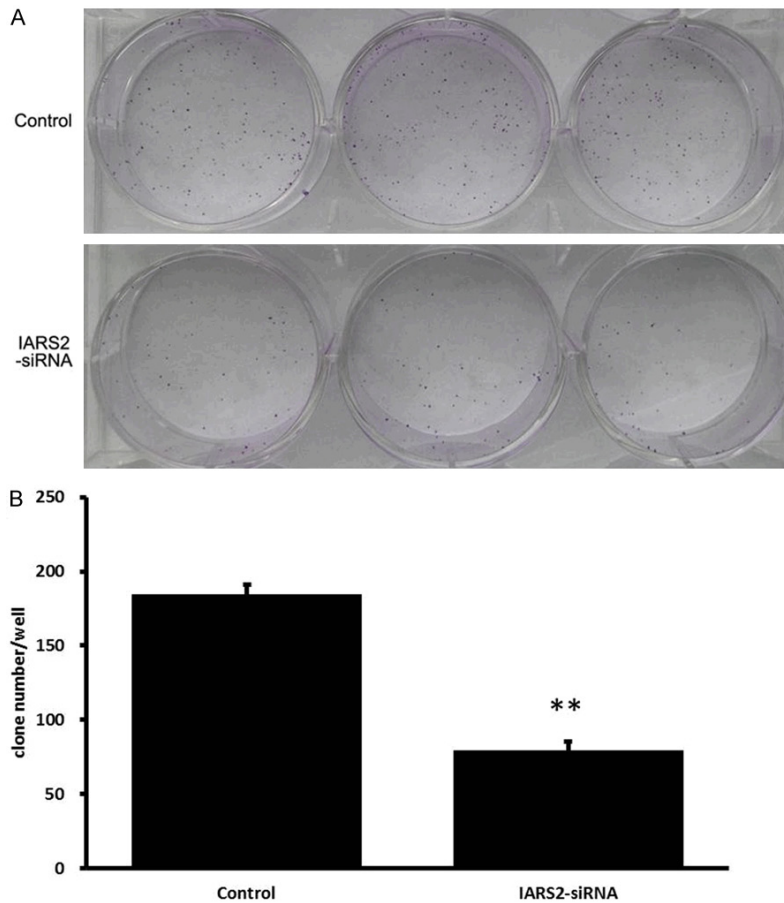


Figure 7. A. Clone formation of cells in the two groups after infection of the RKO cells with lentivirus; B. Mean clone formation of cells in the two groups after infection of the RKO cells with lentivirus (IARS2-siRNA group compared with control group, $n = 3$, $**P < 0.01$).

age of cells in the phase as the Y-axis (**Figure 5A**). It showed that after infection with lentivirus, the number of cells in G0/G1 phase increased in IARS2-siRNA group, and the number of cells in S phase decreased, indicating that knockdown of IARS2 gene could influence the distribution of cell cycle phases.

The cell apoptosis experimental results demonstrated that the percentage of apoptotic cells was significantly higher in IARS2-siRNA group than in control group (**Figure 5B**), indicating that after infection with lentivirus, the RKO cells that experienced apoptosis significantly increased in IARS2-siRNA group; IARS2 gene could inhibit the apoptosis of RKO cells.

Effect of IARS2 gene knockdown on clone formation of the RKO cells

The clonality of cells in the two groups (control group and IARS2-siRNA group) was observed

under microscope. As was shown in **Figure 6**, after infection with lentivirus, the number of colonies of the RKO cells decreased significantly in IARS2-siRNA group compared with control group. After fixation with paraformaldehyde and GIE-MSA staining, the cells were observed for growth in the plates in the two groups. As was shown in **Figure 7A**, the number of clones of the RKO cells decreased significantly in IARS2-siRNA group compared with control group. The analysis results after clone counting were shown in **Figure 7B**, revealing that the number of clones of the RKO cells decreased significantly in IARS2-siRNA group compared with control group, which indicated that the IARS2 gene was significantly associated with clonality of the RKO cells, and IARS2 gene might have promoted the tumor formation ability of the RKO cells.

Discussions

Currently, CRC is one of the most commonly seen diseases and accounts for 10% of all tumors worldwide, and the world's fourth cancer-related cause of death [13-15]. To date, the pathogenesis of CRC has been unclear.

Aminoacyl-tRNA synthetase (AARS) specifically identifies the amino acid side chains and tRNA and enables the genetic information of mRNA to be correctly reflected on the sequence of amino acids of proteins. Studies in recent years have suggested that AARS participates in many other vital activities including apoptosis, angiogenesis, RNA splicing and immunity, in addition to the synthesis of protein. For example, the segments of human tyrosyl-tRNA synthetase have the function of promoting angiogenesis and those of the human tryptophanyl-tRNA synthetase have the function of inhibiting vascular growth. The human glutamyl-tRNA synthetase regulates cell apoptosis through antagonism

with the apoptosis signal-regulating kinase ASK1 and glutamine plays a role of positive regulation. The C-end of human prolyl-tRNA synthetase has the amino acid sequence similar to the angiogenic antagonistic factor (endothelial monocyte activating polypeptide II) and induces the mononuclear macrophages to generate tumor necrosis factors and polymorphonuclear leucocytes to release myeloperoxidase, while the N-end of human prolyl-tRNA synthetase specifically promotes angiogenesis [16]. These effects of aminoacyl-tRNA synthetase on angiogenesis, cell apoptosis and mononuclear macrophages may promote disease course or inhibit disease process in occurrence and development of diseases, especially tumor.

The mitochondrial isoleucine-tRNA synthetase is coded by the IARS2 gene in zone 4 band 1 of chromosome 1 and synthesized in cytoplasm, and then transported into the mitochondrion to catalyze the binding of isoleucine to specific tRNA for completion of mtDNA translation. At present, there are few studies on the association between IARS2 gene and diseases.

Jeremy Schwartzentruber *et al.* [17] reported clinical information of patients with diseases induced by IARS2 gene mutation for the first time in 2014. Three adult patients manifested cataract to different degrees, microsoma secondary to growth hormone deficiency, sensorineural hearing loss, peripheral sensory neuropathy and bone dysplasia. Elena Perli *et al.* [18] reported in 2012 that patients with mtDNA mutation did not have the corresponding positive clinical manifestations due to the presence of aminoacyl-tRNA synthetase in high level. The above two reports were about diseases directly related with mitochondrial isoleucine-tRNA synthetase, but they did not mention the association between IARS2 gene and occurrence and development of tumor.

The study on the chromosomal fragile site FRA1H carried out by Franca Pelliccia *et al.* [19] in 2007 showed that IARS2 gene was expressed in cervical, uterine, ovarian, hepatic and colon tissues and normal lymphocytes; there was no obvious difference in the expression of IARS2 gene between 19 cell strains (including colon cancer cells) and corresponding normal tissue cells. As for the colon cancer cell strains, the DLD1, HCT116, SW620, Caco2, HT29, LoVo and SW48 cells were selected for the experiment. This was one of the few reports about the

relation between IARS2 gene and colon cancer, but it was not focused on the relation between IARS2 gene and colon cancer, and the above finding was just an experimental finding when FRA1H was studied.

It was found out in the present study that the expression level of mRNA of IARS2 gene was higher in colon cancer tissues than in the surrounding tissues; there was expression of mRNA of IARS2 gene in all the 6 cells strains of the cancer, but the expression level was higher in the RKO cells than in the other 5 cell strains. This appeared to be inconsistent with the expression of IARS2 gene in tissues and cells reported by Franca Pelliccia *et al.* [19]. The tissue specimens used by Franca Pelliccia *et al.* were normal colon tissues, while those in the present study was from colon cancer; the colon cancer cell strains used by Franca Pelliccia *et al.* were the DLD1, HCT116, SW620, Caco2, HT29, LoVo and SW48 cells and did not include the RKO cells with the highest expression level of IARS2 gene as detected in the present experiment. Franca Pelliccia *et al.* studied the normal colon tissues but did not observe the difference between colon cancer tissues and normal tissues; they compared the cancer cells and normal tissues in terms of the expression level of IARS2 gene, but did not notice the difference in the expression level of IARS2 gene among different colon cancer cell strains. It is because at present there are few studies on the relation between IARS2 gene and colon cancer, that investigator may carry out preliminary exploration but only come to a conclusion that may represent only one observation perspective.

Further cytological study found out that for the RKO cells with IARS2 gene knockdown, the proliferation rate was inhibited, the proportion of cells in G0/G1 phase increased and that in S phase decreased, there was an increase in apoptosis, and the number of cell colonies decreased. This indicated that IARS2 gene in the RKO cells might have promoted cell proliferation, inhibited cell apoptosis and increased the tumor-forming ability of cells. Is IARS2 gene one of the links in "multiple genes, multiple stages and multiple factors" of colon cancer? This question is worthy of further study and exploration.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jia-Yong Chen, Department of Emergency, The Second Affiliated Hospital of Kunming Medical University, 374, Dianmian Street, Kunming 650101, Yunnan Province, China. Tel: +86-871-65351281; Fax: +86-871-65351281; E-mail: chenjiayongkmmu@163.com

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