Original Article

Evaluation of miR-21 Inhibition and its Impact on Cancer Susceptibility Candidate 2 Long Noncoding RNA in Colorectal Cancer Cell Line

Miganoosh Simonian[1], Mohammadreza Sharifi[1], Reza Nedaeinia[2], Meysam Mosallaie[1], Sharifeh Khosravi[1], Amir Avan[3], Majid Ghayour-Mobarhan[4], Hadi Bagheri[1], Rasoul Salehi[1,5,6]

From the [1]Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, [5]Acquired Immunodeficiency Research Center, Isfahan University of Medical Sciences, [6]Gerfa Namayesh Azmayesh (GENAZMA) Science and Research Institute, Isfahan, [2]Department of Medical Biotechnology, School of Medicine, Mashhad University of Medical Sciences, [4]Biochemistry of Nutrition Research Center, School of Medicine, Mashhad University of Medical Sciences, [3]Molecular Medicine Group, Department of Modern Sciences and Technologies, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Address for correspondence:
Dr. Rasoul Salehi, Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: r_salehi@med.mui.ac.ir

Abstract

Background: Both microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) have been shown to have a critical role in the regulation of cellular processes such as cell growth and apoptosis, as well as cancer progression and metastasis. lncRNAs are aberrantly expressed in many diseases including cancer. Although it is well known that miRNAs can target a large number of protein-coding genes, little is known whether miRNAs can also target lncRNAs. In the present study, we determine whether miR-21 can regulate lncRNA cancer susceptibility candidate 2 (CASC2) in colorectal cancer. **Materials and Methods:** LS174T cells were transfected with locked nucleic acid (LNA)-anti-miR-21 and scrambled LNA for 24, 48 and 72 h. The expression of miR-21 and lncCASC2 was evaluated by quantitative reverse transcriptase polymerase chain reaction. **Results:** However, contrary to what we expected and reported by others, lncCASC2 quantity was significantly reduced in LNA treated LS174T cells compared to the scrambled treated and normal untreated cells ($P < 0.05$). **Conclusion:** The interaction of miRNA and lncRNA are not as simple as suggested by other reports. Moreover, it could be complex molecular mechanisms underlying the communication of various noncoding RNA elements.

Keywords: *Cancer susceptibility candidate 2 gene, colorectal cancer, long noncoding RNAs, microRNA*

Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide.[1] Unfortunately, the occurrence of cancer trends among adults younger than 50 years.[2] Younger patients diagnosed with higher degree of malignancy.[3] Despite the substantial progress in diagnosis and treatment, we still encounter with high mortality rate of advanced CRC. Data presented an increased level of incidence and mortality among patients with advanced cancer.[4]

This means that identification of molecular basis involved to find novel therapeutic options for treatment of these patients is vital.

It has recently been indicated that noncoding RNAs (ncRNA) play crucial roles in the tumorigenic of CRC.[5] The class of long ncRNA (lncRNA) contains a very heterogeneous subclass of ncRNA.[6]

Despite often being represented as transcriptional noise, it has verified that lncRNAs can be regulating nearly every step within the life cycle of a gene.[7-10] There are many reports stating lncRNAs involvement in development and progression of various cancers, including breast cancer,[11,12] gastric cancer,[13,14] bladder cancer,[15,16] lung cancer,[17] and CRC[18,19] and glioma.[20] lncRNAs act to regulate gene expression through various mechanisms.[21] A large number of lncRNAs may function as endogenous sponges for other kinds of RNAs such as various microRNAs (miRNAs) which consequently impede miR-mediated functions.[22] Understanding the detailed molecular mechanisms through which lncRNAs function in various diseases might be leading to serve this ncRNA in novel diagnostic or therapeutic strategies. Cancer susceptibility candidate 2 (CASC2), as a novel lncRNA, was originally known as a downregulated gene in endometrial cancer. It has been characterized as a tumor suppressor in several human malignancies,

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including CRC.[23] Although the CASC2 dysregulated expression points out its tumorigenic properties in CRC patients, precise mechanisms of its mediated process is not clear. miRNAs, another class of ncRNA, function as negative gene regulators through specific complementarily sites at the 3-untranslated region of target messenger RNAs (mRNAs), which result in degradation or translational repression.[24,25] miRNAs have important widely regulatory functions in diverse cellular processes at the transcriptional or posttranscriptional level.[26-28] There is a growing body of evidence showing the aberrant expression of certain miRNAs, in particular, miR-21, is associated with carcinogenesis, tumor size, metastasis, and survival rate of patients with CRC.[29-31] It is indicated that miR-21 contents increase during CRC progression from precancerous conditions to advanced carcinoma.[32] Among the mechanisms, taking part in CASC2 regulation has been demonstrated being miR-21 action, it has been shown that there is a reciprocal repression between CASC2 and miR-21 in several malignancies including human gliomas. Wang *et al*. demonstrated that CASC2 expression is dramatically decreased in glioma tissues and it plays a tumor-suppressive function through negative regulation of miR-21, which may be a novel target for therapeutic modalities. Bioinformatics and luciferase reporter assays provided information that miR-21 binds to CASC2 in a sequence-specific manner and they regulate each other reciprocally. In regard to this repression of each of them could upregulate another one as seen in the previous study on glioma cells.[33] In light of vital role of CASC2 and miR-21 in colon carcinogenesis, in the present study, we inhibited miR-21 using locked nucleic acid (LNA) technology in LS174T colorectal cell line.

Materials and Methods

Cell culture

The Human colorectal adenocarcinoma cell line LS174T was purchased from the National Cell Bank of Iran (Pasteur Institute, Iran). The cells were maintained in Dulbecco modified Eagle medium (Gibco, USA) in humidified incubator containing 5% CO_2 at 37°C.

Cell transfection with locked nucleic acid anti-miR-21

miR-21 inhibitor (LNA-anti-miR-21) transfection was performed according to an established protocol. Briefly, cells were transfected using an X-tremeGENE small interfering RNA (siRNA) Transfection Reagent Kit (Roche, Mannheim, Germany), 24 h after seeding into the well.

LNA-anti-miR-21 was added to the culture media at a final oligonucleotide concentration of 50 pmol. The LNA-anti-miR-21-5P and scrambled LNA molecules were synthesized by Exiqon with fluorescein dye, 6-carboxyfluorescein (6-FAM) moiety at the 5′ end. The following sequences are LNA-anti-miR-21, 5′-FAM-ACATCAGTCTGATAAGCT-3′, and its control oligo, LNA-scramble, 5′-FAM-GTGTAACACGTCTATACGCCCA-3′.

Ten percent fetal bovine serum was added 10 h posttransfection, followed by incubation for an additional 24, 48 and 72 h. Untreated cells as a control and cells transfected with oligo control for LNA-anti-miR-21 (LNA-scramble) were cultured parallel to the LNA-antimiR transfected cells. The evaluation of the transfection was detected by FACSCalibur flow cytometer and fluorescent microscopy.

Cell viability assay

Cell invasion assays were performed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Bio-IDEA, Tehran, Iran). In brief, cells were seeded at a density of 2×10^4 cells/well into six-well plates, following 24 h of incubation. The MTT assay was performed 24, 48, and 72 h after transfection. After adding the MTT solution (12 mM) incubate at 37°C in a dark room for 2 h. Afterward, 200 µl dimethyl sulfoxide was added. The plates were capturing at the wavelength of 570 nm.

Measurement of apoptosis

Apoptotic cells were measured by an Annexin-V-FLUOS Staining Kit (Roche, Mannheim, Germany) according to the manufacturer's protocols. 2×10^5 cells/well were seeded in six-well plates. After 24 h incubation, the procedure was performed at 24, 48 and 72 h after transfection. After collection of floating cells, adherent cells were rinsed with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline and harvested after incubation with 2 mM ethylenediaminetetraacetic acid in phosphate-buffered saline enzyme free for 10 min with gentle movement. After the cells were visibly detached, they were suspended by 5 ml of complete growth medium. Then, both floating and adherent cells were pooled and rinsed twice with phosphate-buffered saline. After centrifuging for 4 min at 190 g at 4°C, the resulting pellet was resuspended in 100 µl incubation buffer with 2 µl Annexin-V and 2 µl propidium iodide for 15 min in a dark room at 4°C. Then, the samples immediately analyzed by using a FACSCalibur flow cytometer with Annexin-V and PI excitation/emission wavelengths (488/525 and 488/675 nm, respectively).

Quantitative reverse transcriptase polymerase chain reaction

Briefly, RNAs from LS174T cells were isolated with a miRCURY RNA Isolation Kit (Exiqon, Copenhagen, Denmark), and the concentration and purity of RNA were measured by spectrophotometry. mRNAs complementary DNA (cDNA) were synthesized by Universal cDNA Synthesis Kit (Exiqon, Copenhagen, Denmark) according to manufacturer's protocol. The amount of miR-21 inhibition determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (SYBR Green

Master Mix Kit) with specific hsa-miR-21-5p and hsa-let-7a-5p primers. As an internal control, hsa-let-7a was used for miR-21 template normalization. For evaluation of CASC2 gene expression, one microgram of total RNA was used to synthesize cDNA using Fermentas Kit. Relative levels of lncRNA were carried out using SYBR® Green Master Mix Kit TM (Exiqon, Copenhagen, Denmark) and normalized to levels of endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA. $2^{\Delta\Delta Ct}$ method was used for calculation of relative gene expression levels.

The primers' sequences for CASC2 were: forward 5′-CGGCAGATGGAGATTCAGAAACATAAGG-3′; reverse 5′GCAACACACTTCTTACTCGGAATAGGG-3′and GAPDH primers were forward 5′-AGTCCACTGGCGTCTTCAC-3′; reverse 5′-AGGCATTGCTGATGATCTTGAG-3′. All PCR reactions were run in triplicate by The StepOnePlus TM Real-Time PCR (Applied Biosystems, USA) instrument.

Statistical analysis

Data are presented as mean ± standard deviation (SD) and analyzed using SPSS v. 22 statistical software (IBM, Chicago, IL, USA) by the two-way analysis of variance test with Fisher's *post hoc* followed by Scheffé multiple comparison test. Statistical significance was accepted at the $P < 0.05$ level.

Results

Locked nucleic acid anti-miR-21 reduced the expression of miR-21

For inhibition of miR-21, the miRCURY LNA miRNA Inhibitor™ was transfected to LS174T cells with the X-tremeGENE siRNA Transfection Reagent™. On the basis of the initial optimization experiments, transfection was performed with 50 pM of LNA-anti-miR in 5 µl transfection reagent in a total volume of 200 µl Opti-MEMI medium (Gibco, USA). The transfection efficacy was >85% in the optimized situation. The expression of miR-21 was evaluated by qRT-PCR after transfection for 24, 48, and 72 h. The results showed downregulated level of miR-21 in LNA-anti-miR treated group. No statistically significant difference was found between values of miR-21 in cells transfected with scrambled LNA and control groups during the incubation ($P > 0.05$). In all three time points, the expression of miR-21 was considerably lower in the LNA-anti-miR group compared to scramble LNA and untreated groups ($P < 0.01$) [Figure 1].

Modulation of cancer susceptibility candidate 2 by locked nucleic acid-anti-miR-21

To assess lncRNA CASC2 as a target gene of miR-21 in LS174T, we evaluated the expression levels of lncRNA CASC2 in 3 groups: LS174T cells pretreated with control oligo (LNA-scramble) or LNA-anti-miR-21 and untreated group by qRT-PCR. As shown in Figure 2, CASC2 expression levels were significantly down-regulated in group transfected with LNA-anti-miR ($P < 0.001$).

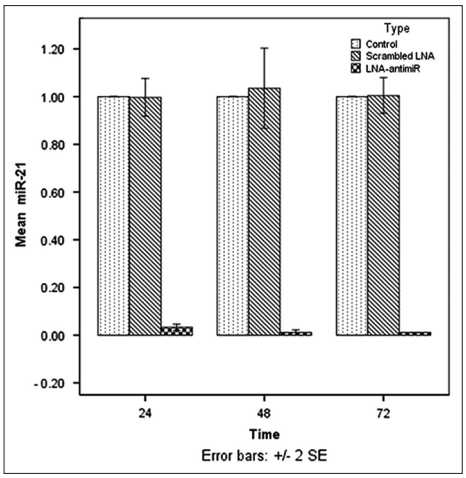

Figure 1: Assessment of the miR-21 level by real-time polymerase chain reaction, 24, 48, and 72 h after transfection. The expression of miR-21 was normalized to let-7a expression using the ΔΔCt method. The untreated group was considered as a reference for each time point. Data were mean ± standard deviation of three independent experiments

Locked nucleic acid-anti-miR-21 reduced viability of LS174T cells

To further examine the functional effect of anti-miR-21 on LS174T cells, we measured cell viability by MTT assay. Treatment of cells with LNA anti-miR-21 showed a significant reduction in cell viability in all their time points compared with the control and scrambled LNA groups, especially after 72 h, with >50% reduction ($P < 0.01$).

Locked nucleic acid anti-miR-21 induces apoptosis in LS174T cells

Because the LNA anti-miR-21 suppressed cell viability, we next sought to determine whether the LNA anti-miR-21 also induced apoptosis. To measure the effect of this treatment on apoptosis we used Annexin-V binding assay, which allows quantification of the level of phosphatidylserine exposure at the outer membrane side, a well-characterized event of early apoptosis.[34] At all three time points, The population of Annexin-V-positive cells increased significantly in LNA anti-miR-21 transfected cells ($P < 0.01$). In addition, we observed the highest amount of apoptosis at 48 h after LNA-anti-miR-21 transfection. LNA-transfected cells show higher percent of apoptosis compared with scrambled LNA-transfected cells and untreated cells ($P < 0.01$) [Figure 3].

Discussion

In recent years, the prevalence of CRC has increased markedly among individuals between ages 20 and 49 years. A significant proportion of young patients present with advanced disease.[3] Even with great development in diagnostic and therapeutic methods, the overall survival rate is still relatively poor and remains unsatisfactory.[35] Hence,

it is imperative to comprehend the pathogenic mechanism of CRC. Nowadays, lncRNAs have gained wide attention as new mediators in tumor biology.[36] Thus, learning the processes by which lncRNA expression is controlled is the first stage toward understanding the basic mechanisms by which they exert their functions in human cancers.

miR-21 is one of the oncogenic miRNAs that are significantly overexpressed in tumor tissue of CRC, which is responsible for the malignant progression of colon cancer.[37] The basic role of miR-21 action may be the consequence of its ability to regulate numerous coding genes[38,39] but growing body of evidence suggested that lncRNA might regulate by similar mechanisms as for protein coding genes.[40]

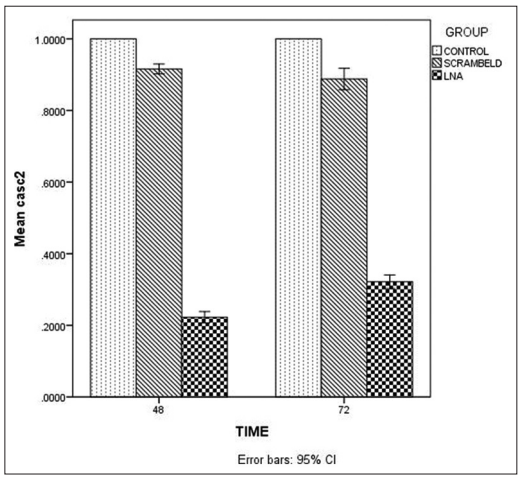

Figure 2: The expression of cancer susceptibility candidate 2 long noncoding RNA in locked nucleic acid-anti-miR, scramble locked nucleic acid and untreated groups in LS174T colon adenocarcinoma cells. The long noncoding cancer susceptibility candidate 2 levels, 48 and 72 h after transfection was analyzed by quantitative reverse transcriptase-polymerase chain reaction. The expression was normalized to glyceraldehyde-3-phosphate dehydrogenase

In this study, we determine whether miR-21 can directly regulate lncRNA CASC2 in CRC and could not find any association. Tumor-suppressor features for the lncRNA CASC2 have been recognized in several cancers. CASC2 was expressed in reduced level in endometrial cancer. Exogenous expression of CASC2 remarkably inhibited the clonal growth in AN3CA endometrial cancer cell line.[23] In the distinct study, lower expression of CASC2 was found in nonsmall cell lung cancer (NSCLC) and significantly associated with advanced tumor node metastasis stage and tumor size. *In vitro* and *in vivo* assays showed inhibited NSCLC cell proliferation through CASC2 overexpression.[41] Cao *et al*. dual-luciferase reporter assays revealed that CASC2 targeted by miR-21 and miR-21 was able to decrease the expression of CASC2 in renal cell carcinoma.[42] In human gliomas, miR-21 could negatively regulate CASC2 function in a sequence-specific manner and therefore destroyed the CASC2-mediated inhibition of glioma cell proliferation and invasion. Moreover, overexpression of miR-21 dramatically suppressed the cell apoptosis caused by CASC2.[33] Despite all these evidence verified the negative regulation of CASC2 by mir-21, surprisingly, our results could not provide any proofs to claim that they are biologically relevant. Indeed, (i) the intricacy of the regulation of this tumor suppressor gene, which may be distinctly, affected depending on the type of cancer and (ii) there are more miRNAs that have a significant negative effect on considered gene. It remains possible that it might be a trans-inhibition of CASC2 results from the synergistic action of multiple miRNAs. Furthermore, extra studies will be needed to gain a full

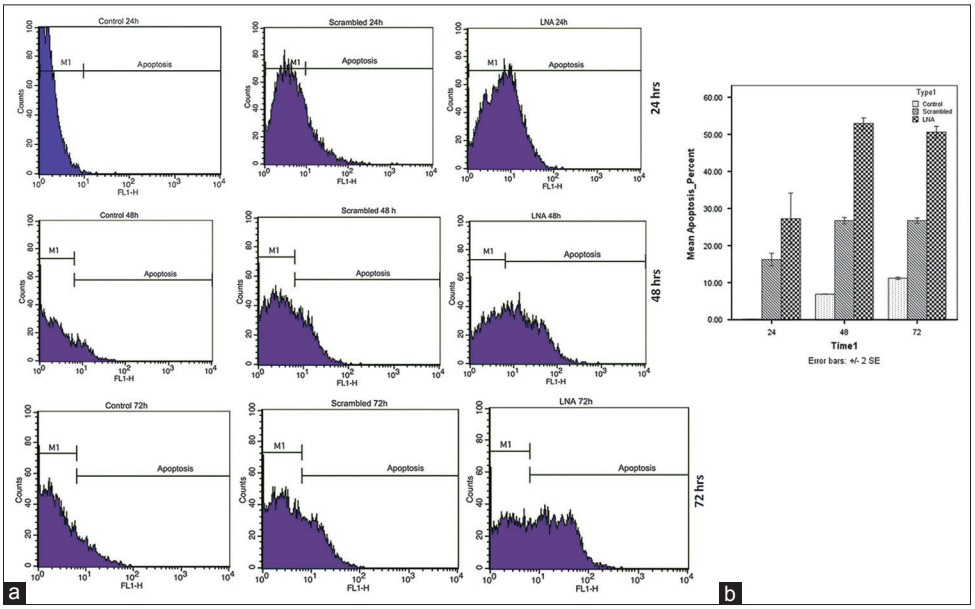

Figure 3: Assessment of apoptosis by Annexin-V-propidium iodide staining performed 24, 48, and 72 h after transfection. Representative cytofluorometric graphs are shown (a); data shown in the graph are mean ± standard deviation of triplicate experiments (b)

understanding of the molecular mechanisms underlying the communication of various ncRNA elements in a network.

Conclusion

Although an inverse correlation between CASC2 and mir-21 levels has been reported in several types of tumors, it had never been investigated in CRC. Thus, further investigation and validation in a larger number of CRC cell models are required to better declare the function and critical mechanisms of lncRNA CASC2 in the progression of colon cancer.

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Conflicts of interest

There are no conflicts of interest.

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