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The possibility of early diagnosis of colorectal cancer through expression of Lncrna UCA1, Lncrna LINC00970, and Wnt genes; an in-vitro study

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ABSTRACT. Colorectal cancer is the 4th cause of cancer death; with considering the growth process of this cancer and the necessity of early diagnosis, the purpose of the research is to state the LncRNA 00970, LncRNA UCA1, and the Wnt gene before and after the treatment by 5-Azacytidine epigenetic medicine, to reach the biomarker in the very first steps of colorectal cancer. In this experiment, the human colon cancer cell line (HT29) treated with different concentrations of 5-aza-2'-deoxycytidine (5-aza-dC) was utilized to induce DNA demethylation; Quantitative PCR (qPCR) was used to measure LncRNA UCA1 and LncRNA LINC00970 and Wnt expression. There was a significant relationship between the expression of LncRNA 00970, LncRNA UCA1, and the Wnt gene and its effects on colorectal ($p < 0.05$). The Wnt gene was treated by 1 and 10 of 5-Azacytidine epigenetic medicine, which then experienced decreases. In LncRNA UCA1 and LncRNA00970 in dose 1 micromolar of 5-Azacytidine had decrement and increment of expression respectively that explains their efficiency but in treatment by dose 10 mM of this medicine, no significant LncRNA expression difference was detected, 5-azacitidine has a direct impact on its target genes and LncRNAs. Therefore, it can be used in the early diagnosis of colorectal cancer.

Keywords: colorectal cancer; long non-coding RNAs; LncRNA UCA1, LncRNA Linc00970, Wnt, 5-azacitidine

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Introduction

Colorectal cancer (CRC) is the third most common cancer (Zhang et al., 2018). Genetic alteration therapies in CRC should be identified in order to discover prognostic biomarkers and therapeutic target cells and methods.

Long non-coding RNAs are a group of regulatory RNAs, RNA polymerase II transcripts (Kong et al., 2016), which lack a significant protein-coding capacity (Lee, 2013), are highly diverse, and are involved in pathobiological pathways of diseases. Although the regulation of LncRNAs expression in human tumors and their mechanism in tumorigenesis is controversial, irregular or mal-expression of LncRNAs has a direct relationship with cancer. As might be expected, many LncRNAs fulfill their roles through cellular interactions with proteins, DNA, and other RNA molecules (Ulitsky & Bartel, 2013). Some studies have suggested that LncRNAs may play a role in cell division, invasion, and cancer progression (Eddy, 2001). LncRNAs also have a function in post-transcriptional settings of genes (Yoon, Abdelmohsen, & Gorospe, 2013). Biological research on LncRNAs indicates their importance in the diagnosis, formation, and progression of cancer (Charles Richard & Eichhorn, 2018). LncRNA coding genes can act as tumor oncogenes or tumor suppressor genes. LncRNAs involved in cancer development have been identified through a variety of methods, including microarrays expression, methylation analysis, to name but a few (Noori-Dalooi & Eshaghkhani, 2015).

LncRNA Linc00970 is situated on 1q24.2 (Zhang et al., 2021). No research has yet been done on LINC00970 in colorectal cancer. The reason for this selection is its association with the Wnt gene (Yang et al., 2018). LncRNA UCA1 with the official full name of urothelial cancer-associated 1, abbreviated as UCA1, located on 19p13.12 (Zhang). UCA1 is an important oncogene in breast cancer (Huang et al., 2014). High expression of UCA1 is correlated with poor prognosis in colorectal Cancer (Han et al., 2014). The expression level of UCA1 in tumors with Mismatch Repair (MMR) defaults in colorectal cancer decreased in contrast to tumors without

such defaults (James de Bony et al., 2018). These differences are in line with the notion that different carcinogenesis pathways define the molecular characteristics of tumors (Renaud et al., 2015).

The Wnt/ β -catenin signaling pathway specifically controls transcription. Inappropriate activation of the proto-oncoprotein β -catenin (Fodde, Smits, & Clevers, 2001; Yamada et al., 2013) or Wnt/ β -catenin complex induces cancer, especially colorectal cancer (Herbst et al., 2014). The Wnt/ β -catenin pathway is important in the development of colorectal cancer. Therefore, controlling this pathway has an important role in preventing the development of colorectal cancer. One of the factors that inhibit this pathway is related to methylation. The epigenetic drug 5-Azacytidine is a demethylating agent that inhibits this pathway, including DKK, which prevents overexpression of Wnt. Wnt activation is frequently seen in many cancers in adults (Polakis, 2000). Wnt/ β -catenin signaling plays an important role in the pathogenesis of CRC, and drugs that target the β -catenin pathway can potentially reduce tumor growth. Thus, inhibition of this pathway and enhancing inhibitors' activity can prevent the development of cancer cells (Yang, Zhang, Zhang, & Rengarajan, 2018).

5-azacytidine, one of the FDA-approved nucleoside-inhibiting drugs, which blocks DNMT1 activity and modifies the process of hypermethylation and re-expression of tumor suppressor genes in cancer cells at low doses, inhibits protein synthesis by placement in DNA structure and alternation in RNA synthesis due to resemblance to the pyrimidine ring of Cytidine (Noroozi Aghide, Soleimani Samarkhazan, & Ahmadnezhad, 2016).

The precise function of LncRNAs in gastrointestinal diseases is not yet fully understood; however, recent studies suggest that they play a critical role in inflammatory cascades, regulating immune function, and the development of autoimmune-related diseases such as Crohn's disease and ulcerative colitis, which display the ability of LncRNAs to regulate protein-coding genes at the level of chromatin remodeling, transcriptional control, and post-transcriptional processes. Impaired expression of proteins' encoding genes and LncRNAs have been reported in both Crohn's disease and ulcerative colitis (Mirza et al., 2015; Chen et al., 2016).

The aim of this research was to evaluate the expression rate of LncRNA UCA1 and LncRNA Linc00970 in colon cancer cell lines before and after being treated by epigenetic drug 5-azacytidine. Consequently, we aimed to evaluate the Wnt gene expression in colon cancer cell line before and after being treated by 5-azacytidine.

Material and methods

Cell culture

Colon cancer cell line (HT29) was purchased from Pasteur Institute (Tehran, Iran), then were cultured in RPMI medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 100 U mL⁻¹ penicillin/streptomycin (Invitrogen, Shanghai, China), 1% non-essential amino acid (NEAA, Gibco), and 10% fetal bovine serum (FBS; Gibco) in a humidified culture chamber of 5% CO₂.

MTT assay

The MTT assay was performed to determine the viability of the cells using the MTT assay (UK, Sigma), based on the manufacturer's instruction. Briefly, MTT powder was dissolved in physiological saline at a concentration of 5 mg mL⁻¹ and kept at -20°C. Firstly, almost 10000 cells mL⁻¹ were seeded in a 96 well culture plate. After 24, 48, and 72 hours cells were treated with 5-Azacytidine. After incubation, DMSO (Germany, Merck) was added to each well to completely dissolve formazan crystals. The insoluble crystals of formazan were dissolved and the maximum optical absorption (OD) was measured at 570 nm by Elisa Plate Reader.

RNA isolation and Real-time PCR

Total RNA was isolated from the cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Then PCR was carried out. In short, in the first step, cDNA was synthesized using AMV reverse transcriptase (TaKaRa, Dalian, China). In Real-time PCR we took advantage of TaqMan PCR kitonan Applied Biosystems 7300 Sequence Detection System (Applied Biosystems). Next, real-time PCR was carried out using RT products, primers, and SYBRGreen dye. Specific primer pairs were added to the gene and LncRNAs, which included forward and reverse primers, as follows: forward and reverse sequence primers of LncRNA Linc00970 are TACGTAATTGGCCTGCCCTGT (forward) and GGCCTCTTCCTGTGGTTTCAG (reverse). Forward and reverse sequence primers of LncRNA UCA1 are CCACACCCAAAACAAAAATCT (forward) and TCCCAAGCCCTCTAACAACAATGAC (reverse). Forward and reverse sequence primers of Wnt are GCCCAGTTTGAATTGAAGC (forward) and TGAGAAAGTCCTGCCAGTTG (reverse). forward and reverse sequence primers of β -catenin are 5'-AAAGCGGCTGTAGTCACTGG-3' (forward) and 5'-GACCTGGGAGGTATCCACATCC-3'

(reverse). forward and reverse sequence primers of *U6* are 5'-CGGGTTTGTTCATTTCT-3' (forward) and 5'-AGTCCCAGCATGAACAGCTT-3' (reverse). Forward and reverse sequence primers of *GAPDH* are 5'-TGCACCACCAACTGCTTAGC-3' (forward) and 5'-GGCATGGACTGTGGTCATGAG-3' (reverse). The SYBRGreen® Mastermix™ II (TAKARA, Japan) was used in this study. Subsequently, the cycle threshold (CT) data were determined. Since we had run each reaction in triplicate, the mean CT was calculated. Afterward, the result of each condition was compared with the controls using the $2^{-\Delta\Delta CT}$ equation.

RNA quality and quantity assays

Total RNAs were extracted using Trizol® reagent (invitrogen). The purity of total RNA was evaluated by measuring the absorbance at 260 and 280 nm. Therefore, we selected an aliquot amount of RNA, which was then measured using optical density determination (OD) by the Nanodrop device at 260 nm.

Statistical analysis

Quantitative RT-PCR and the cell viability assays were performed in triplicate, and each experiment was repeated several times. The data shown are the mean $\pm$ SE of at least three independent experiments. The differences were considered statistically significant at $p < 0.05$ using the Student's t-test. All statistical calculations and graphs were performed by SPSS22 and one way ANOVA software. In this study, all concentrations and their viability were compared with the control group.

Ethics approval

This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for using cell line.

Results

MTT assay

The results of cytotoxic studies of 5-Azacytidine in vitro treatment on HT-29 cell line have been reported as IC50. IC50 is a millimolar concentration of a drug that inhibits the growth and differentiation of 50% of cancer cells compared to the untreated control group of cells. IC50 in MTT assay for 24 and 48 h treatment did not show any appropriate dosage, but for 72 h treatment, 1.25 mM concentration was determined as an appropriate dosage for IC50.

Real-time PCR assessment

Firstly, the quality of RNA was assessed; RNA was isolated and then being placed on the 1.5% agarose gel. Two bands corresponding to 18 and 28s ribosomal RNAs were observed subsequently. Afterwards, the expression level of LncRNAs and the target genes were determined by real-time PCR in C_t format. In this study, *GAPDH* was chosen as the reference gene. There is a difference between healthy and cancerous samples, so we should compare this difference between the C_t of LncRNA Linc00970, LncRNAUCA, and the Wnt gene of the healthy and cancerous samples by normalizing the data. For this, we subtract the C_t of the reference gene from the C_t of the genes under investigation, which yields Δct . It was understood that smaller numbers had a higher expression rate.

The expression level in 5-Azacytidine-treated samples of LncRNA Linc00979 LncRNA UCA1 with a concentration of 1 mM with $p < 0/05$ was meaningful and showed a higher expression than the samples treated with a concentration of 10 mM. Wnt gene expression level was also measured after treatment with different concentrations of the 5-azacytidine drug, the amount of which was meaningful with $p < 0/05$. In other words, Wnt levels were significantly decreased in the cells treated with different concentrations of the drug compared to untreated cells. HT29 cell line was treated with 1 and 10 mM of 5-azacytidine, then RealTime PCR was done in pursue of Wnt gene expression fluctuation level. Interestingly, we realized that at concentrations of 1 and 10 mM of the drug we have had reduced Wnt expression, but to our amazement the decline in Wnt expression at 1mM concentration was much greater than 10 mM. HT29 cell line was treated by 1 and 10 mM of 5-Azacytidine for the aim of investigating the expression changes in LncRNA Linc00970 post-treatment via Real-time PCR. as a result, there were no changes in the expression level of LncRNA linc00970 at 10 mM concentration of 5-Azacytidine but its expression rate had increased at 1 mM of drug concentration. Subsequently, LncRNA UCA1 expression level post-treatment was evaluated. For this reason, the HT29 cell

line was treated by 1 and 10 mM of 5-azacytidine. It was understood from the results that in 10 mM of 5-azacytidine-treated cells the expression rate of LncRNA UCA1 had no change while a decrease was detected in the LncRNA UCA1 expression after being treated by 1 mM of 5-azacytidine, in a contrary manner to LncRNA Linc00970. The fluctuations in the expression level of the Wnt gene, LncRNA UCA1, and LncRNA Linc00970 are presented in Figure 1.

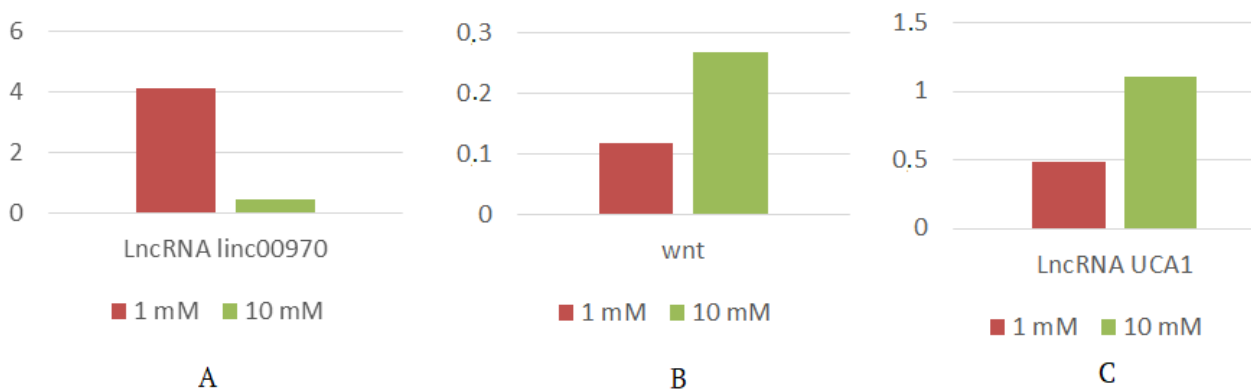


Figure 1. A) the Comparative bar chart study of LncRNA Linc00970 expression rate at concentrations of 1 and 10 mM of 5-azacytidine treatment at which $p < 0.05$ was considered as meaningful. B) the Comparative bar chart study of Wnt gene expression rate at concentrations of 1 and 10 mM of 5-azacytidine treatment at which $p < 0.05$ was considered as meaningful. C) the Comparative bar chart study of LncRNA UCA1 expression rate at concentrations of 1 and 10 mM of 5-azacytidine treatment at which $p < 0.05$ was considered as meaningful.

Discussion

LncRNAs play a critical role in many biological processes and are often dysregulated in CRC. However, further assessments are needed for elucidating each LncRNAs' function. In research conducted by Nikkhoo, Oodi, & Sotoodehnejadnematalahi (2017) on the leukemic NB4 cell line in 2017, it was found that 5-azacytidine drug causes the cell cycle to stop at the S phase. 5-azacytidine is an FDA-approved DNA hypo-methylating agent for the treatment of acute leukemia. However, the aforementioned drug has limitations due to their non-specific function in DNA binding, their limited effect on the S-cell phase, and their inappropriate use of these drugs. Of course, these inhibitors also have poor hypo-methylation potential. Therefore, the use of natural substances that have a hypo-methylation effect on DNA has become the focus of attention nowadays (Nikkhoo, Oodi, & Sotoodehnejadnematalahi, 2017). Consistent with their results, our study on the 5-Azacytidine drug revealed its efficacy in gene expression alteration. In addition, Blum and Marcucci in 2005 endorsed this hypothesis through their study on leukemia treated by the 5-Azacytidine drug. In Leukemia, FLT3 protein mutates, hypermethylation occurs, and this drug causes demethylation of MeCP2 and MBDI-4. One of the causes of cell tumorigenesis is epigenetic pattern change. Epigenetic processes, especially the abnormal pattern of genes, play an important role in cancer. It has been reported in this study that increased methylation has occurred in the regulatory regions of many tumor suppressor genes and consequently leading them to the silencing procedures. Therefore, this drug causes cellular stasis and stoppage in the S phase of the cellular cycle (Blum & Marcucci, 2005).

Based on what Tkach and Théry (2016) concluded LncRNA Linc00970 as well as MALAT1, BCYRN1, Linc00970, and H19 has a major macro-role in lung cancer, which is gaining more and more attention nowadays because of its convenience and cost-effectiveness (Tkach & Théry, 2016). According to Yang et al. (2018), there is a significant direct Relationship between LncRNA UCA1 Expression and Its Impact on colorectal cancer; by tumorigenesis of colorectal cancer the expression level of this LncRNA increases. In line with their findings, Bian et al. (2016) reached the conclusion of the over-expression of LncRNA UCA1 in bladder cancer. As well as the previous researchers, Fang, Chen, and Zhi in 2016 provide that the level of LncRNA UCA1 had increased in MDR gastric cancer. In line with the previous studies, during the experiment conducted in this paper, it was found that the expression level of LncRNA UCA1 would decrease if being treated by 1 mM of 5-Azacytidine, while it would have no changes in its expression when being treated by 10 mM of 5-Azacytidine. It is an oncogenic LncRNA and the epigenetic 5-azacytidine drug affects its inhibitors (EP300, CDKN2B, TGF β -2) and makes their promoter demethylated.

Wu et al. (2011) concluded that the target genes involved in colon cancer have been overexpressed because of increased expression of the Wnt pathway. Thus, by over-activation of the Wnt pathway, the expression level of the genes involved in colon cancer is raised (Wu et al., 2011). In line with their findings, our study observed the accelerated activity of the Wnt pathway which resulted in increased expression of target LncRNAs. To the best of our knowledge, β -catenin plays important role in CRC progression. The expression level of the Wnt gene was declined when it was treated by 1 and 10 mM concentration of 5-azacytidine. There is a significant relationship between its expression and its effect on colorectal cancer. Given that it is a demethylating drug, it is commonly predicted to have increased expression in most of its target genes. 5-azacytidine affects gene expression and is an inhibitory drug. Since Wnt is an oncogene which leads to carcinogenesis and tumor proliferation, its expression level would decrease if being treated by 5-azacytidine. Other studies declare and we endorse them that the Wnt gene plays an important role in colon cancer. This drug demethylates the Wnt gene (DKK) inhibitor. It is observed that in 50% of colon cancer cases where the promoter is demethylated and when being treated with 5-azacytidine, DKK reduces the expression of the Wnt gene.

Conclusion

To the best of our knowledge, there have never been any studies on the correlation of LncRNA Linc00970 and colorectal cancer. This novel research hypothesis has been dealt with in this study. Initially, we detected the correlation between colorectal cancer and LncRNA Linc00970. Next, the HT29 cell line was treated with 1 and 10 mM of 5-Azacytidine for the aim of investigating the expression changes in LncRNA Linc00970 post-treatment via Real-time PCR. as a result, there were no changes in the expression level of LncRNA Linc00970 at 10 mM concentration of 5-azacytidine but its expression rate had increased at 1 mM of drug concentration. Our assessment also revealed the fact of the molecular connection between LncRNA Linc00970 and the Wnt/ β -catenin pathway.

Due to the novelty of this area of research, it is suggested that other researchers study different mechanisms associated with these long non-coding RNAs as well as evaluating their target genes and organs. These novel long non-coding RNAs may have effects on other cancers than colon cancer. Therefore, investigating other cancers' correlation with these properties is highly recommended.

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