Investigating miR-16-5p: The Tumor Suppressor That Influences Cell Cycle Genes in Colorectal Cancer

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ABSTRACT

Background: Colorectal Cancer (CRC) remains a major health challenge, with perturbation in the cell cycle playing a crucial role in its progression. Hsa-miR-16-5p (miR-16-5p) is a crucial tumor suppressor, but its precise role in modulating cell cycle genes, particularly in the presence of ionizing radiation, is not fully understood.

Objective: This study investigated the role of miR-16-5p in modulating the expression of cell cycle genes in colorectal cancer cells exposed to ionizing radiation.

Material and Methods: In this experimental study, HT-29 cells were transfected with miR-16-5p using the polyfectamine transfection reagent. Expression levels of miR-16-5p, *Cyclin-Dependent Kinase 4 (CDK4), Cyclin E1 (CCNE1)*, and *Cyclin D1 (CCND1)* were quantified by real-time Polymerase Chain Reaction (PCR). To assess the changes after irradiation, cells were exposed to 4 Gy.

Results: Ionizing radiation significantly downregulated miR-16-5p compared to controls, while transfection of miR-16-5p significantly increased its expression level. However, irradiation of 4 Gy did not significantly alter *CCND1* or *CCNE1*, but decreased *CDK4* expression. The miR-16-5p transfection significantly suppressed *CCND1*, *CCNE1* and *CDK4* compared to controls. The expression of *CCND1*, *CCNE1*, and *CDK4* significantly decreased when miR-16-5p transfection was performed before 4 Gy irradiation compared to both 4 Gy irradiation alone and the control group.

Conclusion: Our results highlight the role of miR-16-5p in modulating key cell cycle genes in CRC. Increasing miR-16-5p expression could improve radiosensitivity and represent a therapeutic strategy for the treatment of CRC.

Keywords

MicroRNAs; Cell Cycle; Radiation-Sensitizing Agents; Colorectal Cancer

Introduction

Colorectal Cancer (CRC) is one of the most common cancers worldwide, and ionizing radiation is an essential part of the therapeutic approach. Nevertheless, the occurrence of radioresistance can significantly impair the efficacy of treatment and lead to potential treatment failure [1].

In cell cycle regulation, abnormalities significantly contribute to cancer development as important factors in the development of radioresistance. These abnormalities often lead to excessive cell proliferation, which results in hypoxia, increased angiogenesis, altered metabolism,

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changes in the tumor microenvironment and ultimately recurrence, invasion, and metastasis [2]. Several signaling pathways involved in carcinogenesis, including Janus Kinase (JAK) /Signal Transducer and Activator of Transcription (STAT), Wnt/β-catenin, Phosphoinositide 3-Kinase (PI3K)/AKT/ mammalian Target of Rapamycin (mTOR), and mechanisms that allow cancer cells to bypass programmed cell death and senescence, show signs of impaired cell cycle regulation. The successful progression of the cell cycle is characterized by precise genome replication in the S phase and the orderly distribution of chromosomes to the daughter nuclei in the Mitotic (M) phase. This orderly progression depends on a variety of regulatory mechanisms controlled by Cyclin-Dependent Kinases (CDKs) and their corresponding cyclins, which together form complexes that are crucial for cell cycle progression. Consequently, targeting elements of the cell cycle machinery represents a promising therapeutic strategy, potentially suppressing cancer cell proliferation and reversing the effects of various signaling pathways activated in cancer [3, 4].

Small, non-coding RNAs known as microRNAs (miRNAs) play an important role in the control of gene expression at the post-transcriptional level. These key molecules influence essential biological processes, such as proliferation, cell cycle progression, apoptosis, and metastasis [5, 6]. Among them, (miR-16-5p) has attracted attention for its potential role as a tumor suppressor. Previous studies indicate that miR-16-5p can inhibit cell proliferation and promote apoptosis by targeting various oncogenes [7]. However, its specific function in mediating cell cycle arrest and overcoming radioresistance in CRC remains poorly understood.

This study focuses on evaluating the ability of miR-16-5p to promote cell cycle arrest and increase the susceptibility of tumor cells to radiation. We are investigating the role of this tumor suppressor in modulating gene expression changes in CRC cells.

Material and Methods

Cell Culture and Irradiation

In this experimental study, a nutrient-rich culture medium, Roswell Park Memorial Institute-1640 (RPMI-1640), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin, was used to culture the human CRC cell line HT-29. The cells were maintained in a humid environment with 5% CO_2 to ensure optimal cell viability. Irradiation with 4 Gy was performed via the Source-Axis Distance (SAD) technique at a dose rate of 200 Monitor Units (MU)/min using a 6 MV linear accelerator.

miRNA Transfection

Sufficient cells were seeded in 6-well plates to achieve 70-80% confluence on the day of transfection. Using polyfectamine transfection reagent, QIAGEN, and the manufacturer's recommended procedure, miR-16-5p was transfected into the cells. Forty-eight hours later, the efficacy of the transformation was verified by fluorescence.

Ribonucleic Acid (RNA) Extraction

As prescribed by the manufacturer, total RNA was extracted using a kit from Pars Toos Company in Iran. The extracted RNA was immediately stored at -80 °C until the complete Deoxyribonucleic Acid (cDNA) was synthesized. A NanoDrop-2000 (the USA-made) was used to determine the amount of RNA from each group to produce cDNA.

Real-time Polymerase Chain Reaction

A kit from Yekta Tajhiz Azma, Iran, containing 5000 ng of RNA extracted from the groups was used for cDNA synthesis. A specific stem-loop primer for miR-16-5p from Metabion, Germany, with the sequence 5' GTCGTATGCAGAGCAGGGTCCGAGG-

TATTCGCACTGCATACGACCGCCAA 3' was used. Small Nucleolar RNA, C/D box 47 (SNORD47) was used as a control. Its cDNA was synthesized using a stem-loop primer from Metabion, Germany, with the sequence 5' GTCGTATGCAGAGCAGGGTCCGAG-GTATTCGCACTGCATACGACAACCTC 3'. Real-time Polymerase Chain Reaction (real-time PCR) as performed using the CyberGreen Master Mix from Yekta Tajhiz Azma Company, according to the manufacturer's instructions. In this study, the additional primers, purchased from Metabion, Germany, are listed in Table 1.

The study groups to evaluate the relative changes in gene expression included the control group, transfection with miR-16-5p (miR), 4 Gy irradiation (4 Gy), and 4 Gy irradiation followed by transfection with miR-16-5p (miR+4 Gy).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 9.0. Both a two-tailed Student's t-test and a one-way Analysis of Variance (ANOVA) were used. A *P*-value threshold of less than 0.05 was set as the threshold for statistical significance.

Results

Forty-eight hours after transfection with miR-16-5p, transfection efficiency was qualitatively assessed by fluorescence microscopy. Figure 1 shows representative fluorescence images illustrating the uptake of Green Fluorescent Protein (GFP) after transfection.

The expression of miR-16-5p was significantly decreased by irradiation (4 Gy)

Table 1: Primers sequences used in this study

Genes / miRNA	Sense- Sequence	Antisense- Sequence
Cyclin-Dependent Kinase 4 (CDK4)	GCTGCTGCTGGAAATGCTGAC	GCCACTCCATTGCTCACTCC
Cyclin E1 (CCNE1)	AAAGGTTTCAGGGTATCAGTGGTG	TTCTTTGCTCGGGCTTTGTCC
Cyclin D1 (CCND1)	AGCTGTGCATCTACACCGAC	GAAATCGTGCGGGGTCATTG
Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH)	CCTCAAGATCATCAGCAATG	CATCACGCCACAGTTTCC
miR-16-5p	GCCTAGCAGCACGTAAATA	-
Small Nucleolar RNA, C/D box 47 (SNORD47)	ATCACTGTAAAACCGTTCCA	-
Universal R	GCCTAGCAGCACGTAAATA	-

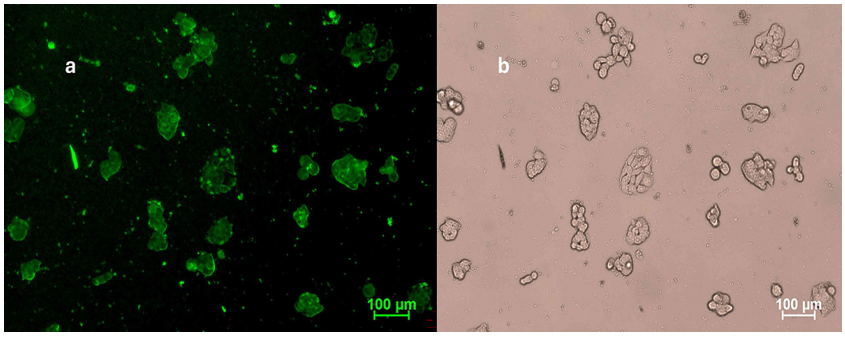

Figure 1: (**a**) Fluorescent microscopy image taken 48 hours post-transfection with miR-16-5p, (**b**) visible light image of HT-29 cells (scale bar: 100 µm).

(*P*-value<0.01). However, transfection with miR-16-5p significantly increased its expression (*P*-value<0.0001), indicating successful transfection. The relative expression of miR-16-5p in the different groups is shown in Figure 2.

Irradiation led to a decrease in the relative expression of *Cyclin D1 (CCND1)*, although this decrease was not statistically significant. Compared to the control group, this gene was significantly suppressed by miR-16-5p (*P*-value<0.001). *CCND1* expression was significantly decreased in the miR+4 Gy group compared to the 4 Gy group (*P*-value<0.0001). Figure 3 shows the *CCND1* gene.

Cyclin E1 (CCNE1) did not change significantly in response to radiation exposure (4 Gy) compared to the control group. This gene was significantly downregulated by miR-16-5p (*P*-value<0.05) compared to the control group. Figure 4 shows the relative changes of this gene in the analysed groups. In addition, the relative expression of this gene was significantly lower in the miR+4Gy group than in the 4Gy group (*P*-value<0.0001).

Based on the relative expression of the *Cyclin-Dependent Kinase 4 (CDK4)* gene, Figure 5 shows that 4 Gy irradiation suppressed CDK4 (*P*-value<0.05). miR+4 Gy significantly decreased *CDK4* expression compared to 4 Gy (*P*-value<0.001). However, the comparison between the miR+4 Gy and

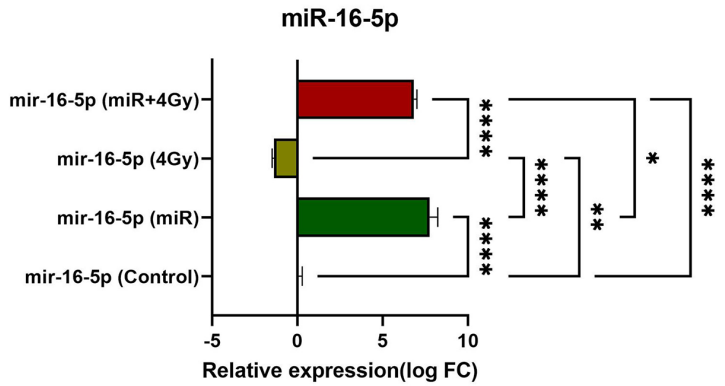

Figure 2: miR-16-5p relative expression in the control, 4 Gy irradiation (4 Gy), miR-16-5p transfection (miR), and combined hsa-miR-16-5p transfection and 4 Gy irradiation (miR+4 Gy) groups. (*P<0.05, **P<0.01, ****P<0.0001).

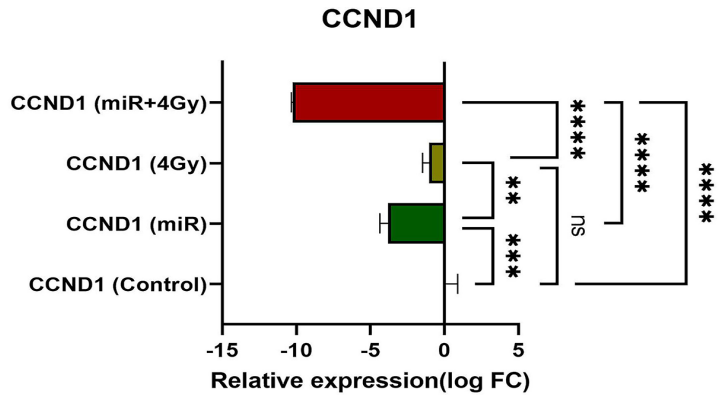

Figure 3: Relative expression of *Cyclin D1 (CCND1)* in the control, miR-16-5p transfection (miR), 4 Gy irradiation (4 Gy), and combined miR-16-5p transfection and 4 Gy irradiation (miR+4 Gy) groups. (ns=not significant, **P<0.001, ****P<0.0001).

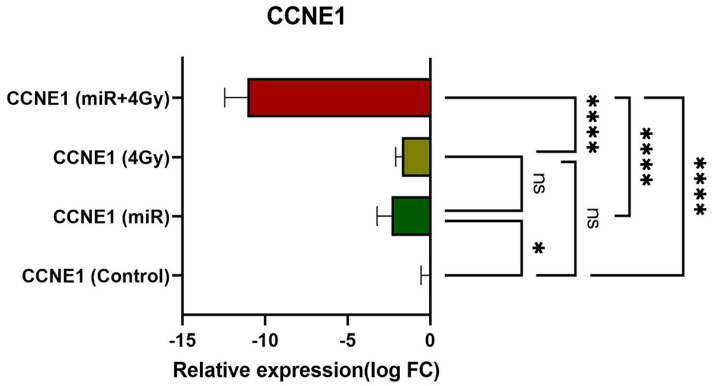

Figure 4: *Relative expression of Cyclin E1 (CCNE1)* in the control, miR-16-5p transfection (miR), 4 Gy irradiation (4 Gy), and combined miR-16-5p transfection and 4 Gy irradiation (miR+4 Gy) groups. (**P*<0.05, *****P*<0.0001, ns=not significant).

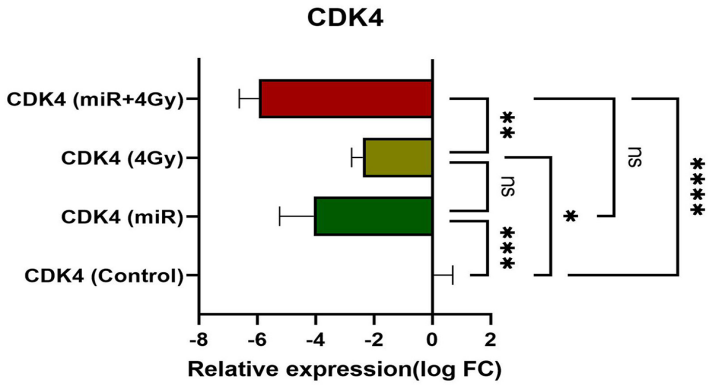

Figure 5: *Cyclin-Dependent Kinase 4 (CDK4)* relative expression in the control, hsa-miR-16-5p transfection (miR), 4 Gy irradiation (4 Gy), and combined hsa-miR-16-5p transfection and 4 Gy irradiation (miR+4 Gy) groups. (ns=not significant, **P*<0.05, **P<0.01, ****P*<0.001, *****P*<0.0001).

miR groups showed no statistically significant changes in *CDK4* expression.

Discussion

Early diagnosis, combined with surgery and adjuvant therapies, such as chemotherapy and radiotherapy, can significantly reduce the mortality rate associated with this disease [8]. Treatment strategies are tailored to the stage of disease, tumor location, lymph node involvement, and metastatic features. Radiotherapy often plays a crucial role in the treatment of CRC, due to its locally effective control of the tumor. The main goal of radiotherapy is to eliminate tumor cells by inducing damage, which ultimately leads to cellular apoptosis [9]. When DNA damage occurs, the cells initiate a series of reactions, as follows: recognizing damage, activating signaling pathways, repairing the cell damage, arresting the cell cycle, or undergoing apoptosis. This response can be either beneficial or detrimental to the elimination of the tumor. Oncogenes can modulate the response to DNA damage and promote tumor cell survival and proliferation by activating downstream proto-oncogenes [10]. Conversely, the activation of certain tumor suppressors can promote tumor cell death. Thus, the pathway involved in tumor cell pathogenesis is crucial to the efficacy of

radiation therapy [11]. This interplay can contribute to radioresistance, which ultimately impairs the efficacy of treatment.

The cell cycle is a carefully regulated sequence of events that contributes to the development and progression of cancer and significantly influences the response of cancer cells to radiotherapy. Research into the relationship between radioresistance and the cell cycle is crucial to improve the effectiveness of cancer treatment [11].

The various phases of the cell cycle are differentially radiosensitive. In particular, cells in the synthesis (S) phase often survive better after radiation damage, which is mainly due to efficient repair by the Homologous Recombination (HR) repair pathway. However, cell cycle checkpoints play an important role in maintaining DNA integrity and ensuring that damaged DNA does not pass into subsequent phases. Among these, the Gap 1 (G1)/S checkpoint is particularly critical. In the presence of Retinoblastoma (Rb), the formation of the *Cyclin-Dependent Kinase (CDK)* and cyclin complex is inhibited, resulting in cell cycle arrest at this stage [12]. Oncogenes can disrupt these checkpoints, impair their functionality, and lead to uncontrolled cell proliferation and associated radioresistance [13]. To improve the efficacy of radiotherapy, a deeper understanding of the interplay between the cell cycle and radioresistance is therefore required [14].

In recent years, miRNAs have gained attention as radiosensitizers. These biological agents can modulate gene expression, potentially overcoming radioresistance and improving the efficacy of radiotherapy [15]. miR-250a-3p, targeting Epidermal Growth Factor Receptor (EGFR), and miR-217, targeting the Mitogen-Activated Protein Kinase (MAPK) signaling pathway, can induce apoptosis in CRC cells [16, 17]. miR-16-5p is a tumor suppressor that affects the activation of oncoproteins in various signaling pathways [18]. It is typically downregulated in CRC, making it a potential biomarker for early diagnosis [19]. In addition, the present study showed that the levels of miR-16-5p significantly decrease after radiation.

Research has explored the potential of miRNAs in mediating cell cycle arrest and inhibiting cell proliferation. For example, a study of 42 pairs of colon cancer tissue and Adjacent Non-Tumor Tissue (ANTT) has shown that miR-195-5p targets *Cyclin-Dependent Kinase 8 (CDK8)*, induces cell cycle arrest in G1 phase, reduces cell growth, and decreases invasion [20]. In addition, miR-6734 was shown to enhance the expression of the p21 gene, leading to cell cycle arrest and apoptosis in colon cancer cells [21]. The miR-193a-5p and miR-146a-5p were identified as regulators of *Mouse Double Minute 2 homolog (MDM2)* that promote G1 arrest in CRC cells via the p53 signaling pathway [22]. For 20 CRC and ANTT pairs, miR-133a-3p targeted *Sentrin/SUMO-Specific Protease 1 (SENP1)* to promote G1/S arrest and inhibit cell proliferation [23]. The miR-1258 was discovered to target E2F *Transcription Factor 8 (E2F8)* by preventing proliferation and promoting G0/G1 arrest in mice and human samples, containing 60 pairs of CRC and ANTT [24]. In contrast, based on findings from eight CRC and ANTT, miR-598 suppresses *Inositol Polyphosphate-5-Phosphatase E (INPP5E)*, promotes G1/S transition, and stimulates cell proliferation [25].

The study also showed that miR-16-5p can effectively suppress gene expression in the cell cycle. In particular, this suppression can be enhanced when the cells are exposed to 4 Gy of radiation, which leads to cell cycle arrest. This prevents the cell from entering the S phase and stops cell proliferation. This mechanism is crucial as it helps to reduce the risk of mutations caused by uncontrolled proliferation and unrepaired DNA damage. Essentially, this maintains cell cycle regulation in CRC cells, contributing to potential therapeutic strategies to control cancer progression.

Furthermore, understanding the intricate

relationships between miRNAs and cell cycle dynamics opens up potential avenues for the development of new therapeutic strategies to manipulate these pathways for better management of cancer cells. The study of miRNAs in the context of cell cycle regulation thus sheds light on fundamental biological processes and holds promise for advances in targeted therapies and precision medicine.

Conclusion

Finally, this study emphasizes the importance of miR-16-5p in controlling the cell cycle dynamics of CRC cells. Our results show that miR-16-5p decreases the expression of essential genes associated with the cell cycle, which is enhanced in response to ionizing radiation. Further genomic instability is prevented, and a strong cell cycle arrest occurs. These results indicate the therapeutic potential of miR-16-5p in the treatment of CRC and suggest that this microRNA may contribute to the treatment of radioresistant cancer cells. The modification of miR-16-5p offers a promising avenue for the development of novel cancer therapies that utilize cell cycle control mechanisms.

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Authors' Contribution

The idea for this article comes from SN. Mousavikia, H. Azimian, and M. M. Matin. The experiments and data analysis were performed by SN. Mousavikia. The research paper was edited and supervised by MT. Bahreyni Toosi and SH. Aghaee-Bakhtiari. All authors have read, revised and approved the final version of the manuscript.

Ethical Approval

The investigation was carried out by Mashhad University of Medical Sciences, and the ethical approval code for this in vitro study is IR.MUMS.MEDICAL.REC.1402.296.

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Conflict of Interest

None

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