Mechanisms of microRNA action in rectal cancer radiotherapy

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Abstract
Preoperative neoadjuvant chemoradiotherapy, combined with total mesorectal excision, has become the standard treatment for advanced localized rectal cancer (RC). However, the biological complexity and heterogeneity of tumors may contribute to cancer recurrence and metastasis in patients with radiotherapy-resistant RC. The identification of factors leading to radioresistance and markers of radiosensitivity is critical to identify responsive patients and improve radiotherapy outcomes. MicroRNAs (miRNAs) are small, endogenous, and noncoding RNAs that affect various cellular and molecular targets. miRNAs have been shown to play important roles in multiple biological processes associated with RC. In this review, we summarized the signaling pathways of miRNAs, including apoptosis, autophagy, the cell cycle, DNA damage repair, proliferation, and metastasis during radiotherapy in patients with RC. Also, we evaluated the potential role of miRNAs as radiotherapeutic biomarkers for RC.

Keywords: MicroRNAs; Rectal cancer; Radiotherapy; Mechanisms

Introduction
Colorectal cancer (CRC) is the third most common cancer and the second most common cause of cancer-related deaths globally, with >1.9 million new cases (10.0%) and 935,000 deaths (9.4%) reported in 2020. According to the NCCN Guidelines (2020), the rectum was defined as below the line from the promontory of the sacrum to the upper margin of the symphysis, as determined by magnetic resonance imaging, rectal cancer (RC) accounts for approximately 39% of CRC cases. Although preoperative neoadjuvant chemoradiotherapy combined with total mesorectal excision has become the standard treatment for advanced localized RC, previous studies have indicated that only approximately 15% to 20% of patients with RC achieved a complete pathological response or no response. Evidence from the previous studies suggests that radiotherapy resistance may be regulated through the mechanisms of apoptosis, autophagy, the cell cycle, and DNA damage repair. Therefore, it is of great importance to explore the mechanisms of radiotherapy resistance in RC, to understand the pathways of tumor cell survival inhibition in patients undergoing radiotherapy, and to develop new therapeutic strategies targeting these mechanisms.

MicroRNAs (miRNAs), a family of small non-coding RNAs, are approximately 22 nucleotides in length and they exert their effects by binding to complementary sequences on the 3′-untranslated regions (3′-UTRs) or the open reading frames of target genes. miRNAs regulate gene expression at the post-transcriptional level, leading to the degradation of target miRNAs or the inhibition of mRNA translation. In some cases, miRNAs interact with long noncoding RNAs to form a network that regulates tumorigenesis. It is believed that up to 30% of human genes are regulated by miRNAs. They play key roles in the regulation of biological processes, such as apoptosis, cell differentiation, development, and cell proliferation. miRNAs are secreted into bodily fluids with minimal degradation; they are highly stable during storage; and they are easy to quantify using quantitative polymerase chain reaction, microarray, bead array, or sequencing approaches.

Findings from many previous studies have indicated that miRNAs may be markers of the response to cancer treatment, which may facilitate the development of new strategies to overcome therapeutic resistance. Recent studies have shown that miRNAs may be involved in the regulation of radiotherapy sensitivity in RC. Zhang et al. found that miR-124 increased the radiosensitivity of CRC cells by blocking the expression of paired related
miRNAs Affect the Response to RC Radiotherapy by Regulating Apoptosis

Apoptosis refers to programmed cell death that is initiated after the damage to DNA or cellular organelles, such as the mitochondria and endoplasmic reticulum. Apoptosis usually occurs after the exposure of cells to stress conditions, such as oxidative stress, IR, chemotherapy drugs, hypoxia, or high temperature. It is modulated via different signaling pathways that affect extrinsic or intrinsic mediators of apoptosis. Previous studies have shown that apoptosis is an indicator of cell radiosensitivity and an important prognostic factor for radiotherapy outcomes. Moreover, miRNAs have been found to regulate apoptosis after IR. Here, we summarize the miRNAs involved in the regulation of apoptosis after IR [Table 1 and Figure 1].

The phosphatidylinositol 3-kinase/AKT (PI3K/AKT) pathway is known to regulate cell survival and increase radiation resistance. Previous studies have shown that phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a natural inhibitor of PI3K, negatively regulates the PI3K/AKT pathway, resulting in apoptosis. There is evidence suggesting that miR-29a, miR-106b, and miR-222 decrease the radiosensitivity of CRC by negatively regulating PTEN expression, to activate the PI3K/AKT signaling pathway. It has also been shown that an miR-221 antisense oligonucleotide enhances IR sensitivity by mediating the upregulation of PTEN. Furthermore, forkhead box O3 (FOXO3a), a member of the FOXO family, is a downstream effector of the PI3K/AKT pathway. Khoshinani et al. showed that FOXO3a is involved in the regulation of the radiation resistance of RC as a direct target gene of miR-155. Moreover, Chen et al. demonstrated that exosomal miR-590-3p derived from cancer-associated fibroblasts (CAFs) increases the radiation resistance of RC by positively regulating the chloride channel accessory 4-dependent PI3K/AKT signaling pathway in vivo and in vitro. Another study showed that, in vitro, the lncRNA, TTN antisense RNA 1, promotes the radiotherapy resistance of CRC cells by negatively regulating miR-134-5p and increasing the expression levels of p21-activated kinase 3, which may be associated with the P21 and AKT/glycogen synthase kinase-3β pathways.

Several miRNAs also affect apoptosis through other signaling pathways and participate in the regulation of the radiotherapy sensitivity of RC. Some members of the Bcl-2 protein family, such as Bad, Bid, and Bax, promote apoptosis, whereas others, such as Bcl-2, Bcl-x, and Bcl-w, prevent apoptosis. Yang et al. reported that miR-100 promotes the X-ray-induced apoptosis of CCL-244 cells and regulates the expression of apoptosis-related proteins, including increasing the expression levels of the pro-apoptotic proteins, P53 and caspase-3, and decreasing the levels of the anti-apoptotic proteins, Bcl-2 and NF-κB, further increasing the radiosensitivity of CCL-244 cells. The forced expression of miR-195 has been shown to induce apoptosis, upregulate Bax and γ-H2AX levels, inhibit the expression of Bcl-2, and increase the radiosensitivity of CRC cells by downregulating coactivator associated arginine methyltransferase 1 in vivo and in vitro. Data from the study reported by Shang et al. implied that overexpression of miR-423-5p promotes radiation-induced apoptosis through downregulation of Bcl-XL, ultimately increasing the sensitivity of HCT116 and RKO cells to IR. The expression level of miR-630 in CRC cells is positively correlated with radiosensitivity and the induction of apoptosis. BCL2L2 and TP53 regulating kinase (TP53RK) have been identified as target genes of miR-630. BCL2L2, also known as Bcl-w, belongs to the Bcl-2 protein family. The kinase, TP53RK, inhibits apoptosis after mitotic stress. Zhang et al. reported that cAMP-response element-binding protein/miR-630/BCL2L2 and TP53RK constitute a novel signaling cascade that modulates the radiosensitivity of CRC cell lines by inducing apoptosis.

Other studies have also reported findings related to the mechanism of apoptosis. CAF-derived exosomes upregulated the level of miR-93-5p and downregulated the level of forkhead box A1, the target gene of miR-93-5p, further inhibiting apoptosis and promoting the resistance of RC to radiation in vivo and in vitro. Ma et al. found that the overexpression of miR-622 induces radiation resistance in vitro. During the radiation response, the retinoblastoma (Rb)-E2F1-P/CAF complex transcriptionally activates pro-apoptotic genes. Rb overexpression has also been shown to reverse radiation resistance induced by miR-622 in vitro. The results reported by Rana et al. suggest that the activation of acid sphingolipids (sphingomyelin phosphodiesterase 1, SMPD1) and the production of ceramides are key processes in the regulation of apoptosis in response to cellular stress, including radiation. Low expression levels of miR-15a upregulate SMPD1 levels, inducing apoptosis and increasing radiosensitivity. Low expression levels of miR-95 have been shown to increase the radiosensitivity of LoVo cells and promote apoptosis, which may be related to the inhibition of forkhead box D1 protein activity. It has been noted that miR-122-5p significantly inhibits cell survival and increases radiotherapy sensitivity and apoptosis by silencing the apoptosis regulator, cell division cycle, and
| miR | Up/Down | Mechanism | Target | Up/Down | Sensitivity/Resistance | Sample type | Reference |
|-----|---------|-----------|--------|---------|------------------------|-------------|-----------|
| miR-15a | Down | Apoptosis | SMPD1 | Up | Sensitivity | Cell lines | [38] |
| miR-29a | Up | Apoptosis | PTEN | Down | Resistance | Cell lines | [26] |
| miR-95 | Down | Apoptosis | FOXD1 | Down | Sensitivity | Cell lines, CDX | [31] |
| miR-134-5p | Down | Apoptosis | PK3 | Up | Resistance | Cell lines | [77] |
| miR-145 | Down | Apoptosis | — | — | Resistance | Tissue, cell lines, PDX, CDX | |
| miR-155 | Up | Apoptosis | FOXO3a | Up | Sensitivity | Cell lines | [27] |
| miR-181a-p | Up | Apoptosis | COS | Down | Sensitivity | Cell lines | [11] |
| miR-205-5p | Up | Apoptosis | — | — | Sensitivity | Cell lines | [78] |
| miR-211-5p | Up | Apoptosis | ErbB4 | Down | Sensitivity | Tissue, cell lines, CDX | |
| miR-222 | Down | Apoptosis | PTEN | Up | Sensitivity | Cell lines | [27] |
| miR-338-5p | Down | Apoptosis | — | — | Resistance | Cell lines | [79] |
| miR-369-3p | Down | Apoptosis | — | — | Resistance | Cell lines | [31] |
| miR-423-5p | Up | Apoptosis | bcl-xL | Down | Sensitivity | Tissue, cell lines | [34] |
| miR-630 | Up | Apoptosis | — | — | Sensitivity | Tissue, cell lines, CDX | |
| miR-622 | Up | Apoptosis, DNA damage | Rb | Down | Resistance | Tissue, cell lines | [57] |
| miR-1 | Up | Apoptosis, migration, invasion | — | — | Sensitivity | Tissue, cell lines | [73] |
| miR-101-3p | Up | Apoptosis, migration, invasion | — | — | Sensitivity | Cell lines | [10] |
| miR-93-5p | Up | Apoptosis, proliferation | FOXA1 | Down | Resistance | Cell lines, tissue, cell lines | [56] |
| miR-770-5p | Up | Apoptosis, proliferation | PBK | Down | Sensitivity | Cell lines, CDX | [44] |
| miR-296-5p | Up | Apoptosis, proliferation, cell cycle | CCAR1 | Down | Sensitivity | Tissue, cell lines, CDX | [42] |
| miR-128 | Up | Apoptosis, proliferation, cell cycle | IGF1R, IGF2 | Down | Sensitivity | Cell lines | [42] |
| miR-129-5p | Up | Apoptosis, DNA damage | CARM1 | Down | Sensitivity | Cell lines, CDX | [33] |
| miR-183-5p | Up | Apoptosis, DNA damage | CLCA4 | Down | Resistance | Tissue, cell lines, CDX | [33] |
| let-7 | Up | Autophagy | beclin-1 | Down | Sensitivity | Cell lines, CDX | [50] |
| miR-451a-3p | Up | Autophagy | — | — | Sensitivity | Tissue, cell lines, CDX | |
| miR-506-3p | Up | Autophagy | — | — | Sensitivity | Tissue, cell lines, CDX | |

CDX: Cell line derived xenograft; DNA: deoxyribonucleic acid; PDX: Patient-derived xenograft; miRNAs: MicroRNAs; RC: Rectal cancer; –: Not applicable.
Apoptosis induced by miR-214 significantly increases the radiosensitivity of CRC cells by downregulating its target gene, ATG5. Liu et al.\(^5\) reported that the overexpression of miR-93 inhibits IR-induced autophagy. Zheng et al.\(^4\) found that the treatment of CAFs with an miR-31 mimic inhibited the expression of ATGs Beclin-1, ATG, DRAM, and LC3, thus increasing the radiosensitivity of CRC cells co-cultured with CAFs. Xu et al.\(^5\) indicated that the overexpression of miR-129-5p inhibits Beclin-1, a key autophagy-related gene, and inhibits autophagy. However, the overexpression of Beclin-1 eliminates the effect of miR-129-5p. These results suggest that miR-129-5p significantly enhances the radiosensitivity of CRC cells by inhibiting Beclin-1-mediated autophagy.

Heterotopic overexpression of miR-18a in HCT116 cells enhances IR-induced autophagy.\(^1\) In addition, evidence suggests that miR-18a overexpression results in upregulation of the expression of the autophagy activator, ataxia telangiectasia mutated, and the inhibition of the mammalian target of rapamycin compound 1 activity. Yang et al.\(^4\) found that the treatment of CAFs with an miR-31 mimic inhibited the expression of ATGs Beclin-1, ATG, DRAM, and LC3, thus increasing the radiosensitivity of CRC cells co-cultured with CAFs. Xu et al.\(^5\) indicated that the overexpression of miR-129-5p inhibits Beclin-1, a key autophagy-related gene, and inhibits autophagy. However, the overexpression of Beclin-1 eliminates the effect of miR-129-5p. These results suggest that miR-129-5p significantly enhances the radiosensitivity of CRC cells by inhibiting Beclin-1-mediated autophagy.

Autophagy is a highly conserved critical regulatory process in which cells degrade aging proteins and damaged organelles through lysosomes, resulting in the circulation of cellular material and the maintenance of homeostasis.\(^4\) The formation of autophagosomes is regulated by autophagy-related genes (ATGs), such as ATG12, ATG5, and microtubule-associated protein light chain 3 (LC3). ATG12 and ATG5 a conjugated complex that plays an important role in autophagosome expansion.\(^1\) Various stimuli, such as starvation, hypoxia, DNA damage, and IR, may activate autophagy. Previous studies have demonstrated that autophagy is tightly linked to various cellular functions and that dysfunctional autophagy leads to various diseases, including cancer.\(^4\) In recent years, there have been many investigations of the mechanism whereby autophagy affects the radiosensitivity of cancer cells [Table 1 and Figure 2].

Figure 1: miRNAs regulate apoptosis through the corresponding target genes and affect the radiation sensitivity of RC. miRNAs: MicroRNAs; RC: Rectal cancer.
the accumulation of harmful genomic damage. The phosphorylation of P53 by ATM induces the expression and phosphorylation of the cyclin-dependent kinase inhibitor, P21. This leads to the inhibition of CDK4/6-cyclin D and CDK1-cyclin B, causing the cell cycle to arrest at G1 and G2, respectively. The efficient induction of cell cycle arrest promotes radiosensitivity, suggesting that cell cycle progression after IR contributes to the radiation resistance of tumors. Several miRNAs have been shown to play a role in cell cycle regulation after IR in CRC [Table 1 and Figure 3].

The let-7 miRNA family of tumor suppressors is downregulated in different types of human malignancies, including CRC. It has been shown that increasing let-7e levels reduces IGF-1R protein levels and inhibits the downstream signaling pathway, resulting in cell cycle arrest at G1, and significantly reducing CRC cell proliferation, survival, and radiation resistance. In addition, miR-296-5p overexpression inhibits cell proliferation and cell cycle progression and promotes apoptosis and radiosensitivity by down-regulating insulin-like growth factor I receptor (IGF1R). Zheng et al. showed that the overexpression of miR-106b decreased the expression levels of the direct targets of PTEN and P21. Meanwhile, the restoration of PTEN or P21 expression in cells stably overexpressing miR-106b reestablishes the effect of miR-106b on CRC cell radioresistance. These findings indicate that miR-106b mediates G1 growth arrest and cellular senescence by targeting P21. In addition, this process is accompanied by enhanced tumor promotion, suggesting that miR-106b may be related to the resistance of RC to radiation therapy.

Relationship Between miRNAs, DNA Damage Repair, and Radiotherapy in RC

DNA double-strand breaks (DSBs) are the most deleterious type of DNA damage as they may initiate genomic
instability, ultimately leading to cancer. Two pathways are specifically dedicated to the repair of DSBs: nonhomologous end-joining (NHEJ) and homologous recombination (HR) repair. The repression of these efficient repair systems permits the accumulation of DNA damage in rapidly dividing cells (such as cancer cells), thus inducing apoptosis. This mechanism plays an important role in the radiotherapy of RC. Recently, the role of miRNAs in DNA damage repair has been recognized, suggesting that miRNAs control the DNA damage response by interacting with DNA repair genes. Here, we summarize the miRNAs involved in DNA damage repair following IR (Table 1 and Figure 4).

There is evidence that changes in miRNA-binding sites in the 3'-UTR of base-resected repair genes may modulate the prognosis and treatment response in patients with RC, due to effects on DNA damage repair. Overexpression of miR-130a has been shown to inhibit the repair of cells after radiation-induced DNA damage. Moreover, there is evidence that SRY-Box transcription factor 4, which is a direct target of miR-130a, increases the activation of ATM signals by increasing the expression level of NBS1 and promoting the interaction between NBS1 and p-ATM, thereby mediating DNA damage repair. In a study of the relationship between miR-100 and radiotherapy for RC, miR-100 was found to be involved in radiation-induced apoptosis and to modulate the sensitization of CCL-244 cells to radiation by enhancing radiation-induced DNA damage repair. miR-122-5p overexpression or CCAR1 silencing, combined with IR, significantly reduces the levels of p-CHK2 and enhances radiosensitivity, indicating that CHK2 is an important regulatory kinase in the DNA damage response. miR-31 suppresses NF-κB signaling pathways by targeting serine/threonine kinase 40, thereby improving RC radiotherapy sensitivity. Moreover, it is possible to improve sensitivity to radiation by promoting DNA damage after radiotherapy. miR-185 regulates radiation-induced HR and NHEJ by targeting IGF1R and IGF2 genes. CAF-derived exosomal miR-590-3p induces radiotherapy resistance in RC through the CLCA4/PI3K/AKT axis, thereby inhibiting the DNA damage response. Overexpression of miR-124 and miR-195, combined with radiotherapy, increases the levels of phosphorylated γ-H2AX. miR-31-5p inhibitors may modulate radiosensitivity through IR-induced DNA damage repair.

**Relationship Between miRNAs, Other Mechanisms, and Radiotherapy in RC**

Unrestricted proliferation is the basis of cancer development. In addition, the invasion and metastasis of tumor cells are the major causes of RC-related deaths. Many studies have shown that miRNAs play important roles in pathways affecting tumor cell proliferation, invasion, and metastasis, thus influencing the efficacy of radiotherapy.
Ruhl et al. found higher expression levels of miR-451a and lower levels of calcium-binding protein 39 (CAB39) and EMSY transcriptional repressor, BRCA2 interacting (EMSY) in patients who responded to radiotherapy, compared with patients who did not respond to radiotherapy. Further exploration revealed that miR-451a was induced by radiation, and it may affect the proliferation of RC through the CAB39 and EMSY pathways. A series of studies have shown that overexpression of let-7e, miR-93-5p, miR-140-5p, and miR-296-5p, miR-506-3p, and miR-770-5p significantly reduces the proliferation of CRC cells after radiotherapy and improves radiotherapy sensitivity.[36,42,44,38,70] Recent studies have suggested that miR-1 may play an important role in RC. The downregulation of miR-1 and metastasis-associated in colon cancer-1 increases MET expression levels and promotes RC metastasis.[71] Furukawa et al. found that the miR-1-notch receptor 3-Asef pathway plays an important role in CRC cell migration. Another study reported that miR-1 mimics promote the expression of Bax and E-cadherin and decrease the expression levels of Bcl-2, MMP2, and MMP9, significantly inhibiting the invasion and migration of CRC cells in conjunction with radiotherapy.[72] miR-1 is thought to increase the radiosensitivity of CRC cells by inducing apoptosis and synergistically inhibiting invasive phenotypes. Ji et al. showed that overexpression of miR-15b inhibits the proliferation, invasion, and metastasis of CRC cells. Liang et al. reported that miR-32-5p may be a prognostic tool for RC. miR-32-5p was found to directly decrease the levels of transducer of ERBB2,1 mRNA by binding to its 3’-UTR, thus sensitizing RC to the effects of radiotherapy and inhibiting metastasis.

Conclusions

With the development of biotechnologies, such as high-throughput sequencing, bioinformatics analysis, genome modification, and mouse models of disease, functional studies can provide new insights into the anticancer activity of miRNAs. By identifying downstream targets, many studies have shown that miRNAs regulate various signaling pathways (such as PI3K/AKT and Wnt/β-catenin) that play roles in a series of various processes, such as RC proliferation, metastasis, autophagy, and apoptosis, and affect the efficacy of radiotherapy for RC. Moreover, miRNAs are often involved in multiple mechanisms to regulate the activity of cancer cells, thus inducing radiotherapy sensitivity or resistance. These studies provide a new theoretical basis for the study of radiosensitivity and the identification of effective biomarkers and promising therapeutic targets for RC. However, many challenges remain, and the targets and downstream pathways of some miRNAs are still being explored.[15,76] In addition, most studies in this field are still at the basic stage, with little progress in in-vivo studies and translational direction. Moreover, most studies have been limited to specific tumor types or treatment modalities. Future studies should focus on exploring these questions. First, for related miRNAs, there is a need to further understand and evaluate genomic and functional approaches for basic and translational research, to help select appropriate and specific targets from a large number of candidates. More importantly, for suitable candidate genes that have been identified and stable delivery vectors that have been developed, there should be a greater focus on clinical studies to assess patients’ responses to miRNA-related therapies. This will improve our understanding of the long-term effects and adverse reactions associated with these therapies and help us to achieve translational results in cancer research.

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Conflicts of interest

The authors declare no competing financial interests.

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