

# miRNA-31 increases radiosensitivity through targeting STK40 in colorectal cancer cells

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## Abstract

**Objective:** To propose and verify that miRNA-31 increases the radiosensitivity of colorectal cancer and explore its potential mechanism.

**Method:** A bioinformatics analysis was performed to confirm that the expression of miRNA-31 was higher in colorectal cancer than in normal colorectal tissue. The expression of miRNA-31 was detected to verify the change in its expression in a radiotherapy-resistant cell line. Methylation was detected to explore the cause of the decrease in miRNA-31 expression. Overexpression or inhibition of miRNA-31 further confirmed the change in its expression in colorectal cancer cell lines. Bioinformatics methods were used to screen the downstream target genes and for experimental verification. A luciferase assay was performed to determine the miRNA-31 binding site in STK40. Overexpression or inhibition of STK40 in colorectal cancer cell lines further confirmed the change in STK40 expression in vitro.

**Results:** The bioinformatics results showed higher expression of miRNA-31 in tumors than in normal tissue, and miRNA-31 mainly participated in the pathway related to cell replication. Next, we observed the same phenomenon: miRNA-31 was expressed at higher levels in colorectal tumors than in normal colorectal tissue and its expression decreased in radiation-resistant cell lines after radiation, implying that miRNA-31 increased the radiosensitivity of colorectal cancer cell lines. No significant change in upstream methylation was observed. miRNA-31 regulated the radiosensitivity of colorectal cancer cell lines by inhibiting STK40. Notably, miRNA-31 played a role by binding to the 3' untranslated region of SK40. STK40 negatively regulated the radiosensitivity of colorectal cancer cells.

**Conclusions:** miRNA-31 increases the radiosensitivity of colorectal cancer cells by targeting STK40; miRNA-31 and STK40 are expected to become potential biomarkers for increasing the sensitivity of tumor radiotherapy in clinical treatment.

**ABBREVIATIONS:** 3' UTR, 3'-untranslated region; CRC, colorectal cancer; DMEM, Dulbecco's modified Eagle medium; EBF3, early B-cell factor 3; FAM134B, family with sequence similarity 134 member B; KANK1, KN motif and ankyrin repeat domains 1; miRNAs, microRNAs; MLH1, mutL homolog 1; MMS19, MMS19 homolog, cytosolic iron-sulfur assembly component; PARP1, poly(ADP-ribose) polymerase 1; qRT-PCR, quantitative real-time polymerase chain reaction; RAD51L3, RAD51 paralog D; SEPHS1, selenophosphate synthetase 1; SMUG1, single-strand-selective monofunctional uracil-DNA glycosylase 1; STK40, serine/threonine kinase 40; WT, wide type

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## KEYWORDS

colorectal cancer, miRNA-31, potential biomarker, radiosensitivity, STK40

## 1 | INTRODUCTION

By 2020, colorectal cancer (CRC) has become the second leading cause of cancer-related death worldwide.<sup>1</sup> Preoperative neoadjuvant chemoradiotherapy and chemoradiotherapy (chemoRT) followed by total mesorectal excision (TME) have become the standard treatment for advanced, localized rectal cancer.<sup>2</sup> However, only approximately 20% of patients achieve complete pathological regression, and the remaining patients achieve an incomplete or no response and even experience side effects of radiotherapy, such as radiation-induced secondary tumors and radioactive enteritis.<sup>3</sup> Clinically, the identification and treatment of radioresistant CRC remains an unsolved problem.

MicroRNAs (miRNAs) are a class of endogenous, 20–22 nucleotide, noncoding RNA molecules that degrade and translationally repress mRNAs by pairing with partially complementary sites in the 3'-untranslated region (3'-UTR) of the targeted genes. On one hand, miRNAs function as oncogenes by targeting tumor suppressor genes such as p53. On the other hand, they also function as antioncogenes by targeting oncogenes such as *Myc*.<sup>4,5</sup> In recent years, miRNAs were reported to participate in the initiation and progression of CRC,<sup>6,7</sup> and they may regulate the radiosensitivity of CRC cell lines to some extent.<sup>8–10</sup>

Researchers have shown that miRNA-31 may play roles in modulating the DNA damage repair response by targeting elements in the DNA damage repair system. However, the relationship between miRNA-31 and radiosensitivity in CRC is still unidentified. In this study, we observed significantly decreased miRNA-31 expression in radioresistant CRC cells compared to untreated cells, and it increased the radiosensitivity of CRC cells. Furthermore, as a novel target in CRC cells, STK40 was involved in the miRNA-31-mediated regulation of radiosensitivity because the effect of miRNA-31 that induced radiosensitivity was weakened upon STK40 knockdown. These results provide *in vitro* evidence that miRNA-31 might be a potential biomarker of the radiation response.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture

HCT116 and LoVo cells were bought from Chinese Academy of Medical Sciences Center, Institute of Basic Medical Sciences. HCT116 and LoVo cells were cultured in DMEM medium with 10% fetal serum. All the cells were incubated at 37°C in 5% CO<sub>2</sub> incubator.

### 2.2 | Radiation conditions

RAD SOURCE RS2000 X-ray biological irradiator was used to give different doses of X-rays (0, 2, 4, 6, and 8 Gy). The absorbed dose rate was

4.125 Gy/min. LoVo cells and HCT116 cells were exposed to radiation (2 Gy/day) for 7 days and the remaining surviving cells were considered as radioresistant cell lines.

### 2.3 | RNA extraction and quantitative real-time PCR and Methylation detection

Total RNA was extracted using Trizol (Invitrogen), and cDNA were synthesized using reverse transcription kit (Tiangen Biochemical Technology Co., Ltd.). Quantitative real-time PCR analyses were performed with SuperReal PreMix (Probe; Tiangen Biochemical Technology Co., Ltd.). The expression of miRNA was quantified by miRcute miRNA qRT-PCR Detection Kit (SYBR Green; Tiangen Biochemical Technology Co., Ltd.) and was normalized by U6 small nuclear RNA. The sequence of the miRNA-31 and U6 was shown in supplemental Table 1. miRNA-31 upstream methylation level was detected by Annoroad Gene Technology (Beijing) Company.

### 2.4 | Transfection

The sequence-specific miRNA-31 mimics, inhibitor, and its corresponding control were purchased from Ribobio (Guangzhou, P.R. China). All siRNA duplexes were purchased from GenePharma (Shanghai, P.R. China). The transfection reagent is lipofectamine2000 (Invitrogen). miRNA mimics or inhibitors, or siRNAs were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacture's instruction.

### 2.5 | Protein extraction and Western blotting

Using RIPA solution to extract cell protein 48 hours after transfection. The proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes. The following antibodies were used: mouse anti-STK40 (1:500 dilution; Abcam), mouse anti-p53 (1:1,000 dilution; Cell Signaling Technology), rabbit anti-p65 (1:500 dilution; Santa Cruz Biotechnology), rabbit anti-phosphorylated-p65 (1:500 dilution; Abcam), rabbit anti-GAPDH (1:1,000 dilution; Cell Signaling Technology). Blots were probed with IRDye<sup>®</sup>700 or IRDye<sup>®</sup>800 conjugated antibodies (1:5,000 dilution; Odyssey), and bands were visualized using Odyssey Infrared Imaging Scanner.

### 2.6 | Clone formation assay

HCT116 and LoVo cells were plated at specific cell densities, corresponding with the IR dose, cells in culture were irradiated with doses of 2, 4, 6, and 8 Gy in 60 mm dishes containing medium and 10% FBS (200 cells for 0 and 2 Gy, 400 cells for 4 Gy, 1,000 cells for 6 Gy, 2,000 cells for 8 Gy). After incubation at 37°C/5% CO<sub>2</sub> for 10–13 days, the cells were fixed with 10% paraformaldehyde and stained with 1%

crystal violet in 70% ethanol. Colonies containing 50 cells or more were counted. Plating efficiency was defined as the percentage of cells seeded that grew into colonies under a specific culture condition. Surviving Fractions (SFs), as a function of irradiation, was calculated as the number of colonies counted/ (the number of cells seeded  $\times$  plating efficiency/100). The SFs after indicated IR doses were used to determine radiosensitivity.

## 2.7 | Luciferase assay

Regions containing the miRNA-31-binding site of the human STK40 3'-UTR was generated by PCR amplification and subcloned into the *NheI/XbaI* sites of the pmirGLO Dual-Luciferase reporter plasmid (E1330; Promega). The mutations were generated with the predicted target site of STK40 3'-UTR using the Q5<sup>®</sup> Site-Directed Mutagenesis Kit (NEB, E0554), according to the manufacturer's instructions. The control luciferase plasmid, pmirGLO-STK40-3'UTR plasmid, or mutation plasmid was cotransfected into 293T cells with miRNA-31 mimics using Lipofectamine 2000 Reagent (Invitrogen, Foster, USA). Luciferase activities were assayed by the Dual-Luciferase Reporter Assay System (Promega) after 48 hours. Firefly luciferase (*luc2*) was used as the primary reporter to monitor miRNA regulation and Renilla luciferase (*hRLuc-neo*) acted as a control reporter for normalization and selection. Reduced firefly luciferase expression indicates the binding of introduced miRNAs to the cloned miRNA target sequence.

## 2.8 | Bioinformatics analysis

The difference analysis of miRNA-31 was from ENCOR database (<http://starbase.sysu.edu.cn/panmirxp.php>). miRNA-31 upstream transcription factors (TFs) were screened out from TransmiR (<http://www.cuilab.cn/transmir>). The downstream target genes were obtained from MirTarBase (<http://mirtarbase.cuhk.edu.cn/php/index.php>). The interaction network visualization was finished by Cytoscape (<https://cytoscape.org/>, version 3.4). The functional enrichment analysis of these genes was performed by the r1760 version of g: ProfileR ([https://biit.cs.ut.ee/gprofiler\\_archive2/r1760\\_e93\\_eg40/web/index.cgi](https://biit.cs.ut.ee/gprofiler_archive2/r1760_e93_eg40/web/index.cgi)). Three prediction algorithms PicTar (<https://pictar.mdc-berlin.de/>), TargetScan ([http://www.targetscan.org/mamm\\_31/](http://www.targetscan.org/mamm_31/)), and miRanda (<http://www.miranda.org/>), were used to predict possible target genes; and GEPIA (<http://gepia.cancer-pku.cn/>), OncoLnc (<http://www.oncolnc.org/>), and Ualcan (<http://ualcan.path.uab.edu/>) were used to predict the relationship between STK40 and clinical prognosis.

## 2.9 | Statistical analysis

All experiments were repeated at least three times. The two-tailed paired Student's *T*-test was used to analyze two groups. *p*-values less than 0.05 were considered statistically significant. All data were analyzed using SPSS.21.0 software.

## 3 | RESULTS

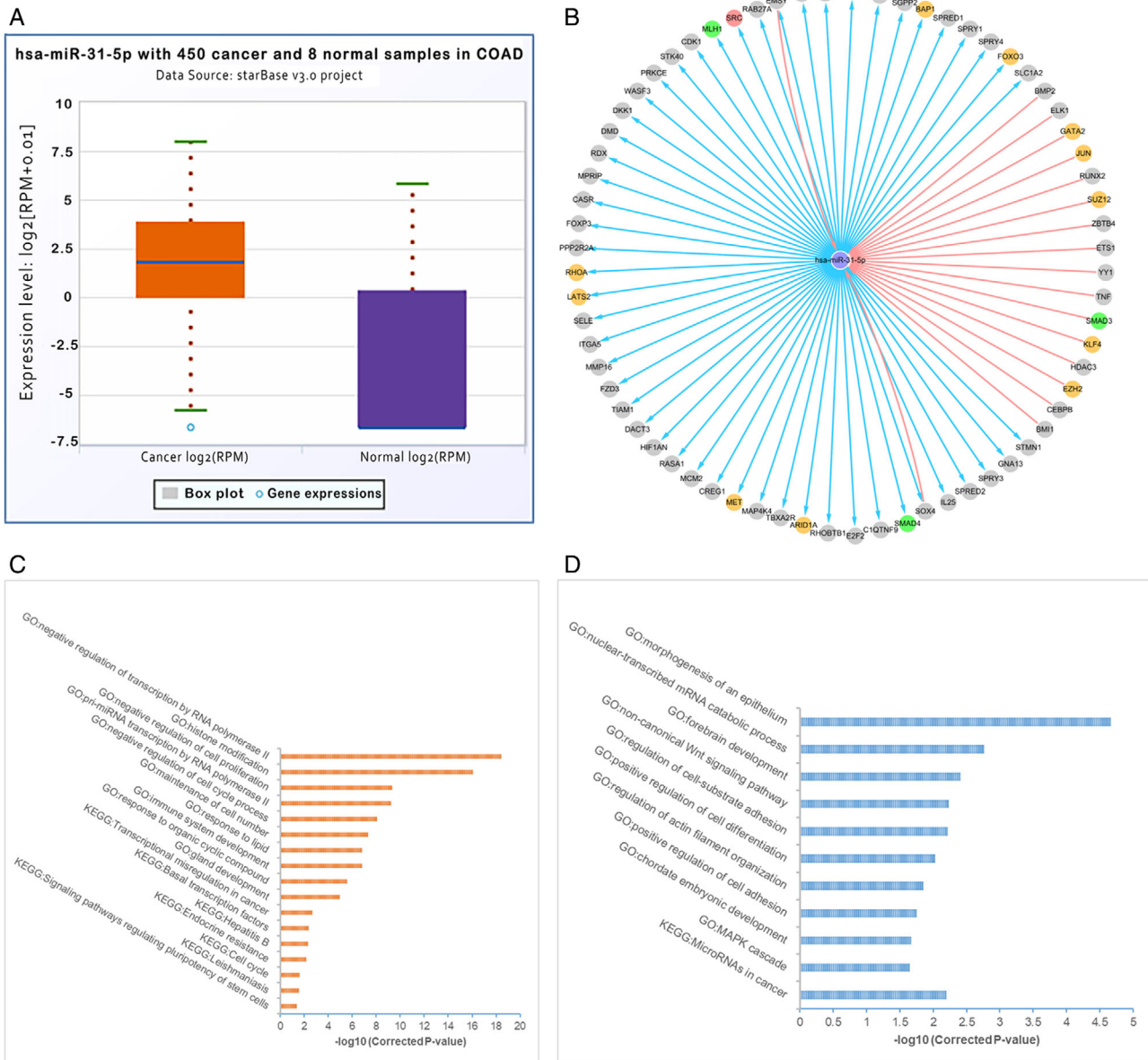
### 3.1 | miRNA-31 is upregulated in tumor tissues, and its contextual regulatory network is involved in multiple pathways, including negative regulation of the cell cycle

According to our previous laboratory results, compared with normal tissues, miRNA-31 was expressed at high levels in CRC. Statistics from The Cancer Genome Atlas (TCGA) shows that miRNA-31 expression in 450 colon cancer samples and 8 normal samples was significantly upregulated in colon cancer tissues (Figure 1A), consistent with previous results. Figure 1B shows the core regulatory network of miRNA-31. Then, we screened the upstream TFs and downstream target genes, and 58 TFs and 220 target genes were identified. We enriched these TFs and target genes, among which upstream enriched TFs were mainly involved in "negative regulation of transcription by RNA polymerase II," "histone modification," and "negative regulation of cell proliferation" (Figure 1C). Enriched downstream target genes were mainly involved in "morphogenesis of an epithelium," "nuclear-transcribed mRNA catabolic process," "forebrain development," and other processes (Figure 1D).

### 3.2 | The expression of miRNA-31 was decreased in radioresistant CRC cells and radiation downregulated miRNA-31 expression

HCT116 and LoVo cell lines were exposed to continuous low-dose radiation (2 Gy/day) for 7 days, and the surviving cells were continuously cultured as radioresistant cells to determine whether miRNA-31 was associated with radiosensitivity in CRC cells.<sup>10</sup> The mean expression level of miRNA-31 was significantly lower in radioresistant CRC cells than in untreated cells, as measured using qRT-PCR. Relative miRNA-31 expression decreased to 37.5% in LoVo cells ( $p < 0.05$ ) and 69.6% in HCT116 cells ( $p < 0.05$ ; Figure 2A and B). Decreased expression of miRNA-31 in radioresistant human CRC cell lines suggested a possible role for miRNA-31 in modulating the sensitivity of CRC cells to radiation.

CRC cell lines were exposed to 0, 2, 4, 6, or 8 Gy of radiation, and the expression of miRNA-31 was examined 48 hours later using qRT-PCR to further determine the relationship between miRNA-31 and radiation in CRC cell lines. The expression of miRNA-31 decreased to 63%, 52%, 43%, and 35% in LoVo cells exposed to 2, 4, 6, and 8 Gy of radiation, respectively, compared to the control group. (Figure 2C,  $p < 0.05$ ). Similarly, miRNA-31 expression was reduced to 63%, 62%, 43%, and 35% in HCT116 cells exposed to the same radiation doses (Figure 2D). The extent of the decrease was dose-dependent within 2–8 Gy. The results further indicated that radiation downregulated miRNA-31 in CRC cell lines.

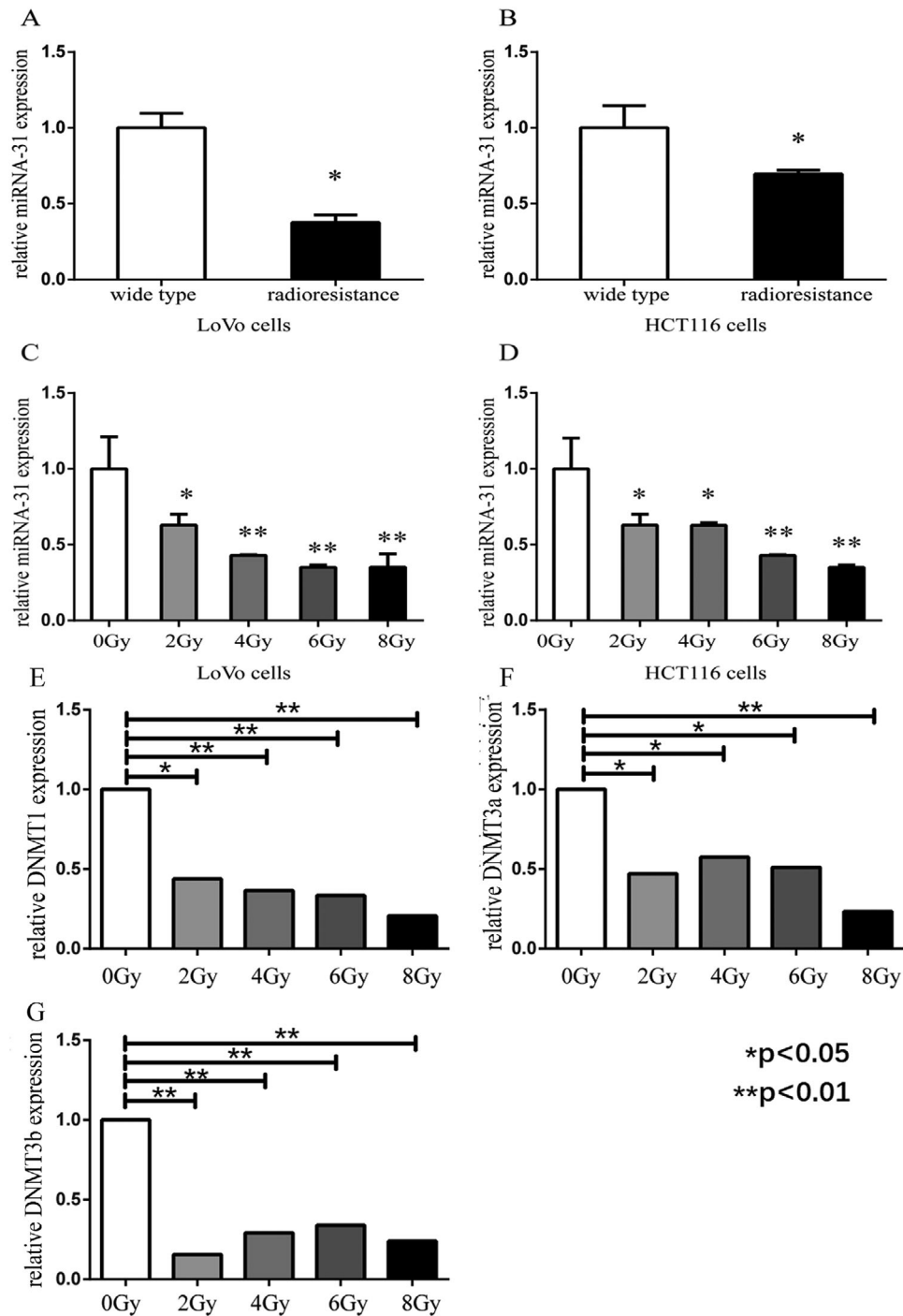


**FIGURE 1** miRNA-31 upregulated in tumor tissue, its context regulatory molecular involved in multiple pathways about cell cycle. (A) Based on the records in ENCORI database, hsa-mir-31-5p is significantly upregulated in colon cancer (COAD) tumor samples from the TCGA cohort (fold change = 2.00, corrected  $p$ -value =  $1.20e-4$ ). (B) The core regulatory interaction network of hsa-mir-31-5p. Only the functional miRNA-target interactions and literature-derived TF-miRNA regulations are shown, which are depicted as blue and red arrow, respectively. The known oncogene and tumor suppressor genes of colon cancer are colored red and green, respectively. Functionally causal genes of other cancer types are shown in yellow. C and D are pathway enrichment of TFs and target genes. (C) 58 upstream regulatory TFs of hsa-mir-31-5p were obtained. The top 10 GO function terms and all significant KEGG pathways are shown in bar plot. (D) 220 target genes of hsa-mir-31-5p were obtained. The top 10 GO function terms and all significant KEGG pathways are shown in bar plot

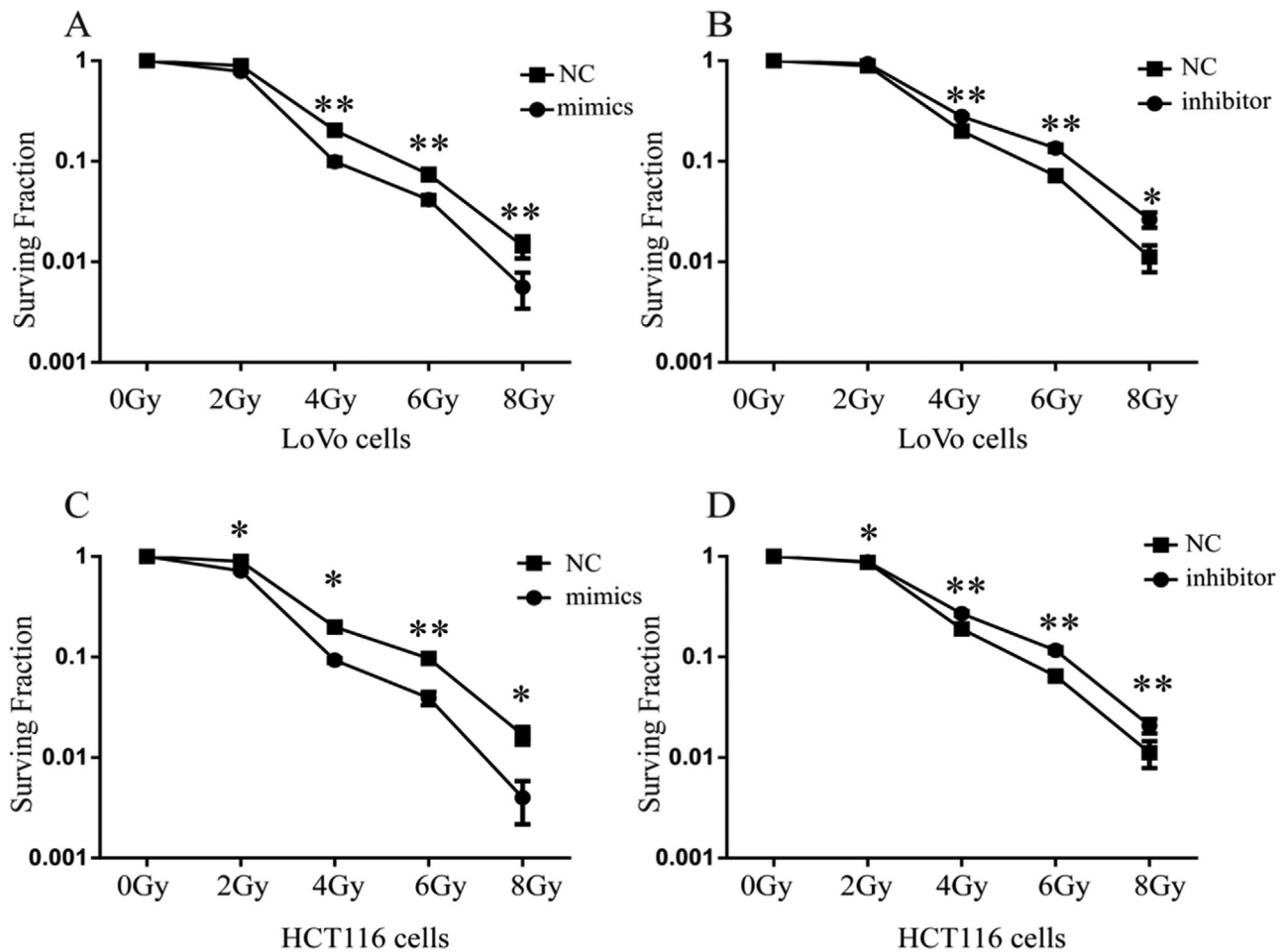
### 3.3 | DNA methylation was not detected in the promoter of miRNA-31 after radiation treatment

The downregulation of miRNAs may be caused by DNA methylation or other transcriptional mechanisms.<sup>11</sup> We first detected the expression of the main DNA methyltransferase in the HCT116 cell line after radiotherapy to explore the pathway. The expression levels of DNMT1, DNMT3a, and DNMT3b were significantly lower in cells exposed to radiation than in those treated without radiotherapy (Figures 2E-G).

We detected the methylation level of the miRNA-31 upstream promoter after radiotherapy to further explore the relationship between miRNA-31 and methylation. The primers were designed to cover the CpG island of the miRNA-31 upstream regulatory region as much as possible. Twenty-three primer pairs were designed. According to the results of the Sequenom EpiDesigner evaluation, the 20th and 22nd primer pairs were selected (Figure S1). In the control group (M13, M14, and M15), HCT116 cells were not irradiated, and total genomic DNA was extracted from the test group (M16, M17, and M18) 48 h



**FIGURE 2** After radiotherapy, miRNA-31 of two cell lines declined and overall methylation level of HCT116 down-regulated. After radiotherapy (2 Gy/d) for 7 days, the expression of miRNA-31 in colorectal cancer cells was detected by qRT-PCR and the remaining cells could be used as radioresistant cells. The fraction of miRNA-31 in LoVo cells (A) and HCT116 cells (B) was 37.5% and 69.6%, respectively. After irradiation, the expression of miRNA-31 was detected by qRT-PCR. (C) and (D) showed that the expression of miRNA-31 in LoVo cells decreased to 63%, 52%, 43%, and 35% after 2, 4, 6, and 8 Gy irradiation, respectively. Similarly, in HCT116 cells, miRNA decreased to 63%, 62%, 43%, and 35%, respectively. (E) and (F) reflected the methylation level of HCT116 cell line. (E), (F), and (G) were the expression levels of methyltransferase DNMT1, DNMT3a, and DNMT3b after irradiation for 2, 4, and 6 Gy for 24 h, respectively. The results showed that the three methyltransferases were decreased in varying degrees after irradiation (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ )



**FIGURE 3** miRNA-31 increases radiosensitivity of CRC cell lines. (A) Clonogenic assay results showed that surviving fractions at each dose (2, 4, 6, and 8 Gy) decreased in miRNA-31 overexpressed cells, indicating miRNA-31 mimics increased sensitivity to radiation in LoVo cells. (B) Surviving fractions increased in miRNA-31 inhibitor group, and under expression of miRNA-31 decreased the radio-sensitivity in LoVo cells. (C) The similar function of miRNA-31 in regulating radiosensitivity was observed in HCT116 cells, which indicated that miR-31 mimics increased radio-sensitivity of HCT116 cells. (D) In contrast, treatment with miRNA-31 inhibitor deprived enhanced radio-sensitivity in HCT116 cells (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ )

after 8 Gy radiation exposure. Compared with the control group, no significant methylation modification was detected in the upstream regulatory region of miRNA-31 in the test group. No significant change in methylation was detected using the 21st primer pair (supplementary Figure 2) or the 22nd primer pair. Based on these results, the decrease in miRNA-31 expression after irradiation may not be related to the methylation of CpG islands in the upstream regulatory region.

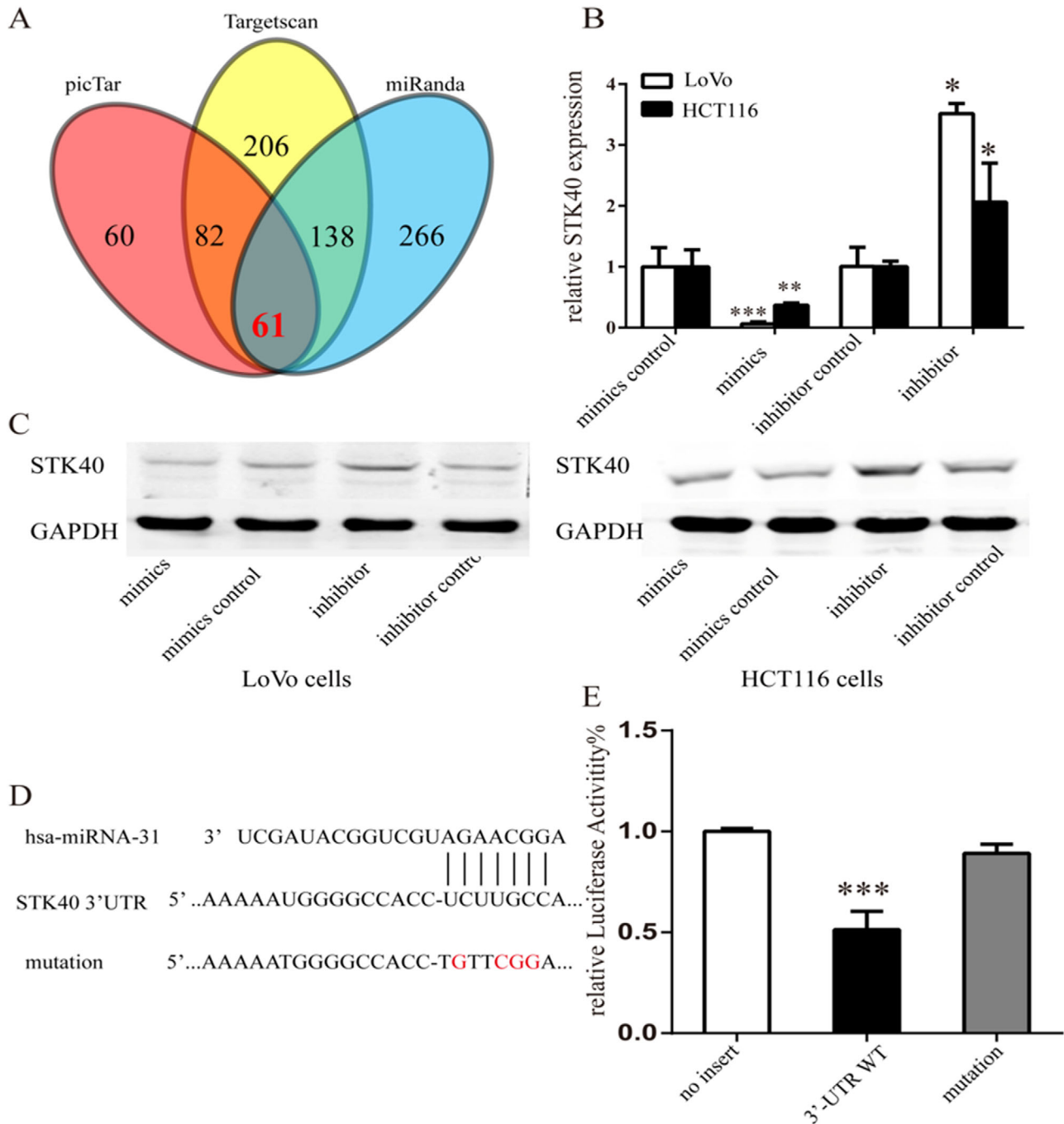
### 3.4 | miRNA-31 sensitized CRC cells to radiation treatment

HCT116 and LoVo cells ( $1 \times 10^6$ ) were seeded into six-well plates and subjected to transfection with an miRNA-31 mimic or mimic control on the next day to determine the effect of miRNA-31 on the sensitivity of CRC cells to radiation. Forty-eight hours after transfection, cells were seeded into different plates, and the plates were irradiated with doses of 0, 2, 4, 6, or 8 Gy. qRT-PCR was conducted to confirm the transfection efficiency by measuring the expression of miRNA-31

upon miRNA-31 mimic or inhibitor transfection. Clonogenic assays showed decreased percentages of surviving miRNA31-overexpressing cells exposed to each dose (2, 4, 6, and 8 Gy), suggesting that the miRNA-31 mimic increased the sensitivity of LoVo cells to radiation (Figure 3A). In contrast, the percentage of surviving cells increased in the miRNA-31 inhibitor group, indicating that the miRNA-31 inhibitor reduced the radiosensitivity of LoVo cell lines (Figure 3B). The function of miRNA-31 in regulating radiosensitivity was similar in HCT116 cells (Figure 3C and D). These results suggested that miRNA-31 conferred CRC cells with sensitivity to radiation treatment.

### 3.5 | miRNA-31 negatively regulated the expression of STK40 by targeting its 3'-UTR in CRC cells

Three prediction algorithms, PicTar, TargetScan, and miRanda, were utilized to predict the possible target genes and understand how miRNA-31 modulated the radiosensitivity of CRC cells to radiation.



**FIGURE 4** miRNA-31 negatively regulated the expression of STK40 via targeting its 3'-UTR in CRC cells. STK40 was a new target of miRNA-31 in CRC cell lines. (A) To explore the mechanism of miRNA-31 in regulating radiosensitivity, three authoritative biological databases were used to predict the target of miRNA-31. The diagram showed that 61 repetitive genes were predicted in picTar, Targetscan, and miRanda databases. (B) Overexpression of miRNA-31 could reduce the expression of STK40 in mRNA level using qRT-PCR in both cell lines. On the contrary, miRNA-31 inhibitor could up-regulate STK40 expression. (C) The expression of STK40 protein was similarly altered by miRNA-31 mimic and inhibitor by Western blot analysis. The results suggested that STK40 had a negative correlation with miRNA-31. (D) The natural sequence of STK40 3'-UTR (WT 3'-UTR) or the mutant sequence (mutation 3'-UTR) were designed and connected into a luciferase reporter vector. The red color indicated the mutation sites. (E) 293T cells were cotransfected with miRNA-31 mimics or mimics control or 3'-UTR WT. (Luciferase activity assay in mimic group cells was significantly decrease compared with control group. Then WT or mutation 3'-UTR vector and miRNA-31 mimics were transfected into 293T cells, and the activity of mutation 3'-UTR group was not changed

Sixty-one overlapping target genes of miRNA-31 were identified by these three predictive databases (Figure 4A). Among the 61 potential targets, a significant inverse correlation was found between the expression levels of miRNA-31 and five predicted target mRNAs in CRC tissues, including EBF3, FAM134B, KANK1, SEPHS1, and STK40 (sample number = 299,  $r < 0$ ,  $p < 0.05$ ; <http://starbase.sysu.edu.cn>, Supplementary Data S1). A negative correlation was observed between STK40 and miRNA-31 expression among these five possible target genes in CRC cell lines using qRT-PCR. (Figure 4B and Figure S4A–S4D). Western blot analysis further confirmed similar alterations in the expression of the STK40 protein upon miRNA-31 mimic and inhibitor transfection (Figure 4C). Moreover, three algorithms from TCGA database were used to detect the relationship between STK40 expression and CRC prognosis (Figure 6), and no significant difference was observed in the CRC prognosis between the algorithms. Subsequently, the same method was used to screen 33 cancers. Notably, five cancer types showed a significant correlation between STK40 expression and the prognosis, including urothelial carcinoma of the bladder, renal clear cell carcinoma, low-grade glioma, hepatocellular carcinoma, and cutaneous melanoma (Table S2 and Figure 6).

The TargetScan analysis revealed a binding site for miRNA-31 in the 3'-UTR of STK40 (Figure 4D). The 3'-UTR fragments of mRNA candidates containing the WT miRNA-31 binding site were subcloned and inserted into a dual-luciferase reporter plasmid to identify the region of SK40 targeted by miRNA-31. Luciferase activity decreased to 49% ( $p < 0.001$ ) after the transfection of a plasmid containing the STK40 3'-UTR with miRNA-31 mimics into 293T cells compared with cells transfected with the WT luciferase reporter plasmid (Figure 4E). Mutation of the target site abolished the effect of miRNA-31 on inhibiting reporter gene expression. Thus, miRNA-31 inhibited STK40 expression by directly targeting the 3'-UTR of the STK40 mRNA.

### 3.6 | STK40 was involved in the mechanism by which miRNA-31 regulated radiosensitivity

STK40 expression was examined in CRC cells exposed to 2, 4, 6, and 8 Gy to determine whether STK40 was involved in the cellular response to radiation. The expression of the STK40 mRNA in LoVo cells increased 1.72- and 1.64-fold compared with that in the control group after 6 and 8 Gy irradiation, respectively (Figure 5A,  $p < 0.05$ ). Similar results were obtained in HCT116 cells (Figure 5B). Western blot analysis showed increased STK40 expression after 8 Gy irradiation compared with that in the groups without radiation treatment (Figure 5C and D).

siRNA of STK40 was designed to knock down STK40 in CRC cells and to determine the role of STK40 in modulating radiosensitivity. The expression of the STK40 mRNA decreased to 37% in LoVo cells and 49% in HCT116 cells transfected with the STK40 siRNA (Figure S4E–S4F). As miRNA-31 increased the radiosensitivity of CRC cells, the miRNA-31 mimic or mimic control was transfected into CRC cells together with the STK40 siRNA, and the plates were irradiated with doses of 0, 2, 4, 6, and 8 Gy 48 h later. Silencing of STK40 expression with an siRNA weakened the effect of miRNA-31 on modulating

radiosensitivity in CRC cells (Figures 5E and F). Based on these results, STK40 might be involved in the miRNA-31-mediated regulation of radiosensitivity in CRC cells.

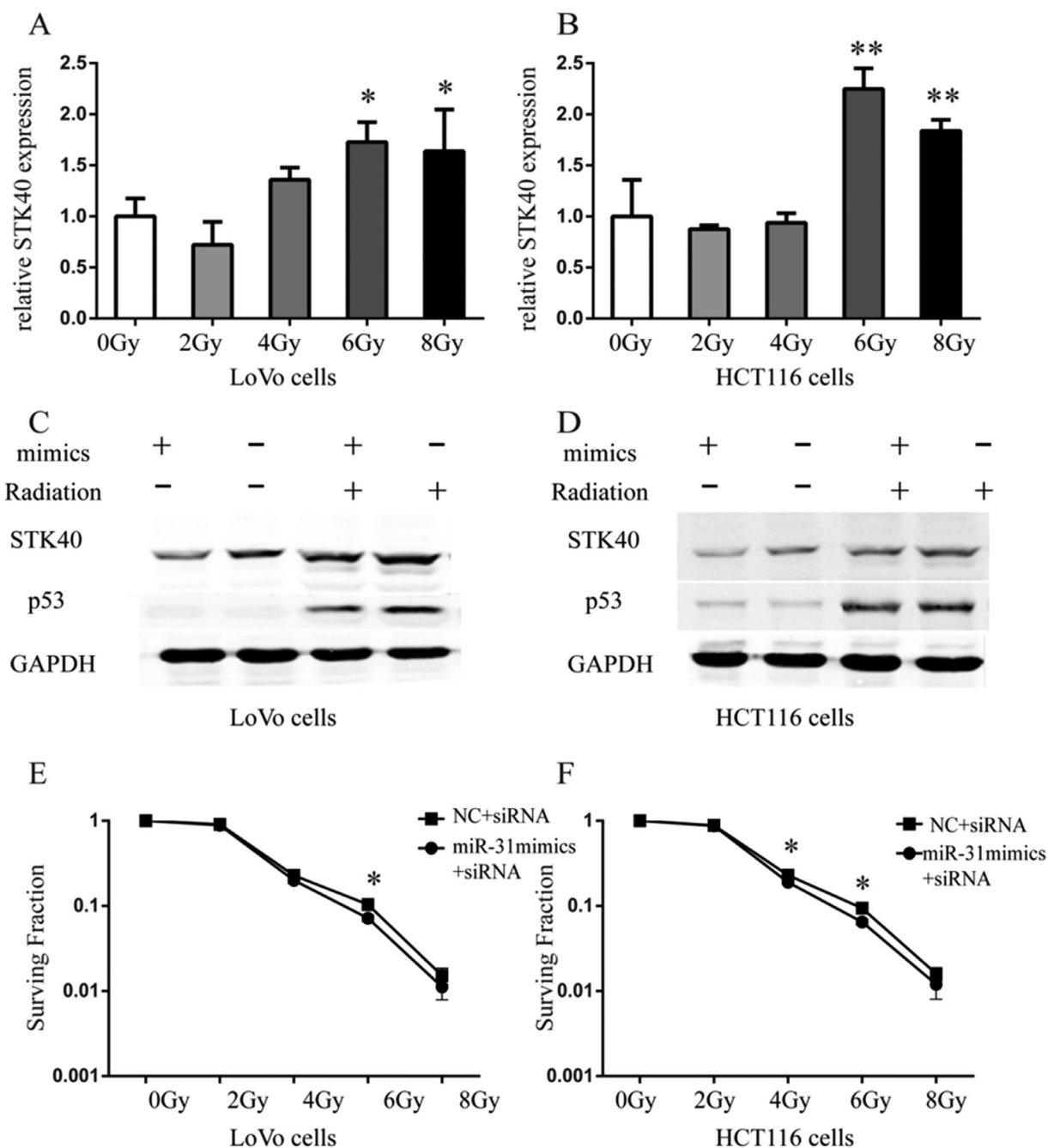
## 4 | DISCUSSION

Radiotherapy is one of the main therapeutic interventions for CRC, and the sensitivity of tumor cells to this treatment may directly determine the prognosis of patients with CRC. Improving the effectiveness of this therapy and reducing the radiation dose to avoid damaging normal tissue have been challenges for radiation oncologists.<sup>10</sup> In colon cancer, suppression of miRNA-31 has been reported to increase sensitivity to 5-FU at an early stage.<sup>12</sup> However, no study has reported the effect of miRNA-31 on the radiotherapy sensitivity of CRC. The role of miRNA biomarkers in predicting the response to anticancer therapy must be elucidated, which is crucial for increasing the efficacy of treatment and improving survival rates. We proposed a model in which miRNA-31 regulates the radiosensitivity of CRC by targeting STK40 (Figure 7) to illustrate the mechanism underlying its effect on radiosensitivity.

By performing a bioinformatics analysis, we observed significantly increased expression of miRNA-31 in colon cancer tissue, consistent with previous results from our laboratory. Based on these results, the function of miRNA-31 in CRC may be more similar to an "OncomiR," which may play a role in radiation resistance during radiotherapy. However, in contrast to our speculation, cells overexpressing miRNA-31 were more sensitive to radiation. One of the main functions of miRNA-31 in mammalian cells is to assist in cell division and participate in the regulation of DNA repair, replication, and chromosome separation. In hepatocellular carcinoma, miRNA-31 inhibits homologous DNA repair by targeting PEX 5 to inhibit the Wnt/ $\beta$  catenin pathway,<sup>13</sup> and miRNA-31 inhibits DNA repair, thus resulting in increased radiosensitivity. According to the GO and KEGG pathways identified for upstream TFs and downstream target genes, miRNA-31-related TFs are involved in the regulation of the cell cycle and cell division and are sensitive to any DNA damage stress. Therefore, on the one hand, miRNA-31, as an OncomiR, is expressed at high levels in colorectal tissue. On the other hand, miRNA-31 also inhibits the repair of radiotherapy-induced DNA damage in tumor cells, leading to an increase in tumor cell death and increased sensitivity to radiotherapy.

Altered miRNA-31 expression has previously been linked to defects in posttranscriptional processing<sup>14</sup> and chromosomal deletions.<sup>15</sup> miRNA-31 is encoded in the chromosome 9p21.3 region, a genomic region frequently lost in human cancers, and the 9p21.3 deletion also potentially contributes to radiation and chemotherapy resistance.<sup>15</sup> Thus, given the role of radiation in inducing genome instability, the downregulation of miRNA-31 may be due to deletions or translocations in this fragile genomic region. We tried to explain the decrease in miRNA-31 expression at the molecular level, but the results showed that although the expression of the main methyltransferases of the HCT116 cell line decreased after irradiation, the methylation level of the miRNA-31 promoter region did not change. Thus, the decrease in miRNA-31 may be caused by mechanisms other than DNA methylation.

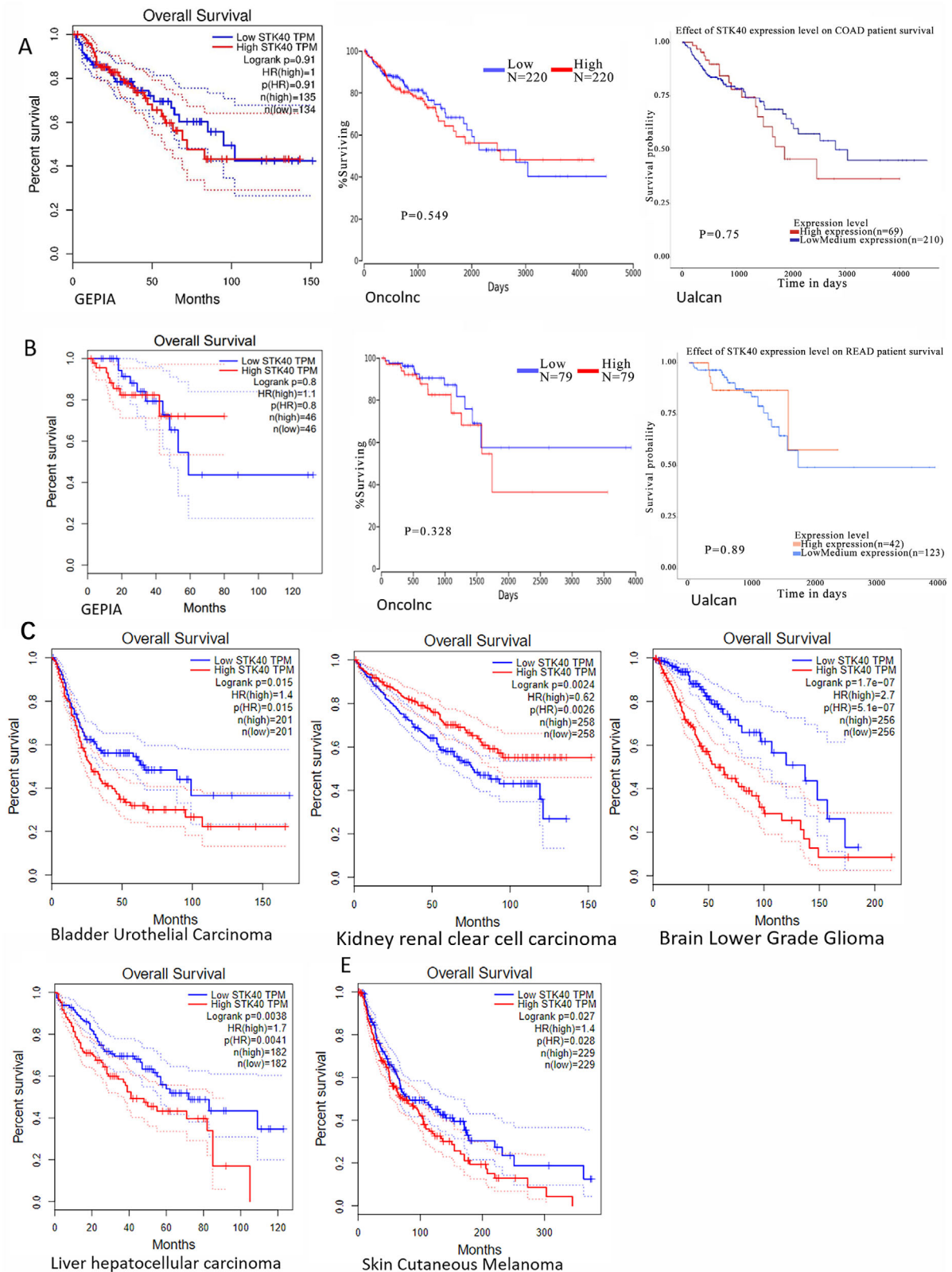




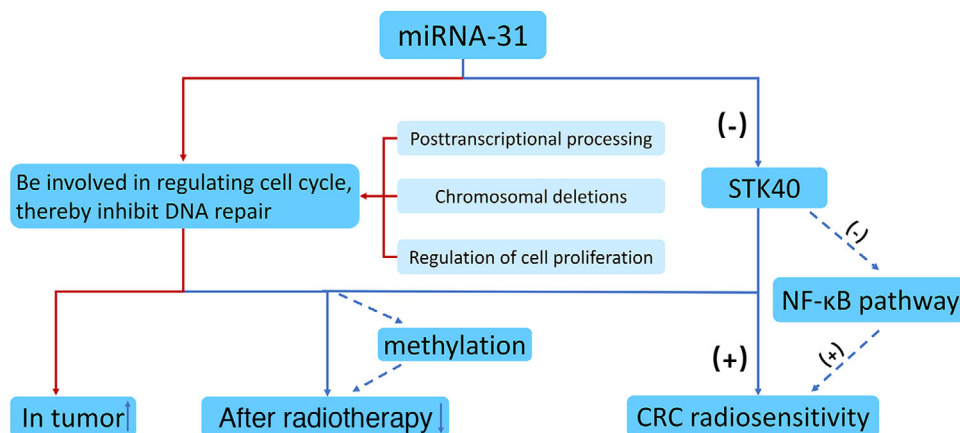
**FIGURE 5** STK40 increased after irradiation and STK40 was involved in miRNA-31 regulating radiosensitivity of CRC cells. (A, B) The expression of STK40 mRNA was increased compared to nonradiation group in response to 6 and 8 Gy irradiation in LoVo cells and HCT116 cells. (C, D) Western blot analysis showed that the protein expression of STK40 increased after 8 Gy radiation compared with groups without radiation, however, the negative regulation of miRNA-31 on STK40 was not obvious in both cells. (E, F) Compared with the results of Figure 3, the obvious function in inducing radio-sensitivity of miR-31 was greatly impaired after down-regulation of STK40

Downregulation of miRNA-31 may therefore represent a mechanism for radioresistance via the increase in the repair of IR-induced DNA damage, which has previously been shown to play an important role in radioresistance.<sup>16,17</sup> In esophageal adenocarcinoma, miRNA-31 increased radiosensitivity by downregulating the expression of 13 DNA repair genes, including PARP1, SMUG1, MLH1, RAD51L3, and MMS19.<sup>16</sup> The majority of these altered genes were involved in base excision repair (BER), mismatch repair (MMR) and nucleotide excision repair (NER) pathways. Moreover, these genes were predicted

to be targets of miRNA-31 by miRNA target prediction programs.<sup>18</sup> Ku80, one of the key enzymes promoting the initiation of NHEJ in DNA damage repair, was also found to be the target of miRNA-31 in squamous cell carcinoma.<sup>19</sup> The overexpression of miRNA-31 significantly increased radiosensitivity by downregulating Ku80 expression in human oral SCC (OSCC) cells. The functions of miRNA-31 reported above provided potential evidence to support the phenomenon we observed in our study that miRNA-31 might increase radiosensitivity by downregulating DNA repair-related genes in CRC cells.



**FIGURE 6** Survival curves of different cancers in databases. (A, B) Cases data from three TCGA algorithms (GEPIA, OncoPrint, Ualcan) showed that STK40 was not related to the prognosis of colorectal cancer. A was colon cancer, B was rectal cancer. (C) Survival curves of top five tumors in Table S2 ( $*p < 0.05$ )



**FIGURE 7** Working model describes the relationship between miRNA-31 and radiosensitivity.

Proposed working model describes the relationship between miRNA-31 and radiosensitivity. The left half mainly describes the results of bioinformatic analysis, which shows that miRNA-31 increases its expression in tumors by regulating cell cycle and DNA repair. The experiment found that the miRNA-31 decreased after radiotherapy, and the possible mechanism was DNA methylation. The right half side describes the miRNA-31/STK40 axis, which shows that miRNA-31 increases CRC radiosensitivity by inhibiting STK40, and the possible pathway was NF- $\kappa$ B pathway. In addition, same as function of cancer promotion, miRNA-31 can increase radiosensitivity of CRC by inhibiting DNA repairing consistent with RT damage. In which red lines represent bioinformatic analysis results, blue lines represent experimental verification results, and dashed lines represent possible mechanisms

As a downstream target of miRNA-31 in CRC, serine/threonine kinase 40 (STK40), also known as SHIK/SgK495, was first reported to induce mouse embryonic stem cells to differentiate into embryonic inner cells.<sup>20,21</sup> Since STK40 was found to inhibit TNF- $\alpha$ -induced NF- $\kappa$ B activation,<sup>22</sup> the analysis focused on its function in regulating the immune pathway by repressing the NF- $\kappa$ B signaling pathway. An miRNA-31 inhibitor suppressed esophageal neoplasia by derepressing STK40 and inhibiting the associated STK40-NF- $\kappa$ B-controlled inflammatory pathway.<sup>23</sup> In addition to esophageal squamous cell carcinoma (ESCC), STK40 was also identified as a direct target of miRNA-31 in ovarian cancer cell lines<sup>24</sup> and individuals with psoriasis.<sup>25</sup> Although the effect of STK40 on regulating radiosensitivity has not yet been reported, a recent study has shown that NF- $\kappa$ B signaling pathways induce DNA double-strand breaks in T cell leukemia to cause cell-cycle arrest or lead to cell senescence and cell death.<sup>26</sup> This result is consistent with our findings that miRNA-31 inhibits NF- $\kappa$ B signaling pathways through the targeted inhibition of STK40, thereby improving radiotherapy sensitivity, and NF- $\kappa$ B signaling pathways in this miR-31-STK4-NF- $\kappa$ B - radiosensitivity regulatory axis may exert a synergistic effect with miRNA-31. It is possible to improve radiotherapy sensitivity by promoting DNA damage after radiotherapy. Moreover, as miRNAs regulate multiple target genes, STK40 might not be the only target gene, and other target genes might be involved in the mechanism by which miRNA-31 regulates the radiosensitivity of CRC cells. In addition, a network of miRNAs has been reported to regulate a single mRNA target and exert combinatorial effects on determining the tumor response to radiation.<sup>27</sup> Therefore, these results may explain why STK40 expression did not increase when miRNA-31 expression decreased upon exposure to 2 and 4 Gy of radiation, which warrants further investigation. In addition, excluding the effects of radiotherapy, STK40 expression was not significantly correlated with the prognosis

of patients with CRC ( $p > 0.05$ ). Interestingly, using the same algorithm, pronounced prognostic effects of STK40 expression were identified on five tumors: bladder cancer, renal clear cell carcinoma, low-grade glioma, liver cancer and melanoma, among which only low-grade glioma was sensitive to radiotherapy, which suggests that STK40 may not be an oncogene and only participate part reason influencing the radiosensitivity in cancer cells. But the limited number of samples may affect the accuracy of screening results. Moreover, the tissue specificity of miRNAs may also be contribute to the differences.

Taken together, we identified a causal relationship between miRNA-31 expression and the radiosensitivity of CRC cells, and STK40, a novel target in CRC, is potentially involved in the radiation response. However, the mechanisms underlying the dysregulation of miRNA-31 in this model remain to be further elucidated. In addition, our study provides cellular evidence that miRNA-31 might represent a potential biomarker to identify responders to radiotherapy and an efficient therapeutic target in patients with CRC.

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#### CONFLICTS OF INTEREST

None of the authors have any conflicts of interest.

#### DATA AVAILABILITY STATEMENT

The authors declare that data supporting the findings of this study are available within the article files and Supplementary Information or available from the corresponding authors upon request.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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