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MicroRNA-1246 regulates the radio-sensitizing effect of curcumin in bladder cancer cells via activating P53

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Abstract

Objectives Radiotherapy is the primary option for bladder cancer patients, but it does not have obvious curative effects. This study was to investigate how to increase radiosensitivity in bladder cancer.

Materials and Methods The curcumin and irradiation treated T24 cells were used for analysis of microRNA expression (miRNA microarray), cell viability (Cell Proliferation Assay Kit), colony formation, apoptosis (Annexin V-FITC/7-AAD flow cytometry), miR-1246 and p53 mRNA (real-time PCR) and protein (Western blot) expression.

Results Microarray assay identified 17 differentially expressed miRNAs (twofold change) in curcumin treated cells compared to control cells. Among them, miR-1246 was the miRNA with the largest change in expression after curcumin treatment. Curcumin significantly decreased T24 cell viability and colony formation in a concentration-dependent manner compared to control cells. miR-1246 expression was significantly higher in T24 cells than in SV-HUC-1 cells and the higher concentrations (10 or 20 μM) of curcumin significantly down-regulated miR-1246 expression in T24 and HT-1376 cells. The combination of 10 μM curcumin and irradiation was more effective in decreasing miR-1246 expression, cell viability and colony formation than curcumin or irradiation alone. Inhibition of miR-1246 significantly decreased cell viability and colony formation in T24 and HT-1376 cells. Transfection with antagomiR-1246 significantly increased the G0/G1-phase of T24 cells and induced apoptosis compared to cells transfected with antagomiR-NC. Luciferase reporter assay showed that the overexpression of miR-1246 suppressed the luciferase activity of the P53 3′-UTR reporter genes.

Conclusion miR-1246 is involved in the anti-cancer effects of curcumin and irradiation through targeting the inhibition of p53 gene translation in bladder cancer cells.

Keywords Bladder cancer · Curcumin · Radiation · MicroRNA · p53

Introduction

Bladder cancer is the most common urinary tract cancer in China (80.5 per 100,000) [1]. More than 90% of bladder cancer is urothelial carcinoma, which is generally classified as non-muscle invasive bladder cancer (NMIBC) or muscle invasive bladder cancer (MIBC). Surgery is a main treatment, but radiotherapy is a primary treatment option for patients with MIBC [2]. However, many patients have no indication for the radical cystectomy; this is due to a median diagnosis age of 73 years or refusal to surgery due to the complexity of the surgery, which is associated with 58% of the early complications [3]. Although radiotherapy becomes an alternative treatment for MIBC, it exhibits a low overall complete response rate of 55% [4]. Thus, there is an urgent need for intense research on the augment radiation effect.

Curcumin is a natural compound from Curcuma longa plants, which are known for their anticancer, anti-inflammatory, pro-apoptosis, and antioxidant effects. Many reports have shown curcumin as a radiosensitizer on various malignancies such as lymphoma, sarcoma, pancreas, liver, colorectal, breast, lung, head/neck, and glioma and

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prostate cancers [5–8]. Although curcumin has direct-anticancer effects on some types of bladder cancer [9–11], whether it acts as radiosenstizer in urothelial carcinoma remains elusive.

MicroRNAs are small, tissue-specific, noncoding RNAs that bind to regulatory sites of target mRNA; this consequently results in decreased protein synthesis through translational repression. Many microRNAs play roles in radiation response processes through the regulations of gene expression [12]. MiRNAs are emerging as promising biomarkers for radiotherapy [13]. Cellular sensitivity to radiation is commonly determined by DNA DSB repair. Recent evidence has suggested that epigenetic alterations are also involved in the anti-tumor properties of curcumin. Among these curcumin-induced epigenetic alterations, miR-21, miR-17-5p, miR-20a, and miR-27a are considered tumor suppressors [14]. Curcumin has been demonstrated to inhibit cell proliferation and induce apoptosis in T24 human bladder cancer cell line which is associated with the upregulation of p53 expression [15]. Co-treatment with curcumin and cisplatin can markedly increase p53 expression in 253J-Bv bladder cancer cell line with wild-type p53 [16].

In this study, we investigated the anti-tumor activity and radio-sensitivity of curcumin in bladder cancer cells, as well as the expression patterns and target genes of miR-1246 involved in the effects of curcumin.

Materials and methods

Cell culture

T24, a cell line derived from human urinary bladder carcinoma, HT-1376, a cell line derived from human grade 3 urinary bladder carcinoma, and SV-HUC-1, a nonmalignant human uroepithelial cell line, were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SV-HUC-1 cells were cultured in F12K medium, while T24 cells were cultured in McCoy's 5A medium with 10% FBS. Cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

Preparation of curcumin

Curcumin was purchased from Sigma-Aldrich (St. Louis, MI, USA) and dissolved in 100% DMSO at 20 mM. The curcumin solution was then diluted in cell culture medium just before use (20 μg/ml in final), and the same concentration of DMSO in medium was used for control.

MiRNA microarray assay

T24 cells were treated with 20 μg/ml of curcumin for 24 h. Small RNAs were isolated from the total RNA of cells and then labeled with Cy3. MiRNA microarray assay was performed using an Agilent-046065 Human miRNA Microarray V19.0 (Agilent). The scanned images were analyzed using Feature Extraction software 10.7.1.1 (Agilent). Raw data were normalized in a quantile algorithm with Genespring 12.0 (Agilent).

Irradiation

Cells in tissue culture flasks were irradiated with 5.0 Gy at a dose of approximately 500 cGy/min using a Mitsubishi linear accelerator. The irradiation dose was selected according to previous publication [17]. The cells were then trypsinized and plated for quantitative RT-PCR, TUNEL assay, and flow-cytometry analysis.

Cell viability and colony formation assay

The T24 cells were plated in 12-well plates at a density of 5×10^5 cells/well and were grown for 24 h. Cell viability was determined at 72 h using the CellTiter 96 AQueousOne Solution Cell Proliferation Assay Kit (Promega), according to the manufacturer's protocol. To perform colony formation assay, cells with different treatments were cultured for 1 week, and colonies were counted with crystal violet.

MiRNA transfection

Cells were cultured in growth medium without antibiotics approximately 24 h before transfections. Transient transfection of agomiR-1246 (a mimic sequence of miR-1246, 1 μg/ml)/agomiR-NC (a sequence does not target any genes, 1 μg/ml) or antagomiR-1246 (an antisense sequence of miR-1246, 1 μg/ml)/antagomiR-NC (an antisense sequence of agomiR-NC, 1 μg/ml) was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol; cells were then continually cultured for 72 h.

Flow cytometry

T24 cells with antagomiR-1246 (1 μg/ml) or antagomiR-NC (1 μg/ml) transfection were harvested, washed with cold PBS, and resuspended in the nuclear stain 40, 6-diamidino-2-phenylindole. Cells were then stained with 7-AAD and Annexin-V-FITC that was provided with

the Annexin V-FITC/7-AAD kit (Beckman Coulter) and immediately analyzed by fluorescence activated cell sorting (FACS; BD Immunocytometry Systems).

mRNA 3′-UTR cloning and luciferase reporter assay

The segments of the human P53 3′-UTR, including the predicted miR-1246-binding site, were PCR-amplified. The PCR products were purified and inserted into the XbaI-FseI site-immediately downstream of the stop codon in the pGL3 control luciferase reporter vector (Promega Corp.); this produced a pGL3-P53 plasmid. T24 cells were transfected with pGL3-P53 plasmid, the pRL-TK renilla luciferase plasmid (Promega Corp.), and agomiR-1246 (1 μg/ml) or agomiR-NC miRNA (1 μg/ml) for 48 h using Lipofectamine 2000 (Invitrogen). The dual luciferase reporter assay system (Promega Corp.) was used to quantify luminescent signal using a luminometer (Glomax; Promega Corp.). Each value from the firefly luciferase assay was normalized to the renilla luciferase value from the co-transfected phRL-null vector (Promega Corp.).

QRT-PCR analysis

QRT-PCR analysis was conducted as described previously [18]. Total RNA was isolated from T24, HT-1376, and SV-HUC-1 cells with different treatments using the TRIzol reagent (Invitrogen); reverse transcription was performed using 1 μg total RNA and SuperScript II (Invitrogen). A 1-μl volume of cDNA was used in each amplification reaction containing SYBR Green PCR Master Mix (PE Applied Biosystems). MiR-1246 was amplified using forward primer: 5′-TGCGGAATGGATTTTTGG3′ and reverse primer: 5′-CCAGTGCAGGGTCCGAGGT-3′. P53 was amplified using forward primer: 5′-AACTGCGGGACGAGACAG A-3′ and reverse primer: 5′-AGCTTCAAGAGCGACAAG TTTT-3′.

Western blot

Western blot was conducted as described previously [19]. Total protein isolated from treated T24 cells were separated on SDS-PAGE gel and transferred to PVDF membranes (Millipore). The membranes were incubated with primary antibody to p53 (ab28; Abcam), or β-actin (ab3280; Abcam), followed by HRP-conjugated secondary antibodies. Blots were developed using an ECL Kit (Santa Cruz) and exposed to X-ray films.

Statistics

Data were presented as mean ± SD (standard deviation). The unpaired, two-tailed Student's *t* tests were used for comparisons between two groups. One-way ANOVA was used for comparisons between multiple groups. All experiments were repeated for at least three times, and representative experiments are shown. A P value < 0.05 was deemed significant.

Results

Curcumin and irradiation downregulate MiR-1246 expression in T24 and HT-1376 cells

To examine the effects of curcumin on miRNAs expression in bladder cancer cells, a miRNA microarray analysis was performed in T24 cells. 17 differentially expressed miRNAs (3 up-regulated and 14 down-regulated) were identified, which displayed at least a twofold change in 10 μM curcumin treated cells compared to control cells (Fig. 1a). Among them, miR-1246 showed the largest change in expression in the microarray assay. The expression of these 17 microRNAs was further validated with qPCR analysis. Four microRNA (miR) exhibited significant differences between control and curcumin group (Table 1). We found that miR-1246 expression was significantly higher in T24 cells than in SV-HUC-1 cells. The dose-dependence assay showed that low concentrations of curcumin (1 or 5 μM) had minimal effects on miR-1246 expression, whereas higher concentrations (10 or 20 μM) significantly down-regulated miR-1246 expression in T24 cells (Fig. 1b). We also compared miRNA expression between control cells and T24 cells received irradiation (Table 2) and curcumin plus irradiation (Table 3) treatments. Irradiation significantly downregulated miR-1246 expression in T24 cells compared to unirradiated control cells. In addition, the combination of 10 μM curcumin and irradiation was more effective in decreasing miR-1246 expression than curcumin or irradiation alone (Fig. 1c). To exclude the possibility that curcumin's regulation on miR-1246 was specific to T24 cells, we measured miR-1246 expression in HT-1376 cells under the treatment of 10 μM of curcumin. qPCR showed that 5–20 μM curcumin significantly decreased miR-1246 expression (Fig. 1d).

The effects of Curcumin and radiation on T24 cells

To examine the effects of curcumin and radiation on bladder cancer cells (T24 cells) with curcumin, irradiation, or the combination of curcumin and irradiation. Significant morphological changes and decreased cell density were observed in cells treated with curcumin, irradiation, or their combination compared to the DMSO control group (Fig. 2a). Curcumin significantly decreased T24 cell viability and colony formation in a concentration-dependent manner compared to control cells (Fig. 2b, c).

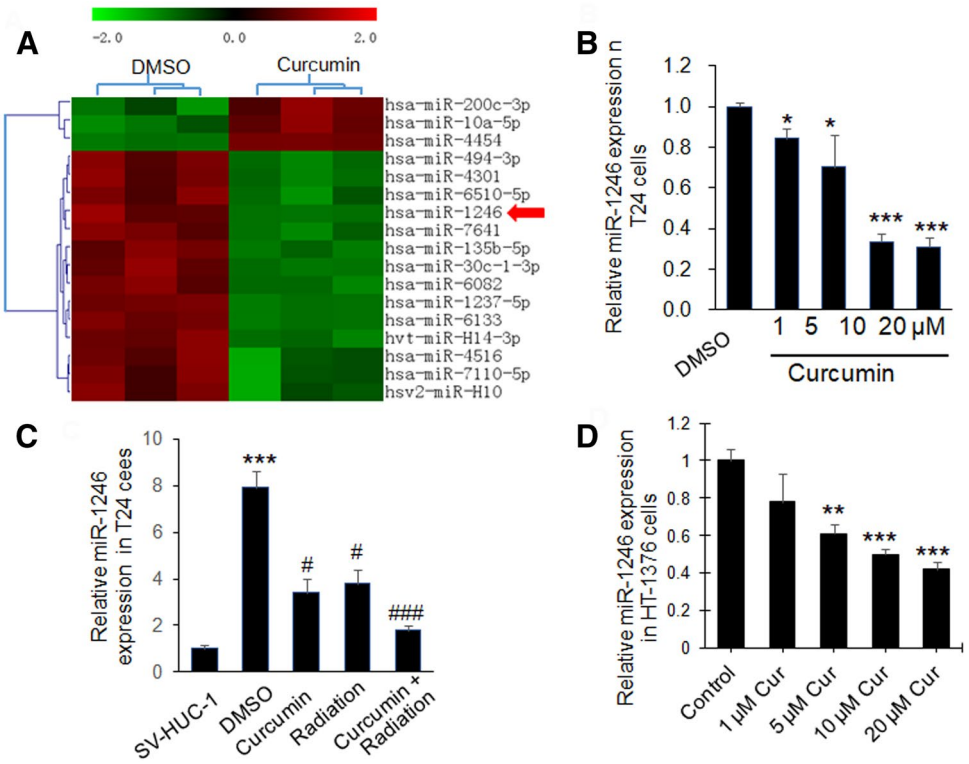

Fig. 1 Curcumin and irradiation downregulated MiR-1246 expression in T24 Cells. **a** The effects of curcumin on miRNAs expression in T24 cells analyzed by miRNA microarray. **b** The relative expression of miRNA-1246 in different doses of curcumin in T24 cells. T24 cells treated with DMSO was serviced as a control. $^{*}P<0.05$, $^{***}P<0.001$ vs. DMSO control. $N=6$. **c** The relative expression of miRNA-1246 in SV-HUC-1 and T24 cells treated with DMSO, curcumin, irradiation or the combination of curcumin and irradiation. miR-1246 levels in SV-HUC-1 cells was regarded as a control and miR-1246 expression in T24 cell was compared with that in SV-HUC-1 cells. $^{***}P<0.001$ vs, SV-HUC-1 cell. $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs. DMSO control, $N=6$. **d** The relative miR-1246 expression in HT-1376 cells under the treatment of curcumin. Control cells were treated with DMSO. *Cur* curcumin. $^{**}P<0.01$, $^{***}P<0.001$ vs. control. $N=6$

Table 1 Microarray assay of microRNA expression between control and curcumin group

Gene	Control	Curcumin	*p* value
hsa-miR-7641	181 ± 37	1682 ± 590	2.45E−03
hsa-miR-1246	66 ± 3	969 ± 252	3.05E−03
hsa-miR-7110-5p	41 ± 19	446 ± 185	9.33E−03
sa-let-7c-5p	853 ± 128	1697 ± 37	1.61E−02

Table 2 Microarray assay of microRNA expression between control and radiation group

Gene	Control	Radiation	*p* value
hsa-miR-7641	181 ± 37	1606 ± 375	1.31E−03
hsa-miR-6510-5p	21 ± 8	405 ± 154	3.29E−03
hsa-miR-200c-3p	1633 ± 140	962 ± 95	5.83E−03
hsa-miR-1246	66 ± 3	2039 ± 1211	8.93E−03
hsa-miR-7110-5p	41 ± 19	493 ± 178	9.37E−03
hsa-let-7c-5p	652 ± 101	1700 ± 408	1.08E−02
hsa-miR-3960	114 ± 11	436 ± 104	1.22E−02
hsa-miR-222-3p	3383 ± 299	2565 ± 119	1.75E−02
hsa-miR-100-5p	1526 ± 118	1135 ± 22	2.32E−02
hsa-miR-5096	260 ± 107	8862 ± 7829	2.56E−02
hsa-miR-4324-5p	417 ± 71	677 ± 82	2.62E−02
hsa-miR-6089	190 ± 11	479 ± 132	3.10E−02
hsa-miR-99a-5p	108 ± 17	408 ± 164	3.26E−02
hsa-miR-221-3p	2066 ± 63	1359 ± 175	3.32E−02
hsa-miR-6087	98 ± 6	572 ± 336	3.92E−02
hsa-miR-103a-3p	663 ± 116	381 ± 86	3.96E−02
has-miR-107	653 ± 112	374 ± 93	4.39E−02

In addition, the combination of curcumin and irradiation was more effective in deceasing cell viability and colony formation than curcumin or irradiation alone (Fig. 2b, c, $P<0.05$). To confirm that the anti-cancer effects of curcumin were directly mediated by miR-1246, T24 cells were treated with curcumin (10 μM) or radiation and agomiR-1246 or antagomiR-1246 transfection. Transfection of agomiR-1246 significantly normalized cell viability whereas antagomiR-1246 transfection further decreased cell viability in curcumin treated cells (Fig. 2d). Similarly, agomiR-1246 transfection significantly normalized

Table 3 Microarray assay of microRNA expression between control and radiation + curcumin group

Gene	Control	Radiation	*p* value
hsa-miR-7641	181 ± 37	1705 ± 841	1.70E−02
hsa-miR-1246	98 ± 6	572 ± 206	1.60E−02
hsa-let-7c-5p	853 ± 128	1654 ± 416	3.74E−02
hsa-miR-6089	190 ± 11	462 ± 86	1.81E−02
hsa-miR-6087	98 ± 6	572 ± 206	1.60E−02
has-miR-23b-3p	1710 ± 111	2181 ± 242	4.72E−02

cell viability, whereas antagomiR-1246 transfection further decreased cell viability in irradiated cells (Fig. 2d). The colony formation analysis showed similar findings in cell viability assay (Fig. 2e).

Inhibition of miR-1246 expression suppresses tumorigenicity in T24 cells

To further determine whether the inhibition of miR-1246 expression can suppress the growth of bladder cancer cells, T24 cells were transfected with antagomiR-1246, and then the cell viability and colony formation ability were measured. Inhibition of miR-1246 significantly decreased cell viability (Fig. 3a) and colony formation (Fig. 3b) in T24 cells compared to control T24 cells. Flow cytometry analysis showed that transfection with antagomiR-1246 significantly increased the G0/G1-phase of T24 cells and induced apoptosis compared to cells transfected with antagomiR-NC (Fig. 3c, d).

MiR-1246 directly targets P53 in T24 cells

miRanda and TargetScan programs were used to predict the targets of miR-1246. Among the predicted genes, p53

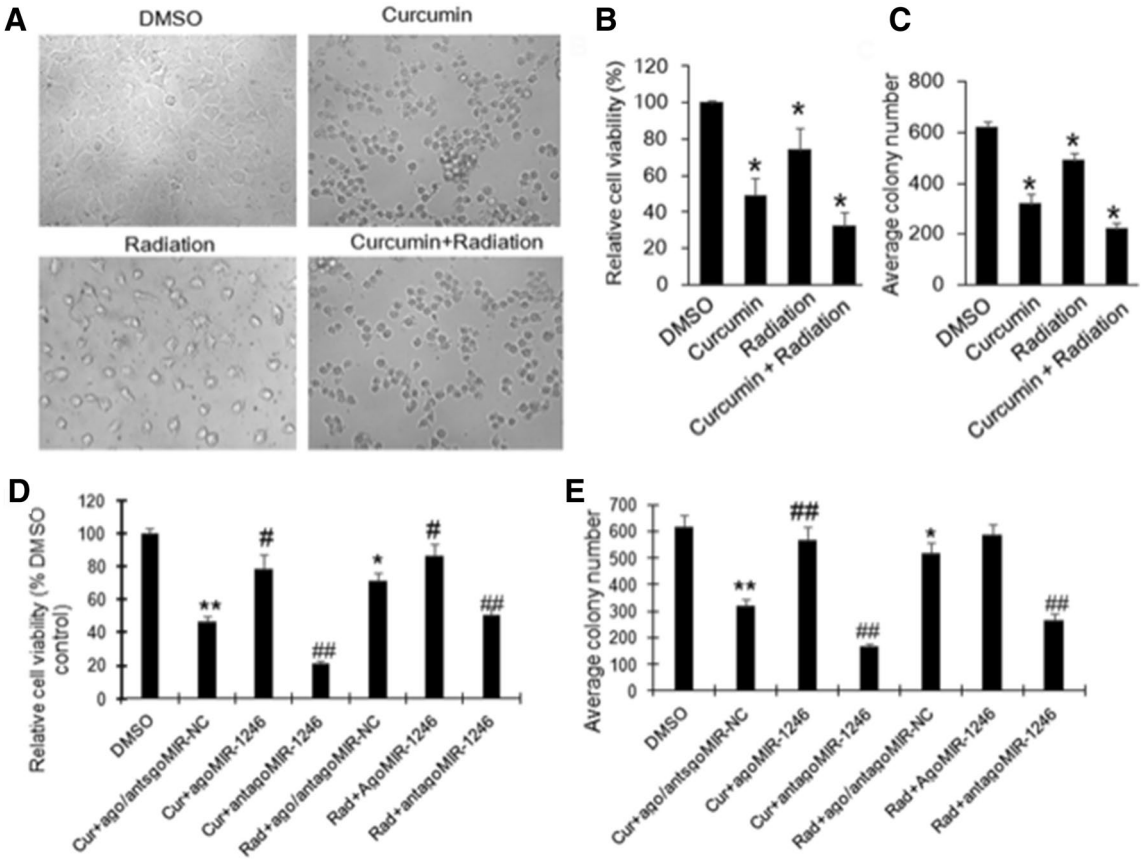

Fig. 2 Curcumin suppresses tumorigenicity of T24 cells. **a** Cell morphological changes and cell density in cells treated with curcumin, irradiation only or their combination compared with DMSO control group (**b**, **c**). The effects of curcumin and irradiation on viability and colony formation ability of T24 cells. $^{*}P<0.05$ vs. control. $^{\#}P<0.05$ vs. curcumin alone and irradiation alone group. $N=6$. **d** Cell viability in T24 cells after treatments of curcumin with agomiR-NC and antagomiR-NC (Cur + ago/antagomir-NC), radiation, agomiR-1246, and antagomiR-1246 transfection. **e** The colony formation analysis of T24 cells after the treatment of curcumin, radiation, agomiR-1246, and antagomiR-1246 transfection. $^{**}P<0.01$ vs. control. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. curcumin with agomiR-NC and antagomiR-NC. $N=6$

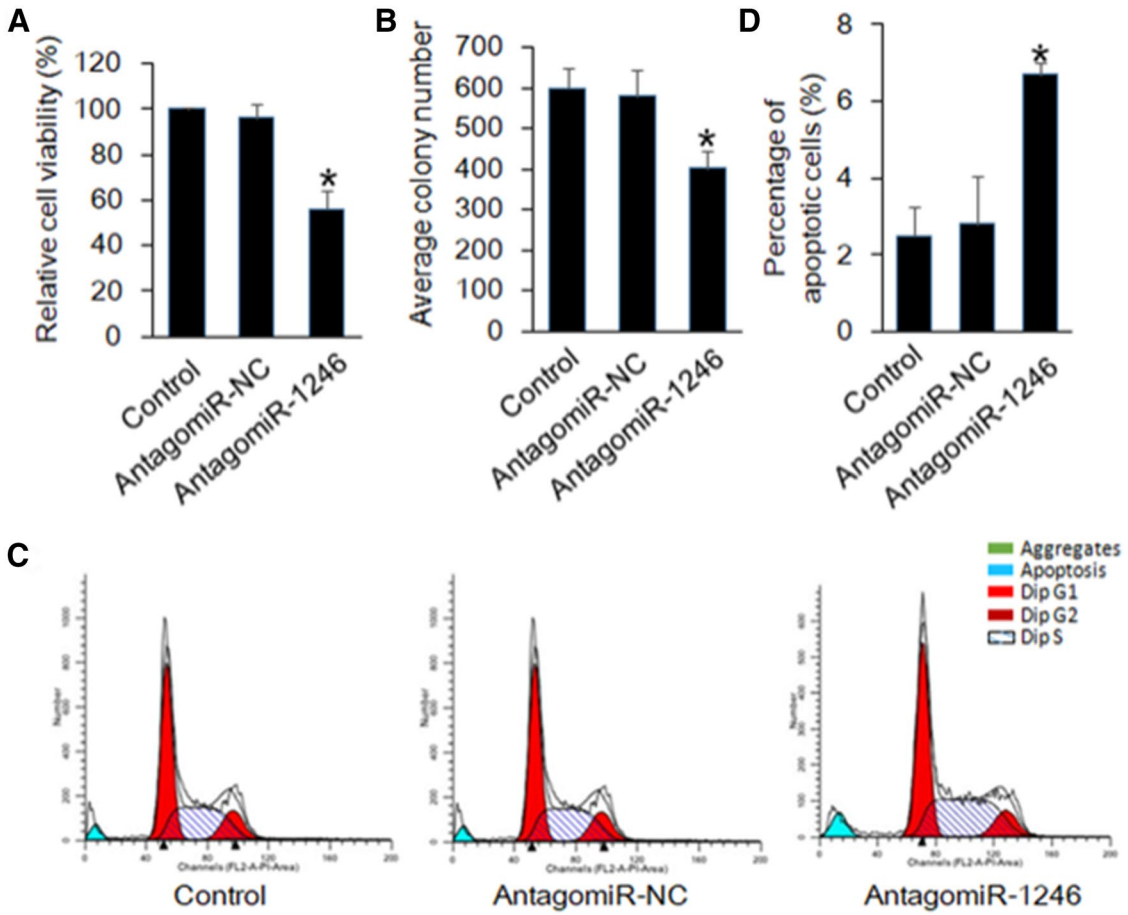

Fig. 3 Inhibition of miR-1246 expression suppresses tumorigenicity in T24 cells. **a** The cell viability assay and **b** colony formation assay of antagomiR-1246 transfected T24 cells compared to control group. **c** The effects of antagomiR-1246 on cell cycle arrest and apoptosis in T24 cell. **d** Percentages of apoptotic cells were measured. The data were presented as the mean ± SD. $^*P < 0.05$

was selected for further analysis. Curcumin (10 μM) and irradiation significantly increased p53 protein levels in T24 cells with irradiation being more effective (Fig. 4a). Transfection of agomiR-1246 (1 μg/ml) or antagomiR-1246 (1 μg/ml) significantly decreased or increased endogenous P53 protein levels in T24 cells (Fig. 4b). However, no significant changes were observed in the mRNA levels of p53 after the transfection of agomiR-1246 or antago-miR-1246 (Fig. 4c). To investigate whether miR-1246 directly targets P53, luciferase reporter constructs containing the predicted miRNA-binding site of P53 (pGL3-P53) was generated. pGL3-P53 was transfected with agomiR-1246 or agomiR-NC into T24 cells, and the level of luciferase enzyme activity was measured. Overexpression of miR-1246 suppressed the luciferase activity of the P53 3′-UTR reporter genes (Fig. 4d). These data indicate that miR-1246 directly targets P53 gene expression posttranscriptionally.

Discussion

Although radiotherapy is the primary option for bladder cancer patients, it does not have obvious curative effects, and it comes with high side effects. How to increase radiosensitivity in bladder cancer is particularly important for clinical research. Curcumin is a natural medicine and has been used as a radiosensitizer for a variety of tumor cells, and a radioprotectant for normal organs [20–23]. The effect of curcumin on radiotherapy has not been reported in patients with bladder cancer. In this study, we found that curcumin and radiotherapy can inhibit proliferation, colony formation, and cell cycle progression, but can also induce cell apoptosis in human urinary bladder carcinoma T24 cells and enhance the radiotherapy effect.

Many studies have shown that miR-1246 can promote the migration and invasion of tumor cells and malignant progression. For example, MiR-1246 has been

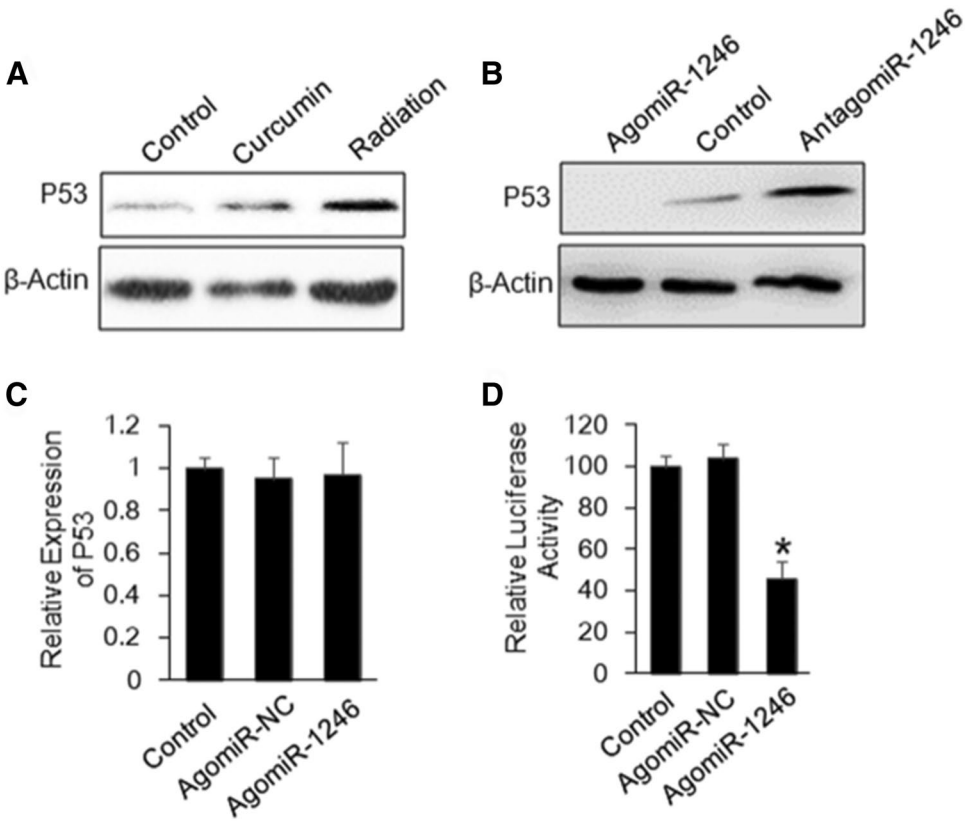

Fig. 4 MiR-1246 directly targets p53 in T24 cells. **a** Representative Western blot analysis of p53 protein expression in T24 cells treated with curcumin (10 μM) and irradiation. **b** Representative Western blot analysis of p53 protein expression in T24 cells transfected with agomiR-1246 (1 μg/ml) or antagomiR-1246 (1 μg/ml). β-Actin was used as a loading control. **c** The mRNA levels of p53. $N=6$. **d** Luciferase activity in T24 cells after the transfection of pGL3-P53 with agomiR-1246 (1 μg/ml) or agomiR-NC (1 μg/ml). $^*P<0.05$. $N=6$

demonstrated to induce the pro-metastatic phenotype and promote the migration and invasion of HOC313-P, TSU, and HeLa cells by binding to 3′UTR of *DENND2D* gene and subsequently inhibit the expression of *DENND2D* gene [24]. miR-1246 can act as an oncogene in colorectal cancer, and its aberrant upregulation promotes the malignant progression of tumors through inhibiting *CCNG2* gene expression by binding its 3′UTR [25]. Our study found that both curcumin and radiotherapy down-regulated the expression of miR-1246, while curcumin combined with radiotherapy was more effective.

P53 is a tumor suppressor gene involved in different cellular functions related to cancer development, progression, and response to therapy, including cell-cycle regulation, apoptosis, DNA repair, and angiogenesis [26]. Previous study found that the p53 family are abnormally expressed in bladder cancer cells [27]. In this study, the overexpression of miR-1246 suppressed the luciferase activity of the P53 3′-UTR reporter genes. However, there was no significant change in the mRNA level of P53. Therefore, we propose that P53 is an important target gene of miR-1246 in bladder cancer cells, and miR-1246 inhibited the translation of p53 gene, but not induced degradation of the mRNA after binding to its 3′-UTR.

We acknowledge the limitations of this study. First, several recent studies reported that p53 can regulate the expression of miR-1246. For example, Zhang et al. [28] study revealed that the overexpression of p53 induced miR-1246 expression in lung cancer cell line. Wild-type p53 was found to regulate miR-1246 expression in human hepatocellular carcinoma HCC cell lines, and the overexpression of p53 subsequently inhibited cell proliferation and colony formation ability [29]. However, our study was only designed to observe the regulative effect of miR-1246 on p53 and provided no evidence whether p53 can, in turn, regulate miR-1246 expression in bladder cancer cells. Second, this study did not investigate whether miR-1246 regulates p53 protein expression involving in the tumor growth in vivo. However, this study presented two novel findings: (1) miR-1246 is involved in the anti-cancer effect of curcumin and sensitizes the radiation; and (2) miR-1246 directly targets p53 and subsequently inhibits the translation of p53, but not degrade mRNA.

In conclusion, miR-1246 is involved in the anti-cancer effects of curcumin and irradiation through targeting inhibition of p53 gene translation in bladder cancer cells. It provided a new target for the treatment of bladder cancer.

Author contribution The manuscript has been read and approved by all the authors and all authors believe that the manuscript represents honest work.

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Compliance with ethical standards

Conflict of interest All authors declared no conflict of interest.

Statement of animals and human participants This article does not contain any studies with animals and human participants performed by any of the authors.

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