

Iodine-125 irradiation inhibits invasion of gastric cancer cells by reactivating microRNA-181c expression

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Abstract. Iodine-125 (¹²⁵I) seed implantation has been widely used for the treatment of unresectable advanced tumors. However, the molecular mechanisms underlying the tumor-suppressive effects of ¹²⁵I irradiation have not been fully elucidated. The present study demonstrated that ¹²⁵I irradiation suppresses cell viability and inhibits cell invasiveness of gastric cancer KATO-III and MKN45 cells. Further mechanistic analysis suggested the involvement of microRNA (miR)-181c in the inhibitory effects induced by ¹²⁵I irradiation. Methylated DNA immunoprecipitation coupled with quantitative-polymerase chain reaction demonstrated that treatment with ¹²⁵I irradiation, at the dose of 4 Gy, induced promoter demethylation of the miR-181c gene in KATO-III and MKN45 cells. Following irradiation, the expression of miR-181c was significantly increased, which may be attributed to the demethylation caused by ¹²⁵I irradiation. In addition, upregulation of miR-181c by administration of miR-181c mimics decreased cell invasion, suggesting the role of miR-181c as a tumor suppressor. More importantly, the tumor-suppressive effects of ¹²⁵I irradiation were significantly compromised by the introduction of miR-181c inhibitors. Overall, these results reveal that ¹²⁵I irradiation inhibits invasiveness of gastric cancer cells by reactivating miR-181c at the epigenetic level, thereby providing important molecular evidence for the anticancer effects of ¹²⁵I irradiation.

Introduction

Gastric cancer is one of the most common human malignancies, globally accounting for ~1 million novel cases and >700,000

cancer-associated mortalities annually (1). Surgical resection remains the primary treatment for gastric cancer; however, ~60% of patients have locally advanced and metastatic disease at the time of surgery, leading to a relatively low therapeutic efficacy (2). Therefore, nonsurgical methods have attracted increasing attention (3).

Iodine-125 (¹²⁵I) seeds have a long half-life and low energy with excellent stability, which has resulted in their extensive clinical use (4). Brachytherapy with low-dose ¹²⁵I seeds has been demonstrated to be an effective salvage therapy for gastric cancer and other malignant carcinomas (5-10). Although ¹²⁵I seed implantation has been successfully utilized in clinics, its radiobiological effect and underlying molecular mechanisms have not been fully elucidated. Recent evidence has indicated that altered DNA methylation patterns have a critical role in tumor inhibition resulting from ¹²⁵I irradiation (3). In addition, consecutive low-energy ¹²⁵I irradiation significantly inhibits the expression of DNA methyltransferases (DNMTs) in cancer cells (11). Following a decrease in DNMT expression, the irradiation-induced DNA demethylation contributes to tumor inhibition by reactivating tumor suppressor genes (11).

MicroRNAs (miRs) are small non-coding RNAs that function as endogenous silencers of numerous target genes. Downregulation of miRs is a common characteristic observed in various types of cancer, suggesting that these molecules may act as a novel class of tumor suppressors (12-18). Previous studies have revealed that a growing number of tumor suppressor miRs are inactivated by promoter hypermethylation in cancers (19-27). One of these miRs, miR-181c, is silenced in gastric cancer by promoter hypermethylation. Furthermore, when miR-181c expression is upregulated, it has been demonstrated to induce growth inhibition of gastric cancer cells, suggesting its role as a potential tumor suppressor in gastric cancer (20). Based on these studies, the present study hypothesized that tumor suppressor miRs, which are epigenetically silenced in cancer, may be activated by irradiation-inducing DNA demethylation and contribute to the anticancer effects of ¹²⁵I irradiation. The aim of the present study was to evaluate whether miR-181c is regulated by ¹²⁵I irradiation and is involved in irradiation-triggering tumor inhibition in gastric cancer cells.

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Materials and methods

Cell culture. Human gastric cancer KATO-III and MKN45 cell lines were purchased from the Shanghai Institute of Cytobiology of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco®; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin. All cell lines were maintained at 37°C in a humidified incubator containing 5% CO₂.

¹²⁵I irradiation treatment of gastric cancer cells. In-house ¹²⁵I seeds were obtained from Beijing Atom and High Technique Industries, Inc. (Beijing, China). An *in vitro* irradiation model was constructed as previously described (28). The absorbed dose was measured and verified as follows: 44, 92, 144 and 204 h were required for doses of 2, 4, 6 and 8 Gy, respectively.

MTT assay. Cell viability was determined by measuring the ability of the cells to transform thiazolyl blue tetrazolium bromide (MTT) to a purple formazan dye as previously described (28,29). Briefly, cells were irradiated and 20 µl MTT solution was added to the cells in each well of a 96-well plate and incubated for 5 h. The medium was replaced with dimethyl sulfoxide to dissolve the purple formazan. The color intensity of the formazan solution, which is positively associated with cell viability, was measured with a microplate spectrophotometer (VersaMax™; Molecular Devices, LLC, Sunnyvale, CA, USA) at 570 nm.

Transwell invasion assay. Invasiveness of gastric cancer cells were measured using a modified Boyden chamber (BD Bioscience, Franklin Lakes, NJ, USA). Briefly, the gastric cancer cell suspensions were obtained 92 h following irradiation at a total dose of 4 Gy. In total, 10⁴ cells were plated in 200 µl RPMI-1640 containing 10% FBS in the upper chambers of the Boyden chamber. The lower chambers were filled with 500 µl RPMI-1640 containing 10% FBS. Subsequently, cells were incubated for 48 h at 37°C, and the membrane was stained with crystal violet and viewed under a microscope to calculate the average number of invasive cells.

Western blotting. Total protein from gastric cancer cells was extracted using RIPA lysis buffer (Cloud-Seq, Inc., Shanghai, China) and quantified using a BCA assay (Pierce™; Thermo Fisher Scientific, Inc.). Protein extracts were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane using RapidBlot Transfer Buffer (Cloud-Seq, Inc.). The membranes were hybridized with the following primary antibodies overnight at 4°C: Mouse monoclonal anti-DNA (cytosine-5)-methyltransferase 1 (DNMT1; catalog no., sc-271729; dilution, 1:200); and mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; catalog no., sc-365062; dilution, 1:200) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequently, membranes were probed with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (catalog no., 115-035-003; dilution, 1:10,000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature, and the immunoreactive signals were detected using a SuperSen Enhanced Chemiluminescence

kit (Cloud-Seq, Inc.). The DNMT1 protein expression levels were normalized to GAPDH. The signal intensities on western blots were semi-quantified using ImageJ version 1.43 software (National Institutes of Health, Bethesda, MA, USA).

Methylated DNA immunoprecipitation (MeDIP)-quantitative polymerase chain reaction (qPCR) assay. The MeDIP assay coupled with qPCR was used to quantitatively evaluate the methylation status of miR-181c in the cells treated without (control) or with 4 Gy of ¹²⁵I irradiation. MeDIP was performed as described previously (3). Briefly, genomic DNA was extracted from cells using DNeasy Blood and Tissue kit (Qiagen, Inc., Hilden, Germany), according to the manufacturer's protocol, and sonicated to produce random fragments ~200-600 bp in size. Following denaturation at 95°C for 10 min, immunoprecipitation was performed using a mouse monoclonal anti-5-methylcytidine antibody (catalog no., 39649; dilution 1:10; incubation, 2 h at 4°C; Active Motif, Carlsbad, CA, USA). Immunoprecipitated complexes were collected with Dynabeads® Protein A (Thermo Fisher Scientific, Inc.), and the methylated DNA fragments were purified by phenol-chloroform extraction and isopropanol precipitation. Subsequently, methylated DNA fragments and input DNA were analyzed by qPCR using an ABI 7900 Real-Time PCR System (Applied Biosystems™; Thermo Fisher Scientific, Inc.) and Rapid SYBR Green PCR Master Mix from Cloud-Seq, Inc. The following cycling conditions were used: 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec, then 60°C for 1 min, concluding with melt curve analysis. The relative changes in the extent of gene methylation were determined by measuring the amount of detected genes in methylated DNA fragments following normalization to the input DNA (30). The primer sequences targeting the promoter of miR-181c were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) as follows: Forward, 5'-GAGGGATGAGGA AATGGA-3' and reverse, 5'-TCACAACAGCGTGAGTGG-3'. The experiment was performed in triplicate.

Transfection of cells with miR-181c mimic and inhibitor. miR-181c was repressed or overexpressed by transfection of cells with miR inhibitor or miR mimics using Lipofectamine® 2000 (Invitrogen™; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. miR-181c mimic and scrambled control miR sequences were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China) as follows: miR-181c mimic, sense 5'-AACAUUCAACCU GUCGGUGAGUUA-3' and antisense 5'-ACUCACCGACAG GUUGAAUGUUUU-3'; miR-181c inhibitor, sense 5'-TAA CUCACCGACAGGUUGAAUGUU-3'; scrambled negative control, sense 5'-UUGUACUACACAAAAGUACUG-3'. All of the oligonucleotides were transfected at a final concentration of 100 nM. Following transfection, the expression of miR-181c was confirmed by qPCR.

Reverse transcription-qPCR. In total, ~5x10⁶ cells were treated without (control) or with 4 Gy of ¹²⁵I irradiation. Total RNA was extracted from cells using ExRNA Reagent (Cloud-Seq, Inc.), according to manufacturer's protocol. Total RNA from each sample was quantified using NanoDrop ND-1000 (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA), and RNA integrity was assessed by standard denaturing

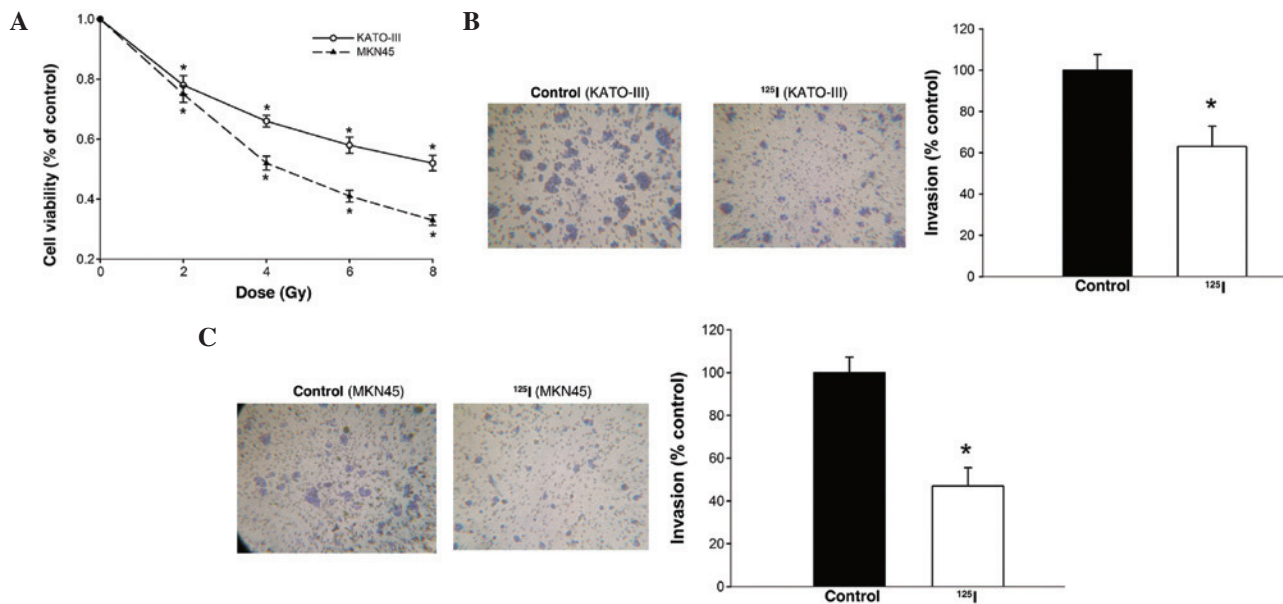


Figure 1. ¹²⁵I seed irradiation inhibits viability and invasiveness of human gastric cancer KATO-III and MKN45 cells. (A) Cell viability of KATO-III and MKN45 cells treated at various doses of ¹²⁵I irradiation was examined by MTT assay. (B and C) Invasive abilities of (B) KATO-III and (C) MKN4 cells treated with 0 and 4 Gy ¹²⁵I irradiation were evaluated by Transwell analysis. The number of cells was counted in 10 fields per chamber to calculate the average number of invasive cells. Data are presented as the mean \pm standard error of the mean (n=3). *P<0.05 vs. 0 Gy treated cells. ¹²⁵I, iodine-125.

agarose gel electrophoresis. miRs and mRNAs were processed into cDNA using a FlashScript Reverse Transcription kit (Cloud-Seq, Inc.) with a miR-181c-specific stem loop primer and oligo dT (18) primer. qPCR was performed with Rapid SYBR PCR Master Mix (Cloud-Seq, Inc.) on an ABI 7900 Real-Time PCR System. Primers were synthesized by Sangon Biotech Co., Ltd. as follows: U6, forward 5'-CTCGCTTCG GCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGC GT-3'; GAPDH, forward 5'-CGGAGTCAACGGATTTGG TCGTAT-3' and reverse 5'-AGCCTTCTCCATGGTGGTGAA GAC-3'; miR-181c, forward 5'-AACATTCAACCTGTCGGT G-3' and reverse 5'-GTCGTATCCAGTGCAGGGTCCGAG GTATTTCGACTGGATACGACACTCAC-3'; miRNA universal, reverse 5'-CCAGTGCAGGGTCCGAGGTAT-3'. For normalization, GAPDH and U6 were used to normalize mRNA and miRNA, respectively. Cycles were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec and annealing/extension at 60°C for 30 sec. Each experiment was repeated at least three times, and the results were calculated according to the $2^{-\Delta\Delta C_q}$ method (31).

Statistical analysis. Statistical analysis was performed with SPSS version 11.0 software (SPSS, Inc., Chicago, IL, USA). Each experiment was repeated three times. Student's t-test was used to assess the statistical significance of differences between groups. Data are presented as the mean \pm standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

¹²⁵I irradiation inhibits viability and invasiveness of gastric cancer cells. To investigate the effects of ¹²⁵I irradiation on the viability of gastric cancer cells, KATO-III and MKN45 cells were treated with continuous ¹²⁵I irradiation at a dose between

0 and 8 Gy. Subsequently, MTT assay was performed to determine the effects of ¹²⁵I treatment. MTT assays indicated that ¹²⁵I treatment significantly decreased the viability of KATO-III and MKN45 cells at a low dose (P<0.05; Fig. 1A). On the basis of these results, 4 Gy was used for subsequent experiments.

To investigate the effects of ¹²⁵I irradiation on the invasion of gastric cancers, KATO-III and MKN45 cells were treated with ¹²⁵I irradiation at 0 (control) or 4 Gy. In total ~48 h later, the invasive ability of the cells was analyzed using Transwell assays. As shown in Fig. 1B, the number of KATO-III cells invading through the Matrigel following ¹²⁵I irradiation was clearly attenuated by 53% compared with the control group. A similar reduction in cell invasive activity was observed in MKN45 cells treated with ¹²⁵I irradiation (P=0.043; Fig. 1C). These data indicate a negative regulatory effect of ¹²⁵I irradiation on the viability and invasiveness of gastric cancer cells.

¹²⁵I irradiation modulates miR-181c activity by inducing DNA demethylation at its promoter region. Previous evidence has demonstrated that ¹²⁵I irradiation alters the expression of DNMT1, leading to epigenetic alterations, which reactivates silenced tumor suppressor genes (11). miR-181c is a potential tumor suppressor that is epigenetically silenced by promoter hypermethylation in gastric cancers (12). Thus, the present study hypothesizes that ¹²⁵I irradiation may modulate the activity of the miR-181c gene by affecting its methylation status.

To confirm this hypothesis, the present study primarily examined the effects of ¹²⁵I irradiation on the expression of DNMT1 protein in KATO-III and MKN45 cells. DNMT1 and GAPDH proteins were detected by western blotting, and protein expression levels of DNMT1 were calculated by normalization to GAPDH density. As indicated in Fig. 2A, ¹²⁵I treatment lead to a significant reduction of DNMT1 protein expression in KATO-III (P=0.040) and MKN45 cells

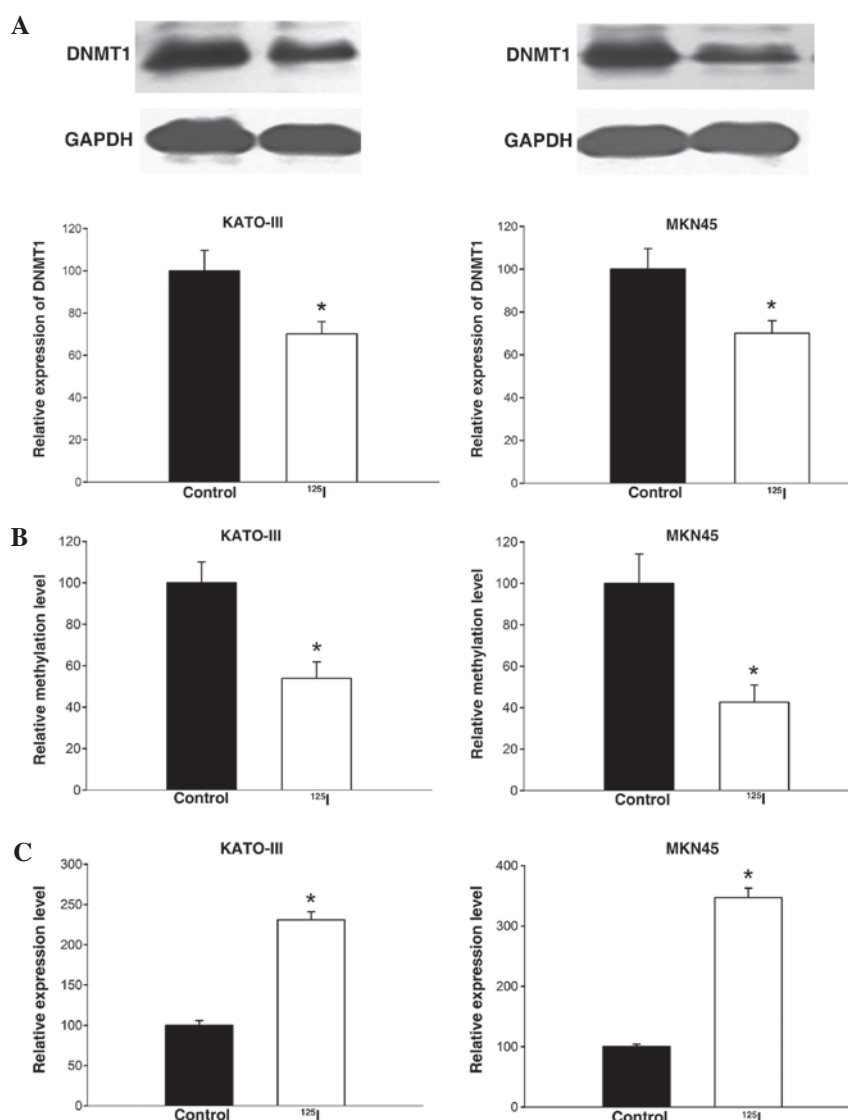


Figure 2. ^{125}I irradiation reactivates miR-181c expression by inducing its promoter demethylation. (A) Protein levels of DNMT1 (180 kDa) and its quantification analysis by GAPDH (37 kDa) in human gastric cancer KATO-III and MKN4 cells was investigated by western blotting. (B) Effects of ^{125}I irradiation on promoter methylation of the DNMT1 gene in KATO-III and MKN4 cells was assayed by methylated DNA immunoprecipitation-PCR. (C) Relative expression of miR-181c was detected using reverse transcription-quantitative PCR. Data are presented as the mean \pm standard error of the mean (n=3). *P<0.05 vs. 0 Gy treated cells. ^{125}I , iodine-125; miR, microRNA; DNMT1, DNA (cytosine-5)-methyltransferase 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction.

(P=0.025). This decrease of DNMT1, which is responsible for the maintenance of DNA methylation patterns, may result in methylation alterations of target genes.

To determine whether ^{125}I irradiation affects the methylation status of the miR-181c gene, a MeDIP-PCR assay was performed in gastric cancer cells treated with and without ^{125}I irradiation (4 Gy). As indicated in Fig. 2B, ^{125}I -irradiated cells exhibited a significantly decrease in the methylation status of miR-181c compared with control cells (KATO-III, P=0.032; MKN45, P=0.025); there was a 53.9% and 42.7% decrease in KATO-III and MKN45 cells, respectively. These results demonstrate that ^{125}I irradiation induces DNA demethylation at the promoter region of the miR-181c gene.

Finally, alterations in the expression of miR-181c in gastric cancer cells following ^{125}I treatment were determined using qPCR. As indicated in Fig. 2C, ^{125}I treatment caused upregulation of miR-181c expression in KATO-III and MKN45 cells,

which was significant compared with the control group (P=0.018 and P=0.014, respectively). Overall, these data suggest that ^{125}I irradiation upregulates miR-181c expression in gastric cancer cells, which may be partially attributed to the irradiation inducing DNA demethylation.

miR-181c exerts a functional role as a tumor suppressor in gastric cancer cells. A previous study reported that there was a decreased expression of miR-181c in gastric cancer, and revealed that miR-181c suppresses cell growth in KATO-III and MKN45 cells (20). To further determine the functional role of miR-181c in gastric cancer cells, the invasiveness of KATO-III and MKN45 cells transfected with miR-181c mimics and scrambled negative control was evaluated by the present study.

The results of a qPCR revealed that miR-181c mimics significantly increased miR-181c expression in KATO-III

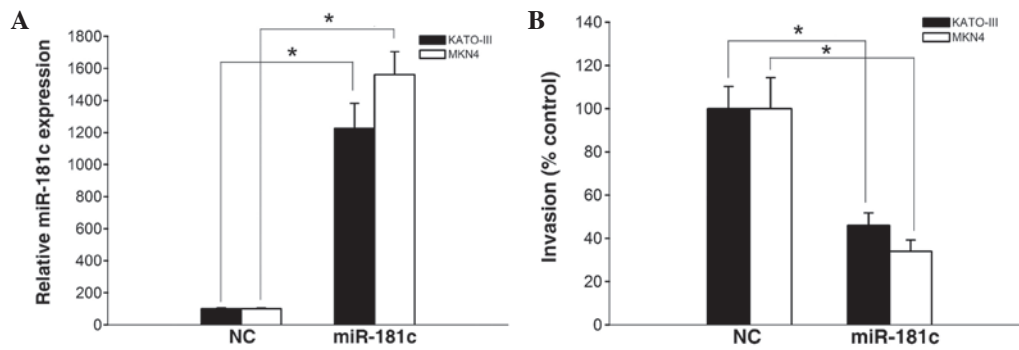


Figure 3. Effects of miR-181c on the invasiveness of gastric cancer cells. (A) Human gastric cancer KATO-III and MKN4 cells were transfected with miR-181c mimics and NC. The corresponding transfection efficiencies were detected by quantitative polymerase chain reaction. (B) Effects of miR-181c overexpression on the invasiveness of KATO-III and MKN4 cells was evaluated by Transwell assay. Data are presented as the mean \pm standard error of the mean (n=3) and normalized to the NC group, which had a value of 100. *P<0.05 vs. NC-transfected cells. miR, microRNA; NC, scrambled negative control.

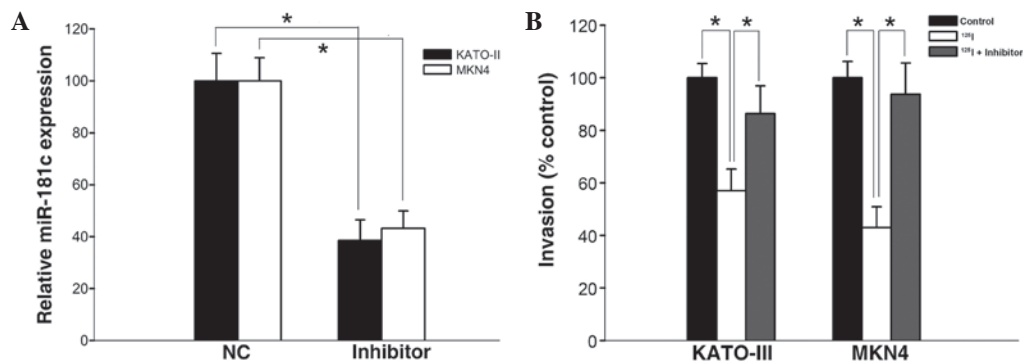


Figure 4. Inhibitory effects of ^{125}I irradiation on cell invasiveness is compromised by miR-181c inhibitors. (A) Human gastric cancer KATO-III and MKN4 cells were transfected with miR-181c inhibitors and NC. Inhibition of miR-181c in KATO-III and MKN4 cells was verified by reverse transcription-quantitative polymerase chain reaction. (B) Transwell assays revealed that miR-181c inhibitor significantly decreased the effects of ^{125}I irradiation on the inhibition of cell invasion. Cells not irradiated with ^{125}I were included for reference. Data are presented as the mean \pm standard error of the mean (n=3) and normalized to the NC group, which had a value of 100. *P<0.05 vs. NC-transfected cells. ^{125}I , iodine-125; miR, microRNA; NC, scrambled negative control.

and MKN45 cells ($P=0.016$ and $P=0.009$, respectively; Fig. 3A), suggesting that miR-181c mimics were efficiently introduced into the cells and upregulated miR-181c expression. Furthermore, the number of gastric cancer cells invading through Matrigel following transfection with miR-181c mimics was remarkably attenuated compared with KATO-III and MKN45 cells transfected with scrambled negative control ($P=0.027$ and $P=0.021$, respectively; Fig. 3B). These data indicate a potential negative regulatory effect of miR-181c on the invasion of gastric cancer cells.

Downregulation of miR-181c compromises the anticancer effects of ^{125}I irradiation. To evaluate the role of miR-181c on the inhibitory effect of ^{125}I irradiation, KATO-III and MKN45 cells were treated with 4 Gy ^{125}I irradiation followed by transfection with miR-181c inhibitors. As shown in Fig. 4A, miR-181c inhibitors significantly decreased the expression of miR-181c in KATO-III and MKN45 cells compared with the scrambled normal control-transfected cells ($P=0.037$ and $P=0.030$, respectively). In addition, the ^{125}I -irradiation-induced inhibition on cell invasion was significantly attenuated in KATO-III and MKN45 cells transfected with the miR-181c inhibitor ($P<0.05$; Fig. 4B). These data implied that miR-181c was responsible for the suppression of cell invasion, which was induced by ^{125}I irradiation.

Discussion

^{125}I seed implantation has been widely used to treat various types of cancer, including gastric cancer, due to its high precision, low trauma to patients, strong lethality and few side effects (9,10). Although ^{125}I seed implantation has been successfully used in clinics, its radiobiological effects and underlying molecular mechanisms are not fully elucidated.

Several studies have indicated that ^{125}I seed irradiation is effective in inducing cell apoptosis in pancreatic and colonic cancer cells (4,11,32). More recently, Tian *et al* (28) revealed that ^{125}I seeds effectively inhibit cell growth and invasion of nasopharyngeal carcinoma. However, the effects of ^{125}I seed irradiation in gastric cancer cell lines have not yet been investigated. The present study evaluated the efficacy of ^{125}I irradiation on the death of KATO-III and MKN45 cells, as assessed by cell viability assays, and demonstrated that a low dose of ^{125}I irradiation (4 Gy) effectively kills gastric cancer cells.

Previous studies have revealed that altered DNA methylation patterns may be critical in tumor inhibition resulting from consecutive low-energy ^{125}I irradiation (11). Ma *et al* (11) demonstrated that low-dose (4 Gy) ^{125}I irradiation causes a significant decrease in DNMT expression in pancreatic cancer cells. Consistent with the present study, a significant

decrease in DNMT1 expression was observed in gastric cancer xenografts implanted with ¹²⁵I seeds (3). In addition, there is a clear and positive association between DNA methylation and expression of DNMTs, since DNMTs maintain DNA methylation patterns (11). As the result of decreased DNMT expression induced by ¹²⁵I seed irradiation, tumor suppressor genes, including BCL2/adenovirus E1B 19kDa interacting protein 3, Wnt family member 9A and germ cell associated 2, are extensively reactivated by promoter demethylation (3).

miRs are known as a class of small noncoding RNAs, which are critical in cancer progression as oncogenes and tumor suppressor genes. It has been reported that numerous tumor suppressor miRs, including miR-9, miR-124 and miR-200 family, are epigenetically silenced by DNA methylation in cancer (33-35). It is reasonable to hypothesize that these miRs may be reactivated by ¹²⁵I-irradiation inducing promoter demethylation, thereby contributing to anticancer effects. Among these tumor suppressor miRs, miR-181c has been reported to be epigenetically silenced in gastric carcinogenesis (20). In the present study, MeDIP-PCR assays were performed to examine the methylation status at the promoter region of miR-181c. As expected, the present results revealed that ¹²⁵I irradiation significantly induced DNA demethylation at the promoter of the miR-181c gene in KATO-III and MKN45 cells. Additionally, a notable upregulation of miR-181c in gastric cancer cells was observed following continuous low-dose ¹²⁵I irradiation. These data suggested that ¹²⁵I irradiation induces DNA demethylation at the promoter region of miR-181c, resulting in the reactivation of miR-181c. Further experiments by the present study demonstrated that overexpression of miR-181c effectively decreased cell invasiveness. In addition, downregulation of miR-181c compromised the anticancer effects observed with ¹²⁵I irradiation. According to these results, it may be concluded that miR-181c is reactivated by ¹²⁵I irradiation through DNA demethylation, and thus, is involved in ¹²⁵I-irradiation induced tumor inhibition.

In summary, the present study provides, to the best of our knowledge, the first demonstration that miRs are involved in the therapeutic effect of ¹²⁵I seed irradiation. The present study provides an illustrative example in gastric cancer with the reactivation of miR-181c by ¹²⁵I irradiation, as well as its functional consequences for tumor inhibition. In addition, the present study has revealed that miR-181c reactivation may be mediated through ¹²⁵I-induced demethylation, emphasizing the critical role of epigenetic regulation underlying the anticancer effects of ¹²⁵I irradiation.

Acknowledgements

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