^{125}I radiation downregulates TRPV1 expression through miR-1246 in neuroblastoma cells

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Abstract. Iodine-125 (^{125}I) seed radiation applied around the celiac ganglion can relieve the refractory pain in pancreatic cancer. In an *in vitro* cell radiation model of human neuroblastoma cell lines, the impact of ^{125}I radiation on the expression of transient receptor potential vanilloid-1 (TRPV1) was investigated. The results indicated that the radiation delivering doses <2.13 Gy did not significantly affect cell growth, whereas the doses >3.12 Gy significantly reduced cell viability. The reduced TRPV1 mRNA level was dependent on the doses, while the reduced protein level occurred at lower doses (2.63 and 4.27 Gy), then returned to normal at an intermediate dose of 5.09 Gy, and decreased again at higher doses (5.91 and 6.73 Gy). The miRNA profiling at the dose of 2.63 Gy revealed 32 and 22 miRNAs that were significantly upregulated and downregulated, respectively. In addition, the upregulated miR-1246 target, regulated the expression of TRPV1, indicating that miR-1246 may be a new therapeutic target for pancreatic pain.

Introduction

Pancreatic cancer (PC) has a very poor prognosis and patients with PC often suffer severe pain. Pain affects ~80% of patients with PC, and half require strong opioid analgesia (1). The spread of tumor cells via the perineural space to the retro-pancreatic region can cause pain, increase the local recurrence rate, and decrease the likelihood of curative-resection. Analgesic therapies have adverse effects, and most importantly, pain relief with analgesic drugs is often inadequate (2). Both celiac plexus neurolysis (CPN) and celiac plexus block (CPB) are efficient on pain relief, however there are a significant number of patients who do not or only partially respond to these drugs and continue to suffer refractory pain (3). Therefore, more efficient therapies are required to treat PC-induced refractory pain.

Implantation of iodine-125 (^{125}I) seeds under the guidance of endoscopic ultrasonography (EUS) has been demonstrated to be a safe alternative therapeutic option for advanced PC (4,5). In a previous study, it was revealed that EUS-guided implantation of ^{125}I around the celiac ganglia is a safe procedure and can induce apoptosis of local neurons in a porcine model (5). It was also revealed that EUS-guided direct celiac ganglion irradiation with ^{125}I seeds reduced the visual analog scale (VAS) score and analgesic drug consumption in patients with unresectable PC (4). However, the mechanisms involved in pain relief are still unclear.

Transient receptor potential vanilloid-1 (TRPV1) is a key transducer of diverse noxious stimuli in pancreatic sensory neurons (6). Increased TRPV1 expression and activity play a key role in pancreatic pain (6-8). PC pain is generally transmitted through the celiac plexus which harbors sympathetic fibers that carry nociceptive information from the pancreas and surrounding organs (9).

Neuroblastoma cells have many sympathetic fibers in aerobic environment. It has been widely used in neuron related researches (10). In the present study, using human neuroblastoma cell lines SK-N-SH and SK-N-BE(2), the impact of ^{125}I administration on the expression of TRPV1 in these cells was investigated, and the possible mechanisms of pain relief were explored.

Materials and methods

Cell culture. SK-N-SH and SK-N-BE(2) human neuroblastoma cell lines were purchased from the American Type

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Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dubecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin at 37˚C and 5% CO_2 in a fully humidified incubator.

CCK-8 (cell viability kit). Cell viability was determined by Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, cells were seeded into plates at $2x10^4$ cells/well in a 96-well plate overnight and treated with or without ^{125}I.

At different time-points following treatment, cells were incubated with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium mono-sodium salt (WST-8) according to the manufacturer's instructions. Absorbance values were measured at 450 nm by enzyme-linked immunosorbent assay (ELISA). All experiments were performed in triplicate.

RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA from cells was extracted using TRIzol reagent (Takara Bio, Inc., Shiga, Japan). RNA was digested for 15 min with DNase followed by purification with an RNeasy kit (Qiagen GmbH, Hilden, Germany). For mRNA detection, 1 μg of purified total RNA was reverse-transcribed with a reverse transcription kit (Takara Bio, Inc.) according to manufacturer's instructions. The amount of TRPV1 in a given sample was normalized by the level of GAPDH in that sample. Each sample was run in triplicate. Primers used in the present study were as follows: TRPV1-F, AATGACGCCGCTGGCTCTG, and TRPV1-R, GCCCACTCGGTGAACTTCCTG; GAPDH-F, AATCCC ATCACCATCTTCCAG, and GAPDH-R, ATCAGCAGA GGGGGCAGAGA. For quantitative miRNA analysis, the Bulge-Loop™ miRNA qPCR Primer Set (Guangzhou RiboBio Co., Ltd., Guangzhou, China) was used to determine the expression levels of miR-1246 and miR-1288-5p by qRT-PCRs with Takara SYBR Premix Ex Taq™. U6 was used as an internal control for miRNA template normalization. The thermocycling settings for both mRNA and miRNA were as follows: 42˚C for 5 min, 95˚C for 3 min, followed by 45 cycles of 95˚C for 5 sec and 60˚C for 40 sec. The relative expression level for each miRNA was calculated using the ΔΔCq method (11). Primers were as follows: miR-1246, AATGGATTTTTGGAGCAGG; miR-1288-5p, GTGGGCGGGGGCAGGTGTGTG; U6, CTCGCTTCG GCAGCACA; universal microRNA, GCTGTCAACGAT ACGCTACCTA.

Western blotting. Total protein from cells were extracted using the ice-cold NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris-HCl, pH 8.0, protease inhibitors). The concentrations of the protein were determined by BCA method. A 50 μg (~5-10 μl) of protein was loaded per lane and was separated by SDS-polyacrylamide gels (8%) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After blocking (5% milk, indoor temperature for 1 h), immunoblots were incubated separately overnight at 4˚C with antibodies against TRPV1 (dilution 1:1,000; cat. no. ab3487; Abcam, Cambridge, UK) and GAPDH (dilution 1:3,000; cat. no. ab181602; Abcam) as a control. Blots were detected with an enhanced chemiluminescence reagent (ECL; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Irradiation with ^{125}I seeds. Irradiation was performed with ^{125}I radioactive seeds (BT-125-I; Shanghai Xinke Medical Company Co., Shanghai, China). Each seed was 4.5 mm in length and 0.8 mm in diameter. The seeds had a radioactive half-life of 60.1 days, with a mean photon energy of 35.5 KeV in γ-rays. The in-house ^{125}-I irradiation model was established based on our previous study (12), SK-N-SH and SK-N-BE(2) human neuroblastoma cells were seeded in a 35-mm culture dish at a density of $5x10^5$/plate for in-house ^{125}-I irradiation. The radiation absorbed dose was validated with thermoluminescent dosimetry measurement using an empirical formula from the American Association of Physicists in Medicine (AAPM). The delivering doses for different exposure time-points were also assessed and ascertained. The exposure time-points for delivering doses of 1.15, 2.13, 2.63, 3.12, 4.10, 4.27, 5.09, 5.91, 6.07 and 6.73 Gy were ~24, 48, 60, 72, 96, 100, 120, 140, 144 and 160 h, respectively.

MicroRNA array analysis and miRNA target gene prediction. To screen miRNA expression after ^{125}I treatment, miRNA profiles were analyzed using the Affymetrix miRNA 4.0 (Shanghai OE Biotech, Inc., Shanghai, China) according to the manufacturer's instructions. Briefly, miRNAs were purified from total RNA extracted from ^{125}I-treated cells or mocked cells and were then labeled using an enzyme-linked oligosorbent assay (ELOSA) and hybridized to the miRNA array. The array data were normalized by global normalization using the miRNA QC tool software (Affymetrix Expression Console software version 1.4.1 (Thermo Fisher Scientific, Inc.). The levels of miRNAs between the ^{125}I-treated cells and control samples were calculated based on the fluorescence intensities. Differential expression levels of miRNAs between the two groups of samples were assessed using one-way ANOVA analysis. TargetScan software analysis (http://www.targetscan.org/vert_71/) was used to miRNA target gene prediction.

miRNA transfection. The miR-1246 and miR-1228-5p mimics, inhibitors, and their negative controls (NCs) were purchased from RiboBio Co., Ltd. Cells were transfected with miR-1246 or miR-1228-5p mimics (50 nM), inhibitors (100 nM), or their negative controls for 48 h using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The miR-1246 and miR-1228-5p mimics (product no. B02001) and inhibitor (product no. B03001) were purchased from the Shanghai GenePharma Co., Ltd. (Shanghai, China).

Construction of the reporter gene system containing TRPV1 3'-untranslated region (3'UTR) and luciferase reporter assay. To construct pGL3-REPORT vectors containing wild-type TRPV1 3'UTR (wild) and corresponding mutant-type (mutant), the wild sequences of 3'UTR of TRPV1 mRNA containing the complementary sequences to the miR-1246 (ENST00000399759.3, site:1327-1333) seed sequence were synthesized, annealed, and ligase into the *Xba*I-*Fse*I sites of the pGL3-Control Vector (GenBank® accession no. U47296;

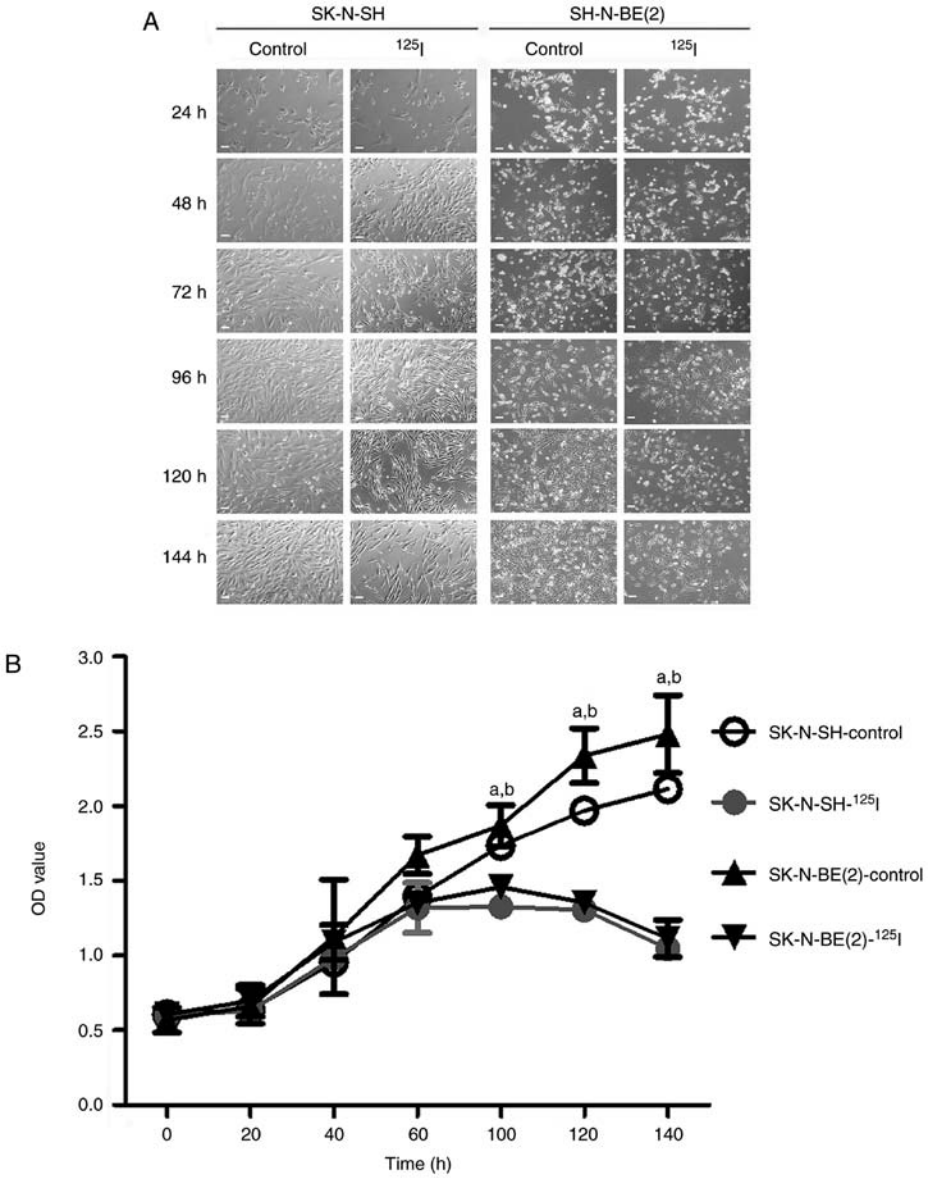

Figure 1. Effect of ^{125}I radiation on neuroblastoma cell proliferation SK-N-SH and SK-N-BE(2) cells were treated with ^{125}I radioactive seeds at various time-points (24, 48, 72, 96, 120 and 144 h; equivalent to delivering doses 1.15, 2.13, 3.12, 4.10, 5.09 and 6.07 Gy). (A) Cell morphology images, and (B) cell growth curve detected by CCK-8. The experiments were dependently repeated three times. Scale bar, 5 μm. ^{a}P<0.01, SH-N-SH-^{125}I vs. SK-N-SH-control; ^{b}P<0.01, SK-N-BE(2)-^{125}I vs. SK-N-BE(2)-control. ^{125}I, iodine-125; CCK-8, Cell Counting Kit-8.

cat. no. selected: E1741; Promega Corp., Madison, WI, USA), while the corresponding mutation sequences of 3'UTR cDNA sequences were produced with a QuikChange XL Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc., North Billerica, MA, USA), and parallelly ligased into the pGL3-Control Vector as the control. For the luciferase assays, SK-N-SH and SK-N-BE(2) cells were co-transfected with wild-type (WT) or mutant (Mut) 3'UTR of pGL3-REPORT vectors and the mimics or inhibitors of miR-1246, along with 0.01 μg of the pRL-TK vector (Promega Corp.). Luciferase assays were performed 48 h later following treatment with the dual luciferase assay kit (Promega Corp.) according to the manufacturer's instructions. The luciferase activities were normalized to the *Renilla* luciferase activity.

Statistical analysis. All quantitative data are presented as the mean ± standard deviation (SD). An independent Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, was conducted to evaluate the one-way layout data. P-values <0.05 were considered to indicate a statistically significant difference. All statistical analyses were performed using the SPSS v18.0 statistics software package (SPSS, Inc., Chicago, IL, USA).

Results

Effect of ^{125}I radiation on the proliferation of neuroblastoma cells. SK-N-SH and SK-N-BE(2) cells were treated with ^{125}I radioactive seeds at a series of time-points (24, 48, 72, 96, 120 and 144 h); equivalent to 1.15, 2.13, 3.12, 4.10, 5.09 and 6.07 Gy. At each time-point, cell morphology was imaged and the cell growth was detected by CCK-8. As revealed in Fig. 1, the lower radiation delivering doses of <48 h (equal to 2.13 Gy) did not significantly affect cell growth,

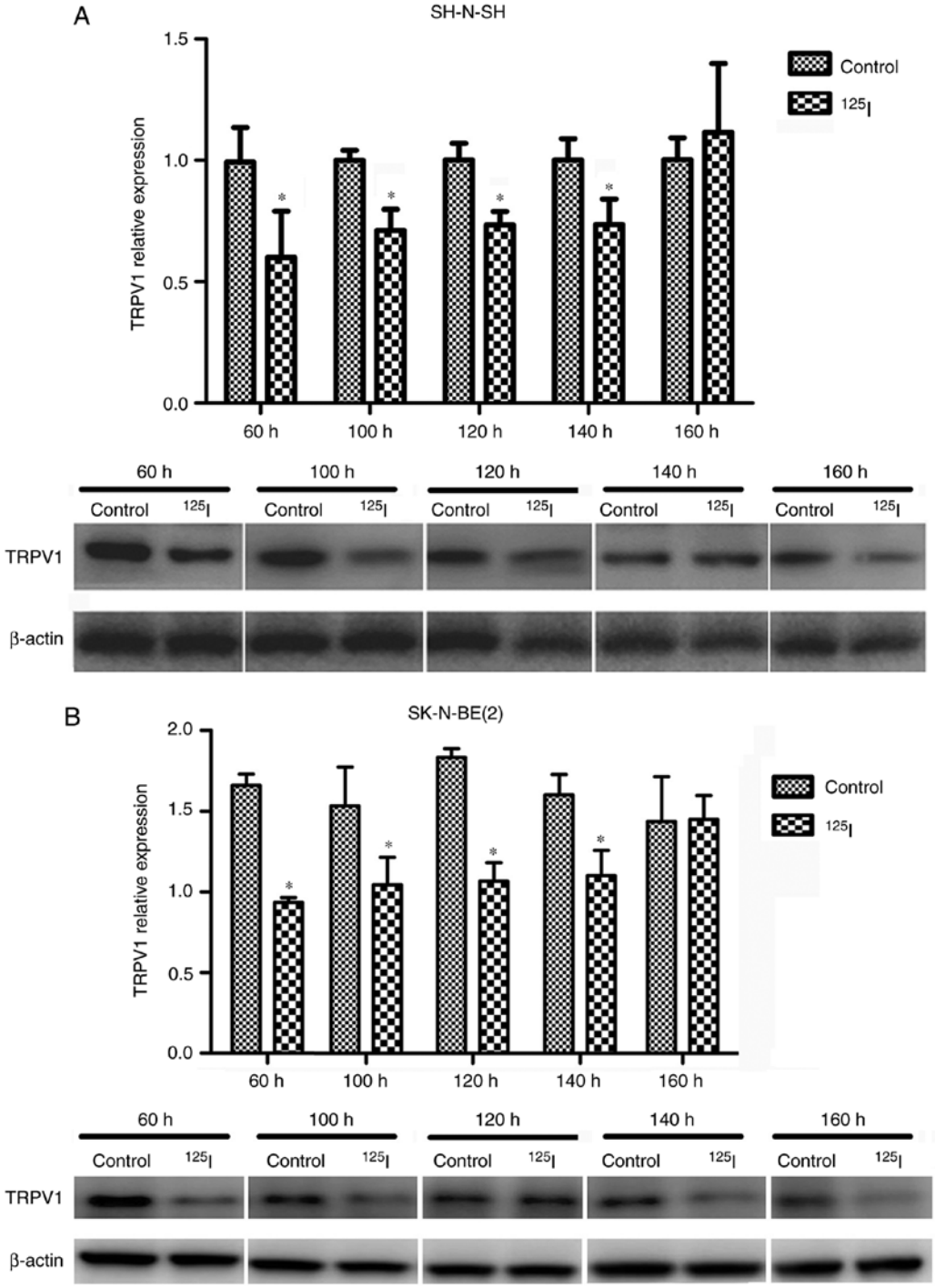

Figure 2. ^{125}I treatment reduces TRPV1 expression. (A) SK-SY-SH and (B) SK-N-BE(2) cells were treated with ^{125}I radioactive seeds at a series of time-points (60, 100, 120, 140 and 160 h; equivalent to 2.63, 4.27, 5.09, 5.91 and 6.73 Gy). The mRNA expression and the protein expression are presented. The experiments were dependently repeated three times. *P<0.05. ^{125}I, iodine-125; TRPV1, transient receptor potential vanilloid-1.

while the radiation delivering doses of >72 h (equal to 3.12 Gy) significantly reduced cell viability. The radiation time-point of 60 h (equal to 2.63 Gy) was therefore determined as the initial effective dose in the present study.

Effect of ^{125}I radiation on TRPV1 expression. To determine the expression of TRPV1 in SK-N-SH and SK-N-BE(2) cells, ^{125}I radioactive seeds were administrated to these cells. As revealed in Fig. 2, the mRNA and protein levels of TRPV1 expression were detected at a series of time-points (60, 100, 120, 140 and 160 h; equivalent to 2.63, 4.27, 5.09, 5.91 and 6.73 Gy). The mRNA expression of TRPV1 was significantly decreased compared with the non-radiated-cells at 60, 100, 120 and 140 h (but not at 160 h); the absolute CT value of the TRPV1 was high (~ >40 CT). The protein levels of TRPV1 were initially extensively reduced at 60 and 100 h, but then returned to the control level at 120 h, followed by downregulation of TRPV1 expression at 140 and 160 h again; it was surmised that the TRPV1 protein was subjected to some type of post-translational regulation over the 140-h irradiation treatment.

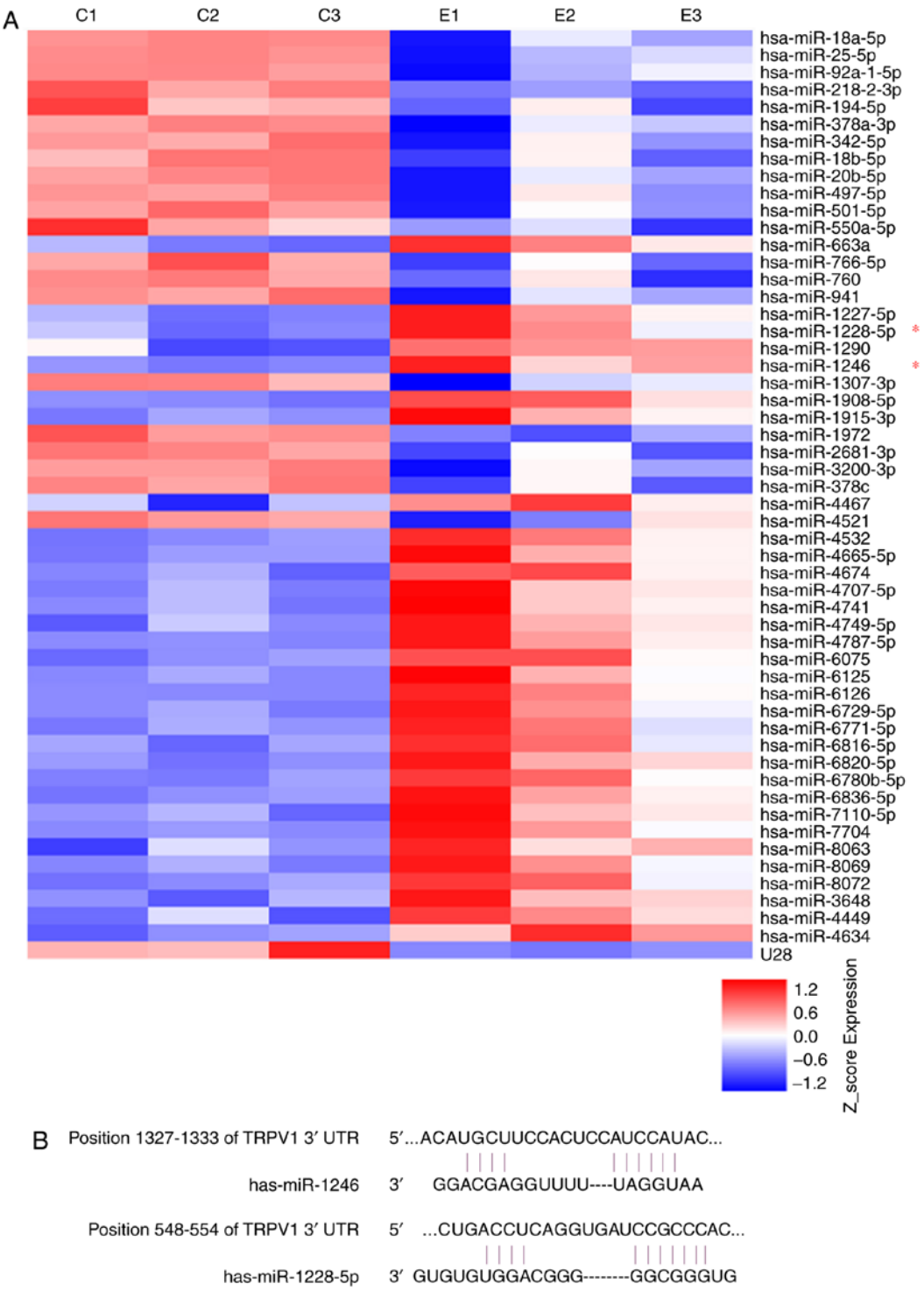

Figure 3. Effect of ^{125}I radiation on the miRNA profiling and the target region of the TRPV1 mRNA 3'UTR determined by TargetScan software analysis. (A) The heatmap of the significant various miRNAs including 32 upregulated and 22 downregulated in the SK-N-SH cells treated with ^{125}I radioactive seeds at the time-point of 60 h (equivalent to 6 Gy). The criteria were FC≥2, P≤0.05. C1, C2, and C3 were the control groups; E1, E2 and E3 were the treatment groups. (B) The upregulated miR-1246 and miR-1228-5p were predicted to target the TRPV1 mRNA 3'UTR as determined by TargetScan software analysis (http://www.targetscan.org/vert_71/). ^{125}I, iodine-125; TRPV1, transient receptor potential vanilloid-1; 3'UTR, 3'-untranslated region.

These data revealed that the effect of ^{125}I radiation on TRPV1 expression was dependent on the ^{125}I radiation dose, and the downregulated mRNA level was continuous at all radiation doses, while the downregulated protein level only occurred at lower doses (2.63 and 4.27 Gy), and then returned to normal at the intermediate dose (5.09 Gy), but further revealed a downregulatory effect at higher doses (5.91 and 6.73 Gy).

Effect of ^{125}I radiation on miRNA profiling. According to the aforementioned results, the time-point of 60 h/2.63 Gy-^{125}I radiation was selected for miRNA profiling in SK-N-SH cells. As revealed in Fig. 3A, 32 miRNAs such as miR-1246 and miR-1228-5p were significantly upregulated and 22 miRNAs downregulated based on the criterion (FC≥2, P≤0.05). As revealed in Fig. 3B the miR-1246 and

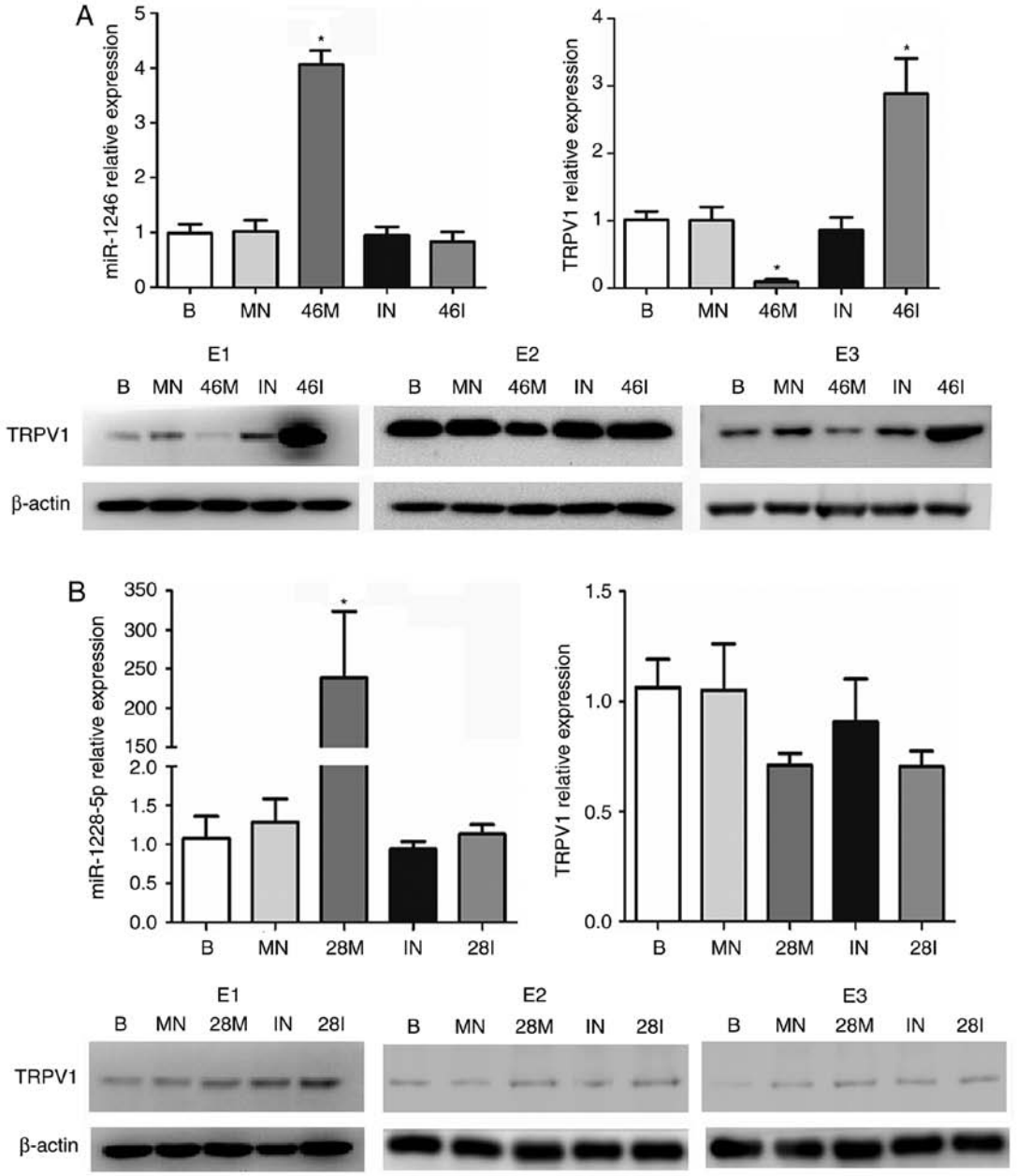

Figure 4. miR-1246, but not miR-1228-5p, downregulates the expression of TRPV1. SK-SY-SH cells were transfected with mimics or inhibitors of (A) miR-1246 or (B) miR-1228-5p. The transfection efficiencies were detected by miR-1246 or miR-1228 expression. Both mRNA and protein levels of TRPV1 were determined. The experiments were dependently repeated three times (E1, E2 and E3). B, blank; MN, mimics negative; 46M, miR-1246 mimics; IN, inhibitor negative; 46I, miR-1246 inhibitor; 28M, miR-1228-5p mimics; 28I, miR-1228-5p inhibitor; *P<0.01, compared with the corresponding negative group. TRPV1, transient receptor potential vanilloid-1.

miR-1228-5p were predicted to target the TRPV1 mRNA 3'UTR by the TargetScan software. Therefore, both miR-1246 and miR-1228-5p miRNAs were selected to validate their function on their regulation of TRPV1 gene expression.

TRPV1 expression is downregulated by miR-1246 but not miR-1228-5p. To validate the effect of miR-1246 and miR-1228-5p on TRPV1 expression, both SK-N-SH and SK-N-BE(2) cells were transfected with mimics or inhibitors of miR-1246 or miR-1228. Blank groups did not undergo any treatment; mimics NC and inhibitor NC were used as controls. As revealed in Fig. 4 for SK-N-SH cells and Fig. 5 for SK-N-BE(2) cells, the transfection of miR-1246 mimic significantly downregulated and miR-1246 inhibitor upregulated the expression of TRPV1 respectively, while neither the miR-1228-5p mimic nor the inhibitor had any effect on the expression of TRPV1.

miR-1246 regulates TRPV1 expression by targeting TRPV1 3'UTR. To explore the mechanism of miR-1246 on the regulation of TRPV1 expression, pGL3-REPORT vectors containing wild-type TRPV1 3'UTR (wild) and corresponding mutant-type (mutant) were constructed, and then transfected into both SK-N-SH and SK-N-BE(2) cells. As revealed in Fig. 6A for SK-N-SH cells and Fig. 6B for SK-N-BE(2) cells, miR-1246 mimics significantly downregulated and miR-1246 inhibitor upregulated luciferase activity, respectively.

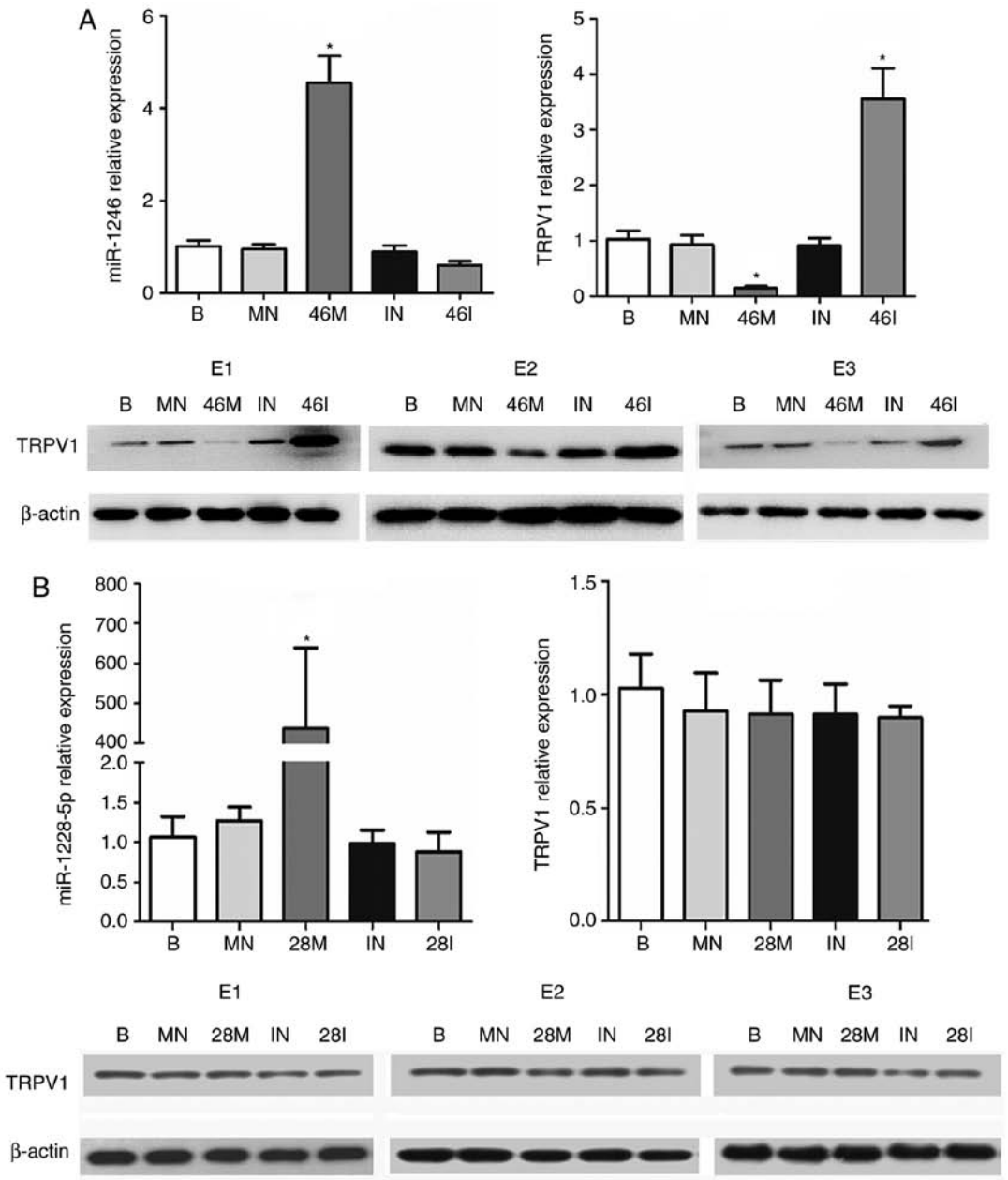

Figure 5. miR-1246, but not miR-1228-5p, downregulates the expression of TRPV1. SK-N-BE(2) cells were transfected with mimics or inhibitors of (A) miR-1246 or (B) miR-1228-5p. The transfection efficiencies were detected by miR-1246 or miR-1228 expression. Both mRNA and protein levels of TRPV1 were determined. The experiments were dependently repeated three times (E1, E2 and E3). B, blank; MN, mimics negative; 46M, miR-1246 mimics; IN, inhibitor negative; 46I, miR-1246 inhibitor; 28M, miR-1228-5p mimics; 28I, miR-1228-5p inhibitor; *P<0.01, compared with the corresponding negative group. TRPV1, transient receptor potential vanilloid-1.

Discussion

TRPV1 is a non-selective cation channel activated by capsaicin (13). It is expressed in human dorsal root ganglia (DRGs), brain, kidney, pancreas, and many other crucial organs (14). TRPV1 expression is also widely distributed in visceral innervation of all organs, and the upregulated expression of TRPV1 is closely correlated with the degree of visceral pain (6,15). The importance of TRPV1 in visceral innervation is also supported by the pain-inducing effects of capsaicin application in several animal models and human studies (16).

Researchers have demonstrated that pancreatic pain has a complicated relationship with TRPV1 (6,7,17), and thus the present study focused on the mechanism of pain in PC. miRNAs are universally involved in the development of tumors, including PC. Previous studies have revealed that miR-1246 aberrant expression is widely involved in many types of cancers (18-24). In PC, the plasma exosome miR-1246 was revealed to be significantly elevated in patients with intraductal papillary mucinous neoplasms (IPMN) (25); increased expression of miR-1246 was detected in pancreatic stellate cells (26), and aberrantly expressed in serum-exosomes (27).

In the present study, it was revealed that ^{125}I treatment could enhance miR-1246 expression, thus downregulating the expression of TRPV1, which plays a key role in pancreatic

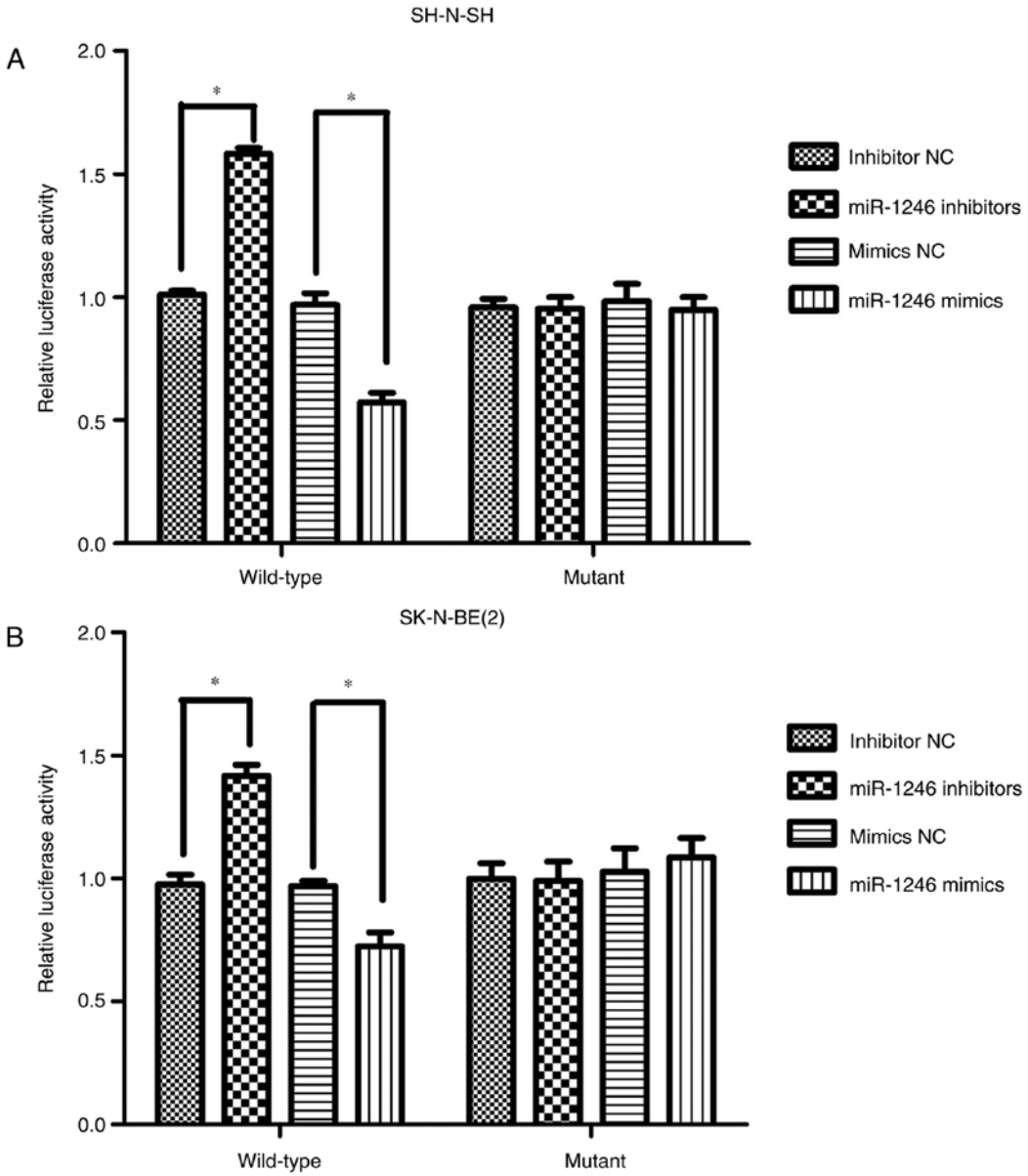

Figure 6. miR-1246 regulates TRPV1 expression by targeting TRPV1 3'UTR. (A) SK-N-SH and (B) SK-N-BE(2) cells were transfected with mimics or inhibitors of miR-1246. Mimics NC and inhibitor NC were used as controls and transfected with wild-type or mutant pGL-TRPV1 BS luciferase report plasmids. In both cell lines, miR-1246 mimics transfection reduced the luciferase activity, while miR-1246 inhibitor transfection upregulated the luciferase activity. *P<0.01. The experiments were dependently repeated three times. Wild, the report plasmids contained the wild-type TRPV1 3'UTR BS; Mutant, the report plasmids contained the wild-type TRPV1 3'UTR BS; 3'UTR, 3'-untranslated region; NC, negative control; BS, binding site.

pain. Concurrently, it was also demonstrated that miR-1246 regulated TRPV1 expression by binding to its 3'UTR. Thus, by targeting miR-1246, an effective treatment for pain in PC patients may have potentially been revealed. In contrast, it was surmised that the downregulated expression of miR-1246 in neurons around pancreatic tissues may be involved in the mechanism causing sustained pain in PC patients.

In the present study, a different effect of ^{125}I radiation on TRPV1 expression was observed between the mRNA and protein. The downregulated mRNA level was continuous at all radiation doses, while the downregulated protein level occurred at lower and then higher doses. It was thus proposed that the undulation of TRPV1 expression was dependent on the radiation dose; in particular, the return to a normal level at an intermediate dose was due to radiation hormesis (28), and was subjected to some type of post-translational regulation over the 140-h irradiation treatment.

It should be noted that there were several limitations in the present study. Although the human neuroblastoma cell lines SK-N-SH and SK-N-BE(2) are closely correlated to the sympathetic nervous system, they are not the same as nerves in PC tissues. In addition, further tests should validate that the TRPV1 abundance regulated by miR-1246 may be sufficient to induce the change of the cation channel activity and the pain in cells and an animal model. With regard to the translational outcomes of these results to clinical experiments in the future, although some studies revealed that miR-1246 promoted angiogenesis in colorectal cancer (29), enhanced cell migration and invasion in hepatocellular carcinoma (22), and promoted tumor progression in cervical (30) and lung cancer (31), the

effect of miR-1246 on pancreatic ductal adenocarcinoma has not been intensively studied. Administration of miR-1246 should weigh the advantages of pain release and disadvantages of cancer progression.

In conclusion, ^{125}I radiation upregulated miR-1246, which downregulated the expression of TRPV1, a key molecule involved in PC pain. The knowledge of this novel mechanism promises a new strategy for pain release in clinical practice.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

DZ, HX and YW collaboratively performed almost all the experiments and the acquisition, analysis, or interpretation of the data, and they were equally contributed to this study. YW and JZ participated in the experiments of the detection of the cell viability and the expression of TRPV1 and miRNAs. LP and BW participated in the experiments of the vector constructions and luciferase reporter assay. Both of KW and JG designed the study, and KW drafted the work and integrated any part of the work appropriately, and JG revised the paper critically for important intellectual content. ZL contributed to the design of the study and was in charge of the agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy and gave the final approval of the version to be published. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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