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Original Article

MiR-32 induces radio-resistance by targeting DOC-2/DAB2 interactive protein and regulating autophagy in gastric carcinoma

Xiao-Yu Wu[1*], Xue-Quan Yao[1*], Zhen-Feng Wu[1], Che Chen[1], Jia-Yun Liu[1], Guan-Nan Wu[1], Fu-Kun Liu[1], Gang Li[2]

[1]Department of Surgical Oncology, Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, Nanjing 210029, China; [2]Department of General Surgery, Jiangsu Cancer Hospital, The Affiliated Cancer Hospital of Nanjing Medical University, Nanjing 210009, China. [*]Equal contributors.

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Abstract: The aberrant expressions of microRNAs have been proved in many solid tumors. MAiR-32 is an oncomiR in gastric cancer (GC); however, the mechanism by which miR-32 functions as a regulator of radiotherapy response and resistance in GC patient is completely uncovered. In the present study, we revealed that DOC-2/DAB2 interactive protein (DAB2IP), the miR-32-dependent tumor-suppressor gene, was down-regulated and induced autophagy, as well as inhibited radiotherapy-induced apoptosis in GC cells. miR-32 expression was up-regulated in GC, and miR-32 inhibited DAB2IP expression through a direct binding site within the DAB2IP 3' un-translated region. MiR-32 mimics enhanced survival and decreased radio-sensitivity, which were reversed by miR-32 inhibitor. Flow cytometric analysis revealed that over-expressed miR-32, consistent with the DAB2IP-knockdown results, reduced ionizing radiation (IR)-induced cell apoptosis, which was restored by 4 nM brefeldin A treatment. More significantly, the over-expression of miR-32 and the knock-down of DAB2IP enhanced autophagy in the IR-treated GC cells. MiR-32 regulated the expression of autophagy-related proteins, such as DAB2IP, Beclin 1 and Light chain 3β I/II, phosphorylation of S6 kinase, as well as mammalian target of rapamycin (mTOR). In conclusion, these data provide novel insights into the mechanisms governing the regulation of DAB2IP expression by miR-32 and their possible contribution to autophagy and radio-resistance in GC.

Keywords: MiR-32, DAB2IP, autophagy, radioresistance, gastric cancer

Introduction

Gastric cancer (GC) is the most frequently diagnosed malignant tumor and the most common death-leading cancers [1, 2]. Current medical treatment for GC ranges from close monitoring for indolent disease to radical treatments, such as surgery or radiation therapy (RT). RT provides excellent local control and increased overall survival rates for GC. However, a significant number of high-risk gastric carcinoma patients will fail therapy, develop resistance and eventually succumb to the disease [3]. With an increased level of knowledge with regard to biomarkers and their effect on therapeutic response, in the future, clinical physicians may be able to personalize care based on a patient's molecular biomarkers.

DAB2 interacting protein (DAB2IP) is a novel member of the Ras GTPase-activating protein family and is down-regulated, with growth inhibitory and apoptosis enhancing activities, in GC [4]. Down0regulation of DAB2IP, mainly due to epigenetic regulation, inversely correlates with tumor grade and predicts GC progression [5]. DAB2IP is a unique scaffold protein that modulates a variety of biological activities, including tumor cell growth, apoptosis and survival via the phosphoinositide 3-kinase-Akt (PI3K-Akt), Wnt-epithelial-mesenchymal transition, ASK-JNK, Ras-mitogen-activated protein kinase, and nuclear factor-κB pathways in GC [6-8]. By knocking down endogenous DAB2IP levels, GC cells could gain proliferative potential and become resistant to stress-induced apoptosis. Previous studies have shown that DAB2IP plays an important role in the radio-resistance and chemo-resistance of GC [4]. DAB2IP regulates clusterin gene expression via cross-talk between Wnt/β-catenin and insulin-like growth

factor-I (IGF-I)/IGF receptor signaling in metastatic castration-resistant GC [9]. DAB2IP loss has been shown to result in resistance to IR due to enhanced DSB repair and apoptosis resistance [10]. Recently, a novel function of DAB2IP was shown in suppressing IR-induced and DNA-PKcs-associated autophagy, and promoting apoptosis in GC cells [11]. However, the regulatory mechanism of DAB2IP on the radioresistance in GC has not been well clarified. Thus, an increased level of knowledge with regard to the molecular mechanisms of DAB2IP in GC therapy resistance could aid in the identification of significant novel therapeutic targets for advanced disease.

MicroRNAs, an abundant class of ~22-nucleotide small non-coding RNAs, post-transcriptionally regulate gene expression through binding to multiple target mRNAs [12-14]. Extensive GC miRNA profiling has shown that a number of miRNAs are differentially expressed between GC and adjacent normal tissues, thus contributing to GC progression. Therefore, understanding the molecular mechanisms by which these miRNAs act in the deregulation of cellular signaling in GC cells may assist in the development of improved therapeutic strategies disease treatment. To date, however, the mechanism behind the involvement of the miRNA-dependent DAB2IP pathway in the radio-resistance of GC has not been investigated. In the present study, the regulatory effect of miR-32 on GC cell survival and apoptosis was determined during radiotherapy, and the involved pathways were analyzed.

Materials and methods

Cells and specimens

GC cell lines (MKN-28 and SGC7901) and the human gastric epithelial cell line (GES-1) were grown in T medium (Invitrogen Life Technologies, Carlsbad, CA, USA) with 5% fetal bovine serum (HyClone, Hudson, NH, USA) at 37°C in a 5% CO_2 humidified chamber. Human GC tissues, adjacent non-tumor tissues (located 2.5 cm from the tumor) and normal GC tissues were obtained from patients diagnosed with GC in Department of Surgical Oncology, Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (Nanjing, China). The specimens were obtained after surgical resection and immediately frozen at -80°C until use. The study methodologies conformed to the standards set by the Declaration of Helsinki. Collection and usage of all specimens were approved by the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (Nanjing, China).

miRNAs and transfection

The *Homo sapien* (has)-miR-32 mimics, has-miR-32 inhibitor and has-miR-32 scrambled negative control were obtained from GeneCopoeia, Inc. (Rockville, MD, USA). miR-32 mimics and miR-32 inhibitor were used to increase and decrease the expression of miR-32, respectively. miRNA transfection was performed using Lipofectamine 2000 transfection reagent. In brief, cells were plated in a 24-well plate and incubated overnight to achieve 80-90% confluence at the time of transfection. In each well, 5 ml miRNA was added to 50 ml Opti-MEM® (31985070; Gibco Life Technologies, Carlsbad, CA, USA). Separately, 2 ml Lipofectamine 2000 transfection reagent was added to 50 ml Opti-MEM and mixed gently. The transfection complex was added to the cells and incubated for 6 h at 37°C in a 5% CO_2 incubator, after which the serum-containing medium was replaced.

To confirm the effect of the miRNAs on the expression of miR-32, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to determine the mRNA expression levels of miR-32 in the GC cell lines. The RT-qPCR primers (hsa-miR-32-5p; cat. no. HmiRQP0404) were obtained from GeneCopoeia, Inc. and the cycling conditions were as follows: Step 1, 95°C for 30 min; and step 2, 40 cycles of 95°C for 15 sec then 58°C for 35 sec. The mRNA copy number results obtained were recalculated per 1 mg total RNA. The transfected cells were the expanded and harvested for the following further analyses.

IR treatment

Cells were irradiated in ambient air using a cesium-137 source at 2 Gy at room temperature for 24 h.

Luciferase reporter assay

The cells were lysed in passive lysing buffer and then analyzed for firefly and Renilla luciferase activities using the commercial Dual-

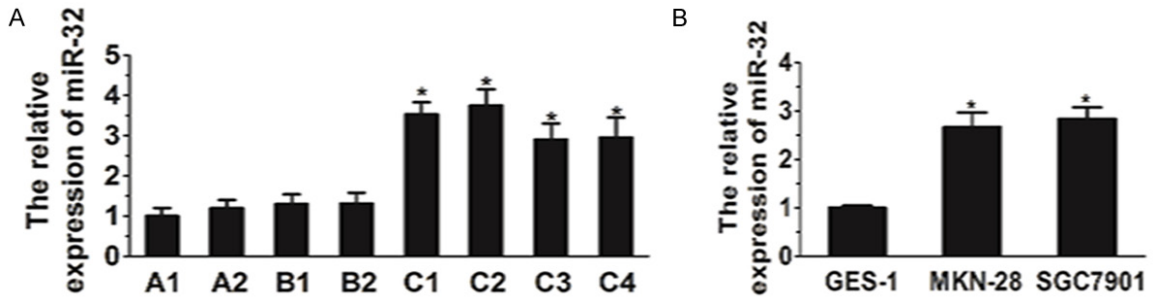

Figure 1. Expression of miR-32 is upregulated in gastric cancer (GC). A. The expression level of miR-32 in normal tissues (A1 and A2), matched adjacent non-tumor tissues (B1 and B2) and GC tissues (C1, C2, C3 and C4) were detected by reverse transcription-quantitative polymerase chain reaction. U6 small nuclear RNA was used as an endogenous control. The miR-32 levels were significantly upregulated in the GC tissues. B. The relative expression level of miR-32 in the normal gastric cell line and two GC cell lines. miR, microRNA.

Luciferase Reporter Assay System (E1910; Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Firefly luciferase activity was normalized to the Renilla luciferase activity.

Western blot analysis

Whole cell extracts were prepared with a cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions, and then the protein was quantified by a bicinchoninic acid assay (Pierce, Rockford, IL, USA). Next, the protein samples were separated by 10% SDS-PAGE and detected using western blot analysis. Mammalian target of rabbit anti-human polycolonal rapamycin (mTOR), phosphor-mTOR (pmTOR, S2448), phospho-S6 kinase (pS6K, T389), Light chain 3β (LC3B), Beclin 1 and DAB2IP antibodies were purchased from Cell Signaling Technology Inc.. Mouse anti-actin monoclonal antibody was purchased from Sigma-Aldrich. Fluorescent dye-conjugated secondary antibodies were obtained from Invitrogen Life Technologies.

RNA isolation and RT-qPCR

Total RNA was extracted using TRIzol (15596-026; Invitrogen Life Technologies), according to the manufacturer's instructions, and then reverse transcribed for quantification using the TaqMan microRNA Reverse Transcription kit (4366596; Applied Biosystems Life Technologies, Foster City, CA, USA) according to the manufacturer's instructions. Mature miRNAs were quantified using 2-step TaqMan RT-qPCR with the TaqMan microRNA kit. The miRNA expression level was normalized using U6 small nuclear RNA (HmiRQP9001) as an internal control.

Cell survival and apoptosis assay

The cells were diluted serially to 5.0×10^5 cells per 60 mm-diameter well and plated into dishes (area, 2,827.43 mm^2) in triplicate. Cells were pretreated with brefeldin A (BFA) to a final concentration of 4 nM. After 3 h of incubation at 37°C in a 5% CO_2 atmosphere, the cells were treated with increasing doses of IR, to a total dose of 2 Gy. After 0 to 48 h, cell viability was estimated by MTT assay [15]. Cell apoptosis was assessed using the ANXA5-FITC Apoptosis Detection kit (556570; BD Pharmingen, San Diego, CA, USA) by flow cytometric analysis, as previously described [16].

Statistical analysis

Each experiment was repeated at least three times. Data are shown as the mean ± standard deviation, and were analyzed using SPSS 18.0. Statistical comparisons between groups were analyzed using the t-test and two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Results

Increased expression of miR-32 in GC

The present study examined the expression of miR-32 in normal prostate tissues, human GC tissues and adjacent non-tumor tissues, as well as two GC cell lines (MKN-28 and SGC7901)

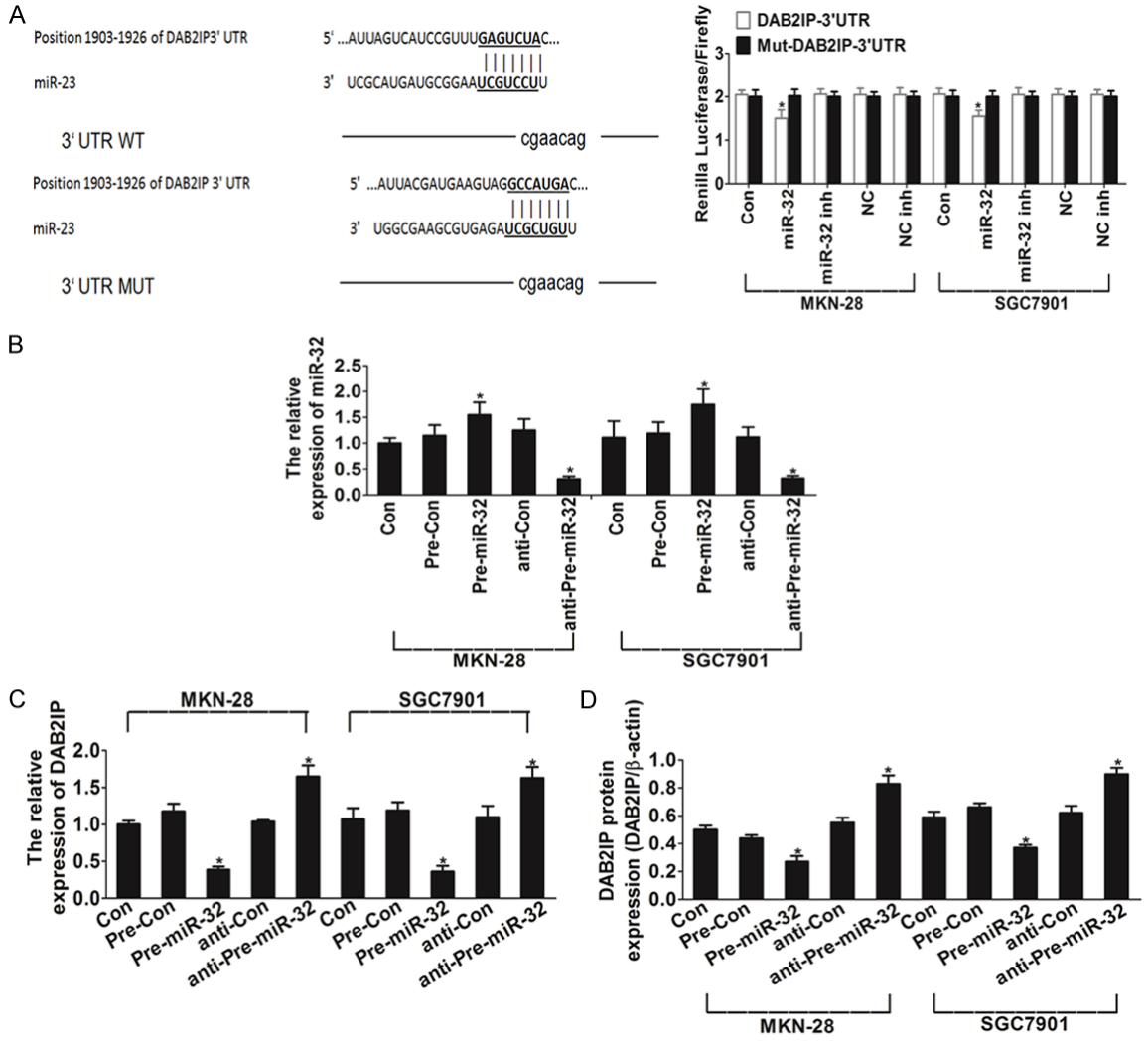

Figure 2. MiR-32 targets DAB2IP. A. Schema representing the functional interaction between miR-32 and the seed sequence (bold) in the 3'-UTR of DAB2IP, as predicted by TargetScan. Luciferase assay of MKN-28 and SGC7901 cotransfected with reporter constructs containing DAB2IP 3'-UTRs with (DAB2IP-3'-UTR) or without (Mut-DAB2IP-3'-UTR) miR-32 binding sites and miR-32 mimic, miR-32 inhibitor or scrambled control miRNA for 72 h. B, C. The level of miR-32 was assayed by TaqMan reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In parallel, the mRNA level of DAB2IP was assayed by qRT-PCR. D. The level of DAB2IP protein expression was assayed by western blotting. MKN-28 and SGC7901 cells were transfected with inhibitor or mimic of miR-32 or inhibitor control or mimic control (100 nM) for 72 h. Error bars represent the standard deviation from the mean. **P*<0.01 vs. control. miRNA, microRNA; DAB2IP, DAB2 interacting protein; NC, negative control of miR-32 group; NC inhibitor, negative control of miR-32 inhibitor group; UTR, untranslated region; con, control; pre-miR-32, miR-32 mimic; pre-con, mimic control; anti-miR-32, miR-32 inhibitor; anti-con, inhibitor control.

and a normal gastric cell line (GES-1), using RT-qPCR. MiR-32 expression was frequently upregulated or overexpressed in the GC tissues and cell lines compared with the normal tissues and gastric cell line (**Figure 1**).

DAB2IP is a target of miR-32 in GC cells

DOC-2/DAB2IP is important in the development of radioresistance in GC through the induction of autophagy. To investigate the diverse and complex network of upstream signaling pathways of DAB2IP-mediated autophagy in radioresistance in GC, the present study screened the candidate miRNAs that may target DAB2IP using TargetScan (www.targetscan.org) and miRanda (www.microrna.org) software. miR-32 was shown to pair well with and target the 3' un-translated region (3'-UTR) of DAB2IP, with strong evidence provided by the

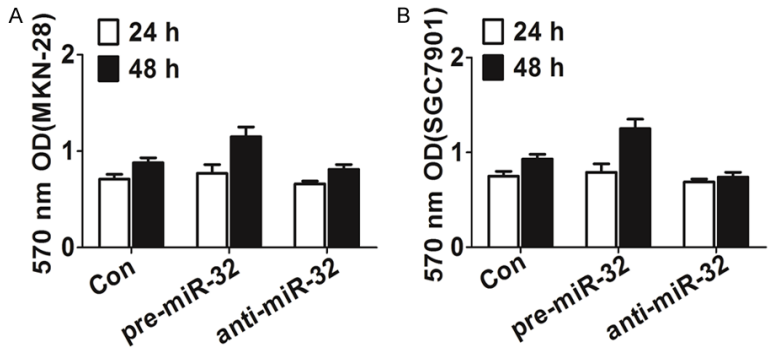

Figure 3. MiR-32 decreases the radiosensitivity in MKN-28 and SGC7901 cell lines. MTT analysis of (A) MKN-28 and (B) SGC7901 cells following combined treatment with miR-32 mimic/inhibitor and IR as indicated. Error bars represent the standard deviation from the mean. **P*<0.01 vs. control. miR, microRNA; OD, optical density. con, control; pre-miR-32, miR-32 mimic; anti-miR-32, miR-32 inhibitor.

reporter assay and RT-qPCR of the present study (**Figure 2A**). To further analyze the targeted regulation of miR-32 on DAB2IP in the IR-treated GC cells, the miR-32 level was manipulated by miR-32 mimic and inhibitor transfection. The miR-32 level was significantly elevated in the miR-32 mimic group, but significantly decreased in the miR-32 inhibitor group of the GC MKN-28 and SGC7901 cell lines (**Figure 2B**). The miR-32 mimic transfection significantly reduced the DAB2IP expression at the mRNA (**Figure 2C**) and protein (**Figure 2D**) levels in the MKN-28 and SGC7901 cells. These results confirmed that miR-32 regulated DAB2IP by targeting its 3'-UTR and suppressing its translation.

MiR-32 contributes to the radio-resistance of GC cells

To test the effect of miR-32 on the radio-sensitivity of GC cells, MKN-28 and SGC7901 cells were employed. As shown in **Figure 3**, the silencing of endogenous miR-32 resulted in a significant radiation-sensitizing effect in the MKN-28 and SGC7901 cells. By contrast, the overexpression of miR-32 increased the radiation resistance of the MKN-28 and SGC7901 cells (**Figure 3**). These *in vitro* results indicated that miR-32 can increase the resistance of MKN-28 and SGC7901 cells to radiation.

MiR-32 inhibits apoptosis by modifying DAB2IP expression

DAB2IP-knockdown cells were resistant to radiation-induced apoptosis in GC. The present study investigated whether miR-32 mimic treatment and the suppression of DAB2IP expression by endogenous DAB2IP knocked down the resistance of GC to radiotherapy. GC cells were treated with 2 Gy IR for 24 h. As shown in **Figure 4A**, cell apoptosis was significantly up-regulated in the miR-32 deficient group (anti-miR-32) and down-regulated in the miR-32 overexpression group (pre-miR-32). The GC cells were further subjected to IR combined with an apoptosis promoter, BFA. Flow cytometry assay clearly showed that BFA treatment restored radiation-induced cell death when miR-32-overexpressing GC cells and BFA-treated miR-32-overexpressing GC cells were compared. Similar to miR-32 overexpression, DAB2IP-knockdown treatment reduced apoptosis (**Figure 4C**). BFA (4 nM) could restore cell apoptosis in the DAB2IP-deficient cells. On the basis of these results, we propose that the increased resistance to IR in miR-32-overexpressing and DAB2IP-knockdown GC cells may be partially due to the inhibition of apoptosis.

MiR-32 induces autophagy via the Mtor-S6K pathway

Given that DAB2IP acts as an autophagy inhibitor in radio-resistant GC cells, the present study investigated the function of miR-32 as a regulator of GC autophagy. Beclin1 and LC3B are autophagy-related markers and are critical for regulating autophagy. LC3B exists in a cytosolic form, LC3B-I, and a form that is conjugated to phosphatidylethanolamine, LC3B-II [17, 18]. Increased LC3B-II levels are closely associated with the number of autophagosomes and serve as a good indicator of autophagosome formation [19]. To investigate the role of miR-32 in autophagy, the expression levels of the microtubule-associated proteins Beclin1 and LC3B were determined. As shown in **Figure 5**, silenced miR-32 impaired the IR-mediated induction of LC3B-II in the GC cells. In addition, DAB2IP-knockdown decreased the expression of LC3B-I and increased the expression of LC3B-II, which were revised by miR-32 inhibitor (**Figure 6A**). Moreover, the higher expression of

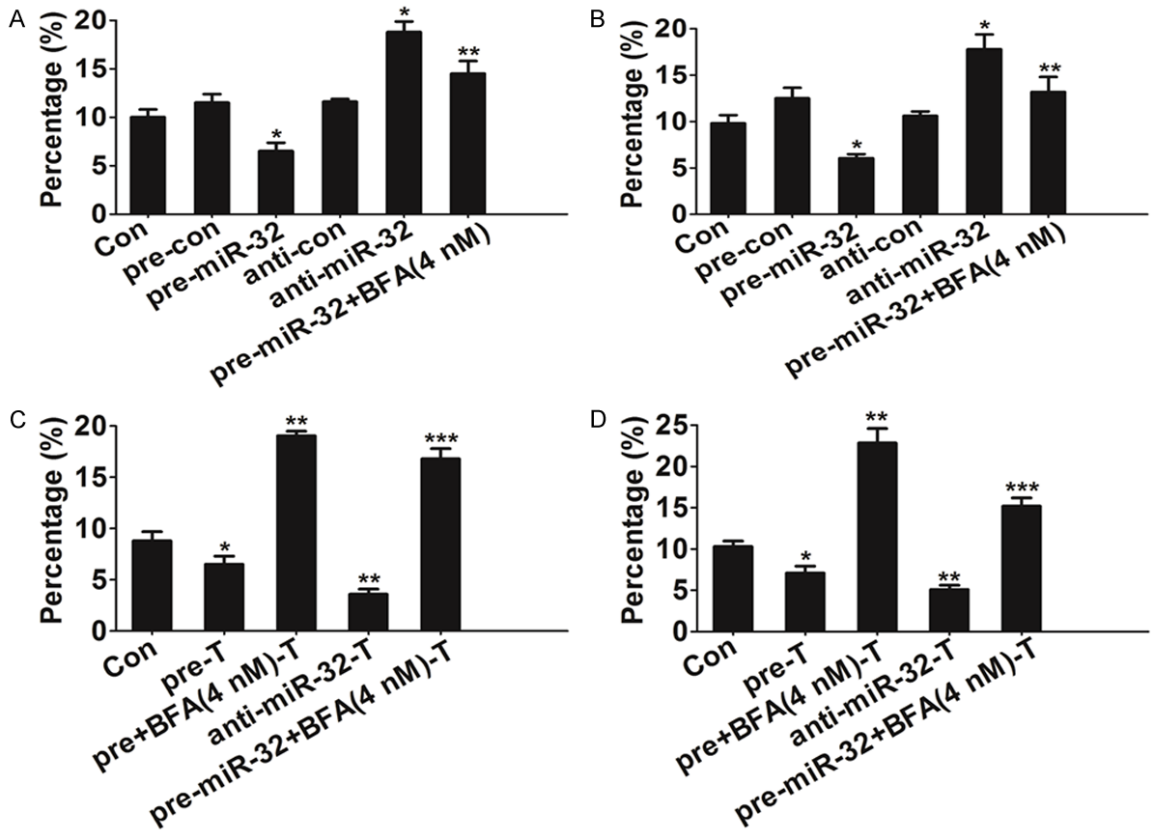

Figure 4. MiR-32 reduces apoptosis through suppression of DAB2IP expression. Apoptosis was analyzed by flow cytometry. (A) MKN-28 and (B) SGC7901 cells were transfected with miR-32 mimic (pre-miR-32), mimic control (pre-con), miR-32 inhibitor (anti-miR-32) and inhibitor control (anti-con). **P*<0.01 vs. control; ***P*<0.01 vs. pre-miR-32 group. (C) MKN-28 and (D) SGC7901 cells were treated with TALEN (T) knockout of DAB2IP. **P*<0.01 vs. control; ***P*<0.01 vs. Con-T group; ****P*<0.01 vs. pre-miR-32-T group and Con+BFA (4 nM)-T group. Error bars represent the standard deviation from the mean. miR, microRNA; DAB2IP, DAB2 interacting protein; BFA, brefeldin A.

Beclin 1 protein was noted in the miR-32 overexpressing and DAB2IP-knockdown GC cells (**Figure 6B**).

It has been reported that the Akt-mTOR pathway negatively regulates autophagy [20]. To investigate how miR-32 regulated IR-induced autophagy in the GC cells, the phosphorylation of mTOR was measured in the two cell lines. **Figure 5** shows the marked inhibition of phosphorylated mTOR in miR-32-silenced cells. Furthermore, studies have shown that S6K is a critical downstream effector of the mTOR signaling pathway [21]. In the present study, increased phosphorylation of S6K was observed in the miR-32-overexpressing GC cells and decreased phosphorylation of S6K was ob served in the miR-32-silenced GC cells (**Figure 5**). Although mTOR-S6K activation is known to suppress autophagy in mammalian cells, emerging studies have indicated that, in certain situations, the mTOR-S6K pathway positively regulates autophagy [22, 23]. Together, the findings indicate that the miR-32 may promote IR-induced autophagy through the mTOR-S6K pathway.

Discussion

GC is the second most frequent diagnosed cancer and one of the most common death-leading cancers in the world. Current medical management for localized GC ranges from close monitoring for indolent disease to treatments such as radiotherapy, chemotherapy or surgery. Treatment with radiation therapy has the advantages of being non-invasive and well tolerated. However, radiotherapy resistance is also a common occurrence and contributes to the failure in blocking disease progression [24].

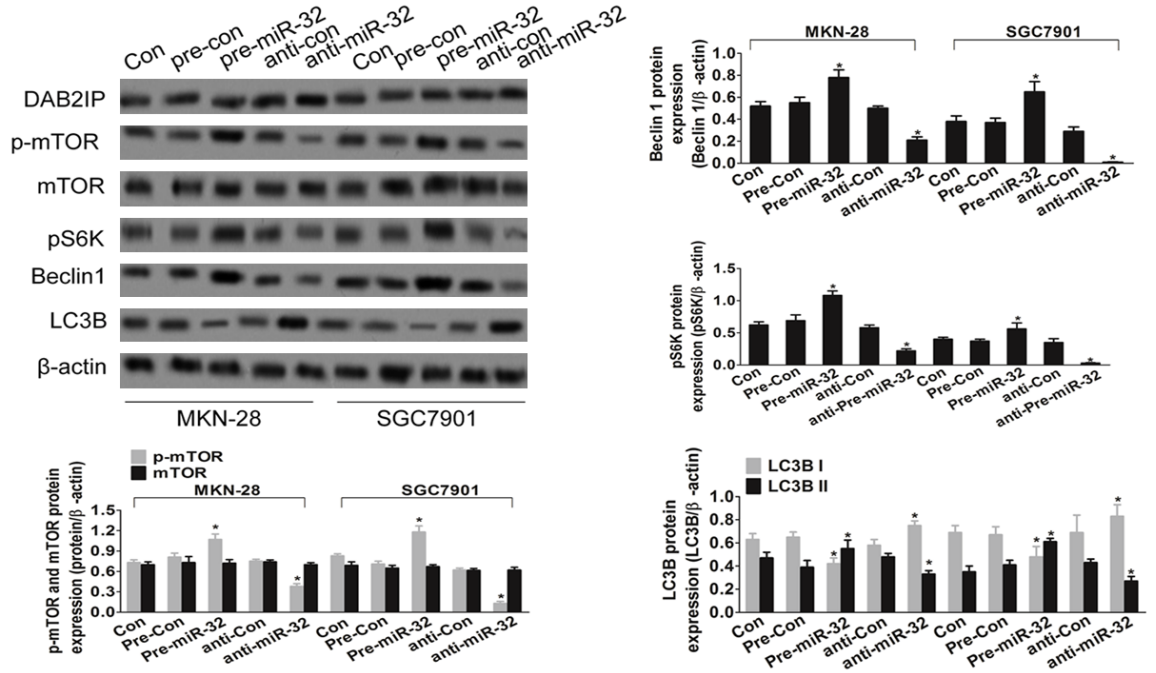

Figure 5. miR-32 induces autophagy via the mTOR-S6K Pathway. The phosphorylation of mTOR and S6K and the expression of autophagy-associated Beclin 1, mTOR, S6K and LC3B were determined by Western blot analysis at 24 h post-IR (2 Gy). **P*<0.01 vs. control. **P*<0.01 vs. control. Error bars represent the standard deviation from the mean. ***P*<0.01 vs. Con-T group. miR, microRNA, p-, phosphorylated; mTOR, mammalian target of rapamycin; S6K, S6 kinase; LC3B, light chain 3β; T, TALEN; pre-miR-32, miR-32 mimic; pre-con, mimic control; anti-miR-32, miR-32 inhibitor; anti-con, inhibitor control.

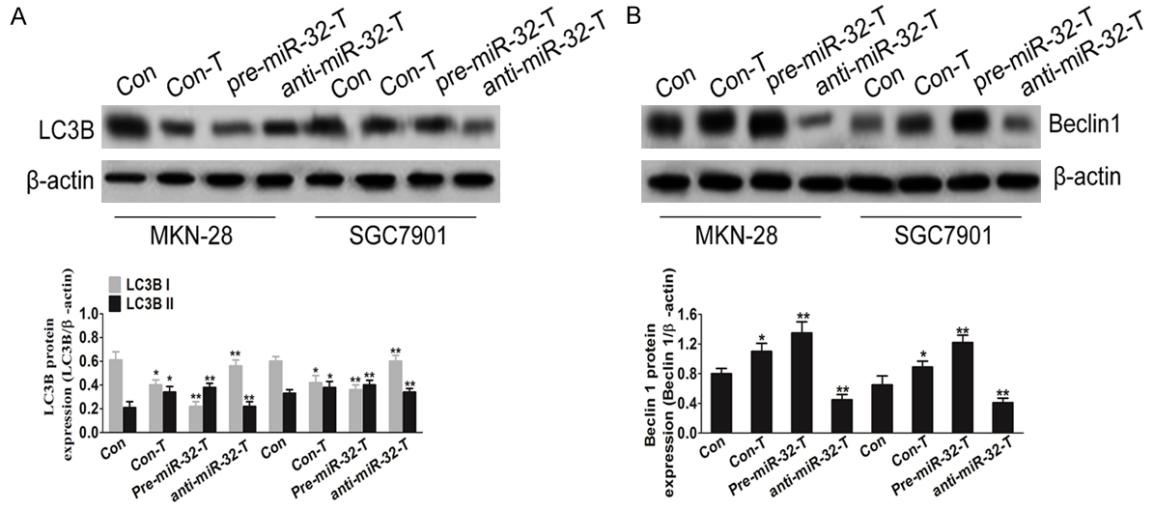

Figure 6. DAB2IP regulates autophagy-associated proteins (A) LC3B and (B) Beclin 1. **P*<0.01 vs. control. Error bars represent the standard deviation from the mean. ***P*<0.01 vs. Con-T group. miR, microRNA, p-, phosphorylated; mTOR, mammalian target of rapamycin; S6K, S6 kinase; LC3B, light chain 3β; T, TALEN; pre-miR-32, miR-32 mimic; pre-con, mimic control; anti-miR-32, miR-32 inhibitor; anti-con, inhibitor control.

Accumulating studies have evaluated the resistance mechanisms and biological factors that are involved [25]. Autophagy is an intracellular self-protective mechanism that functions by preventing the toxic accumulation of damaged components and by recycling these compo-

nents to sustain metabolic homoeostasis [26]. Up-regulated autophagy has been identified in a wide variety of cancer cells that undergo metabolic and therapeutic stress, and the process contributes to the resistance to chemotherapy by a range of tumor types [27]. The blocking of autophagy in cancer cells is emerging as a novel approach to enhance the sensitivity of therapy in cancers [28]. DAB2IP, a potential tumor suppressor gene, is often down-regulated in GC primarily due to altered epigenetic regulation of its promoter. The loss of DAB2IP expression in GC cells greatly increases radiation resistance *in vitro*, and the overexpression of DAB2IP suppresses IR-induced autophagy and promotes apoptosis in GC cells. However, the regulation of DAB2IP, particularly by miRNAs, remains largely unknown. MiRNAs have been detected in association with cancer diagnosis and prognosis as promising biomarkers and regulators of tumor proliferation, invasion, migration and apoptosis. MiR-218, as a tumor-suppresser, inhibits cancer cell migration and invasion via the targeting of SMO in GC [29]. MiR-20a promotes the invasion and migration of GC via the targeting of EGR2 [30]. miR-124 exhibits anti-proliferative and anti-aggressive effects on GC cells via PI3K/Akt and STAT3 signaling pathway [31]. These miRNAs often function as oncogenes by repressing tumor suppressors or function as suppressors by negatively regulating oncogenes, indicating the potential effects on the prognosis and clinical application to GC therapy. The present study established for the first time the important role played by miR-32 in inhibiting the expression of the DAB2IP tumor suppressors in GC. It was demonstrated that miR-32 was well paired with the 3'-UTR of DAB2IP. Functional analysis demonstrated that GC cells post-DAB2IP blockage by miR-32 were more resistant to IR treatment, with increased cell proliferation and reduced cell apoptosis.

DAB2IP mediated the radio-sensitization of GC cells partially through the inhibition of autophagy. DAB2IP was involved in the autophagy pathway and overexpression of this gene attenuated IR-induced autophagy. In the present study, it was observed that LC3B and Beclin 1 were up-regulated in the GC cells with silenced DAB2IP and overexpressed miR-32. The mTOR-S6K pathway is postulated to be a negative regulator of mammalian autophagy [15]. The mTOR-S6K pathway was recorded as inactivated in GC cells with down-regulated DAB2IP expression. Activated mTOR induced mTOR complex 1 substrate S6K phosphorylation, which resulted in the induction of the functional protein translational machinery [32]. Consistent with this study, the present results showed that the phosphorylation of S6K was increased in the miR-32-overexpressed GC cells. Therefore, we speculate that miR-32 enhanced the radioresistance of the GC cells by promoting DAB2IP-related autophagy via the mTOR-S6K pathway.

In conclusion, the present study demonstrated that miR-32 directly targeted DAB2IP in GC, and induced DAB2IP-deficient radioresistant human GC cells. Moreover, the findings demonstrated the critical role of miR-32 in inhibiting the mTOR-S6K pathway and suppressing autophagy by targeting DAB2IP. On the basis of these results, miR-32 appears to be a novel tumor promoter and plays an important role in radiotherapy resistance during the treatment of GC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Gang Li, Department of General Surgery, Jiangsu Cancer Hospital, The Affiliated Cancer Hospital of Nanjing Medical University, Nanjing 210009, China. E-mail: gangli2016@sina.com

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