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MicroRNA-3607 inhibits the tumorigenesis of colorectal cancer by targeting DDI2 and regulating the DNA damage repair pathway

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Abstract

Mutations in the DNA damage repair (DDR) pathway are frequently detected in colorectal cancer (CRC). The dysregulation of miRNAs, such as oncogenes or tumor suppressors, participates in CRC tumorigenesis. A previous study showed that low miR-3607 expression correlated with poor survival in prostate cancer patients, but its role in CRC remains unclear. In this study, we analyzed miR-3607 expression Pan-Cancer data from the NCI's Genomic Data Commons (GDC) and found that miR-3607 was downregulated in lymphatic invasion patients and in recurrent cancer and correlated with Pan-Cancer patient survival. Functional studies indicated that the overexpression of miR-3607 decreased CRC cell proliferation, migration and invasion. Additionally, we used gene set enrichment analysis (GSEA), Gene Ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and a protein–protein interaction network to demonstrate that miR-3607 affects the DDR pathway. Luciferase reporter and apoptosis assays confirmed that DNA damage inducible 1 homolog 2 (DDI2) is the functional target of miR-3607. Therefore, miR-3607 inhibits the tumorigenesis of CRC probably by suppressing the oncogene DDI2, and it might serve as a novel target for CRC prediction and therapy.

Keywords MiR-3607 · Colorectal cancer · DNA damage repair pathway · DDI2 · Apoptosis

Introduction

Colorectal cancer (CRC) is one of the most common cancers, with a higher global mutation burden and a higher mutation frequency in DNA damage repair (DDR) pathways [1–3]. DDR genes play vital roles in maintaining human genomic stability; therefore, DDR pathway dysfunction is a significant determinant of the tumorigenesis and the therapeutic response in general [3]. Interestingly, recent reports have also indicated that some DDR proteins could promote carcinogenesis [4–6].

MicroRNAs (miRNAs) are small noncoding RNAs that posttranscriptionally downregulate gene expression by targeting the 3′ untranslated region (3′-UTR) of mRNAs [7, 8]. As oncogenes or tumor suppressors, miRNAs participate in a variety of cancer types, including CRC [9]. A previous study showed that low miR-3607 expression correlated with poor survival in prostate cancer patients and that the overexpression of miR-3607 inhibited proliferation, apoptosis and invasion by targeting the SRC family kinases LYN and SRC [10]. The activation of SRC family kinases has been reported to be involved in the progression of a variety of cancers, including CRC [11, 12]. Elevated protein levels of SRC and the hyperactivation of LYN have been detected in CRC [13, 14], suggesting that miR-3607 might also be implicated in the tumorigenesis of CRC.

Recently, Chaudhry et al. showed that miR-3607 was downregulated at the 4-h, 12-h and 24-h time points after irradiation of the human lymphoblast cell line TK6 [15]. The result was confirmed by Niamh et al., who showed that miR-3607-5p was significantly decreased in the radioresistant prostate cancer cell line [16]. Irradiation-induced DNA damage triggers DDR [17, 18], and radioresistance is due to preferential activation of the DNA damage checkpoint,

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which promotes survival and repair in cancer stem cells [4]. The downregulation of miR-3607 suggests that the expression of potential target genes increases after irradiation, which might be involved in DDR.

In this study, we showed that the overexpression of miR-3607 decreased CRC cell proliferation, migration and invasion. Additionally, by bioinformatics analysis, we found that miR-3607 affected the DDR pathway. Luciferase reporter and apoptosis assays confirmed that DNA damage inducible 1 homolog 2 (DDI2) is the functional target of miR-3607. Thus, miR-3607 might serve as a new biological marker of CRC.

Materials and methods

Cell lines and transfection

Human CRC cell lines (SW480, HT-29, LoVo and HCT-116) were purchased from the American Type Culture Collection (ATCC) and cultured in complete medium recommended by the ATCC. MiR-3607 mimics, inhibitors and their corresponding negative controls were synthesized (GenePharma, China) and transfected at 100 nM concentration by X-tremeGENE siRNA Transfection Reagent (Roche, USA).

RNA extraction and quantitative RT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen, USA). MiRNA was reverse transcribed by a First Strand microRNA cDNA Synthesis Kit (Sangon, China). cDNA was synthesized with a PrimeScript RT Reagent Kit (Takara, Japan). A SYBR Premix Ex Taq II Kit (TAKARA, Japan) was used to perform quantitative real-time PCR (qRT-PCR) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). mRNA and miRNA levels were normalized to GAPDH and U6 RNA, respectively. The forward primer of miR-3607 was 5′-GCGCGCATGTGATGAAGCAAATC-3′, and the universal reverse primer and primers for U6 were provided in the microRNA cDNA Synthesis Kit (Sangon, China). The qRT-PCR conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s and 65 °C for 35 s.

Cell proliferation

Cell proliferation ability was evaluated using a Cell Counting Kit-8 (CCK8) assay and a colony formation assay. For the CCK8 assay, after 48 h of transfection, cells were seeded into 96-well plates at a density of 1500 cells per well and in 6 replicates. After 24 h, 48 h, 72 h, and 96 h, 10% CCK8 solution (Dojindo, Kyushu, Japan) was added to the cells and incubated for 3 h at 37 °C. The absorbance of viable cells was measured at a wavelength of 450 nm.

For the colony formation assay, 1000 transfected cells per well were seeded into 6-well plates. After 2 weeks, the cells were washed with PBS, fixed with 4% paraformaldehyde, and then stained with 0.1% crystal violet. The number of colonies (> 50 cells) was counted, and three independent experiments were performed for each result.

Migration and invasion assays

For the migration assay, Transwell chambers (Corning, USA) were seeded with 1.5×10^5 SW480 cells (in 200 µl of serum-free medium) in the upper chambers. Then, 800 µl of medium supplemented with 10% FBS was added to the bottom chambers and incubated at 37 °C for 48 h. For the invasion assay, the upper chambers were coated with 50 µl of Matrigel (BD Bioscience, USA). After fixation in 4% paraformaldehyde for 30 min, migrated cells were stained with 0.1% crystal violet. The number of cells was counted from six randomly selected fields. Three independent experiments were conducted for each result.

Western blot analysis

SW480 or HCT-116 cells were harvested after 72 h of transfection. Western blot assays were conducted as previously described [19]. Immunoblotting was carried out with anti-DDI2 (1:5000, Sigma, USA) or anti-GAPDH (1:5000, Abcam, USA) antibodies.

Dual-luciferase reporter assay

The DDI2-3′UTR fragment (328 bp) was constructed into the pmiRGLO dual-luciferase reporter plasmid (Promega, USA). The mutant plasmid (pmiR-DDI2-mut) was constructed by a KOD-Plus Mutagenesis Kit (Toyobo, Japan). SW480 cells were cotransfected with 500 ng of pmiR-DDI2-wt or pmiR-DDI2-mut and 50 nM miR-3607 mimics or a negative control using X-tremeGENE siRNA Transfection Reagent (Roche, USA) in a 24-well plate. After 48 h, cells were lysed using a Dual-Glo Luciferase Assay (Promega, USA) and measured by Luminoskan Ascent (Thermo, USA) according to the manufacturer's instructions.

Apoptosis detection assay

Cell apoptosis was assessed by flow cytometry with an Annexin V/PI Apoptosis Detection Kit (KeyGEN, Nanjing, China) following the manufacturer's instructions. After 72 h of transfection, HCT-116 cells were trypsinized, washed with PBS, and stained with annexin V and PI. Early and late apoptotic cells were detected by a

FACSCalibur flow cytometer (BD Bioscience, USA) and analyzed by FlowJo v10 software (Tree Star, OR, USA).

Bioinformatic analyses

The expression data of miR-3607 in Pan-Cancer and CRC were obtained from the UCSC Xena dataset (https://xena.ucsc.edu/). CRC samples were obtained from The Cancer Genome Atlas (TCGA) database to perform gene set enrichment analysis (GSEA). To identify the function of miR-3607, we used miRDB (http://www.mirdb.org/), TargetScan (http://www.targetscan.org/) and DIANA (http://diana.imis.athena-innovation.gr/DianaTools/) to predict potential target genes. The GO (Gene Ontology) biological processes of these target genes were analyzed by using DAVID analysis (https://david.ncifcrf.gov/). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/anno_iden.php). The STRING database (Version: 10.5) was used to predict protein–protein associations (https://string-db.org/) [20].

Statistical analysis

Data are shown as the mean ± standard error of the mean (SEM) and were analyzed by GraphPad Prism 6 (GraphPad Software Inc.). In cellular experiments of different groups, the significant differences were determined by a nonpaired *t* test. The correlation between the expression of miR-3607 and proliferative or mesenchymal markers was measured using Pearson's test. The standard of statistical significance was considered a P value < 0.05.

Results

MiR-3607 is involved in tumorigenesis

MiR-3607 expression data of Pan-Cancer (including 14 datasets, 27 types of cancer, 20,163 samples) from the NCI's Genomic Data Commons (GDC) were used to determine whether miR-3607 is involved in tumorigenesis. In the TNM staging system, miR-3607 is downregulated in T4 compared to T1 and in lymphatic invasion patients (**P < 0.01, ****P < 0.0001, Fig. 1a). Furthermore, patients with lymph node involvement counts of 30-170 showed decreased levels of miR-3607 compared to those with lymph node counts of 1-10 (****P < 0.0001, Fig. 1b). When we analyzed primary and recurrent cancer, we found that both recurrent blood-derived cancer and recurrent solid tumor showed lower expression of miR-3607 than primary cancer (****P < 0.0001, Fig. 1c). The results were consistent with the overall survival analysis, which illustrated that low expression levels of miR-3607 were related to poor survival (P < 0.0001, log-rank test, Fig. 1d). This evidence suggests that miR-3607 might be implicated in tumorigenesis but is not necessarily involved in the tumorigenesis of CRC.

Therefore, we further analyzed the correlation between miR-3607 and CRC in the TCGA database. Although the difference in TNM staging system was not significant (Fig. 1e), CRC patients with lymphatic invasion counts of 35-109 exhibited lower expression of miR-3607 compared to those with lymph node counts of 1-19 (*P < 0.05, Fig. 1f), suggesting that miR-3607 might participate in CRC cell lymphatic invasion. We further investigated whether miR-3607 was related to epithelial-mesenchymal transition (EMT) in CRC cells, which plays an important role in the metastasis of tumor cells [5]. A set of canonical EMT markers can be used to indicate an increased capacity for migration [6, 21] and CRC tumorigenesis (Fig. S1). MKI67, as a proliferative marker, has a positive relationship with tumor cell proliferation rates [22, 23]. As shown in Fig. 1g, miR-3607 was negatively correlated not only with the proliferative marker (MKI67) but also with EMT markers (VIM, FN1, TWIST1, ZEB1, and MMP9) in CRC patient samples. Together, these results show that miR-3607 might be involved in regulating the proliferation and migration of CRC.

MiR-3607 decreases CRC cell proliferation

In order to assess the role of miR-3607 in CRC proliferation we first analyzed the expression level of miR-3607 in CRC cell lines by qRT-PCR. The results indicated that miR-3607 was expressed in all 4 CRC cell lines, with relatively low expression in SW480 cell lines and high expression in HCT-116 cell lines (Fig. 2a). After we confirmed the transfection efficiency of miR-3607 mimics and inhibitors in SW480 cells (Fig. 2b, c), CCK8 and colony formation assays were used to assess the role of miR-3607 in CRC proliferation. For the CCK8 assay, the overexpression of miR-3607 mimics decreased CRC cell proliferation compared to the negative control (NC) (****P < 0.0001, Fig. 2d), while transfection of the miR-3607 inhibitors promoted cell proliferation (****P < 0.0001, Fig. 2e). The results also confirmed that miR-3607 negatively correlated with the proliferative marker MKI67 (*P < 0.05, Fig. 2f). For the colony formation assay, after 2 weeks of transfection, the number of colonies with miR-3607 mimics was significantly reduced compared to the NC (**P < 0.01, Fig. 2g). In contrast, the transfection of miR-3607 inhibitors promoted colony formation (***P < 0.001, Fig. 2h). These results indicate that miR-3607 suppresses CRC cell proliferation.

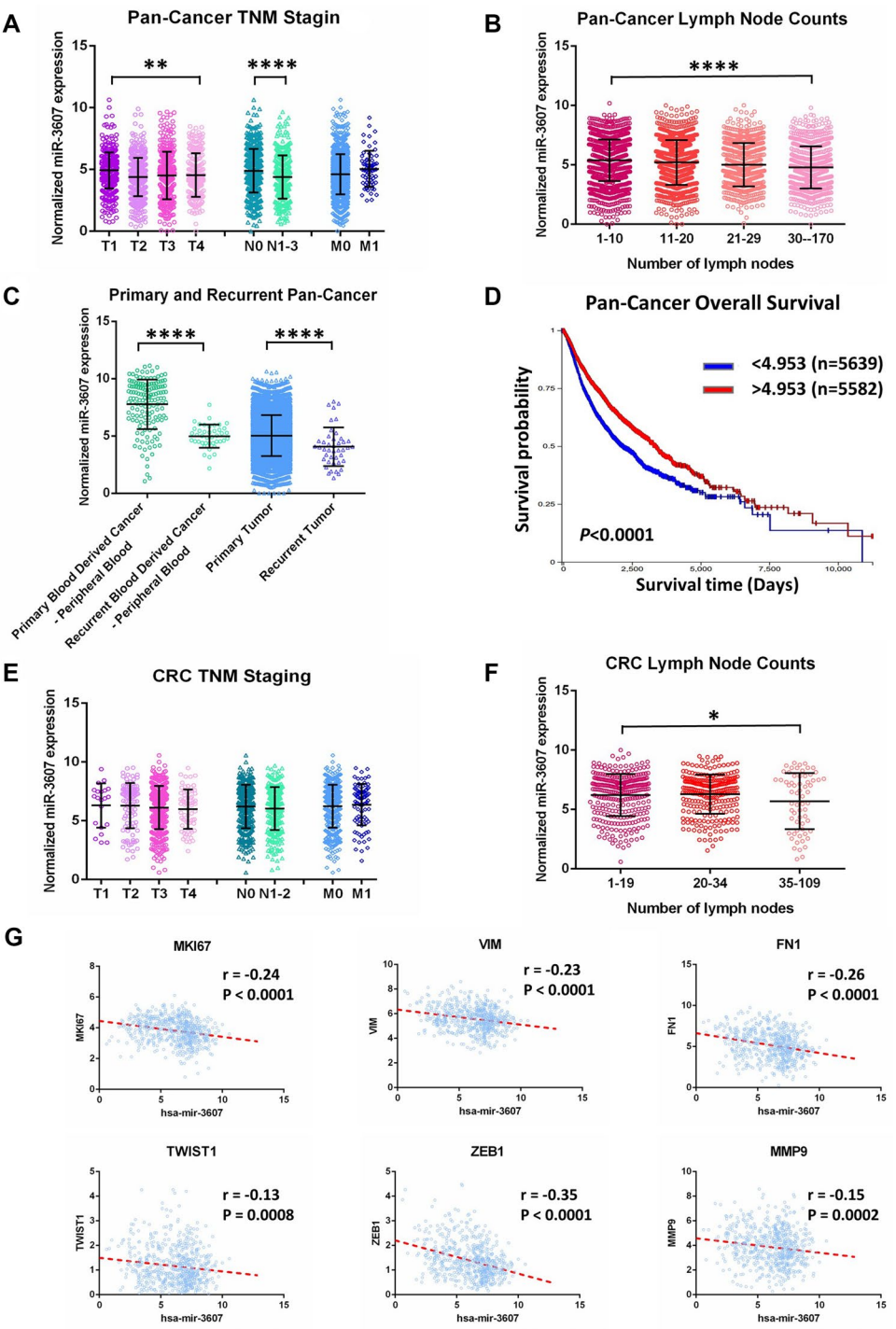

Fig. 1 MiR-3607 is involved in tumorigenesis. **a** MiR-3607 expression analysis in Pan-Cancer using the TNM staging system. MiR-3607 expression data were normalized to reads per million microRNA reads (RPM) and are shown as $\log_2(\text{RPM}+1)$. **b** MiR-3607 expression analysis in Pan-Cancer with different lymph node involvement counts. **c** MiR-3607 expression analysis in Pan-Cancer in primary and recurrent cancer patients. **d** Kaplan–Meier analysis of Pan-Cancer overall survival. Percent survival is shown on the y-axis, and time is shown on the x-axis. Patients were divided into two roughly equal groups according to miR-3607 expression ($\log_2(\text{RPM}+1)$). Patients with higher expression levels of miR-3607 (top, red) had significantly better survival than those with lower miR-3607 expression (bottom, blue) ($P<0.0001$ log-rank test). **e** MiR-3607 expression analysis in CRC using the TNM staging system. **f** MiR-3607 expression analysis in CRC with different lymph node involvement counts. **g** MiR-3607 negatively correlated with a proliferative marker (MKI67) and mesenchymal markers (VIM, FN1, TWIST1, ZEB1, and MMP9) in CRC. Expression data of markers were normalized to fragments per kilobase of transcript per million mapped reads (FPKM) and are shown as $\log_2(\text{FPKM}+1)$. All data are shown as the mean ± SEM, $^*P<0.05$, $^{**}P<0.01$, and $^{****}P<0.0001$ (Color figure online)

MiR-3607 represses CRC cell migration and invasion

We further assessed the role of miR-3607 in CRC cell migration and invasion by the transwell assay. The results showed that the overexpression of miR-3607 mimics in SW480 cells repressed both migration and invasion abilities compared to the negative control ($^{**}P<0.01$, Fig. 3a and $^{***}P<0.001$, Fig. 3b). Conversely, the suppression of miR-3607 promoted both migration and invasion ($^{**}P<0.01$, Fig. 3c, d). These results indicate that miR-3607 inhibits the migratory and invasive capacities of CRC cells.

MiR-3607 affects the DDR pathway in CRC

To further determine which biological processes miR-3607 affects in CRC, GSEA was performed. We downloaded the

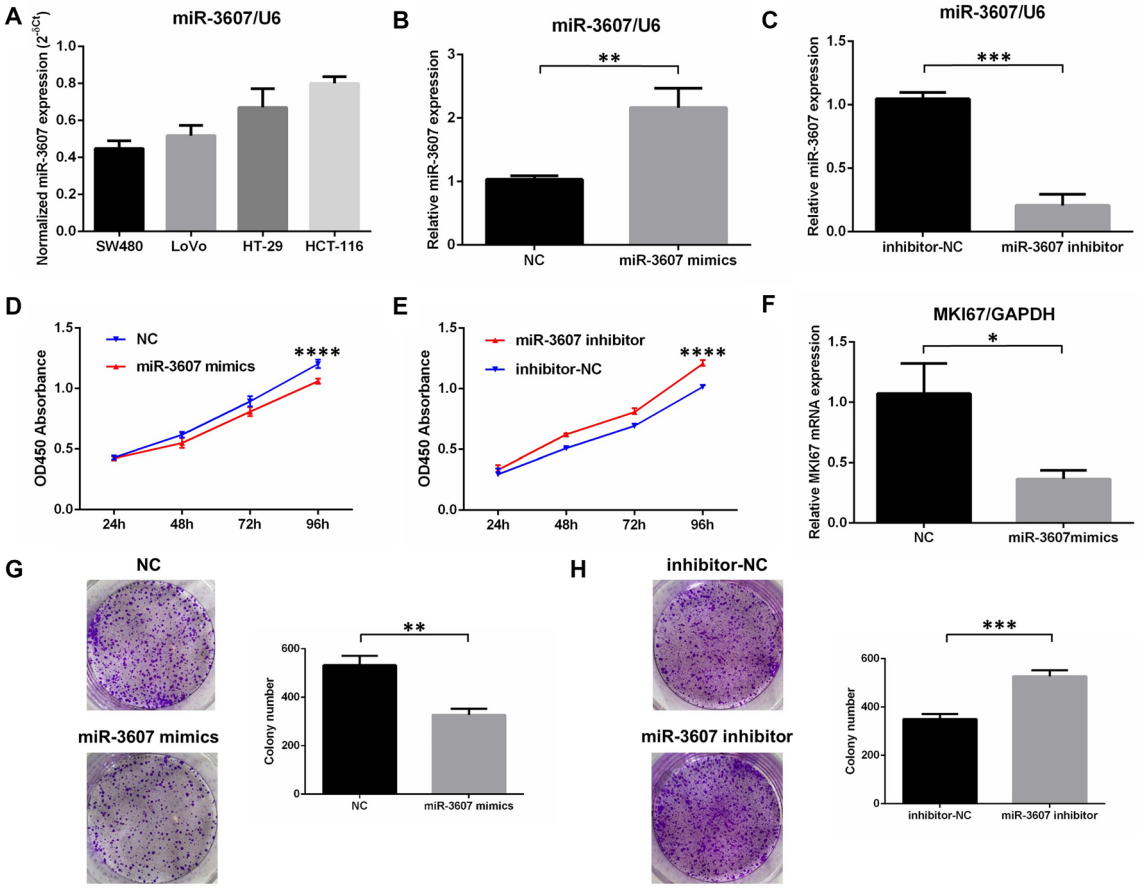

Fig. 2 MiR-3607 decreases CRC cell proliferation. **a** The expression level of miR-3607 in 4 CRC cell lines was analyzed by qPCR. MiR-3607 expression levels were calculated relative to the small RNA, U6, which served as an internal control. To quantify transcript levels, miR-3607 levels were normalized using the delta Ct method (ΔCt = Ct reference gene − Ct target gene). **b**, **c** Transfection efficiency of miR-3607 mimics and inhibitors in SW480 cells was analyzed by qPCR. The data were normalized to U6 and were expressed as fold increase relative to a negative control. **d**, **e** A CCK8 assay was used to assess the role of miR-3607 mimics and inhibitors in SW480 cell proliferation. **f** The mRNA level of MKI67 was analyzed by qPCR in SW480 cells with or without transfection of miR-3607 mimics. MKI67 expression levels were normalized to GAPDH, which served as an internal control, and were expressed as fold increase relative to a negative control. **g**, **h** A colony formation assay was used to assess the role of miR-3607 mimics and inhibitors in SW480 cell proliferation. All data are presented as the mean ± SEM (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001

CRC gene sets from the TCGA database and ranked samples according to the expression of miR-3607 from high to low. As shown in Fig. 4a, the gene sets "SESTO Response to UV" (P = 0.002) and "DAZARD UV Response Cluster" (P < 0.001) were significantly enriched in low levels of miR-3607, and "HAMAI Apoptosis via TRAIL (down)" (P = 0.04) was enriched in high levels of miR-3607. Considering that irradiation-induced DNA damage triggers DDR [17], these data suggest that miR-3607 might be involved in the regulation of the DDR pathway in CRC.

Furthermore, we predicted the potential target genes of miR-3607 based on DIANA, TargetScan and miRDB. A total of 126 overlapping potential target genes of miR-3607 were selected for GO and KEGG pathway analyses (Fig. 4b). The GO biological process analysis showed that miR-3607 participates in the regulation of cell shape, motility and the cell cycle (Fig. 4c). According to KEGG analysis, we found that the target genes of miR-3607 were mainly enriched in "Ubiquitin mediated proteolysis" and "Leukocyte transendothelial migration" (Fig. 4d). Altogether, the GO and KEGG pathway analyses suggest that miR-3607 might be associated with cancer cell migration, proliferation and DDR.

To predict functional associations between the 126 overlapping potential target genes of miR-3607, a protein–protein interaction network was constructed by the STRING database. Based on the interaction score, the key hub nodes were selected (Fig. 5a, red circle), which mainly participate in DDR and ubiquitin-protein transferase activity. Moreover, we analyzed the correlation between miR-3607 and these hub genes in CRC from the TCGA database. Pearson's correlation analysis showed that miR-3607 was negatively

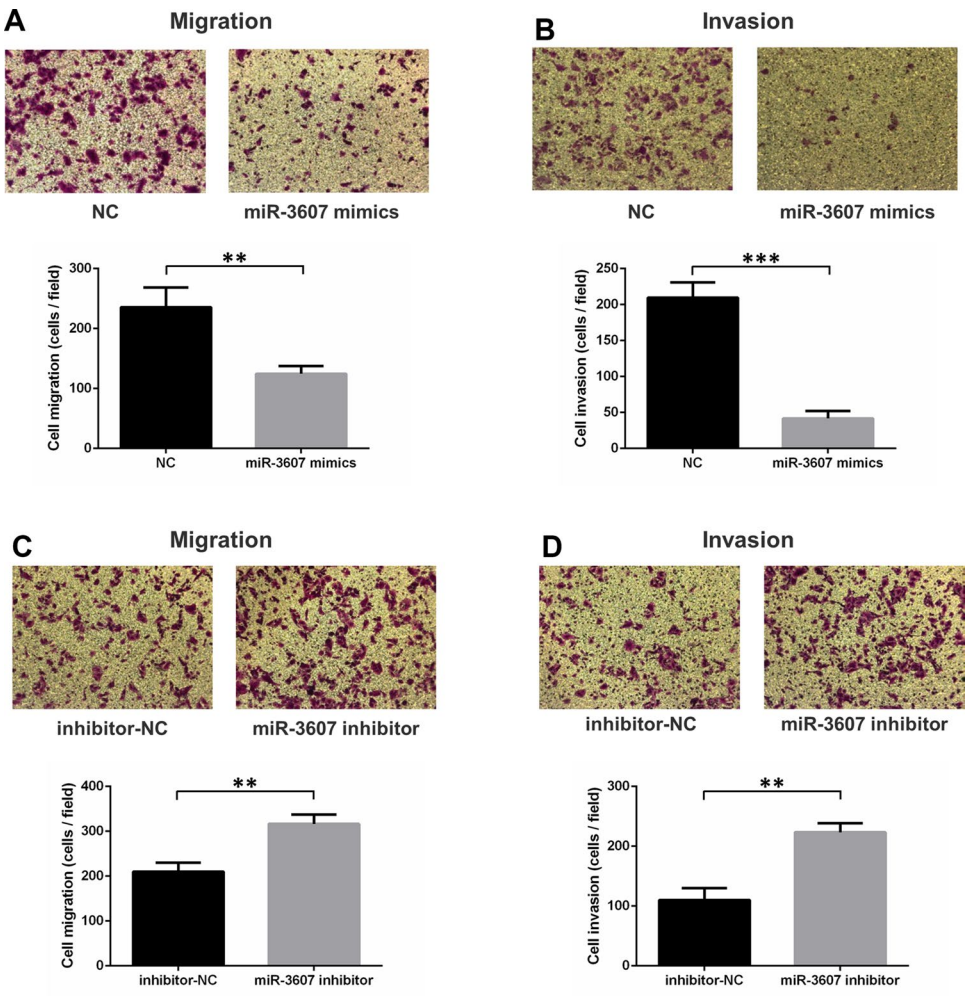

Fig. 3 MiR-3607 represses CRC cell migration and invasion. **a–d** Transwell assays were used to determine the role of miR-3607 mimics and inhibitors in SW480 cell migration and invasion. The overexpression of miR-3607 mimics in SW480 cells repressed both migration and invasion compared to the negative control, while the suppression of miR-3607 promoted both migration and invasion. All data are shown as the mean ± SEM (n = 3), **P < 0.01, ***P < 0.001

associated with cullin 3 (CUL3), ring finger protein 38 (RNF38), baculoviral IAP repeat-containing 6 (BIRC6), DNA damage inducible 1 homolog 2 (DDI2), ubiquitin-conjugating enzyme E2 D3 (UBE2D3), PDS5 cohesion-associated factor A (PDS5A), INO80 complex submit D (INO80D), and junction mediating and regulatory protein, p53 cofactor (JMY) (Fig. 5b).

DDI2 is a direct target of miR-3607

DDI2, which was negatively correlated with miR-3607 after the induction of DNA damage in our recent article (data unpublished), showed the most significant negative correlation with miR-3607 in CRC from the TCGA database ($r = -0.32$, Fig. 5b). Therefore, DDI2 was chosen to determine whether this DDR gene is a direct target of miR-3607. As shown in Fig. 6a, miR-3607 was predicted to complementarily bind to the 3′-UTR of DDI2 (DDI2-wt). We introduced reverse complementary sequence mutations into the seed sites of DDI2 (DDI2-mut). Luciferase activity was measured to determine whether miR-3607 is able to bind to the 3′-UTR of DDI2. The markedly decreased luciferase signal of DDI2-wt compared with the control confirmed that miR-3607 directly targets and downregulates DDI2 (*P < 0.05, Fig. 6b). Moreover, the overexpression of miR-3607 downregulated both the mRNA level and protein expression of DDI2 in SW480 cells. In contrast, the inhibition of miR-3607 increased the mRNA level of DDI2 (Fig. 6c). These results indicate that DDI2 is the direct target gene of miR-3607.

DDI2 could attenuate miR-3607-induced apoptosis

To further assess the contribution of DDI2 to the biological effects of miR-3607, the HCT-116 cell line, which exhibits relatively high miR-3607 expression, was selected to analyze whether the suppression of DDI2 could rescue the effect of the miR-3607 inhibitor. We first confirmed that the transfection of DDI2 siRNA (siDDI2) decreased the protein expression of DDI2 compared with the negative control (siNC) in HCT-116 cells (Fig. 7a). Then, we found that the inhibition of miR-3607 led to increased

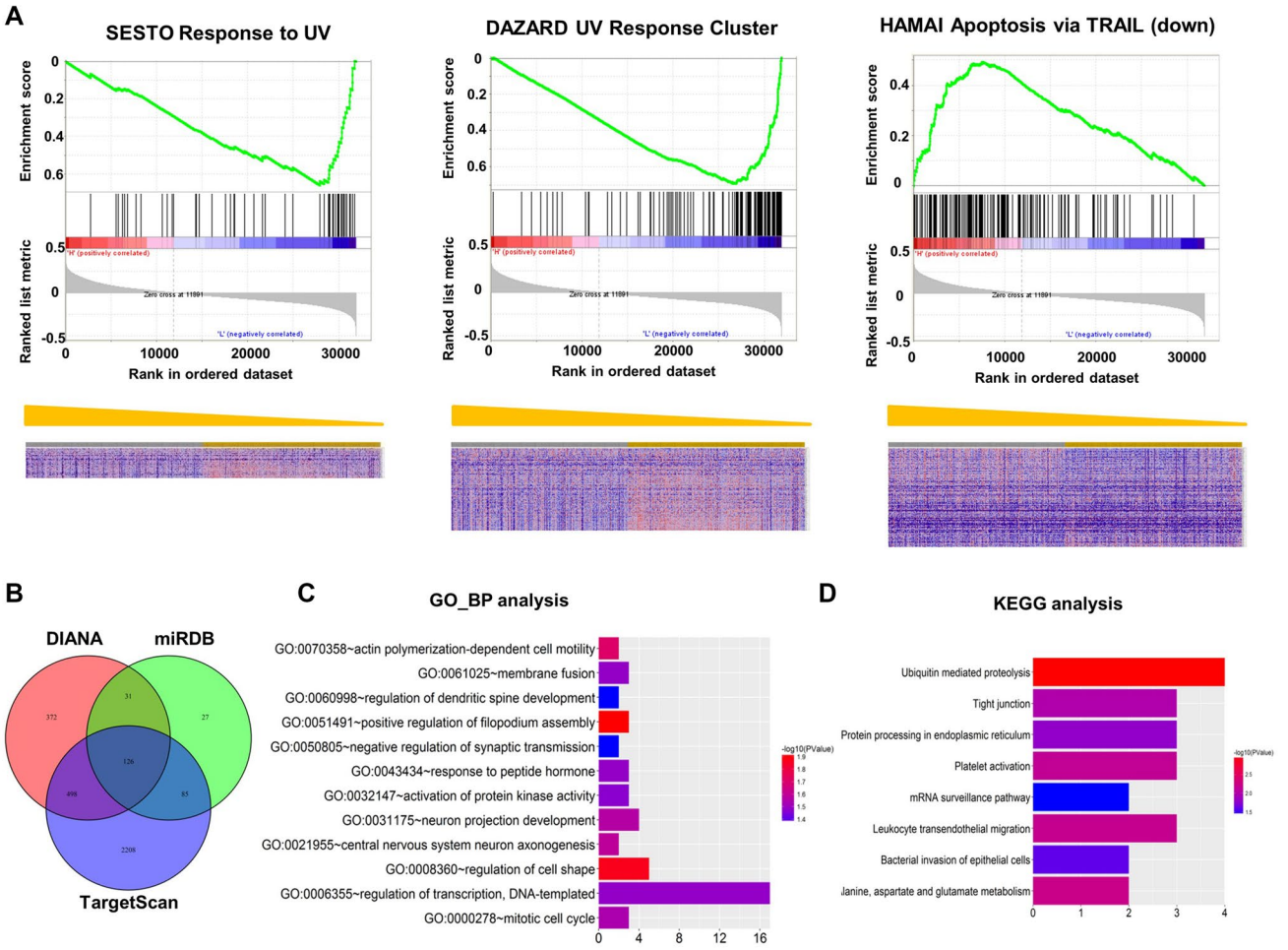

Fig. 4 MiR-3607 affects the DDR pathway in CRC. **a** The GSEA results showed a correlation between miR-3607 levels and the DDR signaling pathway in CRC. The gene sets "SESTO response to UV" (P = 0.002) and "DAZARD UV Response Cluster" (P < 0.001) were significantly enriched in low levels of miR-3607, and "HAMAI Apoptosis via TRAIL (down)" (P = 0.04) was enriched in high levels of miR-3607. **b** The Venn diagram shows the 126 overlapping potential target genes of miR-3607, which were predicted by DIANA, TargetScan and miRDB. **c**, **d** In total, 126 overlapping potential target genes of miR-3607 were selected for GO and KEGG pathway analyses. The color of the bar plot represents -$\log_{10}$(P value), and the number of genes is shown on the x-axis

DDI2 protein expression, while DDI2 levels were reversed by cotransfecting siDDI2 and the miR-3607 inhibitor in HCT-116 cells (Fig. 7a).

After showing that siDDI2 could reverse the DDI2 protein increase caused by the miR-3607 inhibitor, we measured cell apoptosis by flow cytometry. As shown in Fig. 7b, the overexpression of miR-3607 or the inhibition of DDI2 both promoted apoptosis compared with the NC group. The knockdown of both miR-3607 and DDI2 rescued miR-3607 inhibitor-induced apoptosis suppression. The effect of the miR-3607 inhibitor in suppressing cell apoptosis was attenuated by the presence of siDDI2. These results suggest that miR-3607 increases CRC cell apoptosis by downregulating DDI2 expression, further confirming the influence of miR-3607 in regulating DDR.

Discussion

The tumorigenesis of CRC usually lasts for decades, during which normal epithelial cells undergo malignant transformation to metastatic carcinomas [24]. In precancerous lesions, DNA damage activates DDR pathways, which, by inducing apoptosis, raises a barrier to tumorigenesis. If DDR is defective, it would cause genomic instability and, as a result, lead to CRC development [25]. Moreover, recent reports have revealed that some DDR proteins are overexpressed in various tumors and promote carcinogenesis [4–6].

It has been reported that miR-3607 plays a tumor suppressive role in prostate cancer [10]. In this study, we

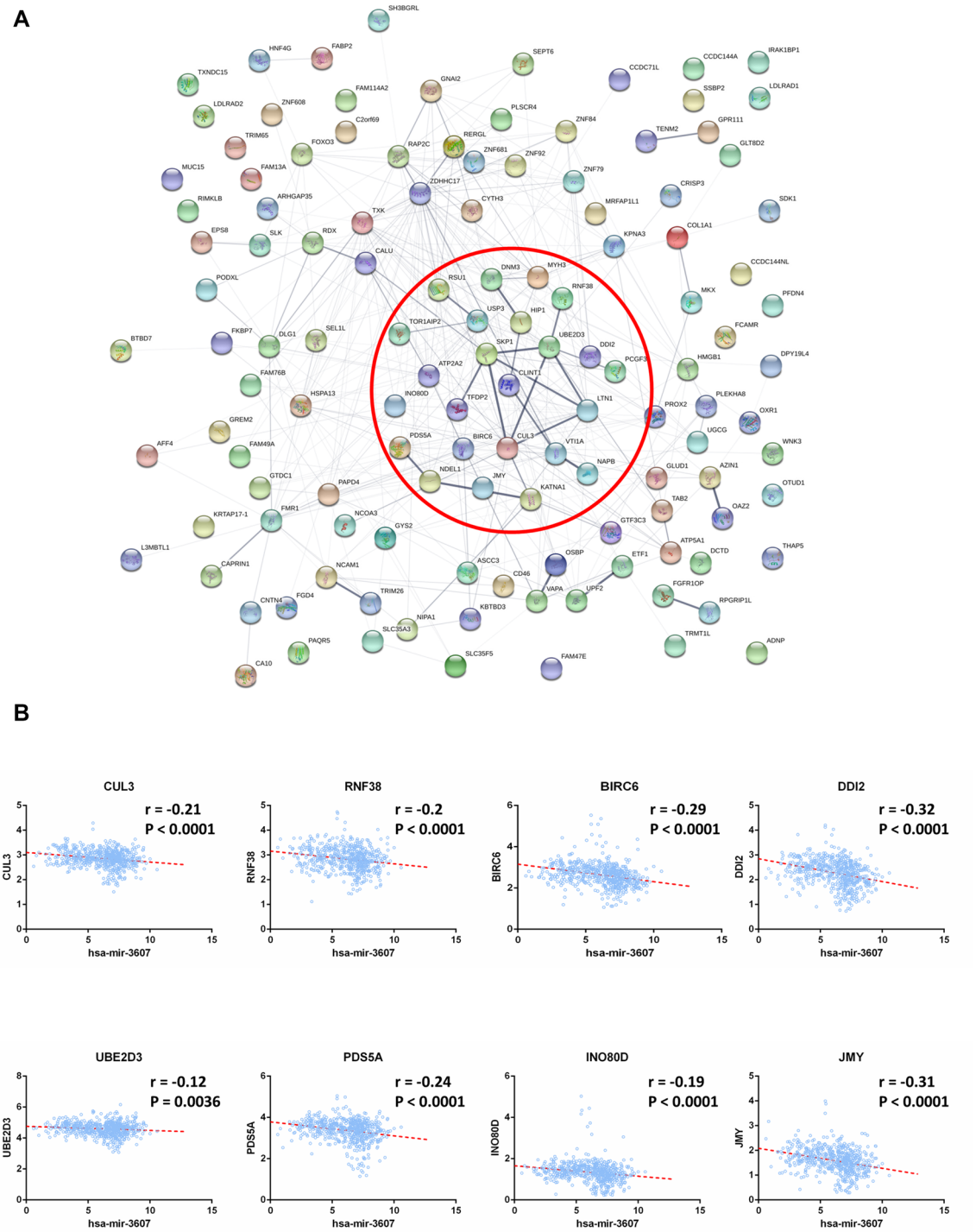

Fig. 5 MiR-3607 is negatively correlated with genes involved in the DDR pathway. **a** The protein–protein interaction network for the potential target genes of miR-3607 was constructed by the STRING database. The key hub nodes are shown in red circles. **b** Pearson's correlation analysis showed that miR-3607 was negatively associated with CUL3, RNF38, BIRC6, DDI2, UBE2D3, PDS5A, INO80D, and JMY. MiR-3607 expression data are shown as $\log_2(\text{RPM}+1)$, and expression data of the target genes are shown as $\log_2(\text{FPKM}+1)$ (Color figure online)

analyzed miR-3607 expression data from Pan-Cancer and found that miR-3607 is downregulated in T4 compared to T1, in lymphatic invasion, and in recurrent cancer. In summary, low expression levels of miR-3607 are related to poor survival in Pan-Cancer. Interestingly, although the difference in miR-3607 in CRC using the TNM staging

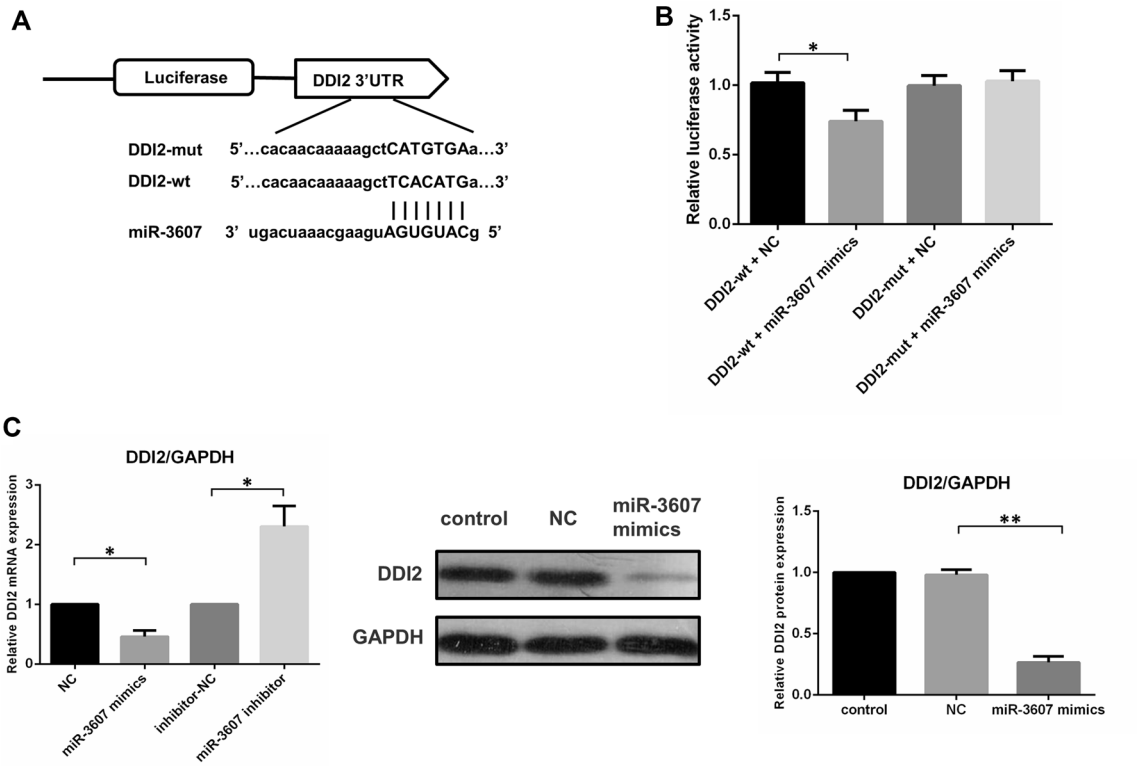

Fig. 6 DDI2 is a direct target of miR-3607. **a** Schematic illustration shows the miR-3607 binding site and the reverse complementary sequence mutations in the 3′-UTR of DDI2. **b** A luciferase activity assay was used to identify the miR-3607 binding site after 48 h of transfection. The results are expressed as normalized luciferase activity relative to DDI2-wt + NC. **c** The mRNA levels of DDI2 were determined in miR-3607 mimics- or inhibitor-transfected SW480 cells by qPCR. DDI2 mRNA levels were normalized to GAPDH and were expressed as fold increase relative to the negative control of the mimics or inhibitors. d The protein expression levels of DDI2 were analyzed after 72 h transfection with miR-3607 mimics or the NC in SW480 cells. Protein expression levels of DDI2 were normalized to GAPDH and were expressed as fold increase relative to the control. All data are shown as the mean ± SEM (n = 3), *P < 0.05, **P < 0.01

system was not significant, we found a negative correlation between miR-3607 and EMT markers in CRC patients. Furthermore, by CCK8, transwell and apoptosis detection assays, we confirmed that the overexpression of miR-3607 inhibited proliferation and invasion and promoted apoptosis in CRC cells.

Emerging studies have reported that miRNAs play a crucial role in the regulation of the DDR pathway [26–28]. It was recently reported that miR-3607 was significantly decreased in the radioresistant prostate cancer cell line [16] and downregulated at the 4-h, 12-h and 24-h time points after irradiation of the human lymphoblast cell line TK6 [15]. Radioresistance is due to preferential activation of the DNA damage checkpoint, which promotes survival and repair [4], and irradiation-induced DNA damage triggers DDR [17, 18]. This evidence suggests that the reduction in miR-3607 might promote DDR gene expression to induce DNA repair after irradiation. In this article, we also found that the gene sets "SESTO Response to UV" and "DAZARD UV Response Cluster" were significantly enriched in low levels of miR-3607. When we constructed the protein–protein interaction network for the potential target genes of miR-3607 by the STRING database, we found that the key hub nodes mainly participate in the DDR pathway. In addition, Pearson's correlation analysis confirmed that miR-3607 was negatively associated with CUL3, RNF38, BIRC6, UBE2D3, PDS5A, INO80D, JMY and DDI2. CUL3, which plays a major role in polyubiquitination, promotes CRC cell growth [3]. RNF38, an E3 ubiquitin ligase, induces gastric cancer cell growth and promotes the proliferation and metastatic capacity of non-small-cell lung cancer cells. [7, 22]. BIRC6, a member of the inhibitors of the apoptosis protein family, is a predictor of a poor prognosis in colorectal cancer [23]. UBE2D3, which functions in the ubiquitination of p53, is implicated in angiogenesis [5] and is increased in colon cancer [12]. PDS5, a cohesion protein, is important for mammalian development [11]. INO80D, an ATP-dependent chromatin remodeling gene, increases proliferation in renal cell carcinoma cells [6]. JMY, a p53 cofactor, predicts poor outcomes in esophageal adenocarcinoma patients [21].

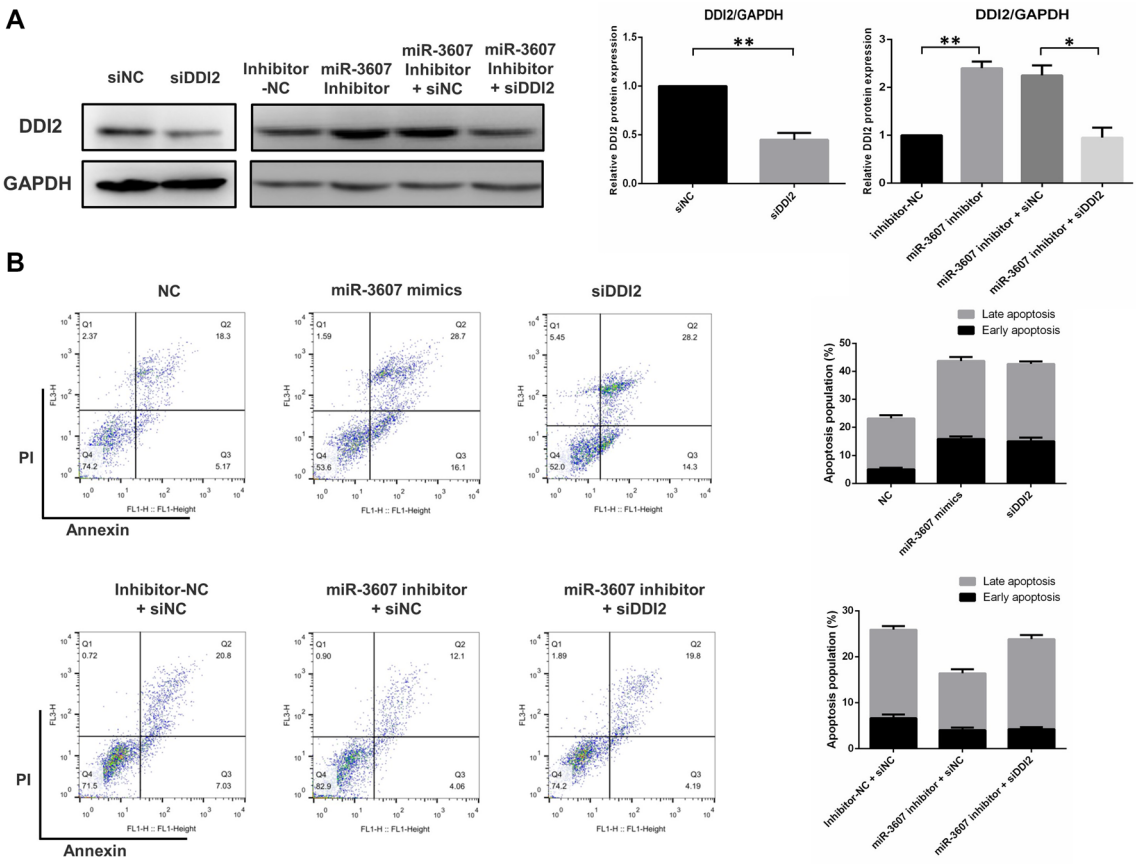

Fig. 7 DDI2 attenuates miR-3607-induced apoptosis. **a** The protein expression of DDI2 was analyzed after 72 h of transfection with siDDI2 or the miR-3607 inhibitor or after cotransfection with the miR-3607 inhibitor and siDDI2 in HCT-116 cells. Protein expression levels of DDI2 were normalized to GAPDH and were expressed as fold increase relative to the negative control (siNC or inhibitor-NC). **b** Cell apoptosis was detected by flow cytometry in HCT-116 cells. The overexpression of miR-3607 or the inhibition of DDI2 both promoted apoptosis compared with the NC group. The knockdown of both miR-3607 and DDI2 rescued miR-3607 inhibitor-induced apoptosis suppression. All data are shown as the mean ± SEM (n = 3), *P < 0.05, **P < 0.01

DDI2, a proteasomal shuttle protein, plays an important role in the maintenance of genomic stability and in the compensation for proteasome dysfunction [18, 29]. In summary, most of the key hub genes are involved in the DDR pathway and promote tumorigenesis.

In our recent article (data unpublished), 5-fluorouracil (5FU), which is routinely employed in the management of CRC through the inhibition of thymidylate synthase or through incorporation into RNA and DNA [30], causes DNA damage-induced apoptosis and antiproliferation in SW480 cells. The results of qRT-PCR showed that miR-3607 was downregulated and DDI2 was upregulated with the increase in 5FU, suggesting that DDI2 is negatively correlated with miR-3607 after the induction of DNA damage. According to the negative correlation between miR-3607 and DDI2 in CRC from the TCGA database (Fig. 5b), we chose DDI2 to determine whether this DDR gene is a direct target of miR-3607. In this article, the luciferase reporter assay and rescue experiment confirmed that DDI2 is the functional target of miR-3607. It was recently reported that DDI2 is required for cellular survival following replication stress [29] and the nuclear translocation of NRF3 (NF-E2-related factor 3 or NFE2L3) [31], which is a useful biomarker candidate for the diagnosis of CRC due to its increased expression in CRC compared with normal tissues [32, 33]. This evidence suggests that DDI2, as an oncogene, promotes the tumorigenesis of CRC. Therefore, miR-3607 inhibits the tumorigenesis of CRC probably by suppressing DDI2 expression. Interestingly, we found that the miR-3607 locus overlaps with an annotated small nucleolar RNA (snoRNA), SNORD138 (PMID: 24174566). To gain further insight into the potential role of miR-3607, we will investigate whether SNORD138 can undergo cellular processing to form smaller snoRNA-derived RNAs (sdRNAs) with miRNA-like functions, such as other snoRNAs [17], in the future.

In summary, our results indicate that miR-3607, as a novel tumor suppressor, decreases CRC cell proliferation, migration and invasion. It also plays a critical role in regulating

the DDR pathway and might serve as a novel target for CRC prediction and therapy.

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