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Epstein-Barr virus-encoded LMP1 increases miR-155 expression, which promotes radioresistance of nasopharyngeal carcinoma via suppressing UBQLN1

F. YANG[1], Q. LIU[2], C.-M. HU[3]

[1]Department of Oncology, Daqing Oilfield General Hospital, Daqing, China
[2]E.N.T. Department, People's Hospital of Zoucheng, Zoucheng, China
[3]Department of E.N.T, Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital, School of Clinical Medicine, University of Electronic Science and Technology of China, Chengdu, China

Fang Yang and Quan Liu contributed equally to this study

Abstract. – OBJECTIVE: Epstein-Barr virus (EBV)-encoded latent membrane protein (LMP1) can drive aberrant expression of miR-155 in nasopharyngeal carcinoma (NPC). In this study, we investigated the regulation of miR-155 expression over UBQLN1 and studied their effects on radiosensitivity of NPC.

MATERIALS AND METHODS: MiR-155, LMP1 and ubiquilin-1 expression were measured in 40 cases of NPC cases. The regulative role of miR-155 over UBQLN1 was investigated using a dual luciferase assay, qRT-PCR and Western blot analysis. The effect of miR-155-UBQLN1 axis on radio-sensitivity was explored using loss-and-gain study. The activation of PI3K/Akt pathway and the expression change of some important genes regulating cell cycle, cell proliferation and epithelial-to-mesenchymal transition (EMT) were measured.

RESULTS: MiR-155 was significantly increased in radio-resistant NPC tissues and was negatively correlated to ubiqulin-1 expression. LMP1 overexpression led to significantly higher miR-155 expression. MiR-155 had two binding sites with 3'UTR of UBQLN1 and could decrease it expression. MiR-155 overexpression increased survival fraction of CNE-2 cells after exposure to 6 Gy and decreased cell apoptosis. It also partly abrogated the inhibiting effect of UBQLN1. Through decreasing ubiqulin-1, miR-155 changed the cell cycle to a more radio-resistant model. The miR-155-UBQLN1 axis affected the activation of PI3K/Akt pathway in NPC cells and changed the expression of some important genes regulating the cell cycle, cell proliferation and EMT.

CONCLUSIONS: This study found that aberrant miR-155 expression driven by LMP1 can modulate radio-sensitivity of the NPC cell at least partly through targeting UBQLN1.

Key words:
Nasopharyngeal carcinoma, miR-155, UBQLN1, Radiotherapy.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy highly prevalent in southern China and Southeastern Asia[1,2]. Since the NPC is usually sensitive to radiotherapy, it *is the primary* treatment for the patients[3]. For the patients in advanced stages, cisplatin-based chemoradiotheray is usually administered[4,5]. However, local recurrence, nodal and distant metastasis still occur in a proportion of patients due to the existence of radio-resistance[6]. However, the molecular mechanism of radio-resistance of NPC has *not been* fully *revealed*.

Epstein-Barr virus (EBV) infection has been reported to be associated with the risk of NPC[7,8]. It may *contribute to* pathological development of NPC through multiple mechanisms. For example, the EBV-encoded latent membrane protein (LMP1) is an oncogenic protein that engages in several signaling pathways including NF-kappaB, JNK/p38 (SAPK), PI3-kinase and ERK-MPK[9]. In addition, some recent studies also reported that LMP1 expression can modulate miRNAs in NPC, such as miR-155[10,11] and miR-10b[12]. MiR-155 *overexpression* can *downregulate* the expression of JMJD1A, a histone *demethylase* in NPC cell lines. In fact, the downregulation of JMJD1A is significantly correlated with poor prognosis of NPC patients[11]. Increased miR-155 expression is also associated with cell proliferation and migration of NPC cell lines[10]. Inhibition of miR-155 can suppress cell migration of the cells by reducing ZDHHC2[13]. However, whether *other* targets are involved in its regulation in NPC *is* not clear.

Ubiquilin-1 is a protein that in human is encoded by the UBQLN1 gene. Loss of UBQLN1 may

Corresponding Author: Chunmei Hu, MD; e-mail: chunmeihu80@tom.com

contribute to tumorigenesis through increasing cell migration and invasion, actin cytoskeleton reorganization and induction of epithelial-to-mesenchymal transition (EMT)[14,15]. Ubiquilin1 can regulate ubiquitination, localization, and stability of anti-apoptotic BCL2-like protein, BCL2L10/BCLb[16]. In breast cancer, miR-200c can inhibit autophagy and enhance radio-sensitivity by targeting UBQLN1[17]. These results *suggest that* UBQLN1 may engage in mechanisms regulating radio-sensitivity of cancer. However, its role in NPC *is* still not *revealed*. In this study, we explored the regulation of high miR-155 expression driven by LMP1 over UBQLN1 and investigated their effects on the radio-sensitivity of NPC.

Materials and Methods

Tissue sampling, radiotherapy and response assessment

This study was approved by the institutional Ethical Review Board of the Sichuan Provincial People's Hospital. A total of 40 patients diagnosed as primary NPC and without previous chemo-or/and radio- therapy were recruited. Tumor staging was performed according to the TNM staging system of the International Union against Cancer.

The patients were administered with 2 Gy daily fractions, 5 days per week, for a total intended dose of 66-78 Gy. The dose of RT was adjusted according to the status of lymph node metastasis (50 Gy for patients with lymph node-negative invaded necks and 60-70 Gy for those with lymph node-positive invaded necks). Besides, for patients with advanced stage, additional chemotherapy using the cisplatin plus 5-fluorouracil (PF) regimen was given. The response to radiotherapy was assessed 1 month after treatment using fiber optic nasopharyngoscopy and MRI. The responses were assessed using the following standards: complete response (CR) (complete resolution of all assessable lesions), partial response (PR) (a reduction by 50% or more of the sum of the lesions and no progression of assessable lesions), no change (NC) (a reduction <50% or increase <25% in tumor size) and progressive disease (PD) (increase of 25% in tumor size or with new lesions). CR and PR were defined as radiosensitive, while the NC and PD were defined as radio-resistant.

Cell culture and transfection

Human NPC cell line CNE-1 and CNE-2 were obtained from the Experiment Animal Center of Sun Yat-Sen University and HEK 293T cells were obtained from ATCC. CNE-1 and CNE-2 cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640), while HEK 293T cells were cultured in Dulbecco's modified Eagle Medium (DMEE) medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin in an incubator with a humidified atmosphere and 5% CO_2 at 37°C.

The LMP1 expressing (pcDNA3.1) vector, UBQLN1 lentiviral expression vectors (with 3'-UTR region) and UBQLN1 shRNA were purchased from GENECHEM (Shanghai, China). The pLV-miR-155 expression plasmid, the pLV-miR-155 locker plasmid and the lentiviral packaging vector mix were purchased from Biosettia (San Diego, CA, USA). Lentiviral particles were produced according to manufacturer's instruction. Briefly, the lentiviral vectors and the packaging mix were co-transfected into HEK 293T cells. 48h after infection, the culture supernatant was collected and the viral titer was determined. CNE-1 and CNE-2 cells were transfected with the LMP1 expressing (pcDNA3.1) vector or UBQLN1 shRNA using Lipofectamine 2000 (Invitrogen; Carlsbad, CA, USA). For lentiviral infection, the cells were infected with the corresponding viral supernatants with the presence of 8 μg/ml polybrene (Sigma-Aldrich; St Louis, MO, USA).

Irradiation

Cells after indicating treatments were irradiated in 25 T flasks. Cells were exposed to 6 Gy using a linear accelerator. 1×10^5 cells were plated in six-well plates immediately after irradiation and were further cultured for another 12 days. The colonies were fixed with glutaraldehyde (6.0% v/v) and stained with crystal violet (0.5% w/v). Colonies (> 50 cells) were counted using a stereomicroscope. The surviving fraction was defined as the number of the colonies/number of plated cells. All the procedures were repeated in triplicate.

QRT-PCR analysis of gene expression

Total RNA from tissue or cell samples was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. MiR-155 expression was quantified using TaqMan miRNA reverse transcription kit and TaqMan miRNA assay kits (Applied Biosystems; Foster City, CA, USA) according to manufacturer's protocol. MiRNA expression was normalized to that of rRNA U6. LMP1 and UBQLN1 expression was

quantified using gene specific primers (LMP1: F: 5'-TCCAGAATTGACGGAAGAGGTT-3', R: 5'-GCCACCGTCTGTCATCGAA-3'; UBQLN1: F: 5'-GTCAGCCATGTCGAACCC-TAG-3', R: 5'- GCTGCCAAGCCAGGAG-TAAA-3') and SYBR® Green PCR Master Mix (Applied Biosystems). GAPDH was used as the endogenous control gene. All qRT-PCR analysis was performed using an ABI Prism 7500 (Applied Biosystems). The results of qRT-PCR analysis were presented using $2^{-\Delta\Delta CT}$ method.

Dual luciferase assay

Putative binding sites between miR-155 and 3'UTR of UBQLN1 were predicted using TargetScan 6.2. The two pairs of wild type or mutant (without miR-155 binding site) human UBQLN1 3'UTR sequences with flanking *SacI* and *XhoI* restriction enzyme digestion sites were chemically synthesized. Then the wild type and mutant sequences were inserted between *SacI* and *XhoI* sites of pGL-3 promoter vector respectively. The recombinant plasmids were named as pGL3-UBQLN1-WT1, pGL3-UBQLN1-WT2, pGL3-UBQLN1-MUT1 and pGL3-UBQLN1-MUT2 respectively. To assess the influence of miR-155 on luciferase expression, HEK 293T cells were cotransfected with 200 ng recombinant plasmids and 50 nM miR-155 mimics or the negative control using Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA). 24h after transfection, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to that of Renilla luciferase.

Western blot analysis

Total protein from tissue or cell samples was extracted using RIPA Lysis Buffer (Beyotime; Shanghai, China) according to manufacturer's instruction. Then, the protein concentration of the lysates were measured by BCA protein assay kit (Beyotime). The lysates were separated on 10% SDS-PAGE and then transferred onto a PVDF membrane. After blocking with 5% nonfat dry milk, the membranes were incubated with primary antibodies (anti-LMP1, 1:250, ab78113; anti-ubiquilin, 1:500, ab128011; anti-PI3K, 1:1000, ab40755; anti-pAKT (phospho S473), 1:500, ab8932; anti-AKT, 1:500, ab8805; anti-c-Myc, 1:500, ab56; anti-E2F1, 1:1000, ab94888; anti-cyclin D1, 1:2000, ab137875; anti-p21, 1:1000, ab7960; anti-snail, 1:1000, ab53519; anti-Vimentin, 1:500, ab8978; anti-E-cadherin, 1:50, ab1416; anti-β-actin, 1: 2000, ab8227) overnight at 4°C. Membranes were washed and incubated with corresponding HRP-labeled secondary antibodies. All these antibodies were purchased from Abcam (Cambridge, MA, USA). The blot signals were visualized using ECL Western blotting substrate (Promega).

Flow cytometry analysis of cell apoptosis and cell cycle

48 hours after indicated treatments, CNE-2 cells were harvested and fixed in 70% ice-cold ethanol at 4°C for 24 hours. To assess the distribution of cells in different phases of cell cycle, cells were stained with 20 μg/mL PI (Sigma-Aldrich) and 100 μg/mL RNase A in phoshate buffered saline (PBS) for 15 min at room temperature. To determine the proportion of cells with active caspase-3, cells were stained using Fluorescein Active Caspase-3 Staining Kit (ab65613, Abcam) according to manufacturers' instructions. DNA content and the proportion of cells with active caspase-3 were analyzed using a FACSCaliber (BD Biosciences; Franklin Lakes, NJ, USA). Data acquisition was done using CellQuest 3.2 software (BD Biosciences). Each test was performed with at least three repeats.

Statistical Analysis

All statistical analysis was performed using SPSS 18.0 software (IBM; Chicago, IL, USA). Group comparison was performed using the unpaired *t*-test. A two-sided p value of <0.05 was considered statistically significant.

Results

MiR-155 was significantly increased in radio-resistant NPC and was negatively correlated to ubiqulin-1 expression

Previous studies reported that miR-155 expression in NPC is partly driven by EBV-encoded LMP1[10,11] and contributes to pathological development of NPC by increasing cell proliferation and migration[10]. In this study, we firstly explored the association between miR-155 and radio-sensitivity of NPC. Based on the 40 tumor samples (8 radio-resistant and 32 radio-sensitive), we observed that the radio-resistant cases had significantly higher expression of miR-155 (Figure 1A). Western blot analysis also confirmed that the radio-resistant cases had a higher LMP1 expression (Figure 1C). In both CNE-1

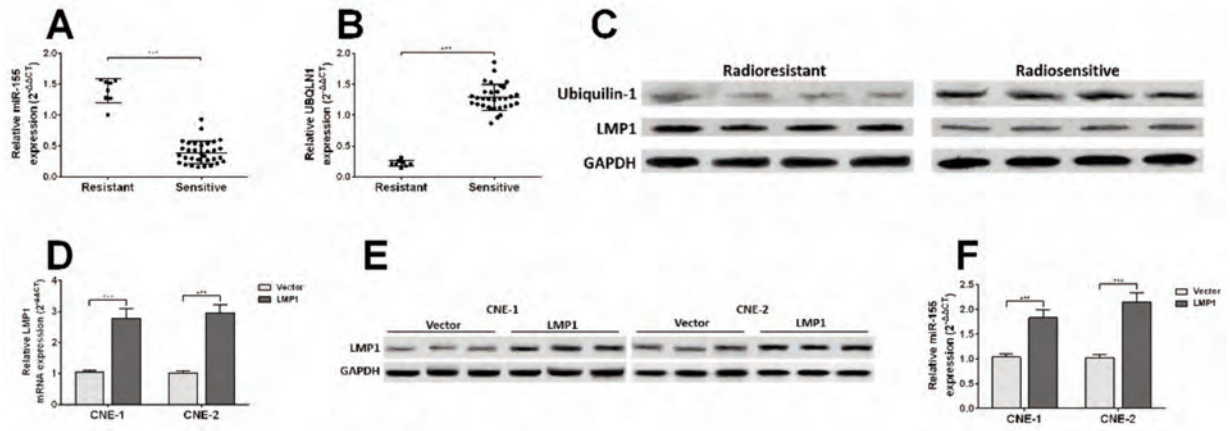

Figure 1. MiR-155 was significantly increased in radio-resistant NPC and was negatively correlated to ubiqulin-1 expression. (**A** and **B**) qRT-PCR analysis of miR-155 (**A**) and QUBLN1 (**B**) expression in tumor tissues of radiosensitive (n=32) and radioresistant (n=8) patients before and after radiotherapy. (**C**) Western blot analysis of LMP1 and ubiquilin-1 expression in tumor tissues of randomly selected radiosensitive (n=4) and radioresistant (n=4) patients. (**D** and **E**) qRT-PCR (**D**) and western blot (**E**) analysis of LMP1 expression in CNE-1 and CNE-2 cells with LMP1 overexpression. (**F**) qRT-PCR analysis of miR-155 expression in CNE-1 and CNE-2 cells with LMP1 overexpression. *p<0.05, **p<0.01, ***p<0.001.

and CNE-2 cells, we confirmed that overexpression of LMP1 (Figure 1D and E) resulted in a significantly higher level of miR-155 (Figure 1F). Recently, our lab also found the radio-resistant NPC had a significantly lower expression of ubiqulin-1 at both mRNA (Figure 1B) and protein level (Figure 1C). Therefore, we decided to explore whether the expression of these two genes are connected in some way.

MiR-155 could directly target UBQLN1 3'UTR and decrease it expression

Through prediction in online databases, we observed that miR-155 has two putative binding sites with UBQLN1 3'UTR (Figure 2A). Considering the inverse expression of these two genes in NPC samples, we decided to verify these binding sites. In CNE-2 cells, overexpression of miR-155 substantially reduced the expression of UBQLN1 mRNA and protein (Figure 2B), while knockdown of endogenous miR-155 showed opposite effects. It increased UBQLN1, similar as transfection with UBQLN1 expression plasmid (Figure 2C). By performing dual luciferase assay with luciferase reporters carrying either wild type or mutant UBQLN1 3'UTR sequences, we found that miR-155 could suppress luciferase activity of the plasmids carrying any one of the predicted binding sequences (Figure 2 D and E). But it had no suppressive effect on the plasmids carrying the mutant sequence (Figure 2 D and E). These results confirmed that miR-155 can directly target UBQLN1 3'UTR and decrease it expression

The miR-155-UBQLN1 axis could modulate radio-sensitivity of NPC cells

To explore the role of miR-155-UBQLN1 axis in CNE-2 cells, the cells were firstly transfected with miR-155, antimiR-155 or simultaneously transfected with UBQLN1 and miR-155 or antimiR-155 (Figure 3 A-C). The cells with indicated treatments were then exposed to 6 Gy irradiation. Enforced expression of UBQLN1 or antimiR-155 significantly inhibited cell survival after the exposure. Co-expression of UBQLN1 and antimiR-155 presented some synergic effect than either UBQLN1 or antimiR-155 alone (Figure 3 D). In contrast, miR-155 significantly promoted cell survival and also partly abrogated the survival inhibiting effect of UBQLN1 (Figure 3 D). Then we studied the role of this axis on cell apoptosis and cell cycle. MiR-155 overexpression reduced cell apoptosis by around 3.4% (Figure 3E). While overexpression of antimiR-155 or UBQLN1 promoted cell apoptosis. MiR-155 partly cancelled the apoptosis inducing effects of UBQLN1, while antimiR-155 enhanced the apoptosis inducing effects of UBQLN1 (Figure 3E). Cells are usually more sensitive to radiation in G2 phase before mitosis and are least sensitive in the mid- to late S and early G1 phases. In cell cycle analysis, we found that miR-155 overexpression increased the proportion of cells in S phase, but decreased the cells in G1 phase (Figure 3F). Either antimiR-155 or UBQLN1 overexpression reduced S phase accumulation and increased G2 phase block (Figure 3F). In contrast,

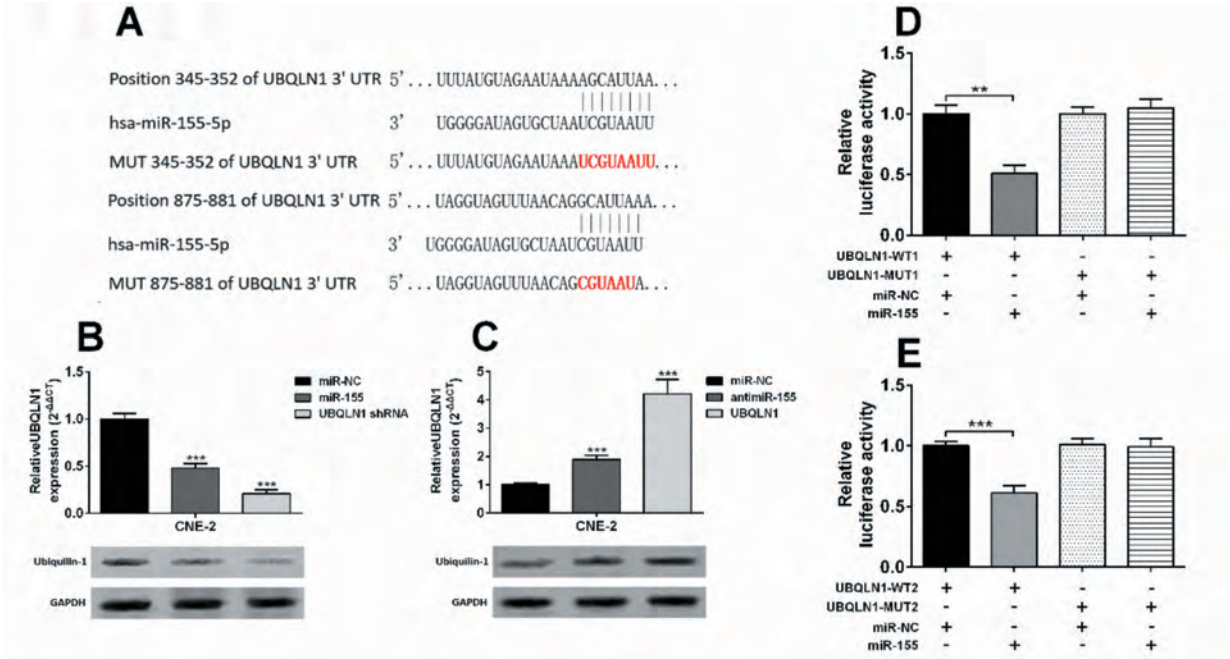

Figure 2. MiR-155 could directly target UBQLN1 3'UTR and decrease it expression. (**A**) Predicted binding sites between miR-155 and 3'UTR of UBQLN1. MUT: designed mutant sequence. (**B** and **C**) QRT-PCR (up) and western blot analysis (down) of UBQLN1 expression in CNE-2 cells infected with the pLV-miR-155 expression plasmid or transfected with UBQLN1 shRNA (**B**) or infected with pLV-miR-155 locker plasmid or UBQLN1 expression vector (**C**). (**D** and **E**) Dual luciferase assay of relative luciferase activity between pGL3-UBQLN1-WT1 (UBQLN1-WT1) and pGL3-UBQLN1-MUT1 (UBQLN1-MUT1) (**D**) or between pGL3-UBQLN1-WT2 (UBQLN1-WT2) and pGL3-UBQLN1-MUT2 (UBQLN1-MUT2) (**E**) in CNE-2 cells transfected with 50 nM miR-155 mimics or the negative control. Firefly luciferase activity was normalized to that of Renilla luciferase. *p<0.05, **p<0.01, ***p<0.001.

miR-155 abrogated the effects of UBQLN1, while antimiR-155 enhanced the effect of UBQLN1 on cell cycle (Figure 3F). These results suggest that the miR-155-UBQLN1 axis can modulate radio-sensitivity of NPC cells.

The miR-155-UBQLN1 axis was involved in multiple signaling pathways related to cell proliferation, cell cycle and EMT

The PI3K/Akt signaling pathway is an important regulator of cancer cell proliferation, metastasis and EMT process. Previous studies reported that there are direct and indirect interactions between TGFβ and PI3K/Akt signaling. Therefore, we investigated whether PI3K/Akt pathway is affected by the miR-155-UBQLN1 axis. Western blot analysis showed that miR-155 could elevate the expression of PI3K and p-Akt, while antimiR-155 and UBQLN1 could decrease the expression of PI3K and p-Akt. MiR-155 partly abrogated the inhibiting effect of UBQLN1 (Figure 4A).

Since the miR-155-UBQLN1 axis is involved in cell cycle regulation, we then investigated the alterations of some proteins related to cell proliferation, cell cycle and EMT process. Notably, miR-155 could increase the expression of c-myc, E2F1 and cyclin D1 but reduce p21 expression in the cells. AntimiR-155 and UBQLN1 showed inverse effects. MiR-155 and antimiR-155 had abrogative and additive effects to UBQLN1 respectively (Figure 4B).

EMT is an important mechanism facilitating cancer cell migration and metastasis, which also promotes radio-resistance of tumor cancer cells[18]. The Western blot analysis also showed that miR-155 resulted in higher transcriptional factor Snail and Vimentin expression, but reduced E-cadherin expression (Figure 4C). AntimiR-155 and UBQLN1 could decrease Snail and Vimentin expression, but increase E-cadherin expression (Figure 4C). Similarly, miR-155 reversed the effect of UBQLN1, but antimiR-155 enhanced the effect of UBQLN1 (Figure 4C).

Collectively, these data suggest that the miR-155-UBQLN1 axis can affect the activation of PI3K/Akt pathway in NPC cells. In addition, some important genes regulating cell cycle, cell proliferation and EMT were also altered by this axis.

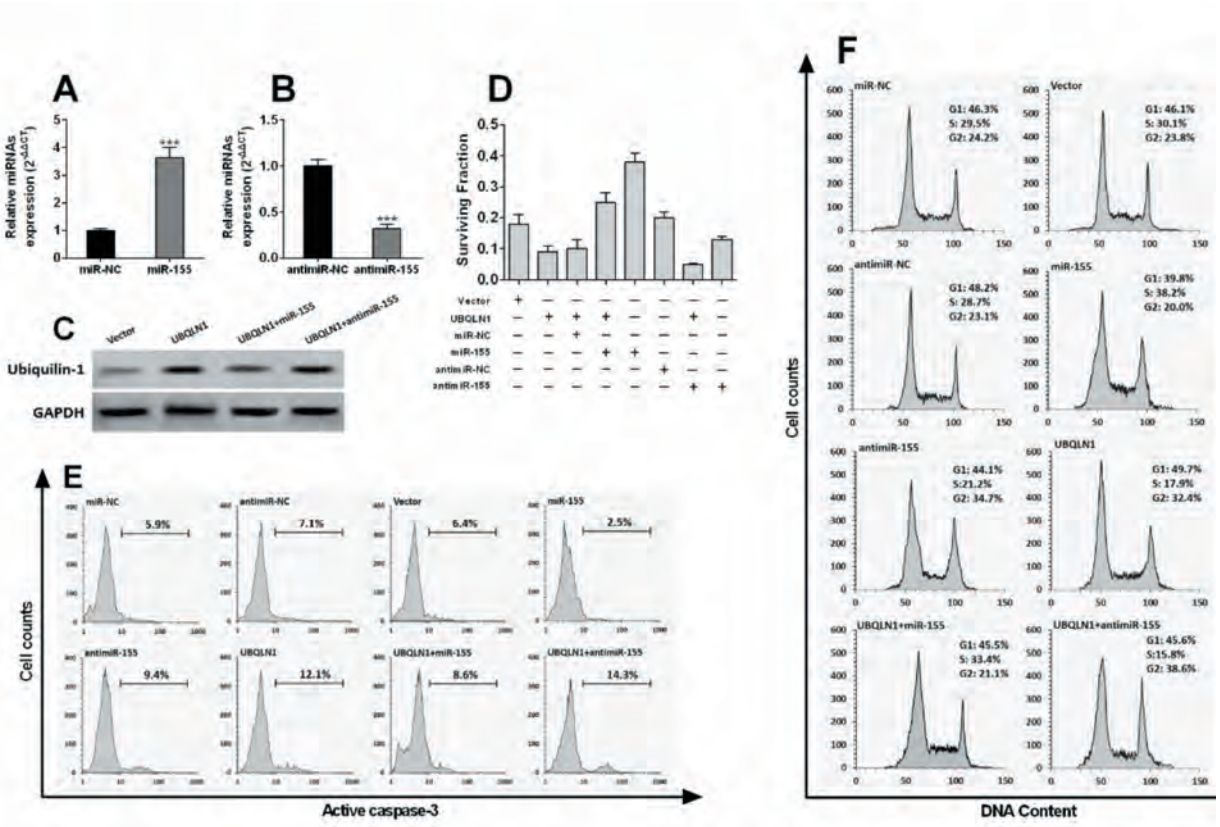

Figure 3. The miR-155-UBQLN1 axis could modulate radio-sensitivity of NPC cells. (**A** and **B**) qRT-PCR analysis of miR-155 expression in CNE-2 cells with miR-155 overexpression (**A**) or knockdown (**B**). (**C**) Western blot analysis of ubiquilin expression in CNE-2 cells with indicated treatments. (**D**) Survival fraction of CNE-2 cells with indicated treatments after exposure to 6 Gy. (**E**) Representative images of flow cytometry analysis of CNE-2 cells with active caspase-3 after indicated treatment. (**F**) Representative images of flow cytometry analysis of cell cycle distribution of CNE-2 cells after indicated treatment. *p<0.05, **p<0.01, ***p<0.001.

Discussion

MiRNAs is a group of small and conserved non-coding RNA. They can modulate gene expression through degrading or inhibiting translation of target mRNAs[19]. EBV infection is quite common in NPC. Some recent studies suggest that EBV infection can induce the expression of many cellular miRNAs, such as miR-10b[20], miR-21[21] and miR-155[11]. In fact, these alterations all participate in the pathological process of NPC. In detail, miR-10b over-expression promoted metastasis of NPC and accelerated death of tumor-bearing nude mice[20]. Another study reported that viral LMP1 can trigger the PI3K/Akt/FOXO3a pathway to induce miR-21 expression, which subsequently decreases the expression of PDCD4 and Fas-L, leading to chemo-resistance in NPC cells[21]. One recent study observed that miR-155 can directly bind to 3'UTR of JMJD1A and BACH1 and downregulate their expression. The follow up study confirmed that downregulation of JMJD1A was significantly correlated with N stage in TNM classification ($p = 0.023$), a lower five-year survival rate ($p = 0.021$), and a lower five-year disease-free survival rate ($p = 0.049$) of NPC patients[11]. These results suggest that the aberrant expression of miRNAs induced by EBV infection may play important roles in the pathological process of NPC. In this study, we confirmed that increased LMP1 expression resulted in higher miR-155 expression. In addition, we also observed that the radio-resistant NPC cases had significantly higher miR-155 expression. Therefore, we decided to further explore whether the increased miR-155 expression is involved in the development of radio-resistance.

Intrinsic and acquired radio-resistance has become a major barrier to effective NPC therapy. The mechanism of radio-resistance is quite com-

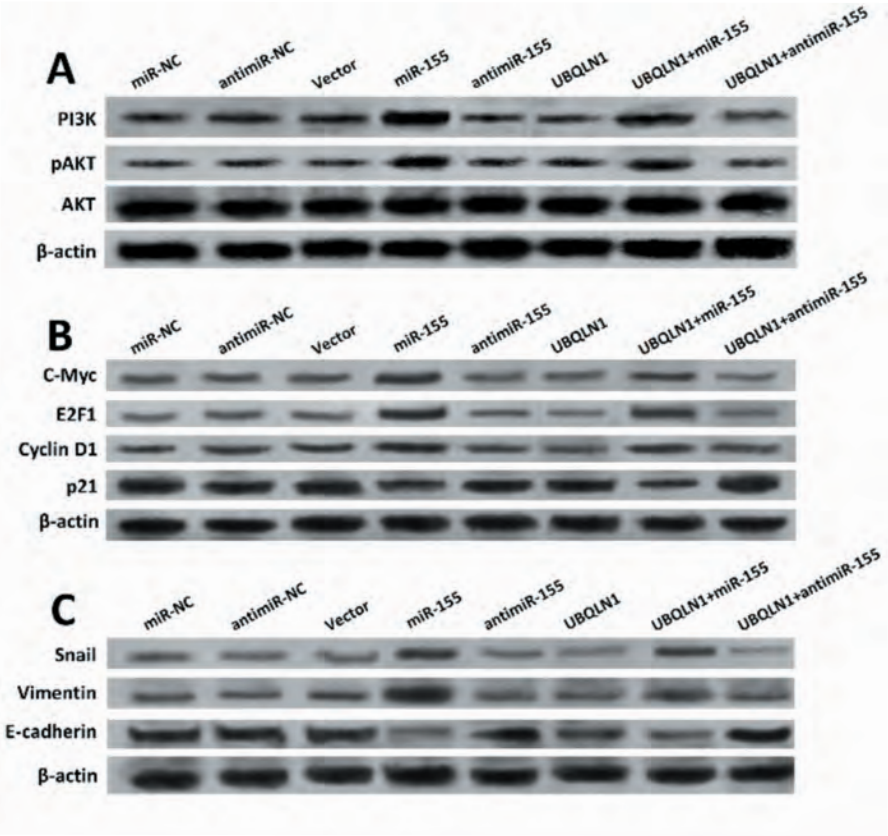

Figure 4. The miR-155-UBQLN1 axis was involved in multiple signaling pathways related to cell proliferation, cell cycle and EMT. (**A**) Western blot analysis of PI3K and p-Akt in the indicated cell groups. (**B**) Western blotting analyses of C-myc, E2F1, cyclin D1, CDK4 and p21 in the indicated cell groups. (**C**) Western blotting analyses of Snail, E-cadherin, and Vimentin in the indicated cell groups.

plex and involves a wide range of dysregulated physiological and pathological processes, such as cancer stem cells[22], overexpression of DNA repair proteins[23] and EMT[24,25]. In NPC, previous studies reported that several miRNAs are involved in regulation of radio-sensitivity through different mechanisms. For example, MiR-185-3p and miR-324-3p can regulate NPC radio-resistance by targeting 3'-UTR and 5'-UTR of WNT2B respectively[24,25], or by targeting SMAD7[26]. MiR-205 can modulate radio-resistance of NPC by directly targeting PTEN[27]. MicroRNA-451 and miR-101 can enhance the sensitivities of NPC cells by targeting Ras-related protein 14 (RAB14) and stathmin 1 respectively[28,29]. In our preliminary study, we observed that miR-155 has two putative binding sites with UBQLN1, a protein with regulative role over EMT and chemo-resistance. In human non-small cell lung cancer cells, one recent study found that loss of UBQLN1 results in a significant decrease in the expression of epithelial markers including E-cadherin and claudin1, whereas expression of mesenchymal markers including Vimentin, Snail and ZEB1 are significantly elevated, suggesting enhanced EMT[14]. Considering the important role of EMT in radio-resistance development, we thereby decided to explore the regulative role of miR-155 over UBQLN1 and their involvement in radio-resistance of NPC. Through performing dual luciferase assay and Western blot analysis, we verified the two predicted binding sites and confirmed that miR-155 can decrease ubiqulin-1 expression. Through decrease ubiqulin-1, miR-155 can enhance NPC cell survival after exposure to irradiation and change cell cycle to a more redio-resistant model. In addition, we further confirmed that miR-155 could activate the PI3K/Akt signaling pathway, which is an important regulator of cancer cell proliferation, metastasis and EMT process. In addition, the miR-155-UBQLN1 axis could also regulate the expression of c-myc, E2F1 and cyclin D1 and the EMT related Snail and Vimentin expression, but reduced E-cadherin expression. In summary, the miR-155-UBQLN1 axis can affect the activation of PI3K/Akt pathway in NPC cells. In addition, some important genes regulating cell cycle, cell proliferation and EMT were also altered by this axis.

Conclusions

This study revealed that aberrant miR-155 expression driven by LMP1 can modulate radiosensitivity of NPC cell at least partly through targeting UBQLN1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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