MicroRNA-145 contributes to enhancing radiosensitivity of cervical cancer cells

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In our study, transcriptome microarrays are used to identify differentially expressed miRNAs and mRNAs in cervical cancer specimens. We find that microRNA-145 (miR-145) expression is significantly decreased in cervical cancer tissues and cell lines, and is associated with advanced cancer stages, large tumor size and moderate/poor differentiation. We show that miR-145 targets the DNA damage repair-associated gene Helicase-like transcription factor (HLTF), which is involved in radio-resistance. Moreover, miR-145 over-expression in cervical cancer cells enhances radiosensitivity in vitro and in vivo. These results indicate that targeting miR-145 may be a novel radiosensitizing strategy for cervical cancer.

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1. Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs that can regulate gene expression through the degradation or translational inhibition of target mRNAs [1]. An increasing number of miRNAs has been demonstrated to be involved in the initiation and progression of various human malignancies [2] and potentially represent novel diagnostic and prognostic markers [3,4]. Moreover, recent evidence has linked several miRNAs with treatment response, such as let-7e [5], miR-375 [6] and miR-200c [7], which play important roles in radiation or chemotherapy sensitivity.

Cervical cancer is one of the leading gynecological malignancies worldwide [8]. Previous studies have reported that many miRNAs can act as oncogenes (e.g., miR-182, miR-205) [9,10] or tumor suppressors (e.g., miR-7, miR-214) [11,12] in cervical cancer. However, our understanding of the potential roles of miRNAs in the prognosis evaluation and treatment response of cervical cancer is still limited. Combining miRNA expression information with clinicopathological characteristics and identifying the lead target mRNAs are helpful in guiding the study of the functions and mechanisms of abnormally expressed miRNAs in cervical cancer.

In our study, to screen aberrant miRNAs and predict the corresponding target mRNAs, high-throughput transcriptome microarrays were used to detect differentially expressed miRNAs and mRNAs synchronously in cervical cancer tissues compared with adjacent non-tumor tissues. From our microarray results, we investigated the most significantly down-regulated (>4-fold) miRNA (Hsa-miR-145-5p, miR-145) in clinical specimens and analyzed the association between miR-145 expression levels and specific clinicopathological characteristics. Then, we combined microarray analyses and bioinformatic predictions to screen candidate target mRNAs of miR-145 effectively and accurately. Helicase-like transcription factor (HLTF) was the best candidate target gene, and it was found to confer radiation resistance in cervical cancer in a recent study [13]. Moreover, we further demonstrated that miR-145 over-expression in cervical cancer cells could enhance radiosensitivity in vitro and in vivo, which showed an additional anticancer function of miR-145 in cervical cancer. These findings might help establish new strategies for therapy decision-making and improving therapeutic effect of cervical cancer.
2. Materials and methods

2.1. Patients and samples

Human cervical tissue samples were collected from patients at the Department of Obstetrics and Gynecology at Changzheng Hospital (Shanghai, China) between 2012 and 2013. A total of 47 paired cervical cancer tissues and adjacent non-tumor tissues (at least 2 cm from each tumor) were obtained from patients with cervical cancer (FIGO stage IB-IIA). All patients recruited in this study were not subjected to preoperative radiotherapy and/or chemotherapy. Besides, three normal cervical epithelial tissue samples were obtained from three premenopausal women (human papillomavirus negative, HPV–) undergoing hysterectomy for myoma. The collection of human tissue samples was approved and supervised by the Ethics Committee of Changzheng Hospital.

2.2. Cell culture and transfection

Human cervical cancer cell lines (HeLa, SiHa, C-33A, Caski) were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China) and maintained as recommended by the American Type Culture Collection (ATCC, USA). Cells were transfected with miR-145 mimic (RiboBio, China) or miRNA negative control (miR-NC) using the riboFECT™ CP transfection reagent (RiboBio, China) according to the manufacturer’s instructions. The final concentration of either mimic was 50 nM in the cell culture medium.

2.3. Microarray analyses and bioinformatic predictions

Briefly, double-stranded complementary DNA (cDNA) was synthesized from 5 cervical cancer tissues and paired adjacent non-tumor tissues through reverse-transcription polymerase chain reactions and then hybridized to Glue Grant Human Transcriptome arrays (Affymetrix, USA) following the manufacturer’s protocol. Affymetrix® Expression Console Software (version 1.3.1) was used for microarray analysis. We identified differentially expressed genes by the random variance model (RVM) t-test and false discovery rate (FDR) analyses, with a predefined P-value threshold of <0.05 [14]. Hierarchical clustering (Cluster 3.0) and TreeView analysis (Stanford University, USA) were performed based on the results of differentially expressed genes. TargetScan, miRdb and miRanda combined with the differentially expressed mRNA identified in the microarray were used to predict the putative targets. The microarray data discussed in this article have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus ( GEO) and are accessible through the (GEO) Series accession number GSE5594 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55940).

2.4. RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissues or cells using the RNeasy Plus reagent (Takara, Japan), and cDNA was synthesized using the SuperScript™ II Reverse Transcriptase kit (Invitrogen, USA). For miRNA, RNA was reverse transcribed using a specific reverse-transcription primer. Quantitative real-time PCR (qRT-PCR) analyses were performed with SYBR® Premix Ex Taq™ (Takara, Japan) using a StepOne-plus Plus Real-Time PCR system (ABI, USA). U6 snRNA or 18S rRNA was used as internal controls to normalize the expression levels of miRNAs or mRNAs, respectively. The relative expression levels of miRNAs or mRNAs were calculated using the 2^(-DDCt) method. The primers for HLTF mRNA and 18S rRNA were as follows: HLTF (forward, 5’-AAC TGG CAA CCT ATT AAA TC-3’; reverse, 5’-TGT GCC TAT TTC CCA AAC-3’); 18S (forward, 5’-AAC TGG CAA TGG CTC ATT AAA TC-3’; reverse, 5’-TGT ATC TGA TAA ATG CAC GCA TC-3’). For miR-145, we used the Bulge-Loop™ miRNA qRT-PCR Primer Set, including a specific reverse-transcription primer and miR-145 forward and reverse primers (RiboBio, China). The sequences of the primers were not provided by the manufacturer.

2.5. Vectors construction and Luciferase reporter assays

The 3’ untranslated regions (3’-UTR) of HLTF mRNA containing the intact miR-145 recognition sequences were PCR-amplified and subcloned into the Sac I and Xba I sites of pmirGLO vector (Promega, USA) for Luciferase reporter assay. The primer sequences were as follows: forward, 5’-CGA CCT CAG TTG AGA ATC CCG-3’; reverse, 5’-GGT GGA AGA CTG TGA A-3’. The pmirGLO-3’ UTR with mutations in miR-145 binding sites was synthesized by GenScript (Nanjing, China). Luciferase assays were performed in HeLa cells. miR-145 mimic or miR-NC was co-transfected with pmirGLO-3’-UTR vector using the riboFECT™ CP transfection reagent. After a 48-h incubation, firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter system (Promega, USA) following the manufacturer’s instructions. Firefly luciferase activity was normalized to Renilla luciferase activity for each sample.

2.6. Western blot assays

Cells were harvested in RIPA lysis buffer (Beyotime, China). Equal amounts of protein were separated by SDS–PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, USA). The membranes were blocked in phosphate-buffered saline/Tween-20 containing 5% non-fat milk and incubated with an antibody against HLTF or β-actin (Santa Cruz Biotechnology, USA). Then, the membranes were incubated with HRP-labeled rabbit anti-goat IgG (KPL, USA) and detected using an Epson Perfection V300 Photo Scanner (Epson, Japan). Quantitative analysis was performed using AlphaEase FC software (Alpha Innotech, USA). Protein levels were normalized to β-actin.

2.7. Irradiation

Cells and nude mice were exposed to 60Co-gamma ray irradiation at different doses (dose rate: 1 Gy/min) in the irradiation center of the Second Military Medical University (SMMU, Shanghai, China), depending on the requirements of the experiment [7].

2.8. Cell viability assay

Cells plated at 60–70% confluence in 6-well plates were transfected with miR-145 mimic or miR-NC and incubated for 24 h. Then, the cells were seeded in 96-well plates (1 × 10^4 cells per well). After overnight incubation, the cells were irradiated at a dose of 0 Gy or 8 Gy. Then, after incubation for another 24 h, cell viability was determined using CCK-8 (Dojindo, Japan) and by measuring absorbance at 450 nm using an ELx800™ plate reader (BioTek, USA), following the manufacturer’s instructions.

2.9. Flow cytometric analysis of cell apoptosis

Cells plated at 60–70% confluence in 6-well plates were transfected with miR-145 mimic or miR-NC and incubated for 24 h. Then, the cells were exposed to 0 Gy or 8 Gy irradiation. At 24 h after irradiation, the Annexin V-FITC cell apoptosis detection kit (Beyotime, China) was used to detect apoptotic cells with a MACS-QuantiCapture™ Analyzer flow cytometer (Miltenyi Biotec, Germany). fol-
lowing the manufacturer’s instructions. The results were analyzed using FlowJo software (Tree Star, USA).

2.10. Tumor xenograft assay

Female nude mice (BALB/C, 5 weeks old) were purchased from Shanghai Laboratory Animal Center (SLAC, China) and housed under specific pathogen-free conditions. All the animal experiments were approved by the Institutional Animal Care and Use Committee of the SMMU (Shanghai, China). To establish the subcutaneous xenograft model, we subcutaneously injected $1 \times 10^7$ HeLa cells in 0.10 ml of phosphate-buffered saline (PBS) into the right thigh of the nude mice. Ten days after tumor cell inoculation, the cells formed palpable tumors, and the mice were divided randomly into three groups (four mice per group) for treatment: (1) 2 nmol miR-145 agomir (cholesterol-conjugated 2’-O-methyl-modified microRNA mimic, RiboBio, China) plus 4 Gy; (2) 5 nmol miR-145 agomir plus 4 Gy; (3) 2 nmol miR-NC plus 4 Gy. For agomir treatment, miR-145 agomir (RiboBio, China) was directly injected intratumorally at a dose of 2 nmol or 5 nmol (diluted in 25 μL of PBS) per mouse in the two treatment groups every 4 days for 6 treatments. miR-NC agomir was directly injected intratumorally at a dose of 2 nmol (diluted in 25 μL of PBS) per mouse in the control group every 4 days for 6 treatments. All of the mice received intratumor injection at days 12, 16, 22, 24, 28, 32, and were exposed to 4 Gy irradiation 24 h after each injection. The tumors were monitored with calipers, and tumor volumes were calculated as length × (width)$^2$/2. At day 34, the mice were sacrificed by cervical dislocation. The xenograft tumors were excised and photographed.

2.11. Statistical analyses

Statistical tests for data analysis included Wilcoxon signed-rank test, Student’s t-test, the Chi-square test, and Pearson’s coefficient correlation. The data are presented as the mean ± S.D. A $P$-value < 0.05 was considered significant. GraphPad Prism5 (GraphPad Software, USA) was used for the statistical analyses.

3. Results

3.1. Microarrays of aberrantly expressed miRNAs and mRNAs in cervical cancer tissues

To investigate potential transcriptome changes in cervical cancer, we performed a gene chip study in five paired cervical cancer & adjacent non-tumor tissues using the Affymetrix Human Transcriptome array. There were 4 up-regulated miRNAs (miR-760, 922, 31-5p, 371b-3p), 8 down-regulated miRNAs (miR-145, 27b-3p, 361-5p, 361-3p, 297, 645, 24-3p, 23b-3p; Fig. 1), and 2189 differentially expressed mRNAs (1288 up-regulated, 901 down-regulated; Supplemental Fig. 1) detected in the microarray. The threshold was a fold change $\geq 1.2$ with $P$ < 0.05. Among these results, miR-145 was the most differentially expressed miRNA.

![Fig. 1](image1.png)

**Fig. 1.** Hierarchical clustering analysis indicates the differentially expressed miRNAs in cervical cancer tissues. A heat map shows 12 differentially expressed miRNAs. The red or green color represents expression values and indicates up- or down-regulation, respectively (c, cancer tissues; ad, paired adjacent non-tumor tissues).

![Fig. 2](image2.png)

**Fig. 2.** MiR-145 expression is decreased in cervical cancer. (A) Significant down-regulation of miR-145 was observed in cervical cancer tissues compared with paired adjacent non-tumor tissues ($*** P < 0.001, n = 42$). The relative expression levels of miR-145 were normalized as ln(2$^{-\Delta CT}$). (B) The expression of miR-145 was much lower in cervical cancer cell lines compared with normal cervical epithelial tissues.
fold change = 4.17). Additionally, recent studies have shown that miR-145 is frequently down-regulated and acts as a tumor suppressor in various human malignancies including cervical cancer [15–18]. Therefore, we chose miR-145 for further investigation of its significance in tumor pathogenesis and clinical treatment.

### 3.2. Association of miR-145 with the clinicopathological characteristics of cervical cancer patients

Recent studies have also shown that miR-145 is down-regulated in cervical cancer; however, the relationship between miR-145 expression and the clinicopathological characteristics of cervical cancer patients remains unclear [19,20]. We first examined miR-145 expression levels in another 42 paired cervical cancer & adjacent non-tumor tissues by qRT-PCR. The miR-145 expression was lower in cervical cancer tissues compared with the corresponding adjacent non-tumor tissues (Fig. 2A). Then, we evaluated the relationship between miR-145 expression and clinicopathological factors (Table 1). The median miR-145 expression level in tumor tissues was used as the cutoff. We found that a lower miR-145 expression was significantly more frequent in cervical cancer tumors with advanced stages, large tumor size and moderate/poor differentiation. Moreover, we found that the expression of miR-145 was much lower in cervical cancer cell lines compared with normal cervical epithelial tissues, which further suggested that the down-regulation of miR-145 was a common phenomenon in cervical cancer (Fig. 2B).

#### Table 1

<table>
<thead>
<tr>
<th>Feature</th>
<th>miR-145 expression level</th>
<th>Chi-square</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>All cases</td>
<td>Low: 21</td>
<td>High: 21</td>
<td></td>
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<tr>
<td>Age, years, &lt;45: 9:12 vs 45: 11:10</td>
<td>0.382</td>
<td>0.357</td>
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<tr>
<td>Tumor size, cm, &lt;4: 6:15 vs 4: 14:7</td>
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<td>FIGO stage, IB:IIA:IIIB 4:9:8</td>
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<td>Degree of differentiation,</td>
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<td>0.039</td>
<td></td>
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<tr>
<td>highly:moderately:poorly</td>
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<tr>
<td>SCC-Ag, ng/ml, &lt;1.5: 7:14 vs 1.5: 24.03</td>
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<td>lymph node metastasis, yes/no 8:13 vs 11:10</td>
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<td>Hb before treatment, g/L, 7.8:6 vs 5.9:7</td>
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Results were considered statistically significant at P < 0.05.

3.3. HLTF is a direct target of miR-145 in cervical cancer

To determine the potential effects and molecular mechanisms of down-regulated miR-145 on the malignant behaviors of cervical cancer cells, we focused on identifying a specific target gene of miR-145. We first utilized three algorithms (TargetScan, miRdb and miRanda) combined with the up-regulated mRNAs from the microarray to predict and analyze the most likely target gene. With this method, 5 potential target genes were identified: HLTF, ONECUT2, BCR, NUFBP2, and ZC3H11A. Among these results, HLTF (GenBank Accession NM_003071.3, transcript variant 1) was the most significantly up-regulated (1.96-fold) mRNA. The qRT-PCR showed that the HLTF expression was higher in cervical cancer tissues compared with normal cervical epithelial tissues, which further suggested that the down-regulation of miR-145 was a common phenomenon in cervical cancer tissues.
sues than that in corresponding adjacent non-tumor tissues (Fig. 3A). Moreover, correlation analysis showed that only HLTF expression significantly inversely correlated with miR-145 in cervical cancer tissues ($r = -0.571$; Fig. 3B). To determine whether HLTF is selectively regulated by miR-145, we performed luciferase reporter assays with the pmirGLO vector carrying the miR-145-complementary sequence of the wild-type or mutant HLTF 3’UTRs (Fig. 3D). The two vectors were co-transfected with miR-145 mimic or miR-NC into HeLa cells. The luciferase assay results indicated that luciferase activity was significantly repressed by miR-145 and was not affected in the mutant 3’UTR group (Fig. 3C). These results showed that HLTF was a direct target gene of miR-145 in cervical cancer.

3.4. Irradiation induces higher miR-145 expression levels in several cervical cancer cell lines

Because the above results suggested that HLTF was a direct target gene of miR-145 in cervical cancer cells, we attempted to determine the potential role of miR-145 in the identified function and mechanism of HLTF. In recent years, several studies have demonstrated that HLTF can act as either a positive or a negative regulator of tumor development, and it is therefore a controversial molecule [21–24]. Moreover, a recent study indicated that HLTF is an important molecule that influences the outcome of radiotherapy in cervical cancer [13]. Therefore, we hypothesized that miR-145 may be associated with radiosensitivity. To test this hypothesis, we first treated several cervical cancer cell lines with increasing doses of irradiation. Then, RT-qPCR analyses showed that the expressions of miR-145 were increased after irradiation in HeLa, SiHa and Caski cells, but not in C-33A cells (Fig. 4). Because HeLa, SiHa and Caski cells contain wild-type p53, but C-33A cells harbors mutant p53. Thus, these results suggested that miR-145 might play a potential role in modulating the sensitivity of cervical cancer cells to radiotherapy and relying on wild-type p53.

3.5. miR-145 over-expression enhances radiation-induced cell viability reduction and apoptosis in cervical cancer cells in vitro

To further investigate whether miR-145 could modulate radiosensitivity by regulating HLTF expression in vitro, we first exogenously up-regulated miR-145 expression in HeLa and SiHa cells by transfecting miR-145 mimic (Fig. 5A and B). The CCK-8 assay and flow cytometric analysis showed that miR-145 mimic could influence cell viability and apoptosis level alone but not very remarkable (Fig. 5C–F, left panel). Nonetheless, radiation-induced cell viability reduction and cell apoptosis both increased significantly by miR-145 mimic (Fig. 5C–F, right panel). These results suggested that miR-145 could enhance radiosensitivity of cervical cancer cells.

3.6. Exogenous miR-145 enhances radiosensitivity of cervical cancer cells in vivo

After validating that miR-145 enhanced radiosensitivity of cervical cancer cells in vitro, we further investigated the radiosensitizing ability of miR-145 in an animal tumor model. We established a HeLa cell subcutaneous xenograft tumor model in nude mice. The mice were treated with irradiation alone (irradiation plus NC agomir) or in combination with the miR-145 agomir. The growth curves and photo of xenograft tumors showed that the tumors in the irradiation-alone group grew faster than those in the combina-

![Fig. 4](https://example.com)
tion group. Moreover, the inhibition was stronger in the combination group with the higher dose of miR-145 agomir (Fig. 6A and B). Additionally, HLTF expression decreased significantly in the corresponding tumor tissues (Fig. 6C). Thus, these results further demonstrated that miR-145 exerted a radiosensitizing effect on cervical cancer cells.

4. Discussion

Previous studies have suggested that miR-145 is down-regulated and acts as a tumor suppressor in various human cancers [25,26]. Recent articles have also reported HPV oncoproteins E6 and E7 could suppress miR-145 expression [20]. In our study, we consistently found that miR-145 expression was remarkably decreased in cervical cancer tissues and cell lines. And our investigation further discovered that decreased miR-145 was significantly associated with clinicopathological features, including advanced cancer stages, large tumor size and moderate/poor differentiation.

Identifying the lead specific target mRNA allows us to study the main functions and molecular mechanisms of miR-145 more effectively. Here, microarray analyses and bioinformatics predictions showed that HLTF was the top candidate target of miR-145. HLTF, belongs to the SNF/SWI family, which plays roles in chromatin remodeling and facilitates trans-factor interactions with nucleosomes [27–29]. In our study, we found that HLTF was up-regulated in cervical cancer tissues and inversely correlated with miR-145.
Furthermore, luciferase reporter assay confirmed that miR-145 could selectively down-regulate HLTF by targeting the 3'UTR of HLTF. Although several studies have identified HLTF as a tumor suppressor gene in digestive tract cancers [30,22], a recent article demonstrated that HLTF confers radio-resistance in cervical cancer by enhancing DNA damage repair capacity of cancer cells [13]. This finding provided us a valuable avenue for further study of the possible role of miR-145 in the treatment response of cervical cancer. As expected, our results showed that miR-145 over-expression enhanced radiosensitivity of cervical cancer in vitro and in vivo through inhibiting cell viability and increasing radiation-induced apoptosis probable by down-regulating HLTF. These results confirmed the previous report of the radio-resistance role of HLTF and showed a additional anticancer function of miR-145 in cervical cancer.

E6 and E7 are the primary HPV oncoproteins, which mainly target p53 and pRB, respectively [31]. It is well known that p53 is a genome guardian acts as a tumor suppressor. Radiation-induced DNA damage could activate p53 and further induce cell cycle arrest and apoptosis [32]. Precious article demonstrated that p53 transcriptionally induces the expression of miR-145 [25]. Moreover, wild-type p53 is critical for the expression of miR-145 in cervical cancer cells [18]. Consistently, we found that radiation had no inducing effect on miR-145 expression in C-33A cells which harbors mutant p53. Furthermore, according to our findings and the conclusions above, we speculated that there might be a potential cascade reaction regarding miR-145 radiosensitization in cervical cancer. The process might consist of: (1) radiation-induced DNA damage activates p53; (2) activated p53 induces the expression of miR-145; (3) up-regulated miR-145 repress HLTF, which ultimately contributes to radiosensitizing effect in cervical cancer cell. Certainly, we need to further study it to excavate more specific mechanism of radiosensitizing effect of miR-145.

In conclusion, our study demonstrates that decreased miR-145 is associated with advanced clinicopathological characteristics of cervical cancer, and that miR-145 contributes to radiosensitizing effect. These findings suggest that miR-145 may be a significant potential biomarker for prognosis evaluation and a novel radiosensitizing target in cervical cancer, which may help establish new strategies for therapy decision-making and improving therapeutic effect of cervical cancer.

5. Conflict of interest

The authors declare no conflict of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.01.037.
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