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Original Article

miR-875-5p counteracts epithelial-to-mesenchymal transition and enhances radiation response in prostate cancer through repression of the EGFR-ZEB1 axis

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ABSTRACT

Radiotherapy is one of the main treatment choices for non-metastatic prostate cancer (PCa), although development of radioresistance limits its effectiveness. Mounting evidence supports the ability of microRNAs to interfere with different radioresistance-associated pathways, suggesting their potential as radiosensitizers. Here, we demonstrate that reconstitution of *miR-875-5p*, whose expression is down-regulated in PCa clinical samples and directly correlates with that of E-cadherin, was able to enhance radiation response in PCa cell lines and xenografts through EGFR direct targeting. Consistent with the established role of EGFR in sustaining epithelial-to-mesenchymal transition (EMT) and promoting DNA repair following radiation-induced nuclear translocation, we found that *miR-875-5p* reconstitution in PCa cells counteracted EMT and impaired DNA lesion clearance. Down-regulation of the EMT-inducing transcription factor ZEB1, which also plays a role in homologous recombination-mediated repair of DNA lesions by regulating CHK1 expression, was found to be a major determinant of *miR-875-5p*-induced radiosensitization, as confirmed by phenocopy experiments showing that siRNA-mediated ZEB1 knock-down was able to reproduce the microRNA radiosensitizing effect. Overall, our data support the clinical interest in developing a novel therapeutic approach based on *miR-875-5p* reconstitution to increase PCa response to radiotherapy.

Abbreviations: CHK1, Checkpoint kinase 1; EGFR, Epidermal growth factor receptor; EMT, Epithelial-to-mesenchymal transition; HR, Homologous recombination; LIN7C, Lin-7 homolog C; miRNA, microRNA; NHEJ, Non homologous end joining; PCa, Prostate cancer; qRT-PCR, Quantitative real-time polymerase chain reaction; SCID, Severe combined immunodeficiency; siRNA, Small interfering RNA; ZEB1, Zinc finger E-box binding homeobox 1.

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Introduction

Radiotherapy, alone or in association with androgen deprivation, represents a standard treatment for non-metastatic prostate cancer (PCa) [1]. Although advancements in treatment planning and delivery allowed radiation dose escalation and consequent enhancement of local tumor control, a significant percentage of patients still undergo recurrence [2]. The onset of radioresistance is a poorly understood, complex phenomenon caused by the activation of both cancer cell intrinsic resistance mechanisms, including the activation of pro-survival and DNA repair pathways, and extrinsic intermediaries of therapy resistance, mainly represented by non-malignant cells and structural components of the tumor stroma [3].

Several genetic and epigenetic abnormalities have been associated with radiation resistance, including the deregulation of microRNAs (miRNAs), endogenous small non-coding RNA molecules that negatively regulate gene expression in a variety of biological processes by translation inhibition, cleavage or degradation of target mRNAs [4]. Mounting evidence supports a functional association between miRNAs and tumor radiation response, indicating the ability of miRNAs to interfere with different radioresistance-associated pathways [5]. In this context, it has been recently reported that specific miRNAs, such as miR-200c, miR-204 and miR-205, which are able to repress epithelial-to-mesenchymal transition (EMT) – a phenotypic switch that promotes the acquisition of a fibroblastoid-like morphology by epithelial tumor cells, resulting in enhanced tumor cell motility/invasiveness, increased metastatic potential and treatment resistance [6,7] – can act as radiosensitizers in selected tumor types [8–10]. Specifically, ectopic expression of such miRNAs targeting the EMT-inducing transcription factor zinc-finger E-box binding homeobox 1 (ZEB1), which also promotes homologous recombination-mediated DNA repair, results in increased tumor radiosensitivity, at least in part, as a consequence of a reduced clearance of treatment-induced DNA damage [9,10].

In this study, by exploiting the correlation with the cell-cell adhesion molecule E-cadherin —the loss of which is one of the hallmarks of EMT [6]— in PCa clinical speciments, we identified *miR-875-5p* as a novel miRNA able to counteract EMT and to increase radiation response in human PCa models through the repression of the epidermal growth factor receptor (EGFR)-ZEB1 axis.

Materials and methods

miRNA and gene expression analysis, Immunoblotting, Immunofluorescence, Immunohistochemistry, Comet assay, Identification of *miR-875-5p* relevant targets and Luciferase assay protocols are described in Supplementary Materials and Methods.

Experimental models

Human PCa cell lines (DU145, PC-3) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Cell lines were authenticated and periodically monitored by genetic profiling using short tandem repeat analysis (AmpFISTR Identifiler PCR amplification kit, Thermo Fisher Scientific Inc, Waltham, MA, USA).

Cell transfection and generation of a miR-875-5p-overexpressing stable clone

Cells seeded at the appropriate density were transfected for 4 h with 20 nM mirVana miRNA mimic and negative control molecules (Thermo Fisher Scientific Inc) or with 20 nM siRNA molecules using Lipofectamine 2000 (Thermo Fisher Scientific Inc), according to the manufacturer's instructions. In miR-Mask experiments, 20 nM EGFR-miScript Target Protector (Qiagen, Hilden, Germany) was transfected alone or in combination with *miR-875-5p* mimic, under the same transfection conditions described above. SiRNA targeting ZEB1 (sense strand 5′-AGGAAGAGGAGGAGGAUAAdTdT-3′) was designed using siMAX Design Software and synthesized by Eurofin MWG Operon (Ebersberg, Germany). A control siRNA with no homology to any known human mRNA was also used (5′-UUAACGAGGUGGACUAAGAdTdT-3′). Hereafter, *miR-875-5p* synthetic mimic will be referred to as *miR-875-5p*, negative mock control oligomer as Neg, EGFR-miScript Target Protector as miR-Mask, ZEB1 siRNA as siZEB1 and control siRNA as siCTRL.

For the generation of a DU145 cell clone overexpressing *miR-875-5p*, pEZX-MR04 vector (GeneCopoeia, Rockville, MD, U.S.A) carrying the 230 bp DNA region encompassing the 76 bp *miR-875-5p* precursor sequence was used to stable transfect DU145 cell line. The plasmid containing a scramble sequence was used as negative control and referred to as Null. Cells were transfected according to Lipofectamine 3000 protocol (Thermo Fisher Scientific Inc). Briefly, cells were seeded in 6-well plate (5×10^5 cells/well) and 24 h later transfected with vectors (10 pg). After 72 h, cells were selected using 0.3 μg/ml puromycin for 5 weeks. Stable transfectants were stored in liquid nitrogen at −196 °C. When cultured, transfected cells were maintained in presence of 0.3 μg/ml of puromycin.

Cell based experiments

Cell morphology was evaluated at day 3 after transfection using an Eclipse TS100 microscope (Nikon, Tokyo, Japan). Images were acquired by a DC290 digital camera (Eastman Kodak Company, Rochester, NY, USA).

Wound healing assay was performed using Ibidi Culture-Insert System (81176 Culture-Insert in μ-Dish 35 mm, high, Ibidi, Munchen, Germany).

Clonogenic assay

Transfected cells were exposed to 2–8 Gy irradiation delivered as a single dose using the 137Cs γ-irradiator IBL-437 (dose rate 5.2 Gy/min). Cells were then seeded at increasing density (500–8000 cells/well), in triplicate, in 6-well plates in RPMI medium containing 10% FBS. After 12 days, colonies were fixed with 70% ethanol, stained with crystal violet in 70% ethanol, and counted. The colony-forming efficiency was calculated as the ratio of the number of colonies (consisting of at least 50 cells) to the number of single cells seeded. The surviving fraction was calculated as the ratio of the colony-forming efficiency of the irradiated sample to that of the non-irradiated one. Triplicate wells were set up for each condition.

In vivo experiments

All animal experiments were approved by the Ethics Committee for Animal Experimentation of Fondazione IRCCS Istituto Nazionale dei Tumori.

Ten million DU145 cells (negative control and stably expressing *miR-875-5p* clones) were injected into the right flank of eight-week-old male SCID mice, and when tumors reached ~300 mm^3 ($Width^2 \times Length/2$), mice were randomly assigned to control or radiation treatment groups (n = 8). Mice received 5 Gy single dose irradiation using a micro-CT/microirradiator (225Cx, Precision X-ray). At day 31 and 80, representative X-ray images were collected. 24 h after irradiation, tumor specimens were removed from 2 additional mice and immediately disaggregated for *ex-vivo* clonogenic assay or fixed in 10% buffered formalin for immunohistochemical analysis.

Statistical analyses

If not otherwise specified, data are presented as mean values ± SD from at least three independent experiments. Statistical analysis was performed by two-tailed Student's *t* test. *P*-values <0.05 were considered statistically significant.

Results

miR-875-5p counteracts epithelial-to-mesenchymal transition

Our previous observation of a direct correlation between E-cadherin mRNA levels and *miR-205*, a miRNA that we demonstrated to exert a prominent role in repressing EMT in PCa cells [11] and that was successively found to act as a tumor radiosensitizer [10], prompted us to exploit correlation with such epithelial marker as a strategy to identify novel miRNAs potentially able to regulate EMT and radiation response. E-cadherin expression levels were measured by qRT-PCR (Table S1) in a subset of samples (n = 44) of the series of PCa specimens and matched normal tissues obtained from radical prostatectomies that we already profiled for miRNA expression (GSE76260). Twenty-six miRNAs showed significant positive correlation with E-cadherin (Table S2), thus representing potential EMT repressors, whereas 18 showed significant negative correlation (Table S2), thus being possible EMT inducers. Among significantly correlated miRNAs, eighteen also showed differential expression in tumors compared to matched non-neoplastic tissues (Table 1).