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miR-205 impairs the autophagic flux and enhances cisplatin cytotoxicity in castration-resistant prostate cancer cells

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ABSTRACT

Compelling evidence suggests that epithelial-to-mesenchymal transition is involved in the resistance of human cancer cells to chemotherapy. We previously reported that the expression of *miR-205*, a miRNA down-regulated in prostate cancer, is further repressed in prostate cancer cells undergoing epithelial-to-mesenchymal transition, suggesting a possible involvement of the miRNA in the acquisition of the chemoresistant phenotype. In the present study, we show that *miR-205* replacement in castration-resistant mesenchymal prostate cancer cells caused an enhancement of cisplatin cytotoxic activity in vitro and in vivo, as a consequence of autophagy impairment. Specifically, the constraints on the autophagic flux were associated to the miRNA-dependent down-regulation of the lysosome-associated proteins RAB27A and LAMP3. These findings suggest that *miR-205*-mediated impairment of the autophagic pathway may interfere with the detoxifying capabilities of prostate cancer cells in their attempt to cope with cisplatin-induced detrimental effects. Overall, our data indicate that (i) loss of *miR-205* may indeed contribute to acquire mesenchymal tracts and concomitantly establish a permissive autophagic milieu that confers a chemotherapy resistant phenotype to prostate cancer cells, and (ii) strategies aimed at restoring *miR-205* expression levels may represent a successful approach to overcome resistance of prostate cancer to platinum compounds.

1. Introduction

Despite the progress in early diagnosis, prostate cancer (PCa) remains the second leading cause of male cancer-specific mortality in western countries [1]. The treatment of PCa varies depending on the stage of the disease. Treatment of advanced or metastatic disease relies on androgen-deprivation therapy [1]. Initial response rates to androgen ablation are high but virtually all patients relapse due to the development of castration-resistant disease (i.e., androgen escape), one of the most significant events in the progression of PCa [1]. In fact, at this stage, the tumor becomes also refractory to conventional chemotherapeutic agents, thus leading to a high rate of cancer-related mortality. Although novel anti-androgen compounds, such as abiraterone acetate and enzalutamide, have been shown to palliate symptoms and prolong life [2,3], metastatic castration-resistant PCa remains incurable. More effective therapies offering extended survival benefit and altering the natural history of the disease are hence urgently warranted. Recent efforts have been focused on the biological and genetic characterization of the disease, with the aim to identify novel targets to be used for the development of innovative and more efficient therapies [4].

MicroRNAs (miRNAs) are a class of small non-coding RNAs that negatively regulate gene expression at the post-transcriptional level. They bind to complementary sequences located on target RNA transcripts, resulting in gene silencing as a consequence of target RNA cleavage, degradation or translational repression [5]. Given that imperfect pairing is sufficient to drive a miRNA/target interaction, it has been largely shown that a single miRNA can

Abbreviations: BafA1, bafilomycin A1; DAPI, 4′,6-diamidino-2-phenylindole; DEVD-pNA, N-acetyl-Asp-Glu-Val-Asp-pNA; EMT, epithelial-to-mesenchymal transition; IPA, Ingenuity Pathway Analysis; miRNAs, microRNAs; PCa, prostate cancer; RQ, relative quantity; siRNAs, small interfering RNAs; TV, tumor volume; TVI, tumor volume inhibition.

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effectively regulate the expression of a number of genes. MiRNAs have been therefore recognized as master regulators of entire biological pathways. Widespread deregulated miRNA expression has been documented in tumors compared to their normal counterparts [6], suggesting that their aberrant expression may have a role in the pathogenesis of cancer. To date, hundreds of human miRNAs have been experimentally identified, and the expression levels of some of them have been proven to correlate with tumor pathobiological features [6]. Moreover, recent evidence showed that the expression patterns of some miRNAs (e.g., *miR-21*, *miR-1*) play a role in tumor response to chemotherapeutics and ionizing radiation [6]. Even if these findings have yet to enter the clinical practice, global miRNA profiling studies have been proposed in tumors to identify responder vs. non-responder patients [6].

We previously reported that *miR-205* is a tumor-suppressor miRNA, the expression of which is reduced or lost in prostate tumors compared to normal tissues, particularly in carcinomas from patients with local-regionally disseminated disease and in overt metastases [7,8]. More recently, we found that *miR-205* is repressed during epithelial-to-mesenchymal transition (EMT) induced in tumor cells by the contact with cancer-associated fibroblasts [8]. Functionally, the restoration of *miR-205* expression in castration-resistant PCa cells results in cell rearrangements consistent with a mesenchymal-to-epithelial transition (i.e., up-regulation of E-cadherin, reduction of cell locomotion/invasion, increased sensitivity to anoikis) [7]. In addition, *miR-205* reconstitution triggers the reactivation of basement membrane deposition thus allowing a 3D organization of tumor cells into normal-like acinar structures [9] and interrupts the pro-invasive circuitries engaged by reactive stroma [8].

Increasing amount of data suggests that EMT and its associated factors may sustain drug resistance in human cancer cells [10]. On the basis of this notion and our observations of *miR-205* role in controlling EMT, in the present study we investigated whether *miR-205* restoration could affect the sensitivity profile of PCa cells to cisplatin (CDDP). The drug was selected based on (i) the documented relevance of EMT on the tumor cell response to it [10], and (ii) the clinical use of platinum-containing regimens in castration-resistant PCa patients [11].

2. Materials and methods

2.1. Cell-based experiments

The human DU145 and PC-3 PCa cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The cell lines were routinely tested for the absence of *Mycoplasma* and authenticated by the AmpFISTR Identifiler PCR amplification kit (PN4322288; Applied Biosystems, Carlsbad, CA, USA). Cells were maintained in the logarithmic growth phase as a monolayer in RPMI 1640 medium (Lonza Milano s.r.l., Treviglio, Italy) supplemented with 10% heat-inactivated fetal bovine serum in a humidified incubator at 37 °C with a supply of 5% CO_2/95% air atmosphere. Four polyclonal cell populations, two expressing *miR-205* (DU145/*miR-205* and PC-3/*miR-205*) and the control DU145/*miRVec* and PC-3/*miRVec* cells, were selected for 1 month in culture medium containing 1 mg/ml of Blasticidin (Life Technologies, Gaithersburg, MD, USA) after Lipofectamine2000™ (Life Technologies)-mediated transfection with a *miR-205* coding vector or a mock vector, respectively (Source BioScience LifeSciences, Nottingham, UK).

Short-term cytotoxicity was assessed in DU145 and PC-3 cell clones seeded in six-well plates and treated for 1 h with increasing concentrations (1–64 μM) of CDDP (Teva Italia s.r.l., Milano, Italy). After drug removal, cells were incubated at 37 °C for an additional 72 h. At the end of treatment, cells were trypsinized and counted in a particle counter (Coulter Counter, Coulter Electronics Ltd., Luton, UK). Each experimental sample was run in triplicate. The results were expressed as the number of adherent cells in treated samples compared with control samples. The in vitro drug activity was expressed in terms of concentrations able to inhibit cell growth by 50% (IC_{50}).

Long-term cytotoxicity was assessed by the clonogenic assay. Briefly, cells were plated at appropriate concentrations (500–1000 cells per well) in plastic dishes, allowed to attach for 24 h and then exposed to increasing concentrations (0.5–10 μM) of CDDP for 1 h. At the end of the treatment, cells were washed with phosphate-buffered saline and incubated in drug-free medium at 37 °C in a 5% CO_2 humidified atmosphere for 14 days. Colonies were stained with 0.5% (w/v) crystal violet and counted under the microscope. Each experimental point was run three times. The plating efficiency was calculated from the number of colonies (made of at least 50 cells) counted and the number of cells seeded. The surviving fraction was calculated according to the following formula: plating efficiency of treated sample/plating efficiency of control. The in vitro effect of the drug was expressed in terms of IC_{50}. For combination experiments, cells were exposed for 1 h to CDDP and 5 nM bafilomycin A1 (BafA1; Sigma Aldrich, St Louis, MO, USA) alone or in combination. The type of drug interaction was assessed in terms of synergistic ratio (R) calculated as (S-exp)/(S-obs), where S-exp is defined as the product of the cell survival observed for BafA1 alone and the cell survival observed for CDDP alone, and S-obs is the observed cell survival for the combination. Synergy is defined as $R > 1$ [12].

Small interfering RNAs (siRNAs) directed against target genes were selected using the online siMAX™ design tool (www.eurofinsdna.com). A BLAST search for siRNA sequences was carried out to exclude any alignment with other sequences in the human genome. Control siRNAs made of mismatched sequences (with no significant homology to any known human mRNA) were included. SiRNAs were manufactured by Eurofins MWG Operon (Ebersberg, Germany) as pre-formed and purified duplexes, made of 19 bp-long RNA oligonucleotides with two extra-thymidine bases forming a 3′ overhang on both strands. Each siRNA was resuspended in sterile RNase-free water, diluted to the appropriate stock solution (5 μM) and stored at −20 °C until use.

RNAi-mediated gene silencing was carried out in DU145/*miRVec* cells seeded at 2.5×10^5 in 25 cm^2 flasks the day before transfection. A given amount of RAB27A (siRAB), LAMP3 (siLAMP) and control (siCTR) siRNAs was mixed with Lipofectamine2000™ for 20 min at room temperature. The mixtures were then applied to the cells in a volume of Opti-MEM I (Life Technologies), giving a final concentration of 25 nM siRNA. After a 4-h incubation, cells were washed with PBS, fed with culture medium supplemented with serum and further incubated at 37 °C for the desired time interval.

2.2. In vivo experiments

Eight-ten weeks-old male SCID mice (Charles River, Calco, Italy) were maintained in laminar flow rooms under constant temperature and humidity conditions. Mice had free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale Tumori of Milan according to institutional guidelines, in compliance with national and international laws and policies. Exponentially growing DU145/*miRVec* and DU145/*miR-205* cells were subcutaneously injected into mouse right flank (10^7 cells/flank). For each cell line, half of the group (8 mice) received saline and half was treated with CDDP. The drug was delivered i.v. at a dose of 4 mg/kg every seven days for three weeks (q7dx3w)