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AUTOPHAGY SIGNALLING IS DIFFERENTIALLY MODULATED BY miR-204 IN CONTEXT OF INNATE IMMUNITY INDUCTION

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Abstract

This study investigates the initiation autophagy complex factor ATG16L and the most common mature autophagosomes marker – LC3 expression levels in conditions of innate immunity challenge and the modulatory role of miR-204 on it. The effect of miR-204 mimic transfection on Gram-synthetic bacterial peptidoglycan challenged human prostate cancer cell lines LNCaP (p53+/+, AR+), and PC3 (p53−/−, AR−) was evaluated using flow cytometric (FCS) and immunofluorescent detection of the expression of ATG16L and LC3. ATG16L and LC3 have an increased fluorescence observation in LNCaP cells treated with ie-DAP for 6 h, while in PC3 cells rather the opposite effect is observed. Flow cytometry demonstrated that ie-DAP and miR-204 have strongest effect on LC3 and lesser prominent one on ATG16L, and no effect when only ie-DAP is applied in LNCaP cells. In PC3 cells, conversely, they exert a rather negative effect with strongest impact of the concomitant application of miR-204 and ie-DAP on LC3 and no effect on ATG16L. Our data suggest that miR-204 differentially impacts autophagy signalling in prostate cancer cells in concordance with the p53 status, raising possibilities for targeted therapy augmentation through autophagy modulation using artificial micro-RNA.

Key words: prostate cancer, ATG16L, LC3, micro-RNA, ie-DAP

Introduction. Autophagy is an evolutionarily-conserved catabolic pathway with multiple roles in carcinogenesis that in some cases is able to inhibit

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the initiation of tumourigenesis through limiting the cytoplasmic damage, genomic instability and inflammation, as the loss of certain autophagy genes could lead to cancer [1]. Conversely, autophagy can also assist cells in dealing with stressful metabolic environments, thereby promoting cancer cell survival [2, 3]. In fact, some cancers rely on autophagy to survive and progress. Furthermore, some tumour cells can exploit autophagy to cope with the cytotoxicity of certain anticancer drugs [4]. Very controversially, it appears that certain therapeutics require autophagy for the effective killing of cancer cells. All these dichotomies converge into the notion that autophagy has an important, although complex, role in cancer. Additionally, the autophagy pathway is connected to the major cancer networks, including those driven by p53, mammalian target of rapamycin, RAS oncogene and the glutamine metabolism [1].

Micro-RNAs are small non-coding RNAs which negatively affect gene expression [5]. Their role in prostate cancerogenesis is still unclear and only a limited number of studies have investigated micro-RNA in prostate cancer [6]. Therefore, a better understanding of the role of specific micro-RNAs for the prostate cancer development and progression is needed. This study investigates the initiation autophagy complex factor ATG16L and the most common mature autophagosomes marker – LC3 expression levels in conditions of innate immunity challenge and the modulatory role of miR-204 on it.

Materials and methods. The effect of miR-204 mimic transfection on Gram-synthetic bacterial peptidoglycan challenged human prostate cancer cell lines LNCaP (p53+/+, AR+), and PC3 (p53−/− , AR−) was evaluated using flow cytometric (FCS) and immunocytochemical detection of ATG16L and LC3 expression.

Cell lines. LNCaP and PC3 prostate cancer cell lines were purchased from ATCC. They are Androgen receptor (AR) responsive (AR+), p53 enabled (p53+/+) and bone marrow metastasis derived p53 null (p53−/−) and AR-unresponsive (AR−) respectively.

Reagents. The minimal bioactive dipeptide present in the peptidoglycan (PGN) of all Gram-negative and certain Gram-positive bacteria-γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) was purchased from Invivogen and applied in concentrations of 4 μg/mL in cell cultures of LNCaP and PC3. miR-204 mimic (Qiagen) is single stranded synthetic micro-RNA resembling in vitro (and in vivo) the effect of mature miR-204 upon transfection in target cells.

Cell culture induction with innate inflammatory ligands and miR-204 mimic transfection. LNCaP and PC3 cell lines were maintained in RPMI and DMEM media (Sigma) respectively, supplemented with 10% fetal bovine serum (FBS), and were experimentally treated for 6 h or 24 h using 4 μg/ml ie-DAP or pre-transfected for 24 h with miR-204 mimic and subsequently antibody treated, and consequently became subject of immunofluorescent staining or flow cytometry analysis using rabbit anti-ATG16L and anti-LC3 polyclonal antibodies

or isotype rabbit IgG, and detected using an anti-rabbit IgG FITC-conjugated secondary antibody (all antibodies were from SantaCruz Biotechnologies).

Immunofluorescence (IFL). Both cell lines were grown on glass cover slips in 12-well cell culture plates and treated for 24 h with ie-DAP. After incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde for 10–15 min and subsequently permeabilized with 0.2% Triton X-100 in PBS for 15 min at RT. After non-specific protein interactions blocking using 1% BSA–PBS incubation at RT for 1 h, the cell-covered cover slips were probed with primary antibody, diluted 1:50 in 1% BSA–PBS and incubated overnight, at 4 °C with anti-ATG16L, antiLC3 antibodies or isotype rabbit IgG. After 3 wash steps with PBS, secondary mouse anti-rabbit IgG-FITC was used at 1:50 dilution in 1% BSA–PBS, for 1 h, RT (dark), and extensively PBS washed and Vector Shield – PI mounting media included. Slides were visualized with an Olympus fluorescent microscope.

Flow cytometry. Control and treated prostate cells were detached with 0.02% EDTA and washed with cold 1% BSA–PBS. For detection of ATG16L and LC3, the above-mentioned antibodies (Abs) were used. After 4% PFA fixation/permeabilization solution wash, and staining solution block (eBiosciences), the specific primary Abs or the appropriate isotype control Abs were used at concentration of 0.5 µg/10^6 cells for 30 min on ice, followed by BSA–PBS wash, secondary antibody incubation at 0.25 µg/10^6 cells for 30 min on ice (in the dark). The cells were gated using forward vs side scatter to exclude dead cells and debris. After washing, the cells were analysed with a BD FACSCalibur flow cytometer (Becton Dickenson). Fluorescence of 10^4 cells per sample was acquired in logarithmic mode for visual inspection of the distributions and for quantifying the expression of the relevant molecules by calculating the median fluorescence intensity (referred to as MFI) in a histogram overlay graphics.

Results and discussion. The choice of investigating the ATG16L and LC3 modulation by micro-RNA in innate immunity induction context is rather purposeful, since not only the chronic inflammation has been implicated in cancerogenesis theories for many years, but Nucleotide Oligomerization Domain receptors signalling has recently been implicated in both NFkB upstream signalling [7, 8] and autophagy pathway molecules recruitment [9]. NFkB is currently regarded as one of the main master transcriptional regulators implicated in multiple types of cancers [10, 11]. Therefore, the initiation of their signalling in prostate cancer cell lines was initially investigated using immune fluorescence with validated commercial antibodies (SCBT) against ATG16L and LC3 upon ie-DAP specific for these receptors ligand challenge. We observed an increase in the fluorescence of ATG16L and especially of LC3 in LNCAP ie-DAP challenged cells in contrast to PC3 cells, where the fluorescent staining of these molecules was surprisingly decreased (Fig. 1). We hypothesized that the innate immunity pathway induction in these cell lines distinct by p53 (positive vs null) as well as by androgen receptor sensitivity (dependent vs independent) have differential effect on autophagy

pathway mainly due to the p53 severe perturbation in PC3s. As one of the mechanisms of micro-RNA is the differential simultaneous modulation of a multitude of gene transcripts [5], we decided to explore further whether a micro-RNA like miR-204, which is under intense study in our lab for its regulatory effects exerted upon several master transcriptional factors, some of which are related to the p53 signalling, is also able to affect the autophagy pathway in these cancer cell line models. By transfecting an exogenous miR-204 synthetic mimic for 24 h we simulated the effect of this micro-RNA artificial upregulation and followed the same 6 h ie-DAP challenge vs non-challenge model on flow cytometry (Fig. 2). We observed that miR-204 upregulation alone resulted in an increase of the Median Fluorescent Index (MFI) of the overlaid histogram of LC3 compared to the non-treated cells. In our experiments we did not pre-treat cells with chloro-quinolne, a vacuole transport blocker usually used in LC3 studies, as this would eventually block the dynamics of the innate pathways as well. We additionally decided to follow the main histogram MFI rather than the high fluorescent populations of cells as we were interested in the general long-term change of LC3 as a marker of the mature autophagosomes. The change was less than several orders of magnitude and this was rather expected as the LC3 population is in constant dynamics and the only approach allowing for more distinguishable differences is through the use of chloro-quinolone, but this would impair the natural processes in other pathways we were interested in. On the other hand, LC3 protein abundance is rather homogenously and almost constantly distributed over the cell, with peri-nuclear and peri-tubular distribution emphasis. Therefore, the observed difference is rather significant and actually demonstrative for an effect of miR-204 on the LC3 mature autophagosome population. Similar, but less demonstrative was the effect of ie-DAP challenge alone, or the combination of ie-DAP challenge in conditions of cells pre-treated with miR-204. Since autophagy is a very complex and controversial process, sometimes having a differential effect in the same cancer type, but different cell lines, it was very interesting to observe that miR-204 actually had a suppressive effect on both ATG16L and LC3, with strongest effect on LC3 after combined miR-204 treatment and ie-DAP challenge. This effect opposing LNCaP has similarity to the observed differential autophagy engagement in prostate cancer treatment studies with PC3, having similarly decreased autophagy. Cao et al. [12] have observed that the mTOR inhibitor rapamycin also sensitizes prostate cancer cells lacking PTEN, like PC3 to radiation by activating autophagy. The need to activate autophagy in PC3 to eradicate them is probably related to the phenomenon of autophagy being a backup "scenario" of the cell-to-cell death faith when apoptosis pathway is "shut off" [13], like in PC3 lacking p53 and therefore devoid of mechanism for DNA damage-induced apoptosis. In contrast to them, LNCaP cells actually have p53 active, and most probably utilize autophagy in mTOR positive scenario, namely, using it for faster energy production after the reverse "Warburg effect", where anaerobic rather than aerobic glycolysis is uti-

lized by the tumour cells as more robust [14, 15]. The differential effect of miR-204 on LC3 in both scenarios suggests differential regulation of genes affected by the p53 changed context, while the lesser ATG16L changes would rather suggest an autophagy pathway induction preserved to some extent, where this molecule plays an important role.

This differential effect should be further explored and possibly utilized in future therapeutic scenarios to boost anti-tumour therapy or to corroborate the therapy to the prostate cancer subtype, as we observed an increased innate immunity signalling of NOD1 [16] and a decreased autophagy in the metastatic AR− cell line (PC3), and an induction in AR+ cell line (LNCaP). The sustained autophagy pathway in LNCaP might suggest that these cells would be more susceptive to therapy with mTOR inhibitors compared to PC3 cells that have the autophagy process even more "switched off" when either miR-204 pro-oncogenic micro-RNA or innate immunity pathway or in some cases both are artificially upregulated/induced.

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