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MIR-15A RECONSTITUTION IN PROSTATE CANCER CELL LINE SUPPRESSES CANCER PROGRESSION THROUGH DOWN REGULATION OF MYB AND ANDROGEN RECEPTOR UPREGULATION

K. Todorova and S. Hayrabedyan

Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences

Summary. Prostate cancer is one of the most common malignancies and the second leading cause of death from cancer in men. MicroRNAs are noncoding RNAs that have a role of post-transcriptional regulators. In this study we investigated how the tumour suppressor miR-15a modulates main transcription factors like cMYB and AR in androgen sensitive prostate cancer cell line LNCaP. The miR-15a inhibitor, mimic, and their negative controls were transfected into LNCaP cells. Real-time PCR analysis was performed in order to estimate the transcript levels of cMYB and AR. Flow cytometry analysis was performed to measure the protein levels of cMYB and AR. A Cell migration assay was done for cells transfected with miR-15a inhibitor and mimic. We found that cMYB is down-regulated and AR is up-regulated by miR-15a on the transcriptional and protein levels. By reconstituting miR-15a, we found that its down regulation in prostate cancer contributes to cMYB-induced cancer progression and reduced androgen receptivity. The ability of miR-15a to suppress cancer cell viability and migration is a very important phenomenon for understanding cancer heterogeneity in regard to adapted therapeutic approach development.

Key words: *prostate cancer, microRNA, AR, cMYB*

INTRODUCTION

Prostate cancer (PCa) is one of the most common malignancies and the second leading cause of death from cancer in men. The proto-oncogene c-MYB has been recently implicated in cancer metastasis [11, 6]. In PCa, AR signalling is crucial for cell survival. AR is fundamental for p53 activation and for the subsequent induction of apoptosis [4]. Its activation can also limit cell proliferation and mediate apoptotic induction under specific circumstances [13], like in conditions of genotoxic stress. MicroRNAs (miRs) are noncoding RNAs that exert a role of post-transcriptional regulators, silencing specific mRNA targets by binding specific seed sequences at their 3' UTR [7, 8]. miRs are mediators of AR function and the existence of a possible feedback loop between miRs, AR, and AR co-repressors [10]. This

implicates them directly in prostate carcinogenesis and progression as a mechanistic factor determining AR signalling specificity in androgen independent prostate cancers. miR-15a acts as putative tumour suppressor and it is implicated in cell cycle, apoptosis and proliferation. In this study we investigated how miR-15a modulates main transcription factors, like cMYB and AR in androgen sensitive prostate cancer cell line LNCaP.

MATERIALS AND METHODS

Cell Lines and MicroRNA Transfection. LNCaP (lymph-node metastasis-derived), AR+, p53 enabled prostate cancer cell line was purchased from the ATCC (US). The miR-15a inhibitor, mimic, and their respective negative controls (MiScript Inhibitor Negative control and AllStars Negative siRNA) (Qiagen) were transfected into LNCaP cells by HiPerFect (Qiagen) for 24h.

Cell Migration Assay. Cell migration assay was done for cells transfected with miR-15a inhibitor, mimic and their respective negative controls in 12-well plate format. We used a p200 pipet tip to create a scratch of the LNCaP cell monolayer after transfection. An inverted bright field Leica microscope, equipped with digital camera was used for the time lapse study. The examination of the cell migration was done on 0, 6, 12 and 18 hours. Using ImageJ software we measured the distance traveled by the cells during the time points and calculated the relative change in the scratch width measured in arbitrary units for each treatment.

Real-Time Reverse Transcription Quantitative PCR Analysis. The expression of cMYB and AR was detected by RT-qPCR. After 24 h mimic, inhibitor and negative control transfection mRNA was isolated from LNCaP cells using Midiprep kit (Qiagen). From each sample 500 ng total RNA was used to synthesise cDNA by Sensiscript Reverse Transcription kit (Qiagen). 500 ng cDNA was used for PCR reactions (SYBR Green QuantiTect RT-PCR MasterMix, Qiagen). RT-PCR Cycler (Agilent Technologies MX3005P, Stratagene) was used in this study. The mRNA transcript expression levels of all studied genes were normalised towards transcript levels of endogenous reference gene phosphoglycerate kinase 1 (PGK1). The following primer sequences, designed by us and produced by Biomers, were used: PGK1 Fw: 5'-att agc cga gcc agc caa aat ag-3', Re: 5'-tca tca aaa acc cac cag cct-3'; MYB Fw: 5'-aag tct gga aag cgt cac ttg-3', Re: 5'-aca tct gtt cga ttc ggg aga ta-3'; AR Fw: 5'-cgc tga agg aca gaa gta -3', Re: 5'-tct cct tcc tcc tgt agt ttc- 3'.

Flow Cytometry. LNCaP cells transfected with mimic, inhibitor and their negative controls were detached with Accutase (eBioscience). Specific monoclonal antibodies (Abs) (Santa Cruz Biotechnology) were used for detection of AR and cMYB. After IC Fixation/Permeabilization solution (eBioscience) wash and flow cytometry staining buffer, the specific primary Abs or the appropriate isotype control Abs were used at concentration of 0.5 μg/106 cells for 60 min, followed by BSA-PBS wash and secondary antibody (rabbit anti-mouse FITC conjugated IgG, Santa Cruz Biotechnology) incubation at 0.25 μg/106 cells for 30 min. Cells were gated using forward versus side scatter to exclude dead cells and debris. The cells were analysed with a BD FACSCalibur flow cytometer (Becton Dickenson). Fluorescence of 104 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 histogram overlay graphics.

Statistical Analysis. One-way ANOVA test with respective multiple comparison post-tests (Greenhouse-Geisser correction for nondata sphericity, Tukey correction post-test, adjusted P value, and family-wise significance, confidence level of 0.05) was used to analyse the data (GraphPad Prism 6). $P < 0.05$ was considered significant.

RESULTS

miR-15a negatively regulates cMYB transcription factor in LNCaP cells

Over-expression of miR-15a using mimic resulted in significant down-regulation of cMYB transcription level in LNCaP cells. We found the same relation on cMYB protein level after miR-15a artificial over-expression. This phenomenon was confirmed when miR-15a inhibitor was used. cMYB was significantly up-regulated on both transcript and protein levels after miR-15a suppression using inhibitor in the prostate cancer cell line (Fig. 1A).

AR regulation by miR-15a in LNCaP cells

AR transcript expression was significantly increased after miR-15a artificial over-expression using synthetic mimic. Transfection of miR-15a inhibitor resulted in non-significant AR mRNA down-regulation. The reconstitution of miR-15a using synthetic mimic produced significant increase in the AR protein levels. Suppression of miR-15a by an inhibitor, did not result in a significant change of the AR protein levels, compared to negative control transfected cells (Fig. 1B).

miR-15a Suppresses Migration in LNCaP Cells

The cell migration was decreased by miR-15a mimic in LNCaP cells (Fig. 2A) and miR-15a inhibitor increased cell migration in the cell line (Fig. 2B).

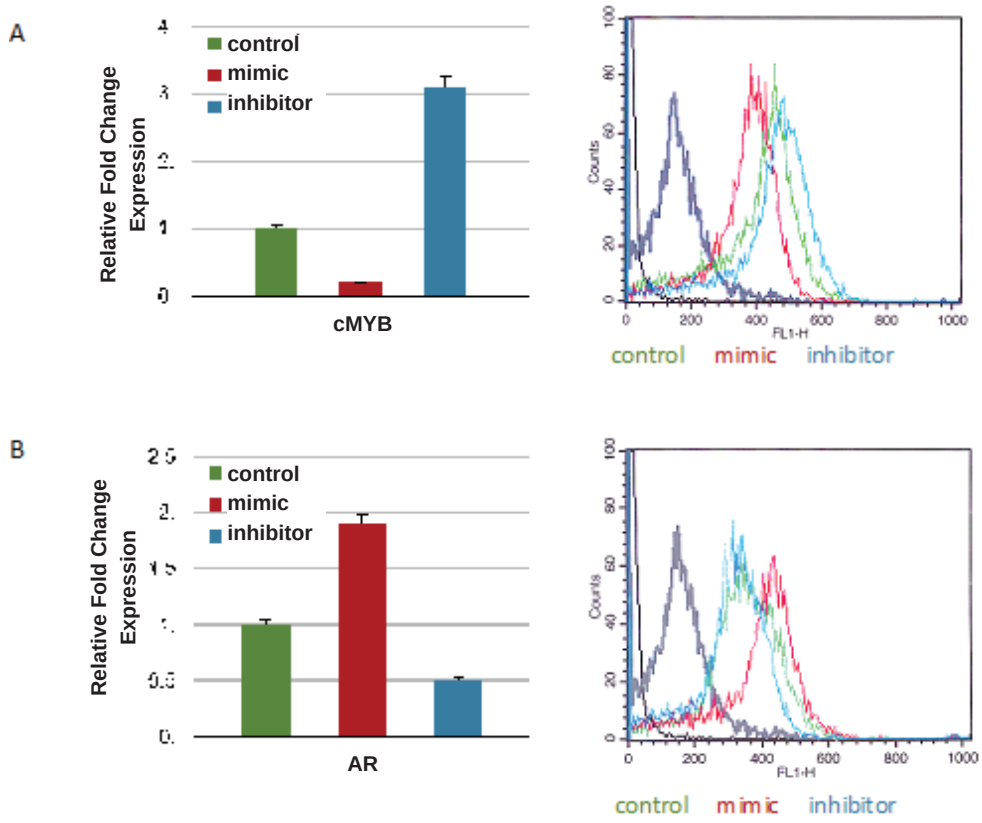

Fig. 1. Effect of miR-15a on cMYB and AR. AllStars Negative siRNA, MiSrcipt Inhibitor Negative Control, miR-15a mimic, or miR-15a inhibitor transfected LNCaP cell line were harvested for total RNA or FCS. qPCR was performed for A) cMYB and B) AR and the relative gene expression (Pfaffl, 2ΔΔCT), normalized to PGK1, was assessed for LNCaP cell line. FCS assay was performed for A) cMYB and B) AR *p < 0.01, *p < 0.05. Error bars represent s.d., n = 3 independent biological repeats

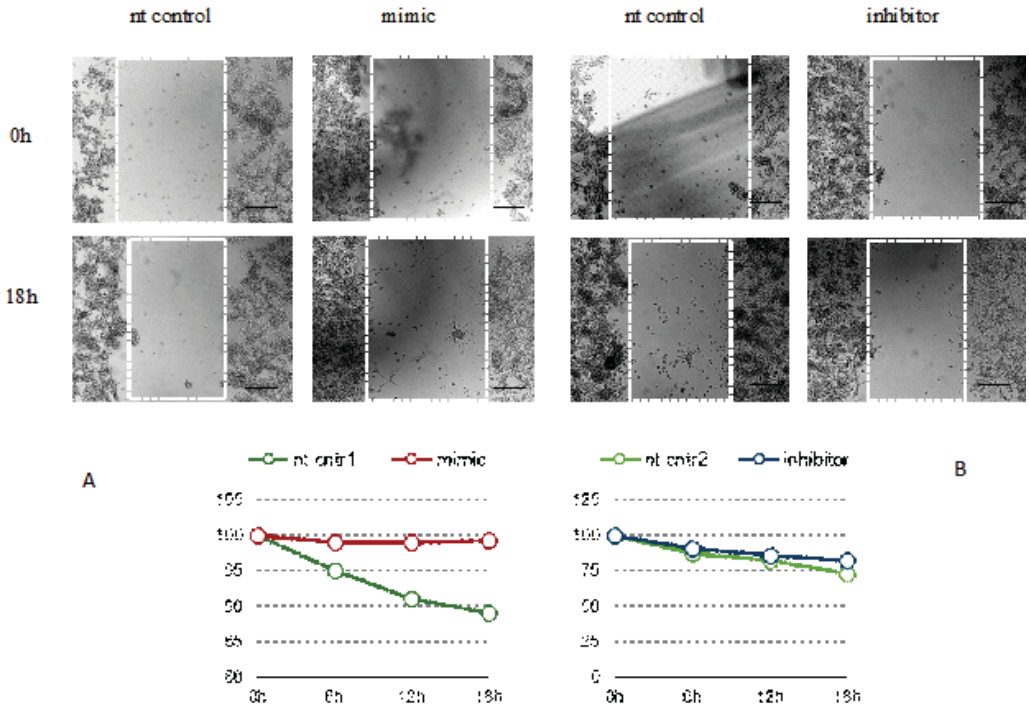

Fig. 2. miR-15a suppresses cell migration in LNCaP cell line. Cell migration assay was done for cells transfected with miR-15a mimic, AllStars Negative siRNA, miR-15a inhibitor, or MiScript Inhibitor Negative control. Morphometric measurement of the scratch width at 0 h and 18 h time points, represented as relative change in arbitrary units for each treatment. Error bars represent s.d., n = 3 independent biological repeats

DISCUSSION

MicroRNAs play an important role in prostate cancer differentiation, proliferation, apoptosis, and invasion. They are especially important for prostate cancer epithelial-mesenchymal transition, cancer stemness, drug sensitivity, cancer microenvironment, energy metabolism, androgen independence transformation, and diagnosis prediction [5]. In the context of PCa, miR-15 and miR-16 are tumour suppressors, at least in regard to tumour and stromal cells [12]. The down regulation of these miRs has been reported in many malignancies including: CLL, pituitary adenoma, and PCa [1, 2, 3]. Our results showed that miR-15a suppresses migration in LNCaP cells. The miR-15/16 family is highly expressed in NK cells. NK cells develop in the bone marrow and complete maturation in peripheral organs, but the factors controlling maturation are incompletely understood. The transcription factor Myb is expressed highly in immature NK cells, nearly absent in mature NK cells, and has been implicated in NK maturation. A study has reported that miR-15/16 dependent Myb regulation is essential for NK cell maturation [9, 14]. c-Myb expression is subject to post-transcriptional regulation by microRNA-15a. Using a luciferase reporter assay, it has found that miR-15a directly binds the 3'-UTR of c-myb mRNA. By transfecting K562 myeloid leukemia cells with a miR-15a mimic, functionality of binding was shown. The mimic decreased c-Myb expression, and blocked the cells in the G1 phase of cell cycle [15]. In this study we investigated how the tumour suppressor miR-15a modulates the master transcription factor cMYB and the AR in an androgen sensitive prostate cancer cell line. Our results showed that miR-15a negatively regulates cMYB transcription factor in LNCaP cells. Our results support the tumour suppressor role of miR-15a in PCa due to its direct relations to oncoprotein cMYB. By reconstituting miR-15a we found that its down regulation

in prostate cancer contributes to cMYB induced cancer progression and reduced androgen receptivity. In conclusion, the suppressive role of miR-15a and its regulatory activity on main master transcript regulators and AR is very important for prostate cancer biology in regard to adapted therapeutic approach development.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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✉ *Corresponding author:*
Krassimira Todorova
Institute of Biology and Immunology of Reproduction
Bulgarian Academy of Sciences
73 Tsarigradsko shose Blvd.
1113 Sofia
✆ +359 894371404
e-mail: krasiot@abv.bg