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THE ROLE OF microRNA-15A IN THE DEVELOPMENT OF PROSTATE CANCER – EFFECTS ON CELL PROLIFERATION AND PRO-INFLAMMATORY SIGNALLING

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Abstract. *Worldwide prostate cancer is the second leading cause of cancer death among men after lung cancer. MicroRNAs are non-coding, endogenous RNAs and they play a role in tumorigenesis, RNA silencing and post-transcriptional regulation of gene expression. In this study we have investigated microRNA-15a impact on transcription factors cMYB and ETS1 in prostate-carcinoma cell line PC3. The PC3 cells were transfected with a synthetic analogue and inhibitor of microRNA-15a. The study was performed using reverse transcription polymerase chain reaction and flow cytometry methods for assessing the transcript and protein levels of cMYB and ETS1, NFκB stable reporter live cell line. Statistical analysis was performed using One–way ANOVA test. We found that cMYB and ETS1 are up-regulated by the synthetic analogue of microRNA-15a at the transcription and protein level. Transfection with microRNA-15a mimic resulted in NFκB transcription factor activation as found by using the live cell reporter system. There was some opportunistic activity exhibited by the synthetic inhibitor, but less pronounced. Our data suggest that microRNA-15a could participate in prostate cancer progression by modulating cell proliferation and pro-inflammatory signaling and paves a way for further in-depth investigation of the gene regulatory networks underneath.*

Key words: *microRNA-15a, prostate cancer, transcription factors cMYB, ETS1, NFκB*

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INTRODUCTION

Prostate cancer is a frequently diagnosed cancer as it is the second leading cause of cancer death in men worldwide [1]. Prostate carcinogenesis is a process involving, among others, activation of oncogenes, and/or inactivating mutations in tumor suppressor genes. Bone metastases are often observed during prostate carcinoma development. The hemopoietic stem cell lineage is a direct target of prostate carcinoma cells during their dissemination and plays an important role in the formation of bone metastases. In this study, we have investigated two transcription factors (TFs) involved in prostate carcinoma metastases. The TF cMyb plays a major role in hematopoiesis and tumorigenesis [2], while the other TF – ETS1, is a major regulator of carcinogenesis and a potential marker for poor prognosis [3]. MicroRNAs, a family of small, ~20-24 nt long, non-coding RNAs, have recently emerged as new tissue and tu-

mor specific biomarkers in the diagnosis of the prostate carcinoma [4]. The tumor-suppressor function of some types of microRNA, such as microRNA-15a, is proposed to suppress various oncogenes. The aim of this study was to investigate the microRNA-15a impact on transcription factors cMYB and ETS1 in prostate-carcinoma cell line PC3.

MATERIAL AND METHODS

Cell Lines and Micro RNA Transfection. We used the androgen-insensitive (AR-), p53 negative (p53 -/-) prostate carcinoma cell line PC3, which has a bone metastasis origin. The cell line was commissioned by the American Type Culture Collection (ATCC). The miR-15a inhibitor, mimic and their respective negative controls (MiScript inhibitor Negative control and ALLStars Negative SiRNA) were transfected into PC3 cell by HiPerFect (Qiagen) for 24h.

Quantitative RT-PCR Cycler (Agilent Technologies MX3005P, Stratagene, USA) was used to detect the expression of the transcription factors cMYB and ETS1.

After transfection of PC3 cells with a synthetic analogue and inhibitor of microRNA-15a, total RNA was isolated with a Quick-RNA MicroPrep kit (Zymo Research). From each sample, 500 ng RNA was isolated for cDNA synthesis by the iScript Select cDNA Synthesis Kit (BioRad). For the PCR reactions, 10 ng of cDNA (Fast EvaGreen, BioRad) was used. The total reaction volume was 20 µL. The concentration of the primers was 0.5 µM (Fw, Re). The following primer pairs (Biomers) were used: c-MYB FW: 5" – aag tct gga aag cgt cac ttg-3' ; Re: 5' – aca tct gtt cga ttc gg gaga ta – 3' ETS1 FW: 5 – agt gct caa gga cat cga gac g-3'.Re:5' – agc cac ttc tgc aca ttg ctg; GAPDH Fw: 5' – aag gtc gga gtc aac gga ttt – 3' , Re: 5' – acc aga gtt aaa agc agc cct g – 3'.

Flow Cytometry. PC3 cells transfected with synthetic analogue and inhibitor of microRNA-15a, or negative controls, were detached with Accutase. Specific monoclonal antibodies (mAbs) were used for intracellular flow cytometry detection of ETS1 and cMYB [9], using a BD FACSCAlibur.

Producing a stable reporter cell line. PC3 cells were transfected with the NFκB reporter plasmid pNiFty2-SEAP (InvivoGen) amplified in E. coli. The cells containing the vector were selected using the antibiotic Zeocin (100 µg/mL) in DMEM medium. Stable clones were sub-selected for sensitivity to activated NFκB (2 months). Selected sub-clones were transfected using Attractene (Qiagen) with a microRNA-15a mimic, and inhibitor and with scrambled siRNA as a negative control. The culture medium was harvested after treatment and NFκB activation was detected by colorimetric secretable alkaline phosphatase (SEAP) reporter assay (InvivoGen). The SEAP reporter system was read on the BMG FLUOStar Optima plate reader at λ = 405 nm.

Statistical Analysis. One–way ANOVA test with respective multiple comparison post-tests (Greenhause – Geisser correction for no data sphericity, Tukey correction post-test, adjusted P value, and family-wise significance level of 0.05) was used to analyze the data (Graphpad Prisim 6) $P < 0.05$ was considered significant.

RESULTS

The amplification and dissociation curves of c-MYB are shown on fig. 1A and 1B with specific primers after RNA to cDNA conversion by reverse transcriptase and amplification by Taq polymerase.

We found that microRNA-15a suppression by synthetic inhibitor significantly increased c-MYB mRNA transcript levels relative to the non-targeting control. Similar but very weak activity was exhibited by microRNA-15a synthetic mimic as well (Fig. 1C). On protein level microRNA-15a mimic and inhibitor increased cMYB level relative to the controls (Fig. 1D).

The amplification and dissociation curves of ETS1 are shown on figures 2A and 2B with specific primers after the RNA reverse transcription to cDNA using reverse transcriptase and Taq polymerase amplification. The exogenous overexpression of microRNA-15a extremely increased ETS1 mRNA transcript levels relative to the non-targeting control and to microRNA-15a suppression (Fig. 2C). On protein level microRNA-15a mimic and inhibitor increased ETS1 level relative to the controls (Fig. 2D).

Using the live cell reporter system, NFκB transcription factor levels were found to be highest compared to the negative control after transfection with a mimic of the microRNA-15a. It has also been demonstrated that NFκB is activated after transfection with a microRNA-15a inhibitor, but significantly less than the use of a synthetic analogue of this microRNA (Fig. 3).

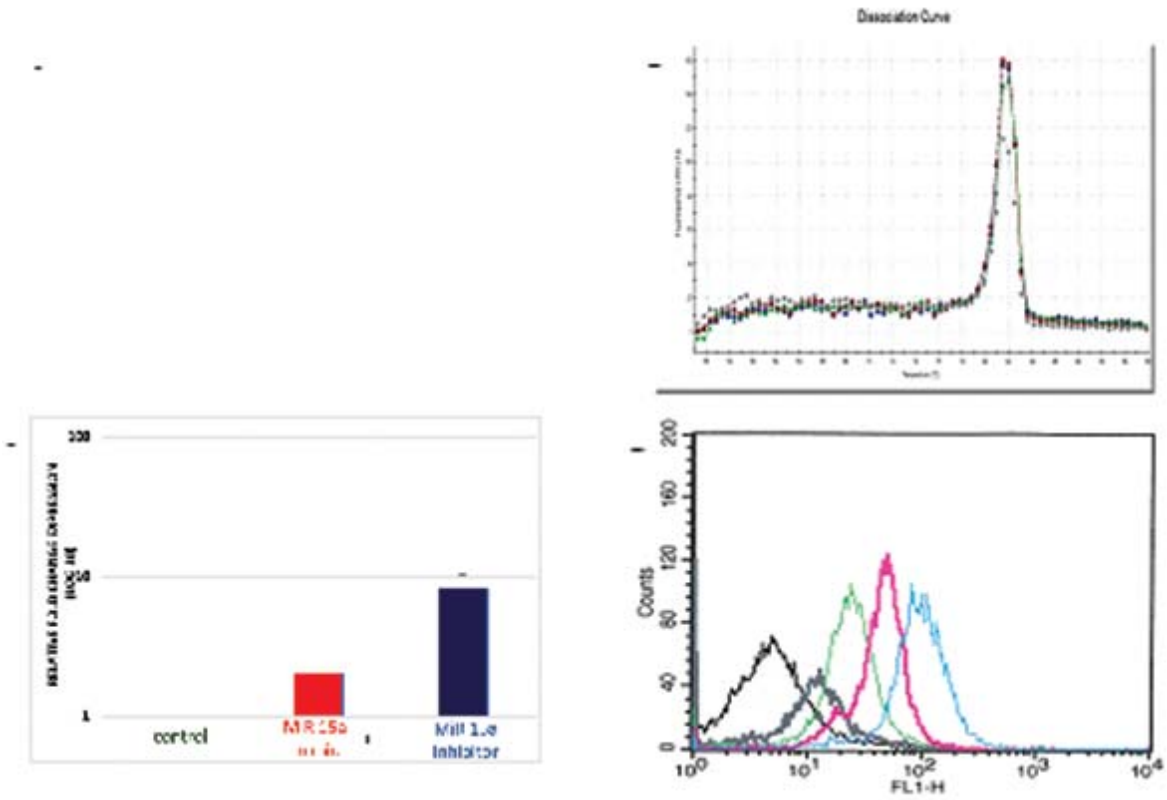

Fig. 1. Effect of microRNA-15a on cMYB. **1A)** Amplification curves. Fluorescence is represented as a derivative of the normalized signal on a linear scale. Exemplary data are amplified with transcripts of the cMYB gene. **1B)** Dissociation curve of the transcripts of the cMYB gene. **1C)** Comparative expression of transcripts of the cMYB gene in modulating the expression of microRNA-15a. **1D)** Flow Cytometry assay was performed for cMYB

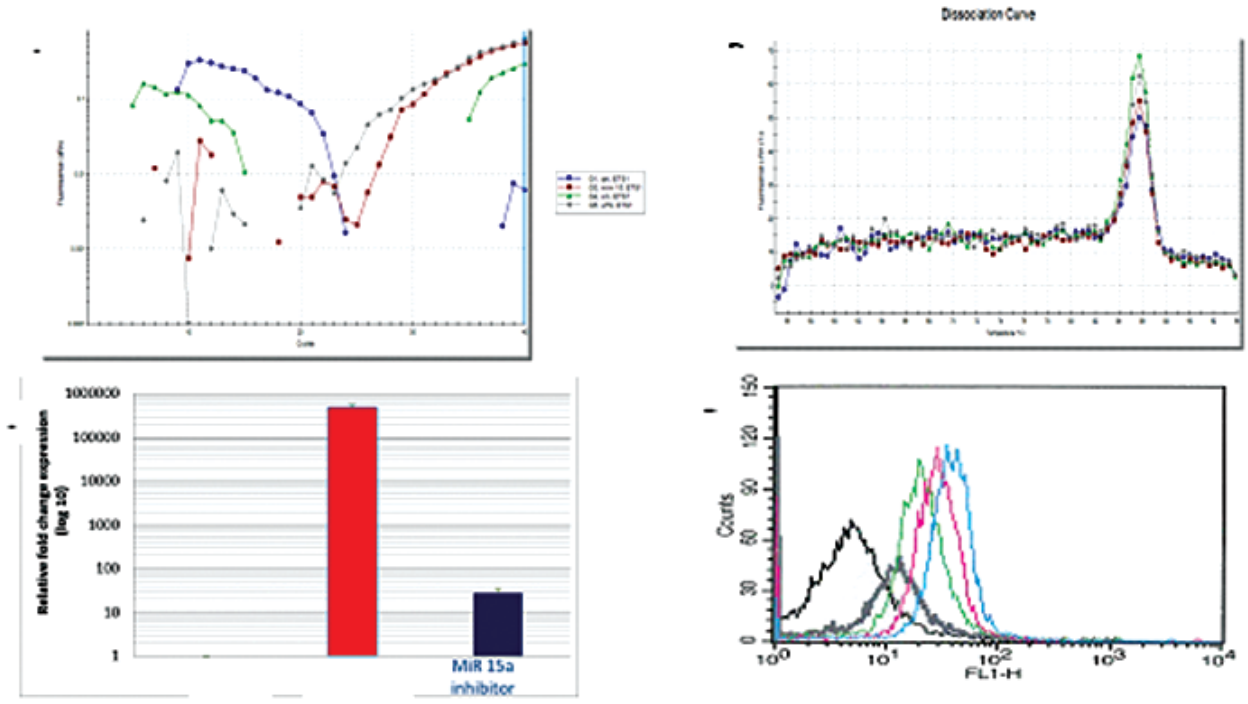

Fig. 2. Effect of microRNA-15a on ETS1. **2A)** and **2B)** show the amplification and dissociation curves of ETS1 with specific primers after the RNA reverse transcription into cDNA using reverse transcriptase and Taq polymerase amplification. **2C)** In PC3 cells, ETS1 mRNA levels were elevated following the action of the transfected synthetic analogue of microRNA-15a. Transcription factor expression levels were also increased by inhibition of the microRNA-15a relative to the control cells, but also significantly reduced by the action of the synthetic analogue of microRNA-15a. **2D)** FSC assay was performed for ETS1

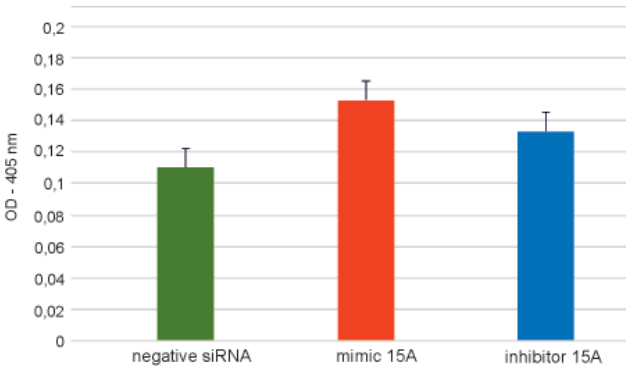

Fig. 3. Active NFκB levels detected by colorimetric SEAP reporter analysis after microRNA-15a transfection into a PC3 stable cell line

DISCUSSION

The disclosure of prostate specific proteins, undergoing quantitative and qualitative changes in malignant transformation, is particularly important for a better understanding of biology and for the diagnosis and treatment of human prostate cancer.

MicroRNAs are direct mediators of androgenic receptivity and their integration into DNA damage, cell death and survival, shows the cell-regulatory complexity of the male reproductive system [5, 6].

In this study, the complex relationship among non-coding microRNA-15a and transcriptional factors c-MYB and ETS1 was investigated as a part of genetic mechanism regulation of transcription factor network supporting the development and metastasis of prostate carcinoma. The role of microRNA-15a in the epithelial-mesenchymal transformation was partly elucidated.

MicroRNA-15a and microRNA-16 are tumor suppressors in tumor epithelial and stromal cells. The decreased expression of these microRNAs has been reported in many malignancies: chronic lymphocytic leukemia, pituitary adenoma and prostate carcinoma [7].

The suppressive role of microRNA-15a and its regulatory activity on major transcription factors is very important for prostate carcinoma biology in connection with the development of individualized therapeutic approaches.

An inducing effect of the microRNA-15a on these transcription factors was established. It was also found that in PC3 cells gene expression of cMYB was increased following the use of a microRNA-15a artificial analogue which significantly increased levels of microRNA in the cell. Suppression of the microRNA-15a using a synthetic inhibitor resulted in a significant change in cMYB mRNA levels relative to the non-target control (a sequence that did not hybridize to any gene), with expression significantly elevated. In PC3 cells, synthetic analogue of microRNA-15a increased the ETS1 mRNA transcript levels. Using the inhibitor of microRNA-15a, the ETS1 transcript levels were increased. Similar values in determining the expression of cMYB and ETS1 were found on the protein level. Probably, this is a rather complex mechanism of action that involves other molecules in the gene-regulatory network. This is indirect regulation of microRNA-15a to the transcriptional factor.

It has been discovered that ETS1 also binds to the promoter of c-MYB, a transcription factor involved in carcinogenesis, originally found overexpressed in some prostate carcinoma cell lines such as LNCaP, PC3, DU145 and C4-2, according to their androgen sensitivity [8].

In our previous study, an overexpression of c-MYB was detected in LNCaP and PC3 cell lines, and the transcriptional factor expression was suppressed in VCaP and H660 cell lines at the transcriptional and protein levels. Using transient gene silencing, positive feedback was found between c-MYB and ETS1 [9].

The promoter hypermethylation is a fundamental mechanism for the expression of c-MYB. Hypermethylation is necessary for the overexpression of c-MYB in lymph node metastases (LNCaP cells), whereas it serves as a limiting mechanism for c-MYB expression in bone marrow metastases (PC3 cells). c-MYB is required for epithelial-mesenchymal transformation in LNCaP and PC3 cells [10].

NFκB plays an important role in the development and invasiveness of many cancers. It provides an important mechanical link between inflammation and oncogenesis. The involvement of microRNA-15a in NFκB regulation relates the inflammatory cell signaling to pro-metastatic transcription factor reprograming, both affected by the same micro-RNA. Current data suggest that innate immune signaling is an effective modulator of the transformation of metastatic carcinoma cells in the prostate, in the direction of survival and transformation to a higher metastatic phenotype.

CONCLUSIONS

In conclusion, the study sought to clarify the role of and mechanisms related to the non-coding microRNAs in prostate carcinogenesis, an aspect of epigenetic regulation. It provides an insight to how knowledge of transcriptional factors signaling and inflammatory signaling could be translated to general pathology and clinical laboratory manifestations of prostate cancer progression. Thus, the study gives an opportunity for elaborating new strategies for disease diagnosis and treatment.

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

Authors' Contribution
Krassimira Todorova and Soren Hayrabedyan designed the study, acquired and analysed the data. Krassimira Todorova did the transfection experiments and FCS. Albena Apostolova, Leyla Sezer and Krassimira Todorova did the cell culturing and qPCR.

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