EXPRESSION OF miR-1247-5p IN PROSTATE CANCER

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HEIKKINEN LAURI: EXPRESSION OF miR-1247-5p IN PROSTATE CANCER

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Mikro-RNA:t ovat keskimäärin 22 nukleotidin pituisia solun lähetti-RNA:ta säätteleviä molekyylejä. Niillä on todettu olevan merkittävä rooli solun aineenvaihdunnan ja erilaistumisen säätelyssä, sekä erilaisissa syövissä.

Eturauhassyövän on todettu ilmentävän normaaliin eturauhaskudokseen verrattuna poikkeavaa mikro-RNA -profiilia ja tämä profiili on hyvin keskeinen eturauhassyöpien eri ominaisuuksien kannalta. Halusimme tietää, onko miR-1247-5p:n sekä kontrollien vesikulaariseen (INTERFERIN™) tekniikkaan perustuvalla transfektiolla vaikutusta LNCaP ja PC-3 syöpäsolulinjojen in vitro miR-1247-5p ekspression ja miR-1247-5p:n potentiaalisen kohde-RNA:n, MYCBP2:n ekspression osalta.

Kartoitimme qRT-PCR -tekniikan avulla solujen miR-1247-5p sekä MYCBP2 lähetti-RNA:n tasojen muutoksia soluissa verraten niitä kontrolleilla käsiteltyihin soluihin. Ekspressioarvojen normalisointiin käytettiin RNU6B -RNA:ta sekä TBP -RNA:ta. T-testiä käytettiin ryhmien välisten erojen havaitsemiseen.

LNCaP -solujen miR-1247-5p ekspressio muuttui merkitsevästi suhteessa kontrolliin. Solulinjojen välille tuli eroja sekä miR-1247-5p ekspressiossa että MYCBP2 ekspressiossa, mutta nämä eivät olleet tilastollisesti merkitseviä. Tulos on osittain ristiriidassa edeltävien aiheesta tehtyjen tutkimusten kanssa. Jatkossa mahdollisten ekspressioerojen osoittamiseksi tutkimus tulisi suorittaa suuremmalla otannalla.

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1. Introduction

1.1 MicroRNA function and biogenesis

MicroRNAs (miRNAs) are small single-stranded non-coding ribonucleic acid (RNA) molecules, consisting of 22 nucleotides on average. They are diversely expressed by eukaryotic cells and they function in the regulation of gene expression. (Bartel) There are several different types of small non-coding RNAs: transfer RNAs (tRNAs), ribosomal RNA (rRNAs), small nucleolar RNA (snoRNAs), small interfering RNA (siRNAs) and miRNA.

Over 60% of protein-coding genes have one or more cross-species conserved miRNA binding sites in their sequence and there is also a host of non-conserved mRNA-miRNA binding sites appearing only in humans. Most of the human protein-coding genome is regulated by miRNA(Minju). Over 2.000 miRNAs have been identified in humans (www.mirbase.org). The primary function of these molecules is considered to be post-transcriptional regulation of mRNA through destabilization, degradation and translational inhibition. (Bartel, Winter) Rarely they are known to up-regulate protein expression (Catto). miRNAs play a prominent role in developmental and oncogenic processes (Winter). The first miRNA was discovered in 1993 in *C. elegans* larvae and was named lin-4. Seven years later, homologues of *C. elegans* let-7 were found in human and fly genomes, and since then a veritable amount of miRNA genes have been identified. (Bartel) Nomenclature has since started to unify. Human miRNAs are named numerically, neighbors in clusters are determined with letter suffixes, and if there are multiple loci for the same miRNA a suffix is added to the end. Finally, depending on whether the mature RNA molecule is formed from the 3′ or 5′ strand of the premature miRNA. For example: miR-125b-1-5p. In this paper I will concentrate on the biogenesis, function and physiological role of miRNA in human cells, with emphasis on the role of miRNAs in prostate cancer.

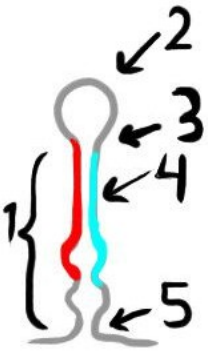

Image 1. Primary miRNA molecule sketch with functional regions. 1. Stem 2. Loop 3. Apical junction area 4. Duplex area of stem 5. Basal junction, with protruding 3′ and 5′ -ends.

1.1.1 miRNA biogenesis and regulation

miRNA synthesis in vivo is known to occur through 4 pathways: the canonical pathway, the Drosha and DGCR-8-independent pathway, the TUTase-dependent pathway and the Dicer-independent pathway. These processes have not yet been completely described in full detail. (Minju, MacFarlane)

The canonical pathway consists of primary transcription, endonuclear and cytosolic cleavage, and finally binding to AGO-proteins to generate an RNA-induced silencing complex (RISC) (Winter). The primary transcription of miRNAs is catalyzed by either RNA polymerases II or III. RNA polymerase II is known to synthesize pre-miRNAs, which have a 5′ 7-methyl-guanosine cap, and a polyadenylated 3′ tail (Lee). RNA polymerase III transcription hallmarks are interspersed Alu-repeats in the miRNA loci (Macfarlane). RNA polymerase III transcribes viral miRNAs, as well as some miRNA-like small RNA molecules derived from tRNA transcripts (Minju). This process results in precursor miRNAs, termed pri-miRNAs of ~70 nucleotides in length, a double strand of RNA consisting of a single ribonucleic acid chain folded upon itself with imperfect complementarity to form a hairpin resembling structure with protruding 5′ and 3′ ends (see image 1). Pri-miRNAs are then processed by an enzyme complex termed Drosha-DGCR8 or 'microprocessor', which cleaves both 5′ and 3′ arms simultaneously, leaving the 3′ arm overhanging by 2 nucleotides. (Minju, Winter, MacFarlane)
In the cytoplasm, the pre-miRNA must bind to an argonaute-protein in order to form RISC (RNA induced silencing complex). It will accomplish this through RLC (RISC-loading complex), which consists of an argonaute protein, RNase-enzyme Dicer, and stabilizing and assisting enzymes TRBP

and PACT. Dicer will cleave off the loop from pre-miRNA, generating approximately 22 nucleotides long duplex of miRNA with protruding 3′ ends. Both 3p- and 5p-strands are intact at this point. RLC will then merge with the functional guide strand, while 96-99% of the time the passenger strand is left for degradation.(Minju) The nucleotides 2 to 7 downstream of the 5′ end are crucial for miRNA target recognition (dubbed 'seed' region), while nucleotides 8 and 13 to 16 often also contribute (Minju). AGO can catalyze RISC forming process by facilitating duplex unwinding and cleaving the passenger strand. For pre-miRNAs displaying high degree of complementarity along the stem, Ago2 can dent the 3′ arm of the hairpin to facilitate its processing by Dicer. (Minju, MacFarlane) Molecules handled in this fashion are dubbed ac-pre-miRNA. There are also some helicase enzymes associated with the unwinding of the duplex into functional and degradable strands, but as in vitro studies of the RISC maturation have proved, helicase activity is not crucial for the reaction. (MacFarlane)

Upon cytoplasmic maturation of miR, the 5p-3p strand duplex subsequently gets unwinded and loaded onto RISC. RNA-induced silencing complex can be classified into 8 subtypes, and can include argonaute proteins 1-4 depending on the type. Ago2 is the only argonaute protein cabaple of directly catalyzing mRNA cleavage when it perfectly complements the guide miRNA, although all four subtypes are cabaple of mRNA translation inhibition. (Minju, Macfarlane) As opposed to many animals, human AGO proteins hardly distuingish mutually between targets, preferring small RNA duplexes with base pair mismatches roughly in the middle (nt positions 8-11). (Minju)

Three alternative pathways have been described for miRNA to reach maturity. Intron-derived miRNAs called mirtrons are spliceosome processed introns, which have the proper amount of nucletotides and can be folded directly by DBR1 into a hairpin pre-miRNA, an example of Drosha-DGCR8 -independent processing. Once the endonuclear processing of the formed pre-miRNA is complete, Exportin-5 will identify it based on the length of the stem and the 3′ end, and will merge with Ran-GTP complex to expel the pre-miRNA from the nucleus. In the cytoplasm, the processing will continue with Dicer cleaving the loop region off, preparing the miRNA duplex for RLC (RISC loading complex). (Minju, Winter, MacFarlane)

Some pre-miRNAs only have one nucleotide overhanging from their 3′ end. These molecules are suboptimal for Dicer processing and need to be monouridylated by either TUT2-, TUT4-, or TUT7-enzymes in the cytoplasm before maturation. This is called the TUTase-dependent pathway. (Minju) For example pre-mir-451 is a Dicer-independent pathway miRNA product. It is produced by Drosha and exported into the nucleus. There it will skip Dicer processing and AGO2 will slice it forming ac-pre-mir-451. The molecule is then further trimmed by PARN enzyme, enabling RLC interaction.

(Minju)

miRNA expression is regulated on every step of the synthesis process, ultimately even the mature RISC complex can be regulated. Tissue-specific regulation is key to bilateralism and embryonic development. Various effectors take part in complex regulation of the transcription enzymes RNA polymerase II and III, which both have their own specificities for recognizing miRNA promoter- and terminator sequences. Same miRNA genes often have multiple transcription start sites. (Minju) Epigenetic regulation occurs through DNA methylation and histone modification. MiRNA genes reside often in clusters, and in close proximity of CpG islands, which enhance epigenetic regulation. miRNA genes often reside in areas of the genome called fragile sites. (Catto) These sites are prone to alterations in their structure, for example sister chromatid exchange, translocation, deletion, amplification or insertion of exogenous DNA. (Croce) Changes in the miRNA synthesis machinery affect miRNA processing to an even greater extent. miRNA expression pattern changes are associated with these events and form the basis of understanding miRNA expression aberrations in human cancers. (Catto, Croce)

MiRNA expression pattern changes can set off carcinogenesis and advance disease state. For example MYC upregulates transcription of miR-17-92 cluster on chromosome 13, which in a pathological cell leads to the overexpression of miR-20a targeting transcription factors E2F1-3. Depending on cell cycle phase, reduced E2F1-3 effect may upregulate cell proliferation or reduce p53 and caspase-mediated apoptosis, initiating carcinogenesis. MiRNA is expression is dynamical, so different physiological circumstances may lead to differing expression of miRNA in a single cell, concerning also tumor cells. Epigenetic and genetic changes both play a major part in miRNA expression. Fragile site genomic alterations have frequently been associated with the onset of cancer. MiRNAs often target hundreds of genes and their mutations are rare in cancer, implying low selection pressure. However, mutations in mRNA target sites for specific miRNA are possibly much more important in carcinogenesis. (Catto) Epigenetic silencing is known to affect miRNA expression in cancer, 20-40% of miRNA are estimated to be situated in close proximity of CpG islands. miRNAs are also able to affect DNA structure epigenetically through histone methylation machinery. Around 1/3 of miRNAs are clustered, while the rest are solitary. Clustered miRNAs are more powerful cellular regulators, since a single change in these clusters can affect the translation of thousands of proteins. (Catto, Macfarlane) Intronic or exonic miRNA expression depends on the host gene expression: in prostate cancer, two of the most highly expressed protein-coding genes contain the highest expressed miRNA sites (MCM7 contains miR-106b-25 cluster and C9orf5

contains miR-32). (Catto) In cancer specimen studies, the miRNA expression patterns are often differing between different sets of tumors, but they often interlap in targeted pathways associated with carcinogenesis. Multiple miRNAs often contribute a synergistic effect on a particular cellular function, which makes the usual physiological effects of miRNAs on any particular mRNA more effective, potentially allowing carcinogenesis more likely. (Catto)

1.1.2 Post-transcriptional editing and degradation of miRNA

Additional post-transcriptional editing of the miRNA base sequence occurs either in the nucleus or cytoplasm, through enzymes ADAR1 and ADAR2 (adenosine deaminases acting on RNA). These enzymes convert adenosine bases into inosine ones. Inosine has similar properties as guanosine, so structural changes of the pri- or pre-miRNA affects their sequence, base-pairing and structural properties. This editing can have a further effect on the cytosolic pre-miRNA, subjecting it to alternate processing and different target protein expression. (Winter) Some proteins can target miRNA for degradation, as lin28 targets let-7 miRNA, inhibiting its Drosha and Dicer processing, and TUTase mediated uridylation, facilitating its decay. (Macfarlane, Minju)

1.1.3 miRNA function.

The primary miRNA function is the regulation of protein expression through mRNA translation inhibition. When the functional guide strand miRNA gets loaded into RISC, it becomes more stable and able to interact with its target mRNAs. In protein translation, the mRNA fuses with ribosomes that synthesize the polypeptide chain that makes up the basic protein molecular structure. RISC is able to bond with mRNA through base-pair interaction. The base-pair binding needed for translational repression commonly occurs between the 3′ UTR of the mRNA and the miRNA seed region (nucleotides 2-8 at 5′ end). This interaction apparently makes it more difficult for ribosomes to bind and sift through the mRNA, without markably stabilizing it, which results in less polypeptide made over time. (Bartel) Usually human miRNAs have partial complementarity with their target sites along the length of the mRNA, making it appear slightly truncated and wrinkled in its bound state, as opposed to a tightly bound perfectly complementary RNA duplex. A single RISC is able to repress multiple mRNAs during its lifespan. If there is a high degree of complementarity between the target mRNA and RISC carrying an AGO2 enzyme, the mRNA can be cleaved. Cleaved mRNA is degraded through deadenylation and exosome exonuclease processing, or Dcp1 and Dcp2 facilitated 5′-3′ degradation by exoribonuclease Xrn1p. (MacFarlane)

1.2 miRNAs in prostate cancer

A host of miRNA gene expression is altered in PCa, associated with pathways controlling apoptosis, androgen independence, cell cycle control, DNA repair, gene expression, signal transduction, drug resistance, cell migration, cell stress response, DNA replication and even the ability of the tumor to tolerate radiation therapy. At least 10 different miRNA pathways facilitating apoptosis avoidance have been associated with prostate cancer to date.
MiR-15a/16-1 are clustered on chromosome 13q14 and downregulated in 4 out of 5 PCas. The primary oncogenic effects exerted through these genes are upregulation of cyclin D1 leading to facilitated G1/S transition, and loss of translational control of WNT3a, leading to oncogenic Wnt pathway activation. Increased miRNA-125b activity facilitates androgen-independent growth in LNCaP (lymph-node derived prostate cancer cell line) cells and reduces apoptosis by targeting BBC3, p53, BAK1. MiR-21 upregulation is shown to reduce apoptosis, induce proliferation, facilitate cell migration and increase androgen insensitivity in PCa. It also seems to correlate well with serum PSA and docetaxel resistance and has an ARE in its promoter. MiRNAs partake in androgen signalling of prostate cancer cells. At least miR-125b, miR-21, miR-146a, miR-141 and miR-331-3p expression aberrations have been implicated in altered androgen responsiveness. (Catto)

2. Materials and methods

2.1 Cell culture and transfection

Cell culture was done with PCa cell lines PC-3 and LNCaP (obtained from the American Type Culture Collection, Rockville, MD), which were cultured according to the recommended conditions. After sufficient amount of cells cultured under stable conditions were obtained, the cells were split into two 24-well plates.
The transfection with miRNA-1247-5p was done with human miRVana™ microRNA mimic or inhibitor miR-1247-5p and respective negative controls at 100nM concentrations (Thermo Fisher scientific/Ambion, Waltham, MA). Transfection reagent in use was INTERFERin™ (Polyplus-transfection, Illkirch, France). Solutions containing 60 pmoles of either miRNA mimic, miRNA mimic control, miRNA inhibitor or miRNA inhibitor control, and 100 microliters of Opti-MEM®

was prepared. 3 microliters of INTERFERIN™ was added to the solutions, and they were immediately vortexed afterwards. Then the solutions were incubated at room temperature for 10 minutes. Meanwhile, the growing medium from the cells which had been transferred to the 24-well plates was gently removed, thus retaining cell adherence onto the plates. 0.5 ml of complete growing medium was added to the wells, after which 100 microliters of the prepared transfection mix was added, and the wells were gently swirled, homogenizing the medium. At 600 microliters of 100nM miRNA transfection solution, the plates were placed in incubation at 37°C for 72 hours.

2.2 Trizol® extraction of RNA

After incubation, the growing medium was gently removed and the wells were washed with 1ml of PBS. 500 microliters of Trizol® was pipeted up and down until the cells had been detached from the plate into the solution, then the extract was stored into separate 1.5ml tubes. In the next phase, the solution was incubated at room temperature for 5 minutes to allow nucleoprotein complex dissociation. 0.1 ml of chloroform was added per tube and was followed by 15 seconds of vigorous shaking of the tube, followed by 3 minutes of incubation at room temperature. Samples were then centrifuged at 12000 * g for 15 minutes at 4°C. The RNA exract at this point had been separated into a colorless upper phase in the tube, which was collected into a separate tube.
In the next phase, 5 micrograms of RNase-free glycogen was added to the RNA extract, followed by 0.5 ml of 100% isopropanol. The solution was incubated at room temperature for 10 minutes, then centrifuged at 12000 * g for 10 minutes at 4°C. The supernatant was then removed from the tube, leaving an RNA pellet in the bottom. The pellets were washed with 0,5ml of 75% ethanol, followed by a brief vortex and centrifugation of 7500 * g for 5 minutes at 4°C. The liquid wash was then removed. Excess remaining liquid was removed by air drying the tubes for approximately 1 hour. The RNA pellets were then resuspended in RNAse free water by pipeting until the pellet had dissolved. After incubating in a water bath at approximately 60°C for 10 minutes, the samples were stored in a freezer at -70°C. Afterwards, the RNA concentrations were determined by photometric absorbance at 260nm wavelenght.

2.3 Reverse transcription of the RNA samples

The reverse transcription for miR-1247-5p was performed using specifically designed TaqMan probes (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer´s protocol. Briefly,

a master mix of 7 µL per sample was prepared containing 0.15 µL 100mM dNTPs, 1.00 µL of MultiScribe™ Reverse Transcriptase, 50 U/µL, 1.50 µL of 10X Reverse Transcription Buffer, 0.19 µL of RNase Inhibitor, 20 U/µL and 4.16 µL of nuclease-free water. 1 to 10 ng of total RNA in a total volume of 5 µL was added, together with 3 µL of reverse transcription miRNA-specific probe per reaction for a final volume of 15 µL per reaction. The 48 samples were pipetted onto a 96-well plate, briefly centrifuged and placed on a thermal cycler with the following program: 30 min. at 16º C, 30 min. at 42º C and 5 min. at 85º C.

The reverse transcription for MYCBP2 and TBP was performed using the Maxima Reverse Trancription protocol (Thermo Fisher Scientific, Waltham, MA), according to manufacturer´s instructions. Briefly, a master mix was prepared containing 0.5 µg of total RNA, 1µL of random hexamer primers, 1µL of dNTPs and water for a total volume of 14.5 µL. After incubation at 65º C for 5 min., 4µL of reverse transcription buffer, 0.5 µL of RNAse inhibitor and 1µL of Maxima reverse transciptase were added per reaction, for a total volume of 20µL. The reactions were pipetted in a 96-well plate and placed in a thermal cycler with the follwing program: 10 min. at 25º C, 30 min. at 50º C and 5 min. at 85º C. The reverse transcription products were subsequently stored at -20º C.

2.4 qRT-PCR

Quantification of RNA expression changes were measured using quantitative real-time PCR. TaqMan assay probes were used for the qRT-PCR of miR-1247-5p and specifically designed primers for MYCBP2 were used, forward MYCBP2 5´-GGGGACGGATTCTACCCAG-3´ and reverse 5´-ATTGAGCGCAGCGGTATAAAT-3´. The expression data were normalized against RNU6B (TaqMan probes) and TBP (forward 5´-GAATATAATCCCAAGCGGTTTG-3´ and reverse 5´-ACTTCACATCACAGCTCCCC-3´) respectively for miR-1247-5p and MYCBP2. A standard reference curve was prepared by using a homogenized sample preparate in a series of six wells in serial 1:5 dilutions. To measure the expression of miR-1247-5p and the reference RNU6B, a master mix was prepared containing 1.00 µL of TaqMan® microRNA Assay probe (20X), 10.00 µL of TaqMan® Universal PCR Master Mix II (2X) and 7.67 µL of nuclease-free water. The samples were place on a thermal cycler with the following program: 50º C for 2 minutes, 95º C for 10 minutes, 95º C for 15 seconds and 60º C for 60 seconds. The steps 3 to 4 were repeated for 40 cycles. For MYCBP2 and TBP, a master mix was prepared containing 12,5 microliters of Maxima™ SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA), 0,125 microliters

of forward and reverse sample-specific primers and 7,25 microliters of RNAse free water all added up by a factor of 60 per study set to account for all samples and pipetting error. 20 microliters of the master mix was used per sample on a 96-well qRT-PCR plate and 2 microliters of template cDNA solution was added per well, amounting up to 54 wells per RNA study set including the standard. The qRT-PCR was then ran with a specific thermal phase sequence depending on the gene that was amplified. Amplification plots including standard curves were retrieved for student's t-test analysis.

3. Results

3.1 Results of the qRT-PCR

The first goal of the study was to measure the effect of transient transfection of PCa cell lines PC-3 and LNCaP with miR-1247-5p mimic and control on the cells' expression of miR-1247-5p. RNU6B was used as a reference expression normalizer gene, to account for individual cell sample differences of RNA expression.

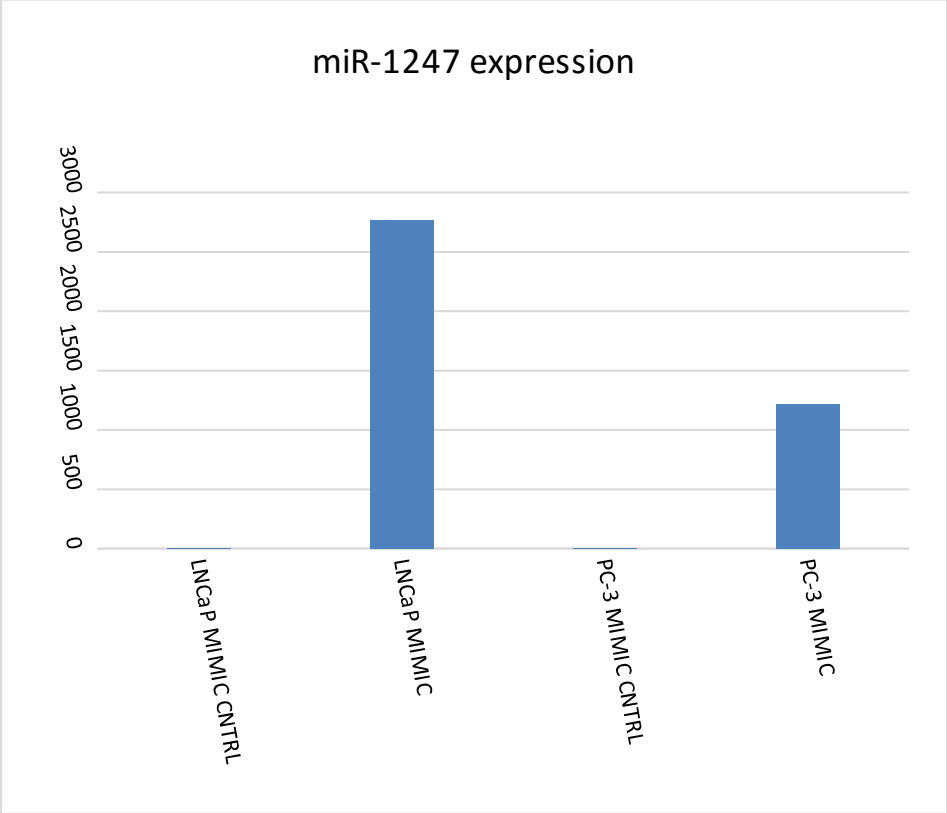

Image 2. qRT-PCR performed on LNCaP and PC-3 prostate cancer cell lines. Expression of miR-1247 measured by TaqMan assay with miR-specific probe and normalized against RNU6B in LNCaP and PC-3 cells transiently transfected with miR-1247-5p mimic or scrambled control.

Student's t-test analysis revealed a dramatically increased expression of miR-1247-5p in LNCaP

mimic vs. control group (p=0,037121). The PC-3 mimic vs mimic control of miR-1247-5p showed similar markedly increased expression, although not statistically signicant in this case (p=0,103238). The results confirm that the transfection of miR-1247-5p was effective.

The second goal of the study was to measure if the transfection of miR-1247-5p mimic or inhibitor (compared to respective controls) had an effect on MYCBP2 mRNA expression. MYCBP2 is a putative target gene of miR-1247-5p. TBP was used in this case as a reference gene for this MYCBP2.

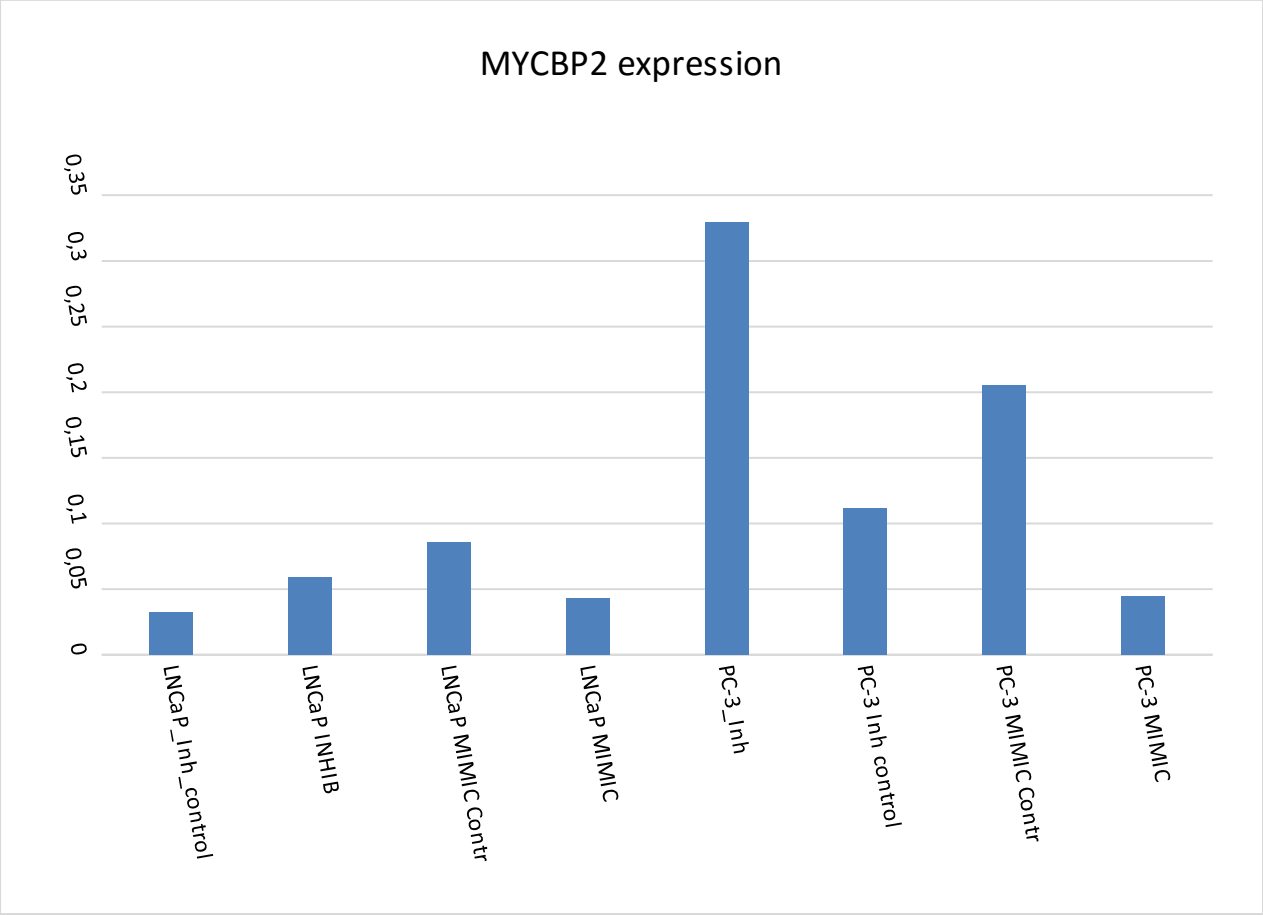

Image 3. qRT-PCR expression of MYCBP2 mRNA normalized against TBP in LNCaP and PC-3 cells transiently transfected with miR-1247-5p mimic or inhibitor and respective controls. The results are the average of 6 replicates. Student´s t-test was used to calculate the statistical significance.

In both cell lines tested a trend of upregulation of MYCBP2 was found in the cells transfected with

miR-1247-5p inhibitor and a trend of downregulation of MYCBP2 was found in cells transfected with miR-1247-5p mimic. These results are consistent with the hypothesis that MYCBP2 is a target gene of miR-1247-5p, although the expression differences were not statistically significant.

4. Discussion

The results revealed that transient transfection of miR-1247-5p was effective, although not statistically signicant (p=0,103238) miR-1247-5p expression level changes were observed in the PC-3 cell line. In both cell lines tested a trend of upregulation of MYCBP2 was found in the cells transfected with miR.-1247-5p inhibitor and a trend of downregulation of MYCBP2 was found in cells transfected with miR-1247-5p mimic, supporting the initial hypothesis that MYCBP2 is a putative target of MYCBP2 in prostate cancer. The lack of statistical significance in MYCBP2 mRNA expression level changes induced by miR-1247-5p overexpression is partially contrary to previous studies performed on the subject, in which a statistically significant downregulation of MYCBP2 mRNA by miR-1247-5p was found. Also MYCBP2 is a high-scoring potential target gene of miR-1247-5p, based on the complementarity of the 3′-UTR of the gene, as determined by online target prediction analysis. (Scaravilli) The possible lack of statistical power could be pinpointed to multiple separate factors affecting the study setup. The efficiency of the standard curve was not optimal, probably due to pipetting error. The cells were cultivated under recommended conditions, although the operator was not very experienced in handling the cells, which may have caused some delays at critical points in the cell culture and transfection process. This could further along affect final RNA output markedly through cell stress. The RNA extraction process was quite laborious with many phases taking part over several different sessions, between which, the samples were always stored under recommended conditions, but the increased handling of the samples always makes them more prone to contamination. Moreover, considerable amount of time passed between cell culture and transfection and the final measurements, during which spontaneous RNA-decaying processes have more time to work. This study would thus warrant further investigation of the MYCBP2 mRNA expression change consistency of PCa cells expressing miR-1247-5p.

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