A pilot study on the expression of microRNAs resident on chromosome 21 in laser microdissected FFPE prostate adenocarcinoma samples

ADRIAN MIHALA1, ANDREEA ANA ALEXA1, CORINA SAMOILĂ1, ALIS DEMA2, ANDA-CORNELIA VIZITIU3, ANDREI ANGHEL1, LIVIU TAMĂȘ1, CĂTĂLIN VALER MARIAN1, IOAN-OVIDIU SIRBU1

1) Department of Biochemistry and Pharmacology, "Victor Babeș" University of Medicine and Pharmacy, Timisoara, Romania
2) Department of Pathology, "Victor Babeș" University of Medicine and Pharmacy, Timisoara, Romania
3) Doctoral School, "Victor Babeș" University of Medicine and Pharmacy, Timisoara, Romania

Abstract

The tremendous research effort of the last decades added a new, epigenetic layer of complexity to the already complex image of prostate cancer pathogenesis. Here we use quantitative real-time polymerase chain reaction (qRT-PCR) to investigate the expression of the microRNAs resident on chromosome 21 (miR-ch21) in laser capture microdissected (LCM) tissues from formalin-fixed paraffin-embedded (FFPE) archived, prostate adenocarcinoma samples. We show a strong, specific down-regulation of miR-ch21 in tumoral epithelia and stromae as compared to normal counterparts, results at odd with the current paradigm on the involvement of these microRNAs in prostate oncogenesis. By comparing this result with the expression of two well-known pluripotency associated microRNA, hsa-miR-372 and mir-373, we suggest that miR-ch21 down-regulation might be the result of specific silencing of miR genes mapped to chromosome 21. Further studies, of larger sample size are needed to confirm our preliminary data.

Keywords: prostate adenocarcinoma, microRNA, laser capture microdissection, formalin-fixed paraffin-embedded, miR-ch21.

Introduction

Despite of the remarkable progress in understanding the molecular pathogenic mechanisms, prostate cancer remains one of the deadliest cancers, topped only by lung cancer [1]. Differences in the incidence of prostate cancer in Scandinavian countries, Japan and China versus Western, occidental countries strongly argue for a multi-factorial pathogenesis, involving genetic, epigenetic and environmental factors. Interestingly, Down syndrome patients seem to be protected against the development of solid tumors [2] due to DSCR1, a gene shown to inhibit the vascular endothelial growth factor (VEGF)-related angiogenesis [3].

In vivo and ex vivo experiments have identified several signaling pathways involved in the development of prostate cancer, including androgen receptor (AR) signaling, PI3K/AKT and MAPK signaling, and TGFβ and Wnt signaling [4]. At least one of these pathways has been shown to act through modulation of expression of microRNAs, small (20–25 nucleotides) non-coding RNA molecules able to modulate gene expression in mammals by altering the stability of target mRNA molecules [5].

Among the most recent developments in the research of prostate cancer pathogenesis, microRNA stand out due to their functional versatility and remarkable stability in fresh and archived tissues or biological fluids [6]. FFPE (formalin-fixed paraffin-embedded) samples analyses found several microRNAs to be associated to prostate tumor biology, most of the times involving down-regulation of microRNAs (e.g., hsa-miR-143, hsa-miR-145), while up-regulation is a much more seldom phenomenon (let-7c, miR-221, miR-182-5p, etc.) [7, 8]. Furthermore, circulating microRNAs like miR-141, miR-200b, miR-375 have been proposed as potential biomarkers for non-invasive diagnostic of prostate cancer [9, 10]. In this context, one of the interesting questions arising is whether the protection against solid tumors of Down syndrome patients associates a change in the expression microRNAs resident on chromosome 21 (miR-ch21): let7c, hsa-miR-125b-2, hsa-miR-155, hsa-miR802, hsa-miR-99a.

Some of the miR-ch21 have already been associated with prostate cancer: miR-125b-2 targets BAK1 and stimulates the growth of prostate cancer cells [11], while miR-99 family of microRNAs directly down-regulates the prostate specific antigen (PSA) and inhibits prostate cancer cells proliferation [12]. However, the full, specific expression profile of miR-ch21 in prostate cancer is not known, neither is their collective role in prostate oncogenesis.

An interesting development arose with the introduction of the cancer stem cells concept, opening the way for testing the role of stem cell specific microRNA in prostate tumor biology [13, 14].

The stem cell concept has dramatically changed our understanding of development, regeneration and (last but not least), tumor biology [15]. Among the microRNAs involved in controlling proliferation and the maintenance of pluripotency of (embryonic) stem cells, three clusters are prominently expressed: miR-290-295 (EEmiRC, early embryonic microRNA cluster), 302 and (in humans) miR-17-92 [8, 16]. The role of the EEmiRC human homologues has been explored in a large panel of cancers, including gastric, ovarian, pancreatic, and prostate cancers, where
it can act both as tumor suppressors or oncogenes [17–20]. However, the results concerning the expression of the hsa-miR-371-3 cluster in prostate cancer are conflicting [21], a situation reflecting differences in the experimental approaches/platforms and differences in the androgen receptors expression and the grades of cancer samples analyzed.

Here we use a real-time polymerase chain reaction (RT-PCR) approach to describe the expression of miR-ch21 in LCM (laser capture microdissected) glandular and stromal tissue from FFPE prostate adenocarcinoma samples. We compared the expression of miR-ch21 with that of two of the stem cell related microRNA known to be associated to prostate cancer biology, hsa-miR-372 and hsa-miR-373. Although the freshly-frozen tissues are always preferable, we have chosen to focus on FFPE samples since it’s the main histology technique in use, and because it has been shown that microRNAs are stable in FFPE samples. The implications of our findings upon our understanding of the prostate tumor biology are further discussed.

Materials and Methods

Statement on ethics

The study was carried out in accordance with the Declaration of Helsinki for Human Research. All the archived FFPE samples included in our study are older than 10 years.

Processing of FFPE samples

Three distinct FFPE prostate adenocarcinoma samples (at least 10-year-old, collection of the Department of Pathology) were sectioned at 10μm using a microtome; the sections were mounted on MMI RNase-free slides (MMI, Switzerland) and subsequently stained with Hematoxylin–Eosin using a standard protocol. The mounted sections were covered with a clean RNase free glass slide to prevent contamination and loss of dissected tissues. The normal and tumoral tissues were diagnosed as such by an trained pathologist and were cut using adequate power and focus for UV laser shots; the cut areas were then captured, pooled (at least three different sectioned tissues for each sample) and placed in RNase free microcentrifuge tubes (mmi IsolationCap tubes with adhesive lid and diffuser, MMI, Switzerland) as described in the manufacturer’s instructions.

RNA extraction

Total RNA from FFPE laser-microdissected tumor (T) and normal (N) tissues and their adjacent stroma (PtS – peritumoral stroma and NS – normal stroma, respectively) was extracted using an RNAeasy FFPE kit (Qiagen), according to the manufacturer’s instructions. Briefly, the samples were mixed with 140μL of optimized buffer and additionally incubated four minutes at 55°C in order to melt the paraffin. The lower clear phase was retrieved and further incubated 15 minutes at 56°C with 10μL proteinase K to release RNA from tissue samples. A final incubation at 80°C for 15 minutes partially reversed formaldehyde modifications and 15 minutes DNase treatment at room temperature was used to eliminate the genomic DNA. The samples, mixed with an optimized buffer and ethanol were then applied to the elution column and eluted (after three washing steps) in 15μL of RNase-free water. All our samples contained in between 10 and 30ng/μL total RNA, as quantified using a Nanodrop spectrophotometer.

qRT-PCR

10ng of total RNA from each sample were reverse transcribed using TaqMan MicroRNA reverse transcription kit (Applied Biosystem) and according to the protocol provided. PCR reactions were carried out in triplicate, each starting from 1.33μL RT-product, with an initial 10 minutes denaturation step followed by 45 cycles (95°C/15 seconds, 60°C/one minute, 72°C/two minutes). The RT-PCR results were analyzed by using the ΔΔCT method, using RNU24 for normalization, as it was previously found to be the most suitable endogenous control for prostate tissue samples [22].

miRWalk analysis

All miR-ch21 were imported into miRWalk2.0 algorithm [23] (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) and used for 3’UTR target predictions (minimum seed length 7, p-value = 0.05). KEGG (Kyoto Encyclopedia of Genes and Genomes) database pathways analysis was used (with Bonferroni’s correction, p<0.05) to retrieve the pathways significantly targeted by at least two of the miR-ch21.

Results

After optimization of laser pulse duration and intensity, we have collected four types of tissue, in triplicates, from each slide: tumoral glands (T), normal glands (N), peritumoral stroma (PtS) and normal stroma (NS). Of note, the PtS was in the close proximity of malignant glands while NS was harvested from areas away from any sign of malignancy. An example of the samples collected is depicted in Figure 1.

Figure 1 – Example of laser microdissected tissue in the prostate cancer FFPE samples (10μm sections). (A and B) Before laser dissection; (A’ and B’) After laser dissection. Areas captured are marked with a yellow star.

qRT-PCR analyses of samples collected from three different FFPE prostate adenocarcinoma specimens shows a strong down-regulation of miR-ch21 expression in tumor...
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As expected, the tumoral epithelia contain (with the exception of hsa-miR-155, fold change = 1) more miR-ch21 compared to the surrounding stroma. Unexpectedly, when we compared the miR-ch21 in normal glandular epithelium with its surrounding stroma, miR-155 and miR-99a were found down-regulated while miR-125b-2 and miR-802 were up-regulated.

In order to understand the biological implications of the alterations in miR-ch21 levels in tumoral tissues, we performed a miRWalk pathway analysis and found that out of the 22 pathways predicted to be impacted by at least two miR-ch21 microRNAs at a Bonferroni’s corrected p-value <0.05, 14 (63.63%) refer to cancer directly or through a signaling pathway (Table 1). This result is interesting as it sheds a new light on the role of this set of microRNAs in prostate cancer in particular and cancer in general.

To further investigate whether the change in microRNA levels in tumoral and peritumoral tissue is specific to miR-ch21, we have chosen to expand our analysis and quantify the expression of two microRNAs, hsa-miR-372 and hsa-miR-373, known to be involved in prostate cancer biology and to regulate the proliferation and pluripotency of stem cells. Our analysis of tumoral epithelia versus normal epithelium shows stunningly divergent results, with an impressive over 200 folds up-regulation of miR-373, while miR-372 is decreased 100 times (Figure 3).

Interestingly, the tumoral content in the two miRs shows no significant changes when compared to the peritumoral stroma, suggesting the existence of a general, common mechanism of regulation of expression in the two tissues.

### Table 1 – The main pathways predicted to be targeted by miR-ch21 (p<0.05)

<table>
<thead>
<tr>
<th>Pathway</th>
<th># miRs involved</th>
<th>Pathway</th>
<th># miRs involved</th>
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<tbody>
<tr>
<td>Chronic myeloid leukemia</td>
<td>7</td>
<td>Adherens junction</td>
<td>4</td>
</tr>
<tr>
<td>Pathways in cancer</td>
<td>7</td>
<td>Pancreatic cancer</td>
<td>4</td>
</tr>
<tr>
<td>Axon guidance</td>
<td>6</td>
<td>Prostate cancer</td>
<td>4</td>
</tr>
<tr>
<td>ErbB signaling pathway</td>
<td>6</td>
<td>Renal cell carcinoma</td>
<td>4</td>
</tr>
<tr>
<td>Neurotrophin signaling pathway</td>
<td>6</td>
<td>Endometrial cancer</td>
<td>3</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>5</td>
<td>Long-term potentiation</td>
<td>3</td>
</tr>
<tr>
<td>Endocytosis</td>
<td>5</td>
<td>Non-small cell lung cancer</td>
<td>3</td>
</tr>
<tr>
<td>Gioma</td>
<td>5</td>
<td>Aldosterone regulated sodium reabsorption</td>
<td>2</td>
</tr>
<tr>
<td>Insulin signaling pathway</td>
<td>5</td>
<td>Apoptosis</td>
<td>2</td>
</tr>
<tr>
<td>Wnt signaling pathway</td>
<td>5</td>
<td>Melanoma</td>
<td>2</td>
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<tr>
<td>MAPK signaling pathway</td>
<td>4</td>
<td>Phosphatidylinositol signaling system</td>
<td>2</td>
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### Discussion

It is currently well understood that basically all cancers associate significant changes in microRNAs expression profiles, suggesting they could play significant roles in cancer development and progression. In prostate cancer, microRNAs have been shown to act both as oncogenes (e.g., hsa-miR-21, hsa-miR-125b, hsa-miR-221, hsa-miR-222) and tumor suppressors (e.g., miR-15a, miR-16, hsa-miR-34 cluster, and hsa-miR-200 family), modulating multiple aspects of cancer biology from cancer stem cells proliferation to epithelial to mesenchymal transition and metastasis [24].
The overall risk of cancer in Down syndrome patients is not changed compared to the normal population [25], however, the risk for solid tumors is considered reduced, due to expression of a tumor suppressor gene on chromosome 21 [26]. Furthermore, all the microRNAs known to be mapped to chromosome 21 are involved in different aspects of cancer biology in general, and of prostate cancer in particular.

Although previous FFPE microarray and in situ hybridization experiments showed a strong down-regulation in prostate cancer, has-let-7c has been recently proposed as a circulating biomarker for prostate cancer [27–29]. Our data also indicate a strong down-regulation of has-let-7c, a microRNA known to regulate AR expression through MYC modulation, raising questions about its possible involvement in androgen resistance [30]. In this context, a recent study [31] points towards the existence of an androgen-dependent regulation loop, with AR regulating the expression of the entire miR-99a/let-7c/miR-125b-2 cluster. However, the biological significance of has-miR-125b-2 de-regulation in prostate cancer remains controversial, given its dual role, pro-proliferative (targets Bak1 and p14ARF) [31, 32] and anti-proliferative (by targeting IGF1) [27].

Up-regulation of has-miR-155 has been found to correlate with alterations of mismatch repair pathways and it has been proposed as part of the predictive signature of prostate adenocarcinoma [33]. This data confirms the general view of miR-155 as part of the so-called solid cancer miRNA signature [34] but is at odds with our results, depicting strong down-regulation of has-miR-155 in both tumoral epithelia and its adjacent stroma laser microdissected (fold change 0.22 and 0.12, respectively). Besides the aforementioned microRNAs, our study advances a novel miR-ch21 down-regulated in malign prostate epithelia: has-miR-802.

Overall, the seemingly global, tissue specific (T vs. PtS) strong down-regulation of miR-ch21 suggests that the active site of synthesis lies in the proliferating glandular epithelia, rather than in the adjacent stroma. In this respect, it would be interesting to investigate the identity of the cells actively synthesizing miR-ch21 and whether any kind of miR transport takes place between the two tissues. When comparing these data to N vs. NS results, it becomes obvious that the relation of the epithelia to the surrounding stroma is more complex and that the oncogenic transformation of the glandular epithelia alters the molecular mechanisms responsible for either biosynthesis, maturation, stability and/or degradation of at least three of the miR-ch21. However, given the small number of analyzed patients, our results should be interpreted with caution until further confirmation on a much larger study.

In this context, our miRWalk analysis confirms and complements our qRT-PCR results, 14 of the 22 relevant pathways being related to cancer, including both solid tumors and signaling pathways like MAPK signaling or Wnt signaling, known to be involved in cancer development and progression. We are fully aware that this might be an effect of the general abundance of microRNA data on cancer; nevertheless, the result is interesting as it sheds a new light on the role of this set of microRNAs in prostate cancer in particular and cancer in general.

Altogether, the global down-regulation of miR-ch21 found in our analysis not only opens a new avenue of research of the molecular basis of Down syndrome “protection” against solid tumors development, but also raise more questions: which is the mechanisms underlying this down-regulation? Is this phenomenon specific to prostate cancer? Which are the main (collective) molecular targets of miR-ch21? Our miRWalk analysis points toward several signaling pathways, including Wnt, insulin growth factor (IGF), VEGF and epidermal growth factor (EGF).

Another interesting result is represented by the divergent behavior of has-miR-372 and has-miR-373; although part
of the same cluster of microRNAs, they belong to two different semi-clusters, the region between which, at least in the mouse homologues, contains separate regulatory sequences [35]. The result is intriguing since EEMiRC is an eutherian stem cell specific cluster and one could speculate that the change in expression we have documented might represent a change in the biology of cancer stem cells. Further experiments involving deeper characterization of the laser-captured cells during microdissection are necessary to shed light on this matter.

Conclusions

Our microRNA expression analysis of carefully selected LCM FFPE samples show a strong, global down-regulation of miR-ch21, hinting towards a specific role of miR-ch21 in prostate cancer biology. Given the opposite behavior of the two EEMiRC homologues analyzed, the miR-ch21 down-regulation herein documented could signal a novel phenomenon involving specific silencing of miRs from chromosome 21 in cancer. In this context, we believe that a systematic re-evaluation of microRNA expression by combining laser micro-dissection with qRT-PCR technique would solve many of the controversies we face in cancer microRNA research nowadays.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

AM selected FFPE samples and sectioned them, laser microdissected tissues and wrote the manuscript; AA, CS and ACV extracted RNA, performed cDNA synthesis and qRT-PCR; AD selected samples and performed pathology diagnostic; AA supervised the technical part and analyzed data; LT analyzed data and wrote the manuscript; CVM and IOS designed the experiments, analyzed data and wrote the manuscript.

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Corresponding author
Ioan-Ovidiu Sirbu, Associate Professor, MD, PhD, Department of Biochemistry and Pharmacology, “Victor Babeș” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041 Timișoara, Romania; Phone +40756–136 272, e-mail: ovidiu.sirbu@umft.ro
Cătălin Valer Marian, Associate Professor, MD, PhD, Department of Biochemistry and Pharmacology, “Victor Babeș” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041 Timișoara, Romania; Phone +40256–204 268, e-mail: cmarian@umft.ro

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