A microRNA signature in urinary exosomes for diagnosis of prostate cancer

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Presented at the AACR conference, April 2016
Introduction

Improved diagnostic tests for prostate cancer are needed

• Current practice: antibody based detection of circulating prostate specific antigen (PSA) in blood
• High rate of false positives and negatives
• Diagnosis requires invasive FNA (Fine-Needle Aspiration) biopsies

A non-invasive test with improved specificity is urgently needed

MicroRNAs as non-invasive biomarkers

• MicroRNAs are stable in a range of biofluids
• Involved in many diseases, including roles as oncogenes and tumor suppressors in cancer
• Used as diagnosis, prognosis, treatment response and safety biomarkers

microRNAs are excellent non-invasive biomarkers for a range of diseases

Exosomes stabilize and transfer microRNAs between cells

• Exosomes are nanovesicles 40 - 140 nm in diameter
• Actively released by a wide range of cell types to the extracellular milieu under normal and pathological conditions
• Present in a wide range of biofluids
• Carry genetic information from the cell of origin, including microRNA

Figure 1.

Urinary exosomes: new liquid biopsies for cancer

• Exosomes from neoplastic cells carry potentially arrays of oncogenic molecules including proteins and microRNAs
• The unique exosomal microRNA signature may reveal the cell of origin and the condition of those cells

Promising non-invasive microRNA biomarkers for early detection of malignancy
Challenges and Solutions for analysis of microRNAs in Biofluids

**Limited amount of microRNA**
- Optimized exosome isolation kit to enhance microRNA signals from dilute biofluid samples
- Optimized RNA isolation kit for biofluids ensures high qPCR performance and reproducibility
- Highly sensitive qPCR detection system

**MicroRNA are challenging targets**
- Short (19-22 nt) microRNAs accurately detected using short, highly specific LNA™ qPCR primers
- Robust detection of all microRNAs regardless of GC content - enabled by LNA™
- Discrimination between highly similar microRNA family members - enabled by LNA™

**Undesired components e.g. PCR Inhibitors**
- RNA spike-ins to monitor RNA isolation efficiency and co-purification of inhibitors

**Pre-analytical variables**
- Optimal experimental design (biological replicates)
- Control sources of technical variation e.g. collection sites

Figure 2.

**Methods**

**Technologies to enable microRNA biomarker analysis in liquid biopsies**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Exosome Isolation</th>
<th>RNA Isolation</th>
<th>microRNA quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 ml cell-free urine</td>
<td>miRCURY™ Exosome Isolation Kit - Cells, urine and CSF</td>
<td>miRCURY™ RNA Isolation Kit – Cell &amp; Plant</td>
<td>miRCURY LNA™ Universal RT microRNA PCR</td>
</tr>
</tbody>
</table>

Figure 3.

Exiqon has developed technologies to fulfil these key requirements:
- Methods suitable for clinical liquid biopsies [serum, plasma, urine, CSF etc.] collected using standard protocols
- Exosome precipitation using a rapid method (< 1 hour), requiring only low speed centrifugation
- Optimized sample preparation to minimize carryover of inhibitory compounds in biofluids
- Procedures for rigorous QC of liquid biopsy samples
- Highly sensitive detection system to handle the very low level of RNA found in biofluids
- Highly specific detection method to discriminate between closely related microRNA family members
- Detection method optimized for detection of short microRNA sequences
miRCURY™ Exosome Isolation Kit

Exosome isolation enables detection of more microRNAs in urine

Exosome isolation enables a larger starting volume of biofluid to be used, increasing signals.

Vesicles of the correct size are recovered in the exosome pellet

Nanosight measurements demonstrate that vesicles of a size range compatible with exosomes are enriched from urine in the pellet.

Figure 4.

Rigorous QC of liquid biopsies

qPCR-based QC procedures optimized for biofluids

A range of RNA spike-ins are detected by LNA™ qPCR assays to monitor RNA isolation efficiency, inhibitors, and detect outlier samples.

Figure 5.
miRCURY LNA™ Universal RT microRNA PCR System

High sensitivity and linearity - Ideal for microRNA analysis in liquid biopsies

• Sensitive assays are crucial, due to the low RNA content of biofluids
• Exiqon’s miRCURY LNA™ microRNA PCR assays are wet-lab validated to have sensitivity and linearity over a wide range of RNA inputs, including biofluids
• Red arrow indicates improvement of microRNA detection from dilute biofluid samples e.g. urine when using the miRCURY™ Exosome Isolation Kit

Figure 7.

A robust system for accurate microRNA analysis - Validated on biofluids

• In the largest cross-platform comparison study ever (miRQC), Exiqon’s PCR system was the only microRNA analysis platform to combine both high sensitivity and specificity (Mestdagh et al., Nature Methods 11(8):809-15, 2014)
• We have used the miRCURY LNA™ Universal RT microRNA PCR System to analyze microRNAs in thousands of biofluid samples including serum, plasma and urine

Figure 8.
Results

Study overview - microRNA biomarker discovery

Urine sample collection and analysis:

**Urine samples collected by hospitals in Denmark**
- 3 ml fresh urine (without stabilizer) was centrifuged to remove cell debris
- Storage in cryotubes at -20 °C (short term) then -80 °C (long term)
- Exosome and RNA isolation followed by microRNA qPCR analysis, using the methods shown in Figure 3

**Urine samples collected by hospitals in the UK**
- Prostate massaged fresh urine (without stabilizers) was centrifuged to remove cell debris
- RNA isolation without exosome isolation followed by microRNA qPCR analysis, using the method shown in Figure 3

Genome wide microRNA profiling of cell-free urine samples:
- Healthy individuals (Benign Prostatic Hyperplasia) and patients with Prostate Cancer (Stage I-IV)
- A subset of relevant microRNAs were selected for subsequent discovery screening

Diagnostic microRNA signatures for prostate cancer in urine

- **Differentially regulated microRNAs** in urine from prostate cancer individuals were identified (Cohort 1, DK)
- **Signatures with diagnostic potential for prostate cancer** have been identified using different combinations of these microRNAs
- **Three-microRNA signature**: high Area Under the Curve (AUC) was validated in two independent cohorts – one using sample collection and isolation protocols identical to the discovery cohort (cohort 2, DK) and one prostate massaged cell free urine without exosome isolation (cohort 3, UK) (Figure 10a)
- The three microRNA signature shows high performance within the intended-use-population (cohort 2 sub-population, DK) (Figure 10b)
A new diagnostic test (non-invasive) for prostate cancer - intended use: men with intermediary PSA levels

Today’s practice - based on blood
PSA – indication for Prostate Cancer:
• Not specific for prostate cancer
• 70 % false positives
• 10-20 % false negatives
• Gray-zone: PSA 3-10 ng/mL, 75 % false positives

<table>
<thead>
<tr>
<th>PSA level</th>
<th>Test result</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 3 ng/mL</td>
<td>Negative</td>
</tr>
<tr>
<td>3 – 10 ng/mL (gray-zone)</td>
<td>Positive</td>
</tr>
<tr>
<td>&gt; 10 ng/mL</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Active surveillance

New test – based on urine
microRNA signature diagnostic for Prostate Cancer:
• Predict positive biopsy, 90 % accuracy
• Reduce number false positives to < 10 %
• Provide guidance for biopsy

TRUS directed biopsy
• Used for tumor grading
• 47 % misdiagnosis
• 30 % false negatives

Figure 10b.

Figure 11.
Conclusions

- **Requirements** for microRNA analysis in biofluids:
  - RT-qPCR system with high sensitivity and specificity
  - Rigorous sample QC and standardization
  - Enrichment of exosomes is preferred in dilute biofluids

- **Exosome isolation** enables detection of more microRNAs in dilute biofluids

- The **methods** developed for sample preparation and LNA™-enhanced microRNA qPCR analysis have been successfully applied in cell-free urine

- MicroRNAs in cell-free urine are promising **non-invasive biomarkers in prostate cancer diagnosis**

- A **three-microRNA signature** has been discovered

- The three-microRNA signature has been **validated in independent cohorts**

Acknowledgements: Nanosight data kindly provided by iNano (Dr. Ken Howard, Aarhus University).

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