Increased levels of serum miR-148a-3p are associated with prostate cancer

SANDRA AMALIE DYBOS,1,2 ARNAR FLATBERG,2 JOSTEIN HALGUNSET,1,2 TROND VISET,1 TORIL ROLFSENG,1 SOLVEIG KVAM1 and HAAKON SKOGSETH1,2

1St. Olavs Hospital, Trondheim University Hospital, Trondheim; 2Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway

Dybos SA, Flatberg A, Halgunset J, Viset T, Rolfseng T, Kvam S, Skogseth H. Increased levels of serum miR-148a-3p are associated with prostate cancer. APMIS 2018; 126: 722–731.

Prostate cancer (PCa) is one of the most common types of cancer and the fifth leading cause of death among men worldwide. The tools for diagnosing PCa have limited value, and to improve correct diagnosis there is a need for markers that can contribute to a more precise diagnosis, which would lead to proper treatment of only those patients who need it. Micro RNA (miRNA) plays a key role in the development of cancer and is therefore a potential marker for PCa. Next-generation sequencing was used to discover differences in miRNA expression between serum samples from PCa patients and healthy controls, and the results were validated by quantitative real-time polymerase chain reaction. Detection of the miRNA of interest was attempted in prostate tissue by *in situ* hybridization. All samples were collected in collaboration with Biobank1. By miRNA sequencing of serum samples, significant expression of some miRNAs in patients with PCa and healthy controls was detected. This study showed that miR-148a-3p is upregulated in men with PCa, and the miRNA is differentially expressed in PCa patients compared to healthy controls. The results also showed that miR-148a-3p is located in prostate tissue.

Key words: miR-148a-3p; microRNA; prostate cancer; next-generation sequencing; qPCR; *in situ* hybridization.

Sandra Amalie Dybos, Biobank1, Medisinsk-teknisk forskningssenter, Olav Kyrres gate 9, 7481 Trondheim, Norway. e-mail: sandra.amalie.dybos@stolav.no

Prostate cancer (PCa) is the second most diagnosed cancer among men and the fifth most frequent cause of death among men worldwide. Overall, PCa is the fourth most common cancer, and 5000 new cases are detected in Norway each year. PCa is the second leading cause of cancer deaths among men in western countries. Moreover, there is a high incidence of PCa in the US, Australia, New Zealand and Canada, while the incidence is low in Asia (1). In 2015, 30% of all newly diagnosed cancers among men were PCa, and one in eight men is expected to be diagnosed during his life (2). In men over 50 years of age, PCa is the most frequent cancer, and in the last decade, the number of diagnoses has doubled due to the aging of the population, and improved diagnostics (1–3). Genetic factors are important in many cancers, and seem to be important in PCa (1).

It has been estimated that more than half of men above 80 years of age have cancer lesions in the prostate, most of them without symptoms and without influencing longevity. Most older men will die for other reasons before the cancer lesions in the prostate become clinically advanced (4). The first part of PCa diagnosis is based on patient symptoms, typically slow urination, difficulty in emptying the bladder, blood in the urine, or back pain. In patients with these symptoms, analysis of prostate specific antigen (PSA) is recommended. PSA is an indicator of the presence of cell changes in the prostate gland. PSA is synthesized by prostate tissue, and normally, only a small part of PSA leaks into the bloodstream. Increased leakage of PSA to the blood is the basis for using PSA to detect and monitor PCa progression (5, 6).

Prostate specific antigen became the most regular tumor marker for PCa early in the 1990s, and the use of PSA led to an increased incidence of PCa in the mid-1990s (7). The majority of the diagnosed
cancers were clinically localized, which led to an increase in treatment intend to cure these early stage cancers (8, 9). Survival from cancer is related to many factors, and the extent of tumor at the time of diagnosis is one of them. Today, there is a 93% 5-year relative survival rate among men with localized PCa. If the cancer has metastasized, the survival rate is approximately 30% (3).

Prostate specific antigen is not a very specific tumor marker since elevated serum levels are detected in benign prostatic hyperplasia, inflammation, and other urinary tract diseases. Thus, this will influence the performance of PSA as a screening test (10).

In many countries, population-based screening of PSA is not recommended due to low specificity and sensitivity. A false negative PSA may cause men to ignore symptoms, while a false positive test can lead to further examinations of patients with a negative impact on the quality of life (11).

A high PSA level will never be used as a cause for surgery, but it will be used for further examination of the patient. This process is very expensive, and further examinations are invasive. If there are early stage cancerous cells in the prostate, some patients will require surgery despite the fact that active surveillance is an option. Moreover, the side effects following a surgical procedure, such as uncontrolled urination and stool, and impotence, may be so harmful to the quality of life that a symptomless life in ignorance of their PCa would have been preferable (12).

At the same time, there are patients with aggressive PCa that receive treatment at a late stage of disease progression.

Many studies have investigated potential diagnostic biomarkers for the precise detection of PCa, and among the most promising are microRNAs (miRNAs) (13–18). miRNA is important in regulating gene expression and plays a key role in the development of cancer (19, 20). miRNA can mediate approximately 30% of protein-coding genes in humans, and one miRNA can regulate multiple mRNAs, which makes this a complicated and complex regulatory network that affect various cell functions. The results from previous studies are varying, and this may be a consequence of the homogeneous expression in the prostate tissue, in addition to methodological challenges (21, 22).

The first study to demonstrate the link between miRNA and cancer was the discovery that miR-15 and miR-16 are lost in most cases of chronic lymphocytic leukemia (23). In PCa, different studies have shown that miRNAs regulate several cellular processes, and some miRNAs have been found to be upregulated and others downregulated in cancer (24, 25). Change in miRNA expression can affect the cell phenotype and the functional expression of some genes involved in various cellular processes, such as proliferation, differentiation, metabolism, stress response, and apoptosis. This can lead to increased growth of cells or increased survival (19, 26, 27).

The processing and mechanisms of miRNA are presented in Fig. 1.

As today’s diagnostic marker in serum is not particularly specific, new precise markers need to be detected, and be able to determine with certainty whether or not the patient has PCa.

The aim of this study was to examine if there are any miRNAs or miRNA expression patterns in the serum that may be potential diagnostic markers for PCa. Next-generation sequencing (NGS) was used to detect which miRNAs are expressed in patients with PCa and whether there are any similarities or differences compared to a healthy control group. The differences in miRNA expression between cancer patients and healthy controls were correlated with the miRNA expression in tissue from the prostate to demonstrate that the current miRNA is located in the prostate.

MATERIALS AND METHODS

Collection of serum samples and prostate tissue

Pre-operative serum samples from patients with PCa were collected in accordance with the approval from regional committees for medical and health research ethics. Samples were stored at −80°C in a freezer equipped with temperature monitoring. The patient cohort is presented in Table 1, and the PSA levels and Gleason sum scores from each patient are presented in Table 2.

Prostate tissue from the same men undergoing radical prostatectomy was formalin-fixed and paraffin-embedded (FFPE) for micro section and further pathological grading. All donors were histologically classified and graded by a clinical pathologist. Grading of the tissue sections is presented in Table 3.

The patient cohort was compared to a control group, which consists of serum samples taken from healthy age-matched blood donors. Hyperplastic tissue was used as control material for the tissue.

Library preparation and NGS

Collected serum was thawed on ice and centrifuged at 4°C, 3000 g for 5 min. Aliquots of 200 µL per sample were transferred to a FluidX tube. Sixty microliters of Lysis solution BF, containing 1 µg carrier-RNA per 60 µL Lysis Solution BF, and RNA spike-in template mixture were added to the sample. The sample was mixed for 1 min and incubated for 7 min at room temperature, followed by the addition of 20 µL Protein Precipitation solution BF. Total RNA was extracted from the samples using miRCURY RNA isolation Kit – Biofluids, Vedbaek, Denmark: high-throughput bead-based protocol v.1

© 2018 The Authors. APMIS published by John Wiley & Sons Ltd on behalf of Scandinavian Societies for Medical Microbiology and Pathology
Exiqon, Vedbaek, Denmark) in an automated 96-well format. The purified total RNA was eluted in a final volume of 50 \( \mu \text{L} \).

The library preparation was done using the NEBNext Small RNA Library preparation kit (New England Bioslabs). A total of 6 \( \mu \text{L} \) of total RNA was converted into microRNA NGS libraries. Adapters were ligated to the RNA, and then, RNA was converted to cDNA. The cDNA was amplified using PCR (18 cycles), and during the PCR, indices were added. After PCR, the samples were purified. Library preparation QC was performed using a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Based on quality of the inserts and the concentration measurements, the libraries were pooled in equimolar ratios. The pool was then size selected using the LabChip XT (PerkinElmer) to select the fraction with the size corresponding to microRNA libraries (~145 nt). The library pool was then sequenced on a NextSeq 500 sequencing instrument according to the manufacturer’s instructions. Raw data were demultiplexed, and FASTQ files for each sample were generated using the bcl2fastq software (Illumina Inc.). FASTQ data were checked using the FastQC tool.

Bowtie 2 (2.2.2) were used for mapping the reads. The mapping criteria for aligning reads to spike-ins, abundant sequence, and miRBase was the reads with perfect match.

**Fig. 1.** miRNA are short, noncoding RNAs (18–25 nt) that regulate gene expression typically by blocking translation of mRNAs. miRNAs can regulate a whole set of different mRNAs by binding to a common sequence in their 3' UTRs. Thus, miRNA can regulate hundreds of different mRNAs. Complete sequence-specific binding will lead to degradation of the target mRNA; incomplete binding induces post-transcriptional silencing of mRNA and downregulation of protein expression (2, 28).
to the reference sequences. Mapping to the genome with no more than one mismatch was allowed in the first 32 bases of the read. No indels are allowed in mapping. Differential expression analysis was performed using the EdgeR statistical software package (Biociconductor, http://bioconductor.org/).

For normalization, the trimmed mean of M-values method based on log-fold and absolute gene-wise changes in expression levels between samples (TMM normalization) was used. The isomiR analysis and putative miRs were done with in-house scripts (exqngs_mircount and exqngs_mirpred using the MiRPara tool).

Principal component analysis (PCA) was performed with R using TMM-normalized quantifications from defined collections of samples as input. The same input was also used to generate a heatmap of expression profiles with R. GO analyses were done with R package TopGO with experimentally verified targets of significantly differentially expressed microRNAs as input.

Quantitative measurement of miRNA by qPCR

RNA was isolated from 500 µL serum using a proprietary RNA isolation protocol. The protocol was the same as for the NGS.

Two microliters of RNA were reverse transcribed in 10 µL reactions using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon). cDNA was diluted 50× and assayed in 10 µL PCR reactions according to the protocol for miRCURY LNA™ Universal RT microRNA PCR; each microRNA was assayed once by qPCR on the microRNA Ready-to-Use PCR, Custom Pick and Mix panel using an ExiLENT SYBR® Green master mix. Negative controls excluding the template from the reverse transcription reaction were performed and profiled such as the samples. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384 well plates. The amplification curves were analyzed using the Roche LC software, both for determination of Cq (by the 2nd derivative method) and for melting curve analysis.

The amplification efficiency was calculated using algorithms similar to the LinReg software. All assays were inspected for distinct melting curves and the Primer Melting Temperature (Tm) was checked to be within known specifications for the assay. Data that did not pass these criteria were omitted from any further analysis. The Cq was calculated as the 2nd derivative. Using NormFinder, the best normalizer was found to be the average of assays detected in all samples. All data were normalized to the average of assays detected in all samples (average – assay Cq).

Table 1. The cohort in this study contains of 38 serum samples, 19 from prostate cancer patients, and 19 controls. The Gleason score sum was classified by a pathologist. Biopsies were taken before prostatectomy surgery.

| Clinical characteristics of donors | Cancer patients | Controls |
|-----------------------------------|----------------|---------|
| Gleason grade                     | 3 + 3, 3 + 4, 4 + 3, 4 + 5, 5 + 4 | Non-cancer |
| Samples (n)                       | 19             | 19      |
| Age (mean)                        | 63             | 64      |

Table 2. Prostate specific antigen (PSA) was measured pre-operatively in all patients at the same time as samples were taken for the analysis of miRNA. All patients are operated at St. Olavs Hospital, and the measurement of PSA was done at the Department of Medical Biochemistry. The reference area for PSA <4.1 µg/L for men between 60 and 70 years. The Gleason grading was done by a pathologist at the Department of Pathology at St. Olavs Hospital

| ID | PSA (µg/L) | Gleason | Gleason sum score |
|----|------------|---------|-------------------|
| 1  | 3.3        | 4       | 3                 |
| 2  | 11.1       | 3       | 4                 |
| 3  | 10.3       | 3       | 4                 |
| 4  | 7.0        | 3       | 4                 |
| 5  | 14.3       | 4       | 4                 |
| 6  | Not measurable | 3 | 4                 |
| 7  | 6.2        | 3       | 4                 |
| 8  | 5.1        | 4       | 5                 |
| 9  | 0.2        | 5       | 4                 |
| 10 | 2.3        | 3       | 4                 |
| 11 | 7.8        | 3       | 4                 |
| 12 | 2.0        | 4       | 3                 |
| 13 | 6.1        | 3       | 4                 |
| 14 | 7.8        | 3       | 4                 |
| 15 | 9.3        | 3       | 4                 |
| 16 | 7.6        | 3       | 4                 |
| 17 | 11.7       | 3       | 3                 |
| 18 | 7.4        | 3       | 4                 |
| 19 | 21.7       | 3       | 4                 |
| Mean | 7.8        | 7       |

Table 3. The primary cancer diagnosis was always verified histologically by pathologists before treatment was initiated. The criteria used to detect malignancy was architectural atypia (invasive growth, perineural infiltration, micro glands, and crib-shaped glands) and cellular atypia (enlarged nuclei with distinct nucleus).

| Hyperplasia | Nodular arrangement of benign glands, regular cylindrical epithelium |
|-------------|------------------------|
| Gleason 3   | Clusters of small, well delineated, rounded glands infiltrating among normal glands. Atypical epithelium without basal cell layer |
| Prostatic intraepithelial neoplasm (PIN) | Sheets of closely spaced, irregularly shaped glands – sometimes fused. Atypical epithelium without basal cell layer |
| Proliferative inflammatory atrophy (PIA) | Glands of unremarkable shape and distribution. Focally thickened epithelium with atypical cells, with intact basal cell layer |
| Prostatic atrophy | Spaced, irregular glands of various size and shape. Thin epithelium with some enlarged nuclei, with intact basal cell layer. Often inflammation in stroma |
RNA isolation, library preparation, NGS, and qPCR were conducted at Exiqon Services, Denmark.

Preparation of tissue micro array block (TMA-block) for use in detection of miRNA in prostate tissue

The FFPE prostate tissue was assessed by a pathologist and divided into five groups (Table 3) based on the pathology for the detection of miR-148a-3p and miR-150-3p.

To collect the tissue of interest from different FFPE-blocks in order to coordinate them in one paraffin block, Galileo TMA CK4500 (semi-automatic) was used to prepare a TMA-block. A hollow needle, 1 mm in diameter, was used to punch cylinders from certain areas of the FFPE material, and the tissue cylinders were then positioned into a donor block. Parallels of prostate tissue that represent the five groups (Table 3) were punched from each patient, provided that the current cell changes were represented in the tissue (N = 152). The amount of tissue per cylinder varies according to the amount of tissue in the donor block. An example of the TMA-block is presented in Fig. 2.

After punching cylinders, assessment of the tissue was done by a pathologist to ensure that the tissue cylinders were taken from the area of interest.

Detection of miRNA in prostate biopsies

Cellular origin, or sub-cellular localization of miRNA, was detected in tissue using *in situ* hybridization (ISH). The detection was performed with protocol and reagents (Double-DIG-labeled scramble miR, 5'-DIG-labeled probe against U6 snRNA, Formamid-free hybridization buffer, Proteinase K) from the miRCURY LNA™ miRNA ISH Kit (Exiqon), and the method was optimized at Biobank1’s research laboratory facility in Trondheim, Norway. Synthesized probes targeting miRNAs of interest (LNA modified and Digoxigenin labeled oligonucleotides; Exiqon) were used for detection (of miRNA).

The principle of miRCURY LNA™ microRNA ISH (FFPE) is presented in Fig. 3.

Tissue sections (4 μm) were deparaffinized, rehydrated, and then unmasked using proteinase K (1000 μL per section). The sections were washed with PBS and dehydrated.

Double-DIG-labeled probes complementary to the miRNA of interest (miR-148a-3p and miR-150-3p) were added to the sections (miR-148a-3p 80 nM and miR-150-3p 100 nM). Sections were incubated for 60 min (miR-148a-3p 50 °C and miR-150-3p 53 °C), and washed in SSC buffer at hybridization temperatures. Blocking solution (DIG Wash and Block Buffer Set; Roche) and enzyme labeled anti-DIG were added (Alkaline phosphatase conjugated anti-Digoxigenin Fab fragments; Roche). Bound probes were detected by subsequent color reaction due to the interaction of enzyme coupled antibodies and the NBT/BCIP reagent (Roche).

Tissue sections were counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA, USA), and dehydrated before the coverslip was applied.

Statistical analyses

The miRNA mature strand expression by RNAseq for PCa tissue miRNA was downloaded from The Cancer Genome Atlas (TCGA) (https://tcga.xenahubs.net/download/TCGA.PRAD.sampleMap/miRNA_HiSeq_gene) along with clinical meta data (https://tcga.xenahubs.net/download/TCGA.PRAD.sampleMap/PRAD_clinicalMatrix). Samples with ‘sample_type’ equal to ‘Solid Tissue Normal’ and subject matched tumor samples were kept for statistical analysis (n = 104). miRNA with matching mature ID in our data was kept and miRNA with more than 50% missing values were filtered out. The remaining missing values were imputed with miRNA mean values.
miRNA sequences with less than one count per million (CPM < 1) in 5 or more samples were filtered out prior to analysis in our data, while 10 or more samples were required for TCGA data. Samples were normalized by the Trimmed Mean of M-values method (TMM). Differential expression was assessed by a paired t-test using the voom method of the limma package (http://bioconductor.org/packages/release/bioc/html/limma.html), and p-values were adjusted for multiple testing using the Benjamini Hochberg false discovery rate correction.

Variance transformed expression data were generated using the DESeq2 package (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) using the rlog transform on TMM-normalized count data. The heatmap for expression data was generated using the heatmap package (https://cran.r-project.org/web/packages/heatmappp) and hierarchical clustering of genes and samples in the heatmap was generated using the Ward’s clustering method and correlation distances. The PCA was performed on variance transformed data (rlog) and visualized using the ‘ggfortify’ package (https://cran.r-project.org/web/packages/ggfortify).

RESULTS

Differential expression of miRNA was detected in cancer patients and healthy controls

Serum from patients with PCa (N = 19) and healthy controls (N = 19) were analyzed by NGS. An overview of the five top hits of miRNA is presented in Table 4.

The characteristic of the data from NGS is summarized in a PCA plot in Fig. 4.

In Fig. 5, a heatmap displays the expression of the five differentially expressed miRNAs from Table 4.

Validation of miRNA associated with PCa

To validate the sequencing data, significantly differentially expressed miRNAs from NGS (p-value < 0.05) were compared to the control group using a t-test. Five candidate miRNAs were proposed, as shown in Table 4: miR-148a-3p, miR-150-3p, miR-379-5p, miR-134-3p, and miR-127-3p. When comparing the miRNA expression data between NGS and qPCR, two miRNAs were found to be differentially expressed using a cutoff of p-value < 0.05. In Table 5, the results for the two differentially expressed microRNAs are presented.

miR-148a-3p and miR-150-3p are represented in prostate tissue

By ISH of miRNA, it is possible to examine the distribution of cells that express the miRNA of interest. In this study, probes targeting miR-148a-3p and miR-150-3p were used.

There was no clearly distinct staining in specific areas of the tissue cylinder, and there was no

Table 4. miRNA with log2 voom transformed expression values above 5 in serum and TCGA tissue. A log FC differential expression above 0.5 in serum, and equal fold change direction in both serum and TCGA tissue differential expression, was kept for further analysis (n = 5)

| ID          | Ave. exp. serum | logFC serum | Adj. p-value serum | Ave. exp. tissue | logFC tissue | Adj. p-value tissue | Status      |
|-------------|-----------------|-------------|--------------------|------------------|--------------|---------------------|-------------|
| hsa-miR-148a-3p | 15.6            | 0.673       | 0.021              | 14.751           | 1.156        | 2.331E-10           | Upregulated |
| hsa-miR-150-3p  | 7.35            | 0.510       | 0.168              | 7.656            | 0.480        | 1.152E-02           | Upregulated |
| hsa-miR-379-5p  | 6.59            | -0.901      | 0.062              | 8.985            | -0.892       | 5.783E-11           | Down regulated |
| hsa-miR-134-3p  | 8.09            | -0.902      | 0.047              | 6.936            | -0.606       | 7.178E-08           | Down regulated |
| hsa-miR-127-3p  | 7.15            | -1.180      | 0.014              | 9.004            | -0.601       | 7.131E-08           | Down regulated |

© 2018 The Authors. APMIS published by John Wiley & Sons Ltd on behalf of Scandinavian Societies for Medical Microbiology and Pathology
apparent correlation to show which cell types express the miRNAs of interest. The expression of miR-148a-3p and miR-150-3p displayed heterogeneity, and the results are presented in Fig. 6.

**DISCUSSION**

Based on the knowledge that current diagnostic markers for PCa are inadequate, this study was conducted to find a more precise marker for PCa diagnosis in order to improve correct diagnosis. The cohort in this study consisted of 38 donors, and all serum samples from cancer patients were taken pre-operatively at the hospital, a short time before radical prostatectomy. The conditions for all sampling were standardized and well documented.

By NGS, miR-148a-3p was upregulated in the serum from patients with PCa. Moreover, the results were confirmed by qPCR and miRNA ISH. We are the first to report these findings.

Previous studies have shown that miR-148a-3p is differently expressed in various types of cancers and is upregulated in some cancers and downregulated in others (30). miR-148a-3p is, for instance, related to cellular differentiation and cellular development and may be involved in tumorigenesis by regulating related signaling pathways. miR-148a-3p promotes the differentiation of activated B cells to plasma cells and improves survival of plasma cells by inhibiting the transcription factors BACH2 and MITF, in addition to the pro-apoptotic factors BIM and PTEN (31). Upregulated miR-148a-3p is also seen in other types of cancers, such as glioma.

Table 5. miR-150-3p and miR-148a-3p were validated and detected by qPCR. Both miRNAs were upregulated in serum samples from patients with PCa. miR-148a-3p is represented in all samples from cancer patients, while some patients did not express miR-150-3p.

| ID            | SD cancer | SD control | Average dCq cancer | Average dCq control | FC  | p-value |
|---------------|-----------|------------|--------------------|---------------------|-----|---------|
| hsa-miR-150-3p| 0.65      | 0.60       | -12                | -13                 | 2.2 | 0.02    |
| hsa-miR-148a-3p| 0.96     | 0.57       | -7.1               | -7.6                | 1.5 | 0.04    |

Fig. 5. Hierarchical clustering of genes and samples generated using the Ward’s clustering method and correlation distances between samples and Euclidean distances between genes. The heatmap cells are colored proportional to rlog expression values.

Table 5.
and osteosarcoma (30). Hua et al. showed that miR-148a was upregulated in glioblastoma by regulating the development and progression of glioma cells (32). Ma et al. found that miR-148a was upregulated in samples from patients with osteosarcoma, and demonstrated that miR-148a could be an indicator of an aggressive cancer (33). Furthermore, a reduction in CAND1 expression has been shown to promote LNCaP cell proliferation, so miR-148a could promote PCa growth by reducing its target CAND1 (31). These are findings that support the hypothesis that notes a potential role for overexpression of miR-148a in PCa development.

The differential expression of miR-148a-3p in various types of cancers indicates that it is more than a general marker of imbalance, such as infections, in the body. By NGS of miRNA, it is possible to examine tissue-specific expression patterns, disease associations, and isoforms of miRNAs and to discover previously uncharacterized miRNAs. The results from NGS express everything contained in the sample, and you do not need to know which sequences you are looking for in advance. The method is precise and gives an accurate expression pattern of the miRNAs in the samples. Based on the results from NGS presented in Table 4, some miRNAs were validated by qPCR, and the results from NGS were confirmed second time.

The results from ISH indicated that both miR-148a-3p and miR-150-3p are expressed in prostate tissue, but it is difficult to differentiate which cell in the tissue express the miRNA of interest. Tumor tissue from the prostate is heterogeneous, which means that there are not only malignant cells in a tissue section. Moreover, cylinders containing hyperplasia were taken as far away from the tumor area as possible. The tissue used in this study was validated by a pathologist before and after preparation of TMA-blocks and that strengthens the selection of the correct tissue areas for ISH. The advantage of using TMA-blocks is that all tissue cylinders are treated under the same conditions, which eliminates technical variation. Despite the strong focus on standardization, it appears that this method could be affected by the time of fixation of...
the tissue, thickness of the tissue slices and washing steps during the procedure. The rinsing of the tissue slices could also cause the miRNA to be washed out of the tissue, and therefore, some miRNAs may end up in the eluate. Overall, the results indicate that miR-148a-3p and miR-150-3p are present in the prostate tissue, but there is no distinct expression in specific cell-types compared to others. There are no clear differences between the divided groups (Table 3), and it appears that the miRNAs are present in hyperplasia as well as Gleason 4.

Due to increasing understanding of the role of miRNAs in PCa development, high-throughput profiling techniques seem to be helpful to identify miRNA expression in biological material. However, there is variation in the reported results. These differences could be linked to the application of various profiling methods (microarray, NGS, qPCR), the preparation of miRNA for analysis, or the analytical tool methods. Obviously, tissue heterogeneity plays a role in replicating an experiment, since the cellular composition will be different between equal histological lesions (25, 34). It is also important that some miRNAs can act as oncogenes in one context, and as a tumor suppressor in another, which could influence the expression profiles. miRNA can also perform differently between various cancer types, and the expression could differ based on tumor size, stage of development, and metastasis (30, 35).

It is unknown how early the serum levels of miR-148a-3p increase in patients with PCa and whether the levels of miR-148a-3p are upregulated before the PSA is increased. It is also unknown whether the levels of miR-148a-3p are normalized after removal of the prostate or if the levels are affected by other cancer treatments. Further work is needed to fully understand the role of miR-148a-3p in accordance to PCa, and these issues must be studied more closely before it would be appropriate to use miR-148a-3p as a marker in the clinic. However, in this study we demonstrate that miR-148a-3p can be a tool for assessing and determining PCa diagnosis. Sampling for the analysis of miR-148a-3p is not an invasive method and the medical examination of PCa can be more cost effective. In conclusion, increased levels of serum miR-148a-3p can be an indicator of PCa, and miR-148a-3p is therefore highlighted as a potential marker for detection of PCa.

CONFLICT OF INTEREST

There are no conflicts of interest.

Thanks to all the patients who allowed samples to be collected for the use in this study.

REFERENCES

1. Stewart B, Wild C. World Cancer Report 2014. Lyon: IARC, 2014.
2. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
3. Cancer Registry of Norway. Cancer in Norway 2015. Institute of Population-Based Cancer Research, 2016. Available from: https://www.kreftregisteret.no/Generelt/Publikasjoner/Cancer-in-Norway/cancer-in-norway-2015/
4. Stensvold A, Dahl AA, Brennhovd B, Smastuen MC, Fossa SD, Lilley W, et al. Other problems in prostate cancer patients after curative treatment. Urol Oncol 2013;31:1067–78.
5. Andriole GL, Crawford ED, Grubb RL, Buys SS, Chia D, Church TR, et al. Mortality results from a randomized prostate-cancer screening trial. N Engl J Med 2009;360:1310–9.
6. Dhanaasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, et al. Delineation of prognostic biomarkers in prostate cancer. Nature 2001;412:822–6.
7. Mettlin C, Jones G, Averette H, Gusberg SB, Murphy GP. Defining and updating the American Cancer Society guidelines for the cancer-related checkup: prostate and endometrial cancers. CA Cancer J Clin 1993;43:42–6.
8. Lu-Yao GL, Greenberg ER. Changes in prostate cancer incidence and treatment in USA. Lancet 1994;343:251–4.
9. Potosky AL, Miller BA, Albertsen PC, Kramer BS. The role of increasing detection in the rising incidence of prostate cancer. JAMA 1995;273:548–52.
10. Lin S, Gregory RI. MicroRNA biogenesis pathways in cancer. Nat Rev Cancer 2015;15:321–33.
11. Hayes JH, Barry MJ. Screening for prostate cancer with the prostate-specific antigen test: a review of current evidence. JAMA 2014;311:1143–9.
12. Alemozaffar M, Regan MM, Cooperberg MR, et al. Prediction of erectile function following treatment for prostate cancer. JAMA 2011;306:1205–14.
13. Leite KR, Tomiyama A, Reis ST, Sousa-Canavez JM, Sanudo A, Camara-Lopes LH, et al. MicroRNA expression profiles in the progression of prostate cancer—from high-grade prostate intraepithelial neoplasia to metastasis. Urol Oncol 2013;31:796–801.
14. Wach S, Nolte E, Szczurba J, Stöhr R, Hartmann A, Orntoft T, et al. MicroRNA profiles of prostate carcinoma detected by multiparameter microRNA screening. Int J Cancer 2012;130:611–21.
15. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857–66.
16. Selth LA, Townley C, Gillis JL, Ochink AM, Murti K, Macfarlane RJ, et al. Discovery of circulating microRNAs associated with human prostate cancer using a mouse model of disease. Int J Cancer 2012;131:652–61.
17. Watahiki A, Wang Y, Morris J, Dennis K, O’Dwyer HM, Gleave M, et al. MicroRNAs associated with metastatic prostate cancer. PLoS ONE 2011;6:e24950.
18. Ambra S, Prueitt RL, Yi M, Hudson RS, Howe TM, Petrozza F, et al. Genomic profiling of microRNA
and mRNA reveals deregulated microRNA expression in prostate cancer. Cancer Res 2008;68:6162–70.
19. Fiorucci G, Chiantore MV, Mangino G, Percario ZA, Alfabras E, Romeo G. Cancer regulator microRNA: potential relevance in diagnosis, prognosis and treatment of cancer. Curr Med Chem 2012;19:461–74.
20. Sood P, Krek A, Zavolan M, Macino G, Rajewsky N. Cell-type-specific signatures of microRNAs on target mRNA expression. Proc Natl Acad Sci USA 2006;103:2746–51.
21. Schaefer A, Jung M, Mollenkopf HJ, Wagner I, Stephan C, Jentzmik F, et al. Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. Int J Cancer 2010;126:1166–76.
22. Schaefer A, Jung M, Kristiansen G, Lein M, Schrader M, Miller K, et al. MicroRNAs and cancer: current state and future perspectives in urologic oncology. Urol Oncol 2010;28:4–13.
23. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 2002;99:15524–9.
24. Turchinovich A, Weiz L, Burwinkel B. Extracellular miRNAs: the mystery of their origin and function. Trends Biochem Sci 2012;37:460–5.
25. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA 2006;103:2257–61.
26. Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL, Visakorpi T. MicroRNA expression profiling in prostate cancer. Cancer Res 2007;67:6130–5.
27. Gregory RI, Shiekhattar R. MicroRNA biogenesis and cancer. Cancer Res 2005;65:3509–12.
28. Ambros V. The functions of animal microRNAs. Nature 2004;431:350–5.
29. Exiqon. miRCURY LNA™ microRNA ISH Optimization Kit (FFPE). https://www.exiqon.com/lst/Documents/Scientific/miRCURY-LNA-microRNA-ISH-Optimization-Kit-manual.pdf.
30. Li Y, Deng X, Zeng X, Peng X. The role of Mir-148a in cancer. J Cancer 2016;7:1233–41.
31. Murata T, Takayama K, Katayama S, Urano T, Horie-Inoue K, Ikeda K, et al. miR-148a is an androgen-responsive microRNA that promotes LNCaP prostate cell growth by repressing its target CAND1 expression. Prostate Cancer Prostatic Dis 2010;13:356–61.
32. Hua D, Mo F, Ding D, Li L, Han X, Zhao N, et al. A catalogue of glioblastoma and brain microRNAs identified by deep sequencing. OMICS 2012;16:690–9.
33. Ma W, Zhang X, Chai J, Chen P, Ren P, Gong M. Circulating miR-148a is a significant diagnostic and prognostic biomarker for patients with osteosarcoma. Tumor Biol 2014;35:12467–72.
34. Ozen M, Creighton CJ, Ozdemir M, Ittmann M. Widespread deregulation of microRNA expression in human prostate cancer. Oncogene 2008;27:1788–93.
35. Mattie MD, Benz CC, Bowers J, Sensinger K, Wong L, Scott GK, et al. Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. Mol Cancer 2006;5:24.