Video Article

miRNA Expression Analyses in Prostate Cancer Clinical Tissues

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URL: https://www.jove.com/video/53123
DOI: doi:10.3791/53123

Keywords: Medicine, Issue 103, MicroRNAs, prostate cancer, clinical tissues, expression analyses, real-time PCR, *in situ* hybridization

Date Published: 9/8/2015

Citation: Bucay, N., Shahryari, V., Majid, S., Yamamura, S., Mitsui, Y., Tabatabai, Z.L., Greene, K., Deng, G., Dahiya, R., Tanaka, Y., Saini, S. miRNA Expression Analyses in Prostate Cancer Clinical Tissues. *J. Vis. Exp.* (103), e53123, doi:10.3791/53123 (2015).

Abstract

A critical challenge in prostate cancer (PCa) clinical management is posed by the inadequacy of currently used biomarkers for disease screening, diagnosis, prognosis and treatment. In recent years, microRNAs (miRNAs) have emerged as promising alternate biomarkers for prostate cancer diagnosis and prognosis. However, the development of miRNAs as effective biomarkers for prostate cancer heavily relies on their accurate detection in clinical tissues. miRNA analyses in prostate cancer clinical specimens is often challenging owing to tumor heterogeneity, sampling errors, stromal contamination *etc.* The goal of this article is to describe a simplified workflow for miRNA analyses in archived FFPE or fresh frozen prostate cancer clinical specimens using a combination of quantitative real-time PCR (RT-PCR) and *in situ* hybridization (ISH). Within this workflow, we optimize the existing methodologies for miRNA extraction from FFPE and frozen prostate tissues and expression analyses by Taqman-probe based miRNA RT-PCR. In addition, we describe an optimized method for ISH analyses formiRNA detection in prostate tissues using locked nucleic acid (LNA)- based probes. Our optimized miRNA ISH protocol can be applied to prostate cancer tissue slides or prostate cancer tissue microarrays (TMA).

Video Link

The video component of this article can be found at https://www.jove.com/video/53123/

Introduction

Cancer of the prostate gland is a commonly diagnosed male malignancy that is one of the leading causes of cancer related mortality among men. In US, an estimated 220,800 new cases and 27,540 deaths will be reported in 2015[1].

Prostate cancer is a heterogeneous disease with highly variable disease course- tumors can be indolent or very aggressive. A critical challenge in prostate cancer clinical management is posed by the inadequacy of currently used methods/biomarkers for disease screening, diagnosis, prognosis and treatment[2]. Current screening methods include prostate specific antigen (PSA) testing and a digital rectal examination (DRE) followed by prostate biopsies[3]. Prostate specific antigen (PSA) is the most widely used prostate cancer biomarker that has significantly revolutionized clinical management and improved survival rates[4]. However, due to inherent limitations of PSA including lack of specificity, PSA-based screening has led to over diagnosis and over treatment of the disease. In view of this, intensive efforts are being directed towards a search for alternate prostate cancer biomarkers, particularly those which can predict the aggressiveness of the disease and drive better treatment decisions[4,5]. Over the last few years, microRNAs (miRNAs) have emerged as promising alternate prostate cancer biomarkers.

MicroRNAs (miRNAs) constitute an evolutionarily conserved class of small non-coding RNAs that suppress gene expression post-transcriptionally via sequence-specific interactions with the 3'- untranslated regions (UTRs) of cognate mRNA targets. It is estimated that >60% of mRNAs are conserved targets of miRNAs[6]. miRNA genes are located in intergenic regions or within introns or exons of protein/non-protein coding genes[7]. These genes are preferentially transcribed by RNA Polymerase II into primary miRNAs (pri-miRNAs, several kilobases long) that form hairpin shaped stem loop secondary structures. These pri-miRNAs are processed into precursor miRNAs (pre-miRNAs, 60-75 nucleotide long) that are exported to the cytoplasm and further processed into mature miRNAs (18-25 nucleotide long)[8-10]. miRNAs regulate key cellular processes including proliferation, development, differentiation and apoptosis[11]. Studies suggest a widespread dysregulation of miRNA expression profiles in various human malignancies including prostate cancer[12-15]. miRNA expression profiles have been reported to be widely dysregulated in primary and metastatic prostate cancer. Altered miRNA expression have been linked with prostate cancer progression, aggressiveness and recurrence highlighting the prognostic potential of miRNAs[12,14,16-19]. A growing body of evidence indicates that miRNAs play important mechanistic roles in prostate cancer initiation, development, progression and metastasis. Overall, miRNAs are emerging as promising alternate biomarkers for prostate cancer diagnosis and prognosis that can distinguish between normal and cancer tissues and aid in stratification of prostate tumors[12]. Also, miRNAs are important targets for development of effective therapeutics against prostate cancer[20].

Owing to their small size and resistance to endogenous RNase activity, miRNAs are stable biomarkers that can be readily detected in formalin-fixed tissues[21] and in prostate biopsies[22]. Moreover, the expression profiles of miRNAs have been compared in frozen and formalin fixed tissues and have been found to be strongly correlated[21]. However, miRNA expression profiling in prostate cancer clinical tissues is often challenging owing to tumor heterogeneity, sampling errors, stromal contamination etc. The development of miRNAs as effective biomarkers for prostate

cancer heavily relies on their accurate detection in clinical tissues. Here we describe a simplified workflow used in our lab for miRNA expression profiling in archived FFPE or fresh frozen prostate cancer clinical specimens. We employ a combination of quantitative real-time PCR and *in situ* hybridization for miRNA analyses of clinical specimens, with the former yielding more quantitative information and the latter for visualizing the differential expression of potential miRNA biomarkers in an array of tissues. Within this workflow, we optimize the existing methodologies for miRNA extraction from FFPE and frozen prostate tissues, expression analyses by Taqman-probe based miRNA RT-PCR and miRNA *in situ* hybridization technique using locked nucleic acid (LNA)-based probes[23]. LNA-based probes offer increased sensitivity and specificity compared to DNA- or RNA- based probes and enables robust detection of all miRNA sequences, regardless of their GC content and also allow discrimination of miRNA families. Our optimized miRNA ISH protocol can be applied to prostate cancer tissue slides or prostate cancer tissue microarrays (TMA), with the latter offering the potential to accelerate miRNA biomarker discovery.

Protocol

Formalin-fixed, paraffin-embedded (FFPE) or fresh frozen prostate cancer samples were obtained from the SFVAMC. Samples were from prostate cancer patients who underwent radical prostatectomy at SFVAMC. Written informed consent was obtained from all patients and the study was approved by the UCSF Committee on Human Research. Alternatively, prostate cancer tissues microarrays were procured from commercial sources and used for miRNA analyses by ISH. Clinicopathological and follow up information for analyzed prostate cancer patients was collected.

1. Tissue Samples

1. Cut prostate cancer tissue samples into 10 µm sections using a microtome and stain with H & E following the manufacturer's protocol.
2. Review stained slides for the identification of prostate cancer foci as well as adjacent normal glandular epithelium.
   Note: A board certified pathologist should review the H & E stained slides and mark for tumor and normal areas. Use the marked slides as guides in the subsequent sections for miRNA analyses from tumor vs normal areas.

2. miRNA Expression Analyses by Quantitative Real-time PCR

Note: This workflow involves the following steps: Laser Capture microdissection, isolation of total RNA (including miRNA and mRNA), assaying of mature miRNAs using the TaqMan MicroRNA expression assays as detailed in the following sections.

1. RNA Extraction
   1. Isolation of miRNA from Prostate Cancer FFPE Tissues
      1. Laser capture microdissection (LCM)
         Note: Perform steps 2.1.1.1.1- 2.1.1.1.4 in a chemical fume hood.
         1. Prepare tissue slides (10 µm sections) for LCM. Deparrafinize tissue sections by soaking in Xylene (2x), for 10 min each, then rehydrate the tissues by incubating the slides for 5 min each in graded ethanol (100%, 95%, 90%, 80%, 70%) followed by distilled water (5 min).
         2. Following rehydration, stain sections with hematoxylin for 30 sec followed by water.
         3. Place tissues in graded ethanol (70%, 95% 90%, 80%, 70%) (5 min each) and xylene (5 min).
         4. Dry slides in a fume hood and then place in the LCM instrument for microdissection.
         5. Perform microdissections with the LCM System in accordance with the manufacturer's instructions[24]. Use pathologist marked up slides as guides for the identification of prostate cancer foci as well as adjacent normal tissue. Use the laser beam at a 10-20 µm diameter pulses with a power of 70-90 mW.
         6. Capture areas of interest with infrared laser pulses onto LCM caps. Combine cells from 2-5 caps for miRNA extraction per sample. To ensure integrity of extracted RNA, immediately process captured cells for miRNA extraction.
         7. Alternatively, lyse cells in the miRNA extraction buffer (according to manufacturer's protocol) and store lysates at -80 ºC.
      2. miRNA Extraction and Analyses from LCM Microdissected Tissues
         1. Extract total RNA from microdissected FFPE tissues using a commercial kit following the manufacturer's instructions.
            Note: The yield of RNA from laser capture miscrodissection is typically low. Therefore, RNA is eluted into low volumes (20-30 µl) and used for expression profiling using Taqman microRNA expression assays following manufacturer's instructions (also described in section 2.2). Optimization of input RNA for real time PCR detection of mature miRNAs suggests that 5 µl of the eluted RNA is optimal for each miRNA-specific reverse transcription reaction. As an endogenous control, RNU48/RNU24 is used. For control reactions, 2 µl of the eluted RNA is used for the reverse transcription reaction.
   2. Isolation of miRNA from Prostate Cancer Frozen Tissues
      1. Homogenize frozen resected prostate tissues by grinding the tissues in liquid nitrogen using a mortar and pestle. Transfer homogenized tissue to a microcentrifuge tube using a cooled spatula. Maintain the mortar/pestle and spatula at the same temperature by dipping in liquid nitrogen so homogenized tissue can be easily transferred.
      2. Add commercial guanidine isothiocyanate-phenol reagent (1 ml/0.1 g of tissue) to the homogenized tissue and incubate at room temperature for 5 min.
         Note: Homogenized tissues in the commercial guanidine isothiocyanate-phenol reagent can be stored at -80 ºC for several months.
      3. Add chloroform to the homogenate (0.2 ml chloroform/ml) and shake vigorously for 30 sec. Incubate samples at RT for 3-5 min followed by centrifugation at 10,000 x g for 20 min at 4 ºC.
      4. Transfer the upper aqueous phase to a fresh sterile RNase-free 1.5 ml tube.

5. Add an equal volume of isopropyl alcohol. Mix and incubate for 10 min at RT followed by centrifugation at 12,000 x g for 20 min at 4 ºC to pellet the RNA.
6. Aspirate the supernatant and wash the RNA pellet with 1 mL of 70% ethanol. Centrifuge at 12,000 x g for 10 min at 4 ºC.
7. Carefully aspirate the supernatant. Dry RNA pellets at RT for 5-10 min. Dissolve RNA in 50-100 µl nuclease-free water.
8. Perform quantification of RNA using a nanodrop spectrophotometer and determine RNA integrity with a bioanalyzer. Adjust RNA concentrations to 10 ng/µl. Use 10-50 ng RNA for miRNA- specific cDNA reaction followed by real-time PCR analyses (Section 2.2).

2. Quantitative Real-time PCR for miRNA Expression Analyses
Note: Assay mature miRNAs using a two-step RT-PCR protocol as described below.
    1. Reverse Transcription (RT)
        1. Reverse transcribe cDNA from RNA using a miRNA reverse transcription kit in accordance with the manufacturer's instructions. Use 10-50 ng of total RNA with miRNA specific primer from the MicroRNA Assays and RT kit. Use RNU48/RNU24/RNU6B as controls. Dilute cDNA 1:5 to 1:10 (depending on the abundance of the analyzed miRNA) and use in Real-time PCR detection as described in the following step.
    2. Real-time PCR for Detection of Mature miRNA
        1. Amplify PCR products from cDNA samples using the MicroRNA assays with a Fast Universal master mix in accordance with manufacturer's instructions. Normalize samples to RNU48/RNU24/RNU6B control. Use the comparative Ct (threshold cycle) method to calculate the relative changes in gene expression on the Fast Real Time PCR System. Analyze each sample in triplicate.

3. miRNA Expression Analyses by *In Situ* Hybridization (ISH)

1. Pre-Treatment of Tissues
    1. Cut 5 µm sections from FFPE prostate cancer tissue blocks using a microtome.
    2. Fix tissue sections by incubating the slides at 56 °C for 1 hr.
    Note: Slides can be stored at RT for several weeks for miRNA analyses.
    3. Deparrafinize the tissues by soaking the slides with xylene (2x), for 15 min each.
    4. Rehydrate the tissues by incubating the slides for 5 min each in graded ethanol (100% (2x), 95%, 90%, 80%, 70%) followed by distilled water (5 min).
    5. Fix the slides with 4% paraformaldehyde in PBS at room temperature for 20 min.
    6. Wash the slides with PBS (2x) at room temperature for 5 min each.
    7. Treat the slides with 10 µg/ml Proteinase K at 37 ºC for 10 min in pre-warmed proteinase K buffer (5 mM Tris-HCL pH 7.4, 1 mM EDTA, 1 mM NaCl).
    8. Following Proteinase-K treatment, rinse the slides with 0.2% glycine in PBS for 30 sec.
    9. Wash slides in PBS (1x) at room temperature for 5 min.
    10. Fix the slides with 4% Paraformaldehyde in PBS for 15 min.
    11. Rinse slides with PBS for 5 min.

2. Hybridization
    1. Pre-hybridize the slides with pre-hybridization solution for 3-4 hr at 55 ºC in a humidified chamber. Use tissue lab wipes soaked in 50% formamide/50% 5x SSC to keep the chamber humidified.
    2. Use 5' digoxigenin labeled probes at a concentration of 20-50 nM in hybridization buffer. Dilute miRNA-specific probe (20-50 nM) and small nuclear RNA U6 control probe (20 nM), heat at 90 ºC for 4 min, place it on ice, and add to ice cold hybridization buffer (2-4 ng/µl).
    3. Remove pre-hybridization solution, add hybridization solution (probe + hybridization buffer, 100 µl per slide), incubate for 12-16 hr at probe-specific hybridization temperature (Hybridization temperature =Tm probe-21 ºC). Perform hybridizations in a humidified chamber (50% formamide/50% 5x SSC).

3. Washing Steps
    1. Wash the slides with 2x SSC for 10 min at 45 ºC.
    2. Wash the slides with 1.5x SSC for 10 min at 45 ºC.
    3. Wash the slides with 0.2x SSC (2x) at 37 ºC for 20 min each.
    4. Incubate slides with 1x blocking solution for 1-2 hr at room temperature

4. Detection
    1. Incubate the slides with 1:100 PBS diluted AP-conjugated anti-digoxigenin antibody for 1-4 hr or overnight at 4 ºC.
    2. Wash the slides with PBS (3x) at room temp for 10 min each.
    3. Wash the slides (2x) with Alkaline Phosphatase buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20) at RT for 5 min each.
    4. Incubate the slides in BM Purple AP substrate in the dark at RT for 1-20 hr.
    5. Rinse the slides with PBS containing 0.1% Tween-20 and wash (2x) in water.
    6. Mount the slides using an aqueous mounting media and examine under a microscope.

Representative Results

Expression profiling of miR-203 in LCM primary prostate cancer clinical specimens by RT-PCR analyses **(Figure 1)**

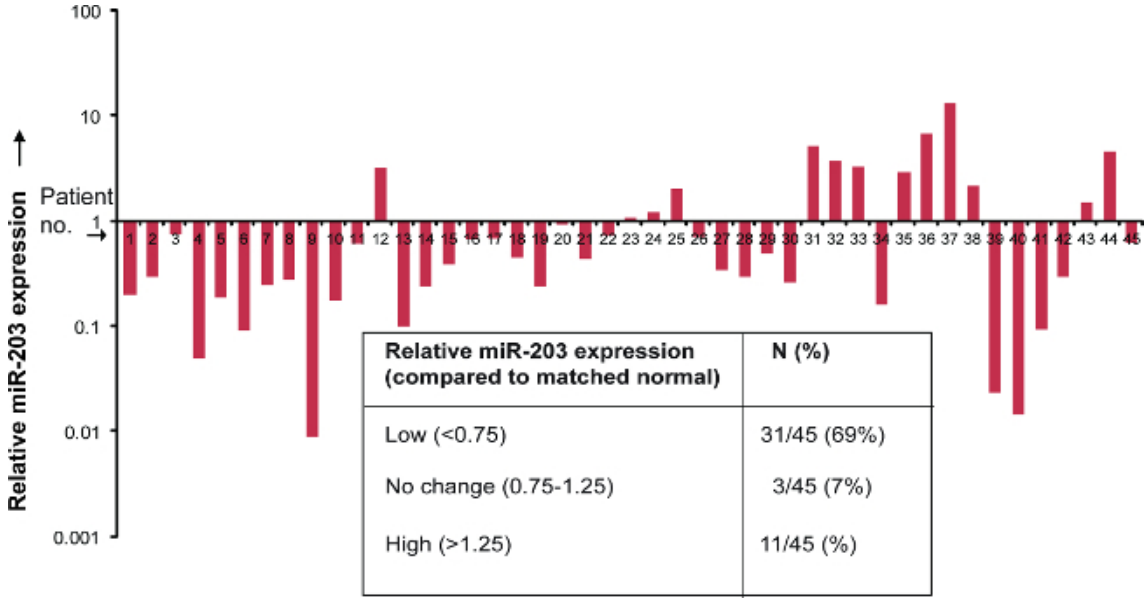
RT-PCR analyses of relative miR-203 expression in LCM primary prostate cancer tissues and the matched adjacent normal regions was carried out as described in Saini *et al.*[15] RNU48 was used as a control. The Table below summarizes the relative miR-203 expression in prostate cancer tumor tissues relative to adjacent normal tissues.

Comparison of miR-383 expression by RT-PCR analyses in microdissected vs laser capture microdissected PCa tissues **(Figure 2)**

Prostate cancer tissues were either microdissected under the microscope or were subjected to LCM followed by RT-PCR analyses of miR-383 expression in tumor tissues relative to matched adjacent normal tissues.Relative miR-383 expression in tumor samples are shown.RNU48 was used as a control. As shown in **Figure 2**, differences were observed when miRNA expression was analyzed in these tissues. LCM has an advantage over microdissection under microscope as it allows isolation of a relatively pure population of prostate tissues and is more reliable.

ISH analyses of miR-203 expression in prostate cancer clinical tissues **(Figure 3)**

In situ hybridization analyses of miR-203 expression in normal and bone metastatic prostate cancer tissues was performed as described in Saini *et al.*[15] Prostate cancer tissue microarray was used for ISH analyses using 5'- DIG labelled LNA probes specific for miR-203 and U6. Representative example of ISH analyses is shown in **Figure 3**, a figure partly reproduced from Saini *et al.* (2011)[15] **Figure 3A** and **3B** showsmiR-203 expression (left) and U6 control expression (right) in the indicated tissues. ISH signals were semi-quantitatively graded based on the intensity of staining and percent of stained cells and assigned a score of 1 to 4. Intensity scores of miR-203 expression were divided by those of U6 expression to obtain normalized miR-203 expression levels that were plotted in **Figure 3C**.



Relative miR-203 expression (compared to matched normal)	N (%)
Low (<0.75)	31/45 (69%)
No change (0.75-1.25)	3/45 (7%)
High (>1.25)	11/45 (%)

Figure 1: Expression profiling of miR-203 in LCM primary prostate cancer clinical specimens by RT-PCR analyses. RT-PCR analysis of relative miR-203 expression in LCM-prostate cancer tissues and the matched adjacent normal regions. RNU48 was used as a control. The Table below summarizes the relative miR-203 expression in prostate cancer tumor tissues relative to adjacent normal tissues. Please click here to view a larger version of this figure.

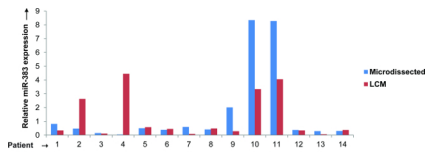


Figure 2: Comparison of miR-383 expression by RT-PCR analyses in microdissected vs. laser capture microdissected PCa tissues. Prostate cancer tissues were either microdissected under the microscope or were subjected to LCM followed by RT-PCR analyses of miR-383 expression in tumor tissues relative to matched adjacent normal tissues. Relative miR-383 expressions in tumor samples are shown.RNU48 was used as a control. Please click here to view a larger version of this figure.

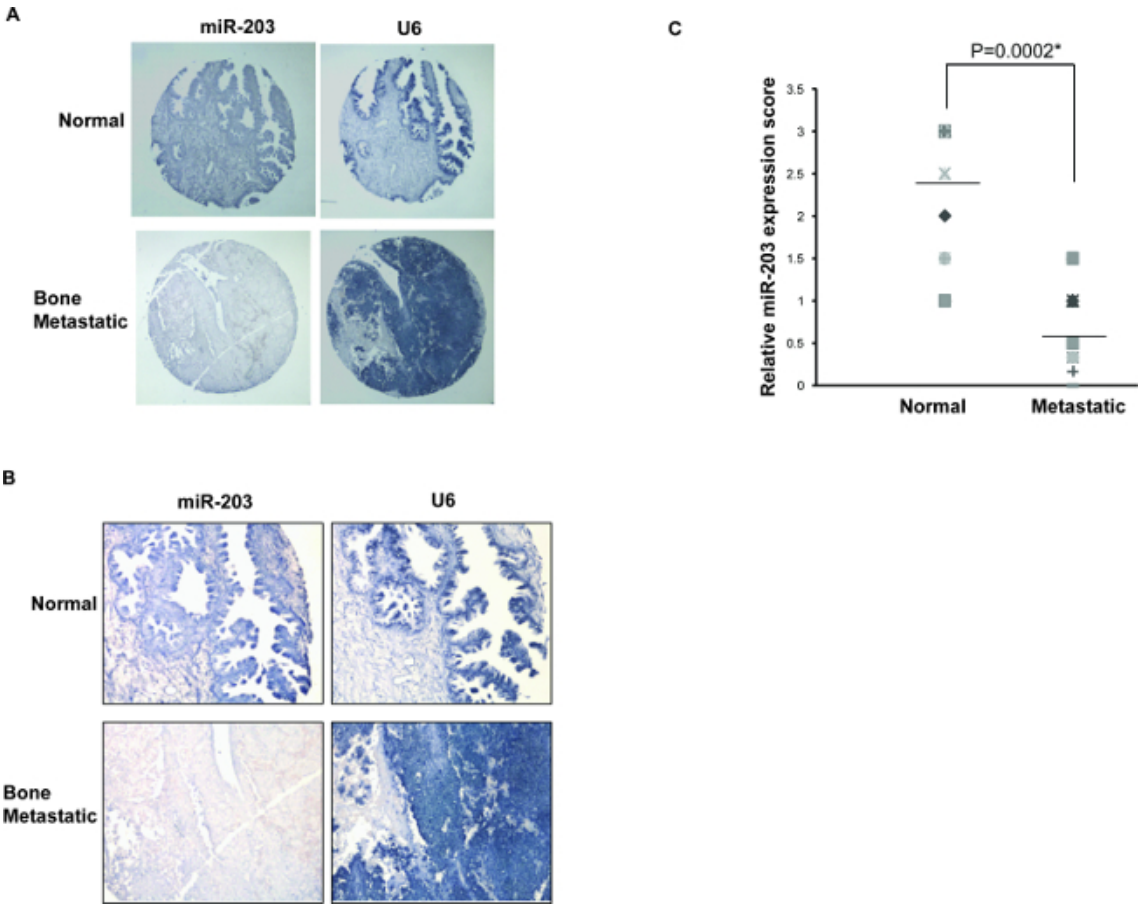


Figure 3: ISH analyses of miR-203 expression in prostate cancer clinical tissues. This figure has been modified from[15]. Tissues were hybridized with DIG-labelled locked nucleic acid (LNA) based probes for mir-203 and U6 (control) as described in[15]. Representative example of ISH analysis of miR-203 expression (left panels) and control U6 expression (right panels) in human prostate cancer normal and bone metastatic tissues showing attenuated miR-203 expression in bone metastatic tissues. Representative ISH examples shown in A at a higher magnification (40X). Relative miR-203 expression levels in normal prostate tissues and bone metastatic prostate cancer tissues as assessed by ISH analysis. miR-203 expression was scored , normalized to U6 scores and plotted. Horizontal line represents the average value. Please click here to view a larger version of this figure.

Discussion

In this article, we describe a simplified workflow for miRNA expression profiling in archived FFPE or fresh frozen prostate cancer clinical tissues. In prostate cancer, several studies suggest an important role of microRNAs in prostate cancer initiation, progression and metastasis. However, conflicting results are often obtained on a specific miRNA[22] since the miRNA extraction and analyses methods differ widely. In view of the emerging evidence supporting the potential application of miRNAs as alternative prostate cancer biomarkers for prostate cancer prognosis, diagnosis and therapy, there is a need to accurately quantitate miRNA expression profiles in clinical tissues.

We employ a combination of quantitative real-time PCR and *in situ* hybridization for miRNA analyses of prostate cancer clinical specimens. Within this workflow, we optimized the existing methodologies for miRNA extraction, expression analyses by Taqman primer based RT-PCR and ISH analyses by LNA probes. During the entire workflow, it is of utmost importance to maintain an RNase free environment. All the reagents/ solutions used should be RNase free and extra caution should be used in handling RNA and other reagents. All real time reactions should be set up on ice to minimize RNA degradation.

Accurate expression profiling in prostate cancer tissues is often hindered by heterogeneity and the multifocal nature of prostate cancer lesions[25]. This can be overcome by using a laser-capture microdisssection technique that allows the separation of benign and malignant epithelial cells and also reduces stromal contamination, allowing accurate downstream molecular analyses of prostate tissues[22,25]. We employ LCM of archived FFPE tissues followed by miRNA expression profiling using Taqman MicroRNA expression assays that has been reported to be reliable. miRNA profiling of LCM tissues has significant potential to enhance miRNA biomarker discovery. In fact, there is considerable interest in analyzing FFPE tissues considering that these tissues are widely used by pathologists for clinical analyses, preservation, and archiving and are readily available[3]. Also, owing to their small size and resistance to endogenous RNase activity, miRNAs are relatively less affected by FFPE dependent degradation and are attractive potential biomarkers[3]. The miRNA expression profiles of formalin fixed tissues have been reported to be strongly correlated to those of frozen tissues[21].

We employed and compared two different commercial kits for miRNA extraction from FFPE tissues. While both kits yielded high quality RNA for RT-PCR analyses, we used the one that uses a simple, streamlined protocol for consistent purification of miRNAs and total RNA from FFPE tissues.

Integrity, purity and concentration of RNA should be tested before RT-PCR analyses. Samples showing low A260/A230 ratio (<1.8) should be re-precipitated and re-analyzed for purity before using in an RT reaction. Also, real-time PCR analyses of clinical tissues may need to be further optimized. Threshold cycle (Ct values) >33 indicate low amplification reflecting low starting cDNA template. In this case, cDNA template inputs can be increased. Also, it is of utmost importance that adequate control is used for normalizing and determining relative miRNA quantifications between normal and tumor tissues. Endogenous control (RNU48/RNU24/RNU6B) is chosen based on the uniformity of amplification signals in normal vs. tumor samples.

Also, we describe an optimized protocol for miRNA ISH analyses of prostate cancer tissues based on LNA- based probes. Our optimized miRNA ISH protocol can be applied to prostate cancer tissue slides or prostate cancer tissue microarrays (TMA), with the latter offering the potential to accelerate miRNA biomarker discovery. This protocol can also be applied to other tissue types. However, the duration of proteinaseKdigestionandparaformaldehydetreatment may need to be optimized. Proteinase K digestion allows the miRNA probe to get into the cells while paraformaldehyde fixation is needed to prevent miRNA loss following permeabilization. This protocol, originally developed for use with LNA probes[26] is optimized to get a specific signal with minimal background in prostate tissues. Probe concentrations and incubation conditions are optimized for prostate cancer tissues. We employ 5'digoxigeninlabeledprobes at a concentration of 20-50 nM depending upon the abundance of miRNA. However, 3'digoxigeninlabeledprobes and dual labeled (5' and 3') labeled probes can be substituted. In fact, dual labeled probes offer the potential for better colorimetric development for microRNA detection and are recommended for low abundance miRNA detection. Also, duration of blocking and antibody incubations may be optimized. Longer blocking is recommended for lower background, particularly with low abundance miRNAs. Thedurationofcolor reaction with BM purple substrate should be closely monitored. Longer incubations can generate background staining. Usually, abundant miRNAs require shorter incubations (1-2 hr) while less abundant miRNA are detected with longer incubations. During ISH, it is important to ensure that the tissue sections do not dry out during any of the steps as this may lead to staining artifacts.

In conclusion, though several studies suggest miRNAs as important prostate cancer biomarkers, the significance of several of these studies is often debated due to conflicting results. Here we have defined an optimized workflow for accurate miRNA expression analyses in prostate cancer clinical specimens that have a potential to accelerate the discovery of miRNA as biomarkers for prostate cancer. While this workflow has significant potential to accurately profile miRNA expression in prostate cancer clinical tissues, there are inherent limitations of the techniques employed. Low abundant miRNAs may be difficult to profile with the optimized miRNA RT-PCR and ISH analyses described here. Though RT-PCR based profiling has a wider dynamic range than ISH[27,28], low RNA amounts obtained from LCM may limit detection of low abundant miRNAs. ISH-based detection of miRNAs yields powerful spatial information, however its limitations are that it is a semi-quantitative technique with a lesser dynamic range[28]. This technique relies on sensitivity and specificity of probes employed and also on the optimization of conditions for hybridization and detection of miRNAs.

Disclosures

The authors have no financial disclosures.

Acknowledgements

We thank Dr. Roger Erickson for his support and assistance with preparation of the manuscript.

This work was supported by *the National Cancer Institute at the National Institutes of Health*

(Grant Number RO1CA177984; RO1CA138642), VA program project on prostate cancer (BX001604).

References

1. Siegel, R. L., Miller, K. D., Jemal, A. Cancer statistics. *CA Cancer J Clin.* **65**, (1), 5-29 (2015).
2. Shen, M. M., Abate-Shen, C. Molecular genetics of prostate cancer: new prospects for old challenges. *Genes Dev.* **24**, (18), 1967-2000 (2010).
3. Sequeiros, T., *et al.* Molecular markers for prostate cancer in formalin-fixed paraffin-embedded tissues. *Biomed Res Int.* **2013**, 283635 (2013).
4. Cary, K. C., Cooperberg, M. R. Biomarkers in prostate cancer surveillance and screening: past, present, and future. *Ther Adv Urol.* **5**, (6), 318-329 (2013).
5. Sartori, D. A., Chan, D. W. Biomarkers in prostate cancer: what's new. *Curr Opin Oncol.* **26**, (3), 259-264 (2014).
6. Friedman, R. C., Farh, K. K., Burge, C. B., Bartel, D. P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **19**, (1), 92-105 (2009).
7. Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L., Bradley, A. Identification of mammalian microRNA host genes and transcription units. *Genome Res.* **14**, (10A), 1902-1910 (2004).
8. Borchert, G. M., Lanier, W., Davidson, B. L. RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol.* **13**, (12), 1097-1101 (2006).
9. Cai, X., Hagedorn, C. H., Cullen, B. R. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA.* **10**, (12), 1957-1966 (2004).
10. Lee, Y., *et al.* MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* **23**, (20), 4051-4060 (2004).
11. Bartel, D. P., *et al.* MicroRNAs: target recognition and regulatory functions. *Cell.* **136**, (2), 215-233 (2009).

12. Gordanpour, A., Nam, R. K., Sugar, L., Seth, A. MicroRNAs in prostate cancer: from biomarkers to molecularly-based therapeutics. *Prostate Cancer Prostatic Dis.* **15**, (4), 314-319 (2012).
13. Hurst, D. R., Edmonds, M. D., Welch, D. R. Metastamir: the field of metastasis-regulatory microRNA is spreading. *Cancer Res.* **69**, (19), 7495-7498 (2009).
14. Saini, S., Majid, S., Dahiya, R. Diet, microRNAs and prostate cancer. *Pharm Res.* **27**, (6), 1014-1026 (2010).
15. Saini, S., *et al.* Regulatory Role of mir-203 in Prostate Cancer Progression and Metastasis. *Clin Cancer Res.* **17**, (16), 5287-5298 (2011).
16. Ambs, S., *et al.* Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. *Cancer Res.* **68**, (15), 6162-6170 (2008).
17. Martens-Uzunova, E. S., *et al.* Diagnostic and prognostic signatures from the small non-coding RNA transcriptome in prostate cancer. *Oncogene.* **31**, (8), 978-991 (2012).
18. Porkka, K. P., *et al.* MicroRNA expression profiling in prostate cancer. *Cancer Res.* **67**, (13), 6130-6135 (2007).
19. Schaefer, A., *et al.* Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. *Int J Cancer.* **126**, (5), 1166-1176 (2010).
20. Maugeri-Sacca, M., Coppola, V., De Maria, R., Bonci, D. Functional role of microRNAs in prostate cancer and therapeutic opportunities. *Crit Rev Oncog.* **18**, (4), 303-315 (2013).
21. Xi, Y., *et al.* Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. *RNA.* **13**, (10), 1668-1674 (2007).
22. Lucas, S. M., Heath, E. I. Current challenges in development of differentially expressed and prognostic prostate cancer biomarkers. *Prostate Cancer.* 640968 (2012).
23. Singh, S. K., Kumar, R., Wengel, J. Synthesis of Novel Bicyclo[2.2.1] Ribonucleosides: 2'-Amino- and 2'-Thio-LNA Monomeric Nucleosides. *J Org Chem.* **63**, (18), 6078-6079 (1998).
24. Suh, S. O., *et al.* MicroRNA-145 is regulated by DNA methylation and p53 gene mutation in prostate cancer. *Carcinogenesis.* **32**, (5), 772-778 (2011).
25. Shukla, C. J., Pennington, C. J., Riddick, A. C., Sethia, K. K., Ball, R. Y., Edwards, D. R. Laser-capture microdissection in prostate cancer research: establishment and validation of a powerful tool for the assessment of tumour-stroma interactions. *BJU Int.* **101**, (6), 765-774 (2008).
26. Nelson, P. T., *et al.* RAKE and LNA-ISH reveal microRNA expression and localization in archival human brain. *RNA.* **12**, (2), 187-191 (2006).
27. Chen, C., *et al.* Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* **33**, (20), e179 (2005).
28. Hanna, J. A., *et al.* Quantitative analysis of microRNAs in tissue microarrays by in situ hybridization. *Biotechniques.* **52**, (4), 235-245 (2012).

