Histol Histopathol (2015) 30: 1379-1396

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

MicroRNA in prostate cancer: Practical aspects

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Summary. In the last decade, microRNAs (miRNAs) have emerged as biomarkers for cancer diagnosis, prognosis, therapy and prediction of treatment response and have earned a promising role in prostate cancer (PCa) management. A plethora of studies have been conducted on miRNA expression in PCa compared to non-neoplastic prostatic tissue, in PCa of different histologic grades and pathologic stages, in castration resistance prostate cancer (CRPC), in metastatic disease and in response to therapy, with evidence pointing towards distinctive miRNAs differentially expressed in each of these phases. In addition to tissue, MiRNA can be detected in blood, serum, and urine. The aim of this review is to survey studies conducted on human prostate tissue and biofluids and to consolidate trustworthy data on the role of miRNA in the occurrence and progression of PCa, with a delineation of differentially expressed miRNAs and an analysis of their association with PCa prognosis, progression to CRPC and metastatic disease, as well as their correlation with response to chemotherapy and hormonal therapy. Changes in circulating miRNAs may represent potentially useful non-invasive biomarkers for PCa diagnosis, staging and prediction of outcome.

Key words: Prostate cancer, Gleason score, Pathologic stage, Prognosis, Castration resistant prostate cancer, Biofluids, Serum, Urine, MicroRNA

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DOI: 10.14670/HH-11-647

Introduction

Prostate cancer (PCa) is the second most common cause of cancer deaths in American men, with 220,800 estimated new cases and 27,540 estimated deaths for 2015 (Siegel et al., 2015). Prostate specific antigen (PSA) has been used as screening test for prostate cancer for over 20 years and has been responsible for a decrease in PCa mortality in the U.S. (Wilt et al., 2012). On the other end, PSA screening has led to potentially unnecessary biopsies and increased detection of indolent low-grade/low-stage PCa that might not affect patients' life if left untreated (overtreatment) (Duffy, 2014). Despite new biomarkers and genetic tests currently undergoing evaluation (Dimakakos et al., 2014), the diagnostic armamentarium is largely deficient in sensitive and specific tools for prostate cancer diagnosis, prognosis and patient follow-up (Fiorentino et al., 2010). The main priority in clinical prostate cancer research is identifying novel biomarkers or combinations of biomarkers to reliably distinguish between low-risk patients, candidate for active surveillance, and intermediate/high-risk patients who need definitive treatment. In addition, a greater understanding of molecular events identifying metastatic potential in PCa would enable us to determine treatment modalities at the time of diagnosis.

In the last decade, microRNAs (miRNAs) have emerged as biomarkers for cancer diagnosis, prognosis, therapy and prediction of response to treatment due to their specificity and ease of detection. Mature miRNAs are small (19-22 nucleotides) non-protein coding RNAs regulating mRNA translation and degradation via mechanisms that are dependent on the degree of complementarity between the miRNA and mRNA

molecules (Carthew and Sontheimer, 2009). The predominant effect of miRNA on genes is inhibitory; hence the mechanism is often referred to as "RNA silencing" (Carthew and Sontheimer, 2009). MiRNA can have the opposing effect to enhance translation during cell cycle arrest, when Adenylate-Uridylate rich elements of target mRNA are in proximity (Vasudevan and Steitz, 2007).

Each miRNA could have multiple target mRNAs/genes and vice versa (Liu et al., 2013). Expression of miRNAs in the cell can be spatial (tissue organ specific) or temporal (stage of development specific). The miRBase lists 1881 precursor and 2588 mature microRNA in humans, which can be grouped into 153 clusters with an inter-miRNA distance of <10kb (Kozomara and Griffiths-Jones, 2014). Computational analysis has revealed that miRNAs may regulate 60% of genes (Lewis et al., 2005; Friedman et al., 2009). MiRNAs are conserved between species (Bartel, 2004) and since the family of miRNAs is relatively small (Munker and Calin, 2013), it holds a possibility of easier biomarker identification compared to the larger biomarker families of mRNA and proteins (Berezikov et al., 2005).

MiRNAs are named with species identified in the first three letters; 'hsa' stands for homo sapiens e.g. hsa-miR-222 (Ambros et al., 2003). The number is assigned in the order of discovery and the same number is used for identical miRNA irrespective of species. When a miRNA with identical sequence is produced by genes from 2 different loci, a numerical suffix is added e.g. hsa-miR-1-1 (chromosome 20) and hsa-miR-1-2 (chromosome 18). Alphabetical suffixes are used when miRNA sequences are minimally different within a species e.g. hsa-miR27-a, hsa-miR-27-b.

MiRNA genes are located in inter-genic non-coding regions and intra-genic coding regions, in intronic regions more commonly than in exonic ones (Melo and Melo, 2014). The miRNA gene is transcribed by RNA polymerase II into a stem loop primary miRNA (approximately 1 kb) (Sun et al., 2014). An RNase III endonuclease complex converts primary miRNA to premiRNA (approximately 70-90 nucleotides) with a 2 nucleotide overhang at the 3'end. An active transport mechanism exports the premiRNA from the nucleus to the cytoplasm. The premiRNA is converted to double stranded miRNA by DICER, another RNase III endonuclease (Sun et al., 2014). The DICER bound miRNA assembles with argonaute and other proteins to form the RNA induced silencing complex (RISC) (Sun et al., 2014). Complementarity of the 5'end of guide strand miRNA with 3'UTR region of target mRNA at the seed sequence (a 6-8 base pairs binding site) determines miRNA target recognition (Hayes et al., 2014). The degree of complementarity with the rest of the sequence of mRNA determines whether decay (perfect complementarity) or repression (imperfect complementarity) occurs (Hayes et al., 2014).

Liang and colleagues revealed the tissue specific microRNA expression and coexpression of microRNAs with related physiologic functions in an elegant study evaluating the distribution of 345 miRNA by RT-PCR in 40 normal human tissues (Liang et al., 2007). Cellular identity signature is preserved in miRNA expression profiles, which supports potential use of miRNA use as a biomarker. MiRNAs have been involved in apoptosis, cell cycle control and several developmental and physiological processes; they have also been implicated in heart and neurological diseases and in a number of cancers. MiRNA play different roles in neoplasia; there are tumor suppressor miRNAs (downregulated in tumors) and oncogenic miRNAs (upregulated in tumors) (Liu et al., 2013). The effects of miRNAs span migration, invasion and cancer stem cells (Liu et al., 2013). Aggressive tumor biology may be associated with changes in miRNA expression.

miRNA expression in PCa

We went through the existing plethora of literature available on PubMed with a good number of studies profiling microRNA in prostate cancer. The cases included in the studies have been drawn from biopsies, radical prostatectomies (RP), transurethral resection of prostate tissue (TURP), and tissue banks from in-house or commercial sources. The study materials used cell lines, human frozen and formalin fixed paraffin embedded (FFPE) tissue. The commonly used methods for miRNA profiling include microarray (often used for high throughput initial identification of differentially expressed miRNA) and qRT-PCR (validation of differentially expressed miRNA). In order to generate clinically useful information we have chosen to exclude studies primarily based on cell lines or evaluating diagnostic uses on animal models and involving less than 5 samples.

miRNA expression in different zones of the prostate

Carlsson and colleagues studied the expression of 667 miRNAs in the normal peripheral (PZ) and transition zone (TZ) of the prostate to investigate if differences in their expression could be a possible explanation for the disparity in propensity of tumors between PZ and TZ (Carlsson et al., 2013). Normal prostate tissue (n=20), collected from men undergoing cystoprostatectomy for bladder cancer, was used to analyze miRNA expression in normal PZ (n=10) and TZ (n=10). Patients (n=16) with prostate cancer were included in the study if they had a tumor with Gleason grade 3 in the PZ (n=5), TZ (n=5), or both (n=3). The authors found that the major differences in miRNA expression occur between neoplastic and non-neoplastic prostatic tissue as opposed to between different zones of the prostate. A single microRNA, miR-433, was significantly differentially expressed between normal TZ and normal PZ; no miRNAs were differentially expressed between TZ tumors and PZ tumors. The

miRNAs differentially expressed between normal and malignant prostate tissue were different for different zones: 149 miRNAs were differentially expressed between normal and malignant TZ, 65 between normal and malignant PZ (Carlsson et al., 2013). When the miRNAs differentially expressed between normal and malignant PZ tissues were compared to the ones differentially expressed between normal and malignant TZ tissues, the correlation revealed that 111 (75%) of the miRNAs differentially expressed in the TZ were unique to TZ but only 27 (42%) of the miRNAs differentially expressed in the PZ were unique to the PZ. For cross validation, the authors selected miRNAs with target genes differentially expressed between normal PZ and TZ tissue previously proven by other studies (Noel et al., 2008; van der Heul-Nieuwenhuijsen et al., 2006) and selected 15 miRNAs with lowest p-values for a more detailed target gene analysis. Six microRNAs (miR-93, miR-95, miR-154, miR-541, miR-539, and miR-28-3p) differentiated normal TZ from normal PZ with 70% accuracy; two miRNAs (miR-187 and miR-19a) differentiated normal PZ from malignant PZ with 100% accuracy; two miRNA (miR-143 and miR-25) differentiated normal TZ from malignant TZ with 94% accuracy (Carlsson et al., 2013).

miRNA expression in non-neoplastic prostate tissue vs. prostate cancer

This section focuses on the differential expression of miRNA by RT-PCR in prostate tumor tissue compared to non-neoplastic prostate tissue. In situ hybridization (ISH) analysis has been mentioned when performed in addition to RT-PCR. Table 1 summarizes the studies included herein. The miRNAs of most significance in distinguishing prostate tumor from non-neoplastic tissue are depicted in Fig. 1.

Hellwinkel and co-authors recently reported a miRNA panel [miR-185, miR-16, let-7a and let-7b] that discriminated non-neoplastic tissue adjacent to PCa (n=31) from non-neoplastic tissue in patients with elevated serum PSA without PCa (n=17) in needle biopsies (Hellwinkel et al., 2013). The area under the curve (AUC) was 0.92 for the entire panel of miRNAs and 0.86 for miR-185. When the authors applied the same miRNA panel to non-neoplastic prostate biopsy tissue from men with normal PSA (n=14) and negative biopsy results, the panel classified 100% of the samples as tumor free (Hellwinkel et al., 2013).

Tong et al. studied tumor and non-neoplastic tissue microdissected from 40 radical prostatectomy (RP) specimens and found downregulation of miR-23b, miR-145 and miR-221/222 with upregulation of miR-135b and miR-194 in tumor (Tong et al., 2009). Majid et al. analyzed miR-23b expression by qRT-PCR in 118 pairs of matched tumor and non-neoplastic prostate tissue samples, along with an unmatched group of 20 tumor and 27 non-tumor samples; in addition a cohort of 48 samples was analyzed by ISH. MiR-23b expression was significantly downregulated in cancer compared to non-

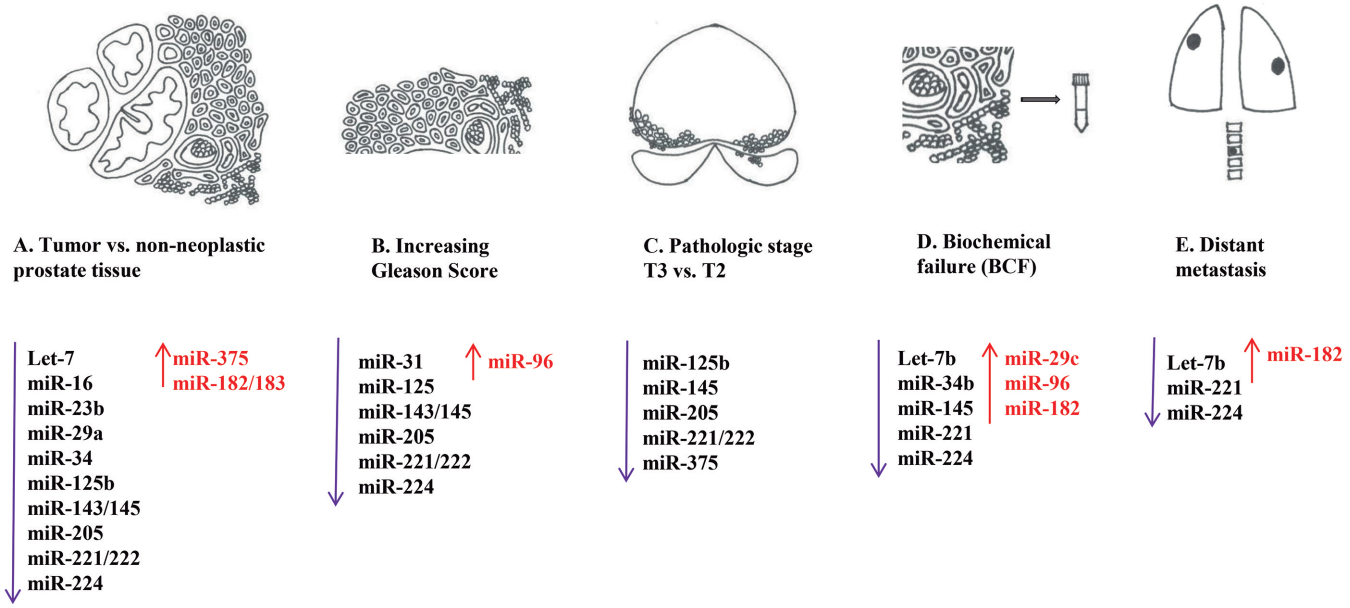

Fig. 1. Diagrammatic representation of miRNAs of significance by RT-PCR in tissue based studies in radical prostatectomy prostate cancer. miRNAs downregulated are listed in black next to the arrow pointing downwards, and those upregulated are listed in red next to the arrow pointing upwards. **A.** Differentially expressed miRNAs in prostate tumor compared to non-neoplastic prostate tissue. **B.** Differentially expressed miRNAs between prostate tumors with increasing Gleason Score. **C.** Differentially expressed miRNAs in prostate tumors with pathological stage 3 (T3) compared to pathological stage 2 (T2). **D.** MiRNAs associated with biochemical failure (BCF). **E.** MiRNAs associated with distant metastasis.

neoplastic tissue, in all samples (Majid et al., 2012).

Multiple studies have reported miR-143/145 downregulation and miR-375 upregulation in prostate cancer versus non-neoplastic prostate tissue. Ozen et al. found downregulation of miR-145b, let-7c, and miR-125b in PCa (n=30) compared to non-neoplastic PZ tissue (n=10) obtained from RP specimens (Ozen et al., 2008). Schaefer and colleagues evaluated tumor and adjacent non-neoplastic tissue from 76 RP specimens and found downregulation of miR-145, miR-16, miR-31, miR-125b, miR-149, miR-181b, miR-184, miR-205, miR-221/222 with upregulation of miR-96, miR-182/183, and miR-375 in PCa compared to non-neoplastic tissue (Schaefer et al., 2010). MiR-205 was able to distinguish prostate cancer from non-neoplastic tissue with an AUC of 0.82; the combination of miR-183 and miR-205 had an AUC of 0.88 (Schaefer et al., 2010). Brase and colleagues reported miR-375 and miR-141 upregulation in PCa compared to non-neoplastic tissue from 36 RP specimens (Brase et al., 2011). Similarly, Wach et al. reported downregulation of miR-143/145 and upregulation of miR-375 in two sets of samples from two participating universities: set 1 included FFPE tumor and non-neoplastic prostate tissue from 50 RP specimens while set 2 consisted of frozen tissue from 26 RP specimens macrodissected to separate tissue containing >70% of tumor and non-neoplastic tissue (Wach et al., 2012). Set 1 showed also upregulation of miR-200c. The sensitivity and specificity of different miRNAs to distinguish tumor from non-neoplastic tissue varied in the two sets of specimens, with higher values for the FFPE tissue compared to the frozen one. Combination of miR-375 and miR-143/145 led to an AUC of 0.95 in set 1, 0.74 in set 2; miR-375/miR-145 ratio distinguished PCa from non-neoplastic tissue with an AUC of 0.92 in set 1 (sensitivity 73%, specificity 96%) and an of AUC 0.93 in set 2 (sensitivity 83%, specificity 95%) (Wach et al., 2012).

Avgeris et al. found downregulated miR-145 expression in PCa from 73 RP specimens compared with non-neoplastic prostatic tissue from 64 patients with benign prostatic hyperplasia (Avgeris et al., 2013). Hart and colleagues found downregulation of miR-145 in PCa compared to adjacent non-neoplastic tissue from 26 FFPE RP specimens (Hart et al., 2013). Subsequently the

Table 1. miRNAs deregulated in prostate cancer compared to non-neoplastic prostate tissue.

Study	Cases	miRNA downregulated	miRNA upregulated
FFPE Tissue or Frozen Tissue*			
Tong et al., 2009	40 RP (T and NN)	miR-23b; miR-145; miR-221/222	miR-135b; miR-194
Majid et al., 2012	118 RP (T and NN); 20 T and 27 NN; 40 T and 8 NN #	miR-23b	
Ozen et al., 2008	30 RP T and 10 NN	let-7c; miR-125b; miR-145b	
Schaefer et al., 2010	76 RP (T and NN)	miR-16; miR-31; miR-125b; miR-145; miR-149; miR-181b; miR-184; miR-205; miR-221/222	miR-96; miR-182/183; miR-375
Brase et al., 2011	36 RP (T and NN)*		miR-141; miR-375
Wach et al., 2012	Set 1: 50 RP (T and NN) Set 2: 26 RP (T and NN)*	miR-143/145 miR-143/145	miR-200c; miR-375 miR-375
Avgeris et al., 2013	73 RP T and 64 NN	miR-145	
Hart et al., 2013	26 RP (T and NN)	miR-145	
Hart et al., 2014	40 RP* (T and NN)	miR-143/145	miR-20a; miR-148a; miR-200b; miR-375
Spahn et al., 2010	12 RP T and 9 NN 92 RP T and 9 NN	miR-16; miR-29a; miR-125b; miR-221; miR-221	
Srivastava et al., 2013	40 RP (T and NN)	miR-99b; miR-205; miR-214; miR-221	
Gandellini et al., 2009	31 RP (T and NN)*	miR-205	
Kalogirou et al., 2013	105 RP T and 10 NN	miR-205	
Li et al., 2012	46 RP (T and NN)	miR-29a	
Casanova-Salas et al., 2014	273 RP T and 10 NN	miR-187	miR-182
Hirata et al., 2013	52 RP (T and NN)		miR-182-5p
Yamamura et al., 2012	10 RP (T and NN)	miR-34a	
Majid et al., 2013	74 RP (T and NN); 20 T and 27 NN; 40 T and 8 NN #	miR-34b	
Ambs et al., 2008	60 RP T and 16 NN*	miR-1	miR-32; miR-106b-25
Mavridis et al., 2013	73 RP T and 66 NN*	miR-224	
Lin et al., 2014	114 T and 20 NN #	miR-224	
Kong et al., 2012	129 RP (T and NN)	let-7 family	

RP: radical prostatectomy; T: prostate cancer; NN: non-neoplastic prostate tissue; #: samples analyzed by in situ hybridization (ISH).

same group validated miRNA expression by northern blotting and RT-PCR on tumor and non-neoplastic prostate tissue obtained from cryopreserved sections from 40 RP specimens and found downregulation of miR-143/145, and upregulated miR-375 and miR-200b, in agreement with Wach and coauthors. MiR-20a and miR-148a were also upregulated in PCa versus non-neoplastic prostate tissue (Hart et al., 2014).

Similarly to other previously mentioned studies (Tong et al., 2009; Schaefer et al., 2010), Spahn et al. found downregulation of miR-221 together with miR-125b, miR-29a, and miR-16 in 12 PCa from RP specimens compared to 9 non-neoplastic prostatic tissue specimens from adenomectomy (Spahn et al., 2010). The downregulation of miR-221 was further corroborated in additional 92 RP specimens with high risk PCa (Spahn et al., 2010). In a study evaluating microdissected prostate cancer and adjacent non-neoplastic tissue from 40 RP specimens, Srivastava et al. showed downregulation of miR-221, miR-205, miR-214 and miR-99b in tumor compared to non-neoplastic tissue (Srivastava et al., 2013). The AUCs for reduced expression of miR-221, miR-205, miR-99b and miR-214 to distinguish PCa were 0.75, 0.83, 0.86 and 0.92, respectively (Srivastava et al., 2013).

Alike aforementioned studies (Schaefer et al., 2010; Srivastava et al., 2013), downregulation of miR-205 in PCa compared to non-neoplastic prostate tissue was detected by Gandellini et al. on 31 tumors and adjacent non-neoplastic tissue obtained from frozen sections of RP specimens (Gandellini et al., 2009), and by Kalogirou and colleagues on 105 FFPE RPs with high risk PCa compared to non-neoplastic prostate tissue (n= 10 adenomectomy specimens for BPH) (Kalogirou et al., 2013). Comparable to Spahn and colleagues, Li et al. reported downregulation of miR-29a in prostate cancer from 46 FFPE RP (Li et al., 2012).

Downregulation of miR-187 and upregulation of miR-182 was detected in PCa from 273 RPs compared to normal prostate tissue from 10 cystoprostatectomy specimens (Casanova-Salas et al., 2014). Upregulation of miR-182-5p in prostate tumor compared to non-neoplastic tissue was detected also by Hirata and colleagues in a recent study analyzing 52 RPs specimens (Hirata et al., 2013).

Majid and colleagues and Yamamura et al. found miR-34b downregulation in prostate cancer compared to adjacent non-neoplastic tissue from 74 and 10 RP specimens, respectively (Yamamura et al., 2012;, Majid et al., 2013). MiR-34b expression was analyzed in 148 laser-captured microdissected matched human tissue samples together with an unmatched group of 20 tumor and 27 non-tumor samples by quantitative real-time PCR. In addition, the authors confirmed downregulation of miR-34b in prostate cancer by performing ISH on commercially available tissue arrays including 40 tumors and 8 non-tumor samples (Majid et al., 2013).

Ambs et al. analyzed 60 fresh-frozen prostate tumors (macrodissected) and 16 non-neoplastic tissue samples from patients with prostate cancer and found that miR-32 and miR-106b were overexpressed and miR-1 was downregulated in tumors when compared with non-neoplastic tissue (Ambs et al., 2008). Downregulation of miR-224 was found in prostate tumor compared to non-neoplastic tissue by quantitative RT-PCR in snap frozen prostate tissue from 73 RP and 66 TURP/open prostatectomy specimens (Mavridis et al., 2013), and by ISH in 114 prostate tumors compared to 20 adjacent non-neoplastic samples included on tissue microarrays obtained from a commercial source (Lin et al., 2014).

The let-7 family of miRNAs was found downregulated in 129 FFPE RP tumors compared to non-neoplastic tissue studied by Kong et al. (2012).

miRNA expression by prostate tumor grade (Gleason score) and pathologic stage

In this section we discuss the significance of miRNAs in relation to tumor grade and pathological stage in studies based on RT-PCR. A summary of the studies discussed in this section is represented in Table 2. The miRNAs of most significance in distinguishing among prostate cancers of different Gleason score (GS) and pathological stage are depicted in Fig. 1.

Walter and colleagues studied 40 prostate cancer tumors microdissection from 37 FFPE RP specimens and found miR-122, miR-335, miR-184, miR-193, miR-34, miR-138, miR-373, miR-9, miR-198, miR-144 and miR-215 upregulation in GS≥8 PCa compared to GS6, while miR-96, miR-222, miR-148, miR-92, miR-27, miR-125, miR-126, miR-27 were downregulated (Walter et al., 2013) (Table 2). On the other hand, Xiong et al. investigated miR-335 expression in 104 clinical PCa samples from a commercial source by ISH and found that low miR-335 expression was significantly associated with high GS [reduced expression in GS≥8 (n=18) vs. GS<8 (n=86)], advanced clinical stage, and positive metastasis [reduced expression in metastatic (n=15) compared to non-metastatic (n=89) tumors], but not with prognosis (Xiong et al., 2013). At the same time miR-335 was found downregulated by both quantitative RT-PCR and ISH in 20 primary PCa specimens relative to pair-matched adjacent non-neoplastic prostate tissue (Xiong et al., 2013). The opposite results on miR-335 reported in these two studies could be due to differences in the study design: Walter et al. microdissected prostate tissue to separate tumor cells and stroma, whereas Xiong et al. used frozen tissue samples provided by a commercial source without specific data regarding the origin of the tissue (RP vs. TURP). Tumors removed by TURP could have different signatures as they are more likely to be arising in TZ as opposed to common location of PCa in PZ.

In a study by Mavridis et al. conducted on snap-frozen tumor tissue samples from 73 patients with PCa who underwent RP, the expression of miR-224 was found to gradually decrease with increasing GS from ≤6 (n=24), 7 (n=39) to >7 (n=9) (Mavridis et al., 2013). Lin

and colleagues conducted an ISH study on PCa tissue microarrays purchased from a commercial source including 114 human PCa tissue samples from patients with detailed clinical information and found, similarly to Mavridis et al., that miR-224 reduced expression was associated with high GS (GS<8 vs. GS≥8) and PCa metastasis (n=23) (Lin et al., 2014).

Spahn and colleagues found miR-221 expression to progressively reduce with increasing GS, advanced tumor stage and clinical progression; they reported greater downregulation in GS≥8 (n=53) vs. GS≤7 (n=39) and in pT4 (n=17) vs. pT2/T3 (n=75) prostate cancer samples (Spahn et al., 2010).

Wach and colleagues evaluated two sets of prostate cancer specimens for miR-375, miR-200c, miR-143 and miR-145; set 1 was mainly characterized by GS7 tumors (82%) while set 2 included a high proportion of GS 8/9 tumors (46%) (Wach et al., 2012). The authors found an inverse correlation between the expression of miR-143 and the primary Gleason component of the tumors, with higher grade tumors expressing less miR-143; however, for miR-145, they did not observe any similar correlation with Gleason sum or Gleason components (Wach et al., 2012). No correlation with pathologic stage was found for miR-375, miR-200c, miR-143 and miR-145 (Wach et al., 2012). In a study by Avgeris et al. conducted on prostate tissue from 73 RP specimens with PCa [GS ≤6 (n=24), GS7 (n=39), GS≥8 (n=10); pT2 (n=45), pT3 (n=28)], the reduction of miR-145 expression in prostate cancer was correlated with higher GS, advanced clinical stage, larger tumor diameter and higher PSA and follow-up PSA levels (Avgeris et al., 2013). The estimation of miR-375 by sequencing (on 20 biorepository samples), northern blot and RT-PCR (both done on 40 cryopreserved samples from RP PCa) showed an increase from normal to organ-confined tumors (pT2, pN0) and a slight decrease in prostate tumors with extraprostatic extension (pT3, pN0); miR-375 was then expressed again at higher levels in lymph node metastasizing tumors (pN1) (Hart et al., 2014).

In a study including RP specimens from 79 patients with prostate cancer [GS≤6 (n=27), GS7 (n=34), GS≥8 (n=18); pT2 (n=54), pT3 (n=25)], Schaefer et al. observed a significant correlation with GS for miR-31, miR-96 and miR-205, and with tumor stage for miR-125b, miR-205 and miR-222 (Schaefer et al., 2010) (Table 2). Lin and colleagues found a significant downregulation of miR-31 with increasing GS in prostate biopsy cores taken from desired regions of frozen tissue blocks; moreover, the DNA methylation level at the miR-31 promoter showed significant differences among GS6 (n=11), GS≥7 (n=27), and metastatic prostate cancer and was inversely correlated with miR-31 expression levels. The authors concluded that both promoter hypermethylation and downregulation of miR-31 could serve as indicators for aggressive behaviors in prostate cancer (Lin et al., 2013).

Table 2. miRNAs deregulated in prostate cancer sorted by Gleason score, pathological stage, lymph node status and distant metastasis.

Study	miRNA downregulated with increasing GS	miRNA upregulated with increasing GS	miRNA downregulated with increasing T stage	miRNA upregulated with increasing T stage	miRNA downregulated in lymph node positive patients
Walter et al., 2013	miR-27; miR-92; miR-96; miR-125; miR-126; miR-148; miR-222	miR-9; miR-34; miR-122; miR-138; miR-144; miR-184; miR-193; miR-198; miR-215; miR-335; miR-373			
Xiong et al., 2013	miR-335		miR-335		
Mavridis et al., 2013	miR-224				
Lin et al., 2014	miR-224				
Spahn et al., 2010	miR-221		miR-221		
Wach et al., 2012	miR-143				
Avgeris et al., 2013	miR-145		miR-145		
Hart et al., 2014			miR-375		miR-375 (primary PCa of node positive patients)
Schaefer et al., 2010	miR-31; miR-205	miR-96	miR-125b; miR-205; miR-222		
Lin et al., 2013	miR-31				
Jiao et al., 2014		miR-663		miR-663	
Formosa et al., 2013	miR-132		miR-132		
Chiyomaru et al., 2013	miR-574-3p		miR-574-3p		
Casanova-Salas et al., 2014	miR-187		miR-187		
Gandellini et al., 2009					miR-205 (primary PCa of node positive patients)
Kalogirou et al., 2013					miR-205

Results on miR-663 expression by ISH in 127 patients with prostate cancer [GS≤6 (n=22), GS7 (n=78), GS≥8 (n=27); pT2 (n=78), pT3 (n=31), pT4 (n=18)] and 10 non-neoplastic controls showed significant difference in staining; miR-663 intensity correlated significantly with increasing GS and tumor stage, and was an independent predictor of clinical recurrence (Jiao et al., 2014).

A dataset analysis of 98 primary prostate tumors revealed negative correlation of miR-132 levels with GS, although the mean miR-132 expression of GS≤7 versus GS≥8 had a decreasing trend. High tumor stage was significantly associated with lower miR-132 expression (Formosa et al., 2013). Methylation analysis of CpG islands upstream of miR-132 in a panel of 50 human prostate carcinomas revealed miR-132 to be methylated in 42% of cases in a manner positively correlated to GS and tumor stage, supporting the previous findings (Formosa et al., 2013).

Chiyomaru and colleagues evaluated the expression levels of miR-574-3p in PCa cases (n=48) [GS6 (n=273), GS7 (n=15), GS≥8 (n=9), GS unknown (n=1); pT2 (n=31), pT3 (n=9), pT unknown (n=8)] and found that low expression level of miR-574-3p was correlated with advanced tumor stage and higher Gleason score (Chiyomaru et al., 2013).

Since miR-182 and miR-187 were reported as the most differentially expressed between prostate tumor and non-neoplastic tissue (Table 1), Casanova-Salas et al. selected these miRNAs for further validation in an independent cohort of 273 FFPE RP cases [GS≤6 (n=107), GS7 (n=134), GS≥8 (n=32); pT2 (n=136), pT3 (n=136)]. MiR-187 expression by quantitative RT-PCR inversely correlated with pathologic stage (reduced in pT3 compared to pT2, p=0.0002) and Gleason score (reduced in GS≥7 compared to GS<7, p=0.003) (Casanova-Salas et al., 2014) (Table 2).

Gandellini et al. found greater downregulation of miR-205 in lymph node positive (n=5) compared to lymph node negative (n=25) PCa patients subjected to radical prostatectomy (Gandellini et al., 2009). Subsequently, the same group studied miR-205 expression pattern in non-neoplastic prostate tissue (n=28), primary tumors (n=96), and metastatic lesions (n=12) from Taylor's dataset (Taylor et al., 2010) and found that miR-205 expression was significantly lower in primary tumors than in non-neoplastic prostate tissue and further reduced in metastatic lesions (Gandellini et al., 2014). Similarly, Kalogirou and colleagues detected lower miR-205 expression in lymph node metastases (n=11) compared to high-risk primary prostate tumors (n=105) from FFPE RP specimens; however primary tumor pathological stage, GS and nodal status at surgery could not be stratified by miR-205 expression (Kalogirou et al., 2013).

miR-1 is encoded by the miR-1-133 cluster which has two copies in the human genome producing identical mature miR sequences for miR-1 and miR-133. Consistent with previous findings, Hudson and colleagues found both miR-1 and miR-133 to be significantly reduced in primary prostate tumors (n=99) compared to non-neoplastic prostate tissue; additional

Table 3. Prognostic significance of miRNA expression in prostate cancer.

Study	miRNA downregulated	miRNA upregulated	BCF	Clinical Progression	DOD/OS
Schaefer et al., 2010		miR-96	Increased and earlier (PSA>0.1ng/ml)		
Avgeris et al., 2013	miR-145		Increased and earlier		
Spahn et al., 2010	miR-221		Increased	Increased and earlier local recurrence and distant metastasis	
Kneitz et al., 2014	miR-221				Increased and earlier DOD in high risk PCa
Amankwah et al., 2013	miR-21		Increased and earlier	Increased and earlier metastasis	Increased and earlier DOD
Casanova-Salas et al., 2014		miR-182	Earlier	Earlier local recurrence, lymph node and distant metastasis	
Hirata et al., 2013		miRNA-182-5p			Reduced OS
Majid et al., 2013	miR-34b		Increased and earlier		Reduced OS
Lin et al., 2014	miR-224		Earlier	Increased distant metastasis	
Mavridis et al., 2013	miR-224		Increased and earlier		
Schubert et al., 2013	let-7b		Increased and earlier	Increased local recurrence and distant metastasis	
Lichner et al., 2013	miR-331-3p	miR-29c	Late ≥36 months		
	miR-152		Early ≤36 months		
Leite et al., 2011		miR-100	Increased and earlier		
Jiao et al., 2014		miR-663	Increased and earlier		
Formosa et al., 2013	miR-132		Earlier	Increased lymph node and distant metastasis	

PCa: prostate cancer; BCF: biochemical failure; DOD: death of disease; OS: overall survival.

reduction in miR-1-133a cluster expression (40-fold) was noted in distant metastatic lesions (n=14) when compared with primary tumors (Hudson et al., 2012).

miRNA expression in relation to prostate cancer prognosis

A number of studies have been conducted to test whether the different expression of various miRNAs in PCa tissues by RT-PCR might have any prognostic value, particularly in predicting biochemical failure (BCF) or progression/disease free survival (PFS). Results of ISH analysis are included when available. The studies discussed in this section are summarized in Table 3. The most significant miRNAs associated with BCF and metastases are depicted in Fig. 1. In most studies BCF has been defined as the rise in PSA ≥0.2 ng/mL on 2 successive follow-up visits; in publications where the criteria differ, the parameters used by the authors have been mentioned in parenthesis.

In a disease recurrence analysis including 75 prostate cancer patients with median follow-up of 50 month (range 1-93), Schaefer et al. reported a significantly decreased recurrence-free interval in patients with high miR-96 expression in their prostate tumor, although not independent of GS (Schaefer et al., 2010). Time of recurrence was defined as the first postoperative PSA value >0.1 μg/l confirmed by at least 1 subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 μg/l) after surgery. When the prognostic impact of miR-96 was validated in a second cohort of 79 patients with median follow-up of 50 month (range 1-99), the authors found that cases with higher miR-96 expression had again significantly higher risk for biochemical recurrence (p=0.039), confirming the prognostic significance of miR-96 (Schaefer et al., 2010). Similarly, Haflidadottir et al. found that miR-96 upregulation was associated with reduced overall survival by up to 1.5 years in a study on prostate cancer detected on TURP, where 13 patients with median survival of 3 years had higher miR-96 than 36 patients with median survival of 4.5 years (Haflidadottir et al., 2013).

A lower miR-145 expression in prostate cancer was found to be related to higher risk for BCF in a study by Avgeris et al. on 73 RP patients. Follow-up ranged from 0 to 74 months in 62 patients with available information. BCF occurred in 75% high and very-high risk, 54% intermediate risk, and 54% low and intermediate risk patients with lower miR-145 expression; as opposed to 70% high and very-high risk, 22% intermediate risk, 28% low and intermediate risk patients with higher miR-145 expression. Underexpression of miR-145 predicted short disease free survival independently of other prognostic factors like GS, clinical stage and serum PSA levels (Avgeris et al., 2013). Chen et al. studied ISH expression of miR-145 and IHC expression of its target BNIP3 on 134 prostate cancer cases (121 biopsies, 14 TURP) and concluded that miR-145 expression is an independent favorable prognostic factor for disease specific and progression free survival (Chen et al., 2010).

Spahn et al. in their study on 92 high risk RP cases found miR-221 downregulation to be associated with BCF and clinical recurrence (local recurrence, distant metastasis) (Spahn et al., 2010). The independent prognostic value for miR-221 was supported by Kneitz and colleagues in a study on 2 groups of high risk PCa patients treated by RP: the study cohort included 134 cases with a median follow-up of 76 months (range 1-154); the validation cohort included 89 patients with a median follow-up of 108 months (range 1-200). Lower tumor miR-221 expression was significantly associated with early cancer specific death (Kneitz et al., 2014). The AUC of miR-221 to predict cancer specific death in the study group was 0.903. After implementation of a cut-off value (ΔCt miR-221 of -0.32) derived from the AUC analysis on the validation group, miR-221 expression correctly classified 97% of patients at low risk for death while identifying 50% of those at high risk for cancer related death (Kneitz et al., 2014).

On the other hand, Kang and colleagues found no significant correlation among miR-96, miR-145 and miR-221 expression and risk of BCF in 73 men treated by radical prostatectomy for prostate cancer with a mean follow-up duration of 19.4 months (+/- 9.8), during which BCF was detected in 19% of patients (Kang et al., 2012). In a study including 65 aggressive prostate tumors (GS≥7 or pT≥3) treated by RP [28 recurrent with median follow-up of 50.9 months (range 2.7-114.5) and 37 non recurrent with median follow-up of 95.1 months (range 9.1-254.3)]. Amankwah et al. concurred with the lack of prognostic value for miR-221/222 in predicting recurrence (defined as rise in PSA ≥0.2ng/mL post-RP or clinical metastasis or death by disease) (Amankwah et al., 2013). However, the authors found that the expression of miR-21 was an independent risk factor for recurrence after radical prostatectomy in obese (BMI>30kg/m^2), but not in non-obese men (Amankwah et al., 2013).

Casanova-Salas et al. found an independent negative prognostic value of miR-182 expression in a study on 273 RP cases with a median follow-up of 92 months (range 2-189) wherein higher miR-182 predicted a worse BCF (>0.4 ng/mL) free survival and worse progression free survival (local recurrence, lymph node and distant metastasis) (Casanova-Salas et al., 2014). Combination of miR-182 with GS was found prognostic for BCF free survival, but not for progression free survival independently of pre-surgery PSA, pathologic stage, margin status, and lymph node positivity (Casanova-Salas et al., 2014). Hirata et al. divided 52 RP cases in two groups based on miR-182-5p expression and found that the miR-182-5p high expression group (n=20) showed significantly lower overall survival compared to the low expression group (n=32) (Hirata et al., 2013).

In a study including 49 TURP specimen with prostate cancer, Hagman and colleagues found lower

miR-205 expression to be related to reduced overall survival (median survival of 2 years vs. 3 years) and metastases (Hagman et al., 2013). On the other hand, Kalogirou et al. did not find any prognostic significance of miR-205 for either BCF or death of disease in 105 high risk PCa cases with a follow-up ranging from 0 to 150 months (Kalogirou et al., 2013).

Majid and co-authors analyzed 94 RP cases with follow-up of 0-160 months and reported that patients with increased miR-34b tumor expression had higher overall survival and low miR-34b expression was associated with higher BCF (defined as single rise in PSA >0.1ng/mL) and a poor BCF free survival (Majid et al., 2013).

Hudson et al. found that lower miR-1 expression independently predicted early recurrence in Taylor's dataset of 99 prostate tumors (Taylor et al., 2010; Hudson et al., 2012). Subsequently, using the same dataset, the authors found that miR-106b-25 expression above median was associated with early BCF and predicted BCF with a hazard ratio of 2.7 (Hudson et al., 2013).

Lin et al. studied miR-224 expression by ISH on 114 PCa included in a TMA obtained from a commercial source and found that high miR-224 expression was associated with longer BCF free survival and miR-224 was an independent prognostic factor for BCF free survival (Lin et al., 2014). Similar findings were reported by Mavridis and colleagues on frozen tissue from 58 RP patients, where tumors with higher miR-224 expression had longer progression free survival. Although miR-224 was associated with good prognosis it was not independent of GS, tumor stage and PSA (Mavridis et al., 2013).

Schubert et al. evaluated two separate cohorts of patients with high risk PCa - a study cohort including 98 RP and a validation cohort with 92 RP specimens - to find that reduced let-7b and let-7c expression in the study cohort correlated with BCF and clinical failure (defined as local recurrence and metastasis). Reduced let-7b and let-7c expression was found to be significant on univariate but not on multivariate analysis. In the validation cohort, let-7b downregulation was an independent prognostic factor for BCF and clinical failure (Schubert et al., 2013).

In a screening set including 41 RP cases, Lichner and colleagues found 130/754 miRNAs differentially expressed between high-risk (27 men with BCF <36 months post-RP) and low-risk (14 men with BCF ≥36 months post-RP) patients (Lichner et al., 2013). Although no single miRNA could distinguish high-risk from low-risk patients, different sets of miRNA could identify high-risk patients: miR-331-3p and miR-152 with an AUC of 0.93; miR-331-3p, miR-152 and miR-135a with an AUC of 0.92; miR-148a and miR-429 classified 100% of high-risk cases correctly, despite an AUC of 0.78. The authors then validated these miRNAs in a set of 64 RP cases (35 high-risk and 29 low-risk) and confirmed that reduced miR-331-3p and increased miR-29c were individually associated with low-risk cases and miR-152 was reduced in the high-risk group. In the validation set, miR-152 alone could predict BCF and the combination of miR-331-3p with miR-152 improved its prediction (Lichner et al., 2013).

Leite and colleagues studied 49 RP patients by selecting 21 with BCF, 28 without BCF in a mean follow-up of 58.8 months and found that increased miR-100 expression predicted BCF and was independent of tumor volume (Leite et al., 2011b).

Jiao and colleagues found that miR-663 upregulation in prostate tumor is an independent poor prognostic factor for BCF; they subdivided 127 RP cases in three groups based on miR-663 intensity of expression (mild, moderate, strong) by ISH and reported significant difference in BCF free survival in the three groups, with higher miR-663 intensity been associated with lower BCF free survival (Jiao et al., 2014).

Formosa et al. divided 98 PCa patients in three groups based on miR-132 expression (low, intermediate, high) and found significantly reduced time to BCF in the low miR-132 expression group compared to the other 2 groups (Formosa et al., 2013).

miRNA expression in castration resistant prostate cancer (CRPC)

Prostate cancer is generally hormonally sensitive. Up to 40% of patients diagnosed with PCa develop metastatic disease that generally responds to initial androgen deprivation therapy (ADT) (chemical or surgical castration). Initially, more than 80% of patients with androgen-sensitive PCa respond favorably to ADT;

Table 4. Changes in miRNA in CRPC compared to hormone sensitive PCa.

Study	Cases	miRNA downregulated	miRNA upregulated
Leite et al., 2011	4 CRPC; 18 high-grade, T3, PCa	miR-let7c; miR-100; miR-218	
Xu et al., 2012	5 CRPC; 15 PCa	miR-146a	
Sun et al., 2012	17 metastatic CRPC; 34 PCa	miR-23b/27b	miR-221/222
Lin et al., 2013	5 CRPC; 38 PCa	miR-31	
Hagman et al., 2013	14 CRPC; 22 PCa	miR-205	
Liu et al., 2014	9 CRPC; 19 PCa	miR-361-5p	

CRPC: castration resistant prostate cancer, PCa: prostate cancer.

however, most tumors relapse within two years. It is not uncommon for PCa to become hormone refractory and eventually progress to castration-resistant prostate cancer (CRPC) with increased invasion, proliferation, and malignancy after which the disease shows poor response to any anticancer therapy and progresses to the lethal stage. CRPC proliferates despite castrate levels of serum testosterone and is a leading cause of cancer-related death in elder men. Early detection of developing hormone resistance during treatment or estimation of effective adjuvant treatment modality at diagnosis would significantly reduce morbidity and mortality in PCa. Since the pathogenesis of CRPC is likely to be regulated by miRNAs, many investigators have studied miRNAs in relation to CRPC and prostate cancer metastasis on cell lines and clinical tissue samples, in search for potential clinical applications. We have noted here and summarized in Table 4 RT-PCR tissue based studies of relevance on miRNAs expression in CRPC.

Leite et al. reported a significant overexpression of miR-let7c, miR-100, and miR-218 in localized high-grade (mean Gleason score 8.6) high-stage (all stage T3) prostate cancer (n=18) in comparison to metastatic CRPC (n=4), and hypothesized that the above mentioned miRNAs may be involved in the process of prostate cancer metastasis (Leite et al., 2011a).

Xu et al. found that miR-146a expression was significantly decreased in five patients with stage IV CRPC compared to fifteen hormone-sensitive prostate cancers (Xu et al., 2012).

Sun and colleagues reported a significant upregulation of miR-221 and miR-222 expression and a significant downregulation of miR-23b and miR-27b in bone metastases of CRPC (n=17) compared to hormone-sensitive PCa (n=34) (Sun et al., 2012). When the authors compared the expression patterns of miR-221/-222 and miR-23b/-27b in individual metastatic CRPC samples relative to their corresponding mean expression level in primary hormone-sensitive tumors they found that more than 90% of CRPC samples examined had both up-regulation of miR-221/-222 (16 of 17) and downregulation of miR-23b/-27b (15 of 17), compared to hormone-sensitive primary tumors (Sun et al., 2012). This finding suggests a potential role for miR-221/-222 and miR-23b/-27b as markers or predictors for prostate cancer progression to CRPC.

Androgen receptor signaling plays a critical role in prostate cancer pathogenesis and persists even during

Table 5. miRNAs deregulated in blood of patients with PCa.

Study	Sample	Compared to healthy controls		Compared to localized PCa		Associated with worse prognosis	
		miRNA downregulated	miRNA upregulated	miRNA downregulated	miRNA upregulated	miRNA downregulated	miRNA upregulated
Mitchell et al., 2008	25 mPCa		miR-141				
Zhang et al., 2013	30 mPCa 20 localized PCa				miR-141		miR-141[1]
Yaman Agaoglu et al., 2011	51 PCa (25 mPCa)		miR-21 miR-221		miR-21; miR-221 miR-141		
Gonzales et al., 2011	21 PCa						miR-141[2]
Brase et al., 2011	Set 1: 45 PCa Set 2: 71 PCa						miR-141[3] miR-375[3]
Bryant et al., 2012	Set 1: 51 PCa Set 2: 47 mPCa; 72 localized PCa		miR-107 miR-574-3p		miR-141 miR-375		
Nguyen et al., 2013	26 mCRPC 28 localized PCa			miR-409-3p	miR-141; miR-375 miR-378*		
Cheng et al., 2013	25 mCRPC		miR-141; miR-375 miR-200a; miR-200c; miR-210; miR-2104				
Huang et al., 2015	Set 1: 23 CRPC Set 2: 110 CRPC						miR-375[5] miR-1290[5]
Chen et al., 2012	Set 1: 25 PCa; 17 BPH Set 2: 80 PCa	let-7e; let-7c miR-30c	miR-622 miR-1285				
Srivastava et al., 2014	40 PCa	miR-628-5p miR-101*					
Santos et al., 2014	45 PCa						miR-7[6] miR-221[6]

PCa: prostate cancer; mPCa: metastatic prostate cancer; CRPC: castration resistant prostate cancer; mCRPC: metastatic castration resistant prostate cancer; BPH: benign prostatic hyperplasia; [1]: Correlated with Gleason Score and number of bone lesions, [2]: Predicted clinical progression outcomes, [3]: Associated with high risk-PCa and positive lymph nodes, [4]: Levels changed with treatment response correlating with PSA changes, [5]: Poor overall survival in CRPC, [6]: Associated with early CRPC.

stringent androgen deprivation therapy. A recent study by Lin and colleagues suggested a complex interaction between the expression of miR-31 and androgen receptor signaling. The authors examined 38 primary prostate cancers with Gleason scores ranging from 6 to 9, and 5 metastatic CRPC cases, and found that miR-31 expression was reduced as a result of promoter hypermethylation. DNA methylation at the miR-31 promoter was positively correlated with prostate cancer progression (Lin et al., 2013).

Hagman and colleagues found that CRPC (n=14) had lower expression levels of miR-205 compared to hormone naïve PCa cases (n=22) and indicated that miR-205 negatively regulates the androgen receptor, and is associated with adverse outcome of prostate cancer patients (Hagman et al., 2013).

Liu and colleagues studied prostate cancer tissue from 19 hormone-sensitive patients who underwent radical prostatectomy for organ-confined PCa and 9 patients on maximum androgen deprivation therapy with stage T4 CRPC who underwent TURP for urinary retention and demonstrated that miR-361-5p expression is downregulated in CRPC compared to hormone-sensitive PCa (Liu et al., 2014). From these results the authors suggested that miR-361-5p might play an important role in the progression of hormone-sensitive PCa to CRPC (Liu et al., 2014).

miRNA detection in biofluids

Biofluids include blood, serum, plasma, urine, CSF, breast milk, saliva, semen, tears etc. These are a heterogeneous mixture of cells shed from organs, tumor cells shed from tumor, and cells that are components of blood. These fluids can be studied as whole, supernatant, cell pellet and circulating tumor cells (CTC). Secreted fluids like breast milk, semen and saliva were found to contain substantially higher miRNA concentration than other biofluids (Weber et al., 2010).

miRNA may entry into biofluids following two distinct mechanisms: 1- passive leakage into biofluids from broken cells after tissue damage or apoptosis; 2- active secretion either through exosomes (microvesicles 50-150 nm in diameter) or bound to proteins or lipids. (Mlcochova et al., 2014). miRNAs are mostly studied in serum and plasma. Although plasma contains a substantial amount of nucleases that make it impossible for free RNA to be present, stable miRNA are detectable in biofluids (Weber et al., 2010). miRNAs are found to be stable in human plasma at room temperature and after freeze thaw cycles (Mitchell et al., 2008). The reasons for miRNA stability include exosomes (Gallo et al., 2012; Vlassov et al., 2012; Huang et al., 2013), complexes with Ago-1 and Ago-2 (Arroyo et al., 2011; Rayner and Hennessy, 2013), transport with HDL (Vickers et al., 2011) and chemical modification like methylation/adenylation/uridylation (Mlcochova et al., 2014).

Exosomes have been found in most body fluids - like CSF, urine, plasma and serum - and perform the function of transferring miRNAs between cells (Stoorvogel, 2012). The content of exosomes seems to represent the tissue of origin (Gonzalez-Begne et al., 2009). Exosomes can carry an estimated 10000 nucleotides equivalent of 500 miRNAs (Vlassov et al., 2012). The serum or plasma has been shown to contain $0.88x10^8$ to $13.38x10^8$ exosomes/ml, and RNA sequencing has revealed about 600 miRNAs in plasma exosomes (approximately 26% of the total listed in miRBase) (Huang et al., 2013).

Since changes in miRNA levels occur in serum and plasma of PCa patients, it is reasonable to speculate that some of these changes may also occur in urine. Due to the unique location of the prostate, urine is a biofluid of choice that contains prostatic secretions and cells shed from the prostate. Urine contains the least concentration of miRNA compared to other biofluids (Weber et al., 2010), and the detection of miRNAs in urine depends on the number and type of cells shed from the urinary tract. Exosomes have been shown to pass the glomerular barrier carrying miRNA into urine; stability of miRNA in urine after 72 hours at room temperature and freeze-thaw cycles, and resistance to proteolytic activity has been shown (Zhou et al., 2006; Cheng et al., 2012; Yun et al., 2012; Hessels and Schalken, 2013). The lesser protein content of urine compared to blood/plasma is a benefit in reducing interference in isolation of miRNA (Mlcochova et al., 2014).

The detection of miRNAs in body fluids represents a promising non-invasive diagnostic utility for PCa. Changes in circulating miRNAs may represent potentially useful biomarkers for the diagnosis, staging

Table 6. MiRNA deregulated in urine of prostate cancer patients.

Study	Cases	miRNA downregulated	miRNA upregulated
Bryant et al., 2012	118 PCa; 17 NN		miR-107; miR-574-3p
Srivastava et al., 2013	36 PCa; 12 NN	miR-205; miR-214	
Haj-Ahmad et al., 2014	8 PCa; 22 NN	miR-484	miR-1825
Guzel et al., 2015	23 PCa; 25 NN	miR-361-3p; miR-133b; miR-221	miR-203

PCa: prostate cancer patients; NN: healthy controls.

and prediction of outcome in prostate cancer.

miRNA detection in serum/plasma of prostate cancer patients

In this section we discuss studies analyzing miRNAs by RT-PCR in blood based samples. A summary of this section in presented in Table 5.

Independent studies have shown that there are two promising miRNAs in the blood circulation, miR-141 and miR-375. Serum miR-141 was found significantly increased in patients with metastatic PCa (n=25) compared with age matched healthy controls (n=25) and could detect metastatic PCa with 60% sensitivity and 100% specificity (Mitchell et al., 2008). Zhang et al. detected higher serum levels of miR-141 in PCa patients with bone metastases (n=30) compared to those without (n=20) and reported that miR-141 serum levels correlated with Gleason score and number of bone lesions (Zhang et al., 2013).

Yaman Agaoglu and colleagues found that an elevated plasma level of miR-21 and miR-221 could distinguish PCa (n=51) from non-neoplastic controls (n=20); in addition miR-21, miR-221, and miR-141 were higher in plasma of men with metastatic disease (n=25) compared to localized PCa (n=26) and miR-141 elevation was the most pronounced with an AUC of 75.5% (Yaman Agaoglu et al., 2011). Gonzales et al. related that plasma miR-141 predicted clinical progression outcomes on periodic follow-up with an odds ratio of 8.3 in 21 PCa patients (Gonzales et al., 2011). Brase et al. reported that increased serum levels of miR-375 and miR-141 were associated with high risk PCa in 45 RP patients (Brase et al., 2011). In a second validation set including 71 patients, elevated serum levels of miR-375 and miR-141 were associated with higher Gleason score (GS≥8 compared to GS≤7) and positive lymph node status (Brase et al., 2011).

Bryant et al. analyzed 742 miRNA using plasma-derived circulating microvesicles of 78 PCa patients and 28 normal control individuals and found that 12 miRNA were differentially quantified in PCa patients, including 9 in patients without metastases, compared with controls (Bryant et al., 2012). Elevated plasma levels of miR-141 and miR-375 could discriminate men with metastatic disease (n=47) from men without metastases (n=72) following radical prostatectomy. MiR-107 and miR-574-3p were increased in plasma of men with non-metastatic PCa (n=51) compared with normal control individuals (n=28) (Bryant et al., 2012).

Nguyen and colleagues reported significant overexpression of miR-141, miR-375, miR-378* and lower levels of miR-409-3p in the serum of patients with metastatic CRPC (n=26) compared to low-risk localized PCa (n=28) (Nguyen et al., 2013). Cheng and colleagues profiled 365 miRNAs in serum and found miR-141, miR-375, miR-200a, miR-200c, and miR-210 elevated in metastatic CRPC patients (n=25) compared to healthy controls (n=25) (Cheng et al., 2013). The levels of miR-210 changed with treatment response, being lower in responsive patients, and directly correlated with changes in PSA (Cheng et al., 2013).

In a recent study, Haldrup et al. confirmed the de-regulation of miR-141 and miR-375 in serum obtained from patients with localized PCa on RP (n=11), lymph node or distant metastasis (n=9), CRPC after neoadjuvant hormonal therapy (n=11), and BPH control patients (n=13) (Haldrup et al., 2014). In addition they identified several new potential serum miRNA markers for PCa and developed three novel and highly specific miRNA panels, the most promising ones being miR-562/miR-210/miR-501-3p/miR-375/miR-551b able to identify 84% of all PCa patients and miR-375/miR-708/miR-1203/miR-200a able to identify 75% of patients with disseminated PCa when compared to localized PCa patients (Haldrup et al., 2014).

Westermann et al. analyzed 133 patients (54 with PCa; 79 non-malignant) and observed that, despite a significant increase of miR-141 in patients with higher GS, the serum levels of miR-141 and miR-26a-1 were similar in patients with positive and negative prostate biopsies (Westermann et al., 2014).

Lately, Huang and colleagues analyzed plasma exosomal miRNA by RNA sequencing on a screening sample of 23 CRPC patients (12 treated with RP, 3 with radiation alone, 2 with RP and radiation, 6 with salvage local treatments after RP) with a mean follow-up of 35.6 months (range 7-47) and found miR-375, miR-1290 and miR-1246 to be associated with overall survival; a validation by RT-PCR on 110 CRPC patients (49 treated with RP, 25 with radiation alone, 8 with RP and radiation, 28 with salvage local treatments after RP) with a mean follow-up of 17.58 months (range 1-48) found increased levels of exosomal miR-375 and miR-1290 to be related to poor overall survival in CRPC (Huang et al., 2015).

Chen et al. investigated whether circulating miRNAs could be used in the diagnosis of prostate cancer in Chinese patients. The authors validated candidates circulating miRNAs selected in a small set of patients [25 PCa and 17 BPH] in a larger independent cohort including 80 PCa patients, 44 BPH, and 54 healthy controls. (Chen et al., 2012). The plasma level of five miRNAs with differential expression [let-7e, let-7c, and miR-30c were downregulated and miR-622 and miR-1285 were upregulated in PCa patients] could accurately discriminate PCa from BPH and PCa from healthy controls (Chen et al., 2012).

Srivastava and colleagues measured serum miRNAs in two cohorts of prostate cancer patients including 36 African American (24 PCa patients and 12 controls) and 36 Caucasian (16 PCa patients and 20 controls) men and found that serum expression levels of miR-628-5p were significantly downregulated in both African American and Caucasian PCa patients when compared with their respective controls, whilst miR-101 was downregulated in Caucasian PCa cases compared to controls (Srivastava et al., 2014).

Wang et al. studied low-risk PCa cases as per UCSF criteria (biopsy GS≤6, <34% cores positive for PCa, <50% single core involvement, PSA≤10ng/mL, clinical stage ≤T2) and reported that pre-surgical serum levels of miR-19, miR-345 and miR-519c-5p could predict surgical GS7 with an AUC of 0.94 (Wang et al., 2014).

Santos et al. studied whole blood miRNA in 45 PCa patients and found miR-7 and miR-221 to be higher in early CRPC (≤20 months); miR-7 seemed particularly associated with earlier time to castration resistance (Santos et al., 2014).

miRNA detection in urine of prostate cancer patients

The limited number of studies on miRNA analysis in urine holds promise that they can be used for urine-based assays. The most interesting results are discussed in this section and summarized in Table 6. The analysis of miRNAs in these studies was conducted by RT-PCR.

An analysis of five selected miRs in urine samples (miR-107, miR-574-3p, miR375, miR-200b and miR-141) found that miR-107 and miR-574-3p were quantified at significantly higher concentrations in cell pellets in the urine of men with prostate cancer (70 localized PCa, 48 advanced PCa) compared with controls (n=17), with miR-107 being 67% sensitive and 43% specific (Bryant et al., 2012).

miR-205, mir-214 were found be downregulated in urine of PCa patients (n=36) compared to ethnically matched healthy individuals (n=12) and together had a sensitivity of 89% and specificity of 80% to distinguish PCa (Srivastava et al., 2013).

Haj-Ahmad and colleagues carried out miRNA expression profiling in urine samples from 8 PCa patients, 12 BPH patients and 10 healthy males and found differential expression of two individual miRNA: miR-1825 was up-regulated in seven of eight PCa samples and it was 60% sensitive and 69% specific to determine PCa; downregulation of miR-484 was found to be 80% sensitive and 19% specific for PCa (Haj-Ahmad et al., 2014).

A panel of miRNAs was profiled in the urine of 33 PCa patients (16 high-risk and 17 low-risk) collected prior to radical prostatectomy and miRNA expression was correlated at a follow-up time of 3.9 years to identify miRNA that could predict clinical response after surgery (Sapre et al., 2014). The combination of miR-16, miR-21 and miR-222, detected at higher levels in the high-risk group, could predict high-risk PCa with an AUC of 0.75. However, in the validation cohort including 36 independent PCa patients (22 high-risk and 14 low-risk) the three miRNAs were surprisingly detected at lower levels in the high-risk group, compared to the low-risk group (Sapre et al., 2014). The authors stressed the need for independent validation cohorts and suggested that urinary microRNA signatures at radical prostatectomy may not be a robust way to predict the course of clinical disease after definitive treatment for PCa.

Guzel et al. studied prostatic secretion samples obtained through prostatic massage in 23 PCa and 25 BPH patients and reported higher levels of miR-203 and lower levels of miR-361-3p, miR-133b, miR-221 in PCa patients compared to BPH patients. (Guzel et al., 2015). MiR-203 (AUC of 0.805), miR-361-5p (AUC of 0.735), miR-133b (AUC of 0.726) and miR-221 (AUC of 0.706) were found to have individually good discriminating ability to identify PCa; all four miRNAs used together resulted in an AUC of 0.95 (Guzel et al., 2015).

Therapeutic importance and upcoming developments

The therapeutic modalities used for treatment of PCa range from active surveillance for very low-risk PCa, radical prostatectomy, radiation therapy and cryotherapy for localized PCa, hormonal therapy for metastatic PCa, and chemotherapy for high-risk PCa. However, it is challenging to determine the perfect line of treatment based on PCa characteristics at diagnosis. The efficacy of each of the treatment modalities used alone and in combination is difficult to monitor on the basis of PSA alone, as with a rising PSA it is difficult to determine recurrence, metastasis or a benign cause. Getting an estimate of sensitivity to hormonal therapy or individual chemotherapeutic agents is an important unmet need in PCa therapy. Extensive work is being done to find out whether molecular techniques like miRNA can be of use to better determine and monitor treatment in PCa. We have compiled some relevant studies in this section. Most of the miRNA tests for therapeutic use have been done on cell lines *in vitro* and *in vivo* on animal models. This section focuses mainly on *in vivo* experiments as they are more relevant and the next step to therapeutic use from *in vitro* studies.

Pre-clinical studies have shown that indole-3-carbinol found in cruciferous vegetables and its *in vivo* metabolite 3,3-diindolylmethane (DIM), especially the formulated DIM (BR-DIM or B-DIM), are potent agents in inhibiting the growth of PCa cells (Heath et al., 2010). Treatment of PCa cell lines with BR-DIM resulted in increased let-7 and reduced enhancer of Zeste homolog 2 (EZH2), causing inhibition of clonogenic process and renewal. Consistent findings were found in a phase II clinical trial where BR-DIM given prior to RP resulted in upregulated let-7 and reduced EZH2, leading authors to believe that BR-DIM has the capacity to reduce PCa aggressiveness by reducing EZH2 and increasing let-7 (Kong et al., 2012). A xenograft study has shown that let-7a mimics inhibit *in vivo* tumor development, and cell line experiments have revealed that let-7a downregulated E2F2 and CCND2 (Dong et al., 2010).

A prostate cancer xenograft model revealed that tail vein injection of atelocollagen in mice could efficiently deliver synthetic miR-16 to tumor cells in bone tissues resulting in significant inhibition of prostate tumor growth in bone. Studies on cell lines have suggested that miR-16 likely suppresses prostate tumor growth by

regulating *CDK1* and *CDK2* gene expression. The systemic delivery of miR-16 could be used to treat patients with advanced prostate cancer (Takeshita et al., 2010).

miR-1 has been found to be epigenetically silenced in human prostate cancer, and its overexpression has led to growth inhibition and down-regulation of genes in pathways regulating cell cycle progression, mitosis, and DNA replication/repair (Hudson et al., 2012). In xenograft experiments a reduction in tumor volume and number of tumors formed was seen in the group transfected with miR-1/133 cluster inoculated cells compared to the control group (Hudson et al., 2012).

miR-18a knockdown resulted in reduced tumor growth in nude mice *in vivo*, and the mechanism of action was induction of apoptosis by dephosphorylation of AKT through STK4 (Hsu et al., 2014).

A study in cell lines revealed that miR-29b induced expression led to anti-metastatic effects by reducing N-Cadherin, Twist and Snail expression; increasing E-Cadherin expression was shown to destroy the capacity of the cells to form metastatic lesions in mice after intravenous injection compared to control cell lines that could form metastases (Ru et al., 2012). miR-23b was found to reduce migration, invasion and showed an anti-metastatic effect by reduction of mesenchymal markers (Vimentin and Snail) with increase in epithelial markers (E-cadherin) on cells lines (Majid et al., 2012). Intra-tumoral delivery of miR-23b to subcutaneous xenograft tumors in nude mice caused reduction in tumor growth (Majid et al., 2012).

miR-34b induced partial demethylation, chromatin modifications and caused G0/G1 arrest, apoptosis and inhibition of proliferation directly acting on Akt (Majid et al., 2013). miR-34b caused inhibition of EMT in PCa cell lines and reduced growth of tumor in nude mice proving its action as tumor suppressor (Majid et al., 2013).

Src tyrosine kinase/EGF inversely regulates miR-30; in turn miR-30 directly acts on ERG a known molecular aberration in PCa. miR-30 overexpression causes inhibition of invasion, cell migration and EMT in PCa cell lines (Kao et al., 2014). miR-30 overexpression inhibits growth of TMPRSS2-ERG dependent VCaP cells *in vivo* and *in vitro* (Kao et al., 2014). EGF-Src/miR 30/ERG axis could be exploited for targeting by Src inhibitors.

miR-125b was proven to be an oncogene supporting PCa cells proliferation (p53 dependent and independent); it was found to regulate p14ARF/MDM2 signaling in an experiment based on lentivirus induced expression of miR-125b in mice xenograft (Amir et al., 2013).

Electroporation to introduce miR-143 in cancer cell xenografts caused reduced tumor growth in mice. Part of the mechanism of action was shown to be by ERK5 inhibition (Clape et al., 2009). Tibial xenograft of human metastatic PCa cell line in mice when transfected by retrovirus with miR-143 and miR-145 for over-expression of these miRNAs led to reduced bone invasion and lower tumor development (Peng et al., 2011).

miRNA-182-5p knockdown reduced growth of PCa cell lines as well reduced *in vivo* prostate tumor growth in nude mice by increasing expression of potential target tumor suppressor genes FOXF2, RECK and MTSS1 (Hirata et al., 2013).

miR-200b expression inhibited formation of subcutaneous PCa in mice xenograft experiment and was found to be a downstream androgen receptor target (Williams et al., 2013).

miR-205 blocked activation of stromal cancer associated fibroblasts (CAF) by PCa as well as counteracted EMT induced in PCa by CAF; premiR-205 transfected tumor cells formed smaller tumors in nude mice compared to premiR205-negative transfected cells (Gandellini et al., 2009, 2014).

Subcutaneous xenografts of PC3 cell line with a high expression of miR-221 and miR-222 were treated with anti-miR-221/222 leading to reduced tumor growth with an effect lasting even at 25 days (Mercatelli et al., 2008). These miRNAs acted through p27 downregulation (Mercatelli et al., 2008). MiR-222 and miR-31 may act as tumor suppressors as they inhibit proliferation, invasion and migration in PCa cell lines (Lin et al., 2013). Xenograft experiments revealed that upregulation of miR-31 reduced AR expression and reduced growth of prostate cancer (Lin et al., 2013).

Conclusions

Although miRNAs appear very promising for exploring as biomarkers and in therapeutics, the current knowledge about miRNAs has been limited and not sufficient to translate to the stage of clinical trials except for LNA-antimir-122 (SPC3649: miravirsen) that successfully underwent phase II trials in the treatment of HCV infections (Janssen et al., 2013). In order to achieve tumor specific identification (as biomarker) and/or delivery (for therapeutics), further understanding of regulatory mechanisms and mechanism of action is required. The detail knowledge of miRNA in PCa can then be tested and validated for practical use.

Take home message

- miRNA downregulation is more common than upregulation in tissue based studies; on the other hand, miRNA upregulation is more frequently reported than downregulation in blood based studies.
- In tissue based studies, miR-143/145, miR-205, miR-221/222, and miR-224 are the most consistently downregulated miRNAs of significance in distinguishing prostate cancer from non-neoplastic tissue, and tumors with different GS, pathological stage and prognosis.
- In tissue based studies, miR-182 is the most consistently upregulated miRNA of significance in distinguishing prostate cancer from non-neoplastic tissue and tumors with different prognosis.

- In blood based studies, miR-141 and miR-375 are the most consistently upregulated miRNAs of significance in discriminating prostate cancer patients from controls, advanced from localized prostate cancer, and tumors associated with worse prognosis.
- Although no particular miRNAs dominated the results on urine based studies, miR-221 and miR-205 are those resonating with results from tissue based studies.

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Accepted July 17, 2015