Research Article

Stability Assessment of Candidate Reference Genes in Urine Sediment of Prostate Cancer Patients for miRNA Applications

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We aimed at assessing the stability of candidate reference genes in urine sediments of men subjected to digital rectal examination for suspected prostate cancer (PCa). Two microRNAs (miR-191 and miR-25) and 1 small nucleolar RNA (SNORD48) were assayed in 35 post-DRE urine sediments of men with PCa and in 26 subjects with histologically confirmed benign prostatic hyperplasia (BPH). The stability of candidate reference genes was assessed through BestKeeper algorithm and equivalence test. miR-200b and miR-452 were used to test for the effect of normalization on target genes. Our results proved miR-191 to be the most stable gene, showing the lowest degree of variation and the highest stability value. miR-25 and SNORD48 values fell beyond the cutoff of acceptability. In conclusion, we recommend the use of miR-191 for normalization purposes in post-DRE urine sediments.

1. Introduction

Prostate cancer (PCa) represents the second most common male cancer [1]. In Western countries, the main diagnostics tools for PCa diagnosis are PSA monitoring together with digital rectal examination (DRE). Although the introduction of PSA testing in clinical practice has dramatically improved the early diagnosis and consequently the treatment of localized disease, the real benefits of an extensive use of PSA remain controversial [2]. Since PSA is a prostate-specific and not a cancer-specific marker, there is an urgent need for new biomarkers able to diagnose real disease and not benign conditions which also cause an increase in serum PSA.

MicroRNAs (miRNAs) represent a class of small, noncoding RNAs which targets complementary sites of messenger RNA (mRNA) and negatively regulates their expression [3]. In the last years, microRNAs have attracted scientific interest upon demonstration of their alteration in response to various diseases: they have been proved to affect the crucial steps of carcinogenesis acting as oncogenes or oncosuppressors [4]. Real-time PCR is an extremely versatile tool to analyse gene

expression: the selection of stably expressed reference genes is a crucial step which inevitably affects data reliability. Often, contrasting results are attributable to improper normalization approaches which impair the objectivity of data. For large RNAs, the stability of several reference genes has been widely reviewed [5–8] and even called into question [9–11]. For microRNAs (miRNAs), the stability of putative reference genes has not yet been completely confirmed and waits for adequate assessments in various biological matrices, though several attempts have been made so far [12–17] also for prostate cancer [18–20]. Small nucleolar RNAs (snoRNAs) [21] are quite often used to normalize tissue miRNA expression; one such example is represented by the SNORD family, which proved to be stable in several biological matrices [22–24]. In particular, SNORD48 has been often used as reference gene in miRNAs expression studies on prostate cancer tissues [25–27]. Starting from these considerations, the present study was aimed at assessing the stability of miR-25, miR-191, and SNORD48 in post-DRE urine sediments of 35 urine sediments of men undergoing radical prostatectomy for prostate cancer. Voided urine obtained immediately after

TABLE 1: Clinicopathological data of subjects involved in the present study.

Clinical parameters	PCa	BPH
Mean age (range)	72 (51–87 ys)	66.5 (48–77 ys)
Mean PSA (range)	6.09 ng/mL (3.16–28.6)	7.44 (1.38–21.2 ng/mL)
Mean prostate volume (range)	53.8 cc (37.5–75.2)	55.6 cc (36.5–80.8)
Clinical stage		
T1c	15	
T2a	10	
T2b	8	
T2c	2	
Pathological Gleason score		
6 (3 + 3)	18	—
7 (3 + 4)	12	
7 (4 + 3)	5	
Pathological stage		
T2a	11	
T2b	15	
T2c	9	

DRE is greatly enriched in prostatic cells (about 80%) and thus represents a challenging starting material for biomarker discovery in oncological research. 26 healthy subjects with benign prostatic hyperplasia (BPH) subjected to the same clinical maneuver were recruited as controls. To date, there are no reports regarding the stability assessment of these genes in post-DRE urine sediments. The two miRNAs were selected as candidate reference genes on the basis of literature data regarding their stability in several biological matrices [18], whereas SNORD48 was commercially available as a reference control. miR-200b and miR-452 were used to test for the effect of normalization on target genes.

2. Materials and Methods

2.1. Patients. 61 subjects subscribing an informed consent were included in the study. Of these, 35 underwent radical prostatectomy for localized prostate cancer and 26 underwent prostate biopsy for suspected PSA levels. Histological examination of tissue biopsy confirmed the absence of cancer in these subjects. Clinical parameters of subjects enrolled in the study are provided in Table 1.

2.2. Post-DRE Urine. First catch voided urine (30 mL) was collected after attentive digital rectal examination and prior to prostate biopsy or surgical intervention. Urine samples were immediately centrifuged (2000 ×g, 10 min, 4°C) and cell pellets were washed twice with phosphate buffered saline. 300 μL lysis and stabilization buffer were added to the pellets and lysates were stored at −80°C until use.

2.3. RNA Isolation. Total RNA Extraction Kit (Norgen Biotek Corp., Ontario, Canada) was used to isolate RNA. After extraction, RNA has been subjected to qualitative assessment through Bioanalyzer and NanoDrop. Total RNA was quantified by Qubit RNA Assay (Life Technologies) and stored at −80°C until use.

2.4. Reverse Transcription and Real-Time PCR. 7.5 ng of total RNA was reverse-transcribed with miRCURY LNA Universal RT microRNA PCR, polyadenylation, and cDNA synthesis kit (Exiqon). 0.5 μL of UniSp6 spike-in control was added to the retrotranscription mix (total volume 10 μL) before incubation as a positive cDNA synthesis control. For real-time PCR amplification, miRCURY LNA specific PCR primer set (Exiqon system) and Exiqon miRCURY LNA Universal RT microRNA PCR SYBR Green master mix were used. Primers were designed based on the mature sequences of genes. Stable Ct values obtained from the amplification of spike-in RNA with LNA control primer set (Exiqon) were used as quality control. Primer sequences, gene symbols, and accession numbers are listed in Additional File 1 available online at http://dx.doi.org/10.1155/2015/973597. Standard curves were generated for each primer using serial dilutions of known quantities of cDNA in triplicate (curves are provided in Additional File 2, together with DNA concentrations used and corresponding efficiencies). PCR reactions were performed on a BioRad iCycler (BioRad, Hercules, CA). 5 μL of SYBR Green master mix was mixed with 1 μL of primer mix and 4 μL of diluted cDNA (1 : 10) (total reaction volume: 10 μL). Thermal cycling conditions included 10 min at 95°C for enzyme activation and 45 cycles of amplification (15 sec 95°C for denaturing double stranded DNA and 1 min at 60°C for annealing/extension steps). Melting curve analysis was performed to assess amplification specificity. Each sample was run in triplicate and the results were averaged; no-template controls were included in the analysis. For each sample, LNA control primer set was used to amplify UniSp6 spike-in positive control. All real-time PCR assays meet

TABLE 2: Analysis of gene stability made through BestKeeper algorithm; Pearson correlation coefficients (left panel) and stability values (right panel).

| | Pearson correlation coefficient (r) | | | Stability value | | | |
	miR-191	miR-25	SNORD48		miR-191	miR-25	SNORD48
miR-25	0.375 ($p = 0.003$)	—	—	Gene stability	0.842	2.212	5.698
SNORD48	0.157 ($p = 0.226$)	0.207 ($p = 0.109$)	—	BestKeeper versus r ($p = 0.01$)	0.616	0.824	0.770

requirements of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [28]. The MIQE checklist is provided in Additional File 3. The $2^{-\Delta Ct}$ method was employed to normalize raw Ct data. Mean Cts of miR-191, miR-24, and SNORD48 from BPH group were used as the calibrator sample (e.g., ΔCt miR-25 = Ct miR-25$_{PCa}$ − Ct miR-25$_{avg,BPH}$).

2.5. Statistical Analysis. The BestKeeper [29] algorithm was used to assess the stability of candidate reference genes. Mean, standard deviation (SD) and coefficient of variation (CV) of quantification cycles (Cq) were calculated. GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA) was used for descriptive statistics of raw Ct data and to calculate variance between PCa and BPH groups. Equivalence test [30] was performed with XLSTAT (Addinsoft).

3. Results

RNA concentrations ranged from 0.5 to 14 ng/μL. No statistically significant difference in RNA yields was observed between PCa and BPH groups ($p > 0.05$). Mean RNA integrity number (RIN) was 8.7 (range 8.3–9.0). 260/280 ratio was 1.84 (range 1.84–2.03).

3.1. Expression Levels of Putative Reference Genes. Ct values of miR-25, miR-191, and SNORD48 ranged between 23 and 26 (Figure 1). Mann-Whitney U test was performed to verify if candidate genes were differentially expressed in the two groups. No statistically significant differences were observed for all genes under analysis (miR-191, p value 0.47; miR-25, p value 0.54; SNORD48, p value 0.25) (Figure 1).

3.2. Relative Expression of Candidate Reference Genes. Ct values of miR-191, miR-25, and SNORD48 from PCa patients were normalized using BPH group as calibrator sample. Relative expression was calculated as ΔCt (e.g., ΔCt miR-25 = Ct miR-25$_{PCa}$ − Ct miR-25$_{avg,BPH}$) (Figure 2).

3.3. Stability of Putative Reference Genes. Stability of selected genes was assayed by means of BestKeeper algorithm. Values are reported in Table 2. Statistical analysis found mir-191 as the most stable gene (0.842), whereas values for miR-25 and SNORD48 exceeded the cutoff of acceptability (2.212 and 5.698, resp.). Equivalence was satisfied only by miR-191 (difference between means 0.277; CI [−0.07, 0.624]), whereas miR-25 (difference between means 1.155; CI [0.275, 2.035]) and SNORD48 (difference between means 0.419; CI [−0.375, 1.212]) fell out of the cutoff limits (Figure 3).
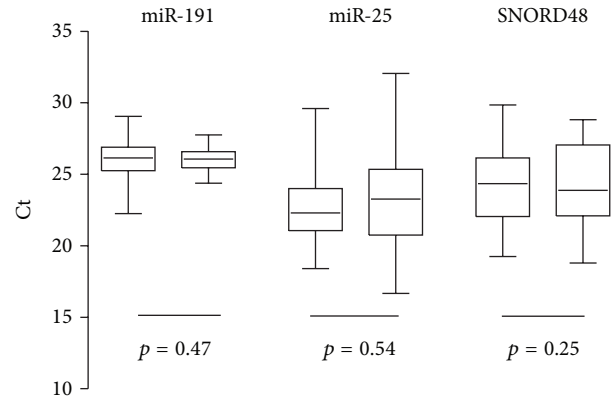

FIGURE 1: Expression levels (as raw Cts) of miR-191, miR-25, and SNORD48 in post-DRE urine sediments from PCa (left) and BPH (right) subjects. Boxes represent lower and upper quartiles; whiskers represent the minimum and maximum value.
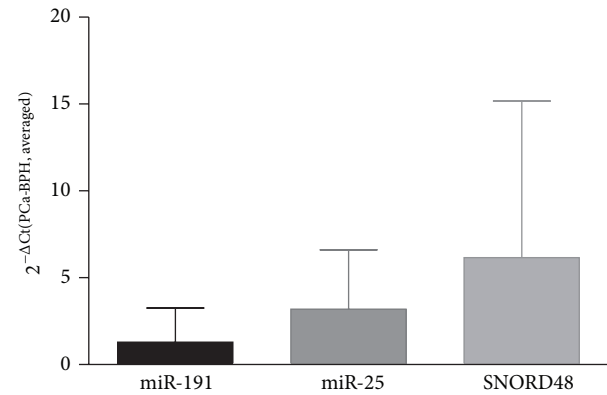

FIGURE 2: Normalized Ct values of miR-191, miR-25, and SNORD48 in post-DRE urine sediments from PCa patients. Mean Ct values from BPH group served as calibrator sample.

3.4. Effect of Normalization on Target Genes. miR-200b and miR-452 were used as target genes to verify the effect of normalization approach on their expression. Results are reported in Figure 4.

4. Discussion

Biomarker discovery in oncological research holds promise to improve the early diagnosis of cancer; in this regard, miRNAs have greatly attracted scientific interest because of their established alteration in response to carcinogenesis [4]. Real-time PCR represents the major technique to explore
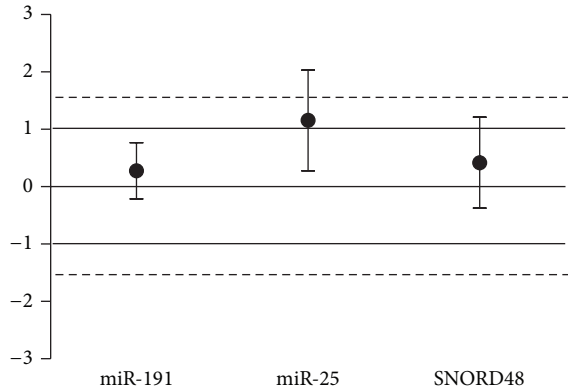
FIGURE 3: Equivalence test for miR-191, miR-25, and SNORD48 in PCa and BPH groups. Solid circles represent the differences of means and bars indicate symmetrical confidence intervals. Logarithmic expression values were used to calculate the relative expression of each gene in PCa compared to BPH group. Fold changes in expression levels between prostate cancer (PCa) and benign prostatic hyperplasia (BPH) groups are plotted on y-axis. Deviation area between solid lines ranging from −1 to 1 corresponds to fold changes ≤ 2, whereas dotted lines indicate a deviation area corresponding to a fold change ≤ 3. Equivalence was satisfied only by miR-191 (difference between means 0.277; CI [−0.07, 0.624]), whereas miR-25 (difference between means 1.155; CI [0.275, 2.035]) and SNORD48 (difference between means 0.419; CI [−0.375, 1.212]) fell out of the cutoff limits.
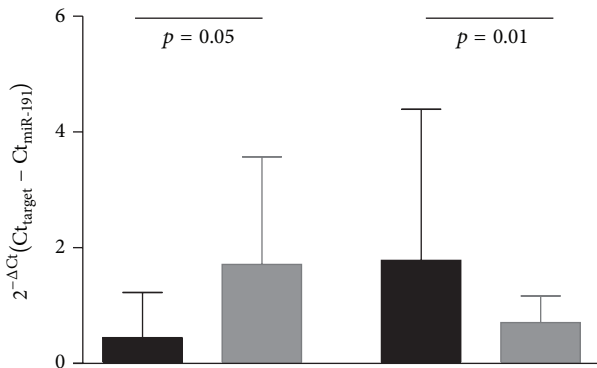

FIGURE 4: Normalized Ct values of miR-200b and miR-452 using miR-191 as reference gene in PCa (black columns) and BPH (grey columns) subjects. Values are reported as mean ± SEM.

variations in gene expression and the most used strategy is the relative quantification approach [5–8]. Here, the gene of interest is normalized against an endogenous control whose expression remains unaltered in the samples under analysis. The proper choice of an endogenous control is a crucial step which inevitably affects the reliability of scientific results [9–11]. Many scientific works have assessed the stability of several miRNAs in diverse biological matrices for several diseases and thus represent a starting point for those dealing with similar experimental conditions [12–19]. One of the most powerful works evaluating the stability of miRNAs across various tissues was made in 2008 by Peltier and Latham [20]. They analysed the stability of several miRNAs

in several normal solid tissues, including miR-191 and miR-25. In this first phase of the study, miR-191 proved to be most consistently expressed gene among almost all healthy tissues. In a second phase of the same study, the authors analysed 5 sets of tumor and normal adjacent tissue pairs to verify the stability of the selected controls upon carcinogenesis. Again, the combined analysis of tumor tissue and normal counterpart showed miR-191 to be the most stable miRNA. Another normalization strategy is the use of exogenous spike-ins: the addition of these miRNAs (primarily cel-miR-39 from *C. elegans*) to the lysis buffer during RNA isolation step would guarantee a proper normalization of PCR data. This approach has been widely used to normalize PCR data of extracellular miRNAs [31–33], although the classical strategy employing reference genes after testing their stability in the sample under analysis is quite often recommended [34] and used [35–37]. While many studies dealt with circulating miRNA profiling as diagnostic tool for urologic diseases, few reports to date delved into their characterization in urine sediment [38–40]. In the present study, we aimed to assess the stability of two microRNAs (miR-191, miR-25) and a small nucleolar RNA (SNORD48) in urine sediment obtained after attentive digital rectal examination. Urine post-DRE is achievable for all men suspected for prostate cancer; thus it represents a minimally invasive and challenging starting material for biomarker discovery. Furthermore, up to 80% of cells of post-DRE urine sediment are of prostatic origin; thus this sample contains enough prostatic cells to make real-time PCR assays feasible. The two miRNAs were chosen on the basis of literature data demonstrating their stability in various tumor tissues compared to healthy counterparts [12, 18, 25]. Our results confirmed the stability of miR-191 in urine sediments, whilst miR-25 and SNORD48 fell beyond the cutoff of acceptability. SNORD48 has been proved to be stably expressed in neoplastic tissues compared to healthy surrounding counterparts [25–27]. Although often stable, a recent study demonstrated the alteration of several snoRNAs, including SNORD48, in many diseases, raising doubt about their reliability as reference genes [41]. Moreover, these products are longer than miRNAs and thus could differ in terms of extraction yield. We choose as target gene miR-200b because it belongs to a family of tumor-suppressive miRNAs and it has been demonstrated to be downregulated in prostate cancer [42–44]. In contrast, miR-452 was proved to be massively upregulated in 6 stem/progenitor cell populations in prostate cancer [45].

5. Conclusions

Normalization of miRNAs is a yet complex and fragmented picture, and although many attempts have been made in this direction, a set of housekeeping genes to be used for PCR normalization has not yet been characterized. This is undoubtedly due to the fact that the study of miRNAs is a relatively young field of research; thus reliable data about invariant products to be used for normalization purposes simply need to grow up. Our work demonstrated the stability of miR-191 in post-DRE urine sediments.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] M. M. Center, A. Jemal, J. Lortet-Tieulent et al., "International variation in prostate cancer incidence and mortality rates," *European Urology*, vol. 61, no. 6, pp. 1079–1092, 2012.

[2] A. Heidenreich, J. Bellmunt, M. Bolla et al., "EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease," *European Urology*, vol. 59, no. 1, pp. 61–71, 2011.

[3] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell*, vol. 136, no. 2, pp. 215–233, 2009.

[4] K. M. Nelson and G. J. Weiss, "MicroRNAs and cancer: past, present, and potential future," *Molecular Cancer Therapeutics*, vol. 7, no. 12, pp. 3655–3660, 2008.

[5] B. Kozera and M. Rapacz, "Reference genes in real-time PCR," *Journal of Applied Genetics*, vol. 54, no. 4, pp. 391–406, 2013.

[6] K. Dheda, J. F. Huggett, S. A. Bustin, M. A. Johnson, G. Rook, and A. Zumla, "Validation of housekeeping genes for normalizing RNA expression in real-time PCR," *BioTechniques*, vol. 37, no. 1, pp. 112–119, 2004.

[7] T. D. Schmittgen and B. A. Zakrajsek, "Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR," *Journal of Biochemical and Biophysical Methods*, vol. 46, no. 1-2, pp. 69–81, 2000.

[8] O. Thellin, W. Zorzi, B. Lakaye et al., "Housekeeping genes as internal standards: use and limits," *Journal of Biotechnology*, vol. 75, no. 2-3, pp. 291–295, 1999.

[9] W. Ruan and M. Lai, "Actin, a reliable marker of internal control?" *Clinica Chimica Acta*, vol. 385, no. 1-2, pp. 1–5, 2007.

[10] T. Montero-Melendez and M. Perretti, "Gapdh gene expression is modulated by inflammatory arthritis and is not suitable for qPCR normalization," *Inflammation*, vol. 37, no. 4, pp. 1059–1069, 2014.

[11] E. M. Glare, M. Divjak, M. J. Bailey, and E. H. Walters, "β-actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels," *Thorax*, vol. 57, no. 9, pp. 765–770, 2002.

[12] K. H. Chang, P. Mestdagh, J. Vandesompele, M. J. Kerin, and N. Miller, "MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer," *BMC Cancer*, vol. 10, article 173, 2010.

[13] P. A. Davoren, R. E. McNeill, A. J. Lowery, M. J. Kerin, and N. Miller, "Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer," *BMC Molecular Biology*, vol. 9, article 76, 2008.

[14] V. Lamba, Y. Ghodke-Puranik, W. Guan, and J. K. Lamba, "Identification of suitable reference genes for hepatic microRNA quantitation," *BMC Research Notes*, vol. 7, no. 1, article 129, 2014.

[15] A. Torres, K. Torres, P. Wdowiak, T. Paszkowski, and R. Maciejewski, "Selection and validation of endogenous controls for microRNA expression studies in endometrioid endometrial cancer tissues," *Gynecologic Oncology*, vol. 130, no. 3, pp. 588–594, 2013.

[16] S. Jurcevic, B. Olsson, and K. Klinga-Levan, "Validation of suitable endogenous control genes for quantitative PCR analysis of microRNA gene expression in a rat model of endometrial cancer," *Cancer Cell International*, vol. 13, no. 1, article 45, 2013.

[17] L. A. Genovesi, D. Anderson, K. W. Carter, K. M. Giles, and P. B. Dallas, "Identification of suitable endogenous control genes for microRNA expression profiling of childhood medulloblastoma and human neural stem cells," *BMC Research Notes*, vol. 5, article 507, 2012.

[18] A. Schaefer, M. Jung, K. Miller et al., "Suitable reference genes for relative quantification of miRNA expression in prostate cancer," *Experimental and Molecular Medicine*, vol. 42, no. 11, pp. 749–758, 2010.

[19] J. Carlsson, G. Helenius, M. Karlsson et al., "Validation of suitable endogenous control genes for expression studies of miRNA in prostate cancer tissues," *Cancer Genetics and Cytogenetics*, vol. 202, no. 2, pp. 71–75, 2010.

[20] H. J. Peltier and G. J. Latham, "Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues," *RNA*, vol. 14, no. 5, pp. 844–852, 2008.

[21] T. Kiss, "Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions," *Cell*, vol. 109, no. 2, pp. 145–148, 2002.

[22] Z. Wotschofsky, H.-A. Meyer, M. Jung et al., "Reference genes for the relative quantification of microRNAs in renal cell carcinomas and their metastases," *Analytical Biochemistry*, vol. 417, no. 2, pp. 233–241, 2011.

[23] J. Sperveslage, M. Hoffmeister, T. Henopp, G. Klöppel, and B. Sipos, "Establishment of robust controls for the normalization of miRNA expression in neuroendocrine tumors of the ileum and pancreas," *Endocrine*, vol. 46, no. 2, pp. 226–230, 2014.

[24] G. Della Vittoria Scarpati, E. Calura, M. Di Marino et al., "Analysis of differential miRNA expression in primary tumor and stroma of colorectal cancer patients," *BioMed Research International*, vol. 2014, Article ID 840921, 8 pages, 2014.

[25] M. Avgeris, K. Stravodimos, E. G. Fragoulis, and A. Scorilas, "The loss of the tumour-suppressor miR-145 results in the shorter disease-free survival of prostate cancer patients," *British Journal of Cancer*, vol. 108, no. 12, pp. 2573–2581, 2013.

[26] S. Saini, S. Majid, S. Yamamura et al., "Regulatory role of mir-203 in prostate cancer progression and metastasis," *Clinical Cancer Research*, vol. 17, no. 16, pp. 5287–5298, 2011.

[27] S. O. Suh, Y. Chen, M. S. Zaman et al., "MicroRNA-145 is regulated by DNA methylation and p53 gene mutation in prostate cancer," *Carcinogenesis*, vol. 32, no. 5, pp. 772–778, 2011.

[28] S. A. Bustin, V. Benes, J. A. Garson et al., "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments," *Clinical Chemistry*, vol. 55, no. 4, pp. 611–622, 2009.

[29] M. W. Pfaffl, A. Tichopad, C. Prgomet, and T. P. Neuvians, "Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—excel-based tool using pair-wise correlations," *Biotechnology Letters*, vol. 26, no. 6, pp. 509–515, 2004.

[30] F. Haller, B. Kulle, S. Schwager et al., "Equivalence test in quantitative reverse transcription polymerase chain reaction: confirmation of reference genes suitable for normalization," *Analytical Biochemistry*, vol. 335, no. 1, pp. 1–9, 2004.

[31] K. M. Foss, C. Sima, D. Ugolini, M. Neri, K. E. Allen, and G. J. Weiss, "MiR-1254 and miR-574-5p: serum-based microRNA biomarkers for early-stage non-small cell lung cancer," *Journal of Thoracic Oncology*, vol. 6, no. 3, pp. 482–488, 2011.

[32] M. Yuxia, T. Zhennan, and Z. Wei, "Circulating miR-125b is a novel biomarker for screening non-small-cell lung cancer

and predicts poor prognosis," *Journal of Cancer Research and Clinical Oncology*, vol. 138, no. 12, pp. 2045–2050, 2012.

[33] A. Hsu, S.-J. Chen, Y.-S. Chang, H.-C. Chen, and P.-H. Chu, "Systemic approach to identify serum microRNAs as potential biomarkers for acute myocardial infarction," *BioMed Research International*, vol. 2014, Article ID 418628, 13 pages, 2014.

[34] Profiling of microRNA in serum/plasma and other biofluids—Guidelines for the miRCURY LNA Universal RT microRNA PCR System, 3rd edition, http://www.exiqon.com/.

[35] M. G. Egidi, G. Cochetti, M. R. Serva et al., "Circulating microR-NAs and Kallikreins before and after radical prostatectomy: are they really prostate cancer markers?" *BioMed Research International*, vol. 2013, Article ID 241780, 11 pages, 2013.

[36] Z.-H. Chen, G.-L. Zhang, H.-R. Li et al., "A panel of five circulating microRNAs as potential biomarkers for prostate cancer," *Prostate*, vol. 72, no. 13, pp. 1443–1452, 2012.

[37] H. C. N. Nguyen, W. Xie, M. Yang et al., "Expression differences of circulating microRNAs in metastatic castration resistant prostate cancer and low-risk, localized prostate cancer," *Prostate*, vol. 73, no. 4, pp. 346–354, 2013.

[38] R. J. Bryant, T. Pawlowski, J. W. F. Catto et al., "Changes in circulating microRNA levels associated with prostate cancer," *British Journal of Cancer*, vol. 106, no. 4, pp. 768–774, 2012.

[39] S. Miah, E. Dudziec, R. M. Drayton et al., "An evaluation of urinary microRNA reveals a high sensitivity for bladder cancer," *British Journal of Cancer*, vol. 107, no. 1, pp. 123–128, 2012.

[40] G. Wang, E. S.-Y. Chan, B. C.-H. Kwan et al., "Expression of microRNAs in the urine of patients with bladder cancer," *Clinical Genitourinary Cancer*, vol. 10, no. 2, pp. 106–113, 2012.

[41] H. E. Gee, F. M. Buffa, C. Camps et al., "The small-nucleolar RNAs commonly used for microRNA normalisation correlate with tumour pathology and prognosis," *British Journal of Cancer*, vol. 104, no. 7, pp. 1168–1177, 2011.

[42] L. V. Williams, D. Veliceasa, E. Vinokour, and O. V. Volpert, "miR-200b inhibits prostate cancer EMT, growth and metastasis," *PLoS ONE*, vol. 8, no. 12, Article ID e83991, 2013.

[43] J. Yu, Y. Lu, D. Cui et al., "miR-200b suppresses cell proliferation, migration and enhances chemosensitivity in prostate cancer by regulating Bmi-1," *Oncology Reports*, vol. 31, no. 2, pp. 910–918, 2014.

[44] M. He, Y. Liu, X. Deng et al., "Down-regulation of miR-200b-3p by low p73 contributes to the androgen-independence of prostate cancer cells," *Prostate*, vol. 73, no. 10, pp. 1048–1056, 2013.

[45] C. Liu, K. Kelnar, A. V. Vlassov, D. Brown, J. Wang, and D. G. Tang, "Distinct microRNA expression profiles in prostate cancer stem/progenitor cells and tumor-suppressive functions of let-7," *Cancer Research*, vol. 72, no. 13, pp. 3393–3405, 2012.