Published in final edited form as:

Cancer Invest. 2011 May ; 29(4): 318–324. doi:10.3109/07357907.2011.554477.

Improvement of Prostate Cancer Detection by Integrating the PSA Test With miRNA Expression Profiling

Yubin Hao^{1,2}, Yuan Zhao¹, Xiaoyun Zhao², Chunnian He², Xiaowu Pang¹, T.-C. Wu^{3,4,5,6}, Joseph A. Califano⁷, and Xinbin Gu⁸

¹Department of Oral Diagnostic Service, College of Dentistry, Howard University, Washington, District of Columbia, USA

²Hebei General Hospital, Hebei, China

³Department of Pathology, The Johns Hopkins University, Baltimore, Maryland, USA

⁴Department of Obstetrics and Gynecology, The Johns Hopkins University, Baltimore, Maryland, USA

⁵Department of Molecular Microbiology and Immunology, The Johns Hopkins University, Baltimore, Maryland, USA

⁶Department of Oncology, The Johns Hopkins University, Baltimore, Maryland, USA

⁷Department of Otolaryngology-Head and Neck Surgery, The Johns Hopkins University, Baltimore, Maryland, USA

⁸Cancer Center, Howard University, Washington, District of Columbia, USA

Abstract

Prostate-specific antigen (PSA) test is limited in prostate cancer diagnosis due to its inaccuracy. A new approach which integrates the PSA test with miRNA profiling was investigated to improve prostate cancer diagnosis. Six prostate cancer-related miRNAs (miR-16, -21, -34c, -101, -125b, -141) were tested in five cultured prostate cell lines and 20 human prostate specimens. We found that the miRNA expression profiles were significantly different between nontumorigenic and tumorigenic cell lines and specimens. Positive predictive value analysis of prostate cancer was increased from 40% to 87.5% by integrating patient PSA blood levels with miR-21 and miR-141 profiles.

Keywords

MiRNA expression profile; PSA; Positive predictive value; Prostate cancer

INTRODUCTION

Prostate cancer is the second leading cause of cancer mortality in men older than 40 years in the United States, with over 190,000 new cases diagnosed annually (1). In general, prostate cancer is a slow growing cancer. Five- and 10-year relative survival rates of early stage

Copyright © Informa Healthcare USA, Inc.

Correspondence to: Xinbin Gu, 600 W Street, NW. Dixon Building, Room 210, Washington, DC 20059, USA. xgu@howard.edu.

DECLARATION OF INTEREST

The authors report no declarations of interest.

prostate cancer are 99% and 95%, respectively (2). In contrast, 46% of patients with metastatic prostate disease die within 22 months after diagnosis and scarcely 32% will reach the 5-year survival mark (3). Thus, diagnosis at an early stage is critical for good prognosis and patient survival. Prostate-specific antigen (PSA), as a primary prostate cancer screening diagnostic marker, is sensitive for detecting prostate cancers at an early stage, but it often leads to a false result. With the standard cutoff of 4.0 ng/mL, the positive predictive value of PSA is only 41.5% (4). In particular, the PSA is not accurate enough to distinguish benign prostate hyperplasia from prostate cancer because many factors can affect the PSA levels, such as pro-statitis and prostatic hypertrophy (5–7). Thus, secondary diagnostic biomarkers are needed to supplement PSA and other tests for accurately detecting prostate cancer.

MicroRNAs (miRNAs) are a class of small, noncoding RNAs that are estimated to regulate at least 30% of all protein encoding genes (8–10). Mature miRNAs regulate gene expression by binding to their target mRNAs to either inhibit translation through imperfect base pairing with the 3'-untranslated region, or to degrade the target mRNA through perfect base pairing (11, 12). In recent years, miRNAs have received much attention as potential cancer diagnostic markers and therapeutic targets. Such a role for miRNAs is supported by numerous miRNA expression profiling studies. For example, profiles of widespread deregulated miRNA expression were reported in various human cancers including B cell chronic lymphocytic leukemias (13), prostate cancer (14), lung cancer (15), and breast cancers (16) by using microarray and RT-PCR methods. Furthermore, miRNA expression profiles have been shown to classify different cancers and identify cancer tissue origins (17, 18). An advantage of miRNA analysis is that their small size reduces their susceptibility to degradation in archived tissue and allows for application of miRNA profiling both on diagnosis and prognosis (19). Thus, miRNAs will likely serve as valuable cancer diagnostic biomarkers for clinical applications.

Except PSA, tumor suppressor p53 gene, Bcl-2 oncogene, and proinflammatory prostaglandin enzyme, cyclooxygenase-2 (COX-2) are often found to be abnormally expressed in prostate cancer tissues. In recent years, many reports indicate that miR-141, -16, -101, and -34c are associated with the regulation of PSA, Bcl-2, COX-2, and p53 expression, respectively (20–23), and that miR-21 and miR-125b are significantly dysregulated in various cancers (24–26). The purpose of this study is to evaluate whether these targeted miRNA expression profiles can improve prostate cancer diagnosis. The expression profiles of miR-141, -16, -101, -34c, -21, and -125b were analyzed from human prostate benign and malignant tissues and cell lines, and their corresponding xenograft tumors. An improvement in the positive predictive value of the PSA test was seen by integrating specific miRNA expression profiles with PSA blood levels.

MATERIALS AND METHODS

Reagents

Chemicals were of the highest available grade from Sigma (St. Louis, MO, USA). The miRNA forward primers were synthesized from Sigma (Woodlands, TX, USA) according to miRBase: Sequences 12.0 as follows: miR-141 (5' TAACACTGTCTGGTAAAGATGG 3'), miR-16 (5' TAGCAGCACGTAAATATTGGCG 3'), miR-101 (5' TACAGTACTGTGATAACTGAA 3'), miR-34c (5' AGGCAGTGTAGTTAGCTGATTGC 3'), miR-21 (5' TAGCTTATCAGACTGATGTTGA 3'), and miR-125b (5' TCCCTGAGACCCCTAAGTTGTGA 3').

Cell lines and cell culture

Benign prostate hyperplasia cell line (BPH1) and tumorigenic cell lines (BPH1^{CAFTD}) were kindly provided by Simon Hayward, Vanderbilt University. Androgen receptor negative cell line (PC3) and androgen receptor positive cell line (LNCap) were obtained from American Type Culture Collection (ATCC, Manassas VA, USA). SV40 immortalized human prostate epithelial cell line (PNT1) was kindly provided by Bernard Kwabi-Addo, Howard University. Cells were grown in RPMI 1640 or other suitable media under 5% CO₂ at 37°C.

Clinical prostate specimens and xenografts

A total of 20 formalin fixed paraffin embedded (FFPE) specimens were collected from Hebei General Hospital, China. The clinical and pathological data of the patients were summarized in Table 1. Of the specimens, eight were prostate cancer tissues (consisting of >60% cancer) and 12 were benign prostatic hyperplasia tissues. The sample collection protocol was approved by ethical committee of Hebei General Hospital. BPH1 and BPH1^{CAFTD} xenograft tissues were collected from our previous studies.

RNA extraction

a) Extraction of RNA from cell lines: Total RNA was extracted from 1×10^6 cells using RNeasy Mini Kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. (b) Extraction of RNA from formalin fixed, paraffin embedded (FFPE) tissues using Recover All total nucleic acid isolation optimized for FFPE sample Kit (Ambion, Austin, TX, USA). A total of 80 µM FFPE sections or 35 µg unsectioned cores were deparaffined in xylene solution for 3 min at 50°C. The deparaffined tissues were digested by protease K for 3 hr at 50°C, and then mixed with isolation buffer. The mixture was passed through a filter cartridge and washed twice. DNase was added to the filter cartridge and incubated for 30 min at room temperature, then washed with wash buffer. The total RNA was eluted by elution solution.

Reverse transcription and real-time quantitative PCR

High-specificity miRNA QRT-PCR Detection kit (Agilent Technologies, Cedar Creek, TX, USA) was used for the tests. MiRNA primers (miR-16, -21, -34c, -101, -125b, -141, and U6) were used to test specific miRNA expression. The average ΔCt of each group was calculated by the following formula: $\Delta Ct = Ct_{miR} - Ct_{u6}$. $\Delta\Delta Ct = \Delta Ct_{Cell\ line} - \Delta Ct_{PNT1}$. The fold-change for miRNA expression levels was calculated using $2^{-\Delta\Delta Ct}$.

Western blotting and immunohistochemistry

Western blotting and immunohistochemistry were performed as previously described (27).

Colony formation assay

BPH1 and BPH1^{CAFTD} cells were seeded at densities of 300 cells per well in a BD Falcon 6-well tissue culture plate (Palo Alto, CA, USA). Colonies were formed after approximately 9 days and were stained with 0.1% trypan blue in 50% ethanol. Colonies containing more than 50 cells were considered to represent a viable clonogenic cell.

Statistical analysis

Values represent the mean $\pm$ standard deviation (SD) of a minimum of three replicate tests. Data were analyzed by the *t*-test when comparing tumorigenic cells to nontumorigenic cells or benign to cancer. Differences were considered to be significant when *p* is .05.

RESULTS

Selection and evaluation of candidate mirnas in various prostate cell lines

To identify miRNAs which may serve as diagnostic markers of prostate cancer, we first measured the expression levels of three key prostate cancer associated proteins, Bcl-2, COX-2, and p53, in five prostate cell lines. These cell lines included a paired set of prostate cell lines (benign prostatic hyperplasia BPH1 and tumorigenic BPH1^{CAFTD}), an androgen receptor positive cell line (LNCap), an androgen receptor negative cell line (PC3), and a nontumorigenic prostate cell line (PNT1). As shown in Figure 1, high levels of Bcl-2 and COX-2, and lowered levels of p53, were detected in BPH1 and in all three tumorigenic cell lines (BPH1^{CAFTD}, PC3 and LNCap) compared with PNT1. On the basis of these results (Figure 1) and literature search, we selected PSA, Bcl-2, COX-2, and p53 protein associated miRNAs (miR-141, -16, -101, and -34c, respectively) and cell proliferation associated miRNAs (miR-21, and -125b) as candidates. The expression profiles of these six miRNAs in prostate cell lines were analyzed by quantitative real time RT-PCR and normalized using U6. Table 2 summarizes the fold changes in miRNA expression levels in benign and tumorigenic prostate cell lines compared to PNT1. Interestingly, miR-21 and miR-125b were upregulated in benign hyperplastic and all tumor cell lines, while miR-101 was downregulated. The other tested miRNAs showed varying expression levels in these cell lines. For instance, miR-34c was overexpressed in BPH1 cells (7.89-fold) and LNCap cells (7.25-fold). In contrast, miR-34c was expressed at low levels in BPH1^{CAFTD} cells (0.8-fold) and PC3 (0.01-fold). All six miRNAs were detectable in the five prostate cell lines and showed dysregulation in benign and cancer cell lines compared to PNT1.

Differences between miRNA expression profiles of benign prostatic hyperplasia cells and tumorigenic prostate cells

Identifying differences between miRNA expression profiles of tumorigenic and nontumorigenic cells is critical for identifying valuable cancer diagnostic miRNA markers. For this purpose, a pair of prostate cell lines, BPH1 cell line and its tumorigenic BPH1^{CAFTD} subline, were tested. Interestingly, we found that all six candidate miRNAs showed expression profiles which were significantly different between the two cell lines ($p < .05$) (Table 3, column 1). MiR-21 and miR-125b were more highly expressed in BPH1^{CAFTD} cells than in BPH1 cells. However, low expression levels of miR-16, miR-34c, miR-101, and miR-141 were observed in BPH1^{CAFTD} cells compared to BPH1 cells. To verify that these expression patterns were consistent between tumor cell lines (*in vitro*) and tumor tissues (*in vivo*), we also tested miRNA expression profiles from BPH1 and BPH1^{CAFTD} xenografts, which were grown in thymus-deficient nude mice. MiRNA expression analysis indicated that miR-21, miR-101, and miR-125b were upregulated in BPH1^{CAFTD} xenografts compared to BPH1 xenografts, whereas miR-16, miR-34c, and miR-141 were downregulated. Thus, with the exception of miR-101, miRNA expression profiles were consistent between cell lines and corresponding tumors (Table 3, column 2).

Improvement of diagnostic accuracy with integration of PSA and miRNA expression profiles

We further measured miRNAs expression levels from 20 human prostate specimens (8 prostate carcinomas and 12 benign prostatic hyperplasias, BPHs). MiR-21 and miR-141 exhibited $\geq$ two-fold upregulation in carcinoma specimens compared to BPH specimens, while the other four miRNAs were downregulated in carcinomas (Table 3, column 3). Analysis of patient blood PSA levels found that only four of the eight (50%) prostate cancer patients had high levels of PSA (>4.0 ng/mL), but six of the 12 (50%) patients with benign hyperplasia had increased levels of PSA (>4.0 ng/mL). The analysis of positive predictive value (PPV) indicated that the PPV of PSA blood levels was only 40% (Table 4). Thus, PSA

levels alone could not be used to distinguish benign tumors from prostate cancer in this group of patients. We further examined whether changes in the PPV occurred when the PSA test was combined with expression profiling of miR-21 and miR-141. Using the PSA standard cutoff of 4 ng/mL, the PPV was increased from 40% to 87.5% when the PSA test was combined with expression profiling of miR-21 and miR-141 together (Table 4).

MiR-21 expression is associated with cell proliferation

MiR-21 was more highly expressed in prostate cancer than in benign hyperplasia when examined in cell lines, xenograft tumor tissues, and human specimens. This consistent overexpression led us to investigate the function of miR-21. Therefore, we studied the correlation between miR-21 expression and cell proliferation *in vitro* and *in vivo*.

In vitro, miR-21 levels were 3.5-fold ($p < .01$) higher in BPH1^{CAFTD} than in BPH1. Colony-forming assay was used to test the differential proliferation abilities of these two cell lines. The average numbers of colony were 238 and 171 for BPH1^{CAFTD} and BPH1 ($p < .05$), respectively (Table 5). Accordingly, the expression of proliferating cell nuclear antigen (PCNA), a cell proliferation biomarker, was 3.7-fold (1.3/0.35) higher in BPH1^{CAFTD} than in BPH1 cells (Figure 1(A)). *In vivo*, the levels of miR-21 were 24.8-fold upregulated in BPH1^{CAFTD} tumors compared to BPH1 tumors. The average tumor weight from 10 samples was 1.21 g for BPH1^{CAFTD} and 0.42 g for BPH1 (Table 5). Immunohistochemistry results revealed that the number of PCNA positive cells were 9.3 and 4.7 per 100 cells for BPH1^{CAFTD} and BPH1 xenograft, respectively (Figure 2(B)). Thus, higher expression of miR-21 was significantly correlated with increased PCNA expression, cell proliferation and tumor growth ($p < .05$).

DISCUSSION

Prostate cancer was one of the most common diseases causing death among American men. Sensitive and accurate detection methods for early diagnosis of prostate cancer will truly help in reducing the prostate cancer mortality and enhancing the quality of life. In this study, we evaluated an improvement in the positive predictive value of the PSA test by integrating prostate cancer-associated miRNA expression profiles with PSA blood levels.

There are commonly found abnormal expression of PSA, COX-2, Bcl-2, and p53 in prostate cancer tissues. Chronic prostate inflammation may lead to initiation and progression of prostate cancer (28). Therefore, COX-2, as a key inflammatory biomarker, is overexpressed in prostate cancer and high expression corresponds with high Gleason scores (29). In addition, the higher level of COX-2 in prostate tumor area is accompanied with higher level of oncoprotein Bcl-2 (30). Reduced expression of p53 protein has also been found in prostate cancer (31). We examined the expression of these three prostate cancer-related proteins (COX-2, Bcl-2, and p53) in five prostate cell lines and found that COX-2 and Bcl-2 were overexpressed, while p53 was underexpressed in benign and cancer cell lines compared to PNT1 (Figure 1). Recent reports indicated that Bcl-2 and COX-2 expressions are targeted by miR-16 and miR-101 (20, 21); PSA and p53 expression are correlated with miR-141 and miR-34c (22, 23), miR-21 and miR-125b are additional miRNAs which are significantly dysregulated in various tumors (25, 26). MicroRNA microarray analysis has revealed that profiles of miRNAs expression can be used to identify origin of cancers and distinguish cancer from normal cells (15, 32). To evaluate the potential of miRNA expression profiling in prostate cancer diagnosis, we therefore selected miR-16, miR-101, miR-141, miR-34c, miR-21 (regulating cell proliferation), and miR-125b as candidate miRNAs. We found that the expression pattern of these six miRNAs varied across cell lines, xenografts, and human specimens. This indicated that the cell environment is involved in miRNA expression. Interestingly, when comparing BPH1 with the BPH1^{CAFTD} cell line, all

six miRNAs showed significantly different expression patterns ($p < .05$, Table 3). We can clearly distinguish benign BPH1 from tumorigenic BPH1^{CAFTD} cells on the basis of miRNA expression patterns. On the other hand, miR-21 and miR-141 were significantly and consistently dysregulated among cell lines, xenografts, and human tissue specimens, suggesting that miR-21 and miR-141 might be critical for prostate cancer and are potential biomarkers for distinguishing carcinoma from BPH. Our results thus agree with Porkka and Szafranska that miRNAs profiling can distinguish benign and malignant tissues (15, 32).

Although many studies have revealed that miRNAs are deregulated in cancer compared to normal tissue and can be used as new markers of diagnosis and therapy, knowledge of miRNA function in cancer is limited. MiR-21 has been reported to be overexpressed in many cancers (24, 33). MiR-21 regulates cell proliferation by downregulating its target genes: programmed cell death 4, WNT1, or TPM1 expression (34, 35). Here, we observed higher PCNA expression in BPH1^{CAFTD} than BPH1 both in cultured cell lines and in tumor xenograft tissues. Accordingly, colony-forming assay and tumor weight showed greater cell proliferation in BPH1^{CAFTD} than BPH1. Analysis of the relationship between miR-21 expression and tumor growth revealed that overexpression of miR-21 in prostate cancer was positively correlated with high level of PCNA and tumor growth. Our data thus suggest that miR-21 may function as an oncogene.

Our study provides the first evidence that the analysis of positive predictive value (PPV) for prostate cancer could be enhanced by integrating serum PSA levels with prostate cancer-associated miRNA expression profiles. Currently, blood PSA screening is commonly used to detect early-stage prostate cancer. The PSA test has a sensitivity of more than 90%, but its cancer specificity is only 25%. This low specificity results in high false negatives (normal results that occur even when cancer is present) or false positives (abnormal results even when cancer is not present) rates. Thompson reported that prostate cancer was diagnosed in 15.2% of men with a PSA level at or below 4.0 ng/mL; 15% of these men had high-grade cancer (36). In our study, 50% of benign prostate hyperplasia patients have a PSA level ≥ 4.0 ng/mL. Unclear test results can cause confusion, anxiety, and even overtreatment. To improve the accuracy of prostate cancer diagnosis, several other tests have been investigated, such as SNP genotyping (4), miRNAs profile (37), and prostate cancer gene 3 (PCA3) (38). To date, there is no study that combines the PSA test with miRNA expression profiling for diagnosis of prostate cancer. In our analysis of prostate cancer and BPH clinical specimens, we found that combining PSA blood levels with miR-21 and miR-141 expression levels resulted in a significantly higher PPV (87.5%) compared to the PSA test alone (40%). For potential clinical application, a large size of samples investigation is being pursued to further confirm the miRNA marker. Our result suggests that integrating the PSA test with prostate cancer related miRNA expression profiling will improve the accuracy of prostate cancer detection and might be more clinically useful than either alone.

In addition, the prostate biopsies are commonly used for prostate cancer diagnosis when PSA levels rise. However, the biopsy cannot exclude the possibility of prostate cancer, because only 1% of the gland is picked up and examined in each needle biopsy. Therefore, the invasive thin needle biopsy also limits the accuracy of prostate cancer diagnosis. MiRNAs are small molecules that reduce their susceptibility to degradation in archived tissue (19) and can be stably detected in human plasma and serum (23). These advantages of miRNAs enable clinical applications for identifying specific miRNA expression profiles in fresh or FFPE human tissue specimens. Furthermore, future investigations of the miRNA expression profiles in peripheral blood will help to develop minimal invasive biomarkers for prostate cancer diagnosis and may greatly benefit prostate cancer patients.

In conclusion, our study demonstrates that combining PSA blood levels with expression profiling of prostate cancer-associated miRNAs can improve the PPV and may be clinically useful in improving the utility of the PSA test in the diagnosis of prostate cancer. In addition, increased miR-21 expression was significantly correlated with the tumor cell proliferation and growth, indicating an oncogenic role of miR-21 overexpression.

Acknowledgments

We thank Dan Zhang for her excellent technical assistance and thank Liang Shan and Xuguang Zhu for valuable discussion. This work was supported in part by grants P20 CA118770, U54 CA091431, and NCRR/NIH 2G12 RR003048 from the National Cancer Institute.

REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010
2. Eastham JA, May RA, Whatley T, Crow A, Venable DD, Sartor O. Clinical characteristics and biopsy specimen features in African- American and white men without prostate cancer. *J Natl Cancer Inst.* 1998; 90:756–760. [PubMed: 9605645]
3. DeMarzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG. Inflammation in prostate carcinogenesis. *Nat Rev Cancer.* 2007; 7:256–269. [PubMed: 17384581]
4. Nam RK, Zhang WW, Trachtenberg J, Seth A, Klotz LH, Stanimirovic A, Punnen S, Venkateswaran V, Toi A, Loblaw DA, Sugar L, Siminovich KA, Narod SA. Utility of incorporating genetic variants for the early detection of prostate cancer. *Clin Cancer Res.* 2009; 15:1787–1793. [PubMed: 19223501]
5. Henderson RJ, Eastham JA, Culkin DJ, Kattan MW, Whatley T, Mata J, Venable D, Sartor O. Prostate-specific antigen (PSA) and PSA density: racial differences in men without prostate cancer. *J Natl Cancer Inst.* 1997; 89:134–138. [PubMed: 8998182]
6. Vilanova JC, Comet J, Barcelo-Vidal C, Barcelo J, Lopez-Bonet E, Maroto A, Arzo M, Moreno A, real J. Peripheral zone prostate cancer in patients with elevated PSA levels and low free-to-total PSA ratio: detection with MR imaging and MR spectroscopy. *Radiology.* 2009; 253:135–143. [PubMed: 19703854]
7. Gu R, Zhou C, Ma Q. Correlation between EPS composition and elevated serum PSA in prostatitis patients. *Zhonghua Nan Ke Xue.* 2004; 10:423–425. [PubMed: 15267204]
8. Lee RC, Ambros V. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science.* 2001; 294:862–864. [PubMed: 11679672]
9. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science.* 2001; 294:858–862. [PubMed: 11679671]
10. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet.* 2004; 5:522–531. [PubMed: 15211354]
11. Vella MC, Choi EY, Lin SY, Reinert K, Slack FJ. The *C. elegans* microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3'UTR. *Genes Dev.* 2004; 18:132–137. [PubMed: 14729570]
12. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. *Genes Dev.* 2004; 18:504–511. [PubMed: 15014042]
13. Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, Shimizu M, Cimmino A, Zupo S, Dono M, Dell'Aquila ML, Alder H, Rassenti L, Kipps TJ, Bullrich F, Negrini M, Croce CM. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc Natl Acad Sci USA.* 2004; 101:11755–11760. [PubMed: 15284443]
14. Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL, Visakorpi T. MicroRNA expression profiling in prostate cancer. *Cancer Res.* 2007; 67:6130–6135. [PubMed: 17616669]
15. Patnaik SK, Kannisto E, Knudsen S, Yendamuri S. Evaluation of microRNA expression profiles that may predict recurrence of localized stage I non-small cell lung cancer after surgical resection. *Cancer Res.* 2010; 70:36–45. [PubMed: 20028859]

16. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Menard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calin GA, Querzoli P, Negrini M, Croce CM. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res.* 2005; 65:7065–7070. [PubMed: 16103053]
17. Gaur A, Jewell DA, Liang Y, Ridzon D, Moore JH, Chen C, Ambros VR, Israel MA. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. *Cancer Res.* 2007; 67:2456–2468. [PubMed: 17363563]
18. Catto JW, Miah S, Owen HC, Bryant H, Myers K, Dudziec E, Larre S, Milo M, Rehman I, Rosario DJ, Di Martino E, Knowles MA, Meuth M, Harris AL, Hamdy FC. Distinct microRNA alterations characterize high- and low-grade bladder cancer. *Cancer Res.* 2009; 69:8472–8481. [PubMed: 19843843]
19. Rosenfeld N, Aharonov R, Meiri E, Rosenwald S, Spector Y, Zepeniuk M, Benjamin H, Shabes N, Tabak S, Levy A, Lebanony D, Goren Y, Silberschein E, Targan N, Ben-Ari A, Gilad S, Sion-Vardy N, Tobar A, Feinmesser M, Kharenko O, Nativ O, Nass D, Perelman M, Yosepovich A, Shalmon B, Polak-Charcon S, Fridman E, Avniel A, Bentwich I, Bentwich Z, Cohen D, Chajut A, Barshack I. MicroRNAs accurately identify cancer tissue origin. *Nat Biotechnol.* 2008; 26:462–469. [PubMed: 18362881]
20. Chakrabarty A, Tranguch S, Daikoku T, Jensen K, Furneaux H, Dey SK. MicroRNA regulation of cyclooxygenase-2 during embryo implantation. *Proc Natl Acad Sci USA.* 2007; 104:15144–15149. [PubMed: 17848513]
21. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A.* 2005; 102:13944–13949. [PubMed: 16166262]
22. Corney DC, Flesken-Nikitin A, Godwin AK, Wang W, Nikitin AY. MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. *Cancer Res.* 2007; 67:8433–8438. [PubMed: 17823410]
23. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Brian KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A.* 2008; 105:10513–10518. [PubMed: 18663219]
24. Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, Zeng YX, Shao JY. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA.* 2008; 14:2348–2360. [PubMed: 18812439]
25. Seike M, Goto A, Okano T, Bowman ED, Schetter AJ, Horikawa I, Mathe EA, Jen J, Yang P, Sugimura H, Gemma A, Kudoh S, Croce CM, Harris CC. MiR-21 is an EGFR-regulated anti-apoptotic factor in lung cancer in never-smokers. *Proc Natl Acad Sci U S A.* 2009; 106:12085–12090. [PubMed: 19597153]
26. Scott GK, Goga A, Bhaumik D, Berger CE, Sullivan CS, Benz CC. Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. *J Biol Chem.* 2007; 282:1479–1486. [PubMed: 17110380]
27. Zhao Y, Hao Y, Ji H, Fang Y, Guo Y, Sha W, Zhou Y, Pang X, Southerland WM, Califano JA, Gu X. Combination effects of salvianolic acid B with low-dose celecoxib on inhibition of head and neck squamous cell carcinoma growth in vitro and in vivo. *Cancer Prev Res.* 2010; 3:787–796.
28. Palapattu GS, Sutcliffe S, Bastian PJ, Platz EA, De Marzo AM, Isaacs WB, Nelson WG. Prostate carcinogenesis and inflammation: emerging insights. *Carcinogenesis.* 2005; 26:1170–1181. [PubMed: 15498784]
29. Wang WD, Chen ZT, Li DZ, Cao ZH, Sun SL, Pu P, Chen XP. Correlation between DNA repair capacity in lymphocytes and acute side effects to skin during radiotherapy in nasopharyngeal cancer patients. *Clin Cancer Res.* 2005; 11:5140–5145. [PubMed: 16033828]
30. Wang W, Bergh A, Damber JE. Chronic inflammation in benign prostate hyperplasia is associated with focal upregulation of cyclooxygenase-2, Bcl-2, and cell proliferation in the glandular epithelium. *Prostate.* 2004; 61:60–72. [PubMed: 15287094]

31. Gumerlock PH, Chi SG, Shi XB, Voeller HJ, Jacobson JW, Gelmann EP, deVere White RW. p53 abnormalities in primary prostate cancer: single-strand conformation polymorphism analysis of complementary DNA in comparison with genomic DNA. The Cooperative Prostate Network. J Natl Cancer Inst. 1997; 89:66–71. [PubMed: 8978408]
32. Szafranska AE, Doleshal M, Edmunds HS, Gordon S, Luttges J, Munding JB, Barth RJ Jr, Gutmann EJ, Suriawinata AA, Marc Pipas J, Tannapfel A, Korc M, Hahn SA, Labourier E, Tsongalis GJ. Analysis of microRNAs in pancreatic fine-needle aspirates can classify benign and malignant tissues. Clin Chem. 2008; 54:1716–1724. [PubMed: 18719196]
33. Ribas J, Ni X, Haffner M, Wentzel EA, Salmasi AH, Chowdhury WH, Kudrolli TA, Egnasubramanian S, Luo J, Rodriguez R, Mendell JT, Lupold SE. miR-21: an androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. Cancer Res. 2009; 69:7165–7169. [PubMed: 19738047]
34. Yamamichi N, Shimomura R, Inada K, Sakurai K, Haraguchi T, Ozaki Y, Fujita S, Mizutani T, Furukawa C, Fujishiro M, Ichinose M, Shiogama K, Tsutsumi Y, Omata M, Iba H. Locked nucleic acid in situ hybridization analysis of miR-21 expression during colorectal cancer development. Clin Cancer Res. 2009; 15:4009–4016. [PubMed: 19509156]
35. Hashimi ST, Fulcher JA, Chang MH, Gov L, Wang S, Lee B. MicroRNA profiling identifies miR-34a and miR-21 and their target genes JAG1 and WNT1 in the coordinate regulation of dendritic cell differentiation. Blood. 2009; 114:404–414. [PubMed: 19398721]
36. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Parnes HL, Minasian LM, Ford LG, Lippman SM, Crawford ED, Crowley JJ, Coltman CA Jr. Prevalence of prostate cancer among men with a prostate-specific antigen level $\leq$ 4.0 ng per milliliter. N Engl J Med. 2004; 350:2239–2246. [PubMed: 15163773]
37. Ambs S, Prueitt RL, Yi M, Hudson RS, Howe TM, Petrocca F, Wallace TA, Liu CG, Volinia S, Calin GA, Yfantis HG, Stephens RM, Croce CM. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. Cancer Res. 2008; 68:6162–6170. [PubMed: 18676839]
38. de Kok JB, Verhaegh GW, Roelofs RW, Hessels D, Kiemeny LA, Aalders TW, Swinkels DW, Schalken JA. DD3(PCA3), a very sensitive and specific marker to detect prostate tumors. Cancer Res. 2002; 62:2695–2698. [PubMed: 11980670]

Figure 1.

Western blot of protein expression of Bcl-2, COX-2, and p53 in benign prostatic hyperplasia BPH1 cell line, tumorigenic BPH1CAFTD, androgen receptor positive prostate tumorigenic LNCap cell line, androgen receptor negative prostate tumorigenic PC3 cell line, and in nontumorigenic human prostatic PNT1 cell line. (A). The relative intensity was measured by using Bio-Rad Quantity one software; the bar shows the fold change of protein levels compared to that in PNT1 (B).

Figure 2.

PCNA expression in BPH1CAFTD and BPH1 cells and their corresponding xenograft tumors. The levels of PCNA expression were detected by Western blot in cultured cell lines, and relative intensity was measured by using Bio-Rad Quantity one software (A).

Immunohistochemical staining was used to measure PCNA expression in xenograft tumors. The number of PCNA positive cells was counted per 100 cells, 200 $\times$ (B).

Table 1

Clinical and pathological characteristics of the study population

	Benign	Carcinoma	All Cases
No. of specimens	12	8	20
Age at prostatectomy	68 (59–77)	66 (53–77)	
Median (range)			
PSA at diagnosis	3.5 (0.16–9.46)	3.1 (0.39–49.8)	
Median (range)			
No. of specimens			
PSA $\geq$ 4 ng/mL	6	4	10
PSA < 4 ng/mL	6	4	10
Gleason sum score			
≥ 6		2	
<6		2	
n/a		4	

Table 2

Variation of miRNAs expression profiling in BPH and three cancer cell lines compared to PNT1

miRNAs	BPH1	BPH1 ^{CAFTD}	LNCap	PC3
miR-21	2.31 ^a	8.17 ^a	14.08 ^a	1.82
miR-125b	8 ^a	54.95 ^a	11.71 ^a	77.7 ^a
miR-101	0.6	0.24 ^a	0.13 ^a	0.51 ^a
miR-141	6.14 ^a	0.54	0.9	0.08 ^a
miR-16	1.77	0.14 ^a	2.46 ^a	0.97
miR-34c	7.98 ^a	0.8	7.25 ^a	0.01 ^a

^aValue is fold change.

Table 3

miRNAs expression in cell line, xenograft, and human prostate specimens

	Cell Line	Xenograft	Specimens
miRNAs	BPH1 ^{CAFTD} /BPH1	BPH1 ^{CAFTD} /BPH1	carcinoma /BPH
miR-21	3.53 [*]	24.8 [*]	2.1 [*]
miR-141	0.09 [*]	0.04 [*]	2 [*]
miR-125b	6.87 [*]	1.2	0.64
miR-16	0.08 [*]	0.92	0.73
miR-34c	0.1 [*]	0.9	0.82
miR-101	0.44 [*]	2	0.91

^{*}
 $p < .05$.

Table 4

PPV was increased by combining PSA blood levels with miRNAs expression profiling

Combine	PSA	Carcinoma	Benign	PPV
miRNA	(ng/mL)	(<i>N</i>)	(<i>N</i>)	(%)
none	≥ 4.0	4	6	40.0
	< 4.0	4	6	
miR-21	≥ 4.0	5	3	62.5
	< 4.0	2	2	
miR-21 and	≥ 4.0	7	1	87.5
miR-141	< 4.0	0	1	

N: indicates number of samples.

Table 5

Correlation of miR-21 Expression with Cell Proliferation and Tumor Growth

	<u>Cultured Cell Line</u>		<u>Tumor Xenograft</u>	
	miR-21(fold)	Ave. Colonies	miR-21(Fold)	Ave. Weight (g)
BPH1	1	171	1	0.42
BPH1 ^{CAFTD}	3.5**	238*	24.8**	1.21*

* $p < .05$ compare BPH1^{CAFTD} with BPH1.

** $p < .01$ compare BPH1^{CAFTD} with BPH1.