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Research Article

# Expression of hsv1-miR-H18 and hsv2-miR-H9 as a field defect marker for detecting prostate cancer

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ABSTRACT

**Background:** Prostate-specific antigen (PSA) is a marker of prostate cancer (PCa), although its efficacy as a diagnostic marker remains controversial. A high false-positive rate leads to repeat biopsy in approximately 70% of patients, which may not be necessary. Epigenetic biomarkers of field cancerization have been investigated widely as promising tools for the diagnosis of patients with suspected tumors. In the current study, we examined the diagnostic value of two microRNA (miRNA) candidates, hsv1-miR-H18 and hsv2-miR-H9, using formalin-fixed paraffin-embedded (FFPE) tissues from patients with PCa or benign prostate hyperplasia (BPH) (as controls) to determine the usefulness of these markers for detecting the presence of cancer.

**Methods:** Expression of hsv1-miR-H18 and hsv2-miR-H9 in 201 FFPE tissues, including 52 primary tumors, 73 surrounding noncancerous tissues, and 90 BPH nontumor controls was examined by real-time PCR.

**Results:** Expression of hsv1-miR-H18 and hsv2-miR-H9 was significantly higher in primary tumors from PCa patients than in BPH controls ($P < 0.0001$). In patients within the PSA gray zone, the two viral miRNAs could distinguish PCa from controls with appropriate sensitivity and specificity. Expression of the two miRNAs did not differ between primary tumors and noncancerous surrounding tissues.

**Conclusions:** The viral miRNAs hsv1-miR-H18 and hsv2-miR-H9 may be associated with field cancerization of PCa and could be promising supplemental biomarkers to the PSA assay to decrease the rate of unnecessary biopsy, particularly in patients within the PSA gray zone.



## 1. Introduction

Prostate-specific antigen (PSA) screening is commonly used to detect early prostate cancer (PCa). However, this assay remains controversial because of its limited diagnostic performance.[1–3] The false-positive rate is particularly high in patients with serum PSA levels of 3.0–10.0 ng/mL, which is known as the 'PSA gray zone'.[3] Approximately 70% of those patients undergo repeat biopsy and are often overdiagnosed and overtreated.[4] Thus, identifying a more accurate biomarker to PSA is an urgent need.

The concept of field cancerization (also known as field defect) was first described by Slaughter et al. in 1953 to explain the development of precancerous changes in histologically normal

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**Table 1**
Clinical characteristics of the study subjects

| Variable | BPH | PCa | | p-value[a] |
|---|---|---|---|---|
| No. of patients | 90 | 125 | | |
| Age (y) | 70.00 ± 6.71 | 69.00 ± 5.41 | | 0.163 |
| PSA (ng/mL) | 2.94 ± 8.36 | 11.42 ± 599.14 | | <0.001 |
| Operation | | | | |
| TURP | 90 (100.0) | 23 (18.4) | | |
| Radical prostatectomy | - | 102 (81.6) | | |
| Gleason score | | | | |
| 6 | - | 2 (1.6) | | |
| 7 (3 + 4) | - | 43 (34.4) | | |
| 7 (4 + 3) | - | 35 (28.0) | | |
| ≥8 | - | 45 (36.0) | | |
| TNM stage | | TURP | RP | |
| Unknown | - | 3 (2) | - | |
| T2≤ | - | 1 (1) | 65 (52) | |
| T3 | - | 19 (15) | 25 (20) | |
| T4 or metastasis | - | - | 12 (10) | |

Values are presented as numbers only, mean ± standard deviation, or number (%).
BPH, benign prostate hyperplasia; PCa, prostate cancer; PSA, prostate-specific antigen; TURP, transurethral resection of the prostate.
[a] All p-values were obtained by the Mann–Whitney *U*-test for comparisons between formalin-fixed paraffin-embedded from non-tumor controls and PCa patients in the study.

tissues.[5] It has been reviewed in various types of cancer, including PCa, as paramount evidence of the multifocality of tumors.[6] Transrectal prostate biopsy is prone to sampling error, which can lead to spurious negative findings, particularly in cases within the PSA gray zone. Therefore, examining preneoplastic molecular alterations has become a promising strategy for the selection of appropriate procedures.[7,8]

MicroRNAs (miRNAs) are small, nonprotein-encoding RNAs of approximately 20 nucleotides in length that regulate gene expression. Many miRNAs have been suggested as biomarkers because of their involvement in biological processes, including field cancerization.[9–11] miRNA profiling can be used to evaluate the likelihood of tumor development and to decrease the rate of histological false-negative results.[12,13]

In the current study, we measured the expression of two miRNAs, hsv1-miR-H18 and hsv2-miR-H9, as relevant epigenetic markers for PCa in formalin-fixed paraffin-embedded (FFPE) tissues to determine their ability to distinguish tumors from nontumor tissues.

## 2. Materials and methods

### 2.1. Cases

A total of 215 FFPE tissues from 125 PCa patients and 90 benign prostate hyperplasia (BPH) controls were obtained from Chungbuk National University Hospital between May 2003 and June 2015 (approval number: 2012-02-017-001) (Table 1). Primary tumor tissues and surrounding noncancerous tissues were collected from PCa patients who underwent radical prostatectomy (RP) or palliative transurethral resection of the prostate (TURP). Control tissues were harvested from patients with BPH who underwent TURP. Fresh tissues were formalin-fixed for >24 h, embedded in paraffin blocks, and stored at room temperature until use. Cases with a serum PSA concentration of 3–10 ng/mL were considered to be in the PSA gray zone. Gleason grades were assigned to samples obtained from TURP or RP. Tumor stage was estimated from biospecimens harvested by RP or from computed tomography, magnetic resonance imaging, or bone scans. The study methodologies conformed to the standards set by the Declaration of Helsinki.

### 2.2. miRNA microarray analysis

The RecoverAll Total Nucleic Acid Isolation Kit (Life Technologies, Carlsbad, CA, USA) was used to extract total RNA from each specimen. The amount and integrity of extracted RNA were examined using the RNA 6000 Pico Chip Kit (Agilent Technologies, Santa Clara, CA, USA) and the Agilent 2100 Bioanalyzer. The Agilent Human miRNA Microarray Release 16.0 platform, which contains 1,205 human and 144 viral miRNAs, was used for miRNA profiling.[14] The protocol used for generating the microarray gene expression datasets have been described previously.[15]

### 2.3. Purification of miRNAs from FFPE tissues

FFPE tissues were cut into 5 μm thick sections with 10–15 slides and placed in 1.5 mL Eppendorf Safe-lock micro-centrifuge tubes and stored at −70°C until use. For extracting miRNA from FFPE sections, a NucleoSpin Total RNA FFPE XS kit (Macherey-Nagel, Düren, Germany) was used according to the manufacturer's protocol.

### 2.4. Synthesis of complementary (c)DNA from miRNA

The concentration of RNA isolated from FFPE tissues was measured using the Quant-IT RiboGreen RNA Reagent and Kit (Invitrogen, Grand Island, NY, USA). The Mir-X™ miRNA First Strand cDNA Synthesis Kit (TAKARA BIO, Otsu, Japan) was used to synthesize miRNA-specific cDNA according to the manufacturer's protocol.

### 2.5. Amplification of miRNAs by real-time PCR (RT-PCR)

For quantifying miRNA expression, RT-PCR amplification was performed using a Rotor-Gene Q (Qiagen, Valencia, CA, USA) apparatus and SYBR Premix EX Taq (TAKARA BIO, Otsu, Japan). Reactions were performed in micro-reaction tubes (Corbett Research Mortlake, Australia) in a final volume of 10 μL. Standard curves were generated using chemically synthesized RNA oligonucleotides (Cosmogenetech, Seoul, Korea) corresponding to the target miRNAs. The standard curves ranged from $2.25 \times 10^5$ to $2.25 \times 10^8$ copies. All samples were run in triplicate, and RT-PCR

conditions followed the manufacturer's protocol. Rotor-Gene Q software 2.3.1.49 was used to capture and analyze spectral data.

### 2.6. Statistical analysis

Receiver operating characteristic (ROC) curves were generated to estimate the optimal cut-off point yielding the highest combined sensitivity and specificity of the two viral miRNAs in FFPE tissues from patients with PCa and from BPH controls. The expression levels of candidate miRNAs in PCa, in surrounding noncancerous tissues, and in BPH controls were examined using the Kruskal–Wallis H and Mann–Whitney *U* tests. Statistical analysis was performed using IBM SPSS version 24.0 (IBM, Armonk, NY, USA) and GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Expression levels of the two miRNAs

The expression levels of hsv1-miR-H18 and hsv2-miR-H9 were similar in PCa and surrounding noncancerous tissues, and significantly higher than in BPH controls ($P < 0.0001$) (Fig. 1A and C). For cases within the 'PSA gray zone', expression of the two viral miRNAs was higher in PCa and surrounding noncancerous tissues than in BPH controls ($P < 0.0001$ and $P = 0.005$, respectively; Fig. 1B and D). These findings indicate that hsv1-miR-H18 and hsv2-miR-H9 are associated with field cancerization of cells surrounding tumors.

### 3.2. Diagnostic performance of the two miRNAs in FFPE samples from PCa, surrounding noncancerous tissue, and BPH controls

ROC analysis was performed to determine whether hsv1-miR-H18 and hsv2-miR-H9 could distinguish patients with tumors from those with BPH. The areas under the curve (AUCs) for the two candidates were 0.800 and 0.706, with a sensitivity of 76.9% and 80.8%, and specificity of 81.1% and 55.6%, respectively (Figs. 2A and 3A). To further examine the diagnostic power of the markers, we compared surrounding noncancerous tissues with BPH tissues and found that the AUCs of the target miRNAs were 0.840 and 0.724, respectively, with a sensitivity of 89.0% and 76.7% and specificity of 75.6% and 60.0%, respectively (Figs. 2B and 3B). In patients within the PSA gray zone, the miRNAs showed improved diagnostic

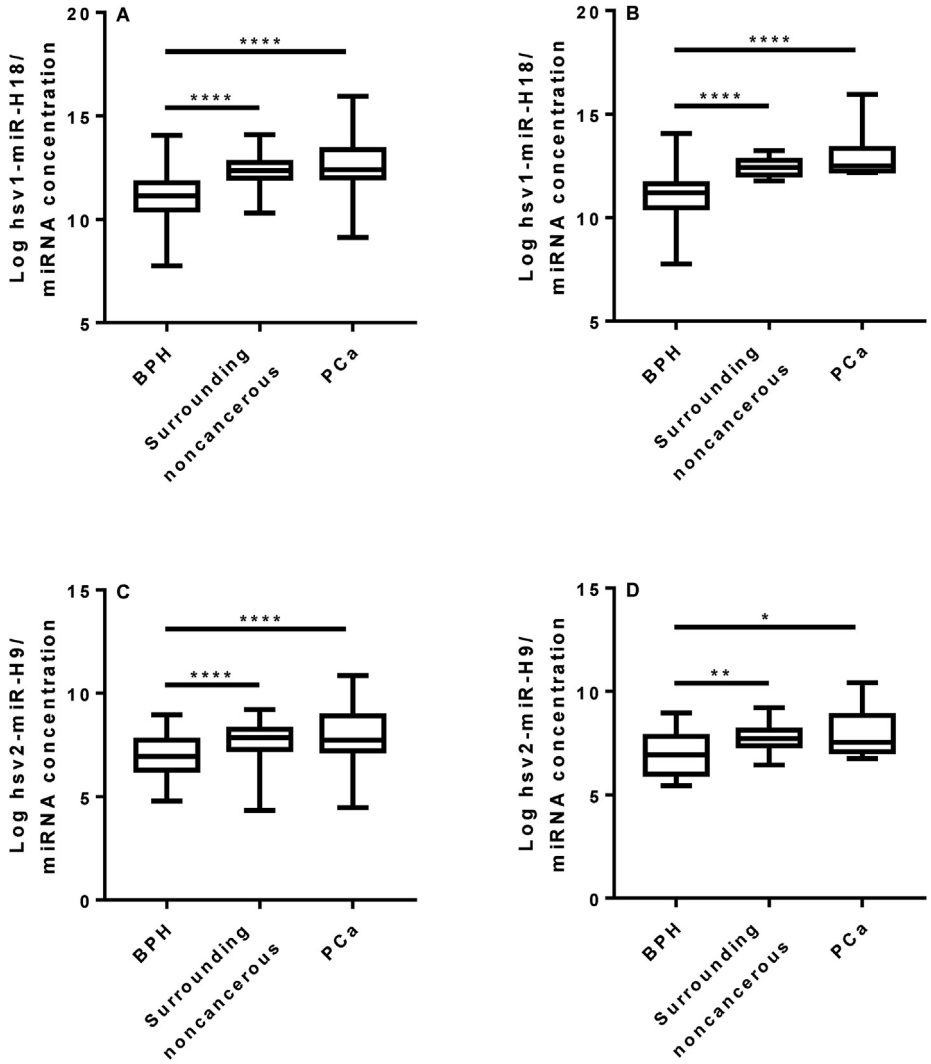


**Fig. 1.** Expression of hsv1-miR-H18 and hsv2-miR-H9 in FFPE tissues from BPH controls, surrounding noncancerous tissues, and primary tumors of PCa patients. Levels of the two viral miRNAs were higher in FFPE tissues from PCa patients than from controls and showed similar expression patterns between surrounding noncancerous tissues and primary PCa tumors. BPH, benign prostate hyperplasia; FFPE, formalin-fixed paraffin-embedded; PCa, prostate cancer; PSA, prostate-specific antigen. P-values were determined by the Mann-Whitney U-test. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$.

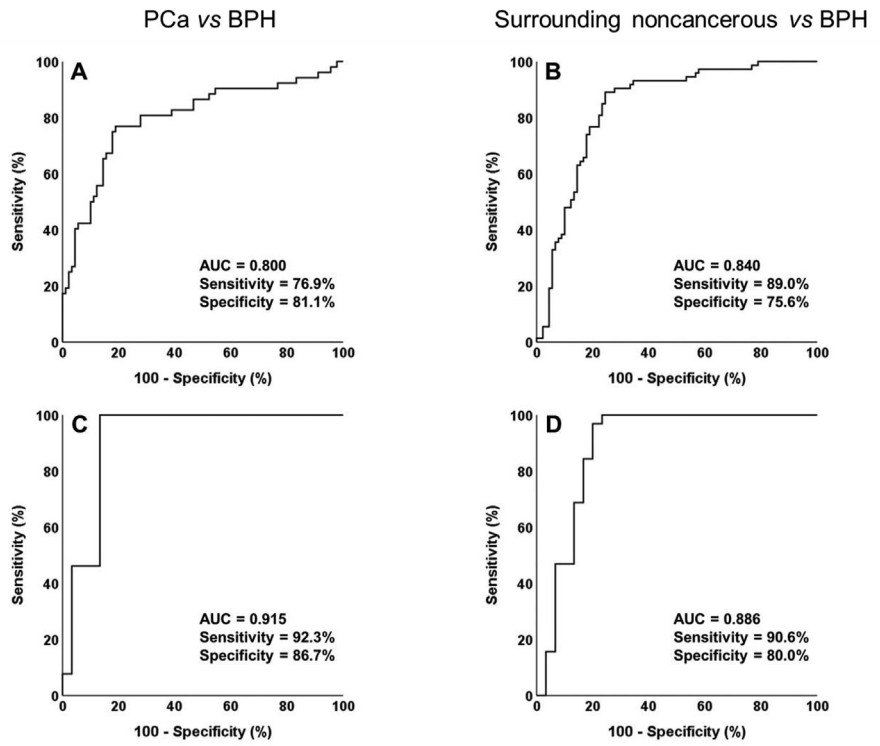


**Fig. 2.** Receiver operating characteristic curves of hsv1-miR-H18 for distinguishing PCa patients from non-tumor controls. (A) and (B) for all patients. (C) and (D) for patients within the PSA gray zone (3.0–10.0 ng/mL). AUC, area under the curve; BPH, benign prostate hyperplasia; PCa prostate cancer; PSA, prostate-specific antigen. *P-value was determined by the Z-score.

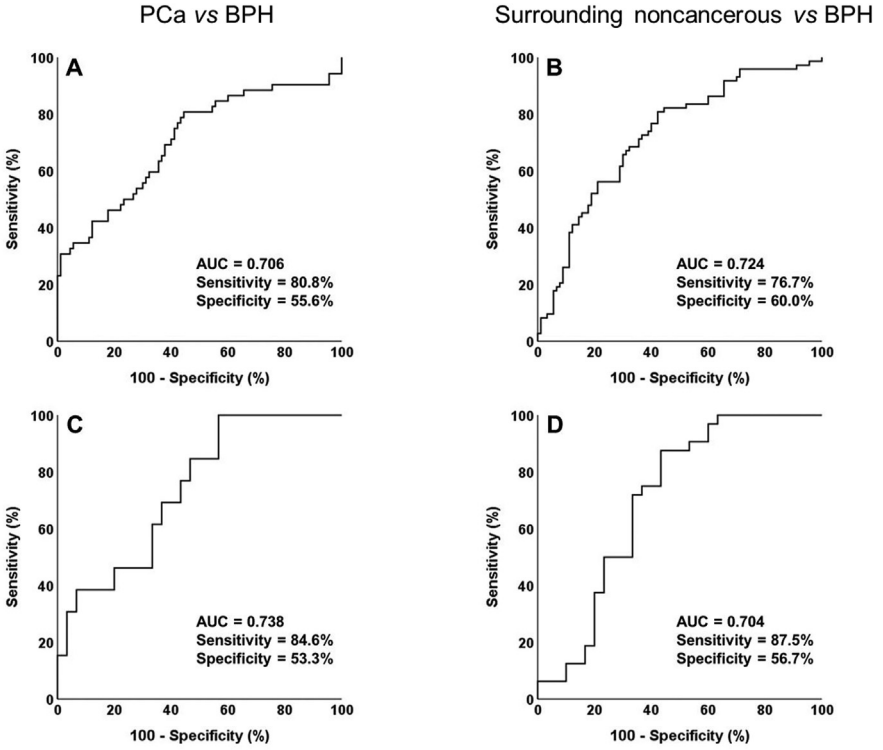


**Fig. 3.** Receiver operating characteristic curves of hsv2-miR-H9 for distinguishing PCa patients from non-tumor controls. (A) and (B) for all patients. (C) and (D) for patients within the PSA gray zone (3.0–10.0 ng/mL). AUC, area under the curve; BPH, benign prostate hyperplasia; PCa prostate cancer; PSA, prostate-specific antigen. *P-value was determined by the Z-score.

performance. When comparing PCa with BPH controls, the sensitivity and specificity of hsv1-miR-H18/hsv2-miR-H9 were 92.3%/84.6% and 86.7%/53.3%, respectively (AUC = 0.915 and 0.738; and $P < 0.0001$ and $P < 0.05$; respectively) (Figs. 2C and 3C). Similar results were obtained when comparing surrounding noncancerous tissues with BPH controls, which showed sensitivity and specificity for hsv1-miR-H18/hsv2-miR-H9 of 90.6%/87.5% and 80.0%/56.7%, respectively (AUC = 0.886 and 0.704; and $P < 0.0001$ and $<0.01$; respectively) (Figs. 2D and 3D). These results suggest that hsv1-miR-H18 and hsv2-miR-H9 can distinguish patients with tumors from those with BPH, thereby preventing miss-diagnosis by biopsy sampling error.

## 4. Discussion

PSA is broadly used as a standard biomarker for the diagnosis of PCa. However, PSA-based PCa screening is limited by poor specificity and sensitivity, especially in patients within the PSA gray zone (3–10 ng/mL).[1,16,17] Patients within the PSA gray zone often undergo repeat biopsy, although the detection rate after this procedure is <30%.[1–4,18,19] Therefore, approximately 70% of these patients are overdiagnosed that resulted in meaningless re-biopsies with no clinical benefits, underscoring the need to identify novel methods for early detection of PCa that can reduce the rate of unnecessary re-biopsy.

Recent studies identified biomarkers that increase the detection rate of PCa; however, there are several limitations. Prostate cancer antigen 3 (PCA3) is a common PCa biomarker, and increased expression of the *PCA3* gene is detected in the urine of PCa patients. However, the threshold value of PCA3 required to distinguish patients at risk of PCa from healthy controls remains unclear, and the cost-effectiveness of this assay was not determined yet, indicating that the performance of PCA3 for clinical practice applications is debatable.[20,21]

The field defect or field cancerization is considered a source of valuable biomarkers for PCa.[9,22] The basic concept of field cancerization, suggested by Slaughter, describes the development of cancer in multifocal areas.[5] There are many studies analyzing field cancerization in head and neck, colorectal, bladder, and other cancers.[23,24] In PCa, methylated *GSTP1* has been studied extensively as a tumor marker.[25] The ConfirmMDx is a quantitative methylation assay used to diagnose PCa. This assay quantifies the DNA methylation rates of three genes, *GSTP1*, *APC*, and *RASSF1*, to diagnose PCa.[26] However it has several limitations; for example, *APC* methylation can be affected by infection or inflammation of tissues, and the performance of this assay in the recent DOCUMENT study was not satisfactory.[27] Many genes have been studied and reported as promising diagnostic biomarkers for PCa; however, most are in the initial stages and require complex protocols that are impractical for clinical application, whereas others failed to show reproducible results in various tests.[28,29]

Recently, many researchers have focused on bacteria and viruses that may associate with infection and inflammation causing PCa.[30,31] Studies suggested that several viruses, including herpes types, encoded their own miRNAs, and they may affect carcinogenesis of the prostate and lead to the transformation of epithelial cells of prostate.[32,33] Although, the exact function of viral miRNA is still unknown, diverse expression levels of viral miRNAs were identified in PCa when compared with BPH controls, indicating that viral miRNA could be a respectable risk factor for PCa.[34]

Previously, we have identified the increased values of hsv1-miR-H18 and hsv2-mir-H9 that may be associated with PCa and suggested that these two viral miRNAs could be relevant diagnostic biomarkers for PCa to decrease biopsy burdens.[16]

Currently, we examined the expression values of hsv1-miR-H18 and hsv2-miR-H9 using FFPE tissues from PCa patients and BPH controls. For further investigation of two viral miRNAs in the biopsy, samples should be prepared as FFPE type, as FFPE biospecimen can be repeatedly used, which could provide molecular and pathological diagnosis at the same time. The hsv1-miR-H18 and hsv2-miR-H9 showed appropriate sensitivity and specificity, independently of histological outcomes, and showed advanced diagnostic performance in cases within the PSA gray zone. These results suggest that hsv1-miR-H18 and hsv2-miR-H9 are upregulated in prostate tumors and show potential as field defect markers for PCa diagnosis. However, a correlation between the two miRNAs and clinicopathological parameters of PCa was not observed (data not shown), which indicates that their ability to predict the aggressiveness of PCa is limited.

The results of this study indicate that hsv1-miR-H18 and hsv2-miR-H9 could serve as diagnostic biomarkers for PCa and may help to decrease the rate of unnecessary biopsy, especially in patients within the PSA gray zone. To apply this diagnosing strategy in clinical practice, validation of these miRNAs in biopsy specimens should be performed as the step for setting precise 'cut-off point' or 'cut-off ranges' to distinguish patients with high potential of PCa presents, then investigated in blinded cohort to determine whether they actually could reduce biopsy sampling errors.

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## Author contribution statement

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Administrative, technical, or material support: Seok Joong Yun and Wun-Jae Kim.

Supervision: Seok Joong Yun and Wun-Jae Kim.

Approval of the final manuscript: All authors.

## Ethics statement

The Ethics Committee of Chungbuk National University approved the protocol, and written informed consent was obtained from each patient. The collection and analysis of all samples were approved by the Institutional Review Board of Chungbuk National University (IRB approval number 2010-02-017-001), and informed consent was obtained from each patient.

## Conflicts of interest

All authors have no conflict of interest to declare.

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