Large-scale circulating microRNA profiling for the liquid biopsy of prostate cancer

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MI and ST are employees of Toray Industries, Inc., the provider of the 3D-Gene® system.

YA is an employee of Dynacom Co., Ltd., the developer of the statistical script used for selecting the best miRNA combination. All other authors have no conflict of interest to declare.
Abstract

Purpose: The high false-positive rate of prostate-specific antigen (PSA) may lead to unnecessary prostate biopsies. Therefore, the United States Preventive Services Task Force recommends that decisions regarding PSA-based screening of prostate cancer (PCa) should be made with caution in men aged 55–69 years, and that men ≥70 years should not undergo PSA screening. Here, we investigated the potential of serum microRNAs (miRNAs) as an accurate diagnostic method in patients with suspected prostate cancer (PCa).

Experimental Design: Serum samples of 809 patients with PCa, 241 negative prostate biopsies, and 500 patients with other cancer types were obtained from the National Cancer Center, Japan. Forty-one healthy control samples were obtained from two other hospitals in Japan. Comprehensive microarray analysis was performed for all samples. Samples were divided into three sets. Candidate miRNAs for PCa detection were identified in the discovery set (n=123). A diagnostic model was constructed using combinations of candidate miRNAs in the training set (n=484). The performance of the diagnostic model was evaluated in the validation set (n=484).

Results: In the discovery set, 18 candidate miRNAs were identified. A robust diagnostic model was constructed using the combination of two miRNAs (miR-17-3p and miR-1185-2-3p) in the training set. High diagnostic performance with a sensitivity of 90% and a specificity of 90% was achieved in the validation set regardless of the Gleason score and clinical TNM stage.

Conclusions: The model developed in the present study may help improve the diagnosis of PCa and reduce the number of unnecessary prostate biopsies.
Translational Relevance

Prostate cancer (PCa) is the most frequently diagnosed tumor among men and the third leading cause of cancer-related death in the United States. PCa screening mainly relies on prostate-specific antigen (PSA) testing. However, the lack of specificity of PSA tests may lead to unnecessary biopsies. Prostate biopsy sampling is an invasive procedure that can cause complications such as urinary retention and infection. Thus, identifying biomarkers for minimally invasive detection is desirable. Circulating miRNAs can provide crucial information about cancerous conditions in a less-invasive manner. Large-scale miRNA microarray analyses were used to establish a model based on a combination of circulating miRNAs to detect PCa in men with suspected PCa with high sensitivity and specificity. This model could help reduce the number of unnecessary biopsies and improve the diagnosis of PCa.
Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in men and the third leading cause of cancer-related death in men in the United States (1), and its incidence and mortality are also increasing in Japan (2). The 5 year relative survival rate in patients with localized PCa is approximately 100% regardless of treatment modality; however, in patients with metastatic disease, the 5 year relative survival decreases markedly to 30% (1) (3). Therefore, early diagnosis before the development of metastatic sites is important to reduce the mortality of PCa. Digital rectal examination (DRE) and serum prostate-specific antigen (PSA) monitoring are the standard methods of PCa screening (4). However, the accuracy of these methods for the detection of PCa is limited. DRE is a subjective test, and the degree of accuracy depends on the experience of the examiner (5). In a meta-analysis, DRE had an estimated sensitivity of 51%, a specificity of 59%, and a calculated overall positive predictive value of 41% for the detection of PCa (6). In addition, PSA has low specificity and a high false-positive rate in patients with benign prostatic hyperplasia (BPH) (7). Therefore, DRE and measuring PSA may lead to unnecessary prostate biopsy and potential complications such as infection, bleeding, urinary retention, and pain. Indeed, PSA testing is estimated to lead to approximately 750,000 unnecessary biopsies for PCa in the United States every year (8). Therefore, the development of efficient and less-invasive biomarkers for the diagnosis of PCa is urgent.

Recently, liquid biopsies based on circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating RNA, or microRNAs (miRNAs), have received increased attention as repeatable and minimally invasive tests for early diagnosis, cancer monitoring, and diagnosis of recurrent disease (9) (10) (11). miRNAs are small non-coding RNAs of 20–25 nucleotides in length that post-transcriptionally regulate the expression of thousands of
genes and thereby play important roles in oncogenesis and metastasis (12). miRNAs secreted from cells are chaperoned by various carriers, such as extracellular vesicles (EVs), RNA-binding proteins, or high-density lipoproteins, and circulating miRNAs can exist stably in body fluids (10). In addition, circulating miRNAs are associated with disease conditions, and the potential of circulating miRNAs as diagnostic biomarkers has been demonstrated (10).

Several studies demonstrated the effectiveness of circulating miRNAs as diagnostic biomarkers of PCa (13) (14) (15) (16) (17). However, the results reported are inconsistent, which may be attributed to the limited number of samples and inconsistencies among detection protocols (18) (19). To resolve this issue, we recently launched a national project in Japan, entitled “Development and Diagnostic Technology for Detection of miRNA in Body Fluids”. The aims of this project are to standardize platforms for the evaluation of serum miRNAs and to characterize the serum miRNA profiles of 13 types of human cancer, including PCa, using a large sample size (N>40,000). In the present study, we used these samples to investigate the efficacy of circulating miRNAs as biomarkers for the diagnosis of PCa in men with suspected PCa.

Materials and Methods

Sample collection

PCa and negative prostate biopsy patients

PCa serum samples were obtained from patients referred to the National Cancer Center (NCC) Hospital (NCCH) who were histologically diagnosed as PCa. Negative prostate biopsy (NPBx) serum samples were obtained from patients who were not diagnosed with PCa based on the results of prostate needle biopsy at the NCCH. These samples were
registered in the NCC Biobank between 2008 and 2016 and stored at -20°C until further use.

Clinical information for each participant was retrospectively obtained from the electronic medical records. Exclusion criteria were as follows: 1) treatment by surgical operation, hormone therapy, chemotherapy, or radiotherapy against PCa before the collection of serum; and 2) simultaneous or previous diagnosis of cancer in other organs.

Healthy controls

Healthy control serum samples were obtained from the National Center for Geriatrics and Gerontology (NCGG) and the Yokohama Minoru Clinic (YMC). The inclusion criteria for these sample sets were no history of cancer and no hospitalization during the last 3 months, and the serum samples were stored at -80°C until further use. Information about urological background, such as serum PSA levels, was not available for most samples. Demographic and clinical characteristics of patients were obtained on the day of sample collection.

Other cancers

To determine the specificity of the identified miRNAs, samples from other cancers were included in the analysis. Serum samples of male patients with 10 solid cancers including glioma (GL), colorectal adenocarcinoma (CC), esophageal squamous cell carcinoma (EC), lung carcinoma (LK), hepatocellular carcinoma (HC), gastric adenocarcinoma (GC), biliary tract cancer (BT), bone and soft tissue sarcoma (SA), pancreatic cancer (PC), and bladder cancer (BL) were collected from the NCCH between 2008 and 2016. The histological diagnosis was retrospectively confirmed using the electronic medical records.

Serum miRNA expression analysis
Total RNA was extracted from 300 µL of serum using the 3D-Gene® RNA extraction reagent (Toray Industries, Inc., Tokyo, Japan). Comprehensive miRNA expression analysis was performed using the 3D-Gene® miRNA Labeling kit and the 3D-Gene® Human miRNA Oligo Chip (Toray Industries, Inc.), which was designed to detect 2588 miRNAs registered in miRBase release 21 (http://www.mirbase.org/) (20). Fluorescent signals for each spot on the microarray were obtained using the 3D-Gene® microarray scanner (Toray Industries, Inc.) and digitized using the accessory digitizing application “Extraction” (Toray Industries, Inc.). For quality control of microarray data, the criteria for low-quality results were as follows: coefficient of variation for negative control probes >0.15; and number of flagged probes identified as an uneven spot image by 3D-Gene® Scanner >10. Samples meeting these criteria were excluded from further analyses. The presence of miRNAs was determined based on a corresponding microarray signal greater than the [mean + 2× standard deviation] of the negative control signal from which the top and bottom 5%, ranked by signal intensity, were removed. Once a miRNA was considered present, the mean signal of the negative controls was subtracted from the miRNA signal. To normalize the signals among the microarrays tested, three pre-selected internal control miRNAs (miR-149-3p, miR-2861, and miR-4463) were used as previously described (21). When the signal value was negative (or undetected) after the normalization, the value was replaced by 0.1 on a base-2 logarithm scale. All microarray data in the present study were obtained in accordance with the Minimum Information about a Microarray Experiment (MIAME) guidelines and are publicly available through the GEO (Gene Expression Omnibus) database (GSE112264). The reproducibility of the microarray analysis was confirmed by performing microarray analyses on the same RNA sample 15 times. A strong correlation between the 15 replicates was indicated by a Pearson’s correlation coefficient (R) of 0.96 (95% confidence interval, 0.94–
Identification of candidate miRNAs

Samples were divided into three groups: discovery, training, and validation sets. The discovery set was used for the selection of miRNA biomarker candidates. First, highly expressed miRNAs with a signal value >2\(^6\) in more than 50% of PCa or NPBx samples were selected in the discovery set. Subsequently, a cross-validation score, which indicates the robustness of discrimination performance between PCa and NPBx samples, was calculated based on Fisher’s linear discriminant analysis for each of the selected miRNAs in the discovery set (Supplementary text). miRNAs with a cross-validation score >0.70 were further selected. Finally, the expression levels of each miRNA were compared between PCa, NPBx, and healthy control samples, and miRNAs with the highest and lowest expression levels in PCa samples compared with the other groups were identified.

Construction of diagnostic models

The residual PCa and NPBx samples were randomly divided into training and validation sets. In the training set, the best combinations of the identified miRNAs were explored using Fisher’s linear discriminant analysis with leave-one-out cross-validation (Supplementary text). Briefly, the best 20 discriminants by one miRNA were selected, one of the residual miRNAs was added to generate two-miRNA discriminants, and the best 20 discriminants by two miRNAs were selected. This method was used to generate 1–10-miRNA discriminants. Subsequently, the best discriminants for each number of miRNAs were listed, as shown in Table 2, and finally the model showing the best AUC with the least number of miRNAs was selected. The solution of the discriminant (an ‘index’) ≥0 indicated the presence of PCa,
whereas an index <0 indicated the absence of PCa. The performance of the diagnostic index was evaluated in the validation set, and the performance of the model was tested in other solid cancers.

Construction of cancer discrimination models

A model was constructed to discriminate PCa from the other cancer types. Candidate miRNAs for model construction were the same as those identified in the discovery set. The residual PCa, NPBx, and other cancer samples were randomly divided into training and validation sets. miRNA combination models were constructed in the training set in the same way, and the performance was evaluated in the validation set.

Statistical analysis

Chi-square test for categorical variables or one-way ANOVA for continuous variables was used to compare the characteristics of patients (Gleason score [GS], serum PSA, age, and clinical TNM stage [UICC2009 7th TNM]) in the three sample sets. The unpaired t-test was used to compare the characteristics (serum PSA and age) of PCa and NPBx patients. Linear discriminant analysis and model selection based on leave-one-out cross-validation were performed using R version 3.1.2 (R Foundation for Statistical Computing, http://www.R-project.org), compute.es package version 0.2-4, hash package version 2.26, MASS package version 7.3-45, mutoss package version 0.1-10, pROC package version 1.8, and STATA version 14 (StataCorp, College Station, TX). Unsupervised clustering and heat map generation using Pearson’s correlation in Ward’s method for linkage analysis, and principal component analysis (PCA), were performed using Partek Genomics Suite 6.6. The limit of statistical significance for all analyses was defined as a two-sided P value of 0.05.
Ethical statement

The study was approved by the NCCH Institutional Review Board (2015-376, 2016-249) and the Research Committee of Medical Corporation Shintokai Yokoama minoru Clinic (6019-18-3772). Written informed consent was obtained from each participant. This study was conducted in accordance with the ethical guideline of "Declaration of Helsinki."

Results

Participants

A total of 1044 PCa and 241 NPBx serum samples were analyzed by miRNA microarray, yielding comprehensive miRNA expression profiles. Among the PCa serum samples, 38 were excluded for lack of patient information, 3 for simultaneous diagnosis of other cancers, 181 for treatment before the collection of serum, and 13 for low-quality microarray results, leaving 809 samples for analysis. PCa and NPBx samples were randomly classified into discovery, training, and validation sets (Figure 1A). There were no significant differences in the characteristics listed in Table 1 between the three sample sets.

The discovery set included 41 PCa and 41 NPBx samples. The training and validation sets included 384 PCa and 100 NPBx samples each. In the discovery set, there was no difference in age between PCa patients, NPBx patients, and healthy controls (P=0.44). In the training and validation sets, PCa patients were older than NPBx patients (P=0.001 and 0.014, respectively). Therefore, age-adjusted analysis was performed after the model construction as described below. Serum PSA levels and family history did not differ significantly between PCa and NPBx samples in each of the three sample sets (Supplementary Table 1).
Forty-one healthy male control serum samples and 50 serum samples obtained from each group of men with 10 other solid cancers, including GL, CC, EC, LK, HC, GC, BT, SA, PC, and BL, were randomly selected from our miRNA database consisting of serum miRNA profiles of more than 15,000 samples.

Selection of circulating miRNA biomarker candidates

The expression levels of the miRNAs were analyzed in the discovery set (41 PCa and 41 NPBx samples). Four hundred eight miRNAs passed the quality check criteria and were selected (Figure 1B). PCA mapping with these 408 miRNAs suggested that the miRNA profiles differed between the PCa and NPBx samples (Figure 1C). We identified 38 miRNAs with a cross-validation score >0.70 between PCa and NPBx in the discovery set (Figure 1B).

To select cancer-specific miRNAs, 41 healthy male controls were included in the analysis, and the expression levels of the 38 miRNAs were compared between the three sample sets (41 PCa, 41 NPBx, and 41 healthy male control samples). The analysis identified 16 miRNAs that were the most upregulated in PCa and 2 miRNAs that were the most downregulated in PCa (Figure 1D). Signal values of these 18 miRNAs >2^6 in more than 50% of PCa or NPBx were confirmed in the training and validation sets (Supplementary Figure 2).

Identifying the best combination of miRNAs for PCa diagnosis

Fisher’s linear discriminant analysis was used to design comprehensive discriminants consisting of 1–10 miRNAs in the training set (Supplementary Table 3). Based on the cross-validation score, the best combinations for each number of miRNAs were selected (Table 2). Based on the area under the receiver operating characteristics (ROC) curve
(AUC) reaching the optimal value (≥0.99), a combination of two miRNAs (miR-17-3p and miR-1185-2-3p) was considered as the best model in the training set (diagnostic index = 0.657×miR-17-3p + 0.385×miR-1185-2-3p - 6.341; AUC, 0.99; sensitivity, 91%; specificity, 97%). Single miRNAs were also statistically significantly effective in distinguishing cancer patients (AUC, 0.97 for miR-17-3p; 0.92 for miR-1185-2-3p) (Figure 2). The diagnostic performance of the model was confirmed in the validation set, which showed that the model was accurate (AUC, 0.95; sensitivity, 90%; specificity, 90%) (Figure 2). Because patient age was not matched between PCa samples and NPBx samples, we performed age-adjusted logistic regression analysis in the validation set. The odds ratios (ORs) of the signal intensity of the two miRNAs and the diagnostic index for the presence of PCa were almost the same before and after adjusting for age (Supplementary Table 3), indicating that the diagnostic index was independently associated with the presence of PCa.

Performance of the diagnostic index according to clinical conditions

The performance of the diagnostic index for each PCa grade was examined in the validation set. GS and cTNM stage were used to assess the performance of the diagnostic index. The diagnostic index showed high performance for all GS values (GS6, 89%; GS3+4, 91%; GS4+3, 92%; and GS ≥8, 89%), T stages (T1c, 93%; T2, 87%; and ≥T3, 92%), N stages (N0, 90%; and N1, 89%), and M stages (M0, 91%; and M1, 85%). In addition, the score of the diagnostic index was significantly lower in GS6 PCa than in other GS groups (P<0.01) (Figure 3).

Comparison of PCa and other solid cancers by the diagnostic index

To investigate whether the serum miRNA profile can distinguish PCa from other solid
cancers, we examined the performance of the diagnostic index in other solid cancers. For this purpose, we randomly selected 50 male serum samples from each group of 10 other solid cancers, and comprehensively analyzed the serum miRNA profiles of these solid cancers. The diagnostic index showed a high performance (≥70%) for all 10 solid cancers (Supplementary Figure 3).

**Potential of serum miRNA profiles to discriminate prostate cancer from other solid cancers**

We investigated whether the serum miRNA profile can distinguish PCa from other solid cancers. For this purpose, PCa, NPBx, and the other cancer samples were randomly divided into training and validation sets (Figure 4A). Using the 18 miRNAs identified in the discovery set, comprehensive discriminants consisting of 1–18 miRNAs were developed in the training set (cancer discrimination model) (Supplementary Table 4). Based on the optimal level of AUC, a combination of 12 miRNAs (miR-6471-5p, miR-17-3p, 1343-5p, miR-4417, miR-1185-1-3p, miR-1202, miR-422a, miR-6877-5p, miR-6076, miR-3185, miR-320b, and miR-1185-2-3p) was considered as the best discrimination model in the training set [cancer discrimination index = 1.059 × miR-6741-5p + 0.207 × miR-17-3p - 1.432 × miR-1343-5p + 0.918 × miR-4417 + 0.163 × miR-1185-1-3p - 0.408 × miR-1202 - 0.161 × miR-422a - 0.350 × miR-6877-5p + 0.279 × miR-6076 + 0.376 × miR-3185 + 0.131 × miR-320b + 0.338 × miR-1185-2-3p - 7.13; AUC: 0.96; sensitivity: 93%; specificity: 87%].

The diagnostic performance of this model was confirmed in the validation set (AUC: 0.91; sensitivity: 91%; specificity: 78%) (Figure 4B). Although this model was able to discriminate PCa from NPBx, CC, SA, EC, and HC with a specificity >80%, it could not successfully distinguish PCa from GL, GC, LK, PC, BT, and BL (Figure 4C). Notably, the index of BL...
samples was similar to that of PCa samples.

Discussion

In the present study, a comprehensive analysis of serum miRNA expression was performed using samples from 809 PCa and 241 NPBx patients on a standardized microarray platform (3D-Gene®, Toray Industries, Inc.). The results showed that PCa patients can be accurately distinguished from NPBx patients according to the serum levels of two miRNAs. To the best of our knowledge, five previous reports demonstrated the potential of circulating miRNAs as diagnostic biomarkers for PCa (13) (14) (15) (16) (17). The largest of these studies included 105 patients with PCa (13). In addition, a comprehensive analysis of all 2588 miRNAs was not performed in these studies. In the present study, we examined the expression profiles of 2588 miRNAs, which constitute all the human miRNAs identified to date according to miRBase rel. 21; this is the largest sample size of PCa reported to date.

The results of the present study identified the combination of miR-17-3p and miR-1185-2-3p in the serum as a biomarker for the detection of PCa. The serum expression profile or function of miR-1185-2-3p has not been reported previously. However, miR-17-3p was previously shown to act as an oncogenic miRNA in PCa. Yang et al. showed that miR-17-5p and miR-17-3p promote PCa proliferation and invasion by targeting the same protein, namely, tissue inhibitor of metalloproteinase 3 (22). Feng et al. reported that the expression levels of miR-17-3p are significantly higher in PCa tissues than in BPH (23). These reports suggest that elevated levels of miR-17-3p in serum are associated with PCa and reflect disease progression. However, in the present study, we were unable to identify the origin of the two miRNAs. We need to think the possibility that these miRNAs are not
released from cancer cells. Huiping et al. reported that miR-17-3p is secreted from immune
cells, and serum levels of miR-17-3p may be helpful to predict the therapeutic benefit of
trastuzumab in HER2-positive breast cancer patients (24). Therefore, the upregulation of
serum miR-17-3p and miR-1185-2-3p in PCa patients could be caused by a type of cells
other than PCa cells in the tumor microenvironment. Further studies are needed to elucidate
the detailed mechanism underlying the upregulation of these miRNAs in PCa patients.

The present results showed that the miRNA profile of PCa is distinct from that of BPH
regardless of the clinical TNM stage. In addition, although the diagnostic index of our model
did not show complete correlation with the GS, the diagnostic index of low-grade (GS6) PCa
was significantly lower than that of high-grade (GS ≥7) PCa. A high GS is associated with
more aggressive disease, whereas a low GS is associated with a more indolent disease
course. Urologists often use the GS to design personalized treatment strategies for their
patients (25). The present results indicate that the diagnostic index of our model may help
identify patients who would benefit from treatments such as radiation therapy or
prostatectomy, although further study is needed to confirm these results.

In the present study, we also investigated whether the two-miRNA diagnostic index could
discriminate between PCa and other types of cancer. The results indicated that the
diagnostic model was not specific for PCa patients. This may be attributed to the fact that
miR-17-3p was included in the miRNA profile. miR-17-3p is a member of the miR-17/92
cluster, which is overexpressed in many human cancers. Circulating miR-17-3p is
upregulated in several types of cancer such as colorectal cancer (26) and lung cancer (27).
Therefore, the possibility of other concomitant cancers needs to be considered in cases
showing an increased diagnostic index of PCa. As most previous studies about circulating
miRNAs in cancer did not demonstrate their specificity for certain types of cancer (10), this is
one of the strengths of the present study.

To investigate whether the serum miRNA profile can distinguish PCa from other solid cancers, we developed another model (cancer discrimination model). We confirmed that it is possible to establish a model to distinguish PCa from other types of cancer. However, even this model could not discriminate PCa from BL, which belong to the same group of urogenital cancers. This is the first report to compare the expression level of serum miRNAs between patients with PCa and those with BL, and the results suggest the existence of a common mechanism mediating the upregulation of these miRNAs in PCa and BL. In addition, the sensitivity of the cancer discrimination model was the same as that of the diagnostic model in the validation set. The need for an increased number of miRNAs could increase the detection costs; therefore, the two-miRNA combination model would be cost effective because its performance is adequate as a clinical application to reduce unnecessary prostate biopsy.

The absence of PCa in healthy controls was defined according to the self-reported medical history, and it was not confirmed by pathological examination. Because the incidence of latent PCa increases with age (28), serum miRNAs from healthy men could not be used as a control group in the discovery and training sets. We therefore analyzed serum samples derived from pathologically confirmed patients to construct the diagnostic model of PCa, and used miRNAs from healthy controls as supportive information to select miRNAs showing higher or lower expression in PCa than in the other sample sets. However, because needle biopsy was mainly performed in patients showing increased PSA levels, the diagnostic power of PSA was low in the present study. As miRNA profiling could discriminate PCa and NPBx patients with a high PSA score, miRNA profiling could be a powerful tool to complement PSA screening, thereby decreasing the number of patients referred for needle
biopsy.

The present study used retrospectively collected samples; therefore, the storage conditions before microarray analysis were not strictly regulated, which may have affected the results. Indeed, several studies reported that miRNAs are affected by various processes (29) (30). Since direct comparison between NCC Biobank samples and healthy control samples could introduce bias, we did not select biomarker miRNA candidates by comparing miRNAs between PCa and healthy samples. Rather, we used healthy samples to further select the miRNA candidates that were upregulated or downregulated both in NPBx and healthy samples compared with malignant samples. This process allowed the exclusion of certain miRNAs showing alterations in serum levels only in NPBx patients but not in PCa patients. In addition, we recently launched a clinical prospective study to validate the general applicability of our data using fresh serum samples, and we will report our results within several years.

In summary, a comprehensive analysis of serum miRNA profiles of 809 cases of PCa and 241 cases of NPBx identified a promising combination of two miRNAs, miR-17-3p and miR-1185-2-3p, for the detection of PCa. The present study is the largest scale study performed to date, and the results indicated that evaluation of circulating miRNAs is a feasible method for detecting PCa in men with suspected PCa. The high sensitivity and specificity of this model could help reduce the number of unnecessary biopsies and improve the accuracy of diagnosis.
Authors’ Contributions

Conception and design:
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing a database):
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References


Figure Legends

Figure 1.

Strategy for the selection of candidate miRNAs

A. Work flow of prostate cancer (PCa) and negative prostate biopsy (NPBx) patients and healthy controls used for developing a diagnostic model. Serum samples were obtained from 1044 PCa, 241 NPBx, and 41 healthy controls. The sample set was divided into three groups: the discovery, training, and validation sets.

B. Flow diagram of miRNAs used for selecting candidate miRNAs.

C. A principal component analysis (PCA) map for 41 PCa samples and 41 NPBx samples with 408 miRNAs.

D. Heat map showing the differences in miRNA expression levels between PCa, NPBX, and healthy control samples. The 16 miRNAs surrounded by a red line were specifically upregulated in PCa, whereas the 2 miRNAs surrounded by a blue line were specifically downregulated in PCa.

Figure 2.

Receiver operating characteristic curve analysis of the diagnostic index

ROC curves for detecting prostate cancer patients using serum PSA levels and the two miRNAs selected for the diagnostic model in the training and validation sets.

Figure 3.

Diagnostic performance of the model at different stages of PCa

Diagnostic performance of the two selected miRNAs at different stages in the validation set.

The diagnostic index showed high performance for all GSs and T, N, and M stages. The
score of the diagnostic index was significantly lower in low-grade (GS6) PCa. The P values were calculated by one-way ANOVA. Diagnostic accuracy (%) is indicated.

Figure 4.

Development of a cancer discrimination model of prostate cancer from other cancers

A. Work flow of the patients included in the development of a prediction model. Serum samples were obtained from 1,500 subjects, including 809 PCa patients, 241 NPBx patients, and 500 other cancer patients. After the selection of candidate miRNAs in the discovery set, the sample set was divided into two groups, a training set and a validation set.

B. ROC curves for detecting PCa patients using the miRNAs selected for the detection model.

C. Diagnostic index using the prediction model in the validation set (PCa, 568; NPBx, 100; sarcoma [SA], 40; colorectal adenocarcinoma [CC], 40; esophageal squamous cell carcinoma [EC], 40; hepatocellular carcinoma [HC], 40; lung cancer [LK], 40; pancreatic cancer [PC], 40; glioma [GL], 40; biliary tract carcinoma [BT], 40; gastric adenocarcinoma [GC], 40; bladder cancer [BL], 40). Diagnostic accuracy (%) is indicated.
Figure 1

A

1285 patients with serum RNA samples
1044 prostate cancer patients (PCa)
241 negative prostate biopsy patients (NPBx)

41 samples with lack of patients’ information
3 samples with simultaneous diagnosis with other cancers
181 samples with treatment before the collection of serum
13 samples with low-quality microarray results

1050 patients with serum RNA samples
809 PCa
241 NPBx

Discovery set
41 PCa
41 NPBx

Training set
384 PCa
100 NPBx

Validation set
384 PCa
100 NPBx

41 Healthy control

B

2588 miRNAs

2180 miRNAs
- signal value >= 2^6 in less than 50% of the samples

408 miRNAs

370 miRNAs
- miRNAs with a cross-validation score < 0.70

38 miRNAs

20 miRNAs
- highest or lowest in non-cancer clusters

18 miRNAs

C

PCA Mapping (43.1%)

D

Heatmap of miRNA expression levels

Figure 2

**Training set**

- **miR-17-3p**
  - AUC: 0.97 (0.96-0.99)
  - Sensitivity: 88%
  - Specificity: 93%

- **PSA**
  - AUC: 0.63 (0.57-0.69)
  - Sensitivity: 55%
  - Specificity: 64%

- **miR-17-3p + miR-1185-2-3p**
  - AUC: 0.99 (0.98-0.99)
  - Sensitivity: 91%
  - Specificity: 97%

**Validation set**

- **miR-17-3p**
  - AUC: 0.91 (0.87-0.95)
  - Sensitivity: 87%
  - Specificity: 83%

- **miR-1185-2-3p**
  - AUC: 0.92 (0.89-0.94)
  - Sensitivity: 86%
  - Specificity: 79%

- **PSA**
  - AUC: 0.60 (0.54-0.65)
  - Sensitivity: 47%
  - Specificity: 72%

- **miR-17-3p + miR-1185-2-3p**
  - AUC: 0.95 (0.92-0.98)
  - Sensitivity: 90%
  - Specificity: 90%
Figure 3

Gleason score

- 3+3
- 3+4
- 4+3
- ≥8

** p < 0.01

T stage

- T1c
- T2
- ≥T3

N stage

- N0
- N1

M stage

- M0
- M1

Diagnostic index
Figure 4

A

1550 patients with serum RNA samples
809 PCa
241 NPBx
500 Other Ca

Discovery set
41 PCa
41 NPBx
41 Healthy control

Training set
200 PCa
100 NPBx
Other Ca 100
(10 cases/each Ca)

Validation set
568 PCa
100 NPBx
Other Ca 400
(40 cases/each Ca)

B

Training set

Validation set

AUC: 0.96 (0.94-0.97)
Sensitivity: 93%
Specificity: 87%

AUC: 0.91 (0.90-0.93)
Sensitivity: 91%
Specificity: 78%

C

Cancer discrimination index

91%
92%
88%
93%
88%
62%
85%
63%
75%
65%
75%
30%

PCa NPBx CC SA EC GL HC GC LK PC BT BL

Type of cancer
Table 1. Characteristics of patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Discovery set (n=123)</th>
<th>Training set (n=484)</th>
<th>Validation set (n=484)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate cancer</td>
<td>41</td>
<td>384</td>
<td>384</td>
<td></td>
</tr>
<tr>
<td>Median age, yr (range)</td>
<td>67 (62-69)</td>
<td>68 (63-73)</td>
<td>67 (62-72)</td>
<td>0.13</td>
</tr>
<tr>
<td>Median PSA, ng/ml (range)</td>
<td>9.4 (5.8-16.4)</td>
<td>9.0 (5.8-17.1)</td>
<td>8.6 (5.8-20.4)</td>
<td>0.30</td>
</tr>
<tr>
<td>Gleason score, n (%)</td>
<td></td>
<td></td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4 (9.8)</td>
<td>45 (11.7)</td>
<td>37 (9.6)</td>
<td></td>
</tr>
<tr>
<td>3+4</td>
<td>15 (36.6)</td>
<td>122 (31.8)</td>
<td>107 (27.9)</td>
<td></td>
</tr>
<tr>
<td>4+3</td>
<td>5 (12.2)</td>
<td>70 (18.2)</td>
<td>84 (21.9)</td>
<td></td>
</tr>
<tr>
<td>8≥</td>
<td>17 (41.5)</td>
<td>147 (38.3)</td>
<td>156 (40.6)</td>
<td></td>
</tr>
<tr>
<td>Clinical T stage, n (%)</td>
<td></td>
<td></td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>T1c</td>
<td>10 (24.4)</td>
<td>124 (32.3)</td>
<td>122 (31.8)</td>
<td></td>
</tr>
<tr>
<td>T2a-c</td>
<td>25 (60.1)</td>
<td>158 (41.1)</td>
<td>171 (44.5)</td>
<td></td>
</tr>
<tr>
<td>T3a-b</td>
<td>5 (12.2)</td>
<td>95 (24.7)</td>
<td>83 (21.6)</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>1 (2.4)</td>
<td>7 (1.8)</td>
<td>8 (2.1)</td>
<td></td>
</tr>
<tr>
<td>Clinical N stage, n (%)</td>
<td></td>
<td></td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>1 (2.4)</td>
<td>26 (6.8)</td>
<td>27 (7.0)</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>40 (97.6)</td>
<td>358 (93.2)</td>
<td>357 (93.0)</td>
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<tr>
<td>Clinical M stage, n (%)</td>
<td></td>
<td></td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>3 (7.3)</td>
<td>28 (7.3)</td>
<td>33 (8.6)</td>
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</tr>
<tr>
<td>M0</td>
<td>38 (93.7)</td>
<td>356 (92.7)</td>
<td>351 (91.4)</td>
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<tr>
<td>Family history, n (%)</td>
<td>0/14 (0)</td>
<td>27/185 (14.6)</td>
<td>19/187 (10.2)</td>
<td>0.16</td>
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<td>Negative prostate biopsy</td>
<td>41</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Median age, yr (range)</td>
<td>66 (61-70)</td>
<td>65 (62-70)</td>
<td>66 (61-70)</td>
<td>0.93</td>
</tr>
<tr>
<td>Median PSA, ng/ml (range)</td>
<td>7.5 (5.2-10.6)</td>
<td>7.1 (5.0-9.8)</td>
<td>7.6 (5.6-10.6)</td>
<td>0.25</td>
</tr>
<tr>
<td>Family history, n (%)</td>
<td>0/17 (0)</td>
<td>4/40 (10.0)</td>
<td>4/41 (9.6)</td>
<td>0.47</td>
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<td>Healthy control</td>
<td>41</td>
<td>N.A.</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>Median age, yr (range)</td>
<td>70 (48-77)</td>
<td>N.A.</td>
<td>N.A.</td>
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Table 2. Discriminant analysis for prostate cancer (diagnostic model)

<table>
<thead>
<tr>
<th>Model</th>
<th>Number of miRNAs</th>
<th>Sensitivity(%)</th>
<th>Specificity(%)</th>
<th>Accuracy(%)</th>
<th>PPV(%)</th>
<th>NPV(%)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>model1</td>
<td>1</td>
<td>88</td>
<td>93</td>
<td>89</td>
<td>98</td>
<td>67</td>
<td>0.97</td>
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<td>model2</td>
<td>2</td>
<td>91</td>
<td>97</td>
<td>92</td>
<td>99</td>
<td>73</td>
<td><strong>0.99</strong></td>
</tr>
<tr>
<td>model3</td>
<td>3</td>
<td>91</td>
<td>97</td>
<td>92</td>
<td>99</td>
<td>73</td>
<td>0.99</td>
</tr>
<tr>
<td>model4</td>
<td>3</td>
<td>95</td>
<td>92</td>
<td>94</td>
<td>98</td>
<td>81</td>
<td>0.98</td>
</tr>
<tr>
<td>model5</td>
<td>4</td>
<td>93</td>
<td>95</td>
<td>94</td>
<td>99</td>
<td>79</td>
<td>0.99</td>
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<tr>
<td>model6</td>
<td>5</td>
<td>91</td>
<td>97</td>
<td>92</td>
<td>99</td>
<td>73</td>
<td>0.99</td>
</tr>
<tr>
<td>model7</td>
<td>5</td>
<td>94</td>
<td>95</td>
<td>94</td>
<td>99</td>
<td>81</td>
<td>0.99</td>
</tr>
</tbody>
</table>

model1: (0.76687)*miR-17-3p-4.05937
model2: (0.657037)*miR-17-3p+(0.384996)*miR-1185-2-3p-6.34099
model3: (0.66011)*miR-17-3p+(0.403526)*miR-1185-2-3p+(0.223082)*miR-197-5p-4.61166
model4: (0.690323)*miR-17-3p+(0.491444)*miR-1185-1-3p+(0.438635)*miR-6819-5p-3.70837
model5: (0.582969)*miR-17-3p+(0.408897)*miR-1185-1-3p+(0.394516)*miR-6076+(0.408373)*miR-197-5p-5.77338
model6: (0.579395)*miR-17-3p+(0.410828)*miR-1185-1-3p+(0.382413)*miR-6076+(0.396207)*miR-197-5p+(0.156305)*miR-1228-5p-4.17594
model7: (0.569247)*miR-17-3p+(0.40399)*miR-1185-1-3p+(0.34074)*miR-6076+(0.423294)*miR-197-5p+(0.0754199)*miR-320b-5p-5.49499

PPV: Positive Predictive Value
NPV: Negative Predictive Value
AUC: Area Under the Curve
Large-scale circulating microRNA profiling for the liquid biopsy of prostate cancer

Fumihiko Urabe, Juntaro Matsuzaki, Yusuke Yamamoto, et al.

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