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Exosomal miR-1290 and miR-375 as Prognostic Markers in Castration-resistant Prostate Cancer

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Abstract

Background—Extracellular microRNAs (miRNAs) embedded in circulating exosomes may serves as prognostic biomarkers in cancer.

Objective—To identify and evaluate plasma exosomal miRNAs for prognosis in castration-resistant prostate cancer (CRPC).

Design, setting, and participants—RNA sequencing was performed to identify candidate exosomal miRNAs associated with overall survival in a screening cohort of 23 CRPC patients. Candidate miRNAs were further evaluated for prognosis using quantitative real-time polymerase chain reaction in a follow-up cohort of 100 CRPC patients.

Outcome measurements and statistical analysis—Cox regression and Kaplan-Meier survival analyses were used to evaluate survival association using candidate miRNAs along with clinical prognostic factors.

Results and limitations—RNA sequencing in screening cohort generated approximately 6.80 million mappable reads per patient. Of those with normalized read counts ≥5, 43% were mapped to miRNAs for a total of 375 known and 57 novel miRNAs. Cox regression analysis identified an association of miR-1290, -1246, and -375 with overall survival (false discover rate <0.05). Of those, higher levels of miR-1290 and -375 were significantly associated with poor overall survival ($p < 0.004$) in the follow-up cohort. Incorporation of miR-1290/-375 into putative clinical prognostic factors-based models in CRPC stage significantly improved predictive performance with a time-dependent area under the curve increase from 0.66 to 0.73 ($p = 6.57 \times 10^{-6}$).

Conclusions—Plasma exosomal miR-1290 and miR-375 are promising prognostic biomarkers for CRPC patients. Prospective validation is needed for further development of these candidate miRNAs.

Patient summary—In this study, we evaluated whether small RNAs circulating in blood could be used to predict clinical outcomes in late-stage prostate cancer patients. We identified two blood-based small RNAs whose levels showed significant association with survival. Our results warrant further investigation because the noninvasive blood-based test has great potential in the management of late-stage prostate cancer.

Keywords

Exosome; microRNA; Extracellular RNA; RNA sequencing; Prostate cancer; Biomarker; Prognosis; Survival

1. Introduction

Prostate cancer (PCa) is the second leading cause of cancer-related mortality in men, with >240 000 newly diagnosed cases and 29 000 deaths annually in the United States [1]. In advanced metastatic stages, androgen deprivation therapy (ADT), a standard first-line therapy, leads to substantial cancer control and palliation that lasts for a median time of 20–30 mo [2,3]. After failure of ADT and emergence of a castration-resistant stage, circulating tumor cell counts (CTCs) are the only prognostic biomarkers available approved by the US Food and Drug Administration. This test is technically challenging and expensive with prognosis adversely affected for patients with >5 CTCs per 7.5 ml blood [4,5]. With the increased availability of therapeutic options for this stage, access and clinical utility of easy-to-use prognostic and predictive biomarkers has become clinically relevant. Cell-free nucleic acid profiling such as exosomal RNA in circulatory fluids offers an attractive potential for prognostic and predictive biomarker development [6].

Exosomes are small (30–100 nm) membrane vesicles released into the extracellular environment on fusion of multivesicular bodies with the plasma membrane [7]. It is reported that exosomes contain RNAs selectively enriched from parent cells [8]. A number of plasma exosomal RNAs are reported as diagnostic, prognostic, or even therapeutic biomarkers in cancer patients [6]. miR-107, miR-574-3p, miR-375 and miR-141 are upregulated in metastatic PCa patients [9]. Circulating microRNA (miRNA)-375 and miRNA-141 are correlated with a high Gleason score or lymph-node positive status [10,11], implying great potential as a PCa biomarker. We recently developed a comprehensive pipeline for exosomal RNA profiling analysis including exosome isolation, RNA extraction, library preparation, sequencing, and bioinformatics analyses [12,13]. In this study, we applied this pipeline to examine exosomal RNA profiles based on the hypothesis that plasma exosomal RNAs can be used for to predict survival in castration-resistant prostate cancer (CRPC) patients.

2. Material and methods

2.1. Patients and samples

Patients consented to participate in an advanced PCa biomarker registry prior to blood collection at the time of ADT failure. For plasma specimens, whole blood collection was first centrifuged at 2000 rpm for 10 min. The supernatant was then fractioned into multiple aliquots for storage at −80°C. A screening cohort (n = 23) and a follow-up cohort (n = 100) were included. ADT failure was defined as the date of the first occurrence of either two serial prostate-specific antigen (PSA) rises during continuous ADT measured at least 1 wk apart with an absolute PSA level >2 ng/ml [14] or the appearance of new image-based metastasis during ADT or initiation of castration-resistant specific therapy to ongoing ADT and in the presence of subcastration testosterone levels (<50 ng/dl). Table 1 lists the demographic characteristics of the screening and follow-up cohorts. For the follow-up cohort, patients were recruited from January 2010 to September 2012. At enrollment time, median PSA, hemoglobin levels, and serum lactate dehydrogenase (LDH) were 20.65 ng/ml (range: 0–2324), 12.7 g% (range: 9.2–15.6), and 192 IU/l (range: 92–500), respectively. Forty-eight patients received additional systemic therapies. A total of 31 patients underwent

one systemic therapy regimen (29 patients with docetaxel chemotherapy alone and 2 patients receiving only abiraterone acetate and prednisone). Thirteen patients received two systemic treatments with nine receiving docetaxel followed by abiraterone acetate, three receiving cabazitaxel as second-line treatment after docetaxel, and one patient receiving mitoxantrone after docetaxel failure. Four patients received three systemic regimens including three patients with docetaxel, abiraterone acetate plus prednisone, and mitoxantrone and one patient with cabazitaxel instead of mitoxantrone as third-line therapy.

To determine the endogenous reference controls for quantitative reverse transcription polymerase chain reaction (qRT-PCR), we examined RNA sequencing profiles from plasma exosomal RNAs in 192 subjects including 50 healthy individuals, 100 colorectal cancer patients, 6 pancreatic cancer patients, and 36 PCa patients (23 were CRPC patients used in the study). Men and women were evenly distributed in these subjects except for PCa. This study was approved by the Mayo Clinic and the Medical College of Wisconsin institutional review board.

2.2. Exosomal RNA extraction and sequencing library preparation

Exosomal isolation, RNA extraction, and sequencing library construction were reported previously [12]. Small RNA libraries were constructed with 2 ng exosomal RNA. Index libraries were equally pooled for sequencing (Illumina HiSeq2000 platform).

2.3. Sequencing data analysis

A graphic user interface-based tool to process the sequencing data has been developed [13]. After initial data cleaning, sequences with a length ≥16 nt were aligned against miRBase (Release 19, 2043 entries) and human genome (Release 103). miRNA profiling was normalized using read counts per million mappable miRNA sequences. miRDeep2 [15] was used to identify novel miRNAs from sequencing data. Predicted miRNAs with scores ≥2 were considered significant.

2.4. Quantitative reverse transcription polymerase chain reaction

To validate miRNAs identified by RNA sequencing, TaqMan MicroRNA Assays (Life Technologies) and miScript SYBR Green PCR Kit (Qiagen, Venlo, The Netherlands) were used to measure expression levels of miR-30a/e-5p, miR-99a-5p, let-7c, miR-1246, miR-1290, miR-16-5p, miR-125a-5p, and miR-375 in the plasma exosomal RNAs. Among those, miR-30a/e-5p, miR-99a-5p, miR-16-5p, miR-125a-5p, and let-7c were evaluated as candidates for normalization control. PCR reaction was performed in triplicate and analyzed using an ABI 7900HT system. Qiagen miScript II RT kit was also used for validation of miR-375, miR-30a/e-5p, and miR-16-5p.

2.5. Identification of endogenous references

In the screening cohort, the normalized sequencing data from 192 individuals (23 with CRPC and an additional 169 healthy and nonhealthy subjects) were first screened for the most stably expressed miRNAs with an overall coefficient of variation (CV) <5%. Selected miRNAs were further processed by NormFinder [16] and BestKeeper [17]. Diagnostic

power with $p < 0.001$ was considered as the reference candidate. In the follow-up cohort, raw C_T values derived from real-time PCR were used to determine their stabilities.

2.6. Survival analysis

Overall survival was recorded from the date of plasma collection after ADT failure to death or last follow-up. Association between exosomal RNA levels and overall survival was assessed using Kaplan-Meier survival curves and Cox regression in Partek Genomics Suite 6. For the multivariate analysis, risk score was calculated by a linear combination of the ΔC_T values from real-time PCR for selected miRNAs and/or clinical factors, weighted by their estimated regression coefficients. Time-dependent receiver operating characteristic (ROC) curve analysis was used to compare the predictive performance of the risk scores for the combined miRNAs and/or clinical variables. The area under the ROC curve (AUC) was used to evaluate survival prediction.

3. Results

3.1. Mapping of RNA sequencing

For the 23 RNA sequencing libraries, we received an average of 13 million raw reads per library, where reads with insert lengths >16 nt (query sequences) were approximately 10 million (76.60%). Of these, 6.7 million reads (51.42%) were mapped to known RNA species and the human genome. Among the mapped reads, mature miRNAs were the most common with an average of 2 784 360 reads (41.72%), followed by piwi-interacting RNA with 1 396 493 (20.92%), long noncoding RNA with 1 347 486 (20.19%), and messenger RNA with 434 955 (6.52%). Six other RNA species collectively constituted 10.64% of the mappable sequences (Fig. 1; Supplementary Table 1). In this study, we reported our analysis on 375 common miRNAs and 57 putative miRNAs with >5 counts per million mappable reads (Supplementary Table 2). The raw sequencing data were deposited in the Gene Expression Omnibus database (accession number: GSE58410).

3.2. Association of overall survival with exosomal microRNAs

To identify survival-related exosomal RNAs in CRPC patients, we further excluded those miRNAs with log2 transformed read counts <5. We performed Cox regression analysis using the remaining 248 known and 19 predicted miRNAs. We identified three miRNAs with a p value <0.0008 (false discovery rate <0.05) including miR-1290 (hazard ratio [HR]: 1.79; 95% confidence interval [CI], 1.30–2.48), miR-1246 (HR: 8.04; 95% CI, 2.36–27.33), and miR-375 (HR: 2.69; 95% CI, 1.52–4.77). In addition, we also observed an association of other RNA types with survival (Supplementary Table 3). For miRNAs, we dichotomized these patients using miRNA mean expression values as a cut-off and performed Kaplan-Meier analysis. We confirmed the significant association of miR-1290, miR-1246, and miR-375 with overall survival ($p < 0.05$) (Supplementary Fig. 1).

3.3. Candidate microRNAs for endogenous normalization

To identify potential miRNA references for exosomal RNA quantification, we analyzed the 173 most abundant known miRNAs from the 192 exosomal RNA sequencing libraries with CV <5%. Based on NormFinder analysis, miR-30e-5p, miR-221-3p, miR-30a-5p,

miR-99a-5p, and miR-181b-5p ranked as the top five most stable miRNAs in ungrouped samples (Fig. 2; Supplementary Table 4). Under BestKeeper, most miRNAs identified by NormFinder were also among the best candidate list ($p < 0.001$), although rank of their diagnostic powers was slightly different(Supplementary Table 5). Based on their expression levels, stability, and diagnostic power, we selected miR-30a/e-5p, miR-99a-5p, let-7c, and miR-125a-5p for further validation. In addition, we also selected miR-16-5p and RNU6B because they have been used for endogenous control in other studies [18–20].

3.4. miR-30a-5p and miR-30e-5p as best endogenous normalizers

To determine the best endogenous RNA references, we examined candidate miRNAs using the TaqMan qRT-PCR assay in the follow-up cohort. RNU6B, a commonly used endogenous control for cellular miRNA quantification, was not detectable by the TaqMan assay. miR-125a-5p was barely detectable, consistent with its relative low read counts in the RNA sequencing data. let-7c, although detectable, significantly varied across technical triplicates, possibly caused by its Ct value >35. We thus excluded RNU6B, miR-125-5p, and let-7 from endogenous candidates. For the remaining four miRNAs, the geometric mean of miR-30a and miR-30e-5p (GM-30a/e) was the best, followed by miR-30e-5p, miR-30a-5p, miR-99a-5p, and miR-16-5p, according to NormFinder (Table 2; Supplementary Fig. 2). Defined by BestKeeper, miR-30a, miR-30e-5p, and GM-30a/e remained significant (cut-off $p = 0.001$). This result was further confirmed separately by the TaqMan assay using a smaller RT primer pool (miR-30a-5p, miR-30e-5p, and miR-16-5p) and Qiagen universal RT primer assay, where a more stable expression pattern was observed in all selected candidates (Table 2; Supplementary Fig. 3). Of note, miR-30a and miR-30e-5p demonstrated better stability than miR-16-5p in these tests. Based on these analyses, miR-30a-5p and miR-30e-5p together with GM-30a/e emerged as the best endogenous references for follow-up validation.

3.5. Association of miR-1290 and miR-375 with overall survival

Based on pretest quality on each assay, we selected the TaqMan assay for miR-1246 and miR-1290, and the Qiagen miScript for miR-375. We first normalized each qRT-PCR value to GM-30a/e to generate ΔC_T and then performed Kaplan-Meier analysis. We observed significant associations of elevated levels of miR-375 and miR-1290 with shorter overall survival ($p = 0.0028$ and 0.0057, respectively) (Fig. 3). No significant association was observed with miR-1246 ($p = 0.13$). The combined effect of higher levels in both significant miRs on survival was more significant than the individual miRs ($p = 9.44 \times 10^{-5}$). The patients with high levels in both miR-1290 and miR-375 ($n = 21$) had a mortality rate of approximately 80% at the 20-mo follow-up; lower levels in both miRs ($n = 40$) had a mortality rate of about 10% over the same follow-up time period. The median overall survival for patients with higher level miRs (7.23 mo) was significantly shorter than patients with lower level miRs (19.3 mo) ($p = 0.0045$).

To evaluate performance of multivariate prediction models, we first estimated the association of individual clinical factors (age, PSA, LDH, and hemoglobin levels at the time of progression to CRPC stage, Gleason score at disease diagnosis, number of treatments after progression to CRPC, time from ADT initiation for hormone-sensitive stage to

treatment failure) with overall survival. Individually, we observed a survival association with PSA levels at the time of CRPC stage ($p = 0.0015$) and ADT failure time from ADT initiation (p=0.041) (Fig. 4; Supplementary Fig. 4). We then computed the risk score for the combination of the selected miRNAs and clinical prognostic factors (multivariate model). We found a significant association of patients' survival with the multivariate model that included miR-1290 and miR-375 levels, ADT failure time, and PSA levels at the time of CRPC stage ($p = 0.00016$). Based on this model, patients with a high risk score had a 2.58-fold higher risk of death than patients with a low-risk score (HR: 2.58; 95% CI, 1.51–4.41). In addition, a time-dependent ROC analysis using the multivariate model generated an AUC of approximately 0.73, which represented significant improvement over a clinical variable-based model (ADT failure time and PSA at CRPC stage) with an AUC of about 0.66 ($p = 6.57 \times 10^{-6}$; two-tailed Student *t* test) (Fig. 4).

4. Discussion

After failure of ADT and with the emergence of castration resistance, the survival period is limited but extremely variable between patients. Knowledge of prognosis for individual patients in this stage has become critical because several therapeutic choices are available to control progression. Such prognostic factors are lacking in advanced PCa therapeutics, barring the presence of CTC counts, which are technically challenging and have not been widely adopted. Clinical prognostic factors used in several nomograms at the CRPC stage have yet to be validated in prospective studies [21]. In this study, we observed a significant association of exosomal miRNA transcripts with overall survival and inculcated clinical prognostic factors using a hospital-based real-world registry. Our results suggest that circulating miRNAs may serve as a sensitive biomarker for the prognosis of CRPC patients.

There are several advantages for developing blood-based prognostic biomarkers in advanced stage cancer patients. Repeated blood measurements are minimally invasive and can provide critical information on a disease's molecular signature status over time, through the course of treatment and recurrence. Although CTC sampling requires reference laboratory work-up and technically challenging equipment for assay performance, a blood-based PCR assay offers an attractive option. Real-time PCR assay is able to detect specific RNAs indicative of disease status from a simple sampling without the need for a biopsy or surgically resected tissue. However, this unmet need will have to take into account several challenges as per the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) [22]. In this initial attempt we attempted to adhere with these recommendations in the context of identifying candidate plasma-based biomarkers for prognosis.

As a well-known oncomiR (miRNA associated with cancer), circulating miR-375 has been implicated in a variety of cancers including PCa [10,11,23,24]. Upregulation of miR-375 has been correlated with PCa metastasis [9] and poor survival in esophageal squamous cell carcinoma patients [25]. Compared with miR-375, exosomal miR-1290 performed slightly better in the outcome prediction. A recent study shows that high levels of miR-1290 in serum discriminate low- from high-stage pancreatic cancer [19]. miR-1290 is involved in estrogen receptor α–positive breast cancer, probably due to targeted inhibition of N-

acetyltransferase 1 arylamine N-acetyltransferase (*NAT1*) and forkhead box A1 (*FOXA1*) [26]; however a direct role for miR-1290 in PCa remains undetermined.

5. Conclusions

This study identified two miRNA candidates (miR-1290 and miR-375) for survival prognosis in CRPC after a systematic attempt for reducing biases from specimen processing and real-time PCR. These results provide strong preliminary evidence to support the notion that circulating exosomal RNAs contain various RNA species and changes in exosomal RNA contents are robust candidates as clinical biomarkers for advanced PCa. Prospective validation studies should be pursued in accordance with REMARK guidelines by the integration of extracellular RNAs and clinical prognostic factors in the future development of a prognostic algorithm for late-stage PCa patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Take-home message

We identified and verified two exosomal microRNAs (miRNAs) showing a significant association with overall survival in castration-resistant prostate cancer patients. The blood-based extracellular miRNA test is noninvasive and has great potential to serve as a prognostic biomarker for late-stage prostate cancer.

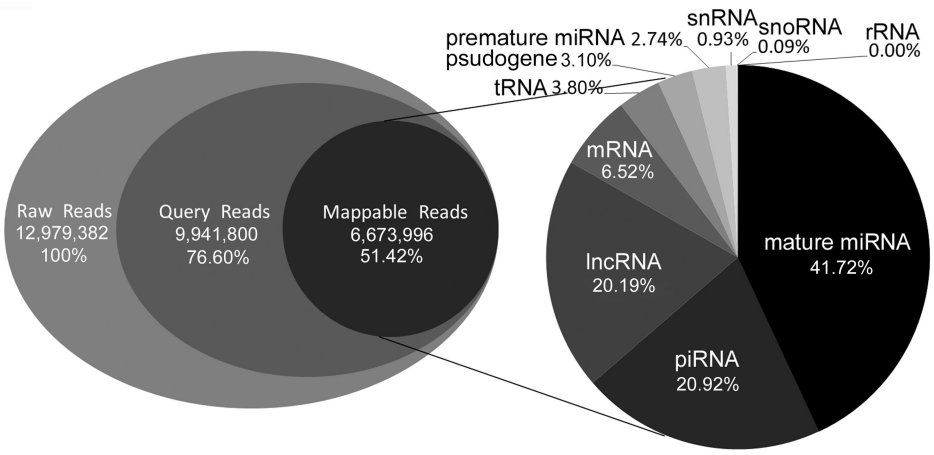

Fig. 1.

RNA species and their distributions in plasma exosomes. Raw reads are the sequences detected by RNA sequencing. Query reads are those after trimming. Mappable reads are those mapped to known human RNA or genome. lncRNA = long noncoding RNA; miRNA = micro RNA; piRNA = piwi-interacting RNA; rRNA = ribosomal RNA; snRNA = small nuclear RNA; snoRNA = small nucleolar RNA; tRNA = transfer RNA.

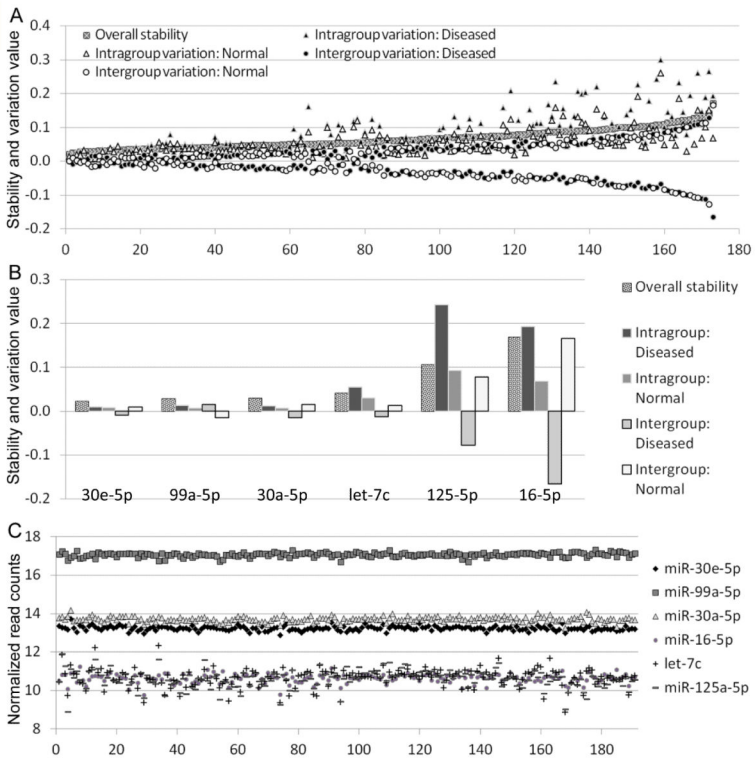

Fig. 2.

Evaluations of microRNA (miRNA) candidates for endogenous normalization. (a) NormFinder-determined intergroup and intragroup stabilities of 173 miRNA candidates; (b) intergroup and intragroup variations of six selected candidates; (c) normalized read counts of six selected candidates in 192 individuals.

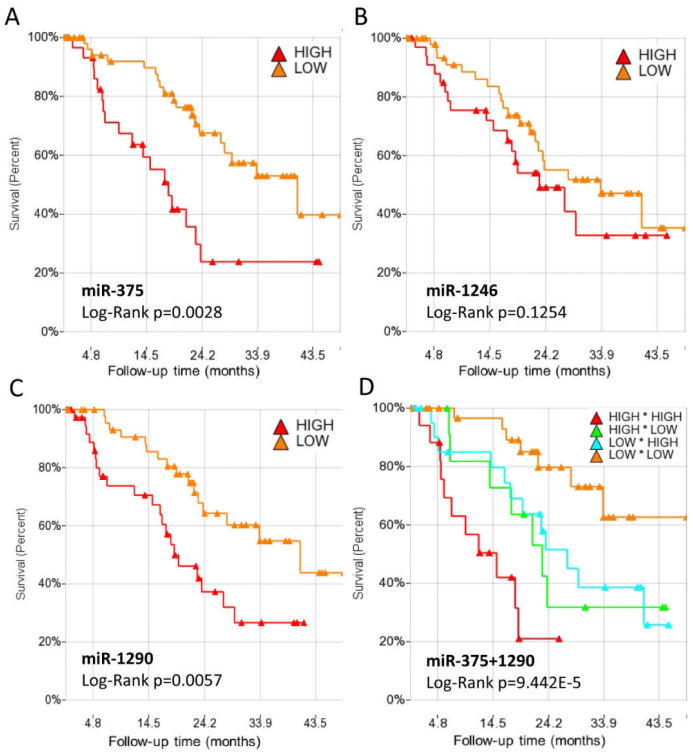

Fig. 3.

MicroRNAs (miRNAs)-based survival analysis in follow-up cohort (time in months). (a, c) Kaplan-Meier curves show that relative expression levels of miR-375 and miR-1290 are associated with overall survival; (b) miR-1246 shows a trend association but not statistically significance; (d) combination of miR-1290 and miR-375 expressions demonstrated a strong synergistic effect.

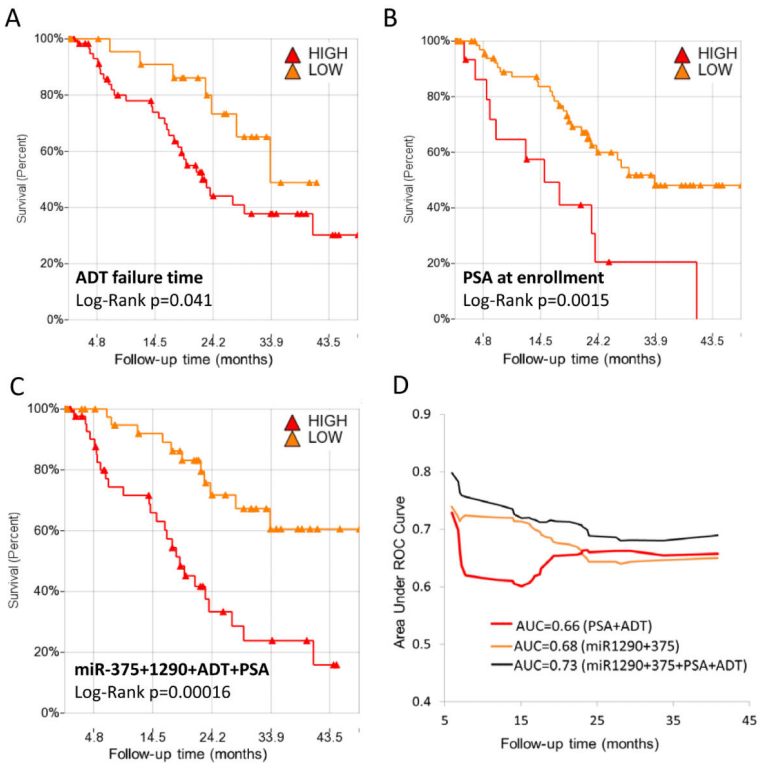

Fig. 4.

Multivariate-based survival analysis in follow-up cohort. Kaplan-Meier curves show significant survival association in androgen-deprivation therapy (ADT) failure time defined as from (a) initiation of ADT for hormone-sensitive stage to development of castration-resistant prostate cancer; (b) prostate-specific antigen (PSA) level; (c) multivariate model. (d) Time-dependent area under the curve (AUC) analysis shows significant improvement of multivariate model over clinical factors-only model.

Table 1

Clinical characteristics of castration-resistant prostate cancer patients in both cohorts

	Screening cohort	Follow-up cohort
Total number	23	100
Age at the time of CRPC and specimen draw, yr, median (range)	69.7 (53–86)	73.3 (43–92)
Gleason score at initial diagnosis		
5	0	3
6	0	10
7	10	32
8	4	19
9	9	26
10	0	3
Unknown	0	7
Clinical TNM staging at initial diagnosis		
T1	0	5
T2	12	36
T3	9	41
T4	1	1
Tx	1	17
N0	7	34
N1	7	15
N2	1	4
Nx	8	47
M0	17	82
M1	6	18
PSA at initial diagnosis, ng/ml, median (range)	10.8 (2.5–136.5)	21.4 (0.1–2324)
No. of patients treated with radical prostatectomy on initial diagnosis	12	49
No. of patients treated with radiation alone on initial diagnosis	3	25
No. of patients treated with radical prostatectomy and radiation on initial diagnosis	2	8
No. of patients who underwent salvage local treatments after primary prostate treatments	6	28
Time from initial treatments for localized stage disease to disease progression, mo, median*	12.94	26.47
Time from initial diagnosis to initiation of ADT for hormone-sensitive prostate stage, mo, median (range)†	26.89 (0–264)	12.2 (0–222.7)

	Screening cohort	Follow-up cohort
Time from initiation of ADT for hormone-sensitive prostate stage to progression for CRPC stage, mo, median (range)‡	19.08 (0.76–104)	19.4 (0.6–202.2)
Follow-up time from date of CRPC specimen collection to last follow-up, mo, median (range)	35.6 (7–47)	17.58 (1–48)
No. of patients dead during follow-up	10	37

ADT = androgen-deprivation therapy; CRPC = castration-resistant prostate cancer; PSA = prostate-specific antigen.

*Disease progression in subjects diagnosed and treated for localized stage disease was defined by treating physician on the basis of either serial PSA rise on two separate occasions after achieving a posttreatment nadir PSA value or appearance of new radiologic disease or with the initiation of a new cancer-specific intervention, whichever came first.

†ADT initiation for hormone-sensitive stage was defined as per treating physician and included either serial PSA rise on two separate occasions after achieving a nadir PSA value or appearance of new radiologic disease or with the initiation of a new cancer-specific intervention, whichever came first.

‡Progression to CRPC stage during ADT for hormone-sensitive stage was defined as per treating physician and included either serial PSA rise on two separate occasions after achieving a nadir PSA value during ongoing continuous ADT or appearance of new radiologic disease or with the initiation of a new cancer-specific intervention, whichever came first.

Table 2

Performance of endogenous control candidates in follow-up cohort

Gene name	NormFinder		BestKeeper		
	Stability	SE	r	*p*	Power, *x*-fold
TaqMan qRT-PCR using 10 RT primer pool					
GM-30a/e*	0.251	0.048	0.766	0.001	1.64
miR-30e-5p	0.311	0.046	0.73	0.001	1.65
miR-30a-5p	0.361	0.046	0.696	0.001	1.63
miR-99a-5p	0.743	0.061	0.592	0.001	1.99
let-7c	0.764	0.062	0.735	0.001	2.68
miR-16-5p	1.129	0.084	0.636	0.001	2.86
Best gene: GM-3-e/a					
TaqMan qRT-PCR using 4 RT primer pool					
GM-30a/e*	0.052	0.05	0.965	0.001	1.81
miR-30e-5p	0.054	0.049	0.967	0.001	1.86
miR-30a-5p	0.212	0.027	0.929	0.001	1.81
miR-16-5p	0.617	0.046	0.913	0.001	2.59
Best gene: GM-3-e/a					
Qiagen universal qRT-PCR assay					
GM-30a/e*	0.075	0.034	0.98	0.001	1.95
miR-30a-5p	0.183	0.023	0.956	0.001	1.84
miR-30e-5p	0.215	0.023	0.957	0.001	2.08
miR-16-5p	0.431	0.032	0.915	0.001	2.1
Best gene: GM-3-e/a					

qRT-PCR = quantitative reverse transcription polymerase chain reaction; SE = standard error.

*Geometric mean of miR-30a-5p and miR-30e-5p.