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Published in:
Carcinogenesis

DOI:
10.1093/carcin/bgz129

Publication date:
2020

Document version:
Accepted manuscript

Citation for pulished version (APA):
Souza, M. F. D., Kuasne, H., Barros-Filho, M. C., Cilião, H. L., Marchi, F. A., Fuganti, P. E., Rogatto, S. R., & Cólus, I. M. D. S. (2020). Circulating mRNA Signature as a Marker for High-Risk Prostate Cancer. *Carcinogenesis*, *41*(2), 139-145. https://doi.org/10.1093/carcin/bgz129

CIRCULATING mRNA SIGNATURE AS A MARKER FOR HIGH-RISK PROSTATE CANCER

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Abstract

Prostate cancer (PCa) is the second most common cancer in men. The indolent course of the disease makes the treatment choice a challenge for physicians and patients. In this study, a minimally invasive method was used to evaluate the potential of molecular markers in identifying patients with aggressive disease. Cell-free plasma samples from 60 PCa patients collected before radical prostatectomy were used to evaluate the levels of expression of eight genes (*AMACR*, *BCL2*, *NKX3-1*, *GOLM1*, *OR51E2*, *PCA3*, *SIM2,* and *TRPM8*) by quantitative real-time PCR. Overexpression of A*MACR*, *GOLM1*, *TRPM8,* and *NKX3-1* genes was significantly associated with aggressive disease characteristics, including extracapsular extension, tumor stage, and vesicular seminal invasion. A trio of genes (*GOLM1*, *NKX3-1*, and *TRPM8*) was able to identify high-risk PCa cases (85% of sensitivity and 58% of specificity), yielding a better overall performance compared with the biopsy Gleason score and PSA, routinely used in the clinical practice. Although more studies are required, these circulating markers have the potential to be used as an additional test to improve the diagnosis and treatment decision of high-risk PCa patients.

Summary

The levels of expression of eight cell-free circulating mRNAs were evaluated in plasma samples from prostate cancer patients. Three of them, *GOLM1*, *NKX3-1*, and *TRPM8* have the potential to identify high-risk prostate cancer.

Introduction

Although prostate cancer (PCa) is the second most common tumor in men worldwide (1), the disease often has an indolent course and the tumor progression is relatively slow (2). Prostate Specific Antigen (PSA)-based screening method can lead to an overdiagnosis, identifying high levels of PSA in patients with a low-grade disease and a favorable outcome (3). The estimate of PCa overdiagnosis ranges from 23% to 42% (4). The lack of markers to identify patients with worse outcome results in a large number of unnecessary surgeries, increases the costs of cancer treatment and morbidities rates (5).

The clinical decision regarding treatment is guided by ultrasonography, PSA levels and histopathological findings in prostate biopsies, which includes the Gleason score (6). The histopathological analysis of the PCa biopsy is considered the gold standard for diagnosis. However, the biopsy procedure presents risks (such as bleeding and sepsis) and, approximately 20% of PCa cases are detected only in the second biopsy (7). In addition, nearly 40% of patients are upgraded before prostatectomy (8) and radical prostatectomy (RP) has been the treatment option for about 60% of low-risk patients (9). The major side effects of RP are urinary incontinence (13.2% to 30.5% of patients) and sexual dysfunction (48.8% to 64.5%) (10,11). These factors demonstrate the relevance of identifying molecular markers able to distinguish patients with an aggressive tumor type from those with an indolent disease that could only be monitored (12).

Minimally invasive methods to identify biomarkers in biological samples, such as blood and urine, have the potential to contribute to treatment decisions. Several biological molecules have been described in plasma (13), including cell-free mRNA (cfmRNAs), which can be found in a free form or in exosomes (14). These molecules can be a promising tool for the development of a "liquid biopsy", which is a non-invasive procedure, and may be more specific and informative than protein biomarkers. Liquid biopsies can also be used for monitoring long-term disease progression, as found in prostate cancer (14). Recent studies have demonstrated the potentiality of cell-free RNAs in assisting the diagnosis and prognosis of breast and pancreatic tumors (15,16). In prostate cancer, only a few reports showed circulating mRNA in blood samples as markers useful for diagnosis (17-19) or poor outcome (20).

In a previous study, we evaluated 102 prostate cancer and 50 cancer-free individuals to identify circulating molecular markers. Among the quantified and analyzed genes, *OR51E2* and *SIM2* and the miR-200c and miR-200b were associated with PCa (21). In the current study, a subset of 60 of these 102 PCa was evaluated to identify circulating markers with potential to stratify the patients according to the risk of the disease (high or low). The ultimate goal was to assist patients and physicians in choosing the most appropriate treatment decision.

Material and methods

Patients

Peripheral blood samples were collected from 60 patients who have undergone radical prostatectomy at the Cancer Hospital of Londrina, Londrina, Paraná, Brazil, from 2014 to 2015. These patients were selected from a previous case-control study composed by 102 PCa and 50 cancer-free individuals (21). The study was approved by the institutional Human Research Ethics Committee (CAAE19769913.0.0000.5231). The patients signed an Informed Consent Form and provided information about their lifestyle and occupational exposures. Ethnic groups were determined according to the Brazilian Institute of Geography and Statistics (22). Clinical and histopathological data are summarized in Table 1. The average age of patients at diagnosis was 64.5±6.9 years. Low-grade PCa (pT1 to pT2c) was found in 60% of the patients.

Gene selection

The sample collection, RNA extraction, RT-qPCR, and data analysis were performed as previously described (21). The gene selection was based on a previous study which used an *in silico* analysis (21). Briefly, we selected differentially expressed genes found in the TCGA PCa cohort (PCa= 425; surrounding normal tissue= 52, t-test P adjusted< 0.001 fold change> 2), gross number of reads obtained from the sequencing data (values > 1,000)

presenting significant association with aggressive clinicopathological features ($P < 0.05$), and described in the literature as involved in prostate cancer.

Eight candidate genes (*AMACR*, *BCL2*, *NKX3-1*, *GOLM1*, *OR51E2*, *PCA3*, *SIM2,* and *TRPM8)* and two reference genes (*GAPDH* and *ACTB*) were tested in plasma samples from prostate cancer patients and cancer-free individuals. The reference genes, *GAPDH* and *ACTB*, are frequently reported as normalizers of RT-qPCR (23) and previously described as an ideal combination to be used in prostate cancer cell lines (24).

Statistical analysis

Statistical analysis was performed with GraphPad Prism Software v.5.0 (San Diego, California, USA) and SPSS v.22.0 (IBM Corp, Armonk, New York, USA). The patients submitted to prostatectomy were categorized into two groups: high-risk PCa: Gleason score $\geq$ 8 and/or tumor stage $\geq$ pT3a; and low-risk PCa: Gleason score < 8 and tumor stage < pT3a (25-27). Students t-test was used to compare mRNA expression levels between high- and low-risk cases and according to histopathological features. Significant genes (two-tailed P value < 0.05) were evaluated using a linear discriminant analysis. Receiver Operating Characteristic (ROC) curve was used to determine the effectiveness of the selected markers in comparison with the conventional pre-surgical tests (PSA > 20 ng/mL and/or a biopsy Gleason score between 8 to10) (25,26), and combined tests (Gleason score $\geq$8, PSA >20

and mRNA plasmatic markers). Sensitivity and specificity were determined using MedCalc Statistical Software v.16.8.4 (MedCalc Software bvba, Ostend, Belgium).

Results

The expression levels of *AMACR*, *BCL2*, *NKX3-1*, *GOLM1*, *OR51E2*, *PCA3*, *SIM2,* and *TRPM8* genes were investigated and compared with the histopathological features in a set of 60 PCa (Table 2). The comparison between high-risk with low risk PCa revealed increased expression levels of *GOLM1*, *NKX3-1*, and *TRPM8* genes in cases presenting extracapsular extension (FC = 14.3, *P* = 0.031; FC = 6.0, P = 0.008; and FC = 20.1, *P* = 0.004, respectively) and tumor stage ≥ pT3 (FC = 11.0, P = 0.041; FC = 4.7, *P* = 0.022; and FC = 16.5, P = 0.007, respectively).

Increased *AMACR* expression levels were found in patients with extracapsular extension (FC = 4.3; P = 0.031) while *GOLM1* overexpression was significantly found in patients that displayed vesicle seminal invasion (FC = 23.2, P = 0.004). Decreased *BCL2* expression levels were found in patients having bilateral tumors (FC = 0.2; P = 0.026). No significant association was found comparing the *OR51E2*, *SIM2* and *PCA3* expression levels with histopathological features.

Three genes were associated with higher risk of developing aggressive PCa: *NKX3-1* (FC = 4.5; P = 0.021), *TRPM8* (FC = 16.1; P = 0.011) and *GOLM1* (FC = 26.8; P =

0.003) (Figure 1A). The discriminant analysis revealed that 71.2% of all positive samples showed correlation among these genes (Figure 1B). The results of the biopsy Gleason score, PSA levels, plasma markers (*GOLM1*, *NKX3-1*, and *TRPM8* genes expression levels), and the combination of these markers to identify patients at high-risk for aggressive PCa are summarized in Table 3.

The association among the biopsy Gleason score, PSA levels, and *GOLM1*, *NKX3-1*, and *TRPM8* plasmatic markers showed 88% of sensitivity and 58% of specificity (Table 3, Figure 2A). A representative flowchart suggesting the stratification of PCa patients (high-risk and low-risk) according to the plasmatic markers analysis for treatment decision is provided at Figure 2A. The plasmatic markers presented a higher area under the curve (AUC) (AUC = 0.76) compared to the Gleason score > 8 (AUC = 0.66) and PSA > 20ng/mL (AUC = 0.66) (Figure 2B).

Discussion

Minimally invasive methods to identify prognostic markers have been highlighted in several tumor types in an effort to minimize the patients' morbidity (14,28). Among these methods, circulating nucleic acids have stood out due to their specificity and stability (14,28). In prostate cancer, circulating markers have the potential to be a useful tool for early

identification of the disease and to contribute for medical decisions, including the identification of patients who could benefit from radical prostatectomy (29).

Herein, the candidate genes *AMACR*, *BCL2*, *NKX3-1*, *GOLM1*, *OR51E2*, *PCA3*, *SIM2* and *TRPM8* and two references (*GAPDH* and *ACTB*) were evaluated by RT-qPCR in plasma samples from prostate cancer patients and cancer-free individuals to identify predictive markers for PCa aggressiveness. Although the reference genes used to measure circulating mRNA are still under debate, the quantification of these RNAs levels relative to a set of reference genes is usually described (15,30). The reference genes *GAPDH* and *ACTB,* used in our study, are frequently described for normalization of RT-qPCR analysis in cfmRNA from blood or tissues samples (30,31). The combination of two or three reference genes is recommended to normalize the gene expression data (32). Previously, we reported two circulating mRNAs (*OR51E2* and *SIM2)* and two miRNAs (miR-200c and miR-200b) differentially expressed (normalized with *GAPDH* and *ACTB* and *RNU6B* and *RNU48,* respectively) in PCa patients compared with health controls (21). These circulating candidate markers presented 67% of sensitivity and 75% of specificity for PCa diagnosis. Additionally, *OR51E2* and *SIM2* presented high sensitivity to detected PCa patients with normal PSA levels (21).

We detected increased expression levels of *AMACR*, *GOLM1*, *NKX3-1*, and *TRPM8* cfmRNAs in PCa patients with histopathological characteristics of an aggressive phenotype

(extracapsular extension, tumor stage ≥ pT3 and/ or seminal vesicle invasion). Patients with pT3 clinical stage present a high-risk of recurrence (25), and those showing extracapsular extension have no benefits of prostatectomy alone and need adjuvant therapy, which is one of the main features associated with tumor aggressiveness (33,34). Furthermore, patients with seminal vesicle invasion have a high risk for cancer progression, both regionally and systemically (35).

Although in our previous study the relative expression of *OR51E2* and *SIM2* was statistically significant in the comparison between patients and controls (21), no difference was observed according to the histopathological characteristics. As prostate cancer is a heterogeneous disease characterized by several genetic and epigenetic alterations during its development (36), it is improbable that the same marker could be used for diagnosis and prognosis.

The *NKX3-1* has been associated with prostate cell differentiation, and its loss of function was reported in prostate cancer initiation (36). On the other hand, *NKX3-1* overexpression has been described in patients with high-grade PCa (37,38). The androgenic cells can activate the expression of *NKX3-1* autonomously through the binding of the androgen receptor (AR) to the 11- region (39). This recent finding reinforces the main role of *NKX3-1* in prostate cancer progression and its potential as a circulating biomarker.

Interestingly, it was recently proposed that *TRPM8* (transient receptor potential cation channel subfamily M member 8) has an important role in cell migration and tumor progression (40). TRP channels are expressed in endothelial cells acting in tumor vascularization. In prostate, TRPM8 acts as a testosterone receptor (41), being highly expressed in prostate cancer cells (42,43). Consequently, the TRP channel has been described as a new target for the development of therapeutic strategies in prostate cancer cells and is a promisor marker for this disease.

Although not fully understood in normal prostate physiology, increased transcript levels of *AMACR* and *GOLM1* have been reported in PCa, suggesting their potential as markers of malignancy (44-46). AMARC protein overexpression has been described in 90% of PCa and extensively described as a diagnostic marker, including in precipitates of urine from PCa patients (47,48). Furthermore, *AMACR* was associated with PCa progression (49). Here, we reported *AMACR* and *GOLM1* overexpression in plasma from PCa patients having a worse outcome. Overall, these genes are potential circulating markers to predict prostate cancer aggressiveness.

Low-risk prostate cancer overtreatment involves health-related costs and increased morbidity. The physicists have difficulty to classify patients with PSA lower than 20ng/mL and Gleason score lower than 8 in high- or low-risk to develop aggressive prostate cancer. In general, high-risk patients are treated and those with low-risk undergo active surveillance.

Thus, there is a critical need for the development of additional prognostic tools to identify patients with aggressive PCa, even though these cancers may initially present a low-risk in the first pathological assessment in biopsies (50,51). In our study, a combination of three circulating mRNAs (*GOLM1*, *NKX3-1,* and *TRPM8*) was able to discriminate high-risk from low-risk prostate cancer patients. The addition of cfmRNA markers with the PSA and Gleason biopsy score increased the sensitivity of 15% to 85% for detecting high-risk patients. Based on these findings, we suggest the use of the trio of cfmRNA markers as a tool to better stratify the risk of developing aggressive PCa.

Although this novel approach could yield important benefits for patients and the overall healthcare system, our study presents some limitations including the analysis using the TCGA dataset, the sample size, and the RT-qPCR as the sole procedure to point out the candidate biomarkers. Although the TCGA prostate cancer dataset is of high relevance to be used as an external validation group, its use must be cautious. In general, the data are not curated, the samples were analyzed by different research groups, and they may come from different ethnical populations with distinct pathological features. In our study, a trio of genes (*GOLM1*, *NKX3-1,* and *TRPM8*) showed differential expression and, therefore, was capable to discriminate PCa patients with high- and low-risk of aggressiveness. However, it is essential to carry out randomized studies using different molecular strategies involving a large number of patients from different populations to verify the reproducibility of

this test. Our study highlighted circulating transcripts as predictive PCa candidate markers with potential applicability in making decisions regarding treatment in the next future.

Funding

This study was supported by grants from the Araucaria Foundation of Support for the Scientific and Technological Development of the State of Parana (185/2014); National Institute of Science and Technology in Oncogenomics (Sao Paulo Research Foundation FAPESP 2008/57887-9 and the Brazilian National Council for Scientific and Technological Development CNPq 573589/08-9); Coordination of Improvement of Higher Education Personnel - Brazil (CAPES/PROAP).

Acknowledgements

The authors would like to thank all patients, Londrina Cancer Hospital and the Intermunicipal Health Consortium of the Middle Paranapanema (CISMEPAR), Brazil.

Conflict of Interest Statement: None declared.

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TABLE AND FIGURES LEGENDS

Table 1. PSA = Prostate-Specific Antigen. NI: not informed

Table 2. PSA: Prostate-Specific Antigen; FC: fold change or relative gene expression; *P*: Student's t-test; In bold: statistically significant values (P < 0.05); *Evaluated in post-surgical material; #Measured before surgery

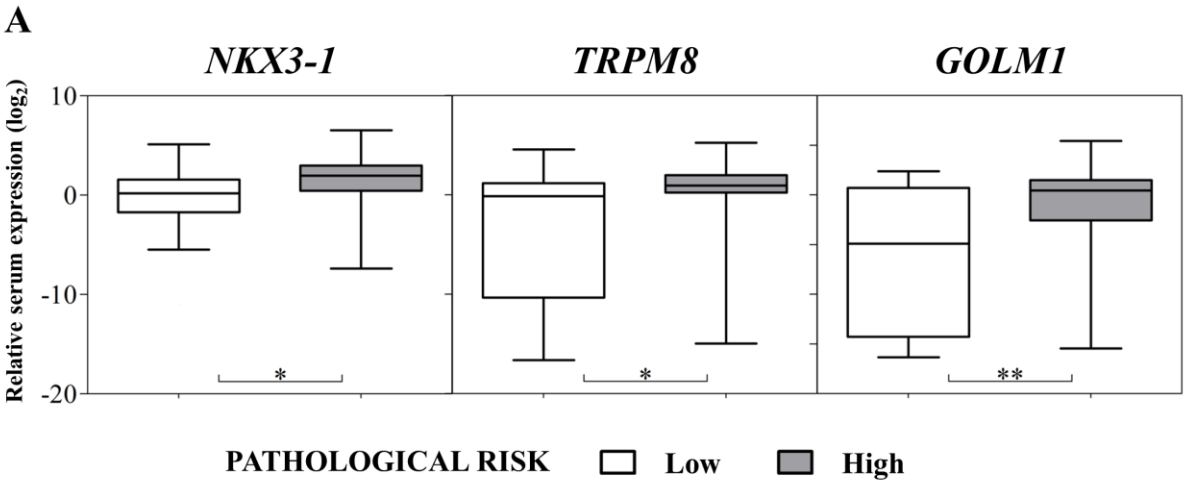
Table 3. [a]Biopsy parameters; [b]combined analysis using Gleason score >8, PSA >20 and mRNA plasmatic markers, PSA = Prostate-specific antigen; PPV=Positive Predictive Value; NPV=Negative Predictive Value

Fig. 1. *NKX3-1, GOLM1, and TRPM8* expression levels were significantly able to differentiate high-risk PCa (Gleason score ≥ 8 and/or tumor stage ≥ pT3a) from low-risk PCa (Gleason score < 8 and tumor stage < pT3a). A) Differential expression of circulating mRNAs in plasma samples. B) Three-dimensional graph representing three makers that differentiate high-

risk from low-risk PCa patients. Data are shown by relative expression log_2. Statistically significant *P < 0.05; **P < 0.01.

Fig. 2. A) Flowchart representative of the suggestive approach to select high-risk and low-risk PCa patients for treatment decision based on the use of the selected plasmatic markers. (one of 60 patients was excluded due to lack of PSA levels information). B) ROC curve representation of the markers used to identify high-risk prostate cancer.

Figure 1



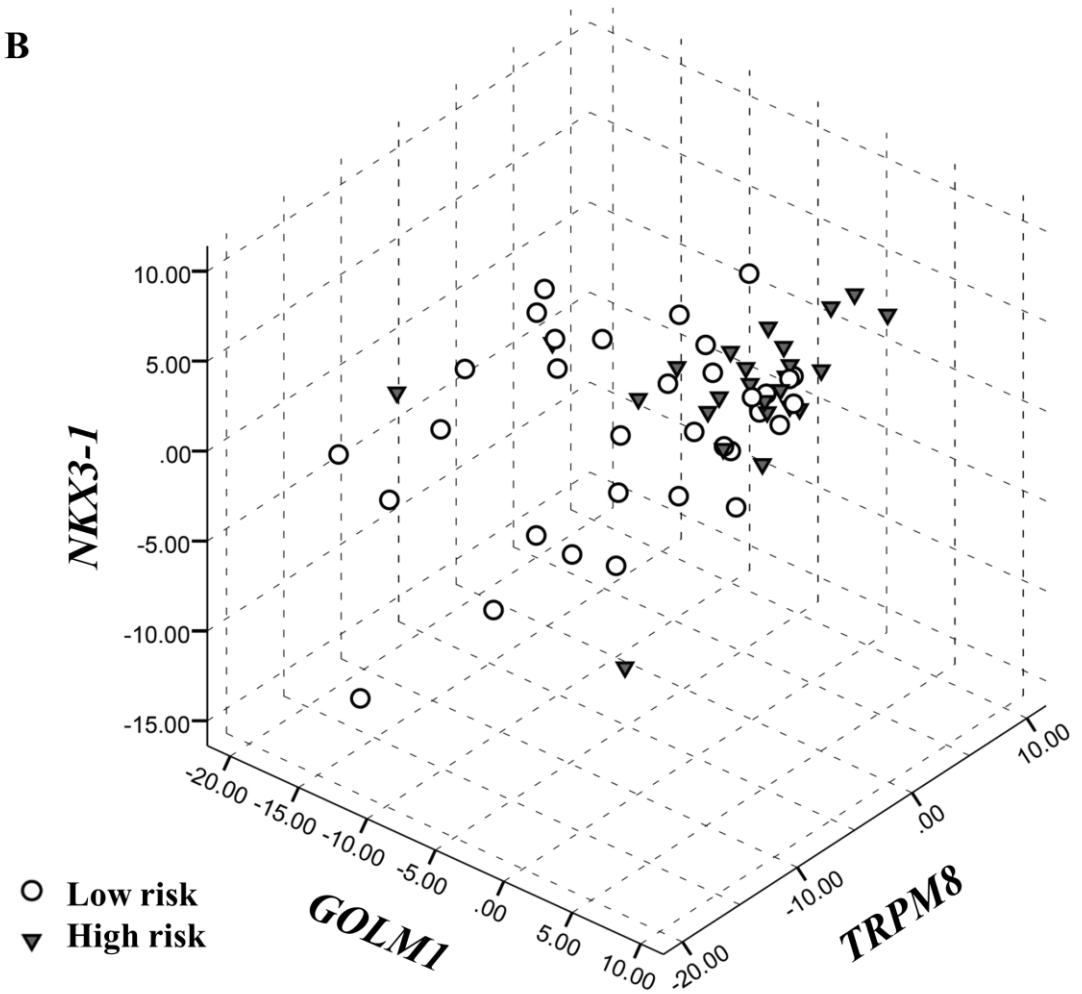


Figure 2

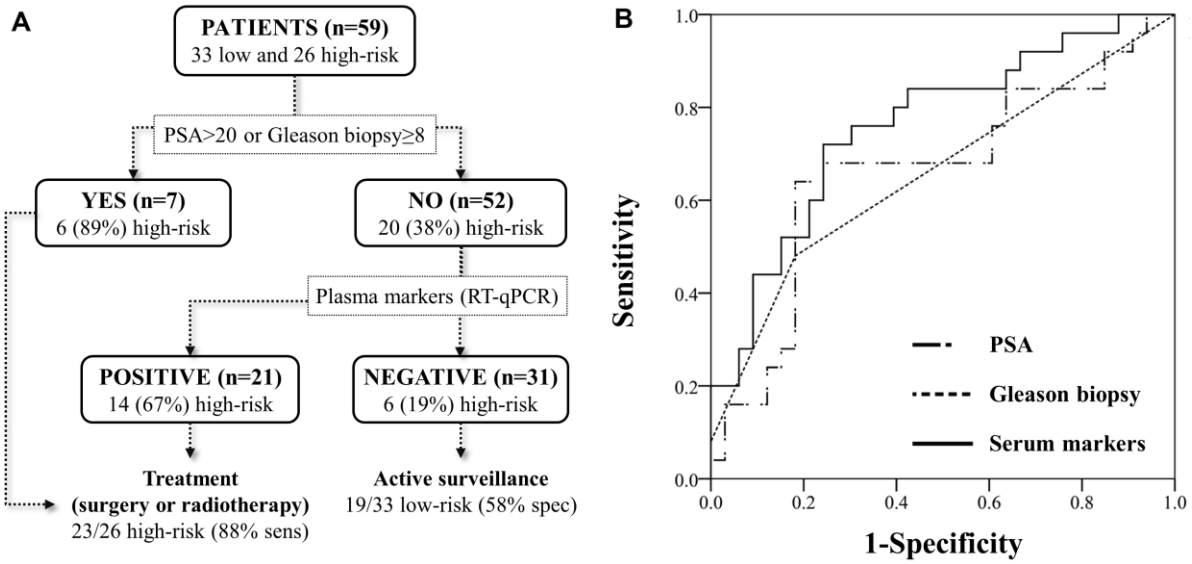


Table 1: Clinical and histopathological characteristics of prostate cancer patients

Characteristics		Patients n (%)
Age (years)	< 65	26 (43.3)
	≥ 65	34 (56.7)
Ancestrally	Caucasian	45 (75.0)
	African	15 (25.0)
Family history of cancer	Yes	40 (66.7)
	Yes, Prostate Cancer	*9 (15.0)*
	No	20 (33.3)
PSA (ng/mL)	≤ 4.0	7 (11.7)
	>4.0 to 10.0	27 (45.0)
	> 10.1 to 19.9	21 (35.0)
	≥ 20	5 (8.3)
Gleason score	6 - 7(3+4)	50 (83.3)
	7(4+3) - 9	10 (9.8)
Extracapsular extension	Presence	21 (35.0)
	Absence	37 (61.7)
	NI	2 (3.3)
Seminal vesicle invasion	Presence	5 (8.3)
	Absence	54 (90.0)
	NI	1 (1.7)
Perineural invasion	Presence	3 (5.0)
	Absence	55 (91.7)
	NI	2 (3.3)
Bilateral tumor	Presence	38 (63.3)
	Absence	21 (35.0)
	NI	1 (1.7)
Lymph node invasion	Presence	2 (3.3)
	Absence	40 (66.7)
	NI	18 (30.0)
Tumor stage	pT1 to pT2c	36 (60.0)
	pT3 to pT4	21 (35.0)
	NI	3 (5.0)

Table 2: Differential gene expression results in plasma samples from prostate cancer patients

Histopathological parameters*		Genes							
		AMACR	***BCL2***	***GOLM1***	***NKX3-1***	***OR51E2***	***PCA3***	***SIM2***	***TRPM8***
PSA (> 20 ng/mL)#	FC	0.8	0.4	3.5	2.4	0.5	5.0	0.2	0.8
	P	0.810	0.401	0.556	0.453	0.625	0.404	0.378	0.916
Gleason score (≥ 8)	FC	0.3	2.5	35.1	0.8	2.9	30.6	0.5	0.5
	P	0.815	0.558	0.183	0.888	0.576	0.159	0.715	0.781
Extracapsular extension (positive)	FC	4.3	3.3	14.3	6.0	1.2	4.0	1.4	20.1
	P	**0.031**	0.094	**0.031**	**0.008**	0.868	0.222	0.696	**0.004**
Seminal vesicle invasion (positive)	FC	1.4	2.4	23.2	3.2	1.4	10.4	10.6	16.2
	P	0.755	0.473	**0.004**	0.319	0.817	0.226	0.139	0.178
Perineural invasion (positive)	FC	0.9	6.8	1.7	0.6	1.1	18.0	21.5	40.3
	P	0.916	0.222	0.849	0.744	0.978	0.243	0.131	0.160
Bilateral tumor (positive)	FC	0.6	0.2	0.4	0.3	0.7	3.5	1.0	0.3
	P	0.509	**0.026**	0.474	0.064	0.706	0.327	0.980	0.372
Lymph node invasion (positive)	FC	24.6	21.9	25.0	13.2	4.2	26.6	12.4	20.9
	P	0.110	0.131	0.337	0.203	0.530	0.263	0.181	0.333
Tumor stage (≥ pT3)	FC	3.7	2.0	11.0	4.7	1.6	3.3	1.0	16.5
	P	0.052	0.333	**0.041**	**0.022**	0.576	0.293	0.995	**0.007**

Table 3: Performance of markers to distinguish high-risk *versus* low-risk prostate cancer

Markers	Sensitivity % (IC95%)	Specificity % (IC95%)	PPV % (IC95%)	NPV % (IC95%)
Gleason score[a] (>8)	8 (1-26)	100 (89-100)	100 (20-100)	59 (56-62)
PSA (>20)	15 (4-35)	97 (84-100)	80 (32-97)	59 (55-63)
mRNA plasmatic markers	85 (65-96)	58 (39-75)	61 (51-71)	83 (65-92)
Combined analysis[b]	88 (85-98)	58 (22-75)	62 (52-71)	86 (68-95)