

1 ARTICLE

2 **Cancer/Testis Antigens Differentially Expressed in**
3 **Prostate Cancer: Potential New Biomarkers and Targets**
4 **for Immunotherapies**

5 Luciane T. Kagohara^{1,\$,¥}, Neil M. Carleton^{1,2}, Sayuri Takahashi^{1,*}, Takumi Shiraishi^{1,#}, Steven M.
6 Mooney^{1,€}, Robert L. Vessella³, Robert H. Getzenberg^{1,∞}, Prakash Kulkarni^{1,€}, Robert W. Veltri¹.

7 ¹ Brady Urological Institute, Department of Urology, Johns Hopkins University, Baltimore, MD;
8 Itsukam1@jhmi.edu (L.T.K.); nmc-42@pitt.edu (N.M.C.); t-sayuri@athena.ocn.ne.jp (S.T.); takumi14@koto.kpu-
9 m.ac.jp (T.S.); s2mooney@uwaterloo.ca (S.M.M.); rgetzenb@nova.edu (R.G.); pkulkarni@coh.org (P.K.);
10 rveltri11@gmail.com (R.W.V.)

11 ² Department of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA; nmc-42@pitt.edu (N.M.C.)

12 ³Department of Urology, University of Washington, Seattle, WA; vessella@uw.edu (R.L.V.)

13 [¥] Correspondence: Itsukam1@jhmi.edu (L.T.K.)

14 Current affiliations:

15 ^{\$}Department of Oncology, Johns Hopkins University, Baltimore, MD

16 ^{*}Department of Urology, The University of Tokyo, Tokyo, Japan

17 [#]Department of Urology, Kyoto Prefectural University of Medicine, Kyoto, Japan

18 [€]Department of Biology, University of Waterloo, Waterloo, ON Canada N2L 3G1

19 [∞]Dr. Kiran C. Patel College of Allopathic Medicine, Nova Southeastern University, Fort Lauderdale, FL

20 ^{CA}Department of Medical Oncology and Experimental Therapeutics, City of Hope National Medical Center, Duarte,
21 CA

22 **Abstract:** Current clinical tests for prostate cancer (PCa), such as the PSA test, are not fully capable of
23 discerning patients that are highly likely to develop metastatic prostate cancer (MPCa). Hence, more
24 accurate prediction tools are needed to provide treatment strategies that are focused on the different
25 risk groups. Cancer/testis antigens (CTAs) are expressed during embryonic development and present
26 aberrant expression in cancer making them ideal tumor specific biomarkers. Here, the potential use of a
27 panel of CTAs as a biomarker for PCa detection as well as metastasis prediction is explored. We
28 initially identified eight CTAs (*CEP55*, *NUF2*, *PAGE4*, *PBK*, *RQCD1*, *SPAG4*, *SSX2* and *TTK*) that are
29 differentially expressed in MPCa when compared to local disease and used this panel to compare the
30 gene and protein expression profiles in paired PCa and normal adjacent prostate tissue. We identified
31 differential expression of all eight CTAs at the protein level when comparing 80 paired samples of PCa
32 and the adjacent non-cancer tissue. Using multiple logistic regression we also show that a panel of these
33 CTAs present high accuracy to discriminate normal from tumor samples. In summary, this study
34 provides evidence that a panel of CTAs, differentially expressed in aggressive PCa, is a potential
35 biomarker for diagnosis and prognosis to be used in combination with the current clinically available
36 tools and is also a potential target for immunotherapy development.

37 **Keywords:** Cancer/testis antigens; prostate cancer; gene expression; immunohistochemistry; biomarker;
38 immunology

39

40 **1. Introduction**

41 Prostate cancer (PCa) is the most prevalent cancer type among men and the second leading cause
42 of male cancer-associated deaths in the United States accounting for an estimated 165,000 new cases

43 and 30,000 deaths in 2018 . While local tumors are successfully treated, metastatic PCa (MPCa) remains
44 an incurable disease with a 30% 5-year survival rate [1]. The most common treatment for advanced PCa
45 consists of androgen ablation to which most patients are responsive; however, a great proportion of
46 men progress with metastatic castration-resistant PCa (mCRPC) and die from the disease. Although
47 much progress in the treatment of mCRPC was made in the last decade, improvements regarding
48 survival are still measured in months [2,3].

49 PCa screening and disease control is largely based on the prostate specific antigen (PSA) test that
50 was initially introduced as a follow-up instrument for the detection of recurrence and progression to
51 metastatic disease. Subsequently, its potential as an early diagnostic tool was explored [4,5] and PSA
52 was accepted as a standard test to identify men at risk of PCa before any symptoms appeared. Thus,
53 PSA was heralded as a promising early detection biomarker [5]. However, PSA screening has been
54 considered a controversial assessment since many men are over-diagnosed and over-treated since PSA
55 is not capable of differentiating more indolent from aggressive disease. It is estimated that 23% to 60%
56 of men, with increased PSA levels present with prostate tumors that would remain clinically
57 insignificant during their lifetime [6]. Unfortunately, these men who present with increased PSA may
58 be submitted to unnecessary aggressive and invasive treatment and its consequent comorbidities [6–8]
59 [6–8]. The use of active surveillance programs in men who are considered to have very low and low risk
60 prostate cancer has had a major impact on over-treatment but one of the major dilemmas in PCa
61 remains to identify patients with aggressive tumors at an early stage so that they can benefit from
62 immediate definitive treatment. PSA based tests such as the Prostate Health Index (phi) and the 4K
63 Score, are options to predict more accurately detect PCa [9]. The first test, that measures total, free and
64 [-2]proPSA [10]; is FDA approved and have shown to be an important tool for risk stratification [11,12].
65 The 4K Score measures four kallikrein markers (total, free and intact PSA and hK2) and presents the
66 same performance and is also associated with the risk of MPCa [13,14]. Still, additional molecular
67 biomarkers for a combined test are crucial to categorize tumors according to their aggressive potential
68 in a more accurate manner and to stratify men with PCa into more appropriate treatment strategies.

69 Cancer/testis antigens (CTAs) constitute an important class of cancer biomarkers that have not
70 been fully explored, especially in PCa [15–18]. CTAs by definition are normally expressed in testis and
71 other developmentally regulated tissues (e.g., placenta) but are aberrantly expressed in many types of
72 cancers [19]. This unique pattern of expression makes these genes attractive candidates as biomarkers
73 and, together with their immunogenic capacity, also good targets for the development of cancer
74 immunotherapy [20–22]. The aberrant expression of CTAs in different cancer types is associated with
75 phenotypic changes that confer cancer cells added advantages for proliferation and survival [23,24]. In
76 a previous study, Takahashi et al. [25] evaluated the expression of 22 CTAs in localized (LPCa) and
77 MPCa. Five of the CTAs (*CEP55*, *NUF2*, *PAGE4*, *PBK* and *SPAG4*) were differentially expressed
78 between the two groups, suggesting that CTAs have the potential as biomarkers for differentiating
79 aggressive PCa. However, since it was a retrospective study, the possibility of using these CTAs as
80 predictors for MPCa could not be assessed.

81 In this study, we used the data generated by Takahashi et al. [25] to create a panel of CTA genes
82 that are differentially expressed between LPCa and MPCa, and used this gene set to develop a panel of
83 biomarkers for PCa screening. We hypothesize that using a panel of genes differentially expressed in
84 advanced PCa early in the screening process would facilitate the early prediction of patients that will
85 develop metastasis. In addition to Takahashi et al. analysis [25], we used a statistical multivariate
86 logistic regression (MLR) model to identify with more stringency, a panel of potential CTA candidates
87 as biomarkers for aggressive tumors. We found that, among the CTAs evaluated in the current study,
88 *PAGE4* is down-regulated (undetectable) in 100% of MPCa cases. Thus, *PAGE4* is a promising
89 candidate to discriminate indolent from aggressive cases. Also, our results showed that the CTAs
90 *CEP55*, *NUF2*, *PBK* and *TTK* were up-regulated in MPCa and their combined pattern of expression was
91 capable of differentiating metastatic from non-metastatic tumors. Finally, we evaluated the expression

92 of this CTA panel in normal and tumor paired tissues from PCa patients who were treated with radical
93 prostatectomy to identify their potential as screening biomarkers. We observed significant variation in
94 mRNA and protein expression levels of all these CTAs, suggesting that the changes in expression occur
95 before metastasis development and could be used as early diagnostic and prognostic biomarkers.

96 **2. Materials and Methods**

97 *2.1. Clinical Samples*

98 Samples from clinically localized PCa (LPCa) (n=20) and soft tissue metastasis (MPCa) (n=20) were
99 obtained at University of Washington from radical prostatectomies and autopsies, respectively. The age
100 range of the patients with clinically LPCa was 48-75 years (median, 58 years) and a preoperative serum
101 PSA median of 7.54 (ng/ml) (range, 2.4-64.0). The Gleason Score was: 6 (n=3), 7 (n=14), 8 (n=1) and 9
102 (n=2). Soft tissue metastasis were obtained from lymph node (n=8), liver (n=5), adrenal (n=1), bladder
103 (n=1), kidney (n=1), lung (n=1) and pancreas (n=1). The specimens were used with the approval of the
104 University of Washington Institutional Review Board. Complete demographic and clinical data are
105 presented on **Supplementary Table 1**. Approximately 30 to 100mg of fresh tissue (with no dimension
106 greater than 0.5cm) was collected and placed in RNAlater Solution (Ambion, Austin, TX). Samples were
107 stored at 4°C for 1-7 days to allow solution to thoroughly penetrate the tissue and then maintained at -
108 20°C until RNA extraction [25].

109 RNA samples from matched tumor and normal adjacent tissues were obtained from the Prostate
110 Cancer Biorepository Network (PCBN). Using the standard operating procedure (SOP) protocols, as
111 previously described in detail [26], RNA was isolated from 24 radical prostatectomy specimens. The
112 grade and stage of each case are listed in **Supplementary Table 2**. Each case consisted of fresh-frozen
113 tumor and benign tissues obtained at radical prostatectomy. Cancer samples were macro-dissected to
114 ensure the presence of at 70% to 90% tumor cells.

115 The paired normal and PCa samples for immunohistochemistry assays were included in tissue
116 microarrays (TMAs). The two TMAs included 80 unique prostate cancer patients representing different
117 Gleason scores (3+3, 3+4, 4+3, and ≥8) with quadruplicates of cancer and cancer-adjacent normal areas.
118 The detailed demographics of the total 80 cases stratified by Gleason scores are shown in
119 **Supplementary Table 3**.

120 *2.2. RNA isolation*

121 RNA from 20 paired normal and PCa from PCBN were obtained using Trizol (Invitrogen). RNA
122 quantification and integrity were assessed by Nanodrop and 2100 Bioanalyzer (Agilent Technologies).
123 Additional information for PCBN SOPs can be found at
124 <http://www.prostatebiorepository.org/protocols>.

125 *2.3. Nanostring gene expression analysis*

126 Nanostring nCounter Gene Expression Assay (NanoString Technologies, Seattle, WA) gene
127 expression data were obtained previously for the LPCa and MPCa cohort [25]. The Nanostring
128 approach was performed for 22 CTA genes (*CEP55, CSAG2, CTAG1B (NY-ESO-1), JARID1B, MAGEA1,*
129 *MAGEA2, MAGEA6, MAGEA12, NOL4, NUF2, PAGE4, PBK, PLAC1, RQCD1, SEMG1, SPAG4, SSX2,*
130 *SSX4, TMEFF2, TMEM108, TPTE and TTK*). The CTA genes were selected by mining publicly available
131 microarray data from the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) in conjunction
132 with our own data [27,28]. *ACTB* was used as the housekeeping gene for normalization.

133 *2.4. qRT-PCR gene expression analysis*

134 One microgram of total RNA was used for cDNA synthesis using the iScript cDNA Synthesis Kit
135 (Bio-Rad Laboratories, Inc., Hercules, CA). The PCR reactions were performed with 0.2 μ l of cDNA
136 template in 25 μ l of reaction mixture containing 12.5 μ l of iQ SYBR Green Supermix (Bio-Rad
137 Laboratories, Inc.) and 0.25 μ mol/L each primer. PCR reactions were subjected to hot start at 95°C for 3
138 minutes followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds,
139 and extension at 72°C for 30 seconds using the CFX96 Real-Time PCR Detection System (Bio-Rad
140 Laboratories, Inc.). Analysis and fold differences were determined using the comparative threshold
141 cycle method. ACTB was the housekeeping gene used for normalization. Primers' sequences for the
142 CTAs evaluated are shown in **Supplementary Table 4**.

143 *2.5. Immunohistochemistry*

144 The TMA slides were deparaffinized using xylene, and tissues were rehydrated in decreasing
145 concentrations of ethanol (100%, 75%, 50%, and 25%; all vol/vol). Antigen retrieval was performed at
146 controlled pH values under heat, followed by endogenous peroxidase inhibition using 0.3% hydrogen
147 peroxidase. TMA slides were incubated for 1h at room temperature with a proprietary protein block,
148 Protein Block Serum Free reagent (Dako). Primary antibody incubation was performed at 4°C overnight
149 using the ideal dilution for each antibody (**Supplementary Table 5**). Primary antibody was washed
150 with 1X PBS, and secondary antibody (1:200) was added to the slides and incubated for 1h at room
151 temperature. Antigen localization was developed using 3,3'-diaminobenzidine chromogen. Tissue
152 samples were counterstained in hematoxylin and dehydrated in ethanol and xylene.

153 For quantitative IHC (qIHC) analysis, slides were scanned using the Aperio Scanscope XT (Leica
154 Biosystems) and the staining quantifications were performed using Aperio Imagescope v12.3 software
155 (Leica Biosystems). Intensity and frequency of positive staining are determined by the pixel count of the
156 delimited area selected for analysis. Intensity (different brown-staining shades) for a determined area is
157 given as the total brown pixel count for that region. The frequency (area of positive staining) is given by
158 the ratio of positive brown region and the total area selected for analysis (positive + negative area).
159 Protein expression differences between the paired normal and tumor areas were compared using the
160 Wilcoxon matched-pairs test. The average for all cores available from each patient for qIHC analysis
161 was calculated, and the values were used to compare medians between the groups (tumor vs. benign).
162 Protein expression (frequency or intensity) was considered significantly different for a *P* value \leq 0.05.

163 *2.6. Statistical analysis*

164 Receiver Operator Characteristic (ROC) curves were used to identify CTAs with a high probability
165 of accurately discriminating between localized and metastatic PCa or tumor and non-tumor cases. Gene
166 expression changes were considered significant when AUC $>$ 0.7. Wilcoxon signed-rank or Mann-
167 Whitney non-parametric test were used to compare CTA gene expression means between LPCa vs.
168 MPCa and benign vs. tumor tissues, respectively. Gene expression differences were considered
169 significant when *P* value \leq 0.05. After the best individual genes were identified, the multivariate logistic
170 regression (MLR) backward stepwise model was used to identify a CTA panel (with high specificity,
171 sensitivity and significant AUC) capable of discriminating LPCa from MPCa or tumor from benign
172 cases. All statistical analyses were performed using STATA version 13.

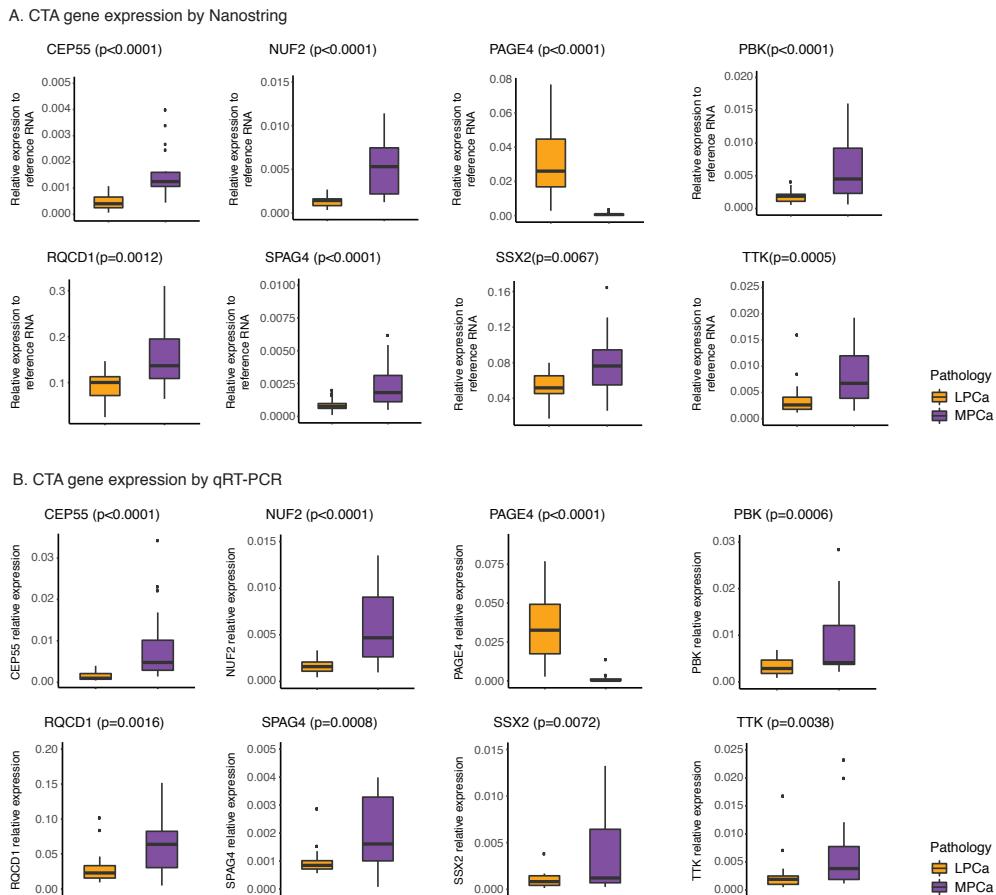
173 **3. Results**

174 *3.1. Differential CTA gene expression in LPCa and MPCa*

175 Nanostring is a digital multiplex approach in which multiple mRNAs can be absolutely quantified
176 making the cDNA synthesis step unnecessary. Using this approach, Takahashi et al. [25] measured the

177 expression of a panel of 22 CTA genes. All analyses were normalized using *ACTB* as a house-keeping
178 gene. Here, we used the previously published dataset to perform a more stringent statistical analysis to
179 identify CTAs that can accurately discriminate LPCa from MPCa.

180 We performed ROC analyses to verify the accuracy of each biomarker expression profile in
181 discriminating LPCa from and MPCa samples. To classify the 22 CTA genes (*CEP55*, *CSAG2*, *CTAG1B*
182 (*NY-ESO-1*), *JARID1B*, *MAGEA1*, *MAGEA2*, *MAGEA6*, *MAGEA12*, *NOL4*, *NUF2*, *PAGE4*, *PBK*, *PLAC1*,
183 *RQCD1*, *SEMG1*, *SPAG4*, *SSX2*, *SSX4*, *TMEFF2*, *TMEM108*, *TPTE* and *TTK*) as good markers to
184 discriminate indolent and aggressive cases, we used a cutoff $AUC \geq 0.7$. ROC curve analysis was also
185 used to determine the highest specificity, sensitivity, positive (PPV) and negative prediction (NPV)
186 values that maximize the cases correctly classified. Expression level means were compared to assure
187 that the differences found were significant. Nanostring multiplex gene expression analysis of the CTA
188 genes showed down-regulation of *PAGE4* and up-regulation of *CEP55*, *MAGEA2*, *NUF2*, *PBK*, *RQCD1*,
189 *SPAG4*, *SSX2*, and *TTK* in MPCa (compared with LPCa) (Figure 1A, Table 1 and Supplementary
190 Figure 1) with AUC above the cutoff established, suggesting that each of the CTAs was capable of
191 discriminating the two groups. *PAGE4* was at undetectable levels in all MPCa cases.



192 **Figure 1** – Cancer/testis antigens (CTA) gene expression analysis in localized (LPCa) and metastatic (MPCa)
193 prostate cancer. Representation of gene expression measured by Nanostring (A) and by qRT-PCR (B). Nanostring
194 relative gene expression is the ratio between CTA and ActinB measured. For the qRT-PCR the relative gene
195 expression calculation was performed using the $2^{-\Delta Ct}$ approach using ActinB as the housekeeping gene. Wilcoxon
196 signed-rank test was used to compare means between LPCa and MPCa groups. Gene expression differences were
197 considered significant when P value ≤ 0.05 . *PAGE4* is down-regulated in MPCa while all other CTAs present
198 increased expression. Nanostring results were confirmed by qRT-PCR in the same cohort (technical validation).

Table 1 - Localized and metastatic prostate cancer gene expression ROC analysis for 22 CTAs.

CTA	NANOSTRING					qRT-PCR				
	AUC	SENSITIVITY	SPECIFICITY	PPV	NPV	AUC	SENSITIVITY	SPECIFICITY	PPV	NPV
PAGE4	0.99	95.00	95.00	95.00	95.00	0.99	95.00	90.00	90.48	94.74
CEP55	0.97	90.00	90.00	90.00	90.00	0.91	80.00	90.00	88.89	81.82
NUF2	0.92	80.00	95.00	94.12	82.61	0.89	70.00	90.00	87.50	75.00
PBK	0.86	65.00	90.00	86.67	72.00	0.81	55.00	85.00	78.57	65.38
SPAG4	0.85	70.00	80.00	77.78	72.73	0.80	65.00	95.00	92.86	73.08
TTK	0.81	65.00	85.00	81.25	70.83	0.76	50.00	90.00	83.33	64.29
RQCD1	0.79	65.00	85.00	81.25	70.83	0.79	60.00	85.00	80.00	68.00
SSX2	0.75	65.00	65.00	65.00	65.00	0.75	50.00	95.00	90.91	65.52
MAGEA2	0.71	45.00	80.00	69.23	59.26	0.63	40.00	65.00	53.33	52.00
SEMG1	0.70	80.00	45.00	59.26	69.23	0.52	35.00	70.00	53.85	51.85
TMEFF2	0.69	55.00	55.00	55.00	55.00	0.63	65.00	45.00	54.17	56.25
MAGEA6	0.69	50.00	85.00	76.92	62.96	0.68	40.00	85.00	72.73	58.62
MAGEA12	0.67	55.00	80.00	73.33	64.00	0.70	50.00	75.00	66.67	60.00
MAGEA1	0.67	50.00	85.00	76.92	62.96	0.75	45.00	80.00	69.23	59.26
CSAG2	0.63	40.00	80.00	66.67	57.14	0.72	50.00	80.00	71.43	61.54
PLAC1	0.57	45.00	75.00	64.29	57.69	0.59	45.00	75.00	64.29	57.69
CTAG1B	0.56	5.00	95.00	50.00	50.00	0.59	5.00	90.00	33.33	48.65
SSX4	0.51	40.00	65.00	53.33	52.00	0.80	55.00	85.00	78.57	65.38
JARID1B	0.51	45.00	45.00	45.00	45.00	0.67	50.00	85.00	76.92	62.96
TPTE	0.50	25.00	80.00	55.56	51.61	0.47	35.00	60.00	46.67	48.00
NOL4	0.46	25.00	70.00	45.45	48.28	0.55	35.00	75.00	58.33	53.57
TMEM108	0.42	40.00	60.00	50.00	50.00	0.68	40.00	75.00	61.54	55.56

CTAs: cancer/testis antigens; ROC: Receiver operating characteristic; AUC: area under curve; PPV: positive predictive value; NPV: negative predictive value.

199 qRT-PCR was used to verify the results obtained using the Nanostring multiplex approach.
 200 Validation was performed for all 22 CTAs using the same sample sets that were examined by Takahashi
 201 et al [25]. Statistical analysis showed significant ROC curves (AUC>0.7) (**Table 1**) and confirmed
 202 overexpression of the CTA genes *CEP55*, *NUF2*, *PBK*, *RQCD1*, *SPAG4*, *SSX2* and *TTK* in MPCa, as well
 203 as the down-regulation of *PAGE4* (**Supplementary Figure 2** and **Figure 1B**). The other selected CTAs
 204 did not show significant expression changes between LPCa and MPCa (data not shown). Of note, in the
 205 study by Takahashi et al. [25], only *CEP55*, *NUF2*, *PBK*, *PAGE4* and *SPAG4* were found differentially
 206 expressed in LPCa vs. MPCa. However, in the present study, a more robust analysis increased the panel
 207 of potential aggressive PCa biomarkers. These data not only support the fact that CTA expression
 208 patterns can be used to discriminate MPCa and LPCa cases, but also corroborates the previous data
 209 using the same biomarkers.

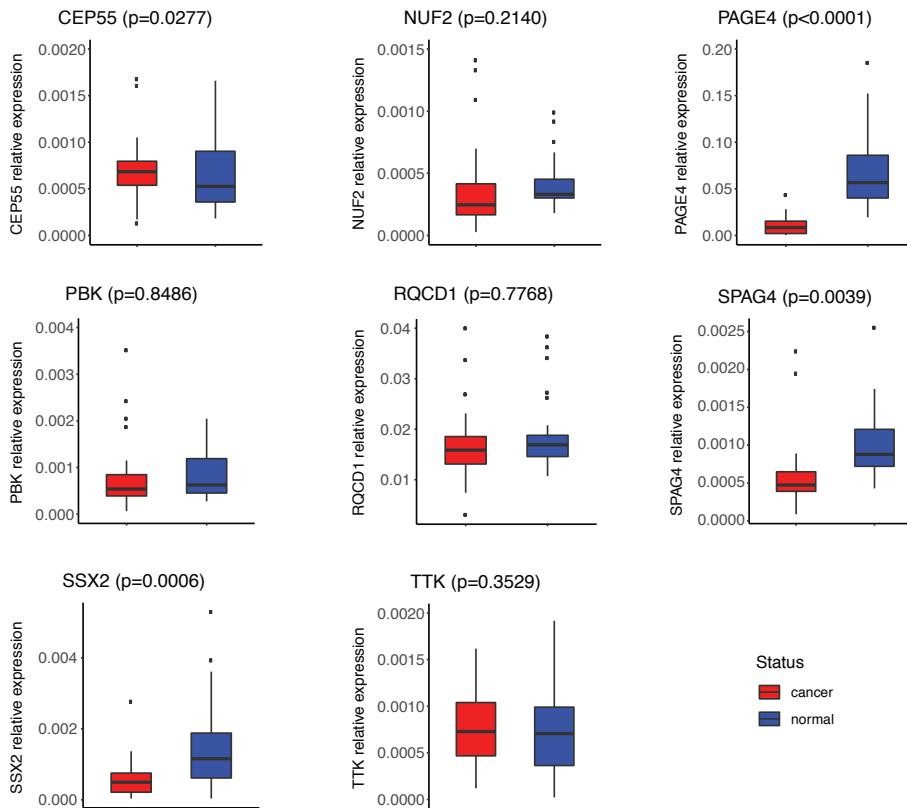
210 3.2. CTA expression in paired tumor and adjacent normal prostate tissue samples reveals differences at the
 211 mRNA and protein level

212 To determine if the CTAs differentially expressed in LPCa vs. MPCa also present different
 213 expression patterns in normal prostate tissue and PCa samples both at the mRNA and protein level,

214 *CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4, SSX2* and *TTK* expression levels were evaluated in paired
215 tumor samples and the adjacent normal tissues obtained from radical prostatectomies. Two distinct
216 cohorts were used, one for gene expression analysis (22 paired samples) and another for protein
217 expression (80 paired samples).

218 Gene expression analysis of the 22 paired tumor and normal samples did not show significant
219 differences for *CEP55, NUF2, PBK, RQCD1* and *TTK* (Figure 2). *PAGE4, SPAG4* and *SSX2* are up-
220 regulated in benign areas of the prostate when compared to tumor tissue. The expression profile of
221 these genes can discriminate with good accuracy normal from PCa samples, as shown by ROC curve
222 analysis (Table 2). These findings suggest that, for the CTAs selected in this study, changes in gene
223 expression occur in advanced stages of PCa progression and are associated with a more aggressive
224 phenotype.

225



226 **Figure 2** – Cancer/testis antigens (CTA) gene expression analysis in paired tumor and normal adjacent samples
227 from patients with prostate cancer. Gene expression was quantified by qRT-PCR. The relative gene expression
228 calculation was performed using the $2^{-\Delta Ct}$ approach using ActinB as the housekeeping gene. Mann-Whitney non-
229 parametric test was used to compare means between LPCa and MPCa groups. Gene expression differences were
230 considered significant when P value ≤ 0.05 . *CEP55* presents increased mRNA levels in PCa compared with normal
231 samples. Up-regulation in normal versus tumor tissue was observed for *PAGE4, SPAG4* and *SSX2*. For the other
232 CTAs no significant changes in expression was noted.

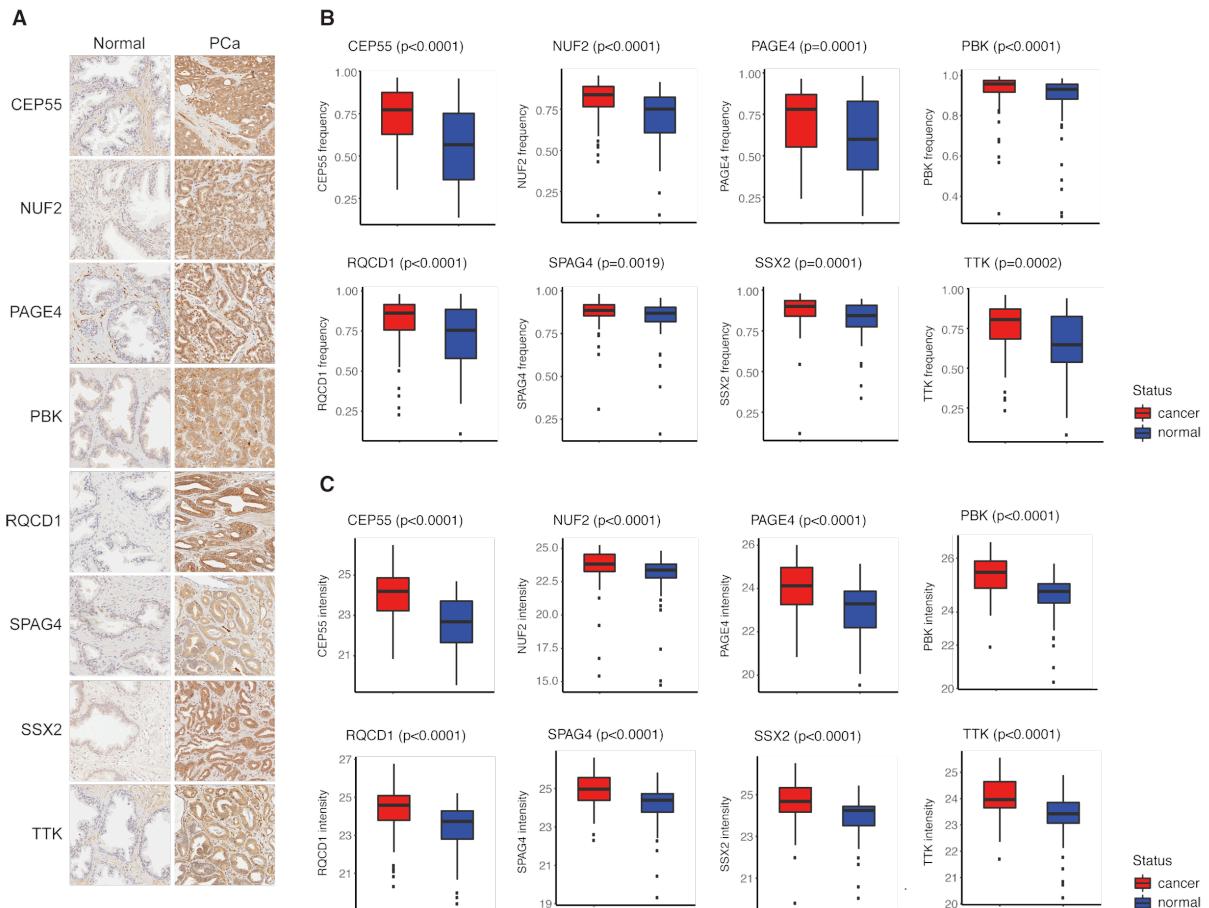
Table 2 - ROC analysis for gene expression profile of paired normal and tumor samples.

CTA	AUC	SENSITIVITY	SPECIFICITY	PPV	NPV
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<i>CEP55</i>	0.58	13.00	92.00	60.00	53.50
<i>NUF2</i>	0.69	13.00	100.00	100.00	55.60
<i>PAGE4</i>	0.98	95.70	92.00	91.70	95.80
<i>PBK</i>	0.58	0.00	100.00	NA	53.20
<i>RQCD1</i>	0.59	52.20	64.00	57.10	59.30
<i>SPAG4</i>	0.83	73.90	76.00	73.90	76.00
<i>SSX2</i>	0.74	78.30	64.00	66.70	76.20
<i>TTK</i>	0.55	100.00	0.00	53.70	NA

ROC: Receiver operating characteristic; AUC: area under curve; PPV: positive predictive value; NPV: negative predictive value.

233 Although gene expression changes were not detected for some of the CTA genes selected, the
234 protein expression analysis by IHC in 80 paired PCa and normal samples revealed significant
235 differences between tumor and normal adjacent tissue from the same patients for all 8 genes (**Figure**
236 **3A**). Using quantitative image analysis, we measured the frequency and the intensity of the staining,
237 independently. For both variables, we observed significant differences in the protein levels when
238 comparing the tumor with its paired adjacent normal tissue (Mann-Whitney non-parametric test with *P*
239 value ≤ 0.05) (**Figure 3B** and C). All CTAs show increased protein expression in PCa versus the normal
240 tissue. In order to verify if the increased protein levels were useful to accurately discriminate tumor
241 from adjacent normal samples we performed ROC analysis. The intensity of staining for all CTAs, but
242 NUF2, is an accurate variable (AUC > 0.70) to discriminate cancer from normal tissue. (**Table 3**). The
243 frequency of positively stained cells was significantly higher among tumor samples when compared to
244 the normal adjacent paired tissue for all CTAs. Although, almost all AUCs were below the cutoff value
245 (**Table 3**), when we compared the means of positive cells between tumor and normal samples the
246 differences are significant (**Figure 3B**). The progressive down-regulation of *PAGE4* in PCa is a
247 distinctive marker of metastasis development and therefore, tracing its loss of expression from the time
248 of PCa diagnosis can provide valuable prognostic information. The observation that gene expression is
249 not different between normal versus PCa and the fact that *SPAG4* and *SSX2* present with higher levels
250 in normal samples suggest that slight changes at the transcriptional level may lead to significant
251 changes in protein expression in cancer cells.



252 **Figure 3** – Cancer/testis antigens (CTA) protein expression analysis by immunohistochemistry (IHC) in paired
253 tumor and normal adjacent tissues. IHC using antibodies against eight CTAs were performed to identify significant
254 differences between normal and tumor areas from the same prostate. Panel A represents the immuno-staining for
255 CTAs in normal and PCa paired samples. Using a computational quantitative approach, it was possible to measure
256 the frequency (B) and intensity (C) of the staining. Mann-Whitney non-parametric test was used to compare means
257 (P value ≤ 0.05). All CTA proteins present increased expression in PCa when compared to the normal paired
258 sample.

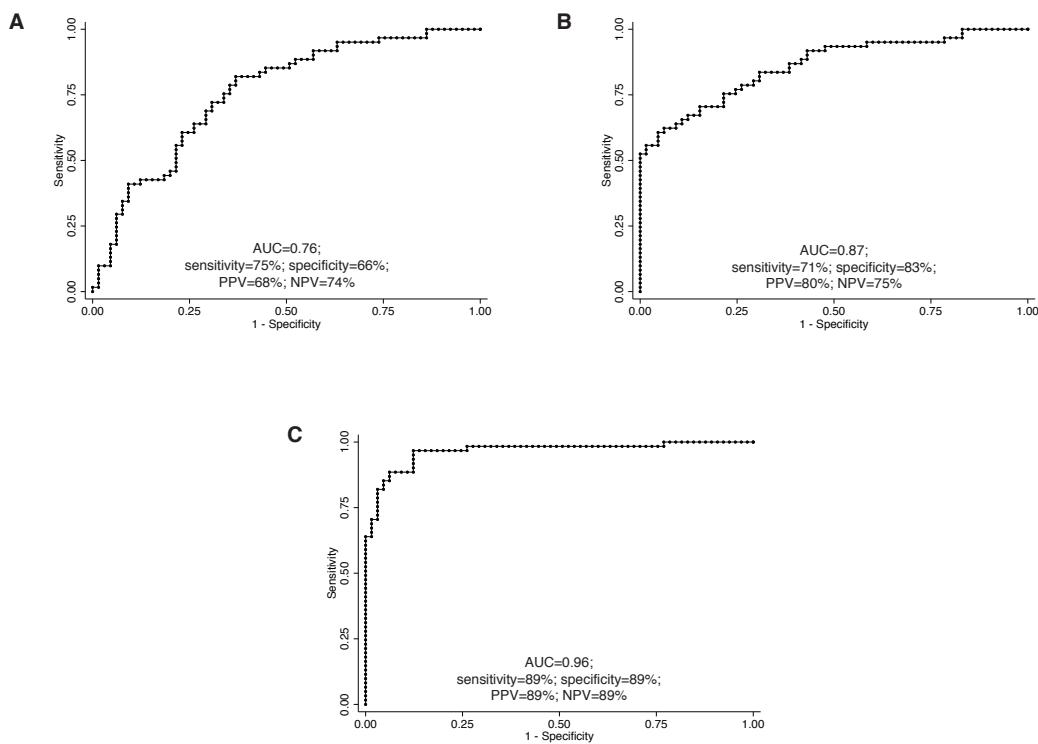
Table 3 - ROC analysis summary for the immunohistochemistry intensity and frequency protein expression analysis.

CTA	INTENSITY					FREQUENCY				
	AUC	SENSITIVITY	SPECIFICITY	PPV	NPV	AUC	SENSITIVITY	SPECIFICITY	PPV	NPV
CEP55	0.79	0.64	0.75	0.71	0.68	0.71	0.70	0.62	0.64	0.68
NUF2	0.67	0.54	0.73	0.66	0.63	0.69	0.75	0.53	0.61	0.69
PAGE4	0.72	0.56	0.78	0.71	0.65	0.61	0.64	0.54	0.57	0.62
PBK	0.78	0.65	0.82	0.77	0.72	0.64	0.71	0.47	0.56	0.64
RQCD1	0.71	0.63	0.73	0.69	0.66	0.64	0.71	0.52	0.59	0.64
SPAG4	0.72	0.61	0.77	0.72	0.67	0.62	0.69	0.49	0.57	0.62
SSX2	0.73	0.56	0.80	0.72	0.66	0.67	0.67	0.56	0.58	0.64
TTK	0.73	0.58	0.76	0.70	0.65	0.67	0.67	0.58	0.61	0.64

ROC: Receiver operating characteristic; AUC: area under curve; PPV: positive predictive value; NPV: negative predictive value.

259 3.3. Identification of a CTA panel as a potential biomarker for aggressive disease

260 In an attempt to identify a panel of CTAs that would be more sensitive than a single CTA to
261 discriminate indolent from aggressive disease, we performed multiple logistic regression (MLR). We
262 identified a panel of CTAs whose combined expression pattern could represent a potential tool for the
263 discrimination of MPCa cases from LPCa. Using the expression profiles determined by qRT-PCR, MLR
264 led us to a panel that included the CTAs *CEP55* and *RQCD1* and that correctly classify MPCa or LPCa
265 in 87.5% of the cases evaluated in the present study (AUC=0.95, sensitivity=85.0%; specificity=90.0%;
266 positive predictive value=89.5%) (Figure 4A).



267 **Figure 4** – Receiver operating characteristic (ROC) curve analysis of the multivariate logistic regression (MLR)
268 performed to identify panels of biomarkers accurate to discriminate localized (LPCa) from metastatic (MPCa)
269 prostate cancer and normal from tumor samples. A. ROC curve analysis for the gene expression levels of *CEP55*
270 and *RQCD1* to discriminate LPCa from MPCa. B. ROC curve analysis for the protein expression analysis. Here,
271 MLR identified a panel including all CTAs staining intensity and 3 CTAs staining frequency as a good panel to
272 discriminate normal from tumor samples. C. ROC curve analysis for *PAGE4* gene expression that alone is capable
273 of discriminating virtually all MPCa cases from LPCa. AUC – area under curve; PPV – positive predictive value;
274 NPV – negative predictive value.

275 For the paired PCa and normal adjacent tissue cohort, the MLR analysis resulted in a panel in
276 which intensity and frequency of the CTA proteins accurately discriminate normal from tumor

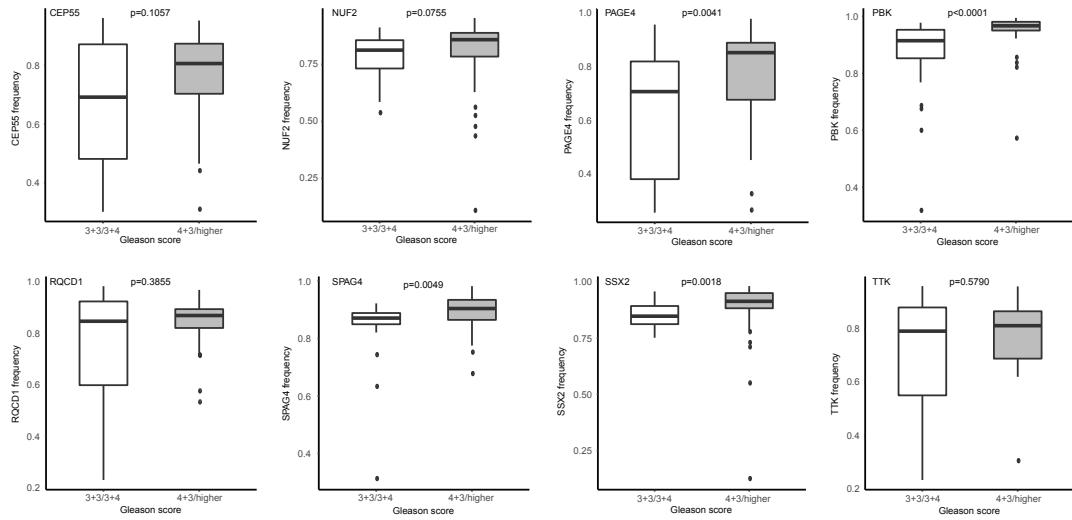
277 samples. The panel including all CTA protein expression intensity and NUF2, PBK, SSX2 and TTK
278 protein expression frequency correctly classified ~89% of the samples (AUC=0.96, sensitivity=88.5%;
279 specificity=89.2%; positive predictive value=89.2%) (**Figure 4B**).

280 Although the combined expression of *CEP55* and *RQCD1* expression presented high accuracy, this
281 panel is not any more accurate than the *PAGE4* pattern of expression alone. *PAGE4* by itself is capable
282 of separating almost all cases (AUC~1) (**Figure 4C**). No other CTA selected from the current study
283 demonstrates the same degree of accuracy to differentiate MPCa and tumor cases from LPCA like
284 *PAGE4*. Therefore, a decrease in *PAGE4* level is an important, and to the best of our knowledge, a
285 unique feature among CTAs known to be expressed in PCa, mainly in MPCa. Therefore, *PAGE4* is a
286 strong candidate as a biomarker for aggressive prostate tumors.

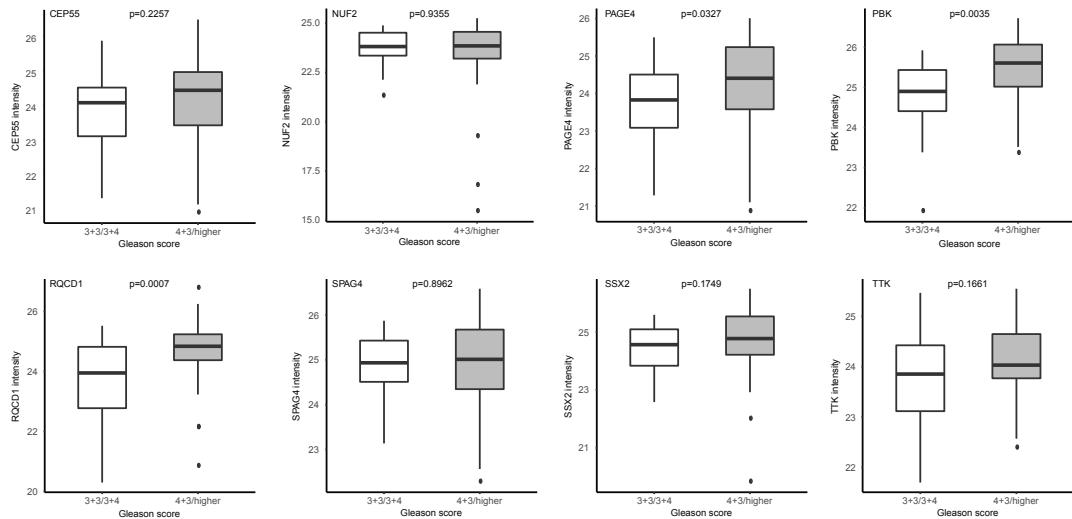
287 *3.4. CTA expression and association with Gleason score*

288 The Gleason score is an important feature considered to determine therapy and prognosis of PCa
289 patients. Due to its relevance, we investigated the CTAs protein expression in 3+3/3+4 and 4+3/≥8
290 Gleason score groups. Although, 3+4 and 4+3 are score 7, it is widely known that the prognosis for these
291 groups of men are significantly different and so we decided to group the first with more indolent
292 tumors (3+3/3+4) and the later with the more aggressive (4+3/higher). Most of the CTAs analyzed by
293 IHC in this study present increased protein levels in patients with higher Gleason scores when
294 compared to lower scores (**Figure 5A and B**). Frequency of *PAGE4*, *PBK* and *RQCD1* positive cells are
295 significantly increased in PCa with Gleason score 4+3/higher (**Figure 5A**). When considering the
296 intensity of the IHC staining, *PAGE4*, *PBK*, *SPAG4* and *SSX2* presented significant stronger staining
297 associated with more aggressive histopathology (**Figure 5B**). These findings suggest that *PAGE4* and
298 *PBK* could be used to determine PCa prognosis since the frequency and protein levels together in the
299 tumor cells present positive association with the Gleason score.

A - Frequency of positive cells and Gleason score



B - Intensity of staining and Gleason score



300 **Figure 5 –** Cancer/testis antigens (CTA) protein expression by immunohistochemistry according to the Gleason
301 score. The PCa samples from the TMAs were grouped in Gleason score 3+3/3+4 and 4+3/higher. The frequency of
302 positive tumor cells (A) and the intensity of the staining (B) were measured in separate and statistical analysis
303 (Mann-Whitney non-parametric test) was performed for each of the staining measurements.

304 **4. Discussion**

305 In this study we used gene and protein expression analysis to identify a panel of CTA biomarkers
306 that are differentially expressed in MPCa but could potentially be used for tumor screening as well. We
307 used the gene expression profiles previously published by Takahashi et al. [25] and performed a new
308 statistical exploration, with ROC curve and MLR analysis, to identify CTA genes that by themselves
309 were capable of discriminating MPCa from LPCa cases and also to create a panel with even better
310 accuracy. The expression of the CTAs CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4, SSX2 and TTK are
311 capable of discriminating aggressive from indolent PCa. Loss of PAGE4 expression was detected in all
312 MPCa cases and this feature alone is enough to distinguish all MPCa from LPCa cases. The up-

313 regulation of *CEP55* and *RQCD1* together is the second most accurate biomarker of MPCa cases. These
314 findings represent good evidence that the changes in CTA gene expression with the progression of PCa
315 can identify men with more aggressive tumors. Unfortunately, since the LPCa and MPCa samples were
316 not paired, it was not possible to determine the evolution of the CTA expression profiles in the same
317 patient. Prospective studies with patient follow-up, from disease diagnosis until metastasis
318 development, would allow a better understanding about the time point where the changes in CTA
319 expression begin during the course of PCa development.

320 One of the main causes of death among men with PCa is metastatic disease [1]. Although PSA is
321 the gold standard for screening, it lacks the ability to predict the development of metastasis [29,30].
322 Since the CTAs we selected from the Takahashi et al. [25] study are differentially expressed between
323 LPCa and MPCa, we analyzed their expression in a cohort of paired tumor and adjacent normal tissue
324 obtained from patients with PCa that underwent radical prostatectomy. Using quantitative IHC, we
325 found that all the selected CTA proteins are up-regulated (intensity and frequency) in the tumor
326 samples when compared to the normal adjacent tissue. As with the above mentioned cohort, we found
327 a panel of CTAs whose intensity and frequency at the protein level are capable of discriminating
328 normal from cancer samples with great accuracy. This observation further highlights the usefulness of
329 CTAs as biomarkers for PCa that could be used during screening together with the PSA test, though
330 further studies with larger cohorts across institutions and demographics are needed to determine the
331 real prediction ability of these biomarkers for the development of MPCa. Studies in the future with
332 prospective cohorts from screening to diagnosis, and the development of metastatic disease, can shed
333 new light on how the expression of these CTAs progress during the course of the cancer.

334 An important contribution of this study is that we show the importance of using a panel of
335 biomarkers for detection or prognosis. In both scenarios, normal vs. cancer, and LPCa vs. MPCa, the
336 strongest predictors were those including more than one CTA. It is widely known that PCa and many
337 other tumors are heterogeneous and composed of different cell populations with unique molecular
338 profiles [31–34]. The use of single biomarkers may not cover the wide range of cell subclones present in
339 the tumor and only capture the most abundant population. On the other hand, a panel of biomarkers is
340 more likely to cover more broadly the different molecular profiles and allow the development of more
341 accurate tests for screening and follow-up [35–37]. In the current study, there is one exception to our
342 biomarker panel hypothesis: namely *PAGE4*. *PAGE4* gene expression was capable of discriminating
343 MPCa from LPCa with 100% accuracy. Metastatic samples from men previously treated for PCa
344 showed loss of this CTA expression compared to patients with local disease at the moment of diagnosis,
345 suggesting that this gene is critical for tumor development but not for the metastasis establishment in a
346 distant sites. This assumption is corroborated by the fact that *PAGE4* protein expression is
347 downregulated in metastatic PCa suggesting that *PAGE4* may actually be a metastasis suppressor [38].

348 Recent studies suggest that *PAGE4* is developmentally regulated with dynamic expression
349 patterns in the fetal prostate and that it is also a stress-response protein that is up-regulated in response
350 to cellular stress [38]. In the present study, we observed loss of expression of *PAGE4* in MPCa cases.
351 Sampson et al. [39] also observed reduced levels of *PAGE4* in MPCa when compared to indolent cases
352 and found that in *PAGE4* positive cells wild-type AR activity is reduced. This suggests that *PAGE4*
353 plays an important role in MPCa, since aberrant activation of the AR pathway is a critical step in the
354 progression to mCRPC after androgen ablation therapy. Loss of *PAGE4* in MPCa might result in
355 activation of the AR signaling pathway, resulting in resistance to androgen-derivation therapy in men
356 with MPCa [40,41]. However, as we recently demonstrated the role of *PAGE4* in mCRPC is dependent
357 on intratumor heterogeneity and downregulating the activity of the AR pathway depends on the co-
358 expression and *PAGE4* phosphorylation by HIPK1 and CLK2 that either potentiate or attenuate the
359 effects on the AR pathway, respectively [42].

360 CTA expression in PCa and the association with disease aggressiveness was assessed previously in
361 the same cohort of LPCa and MPCa by Takahashi et al. [25]. The authors found that *CEP55*, *NUF2*,

362 *PAGE4*, *PBK* and *SPAG4* are differentially expressed in LCPa vs. MPCa. Using the same cohort, we
363 repeated the technical validation and performed new statistical analysis using different tests. Besides
364 the five CTAs previously shown to be differentially expressed between the two groups, we also found
365 that *RQCD1*, *SSX2* and *TTK* are up-regulated in the MPCa samples. In addition, one of these CTAs, not
366 previously predicted to be a biomarker candidate, was relevant when combined with another gene. The
367 combined expression pattern of *CEP55* and *RQCD1* could be a marker for aggressive tumors. Although
368 this panel is not any more accurate than *PAGE4* expression profile alone in local and metastatic tumors,
369 it provides good evidence that a combination panel could be more relevant for prognostication than a
370 single marker, resulting in higher specificity. Besides *PAGE4*, other CTAs such as *PBK* and *SSX2*, were
371 previously detected in PCa. *PBK* expression is absent in vitro and in normal prostate tissue. A gradual
372 increase in *PBK* expression is concurrent with increased disease aggressiveness [43], in accordance with
373 our findings that this CTA is a marker of MPCa. *SSX2* expression was also detected in PCa samples in a
374 few studies [44–46]. Smith et al. observed that *SSX2* higher levels were present in advanced cases,
375 however they also noticed that the pattern of expression across different tumor stages (including
376 benign prostate) was heterogeneous [44]. The immunohistochemistry data and gene expression
377 findings by Bloom & McNeel [47] corroborate our observations that *SSX2* protein is increased in MPCa.
378 The authors also showed that circulating tumor cells expressing the correspondent gene could only be
379 detected in peripheral blood of PCa patients, while undetectable in healthy men [47].

380 One intriguing observation in our study is that the gene expression levels of *PAGE4*, *NUF2* and
381 *SPAG4* are increased in normal prostate tissue relative to the tumor samples. Since the normal tissues
382 were collected adjacent to the prostate tumors it is probable that the up-regulation of CTAs in non-
383 cancer areas is a field effect as previously described by Zeng et al. [48]. They describe the same trend for
384 *PAGE4* when comparing its expression in PCa with the adjacent normal tissue from the same patients.
385 Another plausible reason for the discrepancy in gene expression in the paired tumor and normal
386 samples is that the RNA abundance for these genes does not reflect protein levels, since our IHC results
387 show that *PAGE4*, *NUF2* and *SPAG4* are up-regulated in tumor although the mRNAs are down-
388 regulated when compared to the normal prostate. This would also explain why for the other CTAs no
389 differences in mRNA levels in normal and tumor contrast with significant differences at the protein
390 level. Also, translational machinery activity and temporal mRNA and protein degradation are
391 additional variables that can cause in discrepancies between RNA abundance and protein expression
392 [49,50].

393 CTAs, especially the ones located on the X chromosome (the CT-X-Antigens), constitute a family
394 of genes with great potential as biomarkers in different types of tumors, since they are cancer-specific
395 and rarely expressed by normal tissues. Many of these genes when aberrantly expressed in cancer cells
396 are immunogenic and can induce antibody- and cell-mediated responses that make them good targets
397 for the development of cancer vaccines [24,51,52]. Unfortunately, their role as cancer biomarkers and
398 therapeutic targets has not been appreciated in many tumor types, including PCa. The current study
399 demonstrates that CTA expression profiles might be an important tool to predict, at the time of
400 diagnosis, patients with higher risk to develop metastasis and that would benefit from aggressive
401 treatments from those men with indolent disease who may have a better quality of life receiving
402 adequate active surveillance. Here, we used small cohorts to determine CTA expression profiles; the
403 next step is to evaluate the expression of *CEP55*, *NUF2*, *PAGE4*, *PBK*, *RQCD1*, *SPAG4*, *SSX2* and *TTK* in
404 larger cohorts with follow-up data right from screening to the development of MPCa. Also, the
405 development of less invasive approaches (liquid biopsies) to measure CTAs expression in circulating
406 tumor cells, and even the presence of antibodies against these immunogenic biomarkers would be
407 beneficial for early detection of primary tumors as well as metastasis prediction.

408 **5. Conclusions**

409 To summarize, we have demonstrated that eight CTAs are differentially expressed in PCa. The
410 same CTAs can also be useful to discriminate locally confined tumors from metastatic tumors. These
411 observations were detected at the gene expression and protein levels and in different patients cohorts,
412 which provides validation of our findings across different samples and, groups of patients. CTAs are a
413 group of genes aberrantly expressed in cancer and some present immunogenicity. Cancer specificity
414 and immunogenicity make this class of genes unique potential biomarkers and immunotherapy targets.
415 Here, we demonstrate that a panel of CTAs are aberrantly expressed in PCa and associated with
416 metastatic disease suggesting their potential as biomarkers for screening and patients' follow-up.
417 Further studies involving broader prospective cohorts are needed to prove their usefulness as
418 biomarkers and also the investigation of their immunogenicity is valuable and would result in new
419 immunotherapy strategies for men with PCa.

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