

Expression Screening of Cancer/Testis Genes in Prostate Cancer Identifies NR6A1 as a Novel Marker of Disease Progression and Aggressiveness

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BACKGROUND. Cancer/Testis (CT) genes are expressed in male gonads, repressed in most healthy somatic tissues and de-repressed in various somatic malignancies including prostate cancers (PCa). Because of their specific expression signature and their associations with tumor aggressiveness and poor outcomes, CT genes are considered to be useful biomarkers and they are also targets for the development of new anti-cancer immunotherapies. The aim of this study was to identify novel CT genes associated with hormone-sensitive prostate cancer (HSPC), and castration-resistant prostate cancer (CRPC).

METHODS. To identify novel CT genes we screened genes for which transcripts were detected by RNA profiling specifically in normal testis and in either HSPC or CRPC as compared to normal prostate and 44 other healthy tissues using GeneChips. The expression and clinicopathological significance of a promising candidate—NR6A1—was examined in HSPC, CRPC, and metastatic site samples using tissue microarrays.

RESULTS. We report the identification of 98 genes detected in CRPC, HSPC and testicular samples but not in the normal controls. Among them, cellular levels of NR6A1 were found to be higher in HSPC compared to normal prostate and further increased in metastatic lesions and CRPC. Furthermore, increased NR6A1 immunoreactivity was significantly associated with a high Gleason score, advanced pT stage and cancer cell proliferation.

CONCLUSIONS. Our results show that cellular levels of NR6A1 are correlated with disease progression in PCa. We suggest that this essential orphan nuclear receptor is a potential therapeutic target as well as a biomarker of PCa aggressiveness. *Prostate* 73: 1103–1114, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: **Cancer/Testis genes; castration-resistant prostate cancer; biomarker; NR6A1; nuclear receptor**

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INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed non-cutaneous malignancy among men and the second most common cause of cancer-associated mortality in western countries [1,2]. Up to a third of localized PCa patients develop a locally advanced cancer or metastases within 15 years [3,4]. Hormone (androgen deprivation) therapy is an established treatment to delay the progression of metastatic tumors. However, within 2 years most patients develop the hormonally insensitive disease form called castration-resistant prostate cancer (CRPC) [5]. The high morbidity and mortality of CRPC remains a major public health challenge in part due to the unsatisfactory and ineffective treatment options available. The molecular mechanisms and their interplay governing the initiation and progression to this refractory status are still poorly understood in spite of the intensive research [6]. Therefore improved biomarkers as well as new gene-based therapeutic strategies focusing on PCa progression are eagerly awaited.

The idea to direct the immune system toward malign tumors stems from the initial observation that a patient suffering from skin cancer had developed antibodies against a protein called melanoma antigen (MAGEA1). This protein was present in the tumor and in testis but not in healthy non-testicular tissues [7]. Ever since this discovery was made 254 additional Cancer/Testis (CT) genes have been identified using molecular biological methods and genome biological approaches based on microarrays or serial analysis of gene expression; see [8] for a review and the CTDatabase for a comprehensive list [9]. Following-up on this work, genes such as MAGEA3 are being investigated in clinical trials [10,11]. Apart from their potential usefulness as targets for immunotherapy [12], CT genes are also interesting from the perspective of the etiology and the progression of cancer because gametogenesis and oncogenesis share features including immortality, genetic instability and invasiveness; reviewed in [13]. Consistently, CT gene expression levels are often correlated with tumor progression and clinical outcomes in various types of somatic malignancies [14–19]. This is notably the case for PCa where expression screens of known CT genes yielded information on their stage-specific induction as the disease unfolds [20]; reviewed in [21,22]. It is noteworthy that a genome-wide comparison of gene expression patterns across a large number of healthy and pathological human tissues marked out colon-, and hormone-sensitive prostate cancers as failing to express testis-specific (or testis-selective) genes [23]. In contrast to this finding, three recent landmark studies have associated well-known CT genes with tumor aggressiveness and poor

outcomes [20,22,24]. However, to the best of our knowledge, a systematic screening of CT genes showing altered expression in PCa has never been carried out.

To address this issue in a genome-wide and rigorous manner, we analyzed a large gene expression dataset assembled from our own normal testicular sample data (total testis, seminiferous tubules, spermatocytes, and spermatids) [25] with the output of published studies covering nine normal testes, sixteen healthy prostates, 68 HSPC, four CRPC, and 44 other non-testicular and non-prostatic healthy tissues available via public repositories [26,27]. This led to the identification of 98 genes including known CT genes and novel candidates which discriminate between CRPC and HSPC. Of particular interest are the roles of germline-associated sequence-specific transcription factors in controlling CT gene expression in cancer. Therefore, among the 98 candidates genes, we further validated the orphan nuclear receptor NR6A1 (also called GCNF for germ cell nuclear factor)—a DNA binding repressor essential for embryogenesis, neurogenesis, normal female fertility and testicular gene expression in the mouse [28–32]—at the protein level. Using tissue microarrays prepared from a large cohort of patients suffering from prostate cancers for whom clinicopathological information was available, we subsequently observed NR6A1's cellular presence to be significantly associated with PCa progression. These results demonstrate that NR6A1 is a novel biomarker for aggressive PCa.

MATERIALS AND METHODS

Gene-Based Expression Screening

Gene expression dataset. We assembled a large gene expression dataset from several independent microarray studies generated on Affymetrix GeneChip U133 Plus 2.0 microarray consisting of 21,248 coding genes publicly available at the NCBI's GEO (dataset IDs: GSE2109, GSE3325, GSE6565, GSE7307, GSE11839) [26] and ArrayExpress (dataset ID: E-TABM-130) [27]. It included 613 samples (Supplemental file 1) covering eight previously published testicular samples (total testis, seminiferous tubules as well as enriched populations of pachytene spermatocytes and round spermatids samples) [25] together with nine additional healthy testes (hT), 16 healthy prostates (hP), 508 samples corresponding to 44 different non-testicular and non-prostatic healthy tissues (nTnP), four localized CRPC [33], and 68 localized HSPC (ExpO). PCa samples were from radical prostatectomy series or rapid autopsy program (ExpO).

Raw data pre-processing. The microarray data were pre-processed using AMEN [34]. GeneChip data were

quality controlled and normalized using the Robust Multi-Array Average (RMA) method as published [25].

Statistical filtration of transcripts specifically expressed in HSPC or CRPC. Probesets were selected when their intensity values were above the Background Expression Cutoff (BEC = 5.5, corresponding to the overall median log2-transformed intensity) in their corresponding PCa category and below the BEC in the other category as well as in hP (Fig. 1A). To avoid inclusion of transcripts with signal values close to the BEC, only those whose signals were at least twofold higher were retained. A LIMMA statistical test (*F*-value adjusted with the False Discovery Rate method: $P \leq 0.01$) was employed to discriminate probesets with statistically significant changes across individual samples. Finally, remaining transcripts were categorized into transcripts specifically expressed in HSPC or in CRPC.

Statistical filtration of transcripts preferentially expressed in testis. Probesets were selected if at least one intensity value across hT was above the BEC and below the BEC in hP and nTnP (with a maximum of three nTnP exceptions; (Fig. 1). Only those whose signals were at least twofold higher in at least one hT than in nTnP were retained. Finally, a LIMMA

statistical test ($p[\text{FDR}] \leq 0.01$) was employed to identify probesets with significant changes.

Statistical filtration of novel Cancer/Testis genes (CT genes) associated with PCa. The novel CT genes associated with HSPC or CRPC were identified by intersecting the transcripts specifically expressed in HSPC or CRPC with the transcripts preferentially expressed in testis, respectively (Fig. 1).

Functional data mining. Enrichment of GO terms was estimated using AMEN [34] with the Fisher exact probability (Gaussian Hypergeometric test). A GO term was considered to be significantly enriched when the FDR-corrected *P*-value was ≤ 0.05 and the number of gene bearing this annotation was ≥ 3 .

Patients and Tissues—Tissue Microarrays (TMA)

Two hundred twenty-four cases of HSPC were obtained from patients treated by radical prostatectomy (with negative surgical margins) at the Montrouz Institute of Paris. Patients' characteristics are the following: median age: 63 years (46–77), median preoperative PSA: 8.9 ng/ml (1.5–20), 139 pT2 cases and 85 pT3, 51 Gleason score 6 or less, 161 Gleason score 7, and 12 Gleason score 8 or more (Table I). Fifty-seven normal prostate samples were obtained from

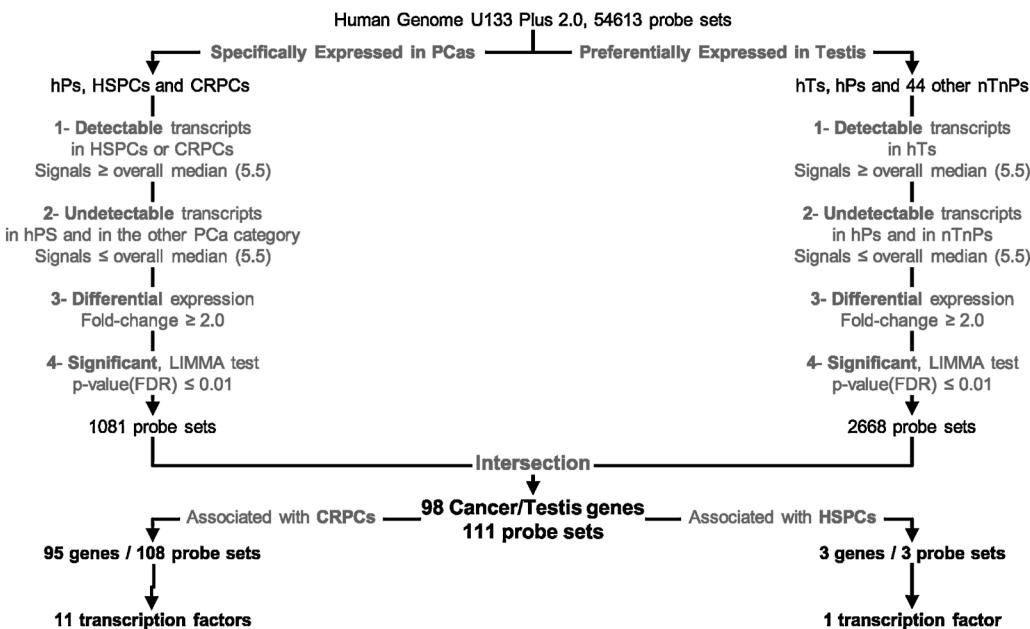


Fig. 1. The expression screening strategy. A flow chart summarizes the systematic screening strategy for Cancer/Testis (CT) genes associated with prostate cancer (PCa) based on healthy prostate (hPs), healthy testicular and germline (hTs), healthy non-testicular and non-prostatic (nTnP), HSPC and CRPC samples as indicated. Potential CT genes were subsequently grouped into two signatures depending on their specific expression pattern in CRPC or HSPC. The numbers of selected genes and probe sets are indicated.

TABLE I. NR6A1 Expression in Cancer Cells (%) According to the Clinicopathological Characteristics of Prostate Cancer Patients

	n	NR6A1 staining (% + cells) median (range)	P-value	Sections with a NR6A1 staining	P-value (chi-square)
Prostate cancer status					
Normal prostate	57	0% (0–90)	<0.0001	5+/57	<0.0001
Hormone-sensitive (HSPCs)	224	5% (0–95)		120+/224	
Castration-resistant (CRPCs)	50	70% (20–95)		50+/50	
Metastases	20	80% (0–100)		18+/20	
TNM (HSPCs)					
Localized prostate cancer (T2)	139	0% (0–95)	0.02	62+/139	0.0006
Locally advanced prostate cancer (T3)	85	25% (0–90)		58+/85	
Gleason score (HSPCs)					
Moderately differentiated tumor (Gleason 5–6)	51	0% (0–95)	0.02	23+/51	0.002
Intermediately differentiated tumor (Gleason 7)	161	5% (0–95)		85+/161	
Poorly differentiated tumor (Gleason 8–10)	12	60% (5–90)		12+/12	

patients with urinary bladder cancer without PCa treated by radical prostatocystectomy (64 years [48–81]). From the same institution, 111 prostate cancers with biochemical relapse, defined as two consecutive increases in serum PSA 0.2 ng/ml or greater, were matched with 111 tumors without recurrence after at least 4 year follow-up. The date of PSA recurrence was defined as the date of the first increase of serum PSA after surgery. Each of these patients was matched with one patient that presented identical age group (i.e., under 50 years old, 50–60, 60–70, and over 70 years old), preoperative PSA rate group (i.e., under 10, 10–15, 15–20 ng/ml), Gleason score, and pathological stage, but free of recurrence after at least the same follow up. Patients' characteristics and follow-up are summarized in Table II.

Fifty cases of CRPC were selected from 323 patients treated with exclusive androgen deprivation therapy (ADT), between 1988 and 2008. Patients were selected if they initially responded to exclusive ADT (decrease in PSA level without clinical or radiological progression), and had post-hormonal relapse tissue sample suitable for analysis. Hormonal therapies were LHRH agonist, steroid or non-steroidal antiandrogen or complete androgen blockage. No patient received chemotherapy, radiation therapy, prostatectomy or 5-alpha reductase inhibitors. Hormonal relapse was defined as two consecutive rises in PSA with at least 1 week interval, and with serum testosterone level under castration level (50 ng/dl). Tissues were collected by trans-urethral resection (TUR), performed in all cases because of lower urinary tract symptoms associated with local tumor progression (Table I).

PCa metastases were obtained from 20 patients treated in the University Hospital of Poitiers, by either

radical prostatectomy or radiotherapy. No hormonal deprivation had been performed before metastases resection. Metastatic tissues were either lymph nodes (n = 10) or bone (n = 10).

Tissue microarrays were constructed as previously described [35] with PCa tissues obtained from the above mentioned normal prostate, HSPC, bone and lymph node metastatic lesions, and CRPC specimens, including 4 cores (0.6 mm diameter) per cancer.

TABLE II. Patient's Clinicopathological Characteristics of the Matched Nested Case–Control Population

	Group 1 (R+)	Group 2 (R−)
Age		
median, range	65 years (55–73)	65 years (520–75)
Pre-operative PSA		
median, range	9 ng/ml (1.5–20)	9 ng/ml (2–20)
Gleason score		
6	n = 29	n = 29
7 (3 + 4)	n = 33	n = 33
7 (4 + 3)	n = 41	n = 41
8 or greater	n = 8	n = 8
Pathological stage		
pT2	n = 75	n = 75
pT3	n = 36	n = 36
Follow-up	20 months (median, range)	86 months (48–128)

R+, biochemical recurrence after radical prostatectomy; R−, no recurrence.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on normal prostate and on PCa Tissue Microarrays (TMA). IHC was performed on de-paraffinized sections and samples were treated with citrate buffer pH 6 for 30 min at 80°C and then kept 20 min at room temperature. The slides were rinsed in 1X phosphate buffered saline (PBS, pH 7.4), treated with 3% hydrogen peroxide in 1X PBS for 5 min. Sections were pre-incubated twice in 5% human serum albumin (Sigma, Saint-Quentin Fallavier, France) for 20 min. Next, the slides were incubated overnight at 4°C with rabbit polyclonal antibodies against NR6A1 (Abcam ab38816) at 1:200, in 5% human serum albumin and 0.1% Tween. IHC staining was performed at room temperature with biotinylated goat anti-rabbit IgG (Dako) and streptavidin-biotin peroxidase (Dako) for 1 hr each at a dilution of 1/500. Slides were then stained for 3 min with 3,3'-diaminobenzidine Enhanced System (Sigma). Finally, the sections were counterstained with 0.2% Hematoxylin. The fraction of anti-NR6A1 stained nuclei/cells was determined on the entire section area. Negative controls included incubation without the primary antibody. The NR6A1 antibody (ab38816) was verified by pre-incubating it with the NR6A1 peptide (ab38815) against which it was raised. This resulted as expected in loss of nuclear staining CRPC (data not shown). IHC staining was also performed on TMA slides using antibodies directed against the proliferation marker Ki-67 (DakoCytomation, dilution 1/50, incubation 30 min). Tissue microarrays were reviewed by two experienced uropathologists (N.R.-L., G.F.) in a blinded fashion. In case of inter-observer variability (different categories in the case of categorical data or variability more than 10% in the case of continuous data), TMA were rescored by both uropathologists until a consensus was reached. For the NR6A1 and Ki-67 nuclear epithelial staining, positive cells were expressed as a percentage of total epithelial cells.

Statistical Analysis

Survival analyses were conducted using Kaplan-Meier method, and curves were compared with the long-rank test. Hazard ratios were calculated using Cox regression for multivariate analysis. To evaluate the association between markers' expression and both Gleason score and pathological stage, either chi-square, non-parametric Mann-Whitney or Kruskall-Wallis tests were used for categorical and continuous variables, respectively. The association between two continuous variables was tested using the non-parametric Spearman test.

RESULTS

Initial Genome-Wide Screening and Functional Analysis

By comparing CRPC, HSPC and healthy prostate (hP) samples through a gene expression screening, we selected 1,046 and 35 transcripts (or Affymetrix probe sets) with a restricted expression in CRPC and in HSPC, respectively (Fig. 1 and Supplemental file 2). In addition, we integrated data obtained with healthy testis (hT) samples and 45 non-testicular normal tissues (including hP samples) we identified 2,668 transcripts (or Affymetrix probe sets) classified as Preferentially Expressed in Testis (PET). Finally, the intersection of both filtration strategies yielded 98 potential CT genes (corresponding to 111 transcripts/probe sets) detected in testicular samples of which 95 (108 transcripts/probe sets) were detected only in CRPC and three (three transcripts) exclusively in HSPC (Figs. 1 and 2A and Supplemental file 2).

We next explored the biological processes associated with the 95 target genes detected only in CRPC by searching for enriched Gene Ontology (GO). We found significant over-representation of terms associated with genes related to cell cycle progression and checkpoint control (CCNE2, CDC25A, CDC25C, CDC6, CEP78, DDX11, MNS1, NEDD1, SESN3, TIPIN) such as regulation of cell cycle arrest (GO:0071156, 5 observed/~1 expected, *P*-value = 0.0469), DNA replication (GO:0006260, 5/~1, 0.0426), cell cycle process (GO:0022402, 10/~3, 0.0426) and more specifically M phase (GO:0000279, 7/~2, 0.0427) and interphase (GO:0051325, 7/~1, 0.0186). We also found genes demonstrated or proposed to be associated with spermatogenesis (SPAG1, MICALC, NR6A1) and more precisely meiosis (MNS1), the acrosome (SPACA3, SPESP1) and sperm motility (DNAH14, DNAH2) as well as loci related to spermatogenesis failures (CNE2, MYBL1) but without significant enrichment for any of these terms.

NR6A1 Transcript Is Up-Regulated in Castration-Resistant Prostate Cancers

Our research interests include the roles of germ-line-associated sequence-specific transcription factors in controlling CT gene expression in cancer. Among the 98 CT gene candidates, 12 encode for DNA binding proteins annotated as known or putative transcription factors (Supplemental file 2). We subsequently ranked them using our Gene Prioritization System (GPSy; <http://gpsy.genouest.org>) capable of prioritizing a given list of genes according to their importance for a biological process based on, among other criteria, expression, sequence conservation, and

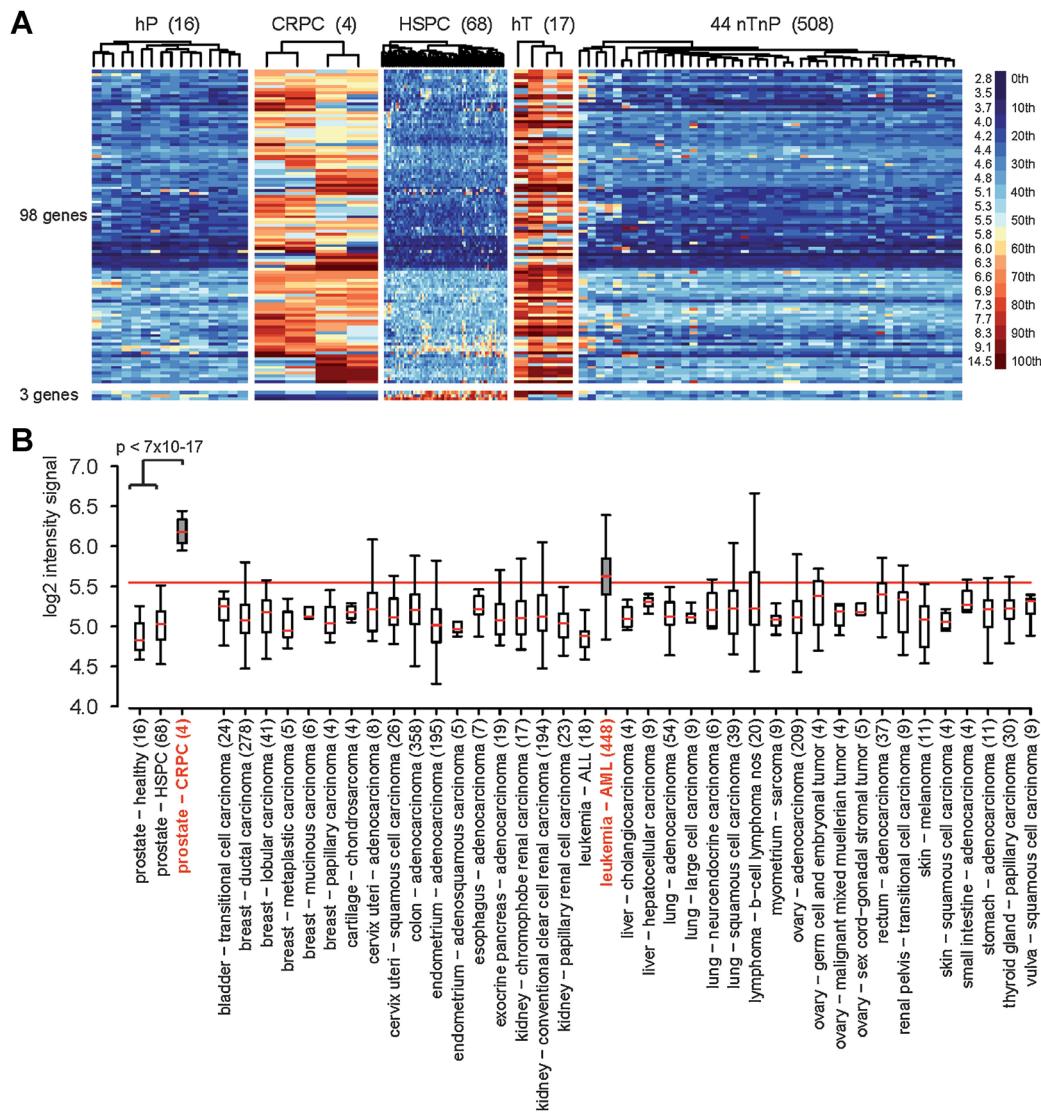


Fig. 2. Resulting Cancer/Testis signatures and NR6A1 transcript expression in prostate cancers. **A:** A false-color heatmap summarizes both CT gene signatures. Each line corresponds to a probe set. The expression data set (grouped into five large columns) corresponds to hP, CRPC, HSPC, hT, and nTnP samples. The number of samples is indicated in brackets. hT data includes four groups of samples including (from left to right) enriched populations of round spermatids, pachytene spermatocytes, total testis and seminiferous tubules. At the top of each large column, the cluster dendrogram of the x-axis reflects the relationship among the samples based on the similarity of relative microarray-based transcript expression (hierarchical clustering). Note that among the 98 CT gene candidates found to be significantly upregulated in CRPC, one third displays a fairly homogeneous detectable signal level. A color scale is shown for log₂ values and percentiles. **B:** Box plots displaying log₂ expression signals (y-axis) of NR6A1 transcript in healthy prostate, HSPC, CRPC, and 39 other cancer types (x-axis) based on the assembled GeneChip expression data. The total numbers of samples are indicated. The large horizontal red line represents the Background Expression Cutoff (BEC = 5.5, corresponding to the overall median log₂-transformed intensity). CRPC and acute myeloid leukemia (AML) samples are highlighted with a gray box and red bold fonts.

function [36]. The top two genes were the forkhead box protein G1 (FOXG1, Affymetrix probe set ID: 206018_at) that was associated with various cancers [37–40], and the germ cell nuclear factor (GCNF/NR6A1, 211402_x_at) [41]. Since NR6A1 had not been associated with human cancer, apart from work done with the murine P19 embryonal carcinoma (EC) cell

line [42], we decided to further investigate NR6A1 expression in PCa. Its transcript (Affymetrix probeset ID: 211402_x_at) was consistently above the threshold of detection in all CRPC (4 out of 4) and testicular samples, and at the lowest concentration in HSPC (64/68) and the other healthy tissues (including normal prostate; Fig. 2B). In addition, we found that

NR6A1's transcript accumulation in CRPC, with the exception of acute myeloid leukemia (AML), did not occur in 38 other cancer types sampled by the Expression Project for Oncology (expO, International Genomics Consortium, <http://www.intgen.org>; R. Mathieu, M. Primig, and F. Chalmel, unpublished observation; Fig. 2B). Finally, we employed the Oncomine database [43] to confirm our finding that NR6A1's transcript accumulates in CRPC. As expected, we observed a higher expression of NR6A1 in refractory samples as compared to naive samples in a PCa microarray study that focus on hormone therapy response status [44]. These RNA profiling results lead us to further examine NR6A1 at the protein level by immunocytochemistry (IHC) analyses.

NR6A1 Protein Accumulates in Castrate-Refractory Disease

We monitored NR6A1 protein accumulation in PCa by IHC using a polyclonal antibody on tissue microarrays (TMA). First, we observed that the protein indeed accumulated in the nuclei of cancer cells in high grade HSPC and CRPC sections (Fig. 3C,D) as compared to normal prostate and low grade tumor samples (Fig. 3A,B). Moreover, the fraction of anti-NR6A1 stained nuclei/cells was found to be significantly higher in HSPC sections (median = ~5%, range = [0–95%]) than in normal prostate (median = 0%, range = [0–90%]) and further increased in CRPC (median = ~70%, range = [20–95%]; $P < 0.0001$, Kruskall-Wallis non-parametric test).

NR6A1 Is Highly Expressed in Cases of Metastatic Prostate Cancer

Next, we asked if NR6A1-positive staining was associated with PCa metastasis as well. Using a TMA containing bone- and lymph node metastatic lesions ($n = 20$), we demonstrated substantially increased NR6A1 staining in all metastatic sites (Fig. 3E,F). The positive nuclear fraction of NR6A1-stained cells was found to be significantly higher in metastatic lesions (median = 80%, range = [0–100%]) than in normal prostate and localized HSPC samples ($P < 0.0001$, Kruskall-Wallis non-parametric test; Table I).

NR6A1 Protein Expression Is Associated With Tumor Progression and High Grade Prostate cancers

To further refine the picture of NR6A1 expression in PCa we examined the correlation of its protein level with traditional clinicopathological indicators (Table I). In the group of HSPC, the positive nuclear fraction of NR6A1-stained tumor cells was significantly lower in localized pT2 tumors (median = 0%,

range = [0–95%]) than in locally advanced pT3 PCa (25%, [0–90%]; $P = 0.02$, Mann-Whitney non-parametric test; Table I). Moreover, the percentage of NR6A1 positive cells was significantly increased in high grade tumors (Gleason 8–10; 60%, [5–90%]) when compared to low (Gleason 5–6; median = 0%, range = [0–95%]) or intermediate (Gleason 7; 5%, [0–95%]) grades ($P = 0.02$, Kruskall-Wallis non-parametric test) (Table I). Finally, no significant association was found between NR6A1 staining and age at diagnosis ($P = 0.5$, Spearman non-parametric test), or preoperative PSA level ($P = 0.6$) in HSPC.

NR6A1 Protein Is Associated With Tumor Cell Proliferation

We subsequently examined correlations of the NR6A1-positive staining in HSPC and CRPC with proliferation as determined by Ki-67 proliferative marker analysis. The median percentage of Ki-67 positive cells was 15% in CRPC (range = [3–90%]) and 1% in HSPC (range = [0–20%]). In the whole patient population, a statistically significant level of co-immunostaining was observed for NR6A1 and Ki-67 ($P = 0.002$, Spearman test).

NR6A1 Protein Is Not Associated With Biochemical Relapse After Radical Prostatectomy

Finally, we examined if NR6A1 protein expression could help predict cancer recurrence, independently from the above-mentioned clinicopathological indicators. To address this issue, we conducted an individually matched nested case-control study: 111 HSPC with biochemical relapse after radical prostatectomy were matched with 111 HSPC with identical age, Gleason score, pathological stage and preoperative PSA but without recurrence (Table II). After adjusting for Gleason score, stage, and PSA level, we concluded that NR6A1 status was not associated with biochemical relapse in HSPC ($P = 0.5$, log-rank test; Supplemental file 3).

DISCUSSION

Genome-wide expression screening strategies using GeneChips are powerful methods for pinpointing transcriptional events of interest despite a number of issues that complicate the interpretation of the data output. For example, in the case of heterogeneous samples it must be kept in mind that the resulting signal is an average of the transcripts present in the different cell types. Furthermore, changes in RNA concentrations across samples may be due to transcriptional effects, varying transcript stability or altered DNA copy numbers (or a combination of all of

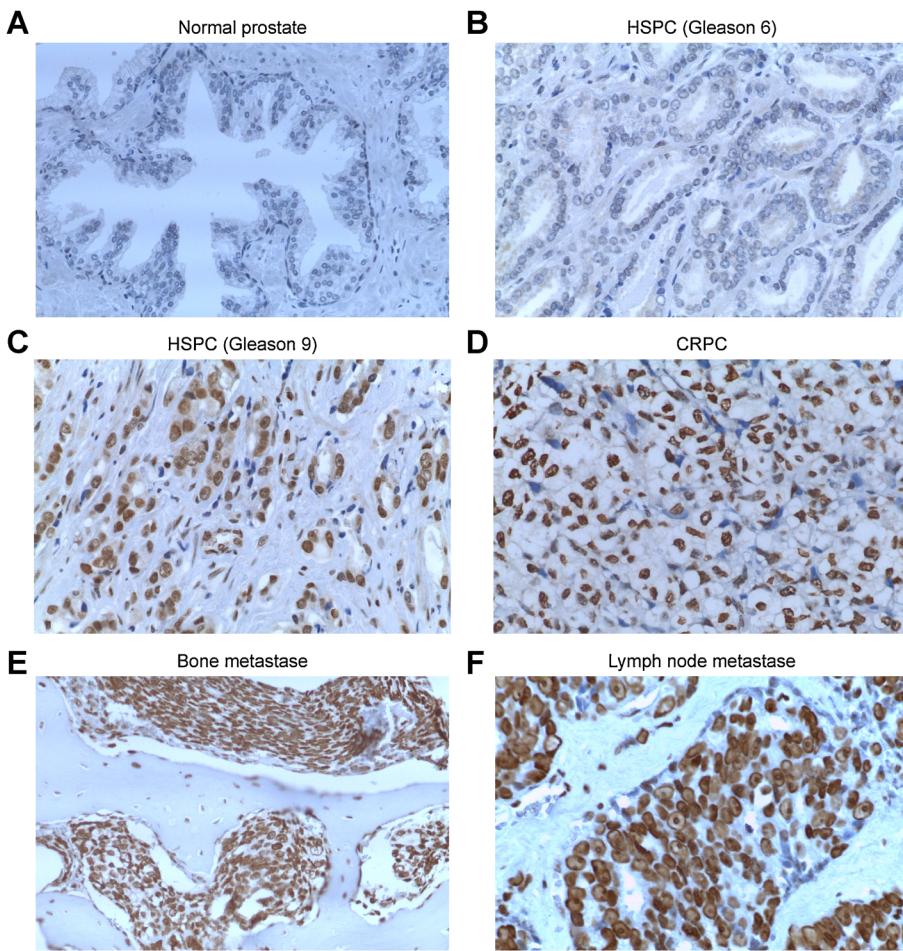


Fig. 3. NR6AI protein expression in prostate cancers Representative immunohistochemical images of human tissue sections from: (A) normal prostate, (B) moderately differentiated (Gleason 6), and (C) poorly differentiated (Gleason 9) HSPCs, (D) CRPC, (E) bone, and lymph node (F) metastases with anti-NR6AI antibody. Positive signal is given by DAB brown stain; tissues were counter-stained with hematoxylin. Bars indicate the scale.

them). Finally, another critical issue especially pertinent for human samples is the extent to which transcript levels are reproducible between individuals. This problem can usually be overcome by analyzing large numbers of samples. This is, however, difficult when they are rare like in the case of CRPC. Gene-based screening strategies based on RNA profiling experiments require in any case follow-up experimental validation by, for example, immunohistochemical (IHC) analysis using tissue microarrays (TMA) [45]. The sequential combination of both array technologies has become a widely used approach to identify and validate candidate biomarkers at the transcript and protein level prior large-scale and expensive clinical trials [46].

In the present study, we report the outcome of a genome-wide expression screen that aimed at the identification of novel Cancer/Testis genes relevant

for PCa. To this end, we assembled a large GeneChip expression data set from normal testicular samples obtained in our own laboratory [25] combined with data from HSPC, CRPC and a range of healthy tissues (including normal prostate) available via public repositories [26,27]. We were able to identify 98 genes for which transcripts were reliably detectable in healthy testicular samples and in either CRPC (95 genes) or HSPC (3) and undetectable in 45 non-testicular healthy tissue controls (including normal prostate). This low yield regarding the three candidates associated with HSPC is not really surprising given a previous observation that this type of PCa does not appear to express testis-selective genes [9]. We observed among our candidates sixteen known CT genes, including five genes belonging to the synovial sarcoma X breakpoint (SSX) family and four genes from the melanoma antigen (MAGE) family

(Supplemental file 2), which validates and supports the validity of our screening approach. Members of the SSX family were recently related with PCa progression and proposed as targets for new therapeutic approaches [47]. Upregulation of MageA subfamily in CRPC was previously reported [20]. Moreover, MAGE-C2 protein was already associated with metastatic PCa and CRPC, and identified as an independent predictor of biochemical recurrence after radical prostatectomy [48]. As expected (since tumors and testes both contain dividing cells), we found that one-tenth of the 98 selected genes was implicated in the control of the cell cycle progression by using functional enrichment analysis. Furthermore, we found a number of poorly characterized genes that thus constitute novel targets potentially relevant for spermatogenesis or PCa (or both); see Figure 2A and Supplemental file 2 and neXtProt for annotation and references [48].

Bearing in mind that transcriptional regulators critical for cell differentiation such as meiosis and gametogenesis may contribute to the etiology and the development of somatic cancer [13], we focused our analysis on genes proposed or known to encode transcription factors. As one of such candidates, one regulator appeared particularly interesting: the orphan nuclear receptor NR6A1, a transcriptional repressor that plays a critical role during embryogenesis, neurogenesis and gametogenesis in mouse models [28–32] for which no IHC data are currently available in the HPA (<http://www.proteinatlas.org>). Its transcript was unambiguously detected in all four CRPC and healthy testicular samples, and it was not found in HSPC samples as well as all healthy non-testicular controls including normal prostate. Moreover, meta-analysis of NR6A1 using both GPSy [36] and Oncomine [43] confirmed our decision to further investigate this gene.

We note that NR6A1 was found to be expressed in maturing male germ cells in the mouse [49–51], in the developing nervous system during embryogenesis [52] as well as in a variety of somatic tissues, including epididymis, oviduct, brain, and pituitary. However, the experiments were done using a very sensitive *lacZ* reporter gene assay [53]. One obvious explanation is that CT gene screens do not include samples from the entire set of human tissues harvested at all stages of pre-, and post-natal development, making it very difficult to categorize a gene as unambiguously testis-specific.

It was previously shown that rodent and human NR6A1 proteins are present in the nuclei of testicular germ cells [54,55], and our validation experiments suggest the same to be true for PCa cells. To further investigate this question and to ask if the protein's

accumulation was significantly linked to cancer progression we monitored it by IHC analysis on TMA (see patients characteristics in Material and Methods Section; Table I). Consistently NR6A1 expression at the protein level indeed mirrors mRNA concentrations obtained by GeneChips. First, we observed that the protein mostly localized to the nuclei of PCa cells which is consistent with the idea that it might unfold its biological activity as a transcriptional regulator during PCa development. Furthermore, we observed that increased NR6A1 expression at the protein level is associated with progression to locally advanced HSPC, CRPC and metastatic lesions. To further refine the picture we examined the correlation of its protein level with traditional clinicopathological indicators (Table I). We showed that the NR6A1's protein accumulation was positively correlated with aggressive PCa phenotypes as defined by traditional indicators such as advanced pT stage and high Gleason score. These results are in keeping with the significant association observed between NR6A1 and tumor cell proliferation determined by Ki-67 marker analysis. Taken together, these results strongly suggest that elevation of NR6A1 (from organ confined to locally advanced tumors, metastatic lesions and CRPC) is a relatively late event in PCa. Focusing on biochemical recurrence after radical prostatectomy, NR6A1 staining has no independent prognostic value in multivariate analysis when adjusting for the validated clinicopathological indicators (including pT stage, Gleason score and preoperative PSA level). This observation may be due to its close association with the established prognostic factors. However, our findings strongly suggest that NR6A1 is a novel biomarker for aggressiveness of PCa.

In the light of our results regarding the positive correlation between NR6A1 staining and PCa aggressiveness, one can raise the question whether it could play a role in the progression of PCa to an advanced stage disease. In addition to its nuclear accumulation in certain forms of HSPC and CRPC, NR6A1 has several key characteristics that support this hypothesis. First, the strong RNA signals we observed in PCa are not simply a consequence of tumor progression towards metastasis in general because the transcript levels were not found to be significantly elevated in 38 other types of cancer (with the exception of acute myelogenous leukemia). This may mean that the gene is not ubiquitously up-regulated in dividing and transformed cells marking out the restricted de-repression of NR6A1 in CRPC and AML as a special property of these malignancies. NR6A1 might therefore at least in part be responsible for a number of specific features observed in both pathologies. Second, this ligand-activated transcriptional repressor

is an orphan member of the nuclear receptor gene superfamily that binds its cognate DNA response elements as a homodimer [56–60]. NR6A1 is unable to form heterodimer with other nuclear receptors [56] but it can recruit nuclear corepressors [41,59,61–64] or bind essential transcriptional activators [31,32,41,56,59,60,63,64]. Thus, NR6A1 regulates the temporal and spatial gene expression of several processes related to stem cell growth and differentiation, neurogenesis and germ cell differentiation—that is to say it can alter cell-fate [49,57,65–67]. Third, neuroendocrine differentiation is known to be involved in PCa proliferation and invasion [68], and several studies have reported that neuroendocrine cell number increases in high grade and high stage tumors, particularly in HSPC and CRPC [69–71]. Since these cells are likely androgen-independent, it is conceivable that hormonal therapy does not eliminate neuroendocrine cancer cells and thus contributes to tumor recurrence [68]. Vitamin A-derived retinoic acid (RA) influences the differentiation of neural stem cells from neural induction through adulthood during the development of the vertebrate nervous system [72]. It was hypothesized that RA-regulated neurogenesis might involve NR6A1-signaling [66] as its transcript was up-regulated during RA-induced neural differentiation of certain EC cell lines [61,62]. We speculate that NR6A1 might contribute the molecular pathways leading to neuroendocrine differentiation in PCa. If this theory is proven, blocking NR6A1-signaling should likely extend the therapeutic window of ADT. Experimental work addressing these issues is under way.

CONCLUSIONS

We discovered that the human orphan nuclear receptor gene NR6A1 is elevated both at the transcript and protein levels in high grade HSPC, CRPC and metastatic lesions, using a gene-based expression screening of Cancer/Testis genes relevant for PCa combined with a TMA-based analysis. To the best of our knowledge, this is the first study to report the association between NR6A1 and PCa progression. Future *in vitro* and *in vivo* studies will investigate whether the pleiotropic function of this transcriptional repressor plays a critical role in the tumor progression and/or aggressiveness or if the observed expression signature is simply a consequence of the disease. These future insights may provide rationale for its use as reliable biomarker of aggressiveness and as a promising target for therapeutic intervention in the clinic.

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REFERENCES

1. Ferlay J, Parkin DM, Steliarova-Foucher E. Estimates of cancer incidence and mortality in Europe in 2008. *Eur J Cancer* 2010;46(4):765–781.
2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009;59(4):225–249.
3. Roehl KA, Han M, Ramos CG, Antenor JA, Catalona WJ. Cancer progression and survival rates following anatomical radical retropubic prostatectomy in 3,478 consecutive patients: Long-term results. *J Urol* 2004;172(3):910–914.
4. Johansson JE, Andren O, Andersson SO, Dickman PW, Holmberg L, Magnusson A, Adami HO. Natural history of early, localized prostate cancer. *JAMA* 2004;291(22):2713–2719.
5. Sharifi N, Gulley JL, Dahut WL. Androgen deprivation therapy for prostate cancer. *JAMA* 2005;294(2):238–244.
6. Xu B, Wang N, Wang X, Tong N, Shao N, Tao J, Li P, Niu X, Feng N, Zhang L, Hua L, Wang Z, Chen M. MiR-146a suppresses tumor growth and progression by targeting EGFR pathway and in a p-ERK-dependent manner in castration-resistant prostate cancer. *Prostate* 2012;72(11):1171–1178.
7. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991;254(5038):1643–1647.
8. Fratta E, Coral S, Covre A, Parisi G, Colizzi F, Danielli R, Marie Nicolay HJ, Sigalotti L, Maio M. The biology of cancer testis antigens: Putative function, regulation and therapeutic potential. *Mol Oncol* 2011;5(2):164–182.
9. Almeida LG, Sakabe NJ, deOliveira AR, Silva MC, Mundstein AS, Cohen T, Chen YT, Chua R, Gurung S, Gnajatic S, et al. CTdatabase: A knowledge-base of high-throughput and curated data on cancer-testis antigens. *Nucleic Acids Res* 2009;37(database issue):D816–D819.
10. Tyagi P, Mirakhur B. MAGRIT: The largest-ever phase III lung cancer trial aims to establish a novel tumor-specific approach to therapy. *Clin Lung Cancer* 2009;10(5):371–374.
11. Atanackovic D, Altorki NK, Cao Y, Ritter E, Ferrara CA, Ritter G, Hoffman EW, Bokemeyer C, Old LJ, Gnajatic S. Booster vaccination of cancer patients with MAGE-A3 protein reveals long-term immunological memory or tolerance depending on priming. *Proc Natl Acad Sci USA* 2008;105(5):1650–1655.
12. Caballero OL, Chen YT. Cancer/Testis (CT) antigens: Potential targets for immunotherapy. *Cancer Sci* 2009;100(11):2014–2021.
13. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/Testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 2005;5(8):615–625.

14. Andrade VC, Vettore AL, Felix RS, Almeida MS, Carvalho F, Oliveira JS, Chauffaille ML, Andriolo A, Caballero OL, Zago MA, et al. Prognostic impact of Cancer/Testis antigen expression in advanced stage multiple myeloma patients. *Cancer Immun* 2008;8:2.
15. Grigoriadis A, Caballero OL, Hoek KS, da Silva L, Chen YT, Shin SJ, Jungbluth AA, Miller LD, Clouston D, Cebon J, et al. CT-X antigen expression in human breast cancer. *Proc Natl Acad Sci USA* 2009;106(32):13493–13498.
16. Gure AO, Chua R, Williamson B, Gonen M, Ferrera CA, Gnijatic S, Ritter G, Simpson AJ, Chen YT, Old LJ, et al. Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. *Clin Cancer Res* 2005; 11(22):8055–8062.
17. Napoletano C, Bellati F, Tarquini E, Tomao F, Taurino F, Spagnoli G, Rughetti A, Muzii L, Nuti M, Benedetti Panici P. MAGE-A and NY-ESO-1 expression in cervical cancer: prognostic factors and effects of chemotherapy. *Am J Obstet Gynecol* 2008;198(1):99 e91–e97.
18. Svobodova S, Browning J, MacGregor D, Pollara G, Scolyer RA, Murali R, Thompson JF, Deb S, Azad A, Davis ID, et al. Cancer-testis antigen expression in primary cutaneous melanoma has independent prognostic value comparable to that of Breslow thickness, ulceration and mitotic rate. *Eur J Cancer* 2011;47(3):460–469.
19. Velazquez EF, Jungbluth AA, Yancovitz M, Gnijatic S, Adams S, O'Neill D, Zavilevich K, Albukh T, Christos P, Mazumdar M, et al. Expression of the Cancer/Testis antigen NY-ESO-1 in primary and metastatic malignant melanoma (MM)-correlation with prognostic factors. *Cancer Immun* 2007;7:11.
20. Suyama T, Shiraishi T, Zeng Y, Yu W, Parekh N, Vessella RL, Luo J, Getzenberg RH, Kulkarni P. Expression of Cancer/Testis antigens in prostate cancer is associated with disease progression. *Prostate* 2010;70(16):1778–1787.
21. Kulkarni P, Shiraishi T, Rajagopalan K, Kim R, Mooney SM, Getzenberg RH. Cancer/Testis antigens and urological malignancies. *Nat Rev Urol* 2012;9(7):386–396.
22. Shiraishi T, Getzenberg RH, Kulkarni P. Cancer/Testis antigens: Novel tools for discerning aggressive and non-aggressive prostate cancer. *Asian J Androl* 2012;14(3):400–404.
23. Axelsen JB, Lotem J, Sachs L, Domany E. Genes overexpressed in different human solid cancers exhibit different tissue-specific expression profiles. *Proc Natl Acad Sci USA* 2007; 104(32):13122–13127.
24. Shiraishi T, Terada N, Zeng Y, Suyama T, Luo J, Trock B, Kulkarni P, Getzenberg RH. Cancer/Testis antigens as potential predictors of biochemical recurrence of prostate cancer following radical prostatectomy. *J Transl Med* 2011;9:153.
25. Chalmel F, Rolland AD, Niederhauser-Wiederkehr C, Chung SS, Demougin P, Gattiker A, Moore J, Patard JJ, Wolgemuth DJ, Jegou B, et al. The conserved transcriptome in human and rodent male gametogenesis. *Proc Natl Acad Sci USA* 2007; 104(20):8346–8351.
26. Barrett T, Troup DB, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippe KH, Sherman PM, et al. NCBI GEO archive for functional genomics data sets—10 years on. *Nucleic Acids Res* 2011;39(database issue): D1005–D1010.
27. Parkinson H, Sarkans U, Kolesnikov N, Abeygunawardena N, Burdett T, Dylag M, Emam I, Farne A, Hastings E, Holloway E, et al. ArrayExpress update—An archive of microarray and high-throughput sequencing-based functional genomics experiments. *Nucleic Acids Res* 2011;39(database issue): D1002–D1004.
28. Chung AC, Cooney AJ. Germ cell nuclear factor. *Int J Biochem Cell Biol* 2001;33(12):1141–1146.
29. Chung AC, Xu X, Niederreither KA, Cooney AJ. Loss of orphan nuclear receptor GCNF function disrupts forebrain development and the establishment of the isthmian organizer. *Dev Biol* 2006;293(1):13–24.
30. Hummelke GC, Cooney AJ. Reciprocal regulation of the mouse protamine genes by the orphan nuclear receptor germ cell nuclear factor and CREMtau. *Mol Reprod Dev* 2004;68(4): 394–407.
31. Lan ZJ, Gu P, Xu X, Jackson KJ, DeMayo FJ, O’Malley BW, Cooney AJ. GCNF-dependent repression of BMP-15 and GDF-9 mediates gamete regulation of female fertility. *EMBO J* 2003; 22(16):4070–4081.
32. Rajkovic M, Iwen KA, Hofmann PJ, Harneit A, Weitzel JM. Functional cooperation between CREM and GCNF directs gene expression in haploid male germ cells. *Nucleic Acids Res* 2010;38(7):2268–2278.
33. Varambally S, Yu J, Laxman B, Rhodes DR, Mehra R, Tomlins SA, Shah RB, Chandran U, Monzon FA, Becich MJ, et al. Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. *Cancer Cell* 2005; 8(5):393–406.
34. Chalmel F, Primig M. The Annotation, Mapping, Expression and Network (AMEN) suite of tools for molecular systems biology. *BMC Bioinformatics* 2008;9:86.
35. Fromont G, Rozet F, Cathelineau X, Ouzzane A, Doucet L, Fournier G, Cussenot O. BCAR1 expression improves prediction of biochemical recurrence after radical prostatectomy. *Prostate* 2012;72(12):1359–1365.
36. Britto R, Sallou O, Collin O, Michaux G, Primig M, Chalmel F. GPSy: A cross-species gene prioritization system for conserved biological processes—application in male gamete development. *Nucleic Acids Res* 2012;40(web server issue):W458–W465.
37. Adesina AM, Nguyen Y, Guanaratne P, Pulliam J, Lopez-Terrada D, Margolin J, Finegold M. FOXG1 is overexpressed in hepatoblastoma. *Hum Pathol* 2007;38(3):400–409.
38. Adesina AM, Nguyen Y, Mehta V, Takei H, Stangeby P, Crabtree S, Chintagumpala M, Gumerlock MK. FOXG1 dysregulation is a frequent event in medulloblastoma. *J Neurooncol* 2007;85(2):111–122.
39. Chan DW, Liu VW, To RM, Chiu PM, Lee WY, Yao KM, Cheung AN, Ngan HY. Overexpression of FOXG1 contributes to TGF-beta resistance through inhibition of p21WAF1/CIP1 expression in ovarian cancer. *Br J Cancer* 2009;101(8):1433–1443.
40. Kim TH, Jo SW, Lee YS, Kim YJ, Lee SC, Kim WJ, Yun SJ. Forkhead box O-class 1 and forkhead box G1 as prognostic markers for bladder cancer. *J Korean Med Sci* 2009;24(3):468–473.
41. Cooney AJ, Hummelke GC, Herman T, Chen F, Jackson KJ. Germ cell nuclear factor is a response element-specific repressor of transcription. *Biochem Biophys Res Commun* 1998; 245(1):94–100.
42. Heinzer C, Susens U, Schmitz TP, Borgmeyer U. Retinoids induce differential expression and DNA binding of the mouse germ cell nuclear factor in P19 embryonal carcinoma cells. *Biol Chem* 1998;379(3):349–359.
43. Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB, Barrette TR, Anstet MJ, Kincaid-Beal C, Kulkarni P, et al. Oncomine 3.0: Genes, pathways, and networks

- in a collection of 18,000 cancer gene expression profiles. *Neoplasia* 2007;9(2):166–180.
44. Best CJ, Gillespie JW, Yi Y, Chandramouli GV, Perlmutter MA, Gathright Y, Erickson HS, Georgevich L, Tangrea MA, Duray PH, et al. Molecular alterations in primary prostate cancer after androgen ablation therapy. *Clin Cancer Res* 2005;11(19 Pt 1):6823–6834.
 45. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998;4(7):844–847.
 46. Brennan DJ, Kelly C, Rexhepaj E, Dervan PA, Duffy MJ, Gallagher WM. Contribution of DNA and tissue microarray technology to the identification and validation of biomarkers and personalised medicine in breast cancer. *Cancer Genomics Proteomics* 2007;4(3):121–134.
 47. Smith HA, Cronk RJ, Lang JM, McNeil DG. Expression and immunotherapeutic targeting of the SSX family of cancer-testis antigens in prostate cancer. *Cancer Res* 2011;71(21):6785–6795.
 48. von Boehmer L, Keller L, Mortezaei A, Provenzano M, Sais G, Hermanns T, Sulser T, Jungbluth AA, Old LJ, Kristiansen G, et al. MAGE-C2/CT10 protein expression is an independent predictor of recurrence in prostate cancer. *PLoS ONE* 2011;6(7):e21366.
 49. Hirose T, O'Brien DA, Jetten AM. RTR: A new member of the nuclear receptor superfamily that is highly expressed in murine testis. *Gene* 1995;152(2):247–251.
 50. Katz D, Niederberger C, Slaughter GR, Cooney AJ. Characterization of germ cell-specific expression of the orphan nuclear receptor, germ cell nuclear factor. *Endocrinology* 1997;138(10):4364–4372.
 51. Zhang YL, Akmal KM, Tsuruta JK, Shang Q, Hirose T, Jetten AM, Kim KH, O'Brien DA. Expression of germ cell nuclear factor (GCNF/RTR) during spermatogenesis. *Mol Reprod Dev* 1998;50(1):93–102.
 52. Susens U, Aguiluz JB, Evans RM, Borgmeyer U. The germ cell nuclear factor mGCNF is expressed in the developing nervous system. *Dev Neurosci* 1997;19(5):410–420.
 53. Lan ZJ, Xu X, Chung AC, Cooney AJ. Extra-germ cell expression of mouse nuclear receptor subfamily 6, group A, member 1 (NR6A1). *Biol Reprod* 2009;80(5):905–912.
 54. Chalmel F, Lardenois A, Evrard B, Mathieu R, Feig C, Demougin P, Gattiker A, Schulze W, Jegou B, Kirchoff C, Primig M. Global human tissue profiling and protein network analysis reveals distinct levels of transcriptional germline-specificity and identifies target genes for male infertility. *Hum Reprod* 2012;27(11):3233–3248.
 55. Hummelke GC, Meistrich ML, Cooney AJ. Mouse protamine genes are candidate targets for the novel orphan nuclear receptor, germ cell nuclear factor. *Mol Reprod Dev* 1998;50(4):396–405.
 56. Borgmeyer U. Dimeric binding of the mouse germ cell nuclear factor. *Eur J Biochem* 1997;244(1):120–127.
 57. Chen F, Cooney AJ, Wang Y, Law SW, O'Malley BW. Cloning of a novel orphan receptor (GCNF) expressed during germ cell development. *Mol Endocrinol* 1994;8(10):1434–1444.
 58. Greschik H, Schule R. Germ cell nuclear factor: An orphan receptor with unexpected properties. *J Mol Med (Berl)* 1998;76(12):800–810.
 59. Greschik H, Wurtz JM, Hublitz P, Kohler F, Moras D, Schule R. Characterization of the DNA-binding and dimerization properties of the nuclear orphan receptor germ cell nuclear factor. *Mol Cell Biol* 1999;19(1):690–703.
 60. Yan ZH, Medvedev A, Hirose T, Gotoh H, Jetten AM. Characterization of the response element and DNA binding properties of the nuclear orphan receptor germ cell nuclear factor/retinoid receptor-related testis-associated receptor. *J Biol Chem* 1997;272(16):10565–10572.
 61. Bauer UM, Schneider-Hirsch S, Reinhardt S, Pauly T, Maus A, Wang F, Heiermann R, Rentrop M, Maelicke A. Neuronal cell nuclear factor—A nuclear receptor possibly involved in the control of neurogenesis and neuronal differentiation. *Eur J Biochem* 1997;249(3):826–837.
 62. Fuhrmann G, Chung AC, Jackson KJ, Hummelke G, Banahmad A, Sutter J, Sylvester I, Scholer HR, Cooney AJ. Mouse germline restriction of Oct4 expression by germ cell nuclear factor. *Dev Cell* 2001;1(3):377–387.
 63. Yan Z, Jetten AM. Characterization of the repressor function of the nuclear orphan receptor retinoid receptor-related testis-associated receptor/germ cell nuclear factor. *J Biol Chem* 2000;275(45):35077–35085.
 64. Yan Z, Kim YS, Jetten AM. RAP80, a novel nuclear protein that interacts with the retinoid-related testis-associated receptor. *J Biol Chem* 2002;277(35):32379–32388.
 65. Mullen EM, Gu P, Cooney AJ. Nuclear receptors in regulation of mouse ES cell pluripotency and differentiation. *PPAR Res* 2007;2007:61563.
 66. Zechel C. The germ cell nuclear factor (GCNF). *Mol Reprod Dev* 2005;72(4):550–556.
 67. Zhao H, Li Z, Cooney AJ, Lan ZJ. Orphan nuclear receptor function in the ovary. *Front Biosci* 2007;12:3398–3405.
 68. Sun Y, Niu J, Huang J. Neuroendocrine differentiation in prostate cancer. *Am J Transl Res* 2009;1(2):148–162.
 69. Ahlgren G, Pedersen K, Lundberg S, Aus G, Hugosson J, Abrahamsson PA. Regressive changes and neuroendocrine differentiation in prostate cancer after neoadjuvant hormonal treatment. *Prostate* 2000;42(4):274–279.
 70. Jiborn T, Bjartell A, Abrahamsson PA. Neuroendocrine differentiation in prostatic carcinoma during hormonal treatment. *Urology* 1998;51(4):585–589.
 71. Puccetti L, Supuran CT, Fasolo PP, Conti E, Sebastiani G, Laccanini S, Mandras R, Milazzo MG, Dogliani N, De Giuli P, et al. Skewing towards neuroendocrine phenotype in high grade or high stage androgen-responsive primary prostate cancer. *Eur Urol* 2005;48(2):215–221; discussion 221–213.
 72. Panchision DM, McKay RD. The control of neural stem cells by morphogenic signals. *Curr Opin Genet Dev* 2002;12(4):478–487.