Co-expression of the androgen receptor and the transcription factor ZNF652 is related to prostate cancer outcome

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Abstract. ZNF652, a DNA binding transcription factor, was previously suggested to be differentially expressed in prostate cancer. This study investigated if the expressions of ZNF652 and androgen receptor (AR) in prostate cancer are associated with prostate specific antigen (PSA) defined relapse. ZNF652 and AR immunoreactivity were evaluated in prostate tissues from a cohort of 121 patients with prostate cancer and associations with disease outcome determined. To assess if ZNF652 can influence AR expression, or vice versa, levels of expression of ZNF652, AR and PSA were determined in the prostate cell line LNCaP following induction of AR activity by 5α-dihydrotestosterone, or knockdown of ZNF652 expression. Two thirds of prostate tumors retained high levels of ZNF652 (71/109 cases) and 50% of tumors high levels of AR (57/113). There was a significant decrease (p=0.005) in relapse-free survival of patients with high expression levels of both ZNF652 and AR and this was independent of preoperative PSA and seminal vesicle involvement. Modulation of either AR or ZNF652 expression levels in LNCaP cells was not associated with any corresponding changes to the levels of either ZNF652 or AR, respectively. High levels of expression of both AR and ZNF652 in clinically organ-defined prostate cancer are associated with a statistically increased risk of relapse. The ZNF652 and AR transcription factors are acting independently and it is proposed that the continued maintenance of expression of ZNF652 in AR positive cells results in a gene expression pattern that contributes to the relapse.

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Introduction

While the incidence of males with cancerous cells in the prostate is estimated to be as high as 70% by the age of 80 years, the majority of this disease remains indolent and 30% of men diagnosed with prostate cancer will eventually succumb to the disease (1). Prostate specific antigen (PSA) is now widely used as a screening tool for prostate cancer but has limitations due to high rates of false positive and false negative findings at diagnosis. PSA levels are also used to predict metastatic disease post-postatectomy, with elevated PSA levels indicating biochemical relapse (1). For example, in a study of men fifteen years following radical prostatectomy, 15% showed increased PSA levels (biochemical PSA relapse) and of these 34% developed metastatic disease with a median time of 8 years (2). Recent studies suggest that in lower grade prostate cancers utilising prostate specific antigen density provides a significant improvement in predicting biochemical relapse compared with a single PSA measurement (3). By incorporating PSA doubling time, together with pathological Gleason score and time from prostatectomy to biochemical relapse, patients can be more accurately stratified according to their risk of prostate-specific mortality (4). Despite these improvements in the monitoring and utilisation of PSA values, identification of additional clinical markers that can more accurately predict cancer progression would have a significant impact in determining the appropriate treatment protocol for each patient.

Molecular profiling studies have been utilised to identify additional markers useful for prostate cancer prognosis. Arising from such approaches α-methylacyl coenzyme A

racemase (AMACR), Ki-67, Rb, MMP-2, MMP-9, p53, E-cadherin ECAD) and others (1) have been identified as potential biomarkers. Retrospective studies to determine if such markers are useful in predicting recurrence of cancer after radical prostatectomy patients suggest Ki-67, (5) Her-2/neu, (6) and EZH2:ECAD (7) have prognostic roles in conjunction with the use of existing or refined clinical parameters. Since none of the identified markers were in common between these studies, it is evident that evaluations of new markers are warranted. The future scenario is likely to be the development of a multi-gene signature that can be used for prognosis. Accordingly, identification of additional genes whose expression is related to prognosis will be a valuable contribution to this ultimate goal.

We have characterised ZNF652, a C_2H_2 classical zinc finger protein that was initially identified in a breast expression library as an interacting partner with CBFA2T3 (MTG16) (8). CBFA2T3 is a breast cancer tumor suppressor (9) and functions as a potent transcriptional repressor complex by recruitment of various co-repressors, for example SIN3A and NCOR1, and histone deacetylases (10). The target gene specificity of the CBFA2T3 complex is determined by recruitment of ZNF652 or other DNA binding zinc finger proteins. An association of CBFA2T3 with cancer is supported by its role as a breast cancer tumor suppressor (9) and involvement in a specific translocation with RUNX1 in acute myeloid leukaemia (AML) (11). Since ZNF652 is a specific effector of CBFA2T3 repressor complex function, the expression of ZNF652 in a limited data set of various tissue normal/tumor pairs was determined (8). In a subsequent immunohistochemistry-based study, there was significant variation in expression of ZNF652 in vulvar carcinoma but no significant relationship with survival was detected (12). Since previous data also suggested ZNF652 expression was markedly variable in prostate cancer, this study was initiated to determine the expression of ZNF652 in a cohort of prostate tumors.

In this study we also determined the expression of the androgen receptor (AR) in prostate cancer, since the development and maintenance of the normal prostate gland is dependent on a functioning androgen-signaling axis, and therefore levels of AR are critical for prostate function (13). The primary components of this axis are the interaction of the androgen hormone 5α-dihydrotestosterone (DHT) with the AR and the subsequent transcriptional regulation of AR target genes. The functional androgen-signaling axis forms the basis of androgen ablation therapy for prostate cancer. Increased expression of AR is associated with the development of castrate resistant prostate cancer and disease progression (14,15). The aims of this study were to assess both ZNF652 and AR protein expression by immunohistochemistry of prostate tumors and to evaluate the relationship of their expressions with biochemical relapse. In addition, whether ZNF652 and AR were functioning independently at the molecular level was determined by modulation of their expression levels in LNCaP cells.

Materials and methods

Patient cohort. The patient cohort consisted of 121 men undergoing radical prostatectomy between 1996 and 2002 for clinically organ confined prostate cancer. They were accrued into the study following informed consent and ethics approval from the Clinical Investigation Committee, Repatriation General Hospital, Daw Park, South Australia. Tissues were provided by the joint Flinders Medical Centre/Repatriation General Hospital tumor bank. The time of PSA relapse was determined as the earliest date that the post-operative serum PSA level was detectable above the sensitivity threshold of the assay (>0.2 ng/ml) on consecutive PSA measurements at least one month apart. In cases where detectable serum PSA levels persisted after surgery, a PSA relapse was defined as the time of the first increase in serum PSA level confirmed by a consecutive measurement. Table I provides a detailed description of the patient cohort.

Table I. Summary of clinical and pathology data of the early stage radical prostatectomy cohort.

Patients (n)	121
Median age at diagnosis (years)	61 (range 44-73, n=119) 2 missing
Median preoperative serum PSA (ng/ml)	9.0 (range 0.2-79, n=113) 8 missing
Median follow-up (months)	79.7 (range 34.7-139.3)
Pathological stage	
pT2	33
pT3	88
Gleason score (n)[a]	
5-6	79
7	30
>7	11
Missing	1
Seminal vesicle involvement	
Negative	112
Positive	9
Extracapsular extension	
Negative	70
Positive	51
Surgical margins	
Negative	72
Positive	49
PSA-failure rate (n)[b]	26/121 (21.5%)

[a]Gleason score determined by a pathologist (J.S.). [b]The presence of relapse at the time of surgery for patients was determined by a PSA-failure, i.e., a return to measurable serum PSA levels on two sequential measurements subsequent to a post-operative level below the sensitivity threshold of the assay (<0.2 ng/ml).

AR and ZNF652 immunohistochemistry. Triplicate cores from each patient were included in tissue microarray blocks and

immunostained for AR and ZNF652. Specificity of the affinity-purified rabbit polyclonal antibodies to the AR sequence (AR-N20, Santa Cruz) and the ZNF652 sequence have been previously confirmed by Western blotting (8,12,16). AR immunohistochemistry was as described previously (16,17). Briefly, the formalin-fixed paraffin sections of the tissue microarrays were deparaffinised and treated with a microwave-based antigen retrieval protocol (5 min 750 W, 15 min 350 W in 10 mM citrate buffer, pH 6.5). The sections were incubated overnight at 4˚C with a 1/1000 dilution of anti-AR antibody. Visualization of AR immunoreactivity was achieved using biotinylated anti-rabbit immunoglobulins (1/400, 1 h RT, Dako), streptavidin-peroxidise conjugate (1/500, 1 h RT, Dako) and diaminobenzidine tetrahydrochloride (DAB) for the immunoperoxidase reaction to yield an insoluble brown deposit. AR immunoreactivity was assessed by video image analysis using established procedures and 113 patients had evaluable cores (17). Video image measurements were confined to epithelial cells. Discrimination between malignant and benign tissue elements was determined by a pathologist using standard cytological and pathological features. Color images for twenty contiguous fields per specimen were collected at a magnification of x400. Video image measurements included the DAB stained area (i.e., positively stained nuclear area in pixel units) and the total nuclear area examined (i.e., positively and negatively stained nuclear area in pixel units, stained with DAB and hematoxylin), respectively. These values were used to derive percentage AR positive nuclear area. Tumors were categorized as having high AR if the highest nuclear positive area of the triplicate cores, that is the median percentage AR positive nuclear area measured in malignant epithelial cells, was >85%.

The ZNF652 immunohistochemistry used the Dako EnVision + System, Peroxidase (K4011; Dako, Carpinteria, CA, USA) and Dako autostainer as described previously (12). Briefly, deparaffinised prostate sections were microwaved in Tris/EDTA buffer pH 9.0 to unmask the epitopes, and treated with 0.03% hydrogen peroxide. The sections were then incubated with polyclonal anti-ZNF652 antibody (1:400, 2.5 μg/ml IgG) overnight at 4˚C, peroxidise labelled polymer conjugated to goat anti-rabbit for 30 min and DAB for 10 min. All series included positive controls, which consisted of human normal vulvar carcinoma that had been shown to express ZNF652 (12). Negative controls included substitution of the polyclonal antibody with the same concentration of normal rabbit IgG. Only distinct nuclear staining was considered positive. Immunostaining was scored on a three-tiered scale for both intensity of (absent/weak, 1; moderate, 2; strong, 3) and extent of staining (percentage of positive tumor cells: 1, <10%; 2, 10-50%; 3, >50%). The scoring results of intensity and extent were multiplied to give a composite score ranging from 1 to 9 for each tumor. Based on the staining pattern observed in normal prostate epithelium and cancer tissue samples, protein expression was defined as high when composite scores were ≥6. The prostate TMAs contained evaluable cores from 109 patients and the ZNF652 expression in the tumor was considered to be retained if at least one of the replicate cores from a patient scored ≥6, and low when composite scores were <6. Sections were scored by a pathologist (J.M.N.) and an experienced senior scientist (R.H.) without knowledge of clinical data.

Statistical analysis. Statistical analysis was performed using the Statistical Package for the Social Sciences version 13.0 (SPSS, Chicago, IL). The χ^2 test and Spearman's correlation tests were used to examine the relationship between ZNF652 or AR immunostaining in tumor foci with various clinical and pathological parameters. PSA relapse-free survival was used as the end-point in Kaplan-Meier analysis to determine whether ZNF652 and AR immunostaining was related to the rate and time of relapse. PSA relapse-free survival was calculated from the date of diagnosis to the date of relapse or date of last follow-up if relapse-free. At time of censore (31 December 2006), 22.6% (26/115) of patients in this archival series were determined to have PSA failure. One patient who died from other causes was censored on the date of death. Statistical significance was set at $p<0.05$.

Cell-based molecular assays. The prostate cell line LNCaP was purchased from ATCC and grown in RPMI medium with 10% fetal calf serum. Before each experiment the cells were grown for 48 h in phenol red-free RPMI media with 10% charcoal stripped FCS. Treatment of LNCaP cells with DHT (0.01-10 nM) was used to increase AR activity. The expression of ZNF652 was knocked down in LNCaP cells by transfection with either the ZNF652-specific (target sequences, siZNF652-2: 5'-GUAGAGAAAGUCAGCG UUA-3' or ZNF652-4: 5'-GAGAAGCACAUGAACGUUA-3'; Dharmacon RNA Technologies) or scrambled control (Qiagen) siRNA at 100 nM using a lipitoid transfection reagent as previously detailed (18). Following treatments cells were harvested and one half used to prepare lysates for Western blot analysis and the other half for isolation of RNA for preparation of cDNA and reverse transcription real-time PCR as previously described (19). Levels of AR and PSA expression was determined by Western blot analysis using either anti-AR (AR-N20, Santa Cruz) or anti-PSA (A0562, Dako) antibodies, respectively. mRNA expression levels were determined by real-time RT-PCR using the following primers: ZNF652 (forward: 5'- CTTCACCAGCAAACAGACTGT GAA and reverse: 5'- TTCTTTTCTGCATATCCATGGACG primers) and PSA (forward: 5'-ACCAGAGGAGTTCTTGA CCCCAAA and reverse: 5'-CCCCAGAATCACCCGAG CAG primers). The housekeeping gene cyclophilin A was used to normalize the expression of ZNF652 and PSA (19).

Results

AR and ZNF652 immunoreactivity predicts PSA failure in prostate cancer. Anti-ZNF652 antibody detects a single band on Western blot analysis of a variety of cell lines and has been used previously for immunohistochemistry studies (8,12). In this study, Western blot analysis of LNCaP cells using anti-ZNF652 antibody detected an extra band (Fig. 3). Since this extra band was not present after co-immunoprecipitation with the anti-ZNF652 antibody, and was not nuclear located (data not shown), this was considered non-specific background. Immunoreactivity for ZNF652 in tissue sections was observed in both the nuclei and to a lesser

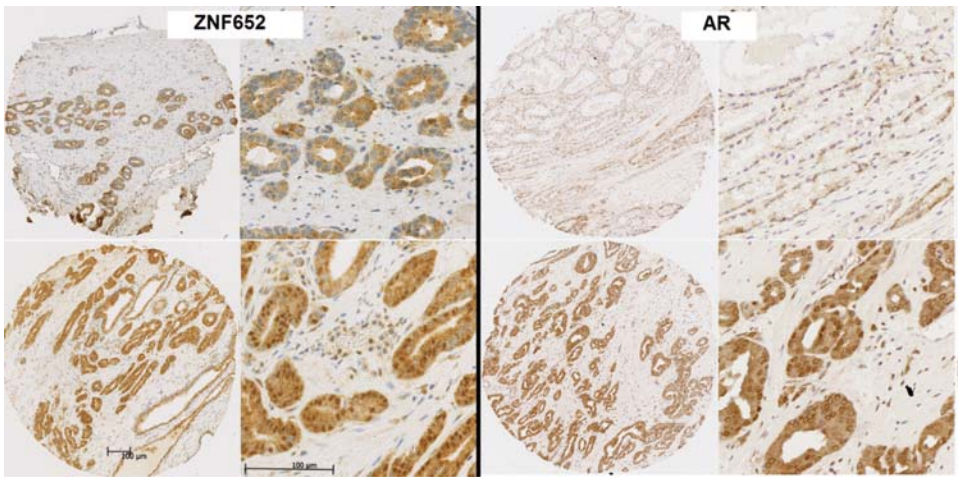

Figure 1. Expression of ZNF652 and AR in prostate tissue. Staining of prostate tumor microarrays with anti-ZNF652 antibody (left four panels) and anti-AR antibody (right four panels). Upper panel shows examples of tissues with low levels of nuclear ZNF652 or AR staining and lower panels show tissues with high levels of nuclear ZNF652 or AR staining. Some non-specific cytoplasmic staining was also present.

Table II. Analysis of relapse-free survival in patients treated by radical prostatectomy.

A. Univariate Cox regression.

Variable	No. of patients	Relative risk	95% Confidence interval	P-value
Age at diagnosis	118	1.00	0.94-1.07	0.985
Preoperative PSA[a]	112	2.52	1.13-5.63	**0.024**
Gleason score[b]	119	6.37	2.68-15.18	**<0.0001**
Surgical margins	120	5.81	2.33-14.47	**<0.0001**
Seminal vesicle involvement	120	3.61	1.36-9.59	**0.01**
ZNF652 score[c]	108	1.09	0.49-2.44	0.837
AR score[d]	111	1.78	0.81-3.92	0.152
ZNF652 and AR score[e]	104	2.73	1.25-5.94	**0.012**

B. Bivariate Cox regression analysis of ZNF652 and AR combined score with significant clinical and pathological parameters.

Variable	No. of patients	Relative risk	95% Confidence interval	P-value
ZNF652 and AR score[a]	97	2.59	1.15-5.80	**0.021**
Preoperative PSA[b]		2.94	1.31-6.61	**0.009**
ZNF652 and AR score	104	2.10	0.96-4.63	0.065
Gleason score[c]		5.66	2.35-13.64	**<0.0001**
ZNF652 and AR score[d]	104	2.31	1.03-5.18	**0.041**
Seminal vesicle involvement		3.38	1.22-9.31	**0.019**
ZNF652 and AR score[e]	104	1.88	0.85-4.17	0.120
Surgical margins		5.25	2.06-13.38	**0.001**

[a]Preoperative serum PSA level (prostate specific antigen, ng/ml) dichotomized by cut-point <10.0 vs. ≥10.0; [b]Gleason score <7 vs. ≥7; [c]AR positive nuclear area measured by video image analysis dichotomized by median value <85 vs. ≥85; [d]Histoscore of ZNF652 immunostaining dichotomized by cut-point ≥6 vs. <6; [e]Combined ZNF652 and AR score, AR positive nuclear area ≥85 and ZNF652 histoscore ≥6 vs. all other groups. P-values highlighted in bold indicate p<0.05.

extent in the cytoplasm. In the present study, only distinct nuclear staining was considered in the analyses due to our findings that some background cytoplasmic staining was also observed in the negative controls where normal rabbit IgG was substituted for the polyclonal anti-ZNF652 antibody. In preliminary immunohistochemistry of paraffin sections of formalin-fixed prostate tissue all five cases of normal prostate epithelia, five cases of prostate hyperplasia and three of five cases of prostate carcinoma expressed ZNF652, while the other two cases of prostate carcinoma had low expression of

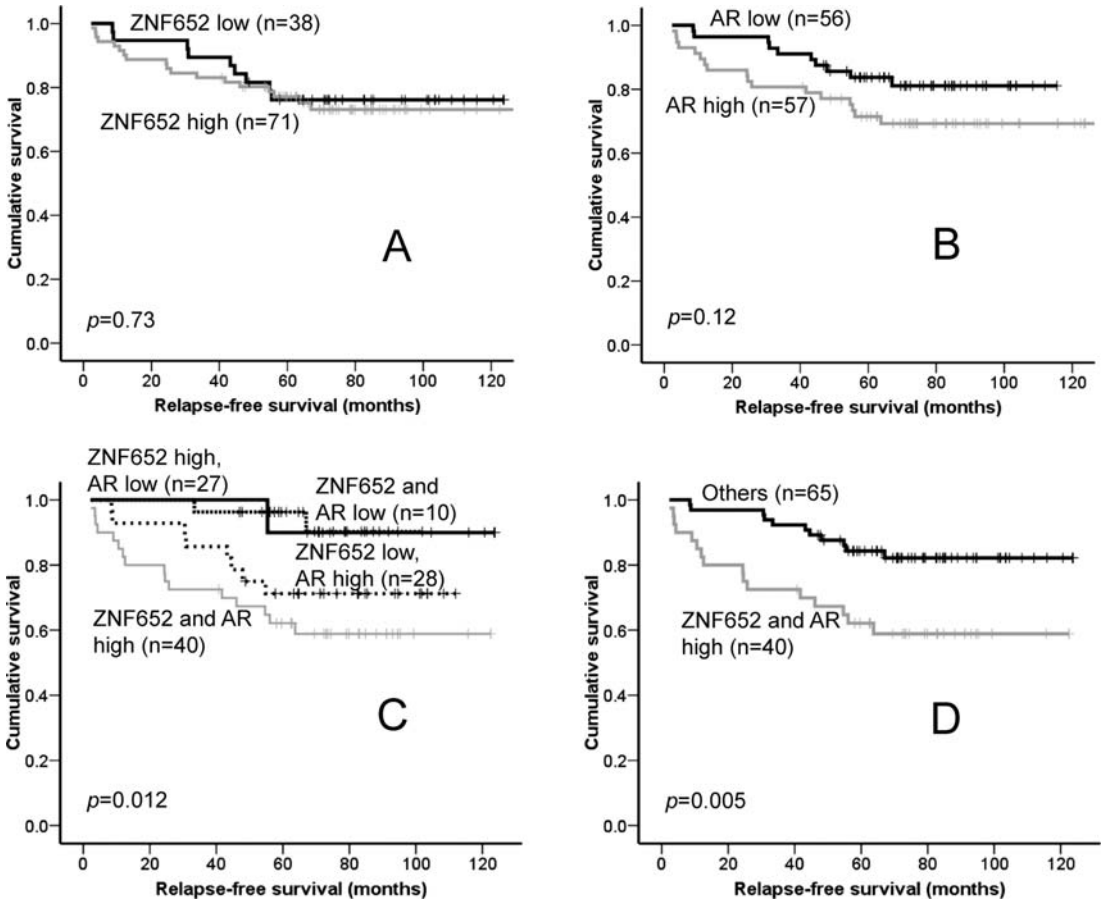

Figure 2. Relationship of ZNF652 and AR expression with PSA-progression in clinically localised prostate cancer. Kaplan-Meier product limit plots of ZNF652 (with low ZNF652 expression defined as a score of <6) and AR (with low AR expression defined as <85% AR positive nuclear area) expression as assessed by immunostaining. Relapse refers to PSA relapse. (A) ZNF652 expression is not significantly associated with PSA relapse-free survival. Log-rank statistic 0.12, p=0.73. (B) AR expression is not significantly associated with PSA relapse-free survival. Log-rank statistic 2.43, p=0.12. [(C) and (D)] High expression of both ZNF652 and AR expression define a group of patients with poor PSA relapse-free survival. (C) Four groups of patients categorised by ZNF652 and AR expression. Log-rank statistic 11.00, p=0.012. (D) High ZNF652 and AR expression compared with other groups pooled. Log-rank statistic 8.03, p=0.005.

ZNF652. To further investigate the potential variation of ZNF652 levels in prostate carcinoma, expression was determined in a prostate tissue microarray and two thirds of evaluable prostate tumors retained high levels of ZNF652 (71/109 cases), representative staining sections are presented in Fig. 1.

Nuclear AR immunoreactivity was present and could be assessed in 113 of the 121 patients. The median highest percentage AR positive nuclear area of the three cores in the non-malignant and malignant epithelial cells assessed by image analysis was 85% (range 11-95%). Fifty percent of tumors (57/113) had an AR positive nuclear area >85% (i.e., high levels as defined in Materials and methods). Representative stained sections showing high and low AR expression are presented in Fig. 1.

Results of the univariate Cox proportional hazards regression analysis are given in Table IIA. The levels of either ZNF652 or AR protein alone were not significantly related to relapse-free survival while Gleason score, surgical margins, seminal vesicle involvement and pre-operative PSA levels were significant predictors of relapse-free survival in the patient cohort. Neither ZNF652 nor AR scores were significantly associated with the clinicopathological parameters including Gleason score, PSA levels at diagnosis, grade, stage, seminal vesicle involvement, extracapsular extension and surgical margin status (data not shown).

When both ZNF652 and AR protein expression were combined in a score, patients with high ZNF652 and high AR levels had a 2.7-fold increased risk of relapse (Table IIA). The significant univariate clinical parameters were further assessed with the combined ZNF652 and AR score using a bivariate Cox regression analysis rather than multivariate analysis due to small sample group (Table IIB). The combined ZNF652 and AR score was found to be independent of pre-operative PSA and seminal vesicle involvement but the significant relationship with relapse-free survival was not independent of Gleason score nor surgical margins. Kaplan-Meier curves for PSA indicated relapse-free survival are presented in Fig. 3 for ZNF652, AR and the combined ZNF652 and AR levels, respectively. While ZNF652 or AR scores alone were not significantly associated with relapse-free survival, there was a significant difference in relapse-free survival when patients with clinically localised prostate cancer were stratified according to levels of both ZNF652 and AR (Fig. 2C, log-rank statistic 11.00, p=0.012). The data set was re-analysed to compare the relapse-free survival between those patients with high expression of both ZNF652 and AR and all other patients (Fig. 2D, log-rank statistic 8.03,

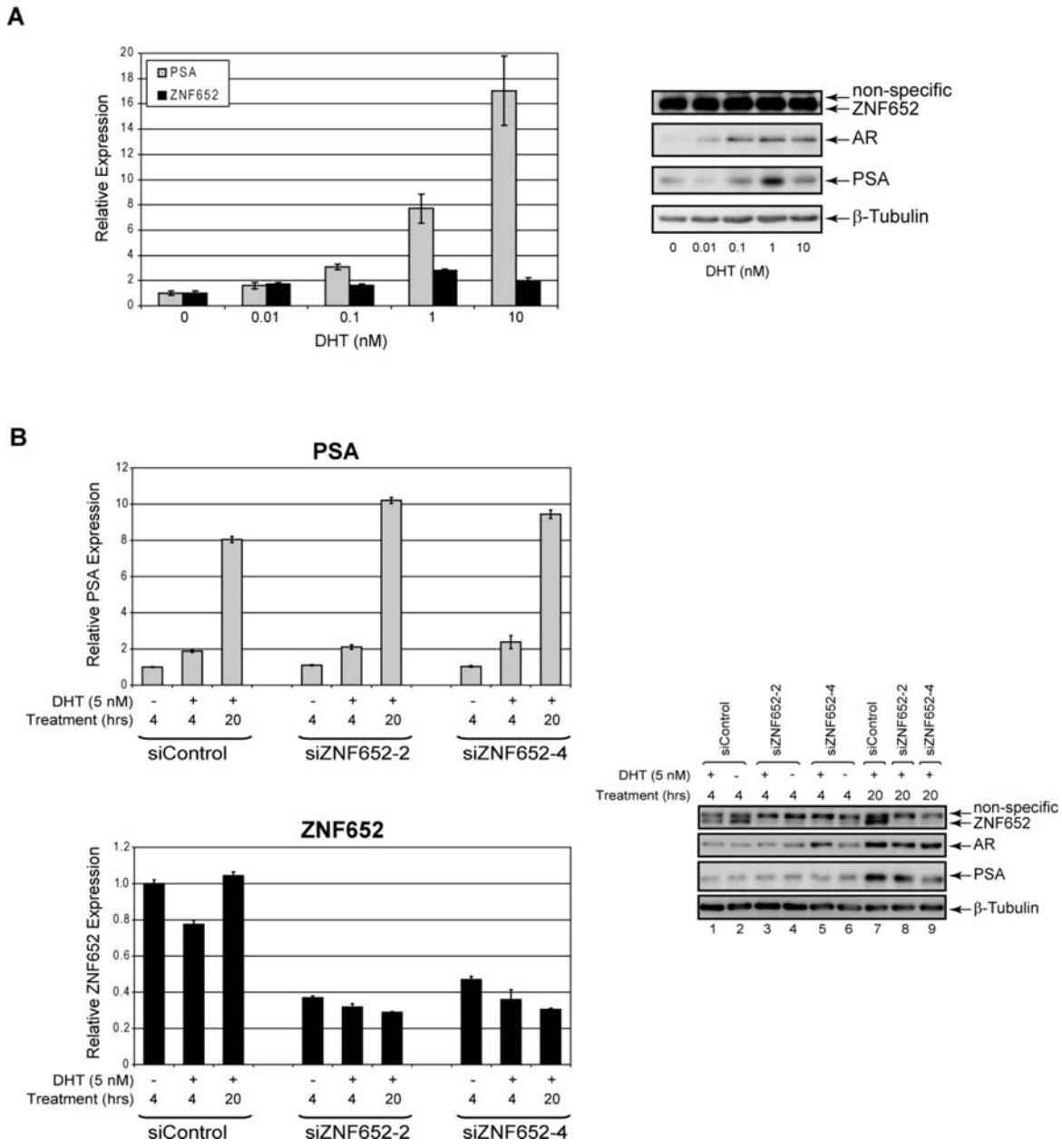

Figure 3. ZNF652 and AR function independently in the prostate cell line LNCaP. (A) Variation in AR expression does not influence levels of ZNF652. The prostate cell line LNCaP was grown in various concentrations of DHT for 48 h. Relative levels of ZNF652 and PSA message were determined by real-time RT-PCR (left panel) and protein by Western blot analysis (right panel). Variation in ZNF652 expression does not influence levels of AR. The prostate cell line LNCaP was transfected with either of two siRNAs against the ZNF652 sequence (siZNF652-2 and siZNF652-4) or a scrambled control and incubated for 48 h. Either 4 or 20 h before harvesting the cells, the cultures were induced with 5 nM DHT. Relative expression of PSA (left upper panel) and ZNF652 (left lower panel) messages were measured by real-time RT-PCR. The levels of proteins were detected by Western blot (right panel).

p=0.005). The proportion of patients with PSA relapse in those with high levels of both ZNF652 and AR (15/40) was also significantly different (p=0.02) from the proportion of relapses in all other patients (11/65).

Are ZNF652 or AR dependently regulated? Patients with high levels of both ZNF652 and AR expression have a lower chance of relapse-free survival. Since both ZNF652 and AR regulate the transcription of downstream genes by binding to their cognate DNA binding sites we determined if either one of these genes can directly or indirectly regulate the other. *In silico* analysis of ZNF652 promoter and intron sequences did not detect a high homology match to the consensus bipartite AR sequence. The consensus binding sequence of ZNF652 has recently been determined (18). At least 2.2 kb 5' to the start site of the AR transcript has been defined as the promoter and upstream regulatory regions (20) and this region together with an additional upstream 3 kb, did not contain a consensus ZNF652 binding sequence. Therefore, from *in silico* analysis, it was considered unlikely that AR or ZNF652 can directly regulate each other's expression.

To further investigate whether there was any direct or indirect relationship between ZNF652 and AR expression, studies were undertaken in the AR-positive prostate cancer cell line LNCaP. AR activity was induced in a dose-dependent manner by treating LNCaP cells with increasing amounts of

DHT. To assess AR activity, prostate specific antigen (PSA), a direct target of AR (21), was assessed by real-time RT-PCR and Western blot analysis. When LNCaP cells were treated with increasing amounts of DHT there were increasing mRNA levels of PSA and protein levels of both AR and PSA, but marginal changes in the mRNA and protein levels of ZNF652 (Fig. 3A). Therefore, there was no evidence that AR can directly or indirectly modulate the expression of ZNF652.

It was then determined if modulation of ZNF652 expression influences the expression of AR (Fig. 3B). The expression of ZNF652 was knocked down in LNCaP cells using two different siRNAs and the cells incubated for a further 48 h. For either the last 4 or 20 h of this incubation, DHT was added to induce AR activity. DMSO, the solvent control, was added in the absence of DHT treatment. Levels of message and protein confirmed that both siRNAs knock down ZNF652. The levels of AR and PSA were induced by DHT treatment but these levels of induction were not influenced in cells with knockdown of ZNF652 (Fig. 3B). Therefore modulations of either AR or ZNF652 expression levels in LNCaP cells were not associated with any major modulation in the levels of either ZNF652 or AR.

Discussion

Expression of either ZNF652 or AR, as assessed by immunohistochemistry in a cohort of 121 prostate tumors, were not significantly related to the occurrence of patient PSA monitored relapse (Table II). However, analysis using a score combining expression of both ZNF652 and AR was found to be independent of pre-operative PSA and seminal vesicle involvement but the significant relationship with relapse-free survival was not independent of Gleason score nor surgical margins. As reported in other studies (22), Gleason score, surgical margins, seminal vesicle involvement and pre-operative PSA levels were significant predictors of relapse-free survival. Kaplan-Meier plots show retention of high levels of ZNF652 together with high levels of AR, are associated with PSA relapse (Fig. 2). ZNF652 has previously been suggested to be a tumor suppressor (8) and therefore the results from these immunohistochemistry studies suggest loss of ZNF652 expression is associated with good prognosis in prostate cancer. It is likely that the role of ZNF652 is complex, since analysis of an expression microarray analysis of prostate shows the levels of expression of ZNF652 is increased in prostate cancer compared with the normal tissue (23). This study showed an average increase of ZNF652 expression in 65 prostate tumors of 43% (59% increase in the median) compared with 18 cases of normal prostate, while in 25 cases of metastatic tumors the average increase was 79% (100% increase in the median). In this study, the expression of ZNF652 and AR were not correlated in 65 prostate tumors ($r=0.139$, $p=0.13$); while in the 25 cases of metastatic prostate cancer there was a significant correlation between ZNF652 and AR expression ($r=0.363$, $p=0.04$). These findings further support the results from our immunohistopathology study.

A number of immunohistochemistry-based studies have investigated the relationship between AR levels in prostate cancer from diagnosis to patient outcome. Comparison between studies is hampered by differing methodologies, antibodies and scoring systems (24). Of the larger investigations of prostate tissues derived from untreated patients, a study of 167 prostate cancers derived from patients with a median follow-up of 88 months failed to find any relationship between AR expression and clinical progression or survival (24). This negative finding is likely to be due to technical issues since latter studies using image analysis to assess AR expression in 551 prostate tumors from patients with a median follow-up of 66 months, (25) and in 53 tissues from patients with a median follow-up of 51 months (17), showed that a high level of AR expression was an independent prognostic indicator of decreased biochemical recurrence-free survival. In this study we determine AR expression using an anti-AR polyclonal antibody and image analysis techniques to provide an unbiased assessment of staining intensity (16). There was a trend of higher AR expression in patients with biochemical relapse, although this was not statistically significant.

In a comprehensive gene expression study that identified AR regulated genes, ZNF652 was not identified as an AR regulated gene (26). *In silico* analysis of ZNF652 and AR promoter sequences also failed to identify possible binding sites for AR or ZNF652, respectively. To determine if there was an indirect relationship between ZNF652 and AR expression, the expression of ZNF652 was knocked down with specific siRNAs in LNCaP cells and AR activity was increased by growth of LNCaP in the presence of DHT (Fig. 3). PSA levels were used to determine the relative activity of AR. The results show that levels of ZNF652 and AR are functionally independent and there is no direct or indirect relationship between their relative expressions.

Both AR and ZNF652 function as transcription factors, with the AR generally being an activator (27), and ZNF652 a repressor of transcription (8,18). The number of verified downstream genes regulated by AR are increasing rapidly with the application of genome-wide chromatin immunoprecipitation to map global AR binding sites (28-31). ZNF652 is a recently characterised transcription factor with a potential role in a variety of cancers (8,12). Although the consensus binding sites for the ZNF652 zinc finger protein are known, TCF12 (HEB), also a transcription factor, is at present the only established target of ZNF652 repression (18). It is suggested that ZNF652 and AR are functioning as master regulators of transcription with the continued maintenance of ZNF652 expression in AR positive cells resulting in a gene expression pattern that contributes to micro- or clinical-metastasis that can be detected as PSA relapse. Studies are in progress to identify the downstream genetic targets of the ZNF652 transcription factor. It is suggested that a subset of the ZNF652 and AR regulated genes relevant to prostate cancer development will contribute to the determination of prognosis for patients with a diagnosis of prostate cancer.

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