Proteins Annexin A2 and PSA in Prostate Cancer Biopsies Do Not Predict Biochemical Failure

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Abstract. *Background/Aim: We previously reported the use
of mass spectrometry and western blotting to identify proteins
from tumour regions of formalin-fixed paraffin-embedded
biopsies from 16 men who presented with apparently localized
prostate cancer, and found that annexin A2 (ANXA2) appeared
to be a better predictor of subsequent biochemical failure than
prostate-specific antigen (PSA). Materials and Methods: In
this follow-up study, ANXA2 and PSA were measured using
western blotting of proteins extracted from biopsies from 37
men from a subsequent prostate cancer trial. Results: No
significant differences in ANXA2 and PSA levels were
observed between men with and without biochemical failure.
The statistical effect sizes were small, d=0.116 for ANXA2,
and 0.266 for PSA. Conclusion: ANXA2 and PSA proteins
measured from biopsy tumour regions are unlikely to be good
biomarkers for prediction of the clinical outcome of prostate
cancer presenting with apparently localized disease.*

Serum prostate-specific antigen (PSA) is a measure widely
used to assist in the early detection of prostate cancer, but on
its own is of limited value in predicting the clinical course
of a prostate cancer. There is therefore a need to identify
biomarkers that better predict tumour behaviour and so
facilitate improved individualized treatment [reviewed in (1-
4)]. Many human studies have focused on discovery of
predictive or prognostic biomarkers from tissue biopsies,

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plasma or serum, exosomes, and urine or prostatic secretions,
including measurement of genes, transcripts, proteins and
metabolites (1-10). The focus for our group has been the
discovery of proteins that could be used to predict the likely
behaviour of prostate cancer at the time of the initial
diagnosis. Access to archival collections of formalin-fixed
paraffin-embedded (FFPE) cancer specimens with associated
clinical details including treatment and disease outcome
provides a resource for potential discovery of biomarkers,
and we have therefore used archival specimens collected as
part of the Trans-Tasman Radiation Oncology Group
(TROG) trials of radiation plus androgen deprivation for
treatment of locally advanced prostate cancer (11, 12).

We previously reported use of mass spectrometry to
identify proteins from FFPE prostate cancer tissue, followed
by western blotting to show that the regulatory protein
annexin A2 (ANXA2) appeared to be the best predictor of
biochemical failure, which was usually due to development
of metastatic disease (13). Our previous examination of
prospective biomarkers was carried out using archival FFPE
specimens from 16 patients from the TROG Trial 96.01 trial
of radiation plus androgen deprivation (11). The TROG Trial
96.01 cases used for protein analysis were chosen to
represent a range of times to biochemical failure. The current
study set out to test the conclusions of the pilot study by
using a new set of otherwise unselected FFPE biopsies from
men entered into the subsequent TROG 03.04 trial, which
was of androgen suppression and radiotherapy with or
without zoledronic acid (12).

Materials and Methods

Biopsy specimens for the current work were from the Wellington
cohort of men enrolled on the TROG 03.04 trial. Proteomic studies
on the biopsies were approved by the Central Ethics Committee of

New Zealand (approval number WGT/03/06/065/AM04). All men on the trial presented with locally advanced prostate cancer without evidence of distant metastases. The presenting tumour characteristics of men on the trial were as described in the first end-point report (12), when the median follow-up of trial patients was 6.5 years.

Prior to protein analysis, archival haematoxylin and eosin (H&E)-stained sections from each case were examined by a pathologist (BD, PF) to confirm the presence of both tumour and normal regions, which were then excised from adjacent unstained sections. Sufficient protein for western blot analysis was excised from each of the tumour and control regions of 37 FFPE specimens. A further 11 blocks contained tumour tissue but insufficient normal tissue to allow both tumour and control amounts of the proteins to be quantified. For western blotting, 10-20 µg of extracted protein from each sample was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) prior to electrophoretic transfer and reaction with antibodies for protein detection.

Biochemical methods, including protein extraction from tumour and control regions from archival FFPE biopsy specimens, and use of multiplexed western blotting to measure ANXA2, PSA and actin, were as previously described (13). Briefly, deparaffinised 10 µm FFPE sections were incubated in 250 µl 40 mM Tris-HCl (pH 8.2), 2% SDS, 3% dithiothreitol for 1 h at room temperature followed by 20 min at 100°C. Extracted proteins were precipitated at −20°C overnight using a ProteoExtract® Protein Precipitation Kit (EMD Millipore, Billerica, MA, USA), then resuspended in 1×lithium dodecyl sulfate (LDS) sample buffer containing sample reducing agent (Invitrogen, Carlsbad, CA, USA) and separated on 4-12% one-dimensional SDS-PAGE gels prior to transfer to Hybond-LFP membranes (GE Healthcare, Pittsburgh, PA, USA) for 1 h at 30 V. After blocking, membranes were incubated overnight with 1:100 anti-annexin A2 (C-10, mouse monoclonal, sc-28385; Santa Cruz Biotechnology, Dallas, TX, USA) and 1:200 anti-PSA (C-19, goat polyclonal, sc-7638; Santa Cruz Biotechnology); followed by sequential incubation with the fluorophore conjugated secondary antibodies AlexaFluor®555 goat anti-mouse IgG (H+L) and AlexaFluor®647 chicken anti-goat IgG (H+L), both at 1:2,500. Actin was measured on the same membranes using a mouse monoclonal anti-actin antibody (MAB1501; Sigma-Aldrich, St. Louis, MO, USA) followed by AlexaFluor®555 goat anti-mouse IgG (H+L) 1:2500. Proteins were quantified using ImageJ™ software (https://imagej.net/ImageJ) after scanning the developed blots with a FLA-5100 fluorescent scanner (FujiFilm, Tokyo, Japan). ANXA2 and PSA abundances were normalized against the actin abundance on the same western blot, and ANXA2 and PSA tumour/control ratios (T/C) were calculated to correct for inter-individual variation in protein amounts (13). For statistical analysis, data were log-transformed and two-sided independent samples t-tests were carried out using IBM SPSS Statistics 23 (IBM Corp., Armonk, NY, USA). Power calculations were performed using G*Power (14).

Results and Discussion

Baseline characteristics for the 37 men with sufficient FFPE tissue for protein analysis of tumour and control regions were Gleason score 7-9, T stage 2b or above, and serum PSA 2.2-47.7 ng/ml. All men had then received external beam radiation therapy in conjunction with either 6 months (short-term) or 18 months (intermediate-term) of androgen suppression with or without 18 months of zoledronic acid (12). Subsequently, at the time of protein analysis, the clinical follow-up of the 37 men in the sample was 9-13 years, and 12 men had experienced biochemical failure (Table I), with a median time to failure of 46 months.

Initial statistical analysis focused on comparison of mean T/C forANXA2 and PSA between patients that sustained biochemical failure and those that did not (Table II). The protein expression data were not normally distributed therefore independent samples t-tests were performed on log-transformed data. There was no significant difference in T/C for ANXA2 between patients that sustained biochemical failure and those that did not [t(35)=0.332, p=0.742], with only a small effect size (d=0.116); nor was there a significant difference in that for PSA [t(35)=0.725, p=0.473, d=0.266]. The effect sizes for T/C for ANXA2 and PSA support an expectation for relatively small effects. For comparison, if the effect size (15) had been large (greater than 1.012) there would have been 80% power to detect it using the current sample set of 37 cases. Alternatively, based on the current results, a sample size of at least 788 men would be required to detect a small effect (d=0.2) with 80% power using a two-sided t-test with α=0.05.

One difference in design between the previous and the current study is that the previous analysis was of FFPE specimens chosen to represent a range of times to biochemical failure (five cases >100 months, five cases ≤40 months, and six 'intermediate' of 50-87 months). That analysis of 16 men from the TROG 96.01 trial, in which 11 out of the 16 cases suffered biochemical failure within 100 months, indicated that a more than 2-fold T/C ANXA2, or 3-fold T/C for PSA predicted time to biochemical failure. For the current TROG 03.04 sample dataset, the estimated odds of biochemical failure occurring were therefore also calculated at the ANXA2 and PSA cut-offs that previously gave significant differences using Kaplan–Meier analysis (13). In the current study, only 7 out of the 37 patients (18.9%) had a more than 2-fold T/C for ANXA2 and only one of these experienced biochemical failure. Patients with T/C for ANXA2 >2 were less likely to experience biochemical failure within the study period: odds ratio=0.288, 95% confidence interval=0.031-2.714. Similarly, there were 14 patients (37.8%) with T/C for PSA of more than 3-fold but only three experienced biochemical failure and patients with T/C PSA >3 were also less likely to experience biochemical failure: odds ratio=0.424, 95% confidence interval=0.092-1.953.

The predictive ratios for ANXA2 and PSA established in the previous work were therefore not supported in this follow-up study, although the number of cases was small. Assuming only 37.8% of patients experience a more than 3-fold T/C for PSA, a sample of at least 235 men would be required to determine there is a significant association between experiencing biochemical failure and a more than 3-fold T/C for PSA with 80% power and α=0.05. Similarly, assuming 18.9% of patients

Table I. *Summary of cases used for protein analysis of tumour and control regions. Archival formalin-fixed paraffin-embedded (FFPE) samples were from men from the Wellington cohort of the Trans-Tasman TROG 03.04 trial of androgen suppression and radiotherapy, with or without zoledronic acid, in men with locally advanced prostate cancer (12).*

Treatment	Number of cases with sufficient FFPE tissue for calculation of protein T/C ratios	Number of cases with biochemical failure
EBRT + STAD	11	3
EBRT + ITAD	9	1
EBRT + STAD + Z	9	3
EBRT + ITAD + Z	8	5

EBRT: External beam radiotherapy, STAD: short-term (6 months) androgen deprivation, ITAD: intermediate-term (18 months) androgen deprivation, Z: zoledronic acid.

Table II. *Tumour/control (T/C) ratios for annexin A2 (ANXA2) and prostate-specific antigen (PSA) in prostate cancer biopsies from 37 men with or without subsequent biochemical failure.*

Biochemical failure	Time to biochemical failure (months)			T/C, mean±SD	
	Mean±SD	Median	Range	ANXA2	PSA
Yes (n=12)	61.17±36.13	45.5	24-120	0.99±0.63	2.86±4.10
No (n=25)	-	-	-	1.17±0.98	6.19±12.11

experience a more than 2-fold T/C for ANXA2, a sample of at least 210 men would be required to determine that there is a significant association between experiencing biochemical failure and a more than 2-fold T/C for ANXA2.

In this study of 37 men from the TROG 03.04 trial, there were no significant differences in the expression of ANXA2 or PSA in tumour samples according to whether or not men subsequently underwent biochemical failure. This can be compared to the findings of the pilot study of 16 men from the TROG 96.01 trial, which demonstrated a statistically significant association between ANXA2 and biochemical failure, and a weak association between PSA and biochemical failure (13). One difference between the studies was that the TROG 96.01 cases were chosen to represent a range of times to biochemical failure, plus sufficient excised control and tumour for western blotting of ANXA2, PSA, and actin. In contrast, in the current analysis, all available archival FFPE samples with sufficient tumour and control regions were used without selection based on disease outcome. One possible confounding factor is that expansion of the tumour mass in patients with metastatic disease (16) may mean that men with high-grade locally advanced cancer who are more likely to develop metastatic disease may have very little control tissue present in archival FFPE blocks.

The findings of this study are important although based on small sample sizes. The treatment and clinical follow-up of men on whom proteomic studies are performed needs to be standardized, and this only occurs within the prospect of a randomized controlled trial. The 37 trial subjects from a single centre with sufficient archival tissue for analysis can be considered a sufficient number for a proteomic study of this type. A much larger sample size could be achieved in a multi-centre prospective study in which extra biopsy material would be taken at the onset to ensure that the great majority of trial subjects could undergo proteomic studies to see if there were correlations with subsequent clinical outcome.

However, given that no significant differences were detected in the current work, ANXA2 or PSA protein measured in excised tissue regions are unlikely to be clinically useful prognostic biomarkers for prostate cancer diagnosed in patients with localized disease, and do not warrant the cost and effort of further investigation in a larger study.

Acknowledgements

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