ARTICLE
Molecular Diagnostics

p53 nuclear accumulation as an early indicator of lethal prostate cancer

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BACKGROUND: After radical prostatectomy (RP) for prostate cancer (PC), p53 alterations predict biochemical relapse (BCR), however, recent evidence suggests that metastatic relapse (MR) not BCR is a surrogate for PC specific mortality (PCSM). This updated analysis of a previously published study investigated the association between p53 aberrations, MR and PCSM in men with localised PC.

METHODS: Two hundred and seventy-one men with localised PC treated with RP were included. RP specimens stained for p53 by immunohistochemistry were scored as (a) percentage of p53-positive tumour nuclei; and (b) clustering, where ≥12 p53-positive cells within a ×200 power field was deemed ‘cluster positive’. Associations between p53 status and clinical outcomes (BCR, MR and PCSM) were evaluated.

RESULTS: Increasing percentage of p53-positive nuclei was significantly associated with shorter time to BCR, MR and PCSM (All $p < 0.001$). Half of the patients were p53 cluster positive. p53 cluster positivity was significantly associated with poorer outcomes at all clinical endpoints (BCR: HR $2.0$, 95% CI $1.51–2.65$, $p < 0.001$; MR: HR $4.1$, 95% CI $2.02–8.14$, $p < 0.001$; PCSM: HR $12.2$, 95% CI $1.6–93$; $p = 0.016$). These associations were independent of other established prognostic variables.

CONCLUSIONS: p53 aberrations in radical prostatectomy tissue predict clinically relevant endpoints of MR and PCSM.

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BACKGROUND
Prostate cancer (PC) is the most commonly diagnosed cancer and the second leading cause of cancer death in men in developed countries.1 Localised treatment is beneficial for a subset of patients but there is clear evidence of overtreatment in others. Understanding the fundamental biology is essential to identify better therapeutic targets while tools to optimally select those patients with potentially lethal disease at the outset will improve outcomes and avoid unnecessary morbidity.

p53 is a potent tumour suppressor, and mutation of the encoding TP53 gene is a central element in the initiation and progression in at least half of all human cancers.2,3 TP53 mutations often result in nuclear accumulation of mutant p53 proteins, making it amenable to immunohistochemical analysis across multiple malignancies including PC.4–6 Somatic TP53 mutations are found in up to 20% of patients with localised PC and are enriched in metastatic castration resistant PC tissue (53%), suggesting they may be a harbinger for aggressive disease.7 In patients receiving radiation and androgen deprivation therapy for locally advanced disease, p53 mutations are associated with poor prognosis, but the effect on response to specific treatments is contradictory across the two major studies.8,9

There is increasing evidence that metastatic relapse (MR) is a more accurate surrogate for PC specific survival than biochemical relapse (BCR).10 Recent work from our own group has shown that markers clearly associated with biochemical recurrence do not necessarily translate into markers of MR with more mature follow-up.11 In this cohort, p53 nuclear accumulation correlates with biochemical recurrence after radical prostatectomy (RP) as described by our group more than 15 years ago.12 With longer follow-up, we aimed to examine whether p53 could also predict MR and PC specific survival.

METHODS
Study population
This retrospective study consisted of 271 men with localised PC treated with radical prostatectomy between 1988 and 1996 in a single institution (St Vincent’s Hospital, Sydney, Australia). The 271 cases for the study were selected from 409 RPs treated in the study period and were chosen due to the availability of archival tissue. They were not stratified for known pre-operative prognostic factors. A detailed description of the patient cohort has been previously reported.13 The study was approved by the St Vincent’s Research Ethics Committee (12/231).

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Pathological examination
Formalin fixed paraffin embedded (FFPE) radical prostatectomy specimens were examined contemporaneously for involvement of surgical margins, extraprostatic extension, seminal vesicle involvement, lymph node involvement, pTNM stage (AJCC 2010) and original (1992) Gleason score (GS). These details were extracted from the Australian Prostate Cancer Research Centre-NSW (APCRC-NSW) bio-resource and database. Histopathology sections were regarded as per the 2005 ISUP modification of the Gleason grading system by three uropathologists blinded to the original GS and patient outcomes. All cases were assigned a GS and a 2014 ISUP grade (also known as grade group) in line with the recommendations of the 2014 ISUP consensus conference.

Immunohistochemical analysis
As described previously, immunohistochemistry was performed on formalin-fixed paraffin-embedded blocks sectioned at 5 microns, mounted on SuperFrost Plus slides (Menzel-Glaser, Germany) and processed within 10 days of sectioning. The mouse monoclonal p53 DO-7 epitope directed antibody (DAKO Corpora, Carpinteria, CA) and avidin-biotin-peroxidase and diamino-benzidine kits (Vector Laboratories, Burlingham, CA) were used according to manufacturer’s instructions. Sections were deparaffinised in xylene and rehydrated through graded ethanol then heated in a pressure cooker in 0.01 M citrate buffer at pH 6.0 for 10 min to enhance antigen retrieval. The sodium citrate buffer was brought to the boil in an open pressure cooker. The slides were placed in boiling buffer, the lid locked, and the pressure cooker was allowed to boil for 4 min before the hot plate was turned off. The pressure cooker was then allowed to cool for 10 min under gently running cool water. When pressure in the pressure cooker fell the lid was removed. The sections were then treated with 2% H2O2 for 10 min at RT to inactivate endogenous peroxidase activity. Following a blocking step with 10% normal horse serum, the sections were incubated with DO-7 antibody diluted to 1:200 in 2% BSA/PBS overnight at 4°C. Subsequently, sections were sequentially incubated with a biotinylated horse antimouse IgG, avidin-biotinylated complex and diaminobenzidine. Counterstaining was undertaken with Whittlock’s haematoxylin and ‘light green’ before dehydration through graded ethanol and xylene and cover slipping. A contiguous section was stained with haematoxylin and eosin. Positive controls for p53 nuclear accumulation used with each run of staining were a paraffin embedded pellet of the prostate cancer cell line DU145, which has a documented p53 mutation, a colon cancer specimen with p53 missense mutation and a tongue cancer specimen with p53 nuclear accumulation. Negative controls used were a paraffin embedded pellet of the prostate cancer cell line PC3, which does not express p53 protein and the above described positive controls processed with the substitution of a non-immune mouse monoclonal antibody for the DO-7 antibody.

Scoring for p53 nuclear accumulation required assessment of all cancer in selected sections from an individual patient as described previously. Counting of a minimum of 200 cancer cells in each cancer (mean 812, range 210–2000 cells, median 607), was undertaken to determine the percentage of nuclei showing accumulation across all areas of cancer present. The target cell count was 500 per case were possible, but cancers with fewer cells than this were not excluded because of the potential for selection bias. Where the cancers had multiple foci, were extensive or heterogeneous, more cells were counted and selected from areas of varying p53 nuclear accumulation to provide a sample representative of staining across the entirety of the cancer. Additionally, assessors scored the cancers cluster positive if 12 or more cancer cell nuclei within any 200 power microscopic field showed p53 accumulation. On arriving at the cluster definition, we relied initially on other workers’ observations using clusters of 15 cells and tested a variety of thresholds for the definition of a cluster within a range of 6–20 cells in the p53 score stratum with >0–2% p53 nuclear positivity (see below). Below 10 cells per ×200 field, there was no correlation with prognosis and positive cells tended to be dispersed throughout the cancer rather than be clustered within the area of a single field, whereas there were few cases with clusters of more than 15 cells within the >0–2% p53 score stratum. This definition differentiated for outcome when between 10 and 15 cells (log-rank p = 0.04 and p = 0.05, respectively, within the >0–2% stratum, whereas use of nine cells produced p = 0.12, and 16 cells produced p = 0.24) were included as thresholds and therefore 12 cells (log-rank p < 0.001) within the field was selected as a mid-point in this range. Sections were scored independently for p53 by two assessors (D.I.Q., S.M.H.) and one pathologist (W.D., J.J.T.), all of whom were blinded to patient outcome. The inter-observer Spearman rank coefficients for p53 score were between 0.92 and 0.96 signifying close agreement between scorers. Chi square test for the p53 cluster status initially assigned by different scorers produced p values of 0.87 and 0.76. Specifically, the two assessors identified 12 p53 cluster-positive cases not identified on initial assessment by the pathologist, while the pathologist identified two cases that were not initially identified by the assessors. All of these cases were deemed cluster positive at consensus review. Representative photomicrographs demonstrating p53 nuclear accumulation in PC are presented in Supplementary Fig. 1.

RESULTS
Clinicopathological characteristics for the 271 men included in the study are detailed in Table 1. At a median follow-up of 15.8 years (range 4 months–24.3 years), 203 patients (75%) had relapsed: 156 patients (57.5%) had a biochemical only relapse, 42 (15%) patients had experienced a metastatic relapse and 25 (9%) patients had died from PC. The all-cause mortality was 45.3%. The median time to BCR was 1.8 years, compared to MR (4.7 years) and PC-specific mortality. Time to BCR, MR and PC-specific mortality were defined as interval between date of radical prostatectomy and the event.

Survival analyses were performed by Kaplan–Meier method. Associations between p53 score, p53 cluster status, established clinicopathological variables (pre-operative PSA, pathological stage, ISUP grade group, seminal vesicle involvement, extraprostatic extension) and clinical outcomes (BCR, MR and PCSM) were measured using Cox proportional hazards models. Only those baseline variables that were significant on univariable analysis were included in multivariable analyses. Mann–Whitney U analysis was performed for all correlations. All reported p-values were two-sided. Statistical analyses were performed using SPSS version 24 (IBM, Armonk, NY, USA).

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0.0001, Fig. 1b) and PC-specific mortality (HR 2.4, 95% CI 1.7–3.3; p < 0.0001, Fig. 1c).

p53 cluster status
Half of all the patients were p53 ‘cluster positive’. All patients with >2% p53-positive nuclei were also ‘cluster positive’.

p53 cluster positivity correlated significantly with higher pathological stage (p < 0.001), Gleason grade (p = 0.001) and baseline PSA levels (p < 0.001) (Table 2).

p53 cluster positivity was also significantly associated with shorter time to BCR (HR 2.0, 95% CI 1.5–2.7; p < 0.0001, Fig. 2a), metastatic relapse (HR 5.1, 95% CI 2.3–11.5; p < 0.0001, Fig. 2b) and PC-specific mortality (HR 21.8, 95% CI 2.9–162; p = 0.003, Fig. 2c). Remarkably, only 1/135 (<1%) patients who were p53 cluster negative died from PC over the follow-up period.

On multivariable analyses, including established prognostic variables (lymph node status, pre-operative PSA and ISUP grade group score), p53 nuclear accumulation was independently associated with all clinical outcomes including MR and PC-specific mortality (Table 2 and Supplementary Table 2). The association with p53 was evident whether it was measured by increasing percentage of p53-positive nuclei as a continuous variable (MR, HR 1.4, 95% CI 1.1–1.8; p = 0.007; PC-specific mortality, HR 1.9, 95% CI 1.2–2.8; p = 0.003), by p53 cluster positivity (MR, HR 3.1, 95% CI 1.1–8.6; p = 0.03; PC-specific mortality, HR 9.4, 95% CI 1.2–76; p = 0.04), or using a dichotomous cut point of 10% or more (MR, HR 2.4, 95%CI 1.0–5.4; p = 0.04; PC-specific mortality, HR 4.9, 95% CI 1.7–15; p = 0.004).

Table 1. Clinicopathological characteristics (n = 271)

| Characteristic                        | Number (% or range) |
|---------------------------------------|---------------------|
| Age at RP (years)                     | 64 (43–76)          |
| Length of follow-up (years)           | 15 (0.3–24)         |
| Pre-operative PSA (ng/mL)             | 11 (1–280)          |
| Pathological T stage                  |                     |
| T2N0                                  | 127 (47)            |
| T3aN0                                 | 87 (27)             |
| T3bN0                                 | 45 (17)             |
| T4N0                                  | 5 (2)               |
| TxN+                                  | 6 (2)               |
| Updated Gleason grade (ISUP grade group) |               |
| ≤6 (1)                                | 49 (20)             |
| 3–4 (2)                               | 108 (45)            |
| 4–5 (3)                               | 47 (12)             |
| ≥6 (4)                                | 12 (5)              |
| Extraprostatic extension              | 138 (51)            |
| Seminal vesicle invasion              | 50 (18)             |
| Margin involvement                    | 140 (52)            |
| Adjuvant therapy                      |                     |
| Radiotherapy                          | 12 (4)              |
| Androgen deprivation therapy          | 39 (14)             |
| Radiotherapy and androgen deprivation therapy | 5 (2)       |
| Clinical outcome                      |                     |
| Biochemical relapse                   | 156 (58)            |
| Metastatic relapse                    | 42 (15)             |
| Prostate cancer death                 | 25 (9)              |
| Death from any cause                  | 123 (45)            |

Fig. 1 Kaplan–Meier analysis of nuclear p53 score strata for a biochemical progression-free survival, b metastasis-free survival, c prostate cancer specific survival
Table 2. Multivariable analysis of p53 nuclear staining and established baseline prognostic variables and their association with biochemical relapse, clinical relapse and prostate cancer-specific survival.

| Variable                        | % of p53-positive nuclei model HR (95% CI), p-value | p53 cluster model HR (95% CI), p-value |
|---------------------------------|-----------------------------------------------------|----------------------------------------|
|                                 | BCR       | MR       | PCSM       | BCR       | MR       | PCSM       |
| Increasing p53 positive tumour nuclei* | 1.2 (1.1–1.3) p = 0.006 | 1.4 (1.1–1.8) p = 0.007 | 1.9 (1.2–2.8) p = 0.003 | 1.7 (1.2–2.4) p = 0.002 | 3.1 (1.1–8.6) p = 0.03 | 9.4 (1.2–76) p = 0.04 |
| p53 cluster                     | 1.4       | 1.8      | 2.8       | 1.7       | 3.1      | 9.4       |
| Positive vs negative            | 0.006     | 0.007    | 0.003     | 0.002     | 0.03     | 0.04      |
| Lymph node involvement**       | 8.3       | 0.9      | 0.9       | 0.3       | 12.4     | 0.8       |
| Present vs absent               | 0.6       | 0.006    | 0.003     | 0.3       | 0.001    | 0.8       |
| ISUP grade group               | 1.7       | 0.9      | 0.9       | 1.7       | 0.9      | 0.8       |
| 1                               |           |          |           |           |          |           |
| 2                               | 1.7       | 0.9      | 0.9       | 1.7       | 0.9      | 0.8       |
| 3                               | 2.2       | 0.2      | 0.2       | 2.1       | 0.2      | 0.4       |
| 4                               | 0.07      | 0.1      | 0.1       | 2.3       | 0.1      | 0.9       |
| 5                               | 0.1       | 0.03     | 0.2       | 0.06      | 0.1      | 0.7       |
| Baseline PSA*, ng/ml            | 0.6       | 0.04     | 0.08      | 0.6       | 0.07     | 0.2       |

HR = hazard ratio, CI = confidence interval, BCR = biochemical relapse, MR = metastatic relapse, PCSM = prostate cancer-specific mortality, ISUP = International Society of Urological Pathology
*Continuous variable
**Lymph nodes involved in 6/271 (2%) patients
*Continuous variable, log transformed

Fig. 2 Kaplan-Meier analysis of nuclear p53 cluster status for a biochemical progression-free survival, b metastasis-free survival, c prostate cancer specific survival.
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AUTHOR CONTRIBUTIONS

D.I.Q., S.M.H. and J.G.G. contributed to the design of the study; AH was responsible for clinical follow up, database and histopathological specimen management; J.G.K., J.G., W.D. and J.J.T. provided histopathological analysis; P.D.S.S. was responsible for patient recruitment and clinical management; K.L.M. performed data analysis; D.I.Q., K.L.M. and L.G.H. interpreted data and wrote the paper. Manuscript. All authors read and approved the final paper.

ADDITIONAL INFORMATION

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Data availability: All the data analysed supporting the study results can be found at the Garvan Institute for Medical Research, Sydney Australia.

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