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Original Article

Utility of tissue microarrays for profiling prognostic biomarkers in clinically localized prostate cancer: the expression of BCL-2, E-cadherin, Ki-67 and p53 as predictors of biochemical failure after radical prostatectomy with nested control for clinical and pathological risk factors

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Abstract

A cure cannot be assured for all men with clinically localized prostate cancer undergoing radical treatment. Molecular markers would be invaluable if they could improve the prediction of occult metastatic disease. This study was carried out to investigate the expression of BCL-2, Ki-67, p53 and E-cadherin in radical prostatectomy specimens. We sought to assess their ability to predict early biochemical relapse in a specific therapeutic setting. Eighty-two patients comprising 41 case pairs were matched for pathological stage, Gleason grade and preoperative prostate-specific antigen (PSA) concentration. One patient in each pair had biochemical recurrence (defined as PSA $\geq$ 0.2 ng mL^{-1} within 2 years of surgery) and the other remained biochemically free of disease (defined as undetectable PSA at least 3 years after surgery). Immunohistochemical analysis was performed to assess marker expression on four replicate tissue microarrays constructed with benign and malignant tissue from each radical prostatectomy specimen. Ki-67, p53 and BCL-2, but not E-cadherin, were significantly upregulated in prostate adenocarcinoma compared with benign prostate tissue ($P < 0.01$). However, no significant differences in expression of any of the markers were observed when comparing patients who developed early biochemical relapse with patients who had no biochemical recurrence. This study showed that expression of p53, BCL-2 and Ki-67 was upregulated in clinically localized prostate cancer compared with benign prostate tissue, with no alteration in E-cadherin expression. Biomarker upregulation had no prognostic value for biochemical recurrence after radical prostatectomy, even after considering pathological stage, whole tumour Gleason grade and preoperative serum PSA level.

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1 Introduction

The early detection of prostate cancer with prostate-specific antigen (PSA) testing allows many patients the option of radical treatment with curative intent. However, up to 30% of patients undergoing radical prostatectomy for clinically localized disease will experience biochemical relapse. In some cases, biochemical relapse represents micro-metastatic disease, undetectable before surgery and almost invariably still undetectable at the time of recurrence of PSA [1]. Pound *et al.* [2] suggested that an increase in PSA within 2 years of radical surgery indicates

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a 90% risk of developing metastatic disease. Prognostic indicators for clinically localized tumours include preoprative serum PSA, clinical stage, biopsy grade and, in men who underwent radical prostatectomy, pathological stage. Prospects for total disease control, even survival advantage, remain uncertain for individual patients.

Biomarkers would be invaluable if they could improve the prediction of undetectable micrometastatic disease, thereby guiding appropriate and effective treatment [3]. Although many potentially prognostic markers have been studied, few have been incorporated into prognostic models or therapeutic decision making [4]. Among the better-characterized biomarkers, Ki-67, p53, BCL-2 and E-cadherin are widely reported to be prognostic in prostate cancer. p53 is a tumour suppressor gene that encodes for a transcription factor involved in cell-cycle control [5]. BCL-2 is an oncogene that codes for a protein that suppresses apoptosis [6]. Ki-67 is a nuclear antigen associated with cell proliferation [7]. E-cadherin is a transmembrane glycoprotein that mediates cell–cell adhesion [8].

Each of these markers becomes dysregulated during the progression of prostate cancer. However, stage progression incurs a prognostic disadvantage and may be associated with other independent non-linear effects on biomarker expression. Thus, an adverse prognosis may be observed with a loss of E-cadherin expression at radical prostatectomy, although aberrant expression is also correlated with high tumour grade and more advanced pathological stage [9–11]. Other studies have questioned the independent prognostic value of E-cadherin expression [12, 13], with the possibility of transient downregulation in localized disease that precedes development of the metastatic phenotype [14].

This study examines the immunohistochemical expression of Ki-67, p53, BCL-2 and E-cadherin in relation to the biochemical outcomes after radical prostatectomy. The study design allows for variability in preoperative PSA, whole tumour Gleason grade and pathological stage within the cohort. Patients were paired according to biochemical relapse status, otherwise matched for pathological stage, preoperative PSA, Gleason sum score and maximum Gleason pattern. Each individual patient who experienced biochemical relapse within 2 years was paired with a matched patient with no biochemical recurrence (and no adjuvant treatment) at the time of latest follow-up, at least 3 years after surgery.

This nested case-control design represents a method for evaluating potential molecular prognostic indicators in a specific and representative therapeutic setting, which allows the meaningful study of biomarker expression and outcome in a relatively small sample size and restricted range of disease characteristics, in contrast with studies of large numbers of cases in a clinically heterogeneous population with a wide range of baseline descriptors. This nested design prevents spurious conclusions resulting from the non-linear effects on prognostic marker expression that may be present within broader cohorts. From a practical perspective, the design avoids the need to extrapolate findings derived from a heterogeneous population to a small subset.

This study assessed the expression of four biomarkers in a relatively small population that can be reproducibly represented by diagnostic criteria and related their expression to distinct and clinically significant differences in treatment outcomes.

2 Materials and methods

2.1 Patient selection

Study patients were identified using the University College London Hospital's NHS Trust pathology database of over 1 200 surgical specimens from men with clinically localized prostate cancer undergoing radical prostatectomy between 1991 and 2002. Approval was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research, in compliance with the International Committee on Harmonisation of Good Clinical Practice (ICH GCP).

Patients were anonymized and those who had received preoperative 5 alpha-reductase inhibitor or neoadjuvant antiandrogen therapy were excluded from further study. Patients having no biochemical evidence of disease at last follow-up and at least 3 years or more after surgery and not receiving further prostate cancer therapy were identified. A second group of patients who developed biochemical relapse (defined as detectable PSA $\geq$ 0.2 ng mL^{-1}) within 2 years of surgery was also identified.

Individual patients with early relapse were paired with individual patients without relapse and matched for Gleason sum score, preoperative serum PSA level and pathological stage subcategory (pT3a or pT3b). Relatively few patients had pT2 disease or Gleason sum score < 6 and developed early relapse, and no patients with Gleason sum score > 8 (or maximum Gleason pattern 5) fulfilled the study criteria for non-relapse status. Forty-one pairs were thereby identified for study, representing pathological stage pT3, pN0 and Gleason sum score 6 or 7 clinically localized prostate cancer (Table 1). Clinical DRE findings were recorded inconsistently and therefore are not included in the analysis. Preoperative PSA levels were categorized into three groups for matching pairs: 0–4.0, 4.1–10 and 10.1–25.0 ng mL^{-1}. All radical prostatectomy specimens were examined by a urological pathologist. Surgical specimens were coded to avoid any bias in the subsequent construction and analysis of the tissue array.

Table 1. Forty-one pairs of prostate cancer patients who had undergone radical prostatectomy, matched for pathological stage, Gleason sum score, preoperative PSA, and paired for relapse and non-relapse status.

Pair No.	Non-relapser Pathological	Relapser Gleason	Pre-operative PSA stage	Follow-up grade	Pathological stage	Gleason grade (years)	Pre-operative PSA
1	pT3a	3 + 4 = 7	9.7	5	pT3a	3 + 4 = 7	9.9
2	pT3a	3 + 4 = 7	6	5	pT3a	3 + 4 = 7	9.8
3	pT3a	3 + 3 = 6	7	5	pT3a	2 + 4 = 6	9
4	pT3a	2 + 3 = 5	18	5	pT3a	2 + 3 = 5	15
5	pT3a	3 + 2 = 5	16	5	pT3a	3 + 2 = 5	19
6	pT3a	4 + 2 = 6	13	5	pT3a	2 + 4 = 6	10.2
7	pT3a	3 + 3 = 6	9	5	pT3a	3 + 3 = 6	4.3
8	pT3a	4 + 3 = 7	9	5	pT3a	4 + 3 = 7	8
9	pT3a	3 + 4 = 7	8	5	pT3a	4 + 3 = 7	8
10	pT3a	4 + 4 = 8	16	5	pT3a	4 + 4 = 8	16
11	pT3a	3 + 4 = 7	17	5	pT3a	4 + 3 = 7	14
12	pT3a	4 + 3 = 7	10	5	pT3a	4 + 3 = 7	12
13	pT3a	3 + 4 = 7	5	5	pT3a	4 + 3 = 7	8
14	pT3a	4 + 3 = 7	6	5	pT3a	3 + 4 = 7	7
15	pT3a	3 + 4 = 7	8	5	pT3a	3 + 4 = 7	8
16	pT3a	3 + 4 = 7	8	5	pT3a	3 + 4 = 7	8
17	pT3a	3 + 2 = 5	7	5	pT3a	3 + 2 = 5	5
18	pT3b	4 + 3 = 7	13	5	pT3b	4 + 3 = 7	12
19	pT3a	4 + 3 = 7	18	3	pT3a	4 + 3 = 7	17
20	pT3a	3 + 4 = 7	6	3	pT3a	4 + 3 = 7	8
21	pT3a	3 + 4 = 7	4	7	pT3a	4 + 3 = 7	8
22	pT3a	3 + 4 = 7	5	7	pT3a	4 + 3 = 7	7
23	pT3a	3 + 4 = 7	6	3	pT3a	3 + 4 = 7	6
24	pT3a	3 + 4 = 7	10	3	pT3a	3 + 4 = 7	10
25	pT3a	3 + 4 = 7	17	7	pT3a	4 + 3 = 7	16
26	pT3a	3 + 4 = 7	6	3	pT3a	4 + 3 = 7	5
27	pT3a	3 + 4 = 7	14	3	pT3a	3 + 4 = 7	14
28	pT3a	3 + 2 = 5	6	7	pT3a	2 + 3 = 5	9
29	pT3a	3 + 4 = 7	7	3	pT3a	4 + 3 = 7	6
30	pT3a	3 + 4 = 7	11	3	pT3a	4 + 3 = 7	18
31	pT3a	3 + 4 = 7	13	3	pT3a	4 + 3 = 7	16
32	pT3a	3 + 4 = 7	6	3	pT3a	4 + 3 = 7	9
33	pT3a	3 + 4 = 7	9	8	pT3a	4 + 3 = 7	8
34	pT3a	3 + 4 = 7	14	3	pT3a	4 + 3 = 7	16
35	pT3a	3 + 4 = 7	20	3	pT3a	4 + 3 = 7	21
36	pT3a	4 + 4 = 8	15	10	pT3a	5 + 3 = 8	14
37	pT3a	3 + 4 = 7	7	10	pT3a	3 + 4 = 7	11
38	pT3a	3 + 4 = 7	10	7	pT3a	3 + 4 = 7	10
39	pT3b	4 + 3 = 7	13	3	pT3b	4 + 3 = 7	18
40	pT3b	3 + 4 = 7	8	3	pT3b	3 + 4 = 7	7
41	pT3b	4 + 3 = 7	22	10	pT3b	4 + 3 = 7	13

Abbreviations: PSA, prostate-specific antigen.

2.2 Tissue microarray block construction

The tissue microarray (TMA) blocks were constructed using archival formalin-fixed, paraffin-embedded radical prostatectomy specimens from the 82 patients. Each radical prostatectomy specimen was cut into 5-mm sections and embedded in paraffin wax. The paraffin block containing the highest Gleason grade of tumour within the largest focus was identified. The corresponding hae-

matoxylin and eosin (H + E)-stained slide of the paraffin block was designated, and benign and cancer areas were marked by the histopathologist. From this section, the corresponding region on the paraffin (donor) block was identified, enabling TMA cores to be taken using an MTA 1 Beechers (Beechers Instruments, Sun Prairie, WI, USA) manual tissue arrayer. Four benign prostate cores were taken from the peripheral zone for zonal equivalence to the tumour. Four prostate cancer cores were taken from the higher of the two most prominent Gleason patterns. The core diameter was 0.6 mm, with a depth of 3 mm.

Cancer and benign cores were sampled from patients with malignancies other than prostate cancer to provide positive and negative controls. In total, over 200 cores were sampled from prostate and control tissue and four replicate TMA blocks were produced, containing all 82 samples and controls.

2.3 Immunohistochemical staining

Three-micrometer sections were cut from the TMAs onto coated slides and dried overnight at 60ºC . All slides were dewaxed using two 3-min xylene washes. The slides were rehydrated by immersion in 100% alcohol for 2 min, in 70% alcohol for 1 min and then in water. For antigen retrieval, two buffer solutions with differing pHs were used, depending on the antibody. Epitope retrieval buffer solution 2 (Novocastra ER2, pH 9.0) was used for Ki-67 and BCL-2 and epitope retrieval buffer solution 1 (Novocastra ER1, pH 6.0) was used for p53 and E-cadherin. The slides were placed in 800 mL of working solution (consisting of 720 mL of distilled water and 80 mL of antigen retrieval solution at 100% concentration) and incubated for 30 min at 95ºC in a microwave oven.

After antigen retrieval, the slides were washed in a buffer solution (composed of 1 L of Tris-buffered saline mixed with 500 μL of 0.05% Tween 20 detergent) for 5 min. Immunostaining was performed on an automated Bond maX™ machine (Vision BioSystems, Newcastle, UK) using the Bond polymer detection system at high contrast (DS9173). Endogenous peroxidase was neutralized using peroxidase block for 5 min. The TMA slides were incubated with protein block for 5 min and then with the primary antibody for 30 min. The primary antibodies were diluted to the concentrations shown in Table 2 with Bond primary antibody diluent (Vision Biosystems), which consisted of Tris-buffered saline, a surfactant and a protein stabilizer.

The sections were then covered in secondary antibody for 15 min, followed by antigoat peroxidase for a further 30 min. Finally, the slides were incubated with 3,3-diaminobenzidine (DAB) working solution (containing 0.5% copper sulphate in saline with surfactant) for 10 min and counterstained with haematoxylin for 5 min, dehydrated in ascending concentrations of ethanol and mounted in synthetic mounting medium.

2.4 Assessment of immunostaining

The extent and intensity of immunostaining for the four markers were scored semiquantitatively by two individuals blinded to clinical outcome. The extent of staining for p53, E-cadherin and BCL-2 was scored as a percentage of the number of cells with positive staining. A staining of 0%–5% was given a score of 1, 6%–25% a score of 2, 26%–50% a score of 3, 51%–75% a score of 4 and 76%–100% a score of 5. Intensity was divided into four groups. Negative staining was given a score of 1, mild staining was scored as 2, moderate staining scored 3 and strong staining received a score of 4. As a marker of proliferation, assessment of Ki-67 immunostaining was performed using a labelling index, wherein the number of positively stained nuclei per 100 nuclei counted was assessed and scored as a percentage. The immunohistochemical scores for each of the four cancer cores taken from the same radical prostatectomy specimen were added and divided by 4 to obtain a mean score representing that particular patient. The same methodology was performed for the benign cores taken. Relative biomarker expression was related to relapse status.

2.5 Statistical methods

The non-parametric Mann–Whitney test was used for overall comparison of relapsed patients with non-relapsed patients (irrespective of the matched pairs). The Wilcoxon signed rank test was performed to determine statistical significance when comparing matched pairs (Tables 3 and 4).

3 Results

3.1 Patients

The mean age of the 82 patients was 61 years (range 49–76). The pathological stage was pT3a in 74 patients (90%) and pT3b in 8 patients (10%). The mean preoperative PSA for all 82 patients was 10.7 ng mL^{-1} (range

Table 2. Proteins assessed for immunostaining, together with antigen retrieval conditions, antibody supplier and the antibody dilution concentrations used after optimization.

Antibody	Antigen	Retrieval	Dilution	Supplier
MIB-1 (Ki-67)	ER2, 30	pH 9.0	1/200	Dako M7240
P53	ER1, 30	pH 6.0	1/100	Dako M7001
BCL-2	ER2, 20	pH 9.0	1/200	Dako M0887
E-cadherin	ER1, 20	pH 6.0	1/25	Dako M3612

Supplier: Dako UK Ltd. (Cambridge House, St Thomas Place, Ely, Cambridgeshire CB7 4EX, UK).

Table 3. The mean ranges ± SE and the *P*-values indicating significance when comparing the immunohistochemical expression between malignant prostate and benign prostate tissue for the four individual markers.

	Relapsers	Non-relapsers	Benign	*P*-value
Ki-67				
LI	5.2 ± 0.5	4.3 ± 0.4	0.5 ± 0.1	< 0.00
E-cad				
Extent	4.98 ± 0.03	4.95 ± 0.10	4.90 ± 0.04	0.315
Intensity	3.90 ± 0.01	3.90 ± 0.10	3.90 ± 0.03	0.119
Ext. × Int.	19.5 ± 0.5	19.4 ± 0.8	19.1 ± 0.3	N/M
BCL-2				
Extent	1.30 ± 0.02	1.2 ± 0.1	1.7 ± 0.1	0.006
Intensity	2.1 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	0.004
Ext. × Int.	2.7 ± 0.2	2.6 ± 0.3	3.5 ± 0.4	N/M
p53				
Extent	2.4 ± 0.2	2.0 ± 0.1	1.20 ± 0.04	< 0.001
Intensity	2.0 ± 0.1	2.1 ± 0.1	1.40 ± 0.04	< 0.001
Ext. × Int.	4.9 ± 0.6	4.3 ± 0.5	1.7 ± 0.1	N/M

Abbreviations: E-cad, E-cadherin; Ext., extent; Int., intensity; N/M, not measured; SE, standard error.

4–22). The mean preoperative PSA for all patients who had relapsed within 2 years of surgery was 10.9 ng mL^{-1} and the preoperative PSA for non-relapsed patients was 10.5 ng mL^{-1}. All 41 non-relapsed patients had undetectable PSA at the last follow-up; of these, 27 were PSA-free at 5–10 years (total mean, 5.0 ± 0.3 years) after radical prostatectomy.

3.2 BCL-2 staining

In benign prostatic glandular epithelium, BCL-2 expression was restricted to the cytoplasm of the basal cell layer (Figure 1C). BCL-2 showed cytoplasmic staining in malignant prostatic epithelium (Figure 1D). In malignant tissue, 18% of TMA cores showed > 5% of cells with positively stained cytoplasm. Another 44% of cores expressed BCL-2, but the extent of staining was between 0 and 5%. In the analysis of staining intensity, 62% of cores exhibited staining that was mild (+2), moderate (+3) or strong (+4). The remaining 38% of prostate cancer cores were negative for this marker.

Statistically significant overexpression of BCL-2 was found in malignant prostate tissue compared with benign prostate tissue ($P < 0.05$) (Table 3). However, there was no statistically significant difference in expression when we compared prostate cancer patients who relapsed and patients who did not relapse in both the overall analysis and the matched paired analysis (Table 4).

3.3 E-cadherin staining

E-cadherin staining was strongly positive in the cell membrane of prostate cells (Figure 1E, F) and only cell membrane staining was considered positive for E-cadherin expression. Expression was consistently strong throughout non-malignant and malignant tissue, where 95% of cores exhibited an extent categorized as +4 (strong staining) and +5 (76%–100% of positively stained cell membranes).

Table 4. Statistical *P*-values when comparing the immunohistochemical expression of relapsed prostate cancer patients with that of patients who did not relapse for the four individual markers. All biomarkers showed no statistically significant difference when expression was compared.

Statistical test used	BCL-2	Ki-67	p53	E-cadherin
Mann–Whitney test	0.68	0.16	0.29	0.53
Wilcoxon signed-rank test	0.43	0.26	0.24	0.59

There were no statistically significant differences in expression between benign and malignant cores or between patients who relapsed and those who did not, in both the overall analysis and the matched paired analysis (Tables 3 and 4).

3.4 p53 staining

p53 expression was confined to the nucleus of both benign and malignant prostate epithelium (Figure 1G, H). Sixty percent of malignant TMA cores stained positive for p53, of which 30% showed expression in > 25% of cells. Ten percent of benign cores were positively stained for p53. The most frequent grades of intensity were +2 or +3 (mild and moderately stained). In contrast to earlier studies [8], we did not observe clustering of nuclei staining for p53.

p53 expression was significantly overexpressed in malignant prostate tissue compared with benign prostate tissue ($P < 0.05$) (Table 3). No statistically significant difference was observed when comparing prostate cancer patients who relapsed with those who did not relapse in either the overall analysis or the matched, paired analysis (Table 4).

3.5 Ki-67 staining

Ki-67 was expressed in the nucleus of both benign and malignant prostate epithelium (Figure 1I, J). The percentage of positively stained nuclei ranged from 0 to 24% for malignant prostate tissue compared with 0 to 8% for non-malignant prostate tissue.

Ki-67 was significantly overexpressed in malignant prostate tissue compared with benign prostate tissue ($P < 0.05$) (Table 3). No statistically significant difference was observed when comparing prostate cancer patients who relapsed with those who did not relapse in either the overall analysis or the matched, paired analysis (Table 4).

3.6 Surgical margins

Of the 82 patients, 19 (23%) had positive surgical margin status, including 13 with early biochemical relapse

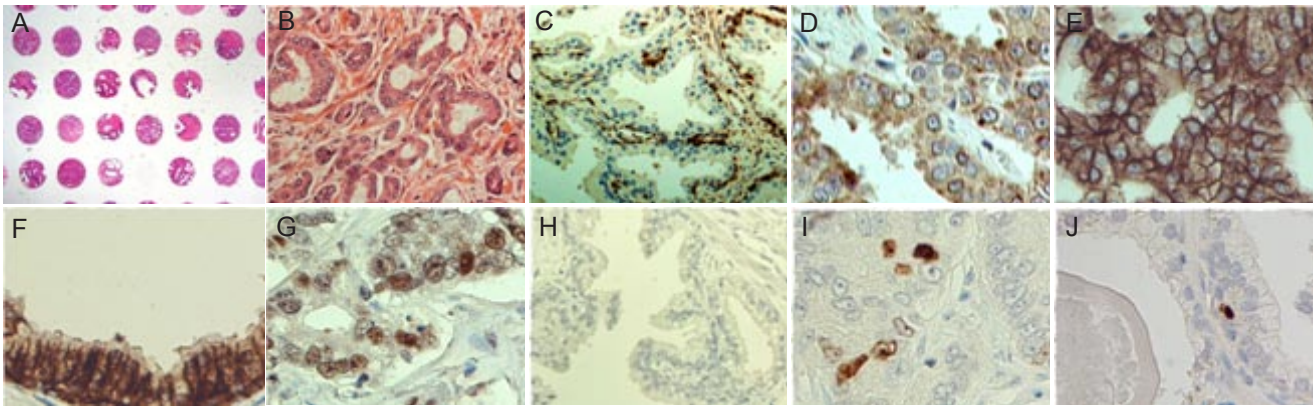

Figure 1. (A): TMA section stained with haematoxylin and eosin (H + E) (× 100). (B): Cancer core stained with H + E (× 400). (C) BCL-2 staining in benign prostate (× 200); (d) BCL-2 staining in malignant prostate (× 400). (E): E-cadherin staining in malignant prostate (× 400). (F): E-cadherin staining in benign prostate (× 400). (G): p53 staining in malignant prostate (× 400). (H): p53 staining in benign prostate (× 200). (I) Ki-67 staining in malignant prostate (× 400). (J): Ki-67 staining in benign prostate (× 400).

and 6 without relapse for 3 years or more. In a subgroup analysis of the 22 pairs that were matched for negative margin status, there were no significant differences in expression of any of the four markers by relapse status.

4 Discussion

This study used TMAs to evaluate the prognostic value of several biomarkers earlier shown in various published studies to correlate with disease stage and outcome. First, expression of these biomarkers was compared in prostate cancer and benign prostatic tissue in the same individual. Overexpression of p53, BCL-2 and Ki-67 in prostate cancer was demonstrated. In contrast, E-cadherin expression was not significantly different in malignant and non-malignant prostate tissue. For each of the four proteins, expression did not correlate with early biochemical relapse after radical prostatectomy, even when taking into consideration pathological stage, whole tumour Gleason sum score and preoperative PSA.

The results of this study suggest that the evaluated biomarkers are unlikely to be of any value for predicting early biochemical failure after treatment of clinically localized prostate cancer. Early biochemical failure is associated with the progression of metastatic disease that is undetectable at the time of treatment. Despite significantly increased expression of p53, BCL-2 and Ki-67 in malignant tissue, there was no significant difference in biomarker expression between tumours likely to be associated with an established metastatic phenotype and tumours apparently cured by treatment. The paired analysis showed that the observed lack of prognostic significance was not related to variation in pathological stage, Gleason grade or preoperative PSA.

Tumours studied were restricted by pathological stage (pT3a and pT3b) and sampled Gleason grade (Gleason 3 and 4) so that tissue pairings were precisely matched to subcategories of pathological stage, highest Gleason grade and preoperative PSA. Biomarker expression in organ-confined cancer (pathological stage pT2 N0) was not studied, because few patients developed early biochemical relapse after treatment. The demonstration of increased biomarker expression for p53, BCL-2 and Ki-67 in prostate cancer confirms the technical utility of using TMAs for examining biomarker expression in archived surgical specimens.

Surgical margin status was not matched in the full-paired analysis. As positive margins were observed more frequently in patients who relapsed, there was a possibility that positive margins rather than micrometastatic disease may account for early biochemical failure in some cases. This could theoretically contribute to the inability of this study to detect altered biomarker expression in tumours with metastatic potential. However, the overall prevalence of positive margins was < 25% and no significant difference in biomarker expression was observed in the analysis of pairs with negative margins. Furthermore, positive margins are not a reliable indicator of residual local disease, indicated by prolonged biochemical disease-free survival in the majority of patients selected for surgery. In this study, of the 41 patients who relapsed early, 28 had negative surgical margins. The prognostic significance of positive margins is substantially reduced when PSA, Gleason grade and pathological stage are also taken into account. Conversely, of the 41 patients who did not relapse early, 6 had positive margins. Therefore, surgical margin status is not a reliable independent determinant of therapeutic outcome.

This study evaluated pathological stages pT3a N0 and pT3b N0 disease (TNM 2002), Gleason 3 + 3, Gleason 3 + 4 and Gleason 4 + 3. Although this permits a degree of stage and grade heterogeneity with respect to prognosis, these subcategorizations cannot be determined precisely without surgical treatment. The variability in pathological sub-stage category and grade was nevertheless taken into consideration by the matched-pair analysis. In patients with a Gleason sum score of 7, cores were taken from the maximum pattern of the dominant tumour focus (i.e., Gleason pattern 4) for equivalence. The differences in prognostic categorization based on subdivision of pathological stage pT3, Gleason sum score 7 and surgical margin status are likely to have had minimal impact, if any, on the findings of this study. Furthermore, if there were any independent prognostic discrimination, it could not be taken into consideration without radical surgery. Ultimately, the purpose of this analysis was to evaluate biomarkers for their utility as pre-surgical prognostic indicators.

The number of replicate cores needed for reproducibility was examined by Rubin *et al.* [15], who concluded that a good correlation exists between TMA cores and histopathological specimens when three or four cores are used. Semiquantitative analysis of immunohistochemical staining similar to that used in this study has been used in many published reports and is useful for screening potential biomarkers. Although the extent of staining appeared reproducible and consistent across four tissue cores, the prognostic value of altered biomarker expression could potentially remain undetected where there is a non-linear relationship to the amount of staining or its categorization.

The nested case–control design of this study enables biomarker expression to be compared between matched cases with distinct outcomes of interest (in this study, early biochemical relapse after radical prostatectomy *vs.* no relapse). By restricting the study cohort using well-defined criteria and allowing for established clinical prognostic factors, the independent prognostic value of candidate biomarkers can be assessed [16]. This methodology offers substantial practical advantages over designs requiring analysis of a large cohort that may encompass a wide spectrum of tumour presentations and stage, for example, in gene [17, 18] and tumour marker analyses [19].

p53 was highly expressed in prostate cancer tissue, whereas the majority of benign tissue was not stained. Expression of p53 has been shown by various studies to have prognostic significance in human prostate cancer [20–28] (Table 5), whereas other studies in patients with clinically localized prostate cancer have shown no predictive value [29–30] (Table 5). p53 is a tumour suppressor protein that may mutate and accumulate within malignant cells with progression of prostate cancer, particularly in the more advanced stages of the disease. Among earlier studies, Moul *et al.* [23] found that p53 protein expression was an independent prognostic biomarker for disease-free survival in patients undergoing radical surgery for clinically localized prostate cancer. Furthermore, Quinn *et al.* [24] stated that the presence of clusters of p53-positive nuclei delineates a group of patients with poor prognosis not identified by traditional scoring methods, supporting the idea that p53 dysfunction may exist within the foci of prostate tumour cells that are clonally expanded in metastases.

In this study, the extent of staining for BCL-2 was less than that for p53, E-cadherin and Ki-67. This is not unexpected because the expression of BCL-2 in patients with organ-confined prostate cancer is less common [23, 25]. BCL-2 is involved in the regulation of apoptosis and tumour response to antiandrogen therapy, as well as in the development of androgen insensitivity [31, 32]. Increased expression of BCL-2 correlates with higher rates of biochemical recurrence in patients who have undergone radical prostatectomy for clinically localized prostate cancer [25]. Further studies have also shown the ability of BCL-2 to be of prognostic value for patients with organ-confined disease [20, 27, 29] (Table 6). However, other studies have shown that BCL-2 does not have prognostic significance in prostate cancer patients [23, 25, 30] (Table 6).

Ki-67 expression ranged between 0% and 24% in our study, which compared favourably with earlier published studies [33, 34]. Ki-67 is a normal nuclear protein expressed in all proliferating cells in all phases of the cell cycle except G_0. Many studies show that the expression of Ki-67 correlates with an adverse prognosis in prostate cancer, among various stages of disease and alternative treatments, with prognostic expression in both whole tumour samples and needle biopsy specimens [23, 25, 28, 33, 35, 36] (Table 7). Multivariate analysis shows Ki-67 expression to be significant across a broad range of tumour characteristics [34, 36], with few studies evaluating a clinically uniform cohort [26, 35]. However, Bettencourt *et al.* [34] contradict Ki-67's utility as a prognostic indicator for biochemical relapse after radical surgery for clinically localized prostate cancer (Table 7).

In this study, E-cadherin expression was strong in virtually all prostate cancer specimens. This could be explained by the grade distribution in our cohort. The majority of patients had a Gleason score between 5 and 7; only a few patients had a Gleason score of 8 and no patient had a Gleason score of 9 or 10. E-cadherin has been proposed as a prognostic indicator in prostate cancer and aberrant expression is associated with higher-grade tumours [37, 38], poor therapeutic outcome [39], circulating prostate cancer cells and postoperative PSA failure [40]. Furthermore, a strong correlation was seen between E-cadherin dysfunction and biochemical relapse ([29] and Table 8). Other studies, however, have shown that expression of

Table 5. Studies of p53 as a prognostic indicator for PSA recurrence following radical prostatectomy in men with clinically localised prostate cancer. Bauer *et al.* [22] also demonstrated statistical significance for disease-free survival ($P = 0.02$). Recurrence range refers to the range in biochemical relapse rate between normal (least) and highest categories of biomarker expression.

Author	No. of patients	Pathological stage range	Gleason score	PSA range	Raw recurrence range (%)	Predictive value for PSA recurrence	
						Univariate	Multivariate
Brewster *et al.* [20]	76	pT1–4	4–10	0.9–37	21–41	Sig. $P = 0.004$	N/P
Yang *et al.* [21]	49	pT1–T2	5–7	1.0–20.0	19–59	Sig. $P < 0.01$	N/P
Bauer *et al.* [22]	139	pT2a–3. N	12–10	N/A	11.5–62.1	Sig. $P = 0.0001$	N/P
Moul *et al.* [23]	162	pT1–4	2–10	N/A	9.1–62.0	Sig. $P = 0.001$	Sig. $P = 0.003$
Quinn *et al.* [24]	263	pT1a–3b	2–10	1–280	N/A	Sig. $P = 0.004$	N/P
Bauer *et al.* [25]	175	pT1a–3b	2–10	N/A	16.1–61.9	Sig. $P = 0.001$	N/P
Inoue *et al.* [26]	52	pT2–T3	5–9	3.8–120	24–57	Sig. $P = 0.009$	N/P
Stackhouse *et al.* [27]	199	pT1a–2c	2–10	N/A	24–45	Sig. $P = 0.004$	N/P
Stapleton *et al.* [28]	47	pT1–3a	5–8	1.5–11.5	23–69	Sig. $P = 0.007$	N/P
Wu *et al.* [29]	70	pT2a + b	2–9	0.4–93.6	26–44	NS	N/P
Merseberger *et al.* [30]	97	pT2b–4a	5–10	N/A	41.9–42.8	NS	

Abbreviations: Sig, significant; NS, non-significant, N/A, not available, N/P, not performed; PSA, prostate-specific antigen.

Table 6. Studies of BCL-2 as a prognostic indicator for prostate-specific antigen (PSA) recurrence following radical prostatectomy in men with clinically localised prostate cancer. Bauer *et al.* [25] also demonstrated statistical significance for disease-free survival ($P = 0.044$). Recurrence range refers to the range in biochemical relapse rate between normal (least) and highest categories of biomarker expression.

Author	No. of patients	Pathological stage range	Gleason score	PSA range	Raw recurrence range (%)	Predictive value for PSA recurrence	
						Univariate	Multivariate
Brewster *et al.* [20]	76	pT1–4	4–10	0.9–37	24–53	Sig. $P = 0.01$	N/P
Stackhouse *et al.* [27]	199	pT1a–T2c	2–10	N/A	27–64	Sig. $P = 0.01$	N/P
Wu *et al.* [29]	70	pT2a + b	2–9	0.4–93.6	28–67	Sig. $P = 0.024$	N/P
Moul *et al.* [23]	162	pT1–4	2–10	N/A	29.7–53.1	NS	Sig. $P < 0.001$
Bauer *et al.* [25]	175	pT1a–3b	2–10	N/A	30.5–50.0	NS	N/P
Merseberger *et al.* [30]	97	pT2b–4a	5–10	N/A	40.5–47.8	NS	N/P

Abbreviations: Sig, significant; NS, non-significant, N/A, not available, N/P, not performed; PSA, prostate-specific antigen.

E-cadherin fails to predict biochemical relapse in patients treated for prostate cancer [20, 12, 41] (Table 8).

We undertook a literature review of published papers that examined the expression of p53, BCL-2, Ki-67 and E-cadherin in patients with biochemical relapse after radical prostatectomy for clinically localized prostate cancer. Papers selected from the literature review are summarized in Tables 5–8. The criteria for selection of these papers included immunohistochemical analysis performed on prostate tissue from patients who underwent radical prostatectomy for clinically localized prostate cancer without lymph node metastases. We excluded studies on tissue from transurethral resection of the prostate (TURP) or prostate biopsy specimens. We also included studies that related biomarker expression to biochemical relapse rate following radical prostatectomy. The papers selected used TMAs or whole paraffin sections.

Tables 5–8 indicate the considerable range of expression that may be associated with biochemical recurrence, in addition to significant biomarker upregulation. Many studies include a wide spectrum of disease characteristics and some include patients who would not be considered favourable candidates for cure. In the present era, a prognostically accurate biomarker must retain its utility within contemporary practice and the more restricted spectrum of disease that is currently treated with radical prostatectomy. For example, the clinical value of a prognostic biomarker would be assured by its ability to predict biochemical recurrence and the potential need for adjuvant therapy or, indeed, by its ability to discriminate tumours of low malignant potential before surgery. This study did not show any clinically useful prognostic value associated with in-

Table 7. Studies of Ki67 as a prognostic indicator for prostate-specific antigen (PSA) recurrence following radical prostatectomy in men with clinically localised prostate cancer. Moul *et al.* [23] and Bettencourt *et al.* [34] also demonstrated statistical significance for disease-free survival ($P = 0.001$ and 0.05, respectively). Recurrence range refers to the range in biochemical relapse rate between normal (least) and highest categories of biomarker expression.

Author	No. of patients	Pathological stage range	Gleason score	PSA range	Raw recurrence range (%)	Predictive value for PSA recurrence	
						Univariate	Multivariate
Moul *et al.* [23]	162	pT1–4	2–10	N/A	14.3–54.8	Sig. $P < 0.0001$	NS
Inoue *et al.* [26]	52	pT2–T3	5–9	3.8–120	14–67	Sig. $P < 0.0001$	N/P
Stapleton *et al.* [28]	47	pT1–3a	5–8	1.5–11.5	N/A	Sig. $P < 0.0390$	NS
Bettencourt *et al.* [34]	180	pT1b–T3	2–10	N/A	16–50	NS	N/P

Abbreviations: Sig, significant; NS, non-significant, N/A, not available, N/P, not performed; PSA, prostate-specific antigen.

Table 8. Studies of E-cadherin as a prognostic indicator for prostate-specific antigen (PSA) recurrence following radical prostatectomy in men with clinically localised prostate cancer. Recurrence range refers to the range in biochemical relapse rate between categories of normal and aberrant biomarker expression.

Author	No. of patients	Pathological stage range	Gleason score	PSA range	Raw recurrence range (%)	Predictive value for PSA recurrence	
						Univariate	Multivariate
Wu *et al.* [29]	70	pT2a + b	2–9	0.4–93.6	13–44	Sig. $P = 0.003$	N/P
Brewster *et al.* [20]	76	pT1–4	4–10	0.9–37	22–37	NS	N/P
Kuczyk *et al.* [12]	67	pT1–4	2–10	N/A	N/A	NS	NS
Rhodes *et al.* [41]	259	pT2–4	2–10	0.5–43	N/A	NS	N/P

Abbreviations: Sig, significant; NS, non-significant, N/A, not available, N/P, not performed; PSA, prostate-specific antigen.

creased biomarker expression in a relatively homogenous cohort of prostate tumours associated with moderate risk of biochemical relapse after surgical treatment.

A number of factors may contribute to the variance between the findings of this study and those of earlier publications. In this study, a well-defined and relatively homogeneous group of patients, matched for known prognostic variables, was used. In many earlier studies, a heterogeneous group of patients, with pathological stage ranging from pT1 to pT4, was used, with some studies including patients with nodal metastases. As a result, substantial variation in the risk of biochemical recurrence would relate to tumour stage and Gleason grade, and these biological differences would contribute to potentially non-linear changes in biomarker expression that may not extrapolate to clinical subgroups.

Molecular pathways that regulate the cell cycle and malignant progression involve many proteins, and it is unlikely that the expression of any single protein will dominate the clinical course of early-stage prostate cancer. Nevertheless, these individual proteins play critical roles in tumour progression, and a better understanding of their interaction with alternative molecular pathways may provide insight into the variable clinical behaviour of prostate cancer and new therapeutic opportunities.

5 Conclusion

This study confirms that p53, BCL-2 and Ki-67 are overexpressed in clinically localized prostate cancer compared with benign prostatic tissue. In contrast, E-cadherin expression was not altered. Altered expression of these biomarkers did not have any significant prognostic value in patients treated by radical prostatectomy, even after taking into account variation of pathological stage, Gleason grade and preoperative PSA level.

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