Pathology International

Pathology International 2010; **60**: 667–672 doi:10.1111/j.1440-1827.2010.02584.x

Original Article

Assessment of circulating tumor cells (CTCs) in prostate cancer patients with low-volume tumors

Amina Ali,[1,2*] Bungo Furusato,[2,3*] Paul O.P. Ts'o,[4] Zhao-Ping Lum,[4] Sally Elsamanoudi,[1,2] Ahmed Mohamed,[1,2] Shiv Srivastava,[2] Judd W. Moul,[5] Stephen A. Brassell,[1,2] Isabell A. Sesterhenn[3] and David G. McLeod[1,2]

[1]*Urology Service, Walter Reed Army Medical Center,* [2]*Department of Genitourinary Pathology, Armed Forces Institute of Pathology, Washington, DC,* [3]*Center for Prostate Disease Research, Department of Surgery, Uniformed Services University of the Health Sciences, Bethesda,* [4]*CCC Diagnostics, LLC, Baltimore, Maryland and* [5]*Division of Urologic Surgery and the Duke Prostate Center, and Department of Surgery, Duke University Medical Center, Durham, North Carolina, USA*

The objective of this study was to assess the incidence of circulating tumor cells (CTCs) in prostate cancer patients with low-volume tumors (less than 0.5 cc) after radical prostatectomy (RP). Blood samples were collected from 64 RP patients to assess the incidence of CTCs following RP. The specimens were processed by whole-mount section. Clinicopathological data (e.g. patient age, race, specimen weight, tumor volume, grade, stage and surgical margin status) and follow-up PSA data were compared to CTC status. Of the 64 RP patients, nine had 'low-volume prostate cancer'. Seven of these patients had detectable levels of CTCs. In two of the seven patients with detectable CTCs, PSA elevation was also observed. Isolation and detection of circulating epithelial cells is possible in low-volume prostate cancer patients. In the setting of low-volume prostate cancer, CTCs may be associated with the presence of detectable PSA levels. However, the detection of CTCs did not predict PSA failure.

Key words: circulating tumor cells, CTCs, insignificant, prostate cancer, small-volume

The level of serum prostate-specific antigen (PSA) after radical prostatectomy (RP) is considered a surrogate marker of 'treatment failure'.[1] The definition of 'indolent' or 'clinically insignificant' prostatic carcinoma in RP specimens varies.[1–10] Although this criterion is still controversial, a tumor with a volume of less than 0.5 cc and without any poorly differentiated elements (Gleason pattern 4 or 5) that is confined to the prostate is currently the most widely accepted definition of clinically 'insignificant' or 'indolent' prostate carcinoma.[11,12] The objective of this study was to assess the occurrence of circulating tumor cells (CTCs) and the potential correlation with serum PSA levels in prostate cancer patients with total tumor volumes of less than 0.5 cc., pathologically organ-confined tumors and negative surgical margins.

MATERIALS AND METHODS

Patients

Between 2000 and 2004, 64 out of the 487 patients who underwent RP at our institution were enrolled in the GP016-Circulating Cancer Cell protocol after institutional review board approval. Blood was collected in seven stages: prior to surgery, 10 min after removal of the prostate, and 48 hours, 6 weeks, 6 months, 12 months, and then annually for 5 years after radical prostatectomy. Clinical data were also collected from all participating patients. The parameters that were collected included the age, race, clinical stage, biopsy grade, and pre- and post-treatment PSA values of the patients.

Prostatectomy specimens

Prostatectomy specimens were sectioned and completely embedded as whole mounts at the Armed Forces Institute of

Correspondence: David G. McLeod, MD, JD, Multi-Disciplinary Prostate Cancer Clinic, Center for Prostate Disease Research, Walter Reed Army Medical Center, Urology Service, Ward 56, 6900 Georgia Ave. NW, Washington, DC 20307, USA. Email: dgmcleod@verizon.net

Disclaimer: The views expressed in this article are those of the authors and do not reflect the official policy of the Department of the Army, Department of Defense, or the U.S. Government.

Received 30 March 2010. Accepted for publication 7 June 2010.

*These authors contributed equally to this work.

Pathology (Washington, DC, USA).[13,14] Each prostate was sectioned at 2.2-mm intervals in a transverse plane perpendicular to the long axis of the posterior surface of the prostate. The volume of each tumor was calculated in three dimensions (apex-to-base, right-to-left and anterior–to-posterior) using the largest dimension in each direction. The tumor volume did not include a shrinkage factor in order to correct for fixation and embedding.[15] The total tumor volume was the sum of the tumor volumes of all measurable foci. The presence of benign glands in the inked surgical margins was also recorded.

Blood collection and cell isolation

For each sample, approximately 15 to 20 mL of venous blood was drawn from the antecubital veins of control subjects and study patients and placed into Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing acid citrate dextrose solution A as an anticoagulant. The samples were processed at room temperature using an isolation procedure within 24 hours of the blood draw. Each whole blood sample was diluted to 30 mL with 1 × phosphate-buffered saline (PBS) in a Corning 50-mL polypropylene tube (Corning Glass Works, Corning, NY, USA). Tubes were capped and mixed gently by inversion three to six times. Using a 20-mL syringe connected to a hub blunt-end stainless steel needle (Popper and Sons, Inc., New Hyde Park, NJ, USA), the bottom of the sample tube was slowly packed with 10 mL of a 1.068-g/mL density gradient, followed by another 10 mL of a 1.083-g/mL gradient beneath the 1.068-g/mL density gradient. The tube was centrifuged at 400 × g for 30 min at 20°C. The upper gradient interface (1.068 g/mL) and the lower gradient interface (1.083 g/mL) were collected separately along with the gradient (about 5 mL for each of the two layers) using a disposable transfer pipette. The two collections were washed with PBS and centrifuged at 250 × g for 10 min. The supernatant in each tube was then gently removed by careful aspiration and the cell pellets were collected.

Magnetic cell sorting and slide preparation

An enrichment system using CD45 mouse anti-human immunoglobulin G magnetic beads was used to remove excess leukocytes and to enrich the target cancer epithelial cells in the blood. The cell pellet from the 1.083-g/mL gradient collection was resuspended in 1.0 mL of 0.1% bovine serum albumin (BSA) and placed on ice for magnetic cell sorting. A total of 200 μL of magnetic Dynal beads (M450; Dynal, Oslo, Norway) were added to a 5-mL polypropylene tube. The beads were washed in 3.0 mL of 0.1% BSA to remove the sodium azide. The cell suspension from the 1.083-g/mL gradient (about 1.0 mL) was transferred to the tube containing the washed beads. The remaining cells were washed from the wall of the original centrifuge tube with another 1.0 mL of 0.1% BSA and transferred to the tube containing the magnetic beads, for a total volume of 2.0 mL. The cells were incubated with the beads at 4–8°C for 30 min at 10 rpm on an Orbitron Rotator (Dynal). The tubes were then placed in a magnetic particle concentrator (Dynal) for two minutes to allow for maximum leukocyte depletion. The 1.068-g/mL cell suspension was transferred to a 5-mL Falcon 2005 tube and kept on ice during incubation of the 1.083-g/mL suspension with anti-CD45 magnetic beads. After leukocyte depletion, the supernatants from the CD45 negative collection of the 1.083-g/mL cell suspension and the 1.068-g/mL cell suspension were combined and washed with 40 mL of 1 × PBS using a centrifugation step at 250 × *g* for 10 min. The cell pellet was resuspended in 300 μL of 0.1% BSA and then loaded evenly onto a 20 × 20 mm area of a slide outlined with a Pap-pen (Research Products International Corp., Chicago, IL, USA). The slides were air-dried for at least three hours or placed on a slide warmer set at 41°C for one hour before staining.

Immunofluorescence staining

Air-dried slides were fixed in 2% paraformaldehyde for 10 min at 4 to 8°C and then rinsed two times with 1 × PBS. Slides were then incubated in PBS for 10 min at room temperature and blotted dry. Cells were incubated with a proprietary fluorescently labeled pan-cytokeratin mAb cocktail that recognizes nine different cytokeratin peptides and a PSA mAb (Dako, Carpinteria, CA, USA) conjugated with Texas Red (Cell Works Inc. Baltimore, MD, USA) at 4°C for at least one hour and up to overnight. The cells were washed twice in PBS at room temperature for 10 min. The slides were blotted dry and mounted with DAPI-containing medium under a coverslip.

Controls

As a positive control, the prostate cancer cell line LNCaP (ATCC, American Type Culture Collection, Manassas, VA, USA) was used in validation tests for tumor cell recovery and immunofluorescence staining. The cell lines were cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (Gemini Bioproducts Inc., Calabasa, CA, USA). Cancer cells were counted after trypsinization from culture, and approximately 100 cells were spiked into approximately 15 mL of blood from healthy control subjects whose blood was found to contain no cytokeratin-positive cells based on CTC analysis. To accurately estimate the

Table 1 Demographic characteristics of patients with small tumors

Case #	1	2	3	4	5	6	7	8	9
Number of CTCs	2	1	5	0	7	0	2	16	2
Postoperative PSA (ng/mL)	No	0.31	No	No	0.25	No	No	No	No
Age	41	53	59	63	62	62	54	61	52
Race	C	C	C	AA	C	Asian	C	AA	AA
Prostate weight (g)	30.0	32.0	38.6	38.5	46.0	53.5	30.7	55.5	45.5
Preoperative PSA (ng/mL)	0.9	4.9	2.6	2.9	5.7	3.7	0.7	6.3	5.6
Clinical T-stage	T2a	T1c	T1c	T2a	T1c	T2a	T1b	T2b	T1c
Pathological T-stage	T2c	T2c	T2c	T2c	T2c	T2c	T2c	T2c	T2a
Index tumor volume (cc.)	0.3	0.3	0.1	0.1	0.2	0.1	0.2	0.1	0.1
Total tumor volume (cc.)	0.4	0.3	0.1	0.1	0.3	0.1	0.3	0.1	0.1
Prostate volume (cc.)	40.0	33.7	58.6	66.4	74.4	66.7	37.9	81.4	55.7
Surgical margin Status	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Benign glands extend to margin status	Yes	No	No	Yes	Yes	No	No	No	Yes
Total number of tumor foci	4	7	5	3	2	9	9	4	3
Gleason score	6	5	6	6	6	6	6	6	6
Tumor differentiation	Well/M	Well	Well	Well	Well	Well/M	Well	Well	Well

No: PSA level of <0.2 ng/mL; C: Caucasian; AA: African American; Well: Well-differentiated; M: Moderately differentiated

number of cells spiked into the blood, two control slides were prepared from the same volume of cell suspension, and cells were verified by cytokeratin-positive staining and counted. The average number of cells from the control slides was used to calculate cell recovery. The spiked blood (i.e. the blood from healthy controls that was injected with LNCaP cells) was subjected to the same complete isolation and staining procedures to which the patient samples were subjected.

As a negative control, blood samples from healthy blood donors without a history of cancer were obtained from BRT Laboratories, Inc. (Baltimore, MD, USA) and other healthy volunteers. A total of 50 healthy blood donors (27 male and 23 female) with ages ranging from 26 to 74 years old served as negative control samples in the present study. Approximately 10 to 20 mL of whole blood from each of the donors was subjected to the same complete isolation and staining procedures to which the patient samples were subjected.

Fluorescence microscopy

Stained slides were examined under a Leica DM RXA microscope (Leica Microsystems, Exton, PA, USA) equipped with a MicroMax Digital CCD Camera System, model 1300YHS (Princeton Instruments, Trenton, NJ, USA). We utilized filter cubes that allowed for the differentiation of up to five fluorescence signals. The wavelengths of the excitation, dichroic, and emission filters that were used in each cube were 360 nm, 400 nm, and 470 nm, respectively, for DAPI and 470 nm, 497 nm, and 522 nm, respectively, for FITC. Digital images of the stained cells were acquired with a 40x objective lens using Image-Pro Plus software (Analytical Imaging Products, San Diego, CA, USA).

Cell recovery and assay specificity

For quality control, one control sample was tested each time that a patient sample was processed. On average, we used one control per three patient samples (range 1 to 7 patient samples) to ensure recovery in the enrichment process and the proper staining procedure. The tumor cells targeted for recovery in this assay stained positive for cytokeratin peptides and were considered CD45-negative since they were not removed from the blood sample when incubated with anti-CD45 magnetic beads in the leukocyte depletion step. The mean recovery rate was 70.4% (range 51%–95%), consistent with the results of our previous validation of this procedure and similar to the reported rates for other enrichment and detection procedures.[16–18]

To evaluate the specificity of the cell recovery and staining procedures, a total of 75 blood samples from 50 healthy individuals were examined as negative controls. These negative control tests were conducted either side by side with patient blood samples or with positive control tests. None of the negative control samples was found to contain cytokeratin-positive cells. The negative control data indicate that the samples from healthy blood donors must contain zero or no more than one positive cell. These results led to the conclusion that samples containing one cell or more would be considered CTC-positive, whereas samples containing no positive cell would be considered CTC-negative.

RESULTS

Overall, 35 of 64 patients had CTCs detected at various time points. Of these 64 patients, 9 had low-volume tumors. The demographic and clinical characteristics of these nine

patients are shown in Table 1. The median duration of PSA follow-up was 39.7 months (range: 9.5–89.1 months). If the four patients with <36 months of follow-up (this arose because they did not return for continuous observation) were excluded, the median follow-up time was 52 months (range: 37.2–89.1 months). The mean age at the time of surgery was 56 years (range 41–63 years). The mean preoperative PSA was 3.9 ng/mL (range 0.9–6.3 ng/mL) and the mean prostate weight was 41.1 g (range 30.0–55.5 g). All patients had multifocal tumors. The mean total number of tumors was 5.1 (range 2–9) and the mean total tumor volume was 0.2 cc (range 0.1–0.4 cc). All tumors were organ-confined and surgical margins were negative. Four of the nine cases had benign glands found at the surgical margin. The distribution of Gleason scores was as follows: Gleason score (GS) less than 6 (one case) and GS 6 (eight cases). In two of the nine cases, postoperative serum PSA levels of greater than 0.2 ng/mL were observed.

CTCs were detected in 7 of 9 patients, including the two patients with elevated PSA. An example image of CTCs from a patient blood sample after radical prostatectomy is shown in Fig. 1. The CTC counts ranged from 1 to 16, with a median count of 2. Four patients had fewer than three CTCs, and three patients had more than 3 CTCs (Table 1). In the two patients with elevated PSA, the detection of CTCs on each follow-up visit varied (Table 2).

We could not observe any trend across the parameters in Table 1, including postoperative PSA, race and margin status, when making comparisons to CTC counts.

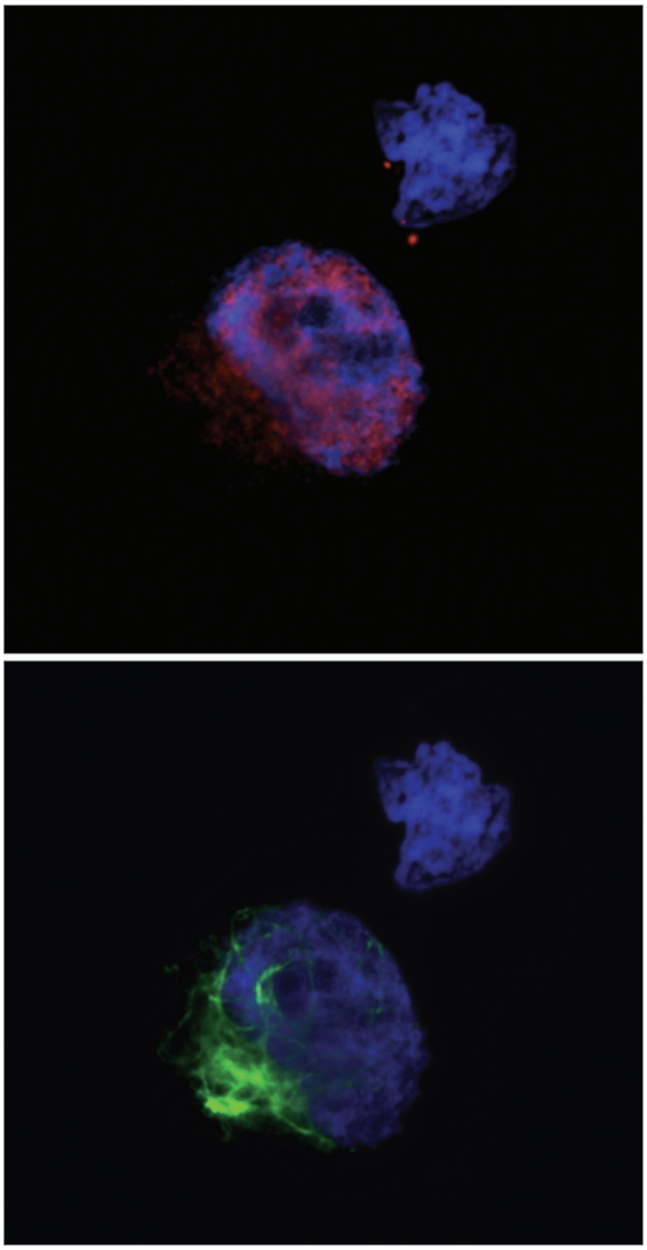

Figure 1 Example image of one prostate circulating tumor cell (CTC) and one white blood cell from a blood sample of a patient who had previously undergone radical prostatectomy. Blue color – DNA staining with DAPI; Red color – PSA staining with Texas-Red; Green color – Cytokeratin staining with FITC.

DISCUSSION

Overall, the pathological characteristics of the low-volume prostate cancer were similar to those described by Cheng *et al.* with respect to mean age, prostate size, tumor volume and tumor grade.[19] As previously reported by our group and others, men with clinically insignificant tumors are rarely at risk for PSA relapse.[13,20,21] We suspect that the serological failures that occurred in three out of the five patients in the study published by Furusato *et al.* were probably due to residual benign prostatic tissue at the surgical margins.[13] However, no such explanation can be offered for the other 2 patients, who experienced biochemical recurrence.[13] We hypothesize that they may represent rare examples of well-differentiated tumors with low volumes that still maintain a biologically aggressive nature, as previously described.[13]

CTCs are found in the peripheral blood and can potentially be disseminated from either primary tumors or metastatic sites. In the prostate research field, the detection of CTCs has been incorporated into different fields of oncology as a prognostic marker, a tool to monitor response to therapy and a method to understand basic tumor characteristics in the prostate.[22] Little is known, however, about whether CTCs can be detected in patients with low-volume prostate cancer.[23] In the current study, we observed CTCs in two patients who had elevated PSAs after surgery. Interestingly, thus far, none of the patients in our study has experienced a recurrence without prior or synchronous identification of CTCs We do not know whether postoperative detection of CTCs in low-risk prostate cancer patients with 'insignificant' tumors indicates that these patients should receive more aggressive follow-up

Table 2 Data regarding PSA follow-up and the number of CTCs cells that were detected in each patient sample

			Number of positive cell found (NT: Not tested)												
			PSA levels												
Case #	Pre-surgery	At surgery	<1 mon	<3 mon	6 mon	12 mon	18 mon	24 mon	30 mon	36 mon	42 mon	48 mon	54 mon	60 mon	>72 mon
1	*0*	*2*	*0*	*0*	NT	*1*	*2*	*0*	*0*	*1*					
	0.73	1.26	1.12	<0.2	0.014	0.023	0.007	0.026	0.011	0.04					
2	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	NT	NT	
	2.08	0	1.06	0.02	0.017	0.008	0.014	NT	0.02	0.31	0.009	0.003	0.01	0.01	
3	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*5*	*0*	NT	NT	NT	NT	NT	NT
	5.32	2.58	2.27	0.006	0.014	0.002	0.032	0.006	0.013	0.003	0.026	0.1	0.01	0.1	0.1
4	*5*	*0*	*0*	*0*	*0*										
	2.94	0	0	0	0										
5	*1*	*0*	*0*	*0*	*7*	*0*	*0*	NT	NT	NT	*0*	NT			
	5.1	2.98	3.2	0.003	0.25	0.062	0.055	0.003	0.062	0.014	0.022	0.003			
6	*0*	*0*	NT	NT	NT	*0*	*0*								
	4.57	0.011	0.009	NT	NT	0.002	0.002								
7	NT	*0*	*0*	*0*	*2*	*0*	NT	NT	NT	NT	NT	NT	NT	*0*	
	0.719	1.23	0.002	0.01	0.005	0.01	0.008	0.003	NT	0.023	NT	0.01	NT	0.05	
8	*0*	*0*	*0*	*0*	*0*	NT	*16*								
	6.86	0	1.84	0.01	0.003	NT	0.007								
9	*0*	*0*	*2*	NT	*0*	*0*									
	6.95	0	3.79	NT	0.104	0.019									

Numbers in *Italics*: Number of CTCs cells identified; Number below the number in *Italics*: serum PSA level (ng/mL); NT: not tested; mon: Month; Blank space: Information not available because the patient did not return for follow-up.

or adjuvant therapies to facilitate a cure. Still, we believe that combining CTC detection with supersensitive PSA testing may allow for the prediction of biochemical recurrence earlier than it could be predicted by conventional PSA testing alone.

One weakness of this study is the small number of patients with low-volume prostate cancer. With continued long-term follow-up, the number of patients showing elevation of PSA may increase, allowing further development of available data. In addition, larger multi-center studies should be applied to this patient group to assess more comprehensively the utility of measuring CTCs in patients with insignificant disease. This study provides a sound methodology for measuring CTCs in prostate cancer patients, and its findings have interesting implications for their treatment.

CONCLUSIONS

In this study, patients with CTCs were found to have detectable PSA levels with and without transient PSA spikes. This study provides further evidence that the isolation and detection of circulating epithelial cells is possible and may be used for follow-up of patients with low-volume prostate cancer. The detection of CTCs, however, did not predict PSA failure using strict criteria (e.g. two consecutive values of elevated PSA ≥0.2 ng/mL) in our patient population.

REFERENCES

1 Stamey TA, Freiha FS, McNeal JE, Redwine EA, Whittemore AS, Schmid HP. Localized prostate cancer. Relationship of tumor volume to clinical significance for treatment of prostate cancer. *Cancer* 1993; **71**: 933–8.

2 Chan TY, Chan DY, Stutzman KL, Epstein JI. Does increased needle biopsy sampling of the prostate detect a higher number of potentially insignificant tumors? *J Urol* 2001; **166**: 2181–4.

3 Epstein JI, Chan DW, Sokoll LJ *et al*. Nonpalpable stage T1c prostate cancer: Prediction of insignificant disease using free/total prostate specific antigen levels and needle biopsy findings. *J Urol* 1998; **160**: 2407–11.

4 Goto Y, Ohori M, Arakawa A, Kattan MW, Wheeler TM, Scardino PT. Distinguishing clinically important from unimportant prostate cancers before treatment: Value of systematic biopsies. *J Urol* 1996; **156**: 1059–63.

5 Irwin MB, Trapasso JG. Identification of insignificant prostate cancers: Analysis of preoperative parameters. *Urology* 1994; **44**: 862–7; discussion 67–8.

6 Noguchi M, Stamey TA, McNeal JE, Yemoto CM. Relationship between systematic biopsies and histological features of 222 radical prostatectomy specimens: Lack of prediction of tumor significance for men with nonpalpable prostate cancer. *J Urol* 2001; **166**: 104–9; discussion 9–10.

7 Augustin H, Hammerer PG, Graefen M *et al*. Insignificant prostate cancer in radical prostatectomy specimen: Time trends and preoperative prediction. *Eur Urol* 2003; **43**: 455–60.

8 Dugan JA, Bostwick DG, Myers RP, Qian J, Bergstralh EJ, Oesterling JE. The definition and preoperative prediction of clinically insignificant prostate cancer. *JAMA* 1996; **275**: 288–94.

9 Elgamal AA, Van Poppel HP, Van de Voorde WM, Van Dorpe JA, Oyen RH, Baert LV. Impalpable invisible stage T1c prostate cancer: Characteristics and clinical relevance in 100 radical prostatectomy specimens – A different view. *J Urol* 1997; **157**: 244–50.
10 Epstein JI, Walsh PC, Carmichael M, Brendler CB. Pathologic and clinical findings to predict tumor extent of nonpalpable (stage T1c) prostate cancer. *JAMA* 1994; **271**: 368–74.
11 Stamey TA. More information on prostate specific antigen and prostate cancer. *J Urol* 2003; **170**: 457–8.
12 Kattan MW, Eastham JA, Wheeler TM *et al*. Counseling men with prostate cancer: A nomogram for predicting the presence of small, moderately differentiated, confined tumors. *J Urol* 2003; **170**: 1792–7.
13 Furusato B, Rosner IL, Osborn D *et al*. Do patients with low volume prostate cancer have prostate specific antigen recurrence following radical prostatectomy? *J Clin Pathol* 2008; **61**: 1038–40.
14 Desai A, Wu H, Sun L *et al*. Complete embedding and close step-sectioning of radical prostatectomy specimens both increase detection of extra-prostatic extension, and correlate with increased disease-free survival by stage of prostate cancer patients. *Prostate Cancer Prostatic Dis* 2002; **5**: 212–18.
15 Ochiai A, Trpkov K, Yilmaz A, Donnelly B, Babaian RJ. Validation of a prediction model for low volume/low grade cancer: Application in selecting patients for active surveillance. *J Urol* 2007; **177**: 907–10.
16 Ts'o PO, Pannek J, Wang ZP, Lesko SA, Bova GS, Partin AW. Detection of intact prostate cancer cells in the blood of men with prostate cancer. *Urology* 1997; **49**: 881–5.
17 Wang ZP, Eisenberger MA, Carducci MA, Partin AW, Scher HI, Ts'o PO. Identification and characterization of circulating prostate carcinoma cells. *Cancer* 2000; **88**: 2787–95.
18 Tkaczuk KH, Goloubeva O, Tait NS *et al*. The significance of circulating epithelial cells in Breast Cancer patients by a novel negative selection method. *Breast Cancer Res Treat* 2008; **111**: 355–64.
19 Cheng L, Jones TD, Pan CX, Barbarin A, Eble JN, Koch MO. Anatomic distribution and pathologic characterization of small-volume prostate cancer (<0.5 ml) in whole-mount prostatectomy specimens. *Mod Pathol* 2005; **18**: 1022–6.
20 Sengupta S, Blute ML, Bagniewski SM *et al*. After radical retropubic prostatectomy 'insignificant' prostate cancer has a risk of progression similar to low-risk 'significant' cancer. *BJU Int* 2008; **101**: 170–4.
21 van Oort IM, Kok DE, Kiemeney LA, Hulsbergen CA, Witjes JA. A single institution experience with biochemical recurrence after radical prostatectomy for tumors that on pathology are of small volume or 'insignificant'. *Urol Oncol* 2009; **27**: 509–13.
22 Panteleakou Z, Lembessis P, Sourla A *et al*. Detection of circulating tumor cells in prostate cancer patients: Methodological pitfalls and clinical relevance. *Mol Med* 2009; **15**: 101–14.
23 Davis JW, Nakanishi H, Kumar VS *et al*. Circulating tumor cells in peripheral blood samples from patients with increased serum prostate specific antigen: Initial results in early prostate cancer. *J Urol* 2008; **179**: 2187–91.