RESEARCH ARTICLE

Extended use of P504S Positive Primary Circulating Prostate Cell Detection to Determine the Need for Initial Prostate Biopsy in a Prostate Cancer Screening Program in Chile

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Abstract

Background: To determine the frequency of primary circulating prostate cells (CPC) detection according to age and serum PSA levels in a cohort of men undergoing screening for prostate cancer and to determine the diagnostic yield in those men complying with the criteria for prostate biopsy. Materials and Methods: A prospective study was carried out to analyze all men evaluated in a hospital prostate cancer screening program. Primary CPCs were obtained by differential gel centrifugation and detected using standard immunocytochemistry using anti-PSA, positive samples undergoing a second process with anti-P504S. A malignant primary CPC was defined as PSA+ P504S+, and a test positive if 1 cell/4ml was detected. The frequency of primary CPC detection was compared with age and serum PSA levels. Men with a PSA >4.0ng/ml and/or abnormal rectal examination underwent 12 core prostate biopsy, and the results were registered as cancer/no-cancer and compared with the presence/absence of primary CPCs to calculate the diagnostic yield. Results: A total of 1,117 men participated; there was an association of primary CPC detection with increasing age and increasing serum PSA. Some 559 men underwent initial prostate biopsy of whom 207/559 (37.0%) were positive for primary CPCs and 183/559 (32.0%) had prostate cancer detected. The diagnostic yield of primary CPCs had a sensitivity of 88.5%, a specificity of 88.0%, and positive and negative predictive values of 78.3% and 94.9%, respectively. Conclusions: The use of primary CPCs for testing is recommended, since its high negative predictive value could be used to avoid prostate biopsy in men with an elevated PSA and/or abnormal DRE. Men positive for primary CPCs should undergo prostate biopsy. It is a test that could be implemented in the routine immunocytochemical laboratory.

Keywords: Prostate cancer screening - primary circulating prostate cells - diagnostic yield

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Introduction

Prostate cancer is the commonest male malignancy in westernized world and the second commonest cause of male cancer related death, with a considerable morbidity directly associated to this cancer (Aus, 1994). The disease is typically associated with the aging male population, in some older patients the disease is relatively indolent whereas in younger men may be surprisingly aggressive. Serum prostate specific antigen (PSA) is the only biomarker routinely used for the early detection of prostate cancer, but it is not a perfect test. Although PSA is highly specific for prostate, an elevated level is not specific for prostate cancer, being increased in benign hyperplasia and prostatitis (Bozeman et al, 2002; Pungalia et al, 2006). An accepted cutoff level of 4.0ng/ml is used to indicate the need for a prostate biopsy, but the PSA test has considerable limitations in its sensitivity and specificity (Schroder et al, 2000). Of men with a PSA level of 4.0-10.0ng/ml the European Randomized Study of Screening for Prostate Cancer (Postma, 2005) determined that 74% of the biopsies were negative for cancer. Furthermore to make matters worse the Prostate Cancer Prevention Trial (Thompson et al, 2003) reported that 39.2% of men with a PSA 2.1-3,0ng/ml, 27.7% of men with a PSA 1.1-2.0ng/ml and 1.3% of men with a PSA <1.0ng/ml had end of trial prostate biopsies with foci of adenocarcinoma. In other words 38% of men with prostate cancer have a PSA <4.0ng/ml and 70% of men with a PSA >4.0ng/ml do not have cancer. It has been estimated that only 50% of asymptomatic men with prostate cancer detected as a result of PSA screening will need active treatment (Draisma et al, 2003). Thus the search for new biomarkers to improve the diagnostic yield is needed. This is especially so as the risks of prostate biopsy are not insignificant, Rietbergen et al (1997), in a study of 5,802 patients undergoing trans-rectal

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prostate biopsy reported an incidence of complications of 0.5% hospitalizations, 2.1% rectal hemorrhage, 2.3% fever and 7.2% persistent hematuria. Although the use of PSA based parameters have been suggested, such as PSA velocity and PSA velocity per initial volume (Zheng et al, 2012), PSA density and dynamics, including velocity and doubling time and PSA molecular forms (De Visschere et al, 2010) none have been shown to be superior to PSA alone. In developing countries the use of DRE and total serum PSA are the standard method of screening when implemented as part of the public health system (Par Kash et al, 2014). In different countries the accepted PSA cutoff value of 4.0ng/ml may not be the most appropriate for the high incidence of benign hyperplasia, as reported from Pakistan (Par Kash et al, 2014).

One possible candidate for improving prostate cancer screening is the detection of primary circulating prostate cells (CPC). In men with prostate cancer there is, at least, one subpopulation of cancer cells that disseminate early, firstly to the neurovascular structures and then to the circulation (Moreno et al, 1992). The number of these cells is very small; however these primary CPC can be detected using immuocytochemistry with a combination of anti-PSA and anti-P504S (methyl-acyl-CoA racemase) monoclonal antibodies. The use of the biomarker P504S, although not prostate specific (Zhou et al, 2002), has facilitated the differentiation between normal, dysplastic and malignant tissues in prostate biopsy samples. Normal or benign cells do not express P504S, whereas cells arising from prostatic intraepithelial neoplasia (PIN) or cancer are positive (1Beach et al, 2002). Benign CPCs can be detected in men with benign hyperplasia and chronic prostatitis (Murray et al, 2013), for this reason it is essential that double immune-labelling is used to detect mCPCs. Double immunomarcation is the sequential use of two monoclonal antibodies and 2 differing systems of detection, thus labeling cells as positive or negative for two different biomarkers. Only cells positive for the two biomarkers are considered as positive. Primary CPCs are defined as those detected before definitive treatment for prostate cancer. This is to differentiate them from secondary CPCs, defined as those detected after primary treatment, and have been shown to indicate a poor prognosis (Fu-Bin Wang et al, 2011) and increase the risk of biochemical failure (Murray et al, 2013a)

A pilot study of patients undergoing first biopsy showed the possible potential of P504S positive primary CPCs as a possible biomarker for the detection of prostate (Murray et al, 2010).

We present a prospective study of the use of primary CPCs as a complementary biomarker to total PSA for the detection of prostate cancer. We look at the frequency of detection with respect to age and serum total PSA, and the diagnostic yield of patients undergoing prostate biopsy.

Materials and Methods

After ethical committee approval of the study for the use of CPC detection, a prospective study was carried out.

Patient selection

Total population: The frequency of CPC detection was analyzed in all men participating in a prostate cancer screening program in the Health System of Carabineros de Chile , independent of age, serum PSA level or whether a biopsy was considered. Immediately before a digital rectal examination (DRE) 8ml of venous blood was collected in EDTA (Beckinson-Vacuntainer®) and sent to a central laboratory.

Biopsy population: All patients with a serum PSA >4.0ng/ml and/or a DRE suspicious of prostate cancer were referred for prostate biopsy. Immediately before the biopsy 8 ml of venous blood was collected in EDTA (Beckinson-Vacuntainer®) and sent to the same central laboratory. Patients were coded and clinical details of serum PSA, age and biopsy results collected. The CPC processing laboratory was blinded to the clinical details. The CPC test was analyzed for specificity, sensibility, positive and negative predictive values.

Detection of CPCs

Mononuclear cells were obtained by differential centrifugation using Histopaque 1,077® (Sigma-Aldrich), washed and resuspended in 100µL of autologous plasma. 25µL aliquots were used to make slides (sialianized, DAKO, USA), dried in air for 24 hours and fixed in a solution of 70% ethanol, 5% formaldehyde and 25% phosphate buffered saline pH 7.4.

CPCs were detected using a monoclonal antibody directed against PSA, clone 28A4 (Novocastro Laboratory, UK) and identified using an alkaline phosphatase-anti alkaline phosphatase based system (LSAB2, DAKO, USA), with new.-fushcin as the chromogen. Positive samples underwent a second process with anti-P504S clone 13H4 (DAKO, USA) and identified with a peroxidase based system (LSAB2, DAKO, USA) with DAB (3,3´diaminobenzidine tetrahydrochloride) as the chromogen.

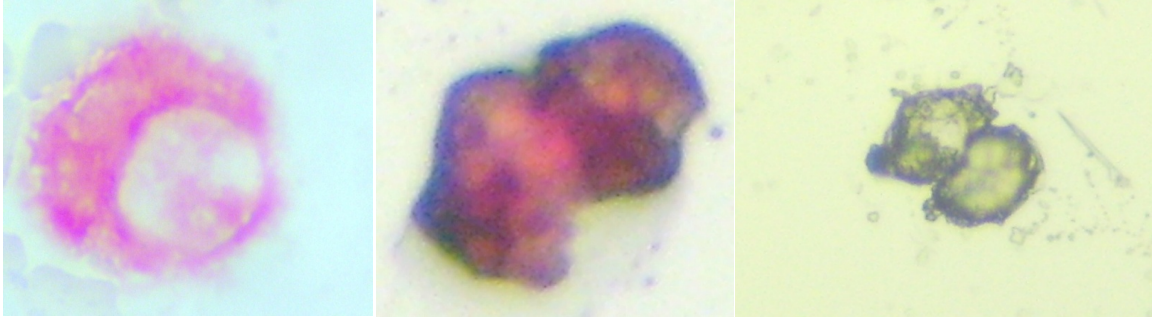

Figure 1. A: Benign CPC PSA (+) P504S (-); B: Malignant CPC PSA (+) P504S (+); C: Leucocyte PSA (-) P504S (+)

A malignant CPC was defined according to the criteria of ISHAGE (International Society of Hematherapy and Genetic Engineering) (Borgen et al, 1999) and the expression of P504S according to the Consensus of the American Association of Pathologists (Rubin et al, 2002). A malignant mCPC was defined as a cell that expressed PSA and P504S, a benign CPC could express PSA but not P504S and leucocytes could be P504S positive or negative but did not express PSA (Figure 1A-C). A test was considered positive when at least 1 cell was detected/4ml blood.

Statistical Analysis

Descriptive statistics were used for demographic variables, expressed as mean and standard deviation in the case of continuous variables with a normal distribution. In case of an asymmetrical distribution the median and interquartile range (IQR) values were used. Noncontiguous variables were presented as frequencies. The Shapiro-Wilk test was used to determine a normal distribution. The Student T-Test was used to compare continuous variables with a normal distribution, the Mann-Whitney test for ordinate and continuous variables with a non-normal distribution and Chi-squared for the differences in frequency. The diagnostic yield for the test detecting CPCs was analyzed using standard parameters. For this purpose patients were classified as having or not having prostate cancer. For the purpose of calculating the diagnostic yield in Group II patients, it was assumed that patients CPC negative and without a biopsy would include patient's false negatives and true negatives in the same proportion as in Group I Statistical significance was defined as a p value less than 0.05 to two-sided. Analysis was performed using the Stata 11.0 program (StataCorp LP, College Station, Texas, USA).

Table 1. Frequency of mCPC Detection According to Serum PSA Level and Age

PSA ng/ml	Number of patients	mCPC detection	Frequency mCPC detection (%)	Relative Risk
0-2	301	11	4	1.00
>2-3	99	8	8	2.32
>3-4	91	19	21	6.96
>4-6	340	153	45	21.57
>6-8	117	54	46	22.60
>8-10	77	38	49	25.69
>10	92	46	50	26.36

Table 2. Frequency of mCPC Detection According to Serum PSA Level and Age

Age (years)	Number of patients	mCPC detection	Frequency mCPC detection (%)	Relative Risk
≤50	96	19	20	1.00
51-55	101	19	19	0.94
56-60	190	54	28	1.61
61-65	195	55	28	1.59
66-70	220	71	32	1.93
71-75	143	46	32	1.92
76-80	112	40	36	2.25
>80	60	25	42	2.85

Table 3. Comparson between mCPC Detection and Prostate Biopsy Results

	Biopsy positive for cancer	Biopsy negative for cancer	Total
mCPC positive	162	45	207
mCPC negative	21	331	352
Total	183	376	559

Table 4. Diagnostic Yield of Primary mCPC to Detect Prostate Cancer at Initial Biopsy

	Diagnostic yield	95% Confidence interval
Sensitivity	88.5%	82.8-92.6%
Specificity	88.0%	84.2-91.1%
PPV	78.3%	71.9-83.6%
NPV	94.0%	90.9-96.2%
Positive LR	7.40	5.60-9.78
Negative LR	0.13	0.09-0.20

PPV=positive predictive value; NPV=negative predictive value; LR=liklihood ratio

Results

Total population

1117 men with a mean age of 64.8±10.3 years participated in the study, with a median serum PSA of 4.32ng/ml (inter-quartile range 1.73-6.10ng/ml. There was an association between the frequency of primary CPC detection and serum PSA level (Chi squared for trends p=0.0001 see table 1).

The frequency of CPC detection also was associated with increasing age (Chi squared for trends p<0.0001 see Table 2).

Men undergoing biopsy based on serum PSA and/or abnormal DRE.

559 men comprised this group with a mean age of 65.1±9.1 years and median serum PSA of 5.49ng/ml (inter-quartile range 4.50-8.03), with a prevalence of 32.7% of men with cancer detected and 207/559 (37.0%) of men were positive for CPCs. Table 3 shows the results of comparing CPC with the biopsy results.

Of the 21 false negative results, 20 complied with the Epstein criteria for active observation (Epstein et al, 1994), the remainder was a Gleason 7 tumor, positive in 1 core, 10% of the core sample infiltrated.

Table 4 shows the diagnostic yield of using primary CPCs for the detection of prostate cancer.

Discussion

The incidence of clinical prostate cancer increases with age, from 0.2 to 0.9% from 50- to 80-year-old men. However, cancer detected as a result of an abnormal DRE or serum

PSA may be underestimated. Latent prostate cancer is defined as an asymptomatic cancer detected only at autopsy, published studies have shown a much higher incidence of latent cancer than in population studies of clinical cancer (Osman et al, 2001). Latent cancer is not linearly associated with age; and there is a plateau in the incidence of latent cancer between 60 and 70 years. The frequency of circulating prostate cells showed a similar trend, increasing up to 55 years and with a plateau between 55 and 75 years before increasing again.

The frequency of CPC detection increases with serum

PSA, increasing markedly in those men with a PSA of >3.0ng/ml, levels of CPC detection frequency in men with a PSA <3.0ng/ml were less than 10%, and similar to the frequency of prostate cancer detected in the Prostate Cancer Prevention Trial (Thompson et al, 2003).

It is important to note that the use of CPC detection is designed as a sequential test, for men with an abnormal PSA or DRE, that positive cases should be evaluated with prostate biopsy and negative cases followed up. That the test is positive or negative with no cut-off point simplifies clinical decisions as to whether proceed to prostate biopsy. This is reinforced by the high negative predictive value of the test, 94% of CPC negative men did not have cancer detected on the initial biopsy, and the fact that the 6% of men with cancer had low grade small volume tumors. These men withCPC negative low grade cancers complied with the Epstein criteria for treatment with active observation. Thus men CPC negative but with an increased serum PSA could be observed rather than evaluated with a prostate biopsy, with all its inherent risks. In comparison with free PSA, PSA velocity and PSA density, the use of primary CPC detection was shown to be superior (Murray et al, 2014)

Studies detecting circulating prostate cells, using different methodologies have been discordant results. Using a dual PSA/prostate specific membrane antigen RT-PCR method Eschwege et al (2009) only found 37% of pre-operative patients to be CPC positive. Davis et al (2008) found no association between CPC detection using the CellSearch® system and the clinical parameters prior to radical prostatectomy or between men with local PC or controls. However, Stott et al (2010) found primary CPCs in 42% of patients with localized cancer, Fizazi et al (2007), using anti-BerEP-4 epithelial antigen combined with telomerase activity, detected primary CPCs in 79% of patients with localized cancer, a similar figure to our study. One possible reason for the wide discrepancy of results is the technology used. Regardless of the system used for isolation or enrichment, detection almost always relies on staining for cells containing cytokeratin (Moll, 2008). In those cases where EpCM has been used for cell enrichment, such as CellSearch®, EpCAM can alternatively be used for detection (Helo et al, 2009). Methods using RT-PCR have utilized anti-EpCAM or anti-cytokeratin based enrichment methods (Schaffer et al, 2007; Helo et al, 2009). The widely accepted concept that all cytokeratin and/or EpCAM positive, CD45 negative cells with a nucleus in cancer pacients are circulating tumor cells (CTCs) has imposed a clear bias on the study of CTCs. Mainly the failure to include tumor cells that have reduced or absent cytokeratin and/or EpCAM expression, the failure to identify such cell types limits investigations into additional tumor types. EpCam is expressed in most but not all tumors ((Went et al, 2004), there is downregulation with cancer progression and metastasis, cytokeratins are heterogeneously expressed in tumor cells and also may be down regulated during disease progression or in poorly differentiated tumors. During the progression of epithelial to mesenchymal transition both markers are downregulated (Paterlini-Brechot et al, 2007), EpCAM may be down regulated to allow epithelial cell dissociation from the tumor and cytokeratin downregulated to facilitate cell plasticity and migration (Raimondi et al, 2011). In this study the use of PSA and P504S to define mCPCs avoids this problem, and the results are similar to that of Fizazi who used also avoided the use of a cytokeratin and/or EpCAM based system. The finding of CTCs that express EpCAM is not in question, but there is concern over false negatives in the failure to detect CTCs that do not express EpCAM. Using a mixture of antibodies against cell surface antigens Mikolajczyk et al (2011) showed in breast cancer patients a higher detection rate of CTCs both qualitatively and quantitatively. In breast cancer 34% of patients had EpCAM negative CTCs detected (Mikolajczyk et al, 2011), and this difference may be one possible explanation for the difference in our findings and those of Fizazi with other studies based on EpCAM and/or cytokeratins.

We believe that part of the difference documented is caused by the relatively high detection in control patients, one explication is that CPC can be found in men with prostatitis, however these CPCs are P504S negative (Murray et al, 2013). This underlies the problem with different methods used to detect circulating tumor cells. This problem has been extensively reviewed as to the advantages and disadvantages of each method (Panteleakou et al, 2009, Fehm et al, 2005). PCR methods have a high rate of false positive results, density gradient centrifugation may be associated with increased lost of circulating cells whereas immunomagnetic separation may not recognize tumor cells which do not express EpCAM and does not differentiate between malignant and benign prostate cells.

We realize that limitations of the study, include the analysis by a trained cytologist, validation with different observers are undergoing, however this could be overcome with training. Equally the DRE and decision to proceed to a prostate biopsy is dependent on the experience of the urologist. The detection of primary CPCs is designed as a sequential test, being requested after the serum PSA and/or DRE, forming a diagnostic test in series. In spite of this the negative predictive value increased. The study did not separately analyze the contribution of the serum PSA and/or DRE in the pre-test determination of detecting prostate cancer, but this constitutes the daily practice of prostate cancer screening, for which it could be viewed as a strongpoint in demonstrating the diagnostic yield of primary CPC detection in the real clinical world.

The test is simple and could be implemented in the routine immunocytochemstry laboratory of a general hospital.

In conclusion, the use of primary circulating prostate cells as a sequential test has a high negative predictive value, which suggests that a prostate biopsy could be avoided in these patients, the cancers CPC negative being small volume low grade tumors. Men positive for CPCs should undergo biopsy for the high risk of prostate cancer.

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