RESEARCH ARTICLE

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10 Year Biochemical Failure Free Survival of Men with CD82 Positive Primary Circulating Prostate Cells Treated by Radical Prostatectomy

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Abstract

Objective: The biological characteristics of circulating prostate cells (CPCs) are probably more important than their mere presence. CD82 is a tumor suppressor, we present the outcome of radical prostatectomy (RP) in men with CD82 positive CPCs. **Methods and Patients:** consecutive men treated with RP were studied, age, total PSA, Gleason, stage, the presence of extra-capsular extension, positive surgical margens and infiltration of the seminal vesicles and lymph nodes were registered. Biochemical failure was defined as a PSA >0.2ng/ml. Immediately before the RP, 8ml of venous blood was taken to detect CPCs. Mononuclear cells were separated using differential gel centrifugation and CPCs identified using immunocytochemistry with anti-PSA and anti-CD82. The men were divided into three groups; 1) CPC (-), 2) CPC (+) CD82 (+) and 3) CPC (+) CD82 (-). The groups were compared with respect to clinical-pathological findings and biochemical free survival using Kaplan Meier and Cox regression models. **Results:** 285 men, mean age 65.9 years participated, 61 (21%) were CPC (-); 57 (20%) were CPC (+) CD82 (+) and 167 (59%) were CPC (+) CD82 (-). Group 1 had low grade small volume cancer, in Group 2, low grade but a larger volume than Group 1 and Group 3 high grade cancer. Kaplan Meier biochemical free survival curves at 36, 60 and 120 months were; Group 1 98%, 96% and 90%; for Group 2 93%, 93% and 69% and for Group 3 62%, 44% and 16% respectively. **Conclusions:** Kaplan Meier survival curves for Group 1 and Group 2 were similar, although Group 2 men had higher PSA values, more advanced staging but a similar Gleason score. Group 3 men had a worse prognosis. The results support that biological characteristics of CPCs are more important than their mere presence identifying men with a high risk of biochemical failure.

Keywords: Prostate cancer- circulating tumor cells- CD82- biochemical failure

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Introduction

The majority of cancer deaths are a result of blood borne dissemination, which occurs at an early stage in cancer development. Prostate cancer cells disseminate first to the neurovascular structures and then on to the circulation (Moreno et al., 1992). The majority of these cells are eliminated by host defenses or destroyed by shear forces as they circulate in the blood and lymph systems (Fidler, 1970) and have been defined as primary circulating prostate cells. The detection of circulating prostate cells (CPCs) provides the potential for detection, enumeration and monitoring of treatments as well as the possibility of being a prognostic factor. However, the biological specificity of a detected cancer cell is more important than its mere presence. The biological potential of a CPC, its capacity to disseminate, implant in distant tissues, survival and proliferate are factors that will determine its role in the formation of possible metastasis. Therefore it is probably more important not only to detect and enumerate the presence of CPCs but to determine important phenotypic characteristics. Tumor cell metastasis is controlled by molecular processes that are distinct from those that control tumorigenesis. Progression of metastasis appears to be controlled in part by a subset of genes that suppress tumor cell dissemination without affecting the development of the primary tumor (Rinker-Schaeffer et al., 2006).

One possible candidate is the tumor suppression gene KAI1 which located in the region p11.2 of chromosome 11 (Dong et al., 1995) and codes for a glycoprotein of the tetraspanin family, CD82. When CD82 is expressed in rat prostatic cancer cells it suppresses metastasis but not tumorigenesis, after gene transfer to induce re-expression of CD82 rat prostatic cancer cells showed decreased invasiveness and motility (Dong et al., 1995). CD82 expression has been reported to be down regulated in a subset of primary human prostate cancers and in all metastasis (Dong et al., 1996). Its expression is inversely correlated to the metastatic potential of that cancer (Udea

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et al., 1996).

In well and moderately differentiated prostate cancer the expression is above that seen in benign hyperplasia, however in poorly differentiated cancer its expression is decreased (Bouras et al., 1999; Lijovic et al., 2002). In men with low grade Gleason 4 or 5, the expression of CD82 has been detected in primary circulating prostate cells, i.e. detected before primary treatment (Murray et al., 2010). The frequency of CD82 expression in primary CPCs is inversely associated with the Gleason score, not being detected in those patients with Gleason ≥7 (Murray et al., 2010).

Expression of CD82 in the primary tumors of non-small cell lung cancer, breast cancer, bladder, pancreatic and colon cancer is associated with a better prognosis (Adachi et al., 1996; Guo et al., 1996; Huang et al., 1998; Maurer et al., 1999; Shiwu et al., 2012; Yu et al., 1997) in comparison with patients with CD82 negative tumors. It has been suggested that although CD82 positive cells can disseminate into the circulation their ability to implant is reduced, thus their presence pre-prostatectomy radical may not signify a poorer prognosis.

The objective of this study was the detection of primary CPCs, the expression of CD82 their association with clinical-pathological features, with biochemical failure after prostatectomy radical.

Materials and Methods

Patients and Methods

A single centre prospective observational study of men who underwent radical prostatectomy as the sole treatment for prostate cancer between 2005 and 2014. The study was approved by the local ethics committee and complied with the Declaration of Helsinki.

For each patient, after giving informed written consent, the following were recorded; date of prostatectomy radical, age and the following clinic-pathological information: Total serum PSA (ng/ml) at the time of diagnosis using the Siemens Advia CentaurXR® assay; percentage of biopsy cores positive for cancer; The pathological study of the surgical piece was performed by dedicated genitourinary pathologists according to the Gleason system; Presence or absence of extra-capsular extension (ECE); Presence or absence of positive surgical margins, defined as one with cancer cells in contact with the inked surface of the specimen; Infiltration of the seminal vesicles and lymph nodes.

Detection of primary circulating prostate cells

Before the surgery all men had a 8ml venous blood sample was taken and collected in a tube containing EDTA (BD-Vacutainer®, USA). Samples were maintained at 4ºC and processed within 48 hours. CPC detection was independently evaluated with the evaluators being blinded to the clinical details.

Collection of CPCs

Mononuclear cells were obtained by differential centrifugation using Histopaque 1,077 (Sigma-Aldrich, USA), washed, and re-suspended in a 100µL aliquot of autologous plasma. 25µL aliquots were used to make four slides (silanized, DAKO, USA), these were dried in air for 24 hours and fixed in a solution of 70% ethanol, 5% formaldehyde, and 25% phosphate buffered saline (PBS) pH 7.4 for five minutes and finally washed three times in PBS pH 7.4.

Immunocytochemistry

Two slides were processed to detect CPCs 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 fuchsin as the chromogen. Positive samples underwent a second process with anti-P504S clone 13H4 (DAKO, USA) and were identified with a peroxidase based system (LSAB2, DAKO, USA) with DAB (3,3 diaminobenzidine tetrahydrochloride) as the chromogen. A primary CPC was defined according to the criteria of ISHAGE (International Society of Hemotherapy and Genetic Engineering) (Borgen et al., 1999) and the expression of P504S defined according to the Consensus of the American Association of Pathologists (Ruben et al., 2001); as a cell expressing both PSA and P504S and detected before definitive treatment for prostate cancer. A test was considered positive for primary CPCs when at least 1 cell/8mL of blood was detected; the number of CPCs detected/8ml blood simple was registered. (Figure 1a: CPC PSA positive(red), P504S positive (brown); Figure 1b: CPC PSA positive (red), P504S negative).

In men with malignant primary CPCs detected, that is PSA (+) P504S (+), the remaining two slides were processed for PSA as previously described, positive samples underwent a second process with anti-CD82 clone 5B5 (Novocastro Laboratory, UK) in a 1:50 dilution and were identified with a peroxidase based system (LSAB2, DAKO, USA) with DAB (3,3 diaminobenzidine tetrahydrochloride) as the chromogen. The expression of CD82 was classified as negative, if no part of the membrane or only part of the membrane showed expression of CD82. If all the cell membrane expressed CD82 the cell was classified as CD82 positive, if >10% of PSA staining cells were CD82 positive, the sample was determined to be CD82 positive (Figure 1c: PSA positive (red) CD82 positive (brown)).

Patients were classified as CPC negative (Group 1) (did not have cells PSA (+) P504S (+) detected) or CPC positive (cells PSA (+) P504S (+) detected). This second group was divided into Group 2 CPC positive expressing CD82 and Group 3 CPC positive not expressing CD82.

Follow-up

All men were followed up with serial total serum PSA measurements; three monthly for two years, then six monthly to detect the presence or absence of biochemical failure (BF) for up to 10 years. BF was defined as a serum total PSA > 0.2ng/ml on two separate occasions, taken at least two weeks apart.

Statistical Analysis

The analysis was performed using the program Stata

(Stata/SE 14.0 for Windows, Stata Corp Lp, 20159, describing according to the nature and distribution of the quantitative and ordinate variables with measurements of central tendency (mean and median) and of dispersion using the inter-quartile range (IQR) and standard deviation. The Shapiro-Wilk Test was used to define the null hypothesis with respect to the normal distribution. The nominal dichotomous variables were described as proportions with their respective confidence intervals.

Age, total serum PSA, pathological Gleason score ≤6 and ≥7, extra-capsular extension, surgical margins, seminal vesicle and lymph node infiltration according to nature of the variables before mentioned, were compared between men negative for CPCs (Group 1); those men positive for CPCs expressing CD82 (Group 2) and those men positive for CPCs but negative for the expression of CD82 (Group 3).

The Cox proportional hazard regression and Kaplan-Meier methods were used to predict biochemical failure for each group at 3, 5 and 10 years post-surgery. A forward sequential technique was used to incorporate CPC data from Groups 2 and 3, which produces less deviation (p-value <0.05) for a significant hazard ratio (p-value <0.05) for the independent variables incorporated into the model (Cleves et al., 2010).

Each model was tested for compliance with the Cox proportional hazards model (log-log plots, Therneau and Grambsch test and testing for a cohort time interaction) (Cleves et al., 2010). In addition Harrell´s C concordance coefficient were used to analyse the respective predictions of biochemical free failure at three, five and ten years.

Results

285 men with a mean age of 65.9 ± 8.8 years underwent radical prostatectomy for prostate cancer between 2004 and 2014. Of these men 61 (21.4%) were CPC negative, 57 (20.0%) had CPCs expressing CD82 detected and 167/285 (58.6%) had CPCs negative for CD82 detected. The clinical-pathological characteristics of each group are shown in Table 1:

Comparing the clinical-pathological features, Group 1 patients had a significantly lower total serum PSA as compared to Groups 2 and 3 (p<0.01). Group 1 consisted of men with predominantly low-grade small volume

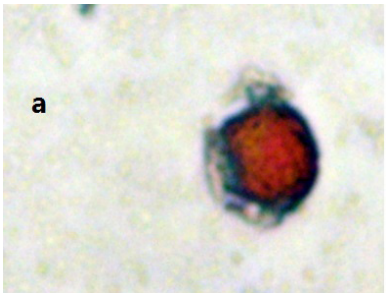

Figure 1a. Circulating Mmalignant Prostate Cell, Positive for PSA (red) and P504S (brown)

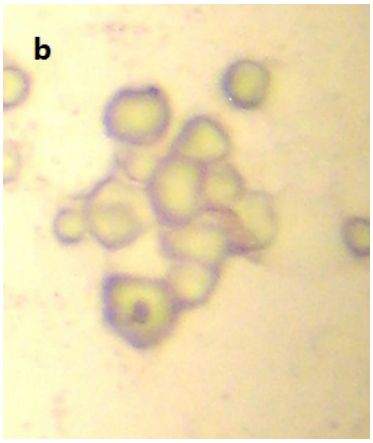

Figure 1b. Leukocytes, Negative for PSA (Red) but Positive for P504S (Brown)

Table 1. Clinical- Pathological Features of 285 Men Treated by Radical Prostatectomy for Prostate Cancer According Presence with and without Expression CD82 of Primary CPCs and to the Absence of Primary CPCs

Variable	Group 1 Negative CPC n=61	Group 2 Positive CPC Positive CD82 n=58	Group 3 Positive CPC negative CD82 n=166	p-value; groups significantly different comparison
Age* (years), Median; IQR	68; 15	65;10	67; 14	0.098a
PSA*, ng/ml, Median; IQR	4.81; 1.99	6.39; 2.67	6.20; 5.29	<0.01[a]; 1 v/s 2 and 1 v/s 3
pGS Score ≤ 6 n (%)	58 (95.08%)	58 (100%)	69 (41.57%)	< 0.01[b]; 1 v/s 3 and 2 v/s 3[c]
ECE, n (%)	7 (11.48%)	19 (32.76%)	107 (64.46%)	<0.01 [b]; 1 v/s 2, 1 v/s 3 and 2 v/s 3 [c]
SM, n (%)	3 (4.92%)	6 (10.34%)	53 (31.93%)	<0.01 [b]; 1 v/s 3, and 2 v/s 3[c]
SVI, n (%)	1 (1.64%)	1 (1.72%)	18 (10.84%)	0.012 [b]; 1 v/s 3, and 2 v/s 3[c]
LNI, n (%)	0 (0.00%)	0 (0.00%)	2 (1.20%)	p-value 1[d]
Pathological stage				
T1	53 (86.9%)	19 (32.8%)	26 (15.7%)	
pT2	5 (8.2%)	34 (58.6%)	84 (50.6%)	
pT3	3 (4.9%)	5 (8.6%)	56 (33.7%)	

CPC, Circulating prostate cells prior to surgery; PSA, serum total PSA at diagnosis; IQR, rango intercuartílico; pGS Score ≤6, pathological Gleason score = ≤6; ECE, extra-capsular extension; SM, positive surgical margins; SVI, Infiltration of the seminal vesicles; LNI, Infiltration of the lymph nodes a Kruskal-Wallis H test; [b], Pearson´s Chi squared test; [c], Marascuilo Procedure (2); [d], Fishers´s exact test

Table 2. Kaplan-Meier Survival Estimates of 285 Men Treated by Radical Prostatectomy for Prostate Cancer According Presence with and without Expression CD82 of Primary CPCs and to the Absence of Primary CPCs.

Time, (Months)	Group 1 Negative CPC, n=61, % survival; 95 % CI	Group 2 Positive CPC, Positive CD82, n=58, % survival; 95 % CI	Group 3 Positive CPC, negative CD82, n=166 % survival; 95 % CI
36	98.11; 87.35 to 99.73	92.69; 81.63 to 97.20*	61.57; 52.94 to 69.09
60	96.11; 85.32 to 99.01	92.69; 81.63 to 97.20*	43.90; 34.58 to 52.82
120	90.35; 75.80 to 96.35	68.66; 38.46 to 86.24	16.16; 8.24 to 26.41

%, percentage; CI, Confidence interval

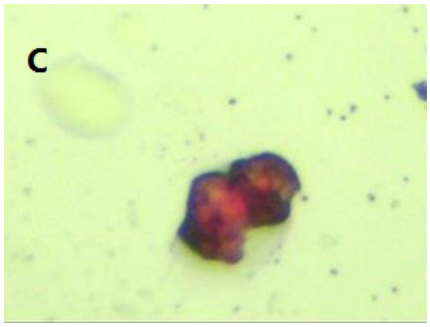

Figure 1c. Circulating Prostate Cell Exressing Both PSA (Red) and Membrane Cd82 (Brown)

tumours, without local extension. Group 2 consisted of men with predominantly low-grade larger volume tumours, and Group 3 predominantly higher-grade tumours with any degree of extension.

After 3, 5 and 10 years of follow up, the Kaplan-Meier biochemical free survivals for the whole group were respectively 76.28 (95% CI: 70.53 to 81.06), 67.05% (95 CI%: 60.52 to 72.75) and 47.34 (95% CI: 38.71 to 55.48). Of the whole population 103/285 (36.1%) had biochemical failure detected within the study period; 5/61 (8.2%) of men CPC negative; 7/57 (13.8%) of men CPC positive CD82 positive and 91/167 (54.4%) of men CPC positive CD82 negative ($p<0.0001$ Chi squared for trends)

Figure 2 shows the Kaplan Meier survival curves for the three groups; for Group 1 the survival at 36, 60 and 120 months were 98.11% (95% CI 87.35 to 99.73), 96.11% (95% CI 85.32 to 99.01) and 90.35% (95% CI 75.80 to 96.35) respectively. For Group 2 these values were; 92.69% (95% CI 81.63-97.20) at 3 and 5 years and 68.66% (95% CI 38.46 to 86.24). For men in Group 3 these values were; 61.57% (95% CI 52.94 to 69.09), 43.90% (95% CI 34.58 to 52.82) and 16.16% (95% CI 8.24 to 26.41) respectively.

Comparison between Groups: When comparing

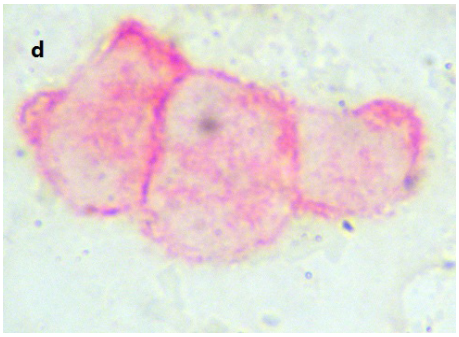

Figure 1d. Circulating Prostate Cell Expressing PSA (Red) but Negative for Membrane CD82

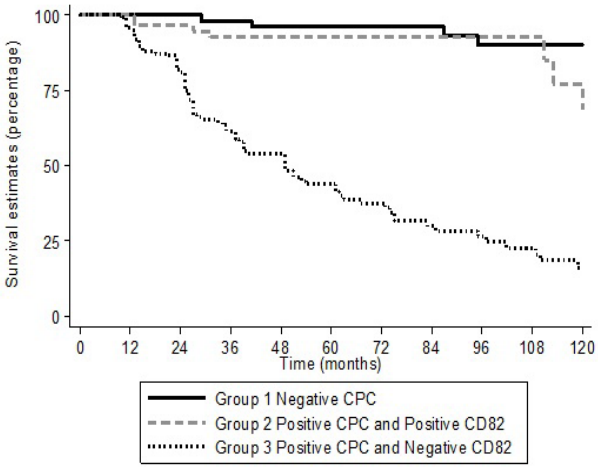

Figure 2. Kaplan-Meier Survival Estimates of 285 Men Treated by Radical Prostatectomy for Prostate Cancer According Presence with and without Expression CD82 of Primary CPCs and to the Absence of Primary CPCs.

Groups 2 and 3, men with CPCs CD82 negative had a higher rate of biochemical failure, HR of 11.84 (95% CI 6.27-22.38 $p< 0.01$) as compared to men with CPCs CD82 positive, with a Harrell´s C discrimination index of 0.72, considered to be adequate.

At 10 years, the comparing predicted (according

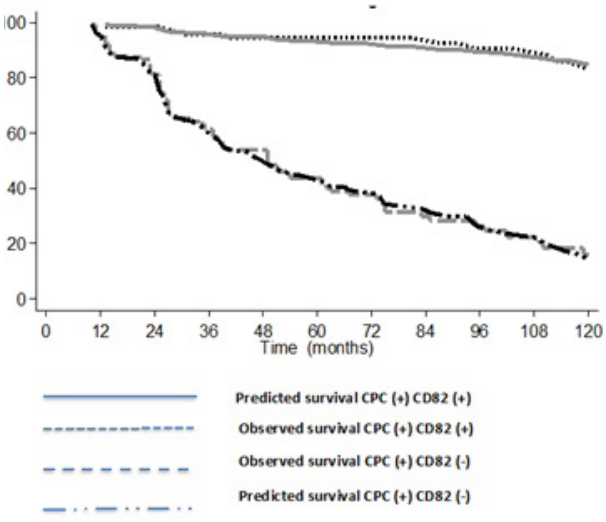

Figure 3. Biochemical Failure Free Progression at Ten Years: Comparing "Predicted Survival" (according to the model of Cox) versus "Observed Survival" (model Kaplan Meier) in 285 men treated by radical prostatectomy. CPC = ≥ 1 circulating prostate cell/ sample detected prior to surgery.

to the model of Cox) versus observed survival (model Kaplan-Meier) showed agreement for the comparison of men CPC (+) CD82 (+) versus men CPC (+) CD82 (-) (Figure 3). Likewise, the confidence intervals for Kaplan-Meier survival curves for the predictors contain the survivals predicted by their respective models cox. The cumulative hazard of Cox-Snell residuals for both models built, showed adequate a goodness of fit. This is summarized in Table 2.

Discussion

There are a number of techniques that have been developed for the detection of circulating tumour cells, which has hindered the comparison of different studies and the consensus of defining these cells. Each method has differing advantages and disadvantages and has been extensively reviewed (Harouaka et al., 2014; van der Toom et al., 2016). This may be due to several reasons; firstly the use of the CPC detection system. Studies using the CellSearch® EpCAM based system have detected the presence of CPCs in between 11-25% of men with pathologically localized prostate cancer (Davis et al., 2008; Eshwege et al., 2009; Meyer et al 2016), whereas a telomerase based method detected CPCs in 80% of cases (Fizazi et al., 2007). Using the ISET (isolation by size of epithelial tumour) system (ISET Rarecells) circulating tumour cells were detected in all cancer patients and in 50% of patients with a normal prostate specific antigen and a positive CTC had cancer detected using PMSA PET scans (Ried et al., 2017). Using a PSA immunocytochemical based method, with double immune-marcation, CPCs were detected in 80% of men with prostate cancer (Murray et al., 2016a).

We used double immune-marcation in our study, firstly to identify patients with malignant CPCs, that is expressing both PSA and P504S, PSA expressing cells can be detected in patients with benign prostate disease, but these cells do not express P504S (Murray et al., 2013). Men positive for this test then underwent testing for cells expressing PSA and CD82. Immunofluorescence is used in some methods to detect positive cells, though there are no studies comparing detection methods.

For a cancer to be able to produce metastasis, the cancer cells have to disseminate from the primary tumour, survive in the circulation and to able to adhere to the vascular endothelium at a distant site before invading the distant tissue. If not all CPCs are able to adhere and invade distant sites, then complete tumour removal at the time of surgery would imply curative therapy and in the clinical would be seen as better survival rates. Recent studies have shown that the mere presence of primary CPCs is not a good prognostic factor for biochemical failure free survival (Meyer et al., 2016; Murray et al., 2016; Murray et al., 2016b). The detection of circulating tumour cells using combined nested reverse transcriptase polymerase chain reaction pre-radical prostatectomy also failed to predict biochemical failure (Thomas et al, 2002).

It has been suggested that the mere enumeration of CPCs may not be ideal and that the phenotypic characteristics of these cells is more important. We chose the co-expression of CD82 as a potential marker as it has been reported to be inversely associated with survival in differing cancers. Low expression of CD82 was negatively correlated with overall survival in colorectal cancer (Wu et al., 2015) and breast cancer (Singh R et al, 2016). However in non-metastatic prostate cancer there is little reported data.

We report that men primary CPC negative had a 10-year biochemical failure free survival of 90%. These men had, in the majority of cases, low-grade (95% having a Gleason score of ≤ 6) small volume tumours (87% T1 tumours) and only 7% having positive surgical margins or seminal vesicle infiltration. This group for the clinical-pathological findings would be expected to have the best survival rates.

Men in Group 3, CPC (+) CD82 (-), had the worst biochemical failure free survival rate, 16% at 10 years, which is consistent with the clinical-pathological findings. That is higher-grade tumours (58% Gleason score ≥ 7), larger tumours 84% pT2 or pT3 and extension outside of the prostate as evidenced by 43% having positive margins, infiltration of the seminal vesicles or lymph nodes.

Group 2 men with CD82 (+) CPCs have a much better biochemical failure free survival similar to CPC (-) men at 5 years (93% versus 96% respectively, and 69% at 10 years. The clinical-pathological features were similar to those found in Group 1 except for a higher frequency of pT2 tumours.

This results support the hypothesis that not all primary CPCs and by inference circulating tumour cells imply a worse prognosis. Those men with CD82 expressing primary CPCs had a prognosis similar to that of men CPC negative.

CD82 is a tumour suppressor gene product; this group of suppressor gene products are primarily operationally defined and rarely mechanistically understood (Zijlstra et al., 2006). CD82 acts by various mechanisms; it has been proposed that CD82 acts with other trans-membrane proteins, especially EW12/PGRL a cell adhesion molecule, to inhibit cell motility Zhang et al., (2003) and also may regulate the intra-cellular signalling pathways that are linked to its associated trans-membrane proteins to suppress cancer dissemination. CD82-Epithelial Growth Factor (EGF) receptor coupling down-regulates EGF receptor mediated signalling by accelerating EGF receptor endocytosis and subsequently inhibits cell migration (Odintsova et al, 2000). More recently it has been reported that CD82 regulates cell migration and invasion by inhibiting the Tumour Growth Factor-ß1/Smad signalling pathway (Zhu et al., 2017) and by down-regulating matrix metalloproteinase 2 expression, important in tumour cell dissemination (Zhu et al., 2017).

If this were the only mechanism, it would not explain the presence of CPCs CD82 (+) in the circulation. The fact that these patients had larger tumours may in part explain why these CPCs had entered the circulation. P504S negative CPCs, defined as benign CPCs may be found in patients with prostatic hyperplasia or chronic prostatitis (Murray et al., 2013), it has been suggested that distortion of the normal architecture or inflammatory cytokines are responsible for this dissemination of

"normal" cells (Coussens and Werb, 2002). Thus these CD82 (+) CPCs may enter the circulation as a result of the distorted architecture of a larger primary tumour or associated inflammatory changes. One limitation of this study was that CD82 expression was not determined in the primary tumour.

Once in the circulation the CPCs have to adhere to the vascular endothelial in order to implant in distant tissues. The trans-membrane protein DARC is a specific CD82 interacting cytokine decoy receptor, which is limited to select cell types, notably endothelial cells. There is a direct interaction between CD82 expressing prostate tumour cells and DARC expressing endothelial cells which leads to suppression of proliferation and induction of senescence in CD82 expressing cells (Bandyopadhay et al., 2006). In a mouse model tumour cells that lacked CD82 expression metastasized equally well in wild type and DARC -/- mice. However tumour cell CD82 expression dramatically suppressed spontaneous and experimental metastasis in wild type but not DARC -/- mice (27). CD82 expression did not lead to reduced primary tumour size, possibly because of limited direct contact between CD82 positive tumour cells and DARC positive vascular cells. The metastasis senescence appears to be due to CD82-DARC interactions occurring in the circulation, that direct physical contact between endothelial cells and circulating tumour cells can control the survival of these disseminating tumour cells (Bandyopadhay et al., 2006). This would also explain why CD82 positive bone marrow micrometastasis are rarely found (Murray et al., 2012). With time and progression CD82 expression may be lost permitting systemic rather than local metastasis.

Conclusions, the expression of CD82 on primary CPCs is associated with a good prognosis, similar to that of men CPC negative. Although CD82 expression does not affect dissemination of primary CPCs to the circulation, possibly as a result of distorted architecture, its expression decreases tumour cell-endothelial cell binding and thus prevents metastatic implantation. Thus the biological characteristics of primary CPCs appears to be more important than their mere enumeration, and would explain would as a prognostic factor the presence of primary CPCs has limited importance. The expression of CD82 in primary CPCs is a good prognostic factor and thus should not impede curative treatment or warrant adjuvant therapy, whereas patients CPC positive CD82 (negative) may require additional treatment to prevent future therapy failure.

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Conflict of Interests

Dr. Murray has received consultancy fees from Viatar CTC Solutions, Boston, USA.

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