DETECTION AND CHARACTERISTICS OF PRIMARY CIRCULATING PROSTATE
CELLS; ASSOCIATION WITH MICROMETASTASIS AND IMPLICATIONS FOR
SURGICAL TREATMENT OF MEN WITH PROSTATE CÁNCER

Nigel P. Murray¹,²,³, Eduardo Reyes⁵, Leonardo Badínez⁴, Nelson Orellana¹, Ricardo Dueñas¹ and Cinthia Fuentealba⁵.

¹Hospital de Carabineros de Chile. Santiago de Chile.  
²Instituto de BioOncología, Santiago de Chile.  
³Facultad de Medicina. Universidad Mayor. Santiago de Chile.  
⁴Fundación Arturo López Pérez, Santiago de Chile.  
⁵Becado Urología Universidad de Santiago. Santiago de Chile.

Summary.- OBJECTIVES: To determine the frequency of primary circulating prostate cells in men with prostate cancer at the time of diagnosis, the association with micrometastasis, sub-classification for CD82 and the relation with pathological stage. To determine their clinical usefulness to identify patients in whom radical prostatectomy would be first choice therapy.

METHODS: Men with the diagnosis of prostate cancer before definitive therapy. Blood and bone marrow samples were taken, mononuclear cells separated by differential centrifugation and prostate cells identified with immunocytochemistry using anti-PSA. Positive samples were sub-classified with anti-CD82. Details of serum PSA, Gleason score and pathological stage were registered.

RESULTS: Of 77 men 58 (75.3%) had primary CPCs detected, there was an association with stage but not Gleason. 31 (40.3%) had micrometastasis with an association with stage and Gleason score. CPC-negative patients had fewer micrometastasis detected, 1/19 versus 30/58 (p<0.003).

There was an inverse relation between CD82 expression and Gleason score, men with CPCs expressing CD82 had fewer micrometastasis. The combined group of CPC negative and CPC positive CD82 positive men showed a sensitivity of 87% and specificity of 73.9% for the absence of micrometastasis.

CONCLUSIONS: The detection of CPCs and sub-classification with CD82 could be clinically useful to identify men with a significantly lower risk of micrometastasis and as a consequence to identify men in whom radical prostatectomy could be the best initial treatment.

Keywords: Prostate cancer. Circulating tumor cells. Micrometastasis. CD82.

Resumen.- OBJETIVO: Determinar la frecuencia de células prostáticas circulantes (CPCs) primaria en hombres con cáncer de próstata en el momento del diagnóstico,
la asociación con micrometástasis ósea y subclasifica-
ción por CD82. Determinar la relación con la estadía
patológica y la eficacia para seleccionar pacientes
para la prostatectomía radical.

MÉTODOS: Se incluyeron hombres con diagnóstico de
cáncer de próstata previo a tratamiento definitivo. Se
obtuvieron muestras de sangre y de médula ósea, las
células mononucleares separadas por centrifugación di-
ferencial y células prostáticas identificadas con inmun-
citoquímica con anti-APE, las muestras positivas fueron
sub-clasificadas con anti-CD82. Se registraron también
los detalles de APE sérico, Índice de Gleason y estadía
patológica.

RESULTADOS: De 77 hombres, 58 (75.3%) tuvieron 1º
CPCs detectadas. Hubo una asociación con estadío
pero no con el Índice de Gleason, 31 (40.3%) tuvieron
micrometástasis. Hubo una asociación significativa con
la estadía patológica y Índice de Gleason. Pacientes
CPC negativa tuvieron una menor frecuencia de micro-
metástasis que los hombres CPC positiva 1/19 versus
30/58 (p<0.0003).

Hubo una relación inversa significativa entre la expresi-
ón de CD82 en CPCs y el índice de Gleason y me-
nor frecuencia de micrometástasis en comparación con
hombres CPC CD82 positivos (p<0.0005).

En el grupo de combinación de hombres CPC negativa
y CPC positiva CD82 positivo la frecuencia de microme-
tastasis fue significativamente menor que el grupo CPC
(+) CD82 (-) 5/39 versus 26/38 respectivamente(p<0
.0000007), con una sensibilidad de 87% y especifici-
dad de 73.9% para la ausencia de micrometastasis.

CONCLUSIONES: La presencia de CPCs implica un
riesgo mayor de desarrollar micrometastasis, la co-
expresión del CD82 es asociada con tumores de bajo
grado, un riesgo disminuido del desarrollo de microme-
tastasis óseas. Como consecuencia, el uso de la detec-
ción de CPCs primarias y su sub-clasificación podrían
ser clínicamente útiles para identificar a los pacientes
los cuales beneficiarán de una prostatectomía radical como
tratamiento de primera línea.

Palabras clave: Cáncer de próstata. Células
prostáticas circulantes. Micrometástasis. CD82.

INTRODUCTION

The combination of serum PSA and rectal
examination are recommended for the early diagnosis
of prostate cancer (1). This has a meant an increasing
number of patients are diagnosed with early stage
localized disease, for which radical prostatectomy
is considered standard treatment (2,3). However,
post surgical pathological findings show that the propor-
tion of cancers potentially cured by surgery
alone are in the range of 40-70% of cases (4) and in
addition 30-60% are pT3 tumours (5), and 20-70%
have positive surgical margins (6). Pathological stage
and positive surgical margins are the 2 factors that
directly influence survival free of biochemical failure
and local and systemic relapse (7).

Despite these limitations as predictors of
pathological stage, Partin et al. (8), together with
clinical stage, Gleason score as a 4 parameter
combination to produce a nomogram in order to
predict the pathological stage. However, inspite
of these methods approximately half the patients
with prostate cancer are understaged (5), which
implies that between 17-70% have positive margins
or extra-capsular extension (6), which are factors
directing affecting outcome and relapse (7). The
high percentage of under staged patients and with
positive margins is one of the reasons that surgery
alone is being considered less as the only treatment
option and motivated methods to improve surgical
cure (9).

The detection of circulating prostate cells
(CPCs) and their role in the development of metastasis
is still controversial. Preliminary reports suggest their
use in orientating treatment options (10). Studies
published in Chile have detected P504S expressing
CPCs in early stage prostate cancer, as well as the
relation between their presence and the presence of
bone marrow micrometastasis with the established
risk factors of cancer dissemination (11,12).

There are 2 types of CPCs; primary CPCs
detected at the time of diagnosis and secondary CPCs
detected after radical treatment. The characteristics of
1º CPCs as well as their presence or absence could
be important in order to identify localized cancer,
where a purely surgical treatment is recommended or
advanced with bone marrow micrometastasis where
a combined therapy would be indicated.

The KA11 tumor suppressor gene is located
in the p11.2 region of chromosome 11 (13) and
codes for a glycoprotein of the tetraspanin family,
CD82. Benign prostate tissue, including benign
hyperplasia express CD82 (13), the expression
increases in benign hyperplasia associated with
low grade prostate cancer, as well as in low grade
prostate cancer with a Gleason score of 3 or 4 (14).
However, its expression is decreased or absent in
high grade cancer and metastasis15. It is postulated
that CD82 acts via protein kinase C, which plays a
crucial role in the cell cycle, migration and invasion
(15).
PRESENTATION

We present a study of the detection of 1° CPCs, the sub-classification with CD82, the relation with the presence of bone marrow micrometastasis and pathological stage in men with prostate cancer.

PATIENTS AND METHODS

A prospective study of all patients diagnosed with prostate cancer during 2008 at the Hospital de Carabineros de Chile and the Instituto de Bio-Oncología Santiago, Chile.

A total of 77 patients with confirmed prostate cancer participated, along with 10 women with hematological disorder acting as controls. Details of age, serum PSA, Gleason score, pathological stage after surgery were registered.

After written informed consent blood and bone marrow samples were obtained;

Sample preparation:

a) blood:

After written informed consent a 4ml blood sample was collected into EDTA (Beckinson-Vacutainer®). The sample was layered onto 2ml Histopaque 1.077® (Sigma-Aldrich) at room temperature, and the mononuclear cells obtained according to manufacturer’s instructions and finally washed 3 times in phosphate buffered saline pH 7.4 (PBS). The pellet was resuspended in 100μl of autologous plasma and 25μl used to prepare each slide (sialinzed DAKO, USA). The slides were air dried for 24 hours and finally fixed in a solution of 70% ethanol, 5% formaldehyde and 25% PBS for 5 minutes and then washed 3 times with PBS.
b) Bone marrow:
Bone marrow biopsy samples were obtained from the posterior superior iliac crest and used to make 3 "touch-preps" using sialinized slides (DAKO, USA) and both types of samples were fixed as previously described. A 4ml bone marrow aspiration was taken at the same location and processed as described for blood samples.

Immunocytochemistry:
Monoclonal antibodies directed against PSA clone 28A4 (Novacastro, UK) in a concentration of 2,5 μg/ml were used to detect prostate cells, and identified using a detection system based on alkaline phosphatase-antialkaline phosphatase (LSAB2 DAKO, USA) with new-fuschin as the chromogen. To permit the rapid identification of positive cells there was no counter staining with Mayer’s hematoxilin. Levisamole (DAKO, USA) was used as an inhibitor of endogenous alkaline phosphatase, with positive and negative controls. Positive samples underwent a second stage of processing, using the monoclonal antibody against CD82 clone 5B5 (Novocastro, UK) and a system of detection based on peroxidase (LSAB2, DAKO, USA) with Vector VIP (Vector, USA) as the chromogen. Endogenous peroxidase was inhibited (DAKO, USA).

Definition of positive samples:
The definition of as circulating prostate cell (CPC) was based on the criteria of ISHAGE 1999, la morphology of a cell with a nucleus positive for PSA. A micrometastasis was defined as bone marrow fragments positive for cells immunostaining positive for PSA (17).

The intensity of CD82 expression was classified using a scale of 0 to +++, with the same definition as the Herceptest®, to give a mean score of 0 to 3.

Statistical analysis:
Descriptive statistics were used to analyze the demographic variables, Student T-Test for the differences of means between groups and Chi-squared for differences in frequency. All tests were 2 tailed. An alpha error of 0.05, a beta error of 0.20 and p<0.05 was considered as significant. The program EpInfo for Windows 98 was used.

Ethical considerations:
The study was directed with complete conformity with the principles of the declaration of Helsinki and approval of the local ethical committee.

RESULTS
During the study period 77 fulfilled the entrance criteria, with a mean age of 68.4 +/- SD 9.7 years and a mean serum PSA of 11.27ng/ml (range 1.5-85ng/ml).

Of the 77 patients, 1° CPCs were detected in 58 (75.3%), the detection frequency in relation to pathological stage and Gleason score are shown in Tables I and II. There were no significant differences of 1° CPC detection frequency with Gleason score, but stage pT2 patients had a frequency of 1° CPC detection significantly less than patients with pT3
DETECTION AND CHARACTERISTICS OF PRIMARY CIRCULATING PROSTATE CELLS

29/42 (69.0%) versus 25/27 (92.5%) respectively (p<0.02 Chi squared 2 tailed).

Of the 77 patients, 39 (50.6%) had a positive bone marrow aspiration and 31 (40.3%) were bone marrow micrometastasis positive (Table III and IV). The presence of prostate cells in the bone marrow aspiration (DTCs) and micrometastasis was associated with pathological stage pT2 versus pT3 for DTCs 15/42 (35.7%) versus 19/27 (70.4%) (p<0.005 Chi squared 2 tailed) and for micrometastasis 10/42 (23.4%) versus 17/27 (63.0%) respectively (p<0.001 Chi squared 2 tailed). There was no significant difference between the frequency of DTC and micrometastasis detection in the same patient with reference to pT2 (p=0.23) or pT3 (p=0.56).

There was no relation between Gleason score and the presence of DTCs in the bone marrow aspirate (Table IV) but there was a significant difference in relation to the presence of bone marrow micrometastasis. Patients with a Gleason score of 4 or 5 had a frequency of micrometastasis significantly lower than those with higher Gleason scores (Table II).

<table>
<thead>
<tr>
<th>Stage</th>
<th>1° CPC positive</th>
<th>1° CPC negative</th>
<th>Total</th>
</tr>
</thead>
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<tr>
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<td>0</td>
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<tr>
<td>Stage 2</td>
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</tr>
<tr>
<td>Stage 3</td>
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<td>Stage 4</td>
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<tr>
<td>Total</td>
<td>58</td>
<td>19</td>
<td>77</td>
</tr>
</tbody>
</table>

Stage 1+2 vs Stage 3+4 p=0.002 (Chi-squared 2 tailed)
Stage 2 vs Stage 3 p=0.02 (Chi-squared 2 tailed)

<table>
<thead>
<tr>
<th>Gleason</th>
<th>1° CPC positive</th>
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<th>Total</th>
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<tr>
<td>Gleason 7</td>
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</tr>
<tr>
<td>Gleason 8 y 9</td>
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</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>19</td>
<td>77</td>
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</table>

Gleason 4 vs 5 p=0.87  Gleason 4 vs 6 p=0.75  Gleason 4 vs Gleason 7,8,9 p=0.71
IV). In patients with Gleason 4, the frequency of micrometastasis detected was significantly lower than for DTCs (p<0.027, Chi squared 2 tailed).

Patients CPC negative were significantly less likely to be micrometastasis positive, 1/19 versus 30/58 (p<0.0003 Chi squared 2 tailed).

<table>
<thead>
<tr>
<th>Aspirate positive</th>
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<th>Biopsy positive</th>
<th>Biopsy negative</th>
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<tr>
<td>2</td>
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<tr>
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<td>32</td>
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<tr>
<td>Stage 3</td>
<td>19</td>
<td>8</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>Stage 4</td>
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<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>37</td>
<td>31</td>
<td>46</td>
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</tbody>
</table>

Stage 1 + 2 versus Stage 3 + 4: aspiration p < 0.001 (Chi squared 2 tailed). Biopsy: p < 0.00005 (Chi squared 2 tailed)
Stage 2 versus Stage 3: aspiration p<0.005 (Chi squared 2 tailed), biopsy p<0.001 (Chi squared 2 tailed).
Aspiration versus biopsy: stage 2 p=0.23  stage 3 p=0.56

Sub-classification with CD82:

All patients with Gleason 4 prostate cancer had CPCs which expressed CD82, CPCs were CD82 positive in 25% (4/15) patients with Gleason 5. Patients with Gleason 6 or higher had CPCs negative for CD82. Patients classified as CD82 positive had

<table>
<thead>
<tr>
<th>Aspirate positive</th>
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<th>Biopsy positive</th>
<th>Biopsy negative</th>
<th>Total</th>
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<tr>
<td>Gleason 4</td>
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<tr>
<td>8</td>
<td>11</td>
<td>2</td>
<td>17</td>
<td>19*</td>
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<tr>
<td>8</td>
<td>13</td>
<td>4</td>
<td>17</td>
<td>21**</td>
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<td>Gleason 6</td>
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<tr>
<td>17</td>
<td>10</td>
<td>17</td>
<td>10</td>
<td>27***</td>
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<td>2</td>
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<td>4</td>
<td>2</td>
<td>6***</td>
</tr>
<tr>
<td>Gleason 8 +9</td>
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<td>0</td>
<td>4</td>
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<td>4***</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>37</td>
<td>31</td>
<td>46</td>
</tr>
</tbody>
</table>

Gleason 4 versus 5: aspirate p=0.80  biopsy p=0.45
Gleason 4 versus 6: aspirate p=0.16  biopsy p<0.0005
Gleason 4 versus 7/8/9: aspirate p=0.36  biopsy p<0.0001
Gleason 5 versus 6: biopsy p<0.002
Gleason 5 versus 7/8/9: biopsy p<0.002 (Fisher, 2 colas)
aspirate versus biopsy: *p<0.027  **p=0.17  ***p=NS

TABLE III. FREQUENCY OF PROSTATE CELLS DETECTED IN BONE MARROW ACCORDING TO STAGE.

TABLE IV. FREQUENCY OF PROSTATE CELLS IN BONE MARROW ACCORDING TO GLEASON SCORE.
a lower frequency of micrometastasis detected in comparison with patients with CPCs CD82 negative (p<0.0005 Chi squared 2 tailed), 4/20 versus 26/38 respectively (Table IV). Combining the groups CPC negative with CPC (+) CD82 (+) and comparing with the group CPC (+) CD82 (−) the frequency of detected micrometastasis was 5/39 versus 26/38 (p<0.0001 Chi squared 2 tailed).

Comparing the 3 groups in order to predict the absence of micrometastasis and therefore a candidate for curative surgery using CPC detection and CD82 sub-classification; patients negative for CPCs were negative for micrometastasis with a sensibility of 94.7% and specificity of 39.1%, patients CPC (+) CD82 (+) had a sensibility of 80.0% and specificity of 57.0%. Combining the 2 groups there was a sensibility of 87.0% and specificity of 73.9%.

**DISCUSSION**

The results of the study ProTECT (Prostate Tumor Early Cancer Test) suggest that at the time of diagnosis it is possible to predict which patients are likely to be micrometastasis negative with a sensibility of 87% and specificity of 74%, independent of stage, serum PSA or Gleason stage. Not all patients 1° CPC positive will have micrometastasis, but the use of

<table>
<thead>
<tr>
<th>N° patients</th>
<th>CPC negative</th>
<th>CPC positive</th>
<th>CPC positive</th>
<th>Total</th>
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<tr>
<td></td>
<td>CD82 negative</td>
<td>CD82 positive</td>
<td>CD82 positive</td>
<td></td>
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<tr>
<td>N° patients</td>
<td>19</td>
<td>39</td>
<td>19</td>
<td>77</td>
</tr>
<tr>
<td>Mean age</td>
<td>68,9 +/- 5,8 years</td>
<td>64,7 +/- 9,9 years</td>
<td>75,3 +/- 8,5 years</td>
<td>68,4 +/- 9,7 years</td>
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<tr>
<td>PSA at time of biopsy</td>
<td>5,83 +/- 2,67 ng/ml</td>
<td>12,93 +/- 11,6 ng/ml</td>
<td>12,41 +/- 12,0 ng/ml</td>
<td>11,27 +/- 10,61 ng/ml</td>
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<td>42</td>
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<tr>
<td>pT3</td>
<td>2</td>
<td>23</td>
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<td>Gleason score</td>
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<td>7</td>
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<td>8 y 9</td>
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<td>0</td>
<td>4</td>
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<tr>
<td>Micrometastasis Positive</td>
<td>1 (5,3%)</td>
<td>26 (66,7%)</td>
<td>4 (20%)</td>
<td>31 (40,3%)</td>
</tr>
<tr>
<td>Relative risk (RR)</td>
<td>1,00</td>
<td>4,80</td>
<td>36,00</td>
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<td>N° of cells detected /ml blood</td>
<td>8 +/- 7 cells/ml</td>
<td>7 +/- 6 cells/ml</td>
<td>8 +/- 7 cells/ml</td>
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</tbody>
</table>
CD82 permits the identification of patients less likely to have bone marrow micrometastasis.

The present study is the first, as far as we know, to report the expression of CD82 in CPCs, and that there is an association with Gleason score and clinical stage as reported in studies using prostate specimens (14,15). However, the findings in this study are different; it has been previously reported that CD82 is thought to inhibit metastasis. We have shown that the expression of CD82 is not sufficient to inhibit dissemination of tumor cells, but appears to inhibit implantation in bone marrow.

Katz et al. (16) have proposed the concept of potential surgical failure, as the presence of positive surgical margins, invasion of the seminal vesicles or lymph nodes, in order to differentiate the patient from those with organ confined disease. However, this definition does not include the group of patients with unfavorable pathology or capsular invasion with negative margins. It has been reported that this latter group have an evolution more favorable than those patients with positive surgical margins (17). In clinically localized cancer the most important predictive factors of extracapsular extension are; serum PSA, Gleason score and clinical stage. Serum PSA has been correlated with tumor volume and consequently with capsular invasion and positive margins. The use of CPC detection and subclassification with CD82 is independent of the other factors and predicts the presence or absence of micrometastasis, it could be utilized in patients to determine those who would most benefit from surgical treatment.

CONCLUSIONS

We propose that:

1) The presence of CPCs implies a greater risk of developing micrometastasis.

2) The co-expression of CD82 is associated with low grade tumors in older men and a decreased risk of bony micrometastasis.

3) Therefore, the use of detecting CPCs and subclassification could be clinically useful to identify patients in whom radical prostatectomy would be the treatment of choice.

4) Patients with CPCs CD82 negative should be evaluated for the presence of micrometastasis and the need for adyuvant therapy.

5) It is a blood test, which is simple non-invasive and could be implemented in the routine laboratory.

The preliminary findings of the ProTECT study warrant further larger studies to confirm the findings.

ACKNOWLEDGMENTS

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REFERENCES AND RECOMMENDED READINGS
(*of special interest, **of outstanding interest)

