TURKISH JOURNAL OF UROLOGY
Turk J Urol 2020; 46(3): 186-95 • DOI: 10.5152/tud.2020.19219
UROONCOLOGY
Original Article

The expression of matrix-metalloproteinase-2 in bone marrow micro-metastasis is associated with the presence of circulating prostate cells and a worse prognosis in men treated with radical prostatectomy for prostate cancer

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Cite this article as: Murray NP, Reyes E, Salazar A, Lopez MA, Orrego S, Guzman E. The expression of matrix-metalloproteinase-2 in bone marrow micro-metastasis is associated with the presence of circulating prostate cells and a worse prognosis in men treated with radical prostatectomy for prostate cancer. Turk J Urol 2020; 46(3): 186-95.

ABSTRACT

Objective: The expression of matrix-metalloproteinase-2 (MMP-2) in the primary tumor is associated with a worse prognosis but little is known at this time regarding the expression in micro-metastasis, the association with circulating prostate cells (CPCs), and outcome.

Material and methods: This was a prospective study of men undergoing radical prostatectomy. Bone marrow and blood samples were taken at one month after surgery. Micro-metastasis and CPCs were identified using immunocytochemistry with anti-prostate specific–antigen and MMP-2 expression determined with anti–MMP-2. Pathological stage, Gleason score, and time to biochemical failure were recorded; meanwhile, Kaplan–Meier biochemical failure–free survival and restricted mean biochemical failure–free survival times for 10 years were determined.

Results: A total of 282 men participated, 54 (19%) of whom had micro-metastasis but not CPCs (group B) and 88 (31%) of whom had micro-metastasis and CPCs (group C). Men in group C had a higher frequency of MMP-2 expressing micro-metastasis at 63% versus 12% ($p<0.001$), and MMP-2 expression in bone marrow micro-metastasis was associated with a higher Gleason score ($p<0.05$) as well as a higher frequency of and shorter time to treatment failure. Also, a 10-year Kaplan–Meier biochemical failure–free survival rate of 0% versus 7.7% (MMP-2 positive versus negative) and a mean time to biochemical failure of 2.6 versus 4.0 years were recorded.

Conclusion: The expression of MMP-2 in bone marrow micro-metastasis is associated with a higher Gleason score, the presence of CPCs, and a higher frequency of and shorter time to failure and could be clinically useful for identifying men at high risk of treatment failure.

Keywords: Biochemical failure; circulating prostate cells; matrix-metalloproteinase-2; micro-metastasis; prostate cancer.

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Submitted:
09.11.2019

Accepted:
21.01.2020

Available Online Date:
11.03.2020

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Available online at
www.turkishjournalofurology.com

Introduction

The presence of metastatic disease will ultimately determine the prostate cancer-specific mortality of patients treated with radical prostatectomy for prostate cancer. The dissemination of tumor cells into the circulation is an early event in the disease process.[1] Few of these circulating prostate cells (CPCs) will survive[2] but those that do will promote micro-metastasis outside the surgical field of the radical prostatectomy. The metalloproteinases are a group of endopeptidases capable of degrading the extracellular matrix and which have an important role in cancer dissemination and the liberation of growth factors.[3] Matrix-metalloproteinase-2 (MMP-2) is a gelatinase and its expression in prostate tissue samples has been reported to be increased among patients with prostate cancer. Its expression is associated with higher-stage prostate cancer, with higher Gleason scores, and as an independent prognostic factor for biochemical failure.[4,5] It is thought to be essential for the active dissemination of tumor cells into the circulation, permitting

tumor cell extravasation through the basement membrane into the circulation.[6] Passive entry into the circulation by cancer cells such as after biopsy does not require MMP-2.[7] These circulating prostate cancer cells continue to express MMP-2 and, finally, home in on the bone marrow, implanting in the premetastatic niche. Here, they interact with bone marrow stromal cells, which have an important role in determining tumor cell behaviors.[8] The majority of bone marrow micro-metastasis in patients with nonmetastatic prostate cancer do not express MMP-2; however, with disease progression, the micro-metastasis may re-express MMP-2.[9] Two subtypes of minimal residual disease (MRD) have been described in non-metastatic prostate cancer, with differing patterns of relapse.[10] Patients positive for the presence of CPCs, independent of whether bone marrow micro-metastasis was present, have a higher risk of early failure, whereas patients only positive for bone marrow micro-metastasis and who are CPC-negative had a higher risk of late failure.[10,11]

We hypothesize that the expression of MMP-2 in bone marrow micro-metastasis permits the dissemination of prostate cancer cells to the circulation (CPCs); these secondary CPCs detected after curative therapy may implant in distant sites and form new micro-metastasis and represent a sign of disease progression. The aim of this study was to determine the expression of MMP-2 in bone marrow micro-metastasis, the association with the presence of CPCs, and outcomes in prostate cancer patients treated with radical prostatectomy as monotherapy.

Main Points:

- Minimal residual disease is the presence of microscopic foci of cancer cells present in an asymptomatic patient after curative treatment. They are not detected by routine tests but with time they may proliferate and cause relapse. There are at least two sub-types; the presence of circulating prostate cells is associated with high risk of early relapse and those with only bone marrow micro-metastasis with late relapse after a mean period of nine years.
- Matrix metalloproteinase-2 (MMP-2) expression permits the dissemination of cancer cells into the circulation, after implanting in bone marrow the majority of these cancer cells do not express MMP-2. Men with MMP-2 expressing micro-metastasis had a very high risk of treatment failure, with shorter times to relapse as compared with men with MMP-2 negative micro-metastasis and had higher numbers of circulating prostate cells detected. This suggests that MMP-2 causes active dissemination of tumour cells from micro-metastasis and represents a more aggressive disease phase. This sub-classification identifies a population with "good risk" Gleason 7 and "poor risk" Gleason 6 patients, this heterogeneity of the biological characteristics of micro-metastasis explains in part the differing results of the presence of micro-metastasis and metastatic behaviour.

Material and methods

This was a prospective, observational, single-center study of men who, between 2000 and 2010, underwent radical prostatectomy monotherapy for prostate cancer. All men with pT2 or pT3 prostate cancer treated with radical prostatectomy were invited to participate in the study. Patients were excluded if the prostatectomy specimen had positive surgical margins, if the patient was to be treated with adjuvant radiotherapy or androgen blockade, or the patient had a positive bone scan. All men had a nadir prostate-specific antigen (PSA) level postsurgery of less than 0.01 ng/mL. The TNM system of the American Joint Committee on Cancer was used to pathologically stage the patients.[12]

Patients were followed up with serial total PSA levels every 3 months for the first year and then every 6 months thereafter. Biochemical failure was defined as a serum PSA level of more than 0.20 ng/mL on two separate occasions. Biochemical failure–free survival time was defined as the time from surgery to the time of reaching a postsurgery PSA level of more than 0.20 ng/mL or time to the last follow-up date.

a) Detection of secondary circulating prostate cells

One-month postsurgery, an 8 mL venous blood sample was taken and collected in a tube containing EDTA (Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ, 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

CPCs were obtained from the mononuclear cell layer of gel differential centrifugation (Histopaque 1.077; Sigma-Aldrich, St. Louis, MO, USA), washed, and re-suspended in a 100-µL aliquot of autologous plasma. Next, 25-µL aliquots were used to make slides (silanized; Agilent Technologies, Santa Clara, CA, USA). The slides were air-dried, fixed, and finally washed 3 times in phosphate-buffered saline (pH 7.4).

Immunocytochemistry

Anti-PSA clone 28A4 (Novocastra Laboratory, Newcastle, UK), a combination alkaline-phosphatase and anti–alkaline-phosphatase system (LSAB2, DAKO, USA) with new fuchsin as the chromogen, was used to detect CPCs. Samples positive for PSA staining cells underwent a second process. Anti–MMP-2 clone 1B4 (Novacastra Laboratories, Newcastle, UK) and a peroxidase-based system (LSAB2; Agilent Technologies, Santa Clara, CA, USBADA) with 3,3 diaminobenzidine tetrahydrochloride as the chromogen was used to detect MMP-2 expression (Figure 1). A secondary CPC was defined according to the criteria of the International Society of Hemotherapy and Genetic Engineering.[13] A test was considered positive for secondary CPCs when at least 1 cell/8 mL of blood was detected (Figure 1).

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Definition of MMP-2 expression: The criteria used for defining a cell expressing MMP-2 were that described by Trudel et al.[5]: A patient was considered to be positive for MMP-2 if >10% of cells expressing PSA coexpressed MMP-2. However, 3 groups of MMP-2 expression were defined: MMP-2 negative, >0–<10% of cells expressing PSA coexpressed MMP-2, and at least 10% of PSA expressing cells coexpressed MMP-2. PSA expressing cells were additionally classified semi-quantitatively as having 0, +1, +2, and +3 intensity of immune staining for MMP-2. A mean MMP-2 score was calculated and defined as total MMP-2 expression/Nº of PSA expressing cells.

b) Bone marrow biopsy

The phenotypic analysis of tumor cells detected in bone marrow aspirates versus those detected in bone marrow biopsy "touch-preps" suggested that those tumor cells detected in aspirates may indicate "true" micro-metastasis but rather are circulating tumor cells detected in the bone marrow compartment.[14] Bone marrow "touch-preps" were used as the sample to test for micro-metastasis.

At the same time as the blood sampling to detect CPCs, a bone marrow biopsy was taken from the posterior superior iliac crest using sedation and local anesthetic in all patients. The bone marrow sample was used to prepare four "touch-preps" using silainized slides (Agilent Technologies, Santa Clara, CA, USA). Samples were processed in the same way as that used for CPCs (Figures 2 and 3). Cells expressing MMP-2 were also classified according to their location, central or periphery of the micro-metastasis, and also regarding whether stromal expression of MMP-2 was present (Figures 2-6).

Definition of MMP-2 expression

The criteria used for defining cell-expressing MMP-2 were that of Trudel et al.[5] similar to as detailed for CPCs.

Classification of patients according to Minimal Residual Disease

Patients were divided into 3 groups: group A was negative for both CPCs and micro-metastasis patients (without evidence of MRD); group B was negative for CPCs but positive for micro-metastasis considered as bony micro-metastasis without dissemination; group C was positive for CPCs and bone marrow micro-metastasis and considered as showing active dissemination from systemic micro-metastasis.

Study endpoint

The primary study endpoint was the presence of biochemical failure and the secondary endpoint was mean time to failure after primary treatment.

Statistical analysis

An analysis was performed using the program Stata (Stata/SE 15.0 for Windows; StataCorp LLC, College Station, TX, USA). Descriptive statistics of central tendency (mean and median) and dispersion (standard deviation and interquartile range) were used to describe patient groups; nominal variables were described as proportions with their respective confidence intervals.

The 3 MRD groups were compared in terms of Gleason score, pathological stage, and MMP-2 expression using the two-tailed chi-squared test for comparing frequencies. A p-value of less than 0.05 was taken to signify statistical significance and all tests were two-tailed.[15]

For each MRD group, a nonparametric biochemical failure–free survival analysis[15] was performed to establish the survival pro-

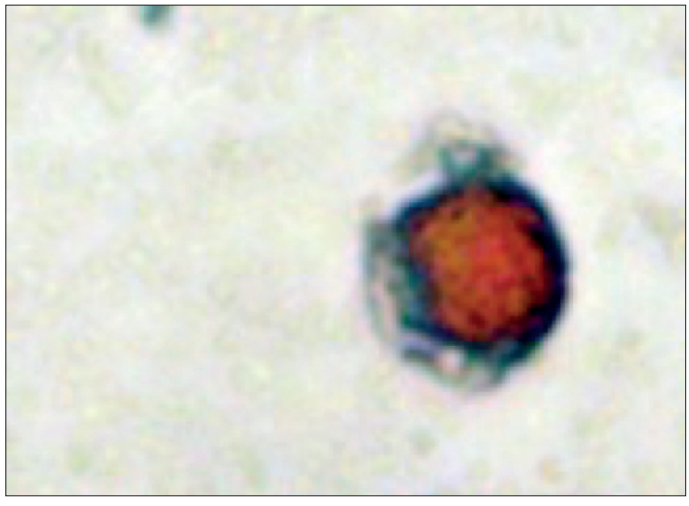

Figure 1. Circulating prostate cell detected in blood, expressing PSA (red) and membrane matrix metalloprtoeinase 2 (brown)

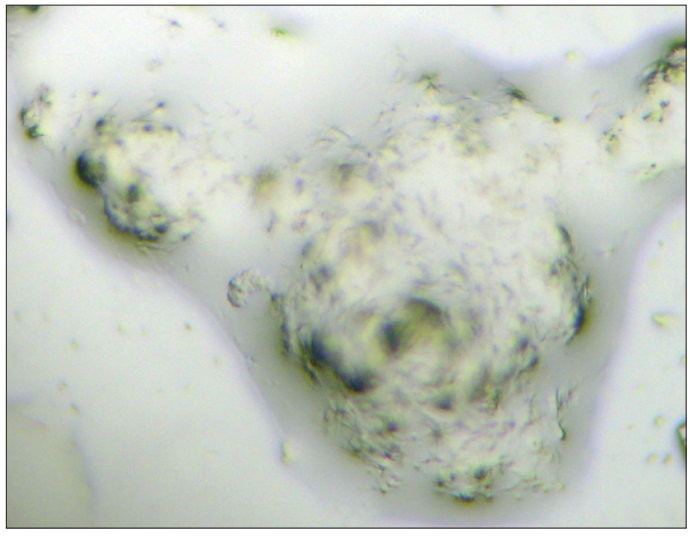

Figure 2. Bone marrow biopsy negative for micro-metastasis

portion of Kaplan–Meier and the restricted mean survival time (RMST) for biochemical failure during the 10-year follow-up.[15,16] The RMST establishes the expected time to biochemical failure during the total observation period and its value is the area under the Kaplan–Meier nonparametric survival curve.[15,16] A nonparametric comparison (log-rank test) of biochemical failure–free survival by MRD group was also performed [15,16]. The comparison between predicted biochemical failure–free survival (Cox regression) versus observed biochemical failure–free survival (Kaplan–Meier) was also performed[15-18] and the Harrell´s C discrimination index was calculated.[19] From the final Cox model for biochemical failure for 10 years, the survival proportion and RMST for biochemical failure were calculated for the 3 groups.[16,20] The same analysis was carried out within groups for patients who were MMP-2 positive and negative. A logistic regression curve analysis was conducted on the number of CPCs detected and the expression of MMP-2 in bone marrow micrometastasis to determine whether there was an association.

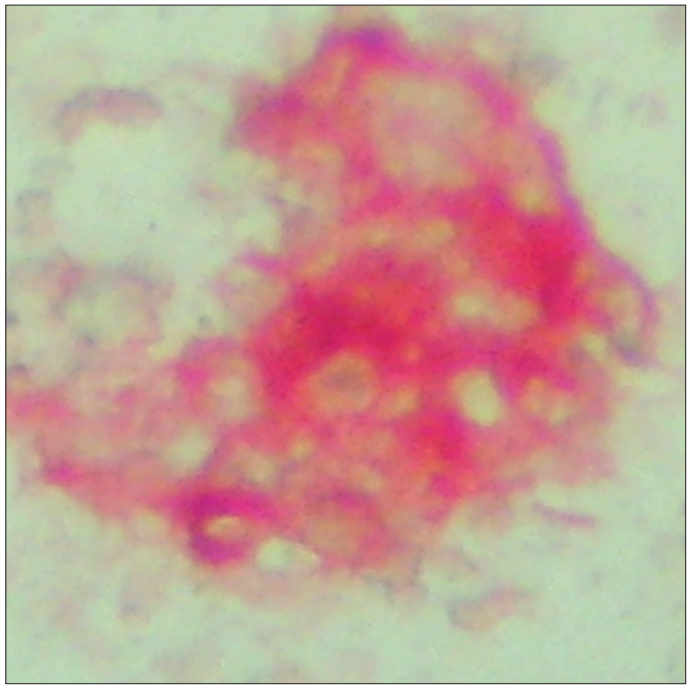

Figure 3. Micro-metastasis in bone marrow, expressing PSA (red) but negative for membrane matrix metalloproteinase 2

Ethical considerations

The present study was approved by the local ethics committee, Hospital de Carabineros de Chile, (Resolution 45-1/5/2008) and in complete agreement with the Declaration of Helsinki. All patients provided written informed consent.

Results

A total of 282 men with a mean age of 66.3±8.2 years and a median serum total PSA at the time of diagnosis of 5.51 ng/mL (interquartile range (IQR): 3.27-7.75 ng/mL) were included. The clinicopathological features are shown in Table 1. Patients with micro-metastasis and CPCs (group C) had a significantly higher PSA level at diagnosis, a higher pathological stage, higher Gleason score, and micro-metastasis that had a higher frequency of MMP-2 expression and higher MMP-2 expression score relative to those patients with no MRD detected or only with bone marrow micro-metastasis. The expression of MMP-2 was located at the edge of the micro-metastasis rather than in the center. All CPCs expressed MMP-2 with a 3+ intensity.

The Kaplan–Meier (observed) biochemical failure–free survival curves for the whole group at five and 10 years were 69.6%

Table 1. Clinicopathological features of the 3 prognostic groups of 282 men treated by radical prostatectomy

	Group A: absence CPC and mM N=140	Group B: absence CPC presence mM N= 54	Group C: presence CPC presence mM N=88	p-value two tailed
Age at diagnosis (years)	64.5±8.0	66.0±8.4	66.5±8.7	0.32[a]
PSA at diagnosis (ng/mL)	5.18 (IQR 1.25)	5.59 (IQR 2.33)	6.87 (IQR 2.62)	< 0.01[b]
pT2	114	39	26	<0.01[b]
pT3	26	15	62	
Gleason 6	123	44	36	<0.01[c]
Gleason 7	17	10	52	
MMP-2 (≥10%)	N/A	2 (4%)	18 (21%)	<0.01[c]
MMP-2 (≥ 0%-< 10%)	N/A	3 (6%)	38 (43%)	<0.001[c]
MMP-2 score		0.36±0.21	1.84±0.37	<0.01[b]

[a]one-way analysis of variance; [b]Kruskal–Wallis test; [c]chi-squared test. CPC: circulating prostate cell; mM: micro-metastasis; MMP-2: matrix metallopñroteinasew-2; N/A: not applicable

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[95% confidence interval (CI): 65.2%–74.3%] and 47.5% (95% CI: 40.7%–53.6%), respectively. The Kaplan–Meier (observed) and flexible parameter model (predicted) biochemical failure–free survival results for each group are shown in Table 2. The restricted mean biochemical failure–free survival times for up to 10 years of follow-up are also presented in Table 2.

There was agreement when comparing the predicted biochemical failure–free survival (model of Cox) with the observed biochemical failure–free survival (Kaplan–Meier), with a Harrell`s C discrimination index of 0.92 (classified as very good agreement). The Kaplan–Meier biochemical failure–free survival of 100% in groups A, B, and C were 2.17, 2.58, and 0.83 years, respectively, and there was no significant difference between groups A and B, but 100% of biochemical failure–free survival time was significantly shorter in group C patients when compared with in groups A and B. Figure 7 shows the biochemical failure–free survival curves for the 3 groups.

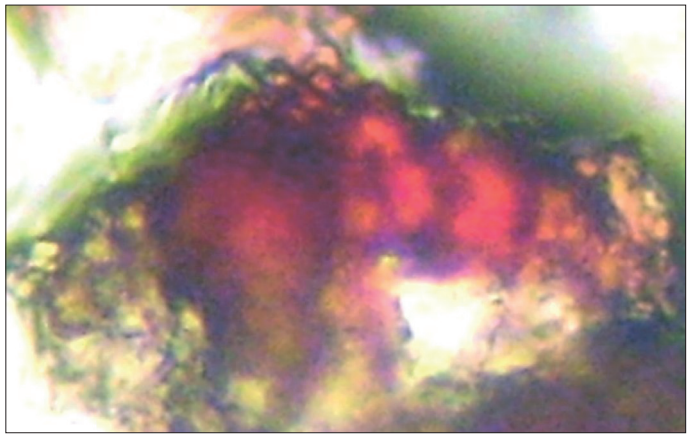

Figure 4. Bone marrow micro-metastasis expressing PSA (red) and membrane matrix metalloproteinase- 2 (brown)

As can be seen, there are three different biochemical failure–free survival curves; patients who were MRD-negative (group A) had the best prognosis with a 92% biochemical failure–free survival at 10 years. Those patients in group B (only bone marrow micro-metastasis) have a similar biochemical failure–free survival rate for up to 5 years and 100% progression-free survival at 2.5 years relative to group A patients. Thereafter, there is an increasing biochemical failure rate observable, with only 58% of patients being free from biochemical failure at 10 years. However, the restricted mean biochemical failure–free survival time was similar to that of group A patients. Group B patients are at risk for late disease progression.

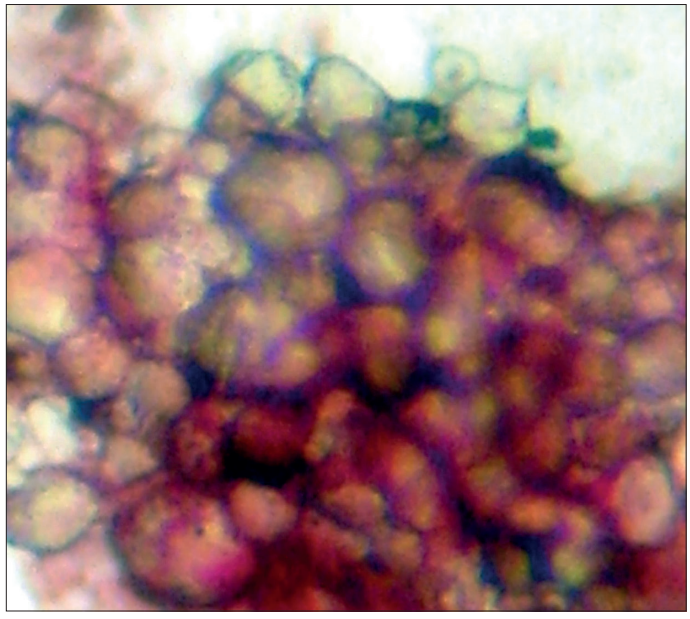

Figure 5. Bone marrow micro-metastasis expressing PSA (red) and matrix metalloproteinase 2 (brown). Adjacent stromal cells negative for PSA and positive for matrix metalloproteinase-2 (brown)

Table 2. Restricted mean biochemical failure–free survival time at 10 years model for 282 men with and without biochemical failure treated by radical prostatectomy for prostate cancer followed for 10 years

	Survival to 5 years (%) (95% CI) Observed Predicted	Survival to 10 years (%) (95% CI) Observed Predicted	Restricted Mean Survival time for 10 years (FP model). (95% CI)
Group A	94.7% 96.1%	92.7% 82.5%	9.47 years (9.24–9.69)
	(89.2–97.4) (92.9–97.8)	(86.3–96.2) (74.3–88.3)	
Group B	98.2% 94.2%	55.8% 75.2%	9.23 years (8.87–9.58)
	(87.6–99.7) (89.4–96.9)	(37.2–70.9) (63.4–83.6)	
Group C	26.1% 24.4%	5.0% 3.6%	3.57 years (3.52–3.63)
	(17.5–35.6) (17.0–32.6)	(1.6–11.1) (1.3–7.7)	

Determining RMST on the curves of Kaplan–Meier, to determine the RMST using flexible parametric survival final model on a hazard scale, incorporates the presence of CPC and mM, with three degrees of freedom for baseline hazard function (DF3) and CPC as a variable time-dependent effect (TVC) with one degree of freedom (DFTVC1); RMST, restricted mean survival time at 10 years; aone-way analysis of variance; for the Bonferroni correction, to adjust for multiple comparisons showed significant difference ($p<0.01$) between groups: A versus C, and B versus C.

Regarding group C patients, those with micro-metastasis and CPCs had a worse prognosis with the lowest biochemical failure–free survival seen at five and 10 years and a significantly shorter restricted mean biochemical failure–free survival time. These patients have a high risk of early disease progression.

Group C subanalysis for the expression of MMP-2

Roughly 43% (38/88) of patients in group C had bone marrow micro-metastasis with some MMP-2 expression, with 20% (18/88) of all group C patients being classified as positive for MMP-2 according to the criteria of Trudel (7). The percentage of tumor cells expressing MMP-2 was significantly associated with a higher Gleason score. Patients with bone marrow micro-metastasis negative for MMP-2 had a significantly lower frequency of a Gleason score of 7 points (Gleason 7) and pT3 tumors. With respect to pathological stage, there was a higher frequency of MMP-2–positive bone marrow micro-metastasis in pT3 tumors, but there was no significant difference with respect to the percentage of tumor cells expressing MMP-2. This suggests that higher-grade tumors have a higher expression of MMP-2 or that the intrinsic characteristics of higher-grade tumor cells permit the expression of MMP-2. Tumor size was not associated with the percentage of cells expressing MMP-2 but was associated with a higher frequency of MRD, which may be expected. Five and 10-year Kaplan–Meier biochemical failure–free survival curves (Table 3 and Figure 8) showed decreasing biochemical failure–free survival outcomes with increasing MMP-2 expression and shorter restricted mean biochemical failure–free survival times with higher MMP-2 expression.

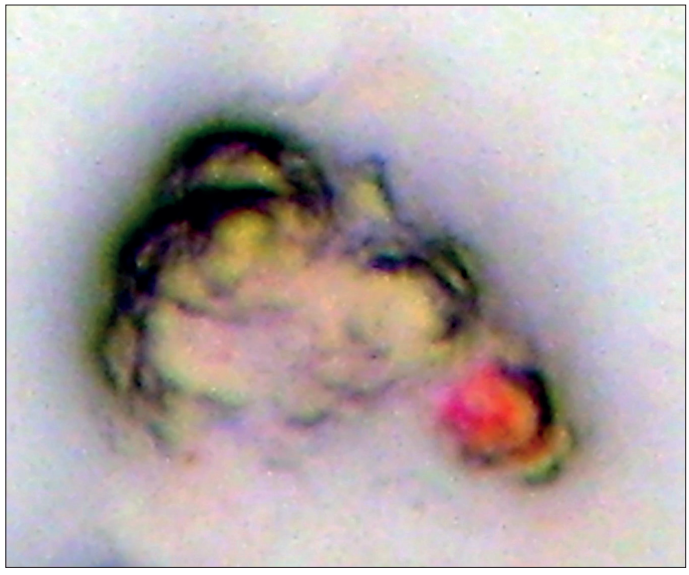

Figure 6. Bone marrow negative for micro-metastasis and tumor cell in the inter-trabecular space expressing PSA (red) and membrane matrix metalloproteinse 2 (brown)

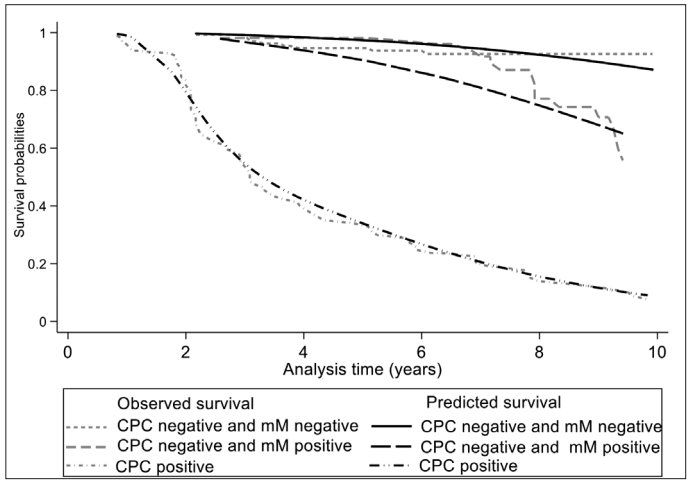

Figure 7. The Kaplan–Meier (observed) Biochemical Failure–Free survival curves and Flexible Parameter Model (Predicted) Biochemical Failure–Free Survival curves for Groups A, B, and C for up to 10 years of follow-up

Group A CPC and micro-metastasis negative; Group B, CPC negative micro-metastasis positive; Group C CPC positive.

The number of CPCs detected was significantly lower in patients with micro-metastasis negative for MMP-2 expression, with a median number of CPCs of 4 CPCs/sample (IQR: 3–7) versus 9 CPCs/sample (IQR: 6–19) ($p<0.001$). In patients with

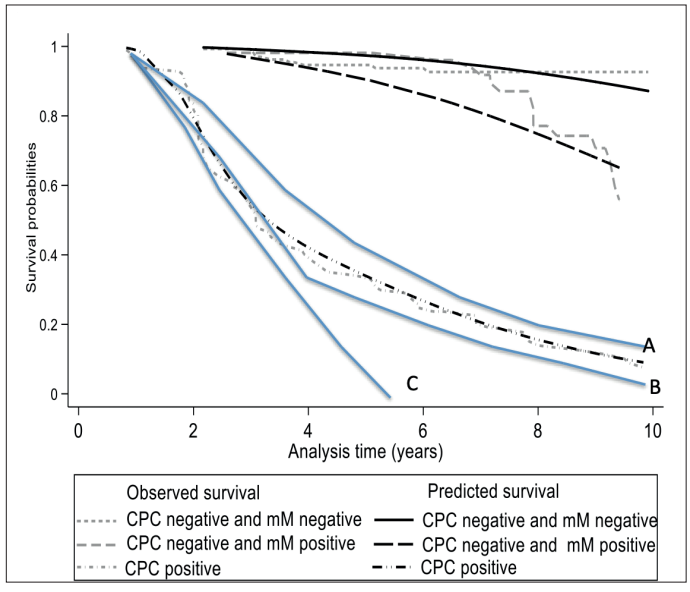

Figure 8. Kaplan-Meier survival curves at 10 years, showing sub-classification of patient's bone marrow positive for micro-metastasis and CPC positive according to bone marrow micro-metastasis MMP-2 expression

A: micro-metastasis positive, MMP-2 negative and CPC positive
B: micro-metastasis positive, MMP-2 >0%-<10% and CPC positive
C: micro-metastasis positive, MMP >10% and CPC positive

CPC: circulating prostate cell; mM: micro-metastasis; MMP-2: matrix metalloproteinase 2

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micro-metastasis positive for MMP-2 expression, there was an association between the number of CPCs detected and MMP-2 expression (Figure 9) with a correlation coefficient of r=0.728, considered to indicate a strong correlation.

The 2 patients who were positive for MMP-2 in group B did not amount to a sufficient size to permit an analysis; both patients progressed to biochemical failure within 4 years.

Discussion

The expression of MMP-2 in the primary tumor has been reported to be associated with a worse prognosis.[4,5] The explication is that increased expression of MMP-2 permits tumor cells to degrade the basement membrane and extracellular matrix and migrate into the circulation. Those tumor cells that survive in the bloodstream utilize MMP-2 to invade distant tissues, where they implant. The characteristics of these tumor cells and the interactions with surrounding stromal cells will determine the prognosis of the patient and the continued expression of MMP-2.

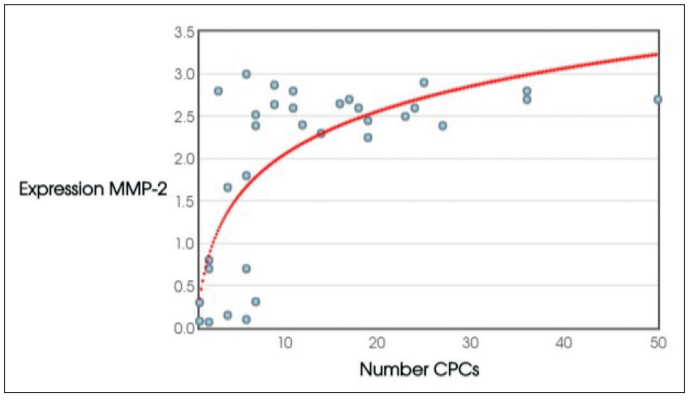

Figure 9. Logistic regression curve of the number of circulating prostate cells/blood sample detected and the expression of membrane matrix metalloproteinse-2 in bone marrow micro-metastasis

The 2 groups, B and C, that were positive for bone marrow micro-metastasis, had different failure kinetics. Patients with only bone marrow micro-metastasis had a similar disease-free progression to that of patients negative for MRD for the first 5 years postsurgery and, thereafter, experienced an increasing failure rate. These patients were at risk for late failure, with a mean restricted time to failure of approximately 9 years. In other words, there was a latency period of more than 5 years before biochemical failure was noted. Differing from this pattern were patients positive for micro-metastasis and CPCs (group C). These patients have a high risk of early failure, with a RMST of only 3.5 years.

Of note, we have previously reported this pattern of failure in men treated for pT2 disease with radical prostatectomy. In these patients, for each MRD group, patients with Gleason 7 had a worse biochemical failure–free survival and shorter restricted mean biochemical failure–free survival time relative to those with Gleason 6, which is not surprising. However, patients with a Gleason 7 and MRD-negative disease had a 100% biochemical failure–free survival at 5 years as compared with 79% in patients with Gleason 6, CPC-positive disease.[21] Men with micro-metastasis positivity who were CPC-negative with Gleason 7 had a 10-year biochemical failure–free survival of 63% versus 19% in men with CPC-positive, Gleason 6 prostate cancer.[21]

Table 3. Analysis of pathological findings and biochemical failure–free survival at five and 10 years according to MMP-2 expression in micro-metastasis

	MMP-2 (-) N=50	MMP-2 >0%-<10% N=20	MMP-2 ≥ 10% N=18	Cohort N=88
Gleason 6	27	8	1	36
Gleason ≥ 7	23	12	17	52
				p=0.0016[a]
				p=0.035[b]
pT2	23	1	2	26
pT3	27	19	16	62
				p=0.005[a]
				p=0.92[b]
K-M survival 5 years	32.4%	23.6%	15.2%	26.1%
K-M survival 10 years	7.7%	3.2%	0%	5.0%
MRST 10 years	4.01	2.95	2.56	3.57

K-M: Kaplan–Meier; MRST: mean restricted survival time; MMP-2: matrix-metalloproteinase-2; [a]comparison between all groups; [b]comparison between micro-metastasis expressing MMP-2

Thus the biological characteristics of prostate cancer, although having the same morphological features (Gleason score), are heterogeneous. More recently, a 30-gene messenger RNA expression signature improved the prediction of indolent and lethal outcomes in men with intermediate-risk Gleason 7, independent of whether the patient had Gleason 3 + 4 or 4 + 3.[22]

The aim of this study was to subdefine men with micro-metastasis and CPCs detected after radical prostatectomy with regard to MMP-2 expression. There was a significant difference in the expression of MMP-2 in the micro-metastasis between group B and C patients. Only 4% of group B patients were classified as MMP-2–positive, both of whom experienced treatment failure before 4 years, while, in patients with CPCs detected, 21% of patients were classified as MMP-2–positive. The expression of MMP-2 was limited to the border of the micro-metastasis, corresponding to the invasion front. In 3 patients, adjacent stromal cells also expressed MMP-2. In vitro experiments have reported the importance of cell-to-cell contact between stromal and tumor cells, that cancer-associated fibroblasts secrete pro-MMP-2 (the inactive form) and activation requires tumor cell membrane–located MMP-1, and that the expression of MMP-2 was found at the periphery of tumors and not in the center and in the invasion front.[23] Cell–cell contact results in increased production and messenger RNA expression of tumor MT1–MMP, which causes a sequential increase in the activation of fibroblast proMMP-2 and the formation of an MT1–MMP–TIMP-2–MMP-2 complex on the tumor cell surface.[23] The detection of MMP-2 at the micro-metastasis–stromal interface in this study is consistent with experimental data. Few stromal cells express MMP-2; those that do so are generally in contact with tumor cells. In this study where bone samples were taken early after treatment, this low stromal MMP-2 detection rate contrasted with the high stromal MMP-2 detection rate reported in patients treated with androgen depletion after treatment failure.[9]

This stromal–tumor cell interaction is responsible for the clinical behavior of the micro-metastasis; patients in group B had a latent period of over 5 years, whereas those in group C had a more aggressive behavior with a short time to failure, with secondary dissemination of CPCs. This stromal–tumor cell cross-talk determines tumor cell behavior. It has been reported that more than 80% of patients with solid tumors harbor Ki-67-negative micro-metastasis with very low or no detectable pAKT levels and that thrombospondin-1 secretion induces a sustained quiescence of cancer cells.[24]

In patients with only micro-metastasis (group B), few patients were positive for MMP-2 expression (4%); in comparison, those patients with CPCs detected (group C) included a significantly higher frequency of patients who were MMP-2–positive and with higher MMP-2 scores. However, not all patients with CPCs had micro-metastasis expressing MMP-2.

In the subanalysis of group C patients, the expression of MMP-2 was associated with a higher Gleason score in the primary tumor and a higher MMP-2 score. This is similar to that found in primary tumors, where there is an association between higher MMP-2 expression and increasing Gleason score.[25] The frequency of patients with micro-metastasis classified as positive for MMP-2 expression (>10%) was associated with tumor stage, again similar to that found in primary tumors.[25] However, the percentage of cells expressing MMP-2 in the micro-metastasis was similar in pT2 and pT3 tumors. Patients with increasing MMP-2 expression in bone marrow micro-metastasis had higher rates of biochemical failure and shorter times to biochemical failure. MMP-2 expression in the primary tumor has been associated with prognosis[25] and plasma concentrations of MMP-2 are higher in patients with metastasis.[26]

The fact that all patients with CPCs did not have MMP-2–positive micro-metastasis may be explained in terms of active and passive dissemination. Tumor cells are thought to enter the circulation actively as single cells, cell clusters, or strands, and MMP-2 is thought to be important in this process. Tumor cells may also enter the circulation passively as a result of growth into the intertrabecular space of the bone marrow or be moved through micro-tracks created by actively migrating tumor cells.[27] That all CPCs expressed MMP-2 independent of the expression of MMP-2 in bone marrow micro-metastasis suggests that bone marrow stromal cells inhibit the expression of MMP-2. Once tumor cells have escaped into the bone marrow intertrabecular space, the inhibition is lifted and CPCs express MMP-2. This suggests that the micro-metastases that express MMP-2 overcome the inhibition produced by stromal cells and, as such, are more "aggressive" which, in clinical practice, is seen as a higher frequency of relapse and a shorter time-interval to relapse. This was seen more frequently in Gleason 7 tumors, which are known to be associated with a worse prognosis.

Those patients with MMP-2 expressing micro-metastasis had a poor prognosis with a very high risk of treatment failure and shorter times to failure as compared with patients with MMP-2 negative micro-metastasis and higher numbers of CPCs detected in the circulation. This suggests that active dissemination represents a more aggressive tumor. If group C patients have a short time to and high risk of biochemical failure, such raises the question of whether early adjuvant therapy is warranted in these patients. It is beyond the scope of this study to answer this question; however, it has been reported that androgen blockade can eliminate bone marrow micro-metastasis[28] and that bisphosphonates also may eliminate micro-metastasis as well as decrease MMP-2 expression.[29]

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In clinical practice, this subclassification of MRD by CPCs and micro-metastasis and expression of MMP-2 identifies patients with very poor outcomes who could benefit from early treatment, those who could benefit from later treatment, and those with an excellent prognosis. This subclassification identifies populations with "good-risk" Gleason 7 and "poor-risk" Gleason 6 tumors. This heterogeneity of bone marrow micro-metastasis explains why there are differing reports of their ability to predict metastatic behavior. With time, there may be successive clonal expansions and a parallel progression that leads to new tumor cell variants. More recently, the SRD5A2 and 11 mitosis and cell-cycle transcripts were reported to predict outcome in Gleason 7 patients, identifying at the molecular level subtypes of Gleason 7 patients with varying prognostic outcomes.[30]

The genetic and phenotypic characteristics of tumor cells found in bone marrow micro-metastasis and that of the tumor microenvironment will determine the fate of the patient. Subclassifying patients after surgery based on CPC and bone marrow micro-metastasis detection and conducting subsequent subclassification using MMP-2 expression in tumor cells can provide clinically useful prognostic information. Not all patients classified by primary tumor characteristics behave in the same way; larger studies are warranted to confirm the reported data.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of the Hospital de Carabineros de Chile Resolution (45-1/5/2008).

Informed Consent: Written informed consent was obtained from each patient who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – N.P.M.; Design – N.P.M., E.R.; Supervision – N.P.M., E.R., A.S.; Resources – N.P.M.; Materials – N.P.M.; Data Collection and/or Processing – E.R., A.S., M.A.L., S.O., E.G.; Analysis and/or Interpretation – N.P.M., E.R., A.S., M.A.L., S.O., E.G.; Literature Search – M.A.L., S.O., E.G.; Writing Manuscript – N.P.M., E.R., A.S.; Critical Review – M.A.L., S.O., E.G.

Acknowledgements: The authors wish to thank Mgrs. Ana Maria Palazuelos for her help in the writing of the manuscript.

Conflict of Interest: Dr. Murray reports having received consultancy fees from Viatar CTC solutions, Boston; there were no other conflicts of interest reported.

Financial Disclosure: A West Metropolitan Health Service Research Grant, Santiago, funded the study.

Availability of data and materials: The datasets generated and/or analyzed during the current study are not publically available due to the Chilean Patient's Rights Law.

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