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Differential expression of E-Cadherin and P-Cadherin in pT3 prostate cancer: correlation with clinical and pathological features

Catarina Ribeiro da Cunha Ferreira

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Guidelines and Submissions

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Resumo

Introdução

Atualmente, o ‘sobrediagnóstico’ e consequente ‘sobretreatamento’ representam um dos principais desafios da abordagem do cancro da próstata. Especificamente nas neoplasias com estadio pT3, com frequência classificadas como de alto risco, admite-se que nem todos os doentes apresentem um prognóstico uniformemente mau.

Como consequência, a pesquisa de novos biomarcadores de prognóstico tornou-se um dos pontos fundamentais da investigação em cancro da próstata. As Caderinas são uma família de proteínas transmembranares cuja expressão alterada tem sido implicada na progressão desta patologia. Mais concretamente, a diminuição da expressão de Caderina E associou-se a pior prognóstico, enquanto que o papel da Caderina P ainda não foi completamente elucidado.

Objetivos

Caracterizar a expressão das Caderinas E e P numa amostra de neoplasias da próstata estadio pT3 e avaliar: a sua associação com variáveis clinicopatológicas descritas na literatura; o seu valor prognóstico potencial; e o seu impacto na sobrevivência.

Metodologia

Amostras de prostatectomia radical de 102 doentes diagnosticados com cancro da próstata estadio pT3, tratados entre 1991 e 2014 na mesma instituição, foram utilizadas para análise da imuno-expressão das Caderinas E e P. Um bloco representativo de casa amostra foi selecionado para a construção do respetivo *tissue micro-array*, com um total de três cores por doente. A imuno-expressão da Caderina E foi avaliada com recurso a um sistema de análise digital de imagens. Para a Caderina P foram utilizados os critérios de classificação do HER2 no cancro gástrico. Os registos clínicos dos doentes foram consultados para obtenção de dados relativos a características clínicas/patológicas iniciais e seguimento.

Resultados

O grupo de doentes com expressão diminuída de Caderina E apresentou pior sobrevida específica de doença, embora sem significância estatística (HR: 2.65, 95%CI: 0.81-7.88). No entanto, ao considerar apenas o grupo de doentes com estadio pT3b, a presença de expressão diminuída de Caderina E associou-se

significativamente com pior sobrevida global e sobrevida específica de doença (HR: 3.69, 95%CI: 1.18-11.50; HR: 5.90, 95%CI: 1.40-24.81). Não foram encontradas associações estatisticamente significativas para a expressão de Caderina P. Adicionalmente, uma associação entre a expressão das Caderina E e P foi observada ($p=0.019$): no grupo de doentes com expressão diminuída de Caderina E, 99.6% apresentavam expressão negativa de Caderina P.

Conclusões

A expressão diminuída de Caderina E discriminou, no grupo de doentes com estadio pT3b, quais apresentam pior sobrevida e que poderão beneficiar de terapia direcionada. O papel da Caderina P poderá estar dependente do contexto no qual é avaliado, merecendo investigação adicional.

Abstract

Cadherins seem to play an important role in prostate cancer progression. Whereas E-Cadherin loss of expression has been associated with poor prognosis, P-Cadherin's role is still elusive. Although pT3 prostate cancer is often considered 'high-risk cancer', it does not exhibit a uniformly poor prognosis. Herein, we assessed the prognostic value and survival impact of E-Cadherin and P-Cadherin immunoexpression in pT3 prostate cancer.

Radical prostatectomy (RP) specimens from 102 pT3 prostate cancer patients treated between 1991 and 2014 in a single institution were designated for E-Cadherin and P-Cadherin immunoexpression analysis. A representative block from each specimen was selected for tissue micro-array construction, using 3 cores *per* case. E-Cadherin immunoexpression was assessed via a digital image analysis system. For P-Cadherin, scoring criteria for HER2 in gastric cancer were used. Clinical records of all patients were reviewed for baseline clinical/pathologic characteristics and follow-up data.

E-Cadherin-low prostate cancer patients displayed worse disease-specific survival, although not reaching statistical significance (HR: 2.65, 95%CI: 0.81-7.88). However, considering the pT3b group only, those with low E-Cadherin immunoexpression displayed significantly worse overall-survival and disease-specific survival (HR: 3.69, 95%CI: 1.18-11.50; HR: 5.90, 95%CI: 1.40-24.81). No significant differences in survival were found for P-Cadherin differential immunoexpression. Furthermore, an association between E-Cadherin and P-Cadherin immunoexpression ($p=0.019$) was found, as among E-Cadherin-low prostate cancer, 96.6% were P-Cadherin negative.

We demonstrated that low E-Cadherin immunoexpression discriminates among pT3b prostate cancer patients those with poorer survival and which might benefit from specific therapy. The role of P-Cadherin in prostate cancer seems context-dependent, deserving further investigation.

Keywords

Prostate cancer; E-Cadherin; P-Cadherin; Survival

Abbreviations

AJCC - American Joint Committee on Cancer

CI - Confidence interval

CRCP - Castration-resistant prostate cancer

DFS - Disease-free survival

DSS - Disease-specific survival

EMT - Epithelial-mesenchymal transition

IHQ - Immunohistochemistry

IQR - Inter-quartile range

HR - Hazard ratio

OS - Overall survival

PCa - Prostate cancer

PSA - Prostate-specific antigen

TMA - Tissue micro-array

USA - United States of America

WHO - World Health Organization

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Introduction

Prostate cancer (PCa) is widely recognized as one of the most relevant medical conditions in the male population. It represents the most common malignancy and a major cause of cancer-related death among men[1]. In the United States of America (USA), an estimated 161,360 new cases of PCa were diagnosed and 26,730 deaths due to PCa were expected in 2017[2]. PCa awareness has increased since the introduction of serum prostate-specific antigen (PSA) testing as a screening tool in the late 1980s[3-5], which remains the only clinically implemented diagnostic and follow-up biomarker for PCa[6]. However, due to the frequently indolent course of the disease and the unspecific nature of the test, increased incidence of PSA-detected PCa has been largely associated with cancers that would not progress to an advanced stage and that are considered clinically insignificant[7]. Overdiagnosis has, thus, become a major challenge and ensues overtreatment consequences.

Nonetheless, PCa encompasses a wide spectrum of clinical outcomes and its natural history is highly variable[8]: in some cases, it progresses to metastatic disease in patients with localized tumour treated radically, whereas in other cases the disease does not progress at all[9-11]. Patients are advised on treatment based on risk assessment and model equations are often used for this purpose, taking into account preoperative serum PSA, postoperative Gleason score and pathologic stage [12]. However, prediction of progression for the individual patient using these models is still not precise, and additional markers are needed to more accurately target high-risk patients and establish a treatment course[13]. This is well illustrated by locally advanced prostate cancer, which is defined as a tumor that has extended beyond the prostatic gland or into seminal vesicles, but without lymph node involvement or distant metastases, corresponding to pT3 stage in the American Joint Committee on Cancer (AJCC) classification[14]. Although these tumors are often referred to in the literature as high risk cancer[15], it has been argued that these patients do not present a uniformly poor prognosis after radical prostatectomy[16].

One group of proteins whose altered expression has been associated with tumor invasiveness, metastatic dissemination, and poor patient prognosis is the cadherins *superfamily*. Cadherins are a large multigene family of transmembrane glycoproteins with a crucial role in homophilic cell-cell adhesion, cell polarity, cell proliferation, migration, and differentiation[17,18]. E-Cadherin has been

considered the paradigmatic classical cadherin and is mainly expressed in epithelial tissues. It likely functions as an invasion suppressor gene/protein since its loss of expression, abnormal function, or both, leads to an increased ability of cells to invade neighboring tissues[19]. In addition, E-Cadherin downregulation is considered a main indicator of epithelial/mesenchymal phenotype switch, which occurs during epithelial-mesenchymal transition (EMT)[20]. EMT allows for the detachment of tumor cells from the primary site, followed by intravasation into the blood stream, extravasation into distant target organs, and metastases formation[21-23]. E-Cadherin has been consistently demonstrated to be downregulated, silenced or aberrantly expressed in multiple cancer types[24] and its fundamental role in tumorigenesis has been recognized, particularly in breast[25], prostate[26,27] and gastric[28] carcinomas. Indeed, several studies have associated the loss of E-Cadherin expression on cell membrane with PCa high histological grade and advanced tumor stage, and therefore with poor prognosis. P-Cadherin is another classical cadherin with a crucial role in the conservation of epithelial tissues' structural integrity. It shares about 67% of homology with the E-Cadherin protein, differing mainly in its extracellular portion[29,30]. The cancer-related function of P-Cadherin is still an object of debate, as it is considered to be context dependent. Opposite effects have been found for P-Cadherin in PCa[31] and the prognostic implications of these findings are still unknown.

It is still unclear whether the evaluation of E-Cadherin and P-Cadherin expression levels in PCa specimens provides prognostic information, independently of well-recognized prognostic parameters. Thus, we sought to characterize E-Cadherin and P-Cadherin expression in a series of pT3 stage PCa patients and further evaluate its association with standard clinical and pathological findings, to assess its potential prognostic value and impact on patient survival.

Material and Methods

Patients and samples

In this retrospective study, 102 radical prostatectomy specimens of PCa patients (1999-2014) with pT3 disease were collected from the archives of the Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto), Portugal. All patients were clinically evaluated and treated by the same multidisciplinary team. Histological slides were reviewed, and Gleason score and prognostic grade groups (based on the 2016 WHO criteria) were assigned by dedicated Uropathologists. The block representing the highest Gleason score (index tumor), or the one with the greatest amount of tumor when various blocks presented the same Gleason score, was selected as the representative block for tissue microarray (TMA) construction. TMAs were built using three representative tumor cores per case. Four- μ m sections were then cut from each block for immunostaining. Clinicopathological data was retrieved from the patients' files, including: age and PSA at diagnosis, macroscopic features, tumor size, histological type, perineural and vascular invasion, tumor stage[14], Gleason score and grade group, and also disease recurrence and last follow-up. Biochemical recurrence was considered as previously described[32].

This study was approved by the institutional ethics committee of Portuguese Oncology Institute Porto (Comissão de Ética para a Saúde do IPO Porto – CES 235/2017). Because the study was based on retrospective analysis of archival material, it was exempted from informed consent.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using Ultraview Universal DAB Detection Kit (Ventana), in Benchmark Ultra Platform. Antigen retrieval was performed for 64 minutes with CC1 buffer (for P-Cadherin) and for 56 minutes with CC2 buffer (for E-Cadherin). Primary monoclonal antibodies for E-Cadherin (Clone NCH38, 1:50 dilution, Reference M3612, DAKO) and P-Cadherin (Clone 56C1, ready to use, Reference ab75442, ABCAM) were used. Hematoxylin was used for nuclear counter staining. Appropriate positive controls were used for each antibody and negative control consisted on omission of primary antibodies. Because all cases analyzed contained non-neoplastic prostatic epithelium, this served as (internal) control as appropriate. However, when the IHC protocols were developed, negative external controls were used.

E-cadherin immunoexpression was assessed using a digital image analysis system (GenASIs™, Israel), which quantified membrane immunostaining. Staining intensity was scored 0 to 3, with 0 representing negative staining, 1 representing weak staining, 2 representing intermediate staining, and 3 representing strong staining; the percentage of cells with each staining intensity was assessed, as well. The H-Score for each TMA core was calculated (sum of the products of each immunostaining score by its proportion) and a mean H-Score was attributed to each case. In the absence of a validated scoring system to evaluate P-cadherin immunoexpression, the scoring criteria for HER2/neu immunoexpression in gastric adenocarcinoma was adopted [33]. Using these criteria, P-Cadherin immunoexpression was scored as positive, negative or equivocal by two researchers blinded to clinical outcome.

Statistical analysis

Statistical analysis was performed using SPSS Statistics for Windows, version 25.0 (SPSS, Chicago, IL). The chi-square test was used for evaluating associations between biomarkers and clinicopathological variables. Distribution of continuous variables between groups was compared using non-parametric tests (Mann-Whitney test). Survival curves were calculated using the Kaplan-Meier method and Log-Rank test was used to compare survival curves. Hazard ratios (HR) and respective 95% confidence intervals (CI) were estimated using Cox-regression models. Statistical significance was set at $p < 0.05$.

Results

Cohort characterization and biomarker immunoexpression

From the initial study population of 102 tumor specimens, two cases were dismissed from analysis due to lack of sufficient tumor cells in the TMA. The clinicopathological features of the remainder 100 patients are displayed in Table I. Median age at diagnosis was 64 years (IQR: 60-68) and median pre-operative PSA was 8.6 ng/ml (IQR: 6.1-11.6). Upon histological examination, 53% of specimens were graded Gleason score ≤ 7 and 71% displayed pT3a disease. Lymphadenectomy was performed in two patients and one of them presented regional lymph node metastasis.

Illustrative examples of E-Cadherin and P-Cadherin immunostaining are shown in Figure 1. The median number of tumor cells and of 200x fields evaluated per case, as well as respective P30 value (30th percentile) for E-Cadherin mean H-score are provided in Table I. For subsequent analyses, PCa cases displaying E-Cadherin immunostaining bellow P30 were considered as having “Low E-Cadherin immunoexpression”, and those above P30 as presenting “High E-Cadherin immunoexpression”. Furthermore, grade groups 1 and 2 were designated as “Low grade groups”, and grade groups 3, 4 and 5 as “High grade groups”.

Cohort validation

Considering the whole cohort, Gleason score > 7 was associated with positive surgical margins ($p=0.046$). As expected, patients with pT3b stage presented significantly worse disease-specific survival (DSS) compared to pT3a group (HR: 4.58, 95% CI: 1.49-14.05) (Figure 2a). Moreover, patients with pT3b stage displayed worse overall survival (OS), although it did not reach statistical significance (HR: 2.03, 95% CI: 0.97-4.25). Patients with high grade group PCa displayed significantly worse DSS compared to those with low grade group disease (HR: 9.65, 95% CI: 1.25-74.89) (Figure 2b).

Associations between E-Cadherin and P-Cadherin and clinicopathological features

An association was found between E-Cadherin and P-Cadherin immunoexpression. In the group of patients with low E-Cadherin immunoexpression, 96.6% of patients were also P-Cadherin negative. Additionally, in the group of patients with equivocal/positive P-Cadherin

immunoexpression, 93.8% presented high E-Cadherin immunoexpression ($p=0.019$).

Low E-cadherin immunoexpression associated with higher serum PSA at diagnosis in pT3a stage patients ($p=0.032$). No other significant associations were found between E-cadherin or P-Cadherin immunoexpression and age, serum PSA, pT stage, grade group, surgical margins, and perineural or vascular invasion.

Survival analysis

The median follow-up time of the PCa patients enrolled in this study was 150 months and a total of 16 (16%) patients died from PCa. Disease free survival (DFS) was 41.9% at 120 months (44.3% in pT3a patients vs. 35.0% in pT3b patients) and 39.6% at 150 months. Eleven patients were not considered to have remission after curative-intent radical prostatectomy.

Univariable analysis:

Considering the whole cohort

Patients with low E-Cadherin immunoexpression displayed worse DSS, although it did not reach statistical significance (HR: 2.65, 95% CI: 0.81-7.88). No significant association with OS or DFS was found. Regarding P-Cadherin immunoexpression, no significant difference was found for OS, DSS and DFS between patients with negative or equivocal/positive immunoexpression.

Considering only pT3a or pT3b subgroups

Both E-Cadherin (Figure 3a) and P-Cadherin (Figure 3b) immunoexpression were not significantly associated with OS, DSS or DFS in patients with pT3a disease.

However, considering only patients with pT3b disease, those with low E-Cadherin immunoexpression displayed significantly worse OS and DSS (HR: 3.69, 95% CI: 1.18-11.50; and HR: 5.90, 95% CI: 1.40-24.81, respectively), but not DFS, compared to patients with high E-Cadherin immunoexpression (Figure 4a). Concerning P-Cadherin immunoexpression, no significant differences were found in OS, DSS and DFS (Figure 4b).

Multivariable analysis:

Owing to the limited number of events, multivariable analysis was not accomplished.

Discussion

PCa is one of the leading causes of cancer-related morbidity and mortality in men, constituting a major health concern and economic burden. Given its heterogeneous course, outcome prediction remains a challenge. The current focus in PCa biomarker research includes the definition of valid prognostic biomarkers with clinical utility, which may accurately predict patients' outcome after surgical resection and support clinical decision-making for the individual patient, considering the tumor's potential aggressiveness[34,1]. Specifically for locally advanced prostate cancer, classified as high risk cancer, outcome after radical prostatectomy varies greatly[16], demonstrating the importance of identifying biomarkers able to discriminate patient outcome, even among patients with unfavorable prognosis. Considering the role of cadherins deregulation in cancer progression and previously reported findings in PCa, we aimed to investigate the prognostic impact of E-Cadherin and P-Cadherin immunoexpression in a cohort of pT3 stage PCa patients submitted to curative-intent radical prostatectomy.

Our study analyzed 100 prostatectomy specimens from patients evaluated and treated in a single tertiary hospital, with a median age at diagnosis of 64 years, which is similar to the 66 years reported in literature[35]. Prognostic grade grouping stratified patients according to DSS, as expected[36,37], although not reaching statistical significance (Figure 5), probably due to sample size. Indeed, when prognostic grade groups were lumped together into high and low, statistical significance emerged concerning DSS. Tumor stage also provided a statistically significant stratification of patients, as pT3b patients experienced significantly worse DSS comparing to pT3a patients, in accordance with the literature[38]. Furthermore, DFS at 120 months was 44.3% in pT3a and 35.0% in pT3b patients, also paralleling previous findings[39]. Thus, despite its limited sample size, this series is validated according to standard clinicopathological parameters.

Interestingly, we showed that pT3b PCa with low E-Cadherin immunoexpression displayed worse DSS in univariable analysis, although this was not replicated for pT3a PCa patients. To the best of our knowledge, this is the first study to specifically consider the role of E-Cadherin immunoexpression in prognosis stratification of pT3 PCa. Previous studies have demonstrated an association

between abnormal or low immunoexpression of this biomarker and poor prognosis in PCa, without stratifying, however, for tumor stage[40-45,34]. These findings might be explained by the pivotal role of E-Cadherin in critical morphogenetic and differentiation processes during development, and in maintaining integrity and homeostasis in adult tissues, including the prostate gland[46]. Indeed, E-Cadherin has been identified as an invasion suppressor gene/protein, and its altered expression is believed to increase the ability of cells to invade neighboring tissues[19]. Moreover, its downregulation is considered a main indicator of EMT, which precedes the detachment of tumor cells from the primary site, and the formation of secondary lesions and metastases[46]. In this line, quantitative E-Cadherin immunoexpression might add valuable independent information to the existing prognostic tools for the pT3b group of patients, allowing for discrimination of more aggressive PCa and assisting in individual clinical management.

The lack of prognostic significance of E-cadherin immunoexpression in pT3a PCa patients might be due to an increasingly important role of E-cadherin underexpression along tumor progression. It is widely acknowledged that pT3a PCa portrays a significantly better prognosis than pT3b PCa, thus suggesting that extension into the seminal vesicles is not just a matter of increased tumor invasion capabilities, but also of systemic dissemination. Thus, owing to the pivotal role of EMT in invasion and metastization, we are tempted to speculate whether critically low E-Cadherin expression levels might only be attained at more advanced disease stages, including pT3b. This is in accordance with previously reported associations between abnormal or low E-Cadherin immunoexpression and high histological grade and advanced tumor stage in PCa, as well as poor prognosis[26,27,40-45,34]. It should be emphasized, however, that previously published studies on E-Cadherin's role in PCa display a considerable heterogeneity among them, including different staining interpretation methods, cutoffs (ranging from 10-70%), cohort types and sample sizes, precluding definitive conclusions on this subject. In our study, the use of a digital image analyzer, which is uncommon in precedent literature, provided a quantitative and less biased assessment of E-Cadherin immunoexpression and might have offered more precise results. Importantly, this might also facilitate its translation into routine diagnosis settings, improving the homogeneity of assessment, without substantially delaying diagnostic workup.

No association was found between E-Cadherin immunoexpression and clinicopathological features, except for higher serum PSA at diagnosis in patients with low E-Cadherin immunoexpression, when considering the pT3a group alone. Bussemakers *et al*[47] first reported an association between the reduction of E-Cadherin expression and PCa metastatic potential, followed by Umbas *et al*[48], who demonstrated an association of this feature with tumor grade and prognosis. Since then, several published studies have examined the association between E-Cadherin expression and PCa clinicopathological features, showing that loss of cell membrane E-Cadherin expression is associated with high PSA level at diagnosis, high histological grade and advanced tumor stage, as well as poor prognosis[13, 26, 34, 40-42, 44, 45, 48]. The absence of association between E-Cadherin immunoexpression and the aforementioned clinicopathological features in our study could be explained by the strong prognostic stratification provided by tumor stage. This finding further enhances the importance of the discriminative power of E-Cadherin immunoexpression as prognostic biomarker in pT3b patients. Unfortunately, multivariable analysis was not possible owing to the limited number of cases, as the sample was already stratified by tumor stage. Nevertheless, given that no significant association was found between E-Cadherin immunoexpression and the remainder clinicopathological features, it is not expectable that these variables might interfere with the significant association found between low E-Cadherin immunoexpression and worse DSS in pT3b PCa patients.

Concerning P-Cadherin immunoexpression, no associations were found with PCa clinicopathological features, DSS, OS and DFS, as well. Comparing to E-Cadherin, P-Cadherin has been scarcely studied and opposite effects have been reported for this cadherin in PCa. Indeed, Arena *et al*[49] concluded in their study that P-Cadherin immunoexpression was increased compared to normal prostate tissue, and Gravdal *et al*[50] demonstrated that positive membranous staining was significantly associated with higher Gleason score, poorly differentiated cancer and shorter time to bone metastases. Inversely, Jarrard *et al*[51] showed that P-Cadherin expression was absent in all of the poorly differentiated PCa specimens enrolled in their study. Bearing in mind that our specimens were obtained from clinically localized disease, it is possible that PCa exhibits an altered expression of P-Cadherin in later stages of the spectrum of disease, which might explain our negative results. Remarkably, P-cadherin is frequently over-expressed in high-

grade invasive breast carcinomas and has been reported to correlate with tumor aggressiveness, being considered an established indicator of poor prognosis[52]. It is noteworthy that all cases considered positive in our sample set exhibited a relatively weak P-Cadherin immunoexpression, which determined the need to use a qualitative estimation analysis, considered to be more subjective when compared to a quantitative technique.

An association was found between E-Cadherin and P-Cadherin immunoexpression, as most tumors with low E-Cadherin immunoexpression were also P-Cadherin negative, and those classified as P-Cadherin equivocal/positive presented high E-Cadherin immunoexpression. E-Cadherin and P-Cadherin are co-expressed in a number of adult tissues, including the prostate gland [53]. On the contrary, the induction of P-Cadherin in some tumors has been associated with E-Cadherin downregulation, which might be part of a process called cadherin switch, which occurs during EMT and correlates with tumor progression[54,55]. The same has been reported in PCa[50]. In our study, a true cadherin switch was not observed. Once again, such results might be explained by the specific spectrum of the disease in our cohort. It is possible that in our cases E-Cadherin is already being downregulated, while P-Cadherin's expression would only rise in later stages, allowing for the cadherin switch to be discernible by immunohistochemistry.

This study presents several limitations, given its retrospective nature and small cohort size, which may potentially bias the results. In fact, multivariable analysis was not possible owing to this. Moreover, although we have presented data regarding OS, the potential impact of comorbidities was not assessed, as we mostly focused on DSS. Nevertheless, the fact that both pT stage and grade grouping stratified PCa patients according to prognosis further supports the validity of our findings. In addition, this study is based in a series of patients that were evaluated and treated in a single institution by the same multidisciplinary team, making results extrapolation more difficult, but allowing for homogeneity in patients' staging and clinical management. IHC is a widespread and accessible technique, but its qualitative analysis carries a considerable inter- and intra-observer variation. In our study, variation was diminished using a quantitative analysis method, which improved biomarker quantification and reduced evaluation subjectivity. Unfortunately, this was not possible for P-Cadherin immunoexpression assessment. Finally, the use of TMA might be limiting owing

to tumor heterogeneity, but we are convinced that assessing three tissue cores from each patient can overcome this potential bias.

Conclusion

In our dataset, low E-Cadherin immunoexpression predicted poor outcome in pT3b PCa patients submitted to radical prostatectomy. In this setting, E-Cadherin might be a promising disease biomarker, since its assessment might assist in discriminating more aggressive from less aggressive PCa among pT3b patients. In our series, the role of P-Cadherin in PCa could not be determined, but it might be context-dependent. The role of E-Cadherin and P-Cadherin in PCa deserves further investigation and validation in future studies, as it may allow for the improvement and personalization of PCa patient management.

Table 1 - Clinicopathological features of prostate cancer patients and E-Cadherin and P-Cadherin immunoexpression.

Age at diagnosis (y, median [IQR] ^a)	64 (60-68)
PSA at diagnosis (ng/ml, median[IQR])	8.6 (6.1-11.6)
Gleason grade, prostatectomy specimen	
3+3	9/100 (9%)
3+4	28/100 (28%)
4+3	16/100 (16%)
3+5	7/100 (7%)
4+4	11/100 (11%)
5+3	2/100 (2%)
4+5	20/100 (20%)
5+4	7/100 (7%)
Prognostic grade groups, prostatectomy specimen	
Group 1	9/100 (9%)
Group 2	28/100 (28%)
Group 3	16/100 (16%)
Group 4	20/100 (20%)
Group 5	27/100 (27%)
pT Stage	
pT3a	71/100 (71%)
pT3b	29/100 (29%)
Biochemical recurrence	
No	40/100 (40%)
Yes	49/100 (49%)
Absence of remission	11/100 (11%)
Extraprostatic extension	
No	1/100 (1%)
Yes	99/100 (99%)
Seminal vesicle invasion	
No	71/100 (71%)
Yes	29/100 (29%)
Vascular invasion	

Present	75/100 (75%)
Absent	25/100 (25%)
Perineural invasion	
Present	2/100 (2%)
Absent	98/100 (98%)
Surgical margins	
Negative	77/100 (77%)
Positive	23/100 (23%)
E-Cadherin	
P30 ^b (H-Score cut-off value)	46.11
Tumor cell count/case(median[IQR])	2850.8 (484.0 - 5198.0)
200x fields/case (median[IQR])	5 (2 - 6)
P-Cadherin	
Negative	84/100 (84%)
Equivocal/Positive	16/100 (16%)

Figure 1

Immunohistochemical expression of E-Cadherin and P-Cadherin in prostate cancer. (a-b) E-Cadherin membrane immunoexpression: (a) High immunoexpression (200x) and (b) Low immunoexpression (200x). (c-e) P-Cadherin membrane immunoexpression: (c) positive staining (200x); and (d) equivocal staining (200x); and (e) negative staining (200x).

Figure 2

Cohort stratification according to WHO prognostic grade groups and pT stage, concerning disease-specific survival. (a) Disease-specific survival according to pT stage; (b) Disease-specific survival according to WHO prognostic grade groups.

Figure 3

Disease-specific survival according to biomarker expression levels in pT3a prostate cancer patients: (a) E-Cadherin; and (b) P-Cadherin.

Figure 4

Disease-specific survival according to biomarker expression levels in pT3b prostate cancer patients: (a) E-Cadherin; and (b) P-Cadherin.

Figure 5

Cohort stratification according to WHO prognostic grade groups, concerning disease-specific survival.

Supplementary Material

A general overview on Cadherins' role in Prostate Cancer

PCa is widely recognized as one of the most relevant medical conditions in the male population. It represents the most common malignancy and a major cause of cancer-related death in men[1]. An estimated 161,360 new cases of PCa were diagnosed in 2017 and 26,730 deaths due to PCa were expected in the USA[2]. PCa represents almost 1 in each 5 new diagnoses of cancer in men[2] and, despite substantial progress in understanding its biological mechanisms and improvements both in diagnosis and treatment, it still accounts for 1-2% of deaths in men[1].

PCa is a worldwide major health and economic burden in our currently aging population. In fact, the best established risk factor for PCa is aging - at 80 years of age, the estimated incidence reaches 80%[56]. Other established risk factors include race and family history. Obesity, diet and other environmental factors have also been associated with PCa[57,58,1].

Acinar adenocarcinoma is the most common histological type of PCa, representing more than 90% of all cases. Other histological types and subtypes include mucinous, ductal, signet ring cell, small cell, clear cell, squamous cell and urothelial carcinomas, as well as sarcomas and lymphomas[59].

PSA screening has increased PCa awareness since its implementation as a screening tool in the late 1980s[3-5] and remains, until now, the only clinically implemented diagnostic and follow-up biomarker for PCa[6]. However, due to the frequently indolent course of the disease and the unspecific nature of the test, increased incidence of PSA-detected PCa has been largely associated with cancers that would not progress to an advanced stage and that are considered clinically insignificant[7]. The introduction of screening programs has allowed for the diagnosis of more organ confined lesions, but it is unclear whether this has improved overall survival[48]. Overdiagnosis has, thus, become a major challenge and ensues overtreatment consequences. The high risk of side effects associated with the current treatment techniques exacerbates these consequences, as it may compromise the patients' quality of life with little or no survival benefit[7].

Early-stage localized tumors are potentially treated by surgery, radiation and androgen-deprivation therapy[60]. However, even though PCa is traditionally considered a disease of elderly men, it presents with increasing tendency in men of pre-retirement ages[57]. Therefore, at present, active surveillance also represents state-of-the-art care for patients with localized PCa and a long life expectancy, possibly postponing definitive therapy[61].

In more advanced stages, these treatments are frequently unsuccessful, and after 18-24 months the disease progresses to its lethal stage, characterized by insensitivity to hormone deprivation (castration-resistant prostate cancer, CRPC), metastases and a short survival period[62]. In fact, PCa-related mortality is largely due to its high metastatic potential for bone and/or other organs[63,64], but fortunately this represents the minority of the patients[7].

Nevertheless, PCa encompasses a wide spectrum of clinical outcomes and its natural history is highly variable[8]: in some cases, it progresses to metastatic disease in patients with localized tumors treated radically; while in other cases the disease does not progress at all[9-11]. Patients with localized disease can

have very different prognoses and are advised on treatment based on risk assessment. Model equations are often used for this purpose, taking into account preoperative serum PSA, Gleason score and pathologic stage[12]. The National Comprehensive Cancer Network guidelines define high-risk localized PCa as initial PSA greater than 20 ng/mL, biopsy Gleason score equal or greater than 8 and clinical stage greater than or equal to T3a[65]. However, prediction of progression for the individual patient using these models is still not precise, and additional markers are needed to more accurately target high-risk patients and establish a treatment course[13].

A search for new biomarkers: The Cadherins

In recent years, efforts have been directed towards utilizing a combination of biological rather than clinical markers that may not only diagnose PCa, but also distinguish between indolent and clinically significant disease, predicting prognosis and response to therapy at the initial biopsy or surgical specimen[66,6]. More specifically, the current focus of PCa biomarker research is to identify a valid biomarker to complement PSA for screening, to define prognostic biomarkers with clinical utility, and to establish molecular stratification methods[1,66].

The identification of prognostic biomarkers gained considerable attention in recent medical literature and many molecules have been correlated with PCa progression and metastatic potential.

One group of proteins whose altered expression has been associated with tumor invasiveness, metastatic dissemination, and patient prognosis is the cadherins *superfamily*. Cadherins are a large multigene family of transmembrane glycoproteins with a crucial role in homophilic cell-cell adhesion, cell polarity, cell proliferation, migration, and differentiation[17,18].

It is possible to divide the cadherins *superfamily* into classical cadherins, which are the major components of cell-cell adhesive junctions; non-classical cadherins, which include desmosomal cadherins; and protocadherins, which are implicated in neuronal plasticity[67,68]. Cadherins are very diverse both in structure and function, influencing biological responses in multiple and versatile ways[69].

The classical (or type I) cadherins have five extracellular cadherin repeats, a single transmembrane domain and a cytoplasmic domain[69]. The extracellular regions bind to other cadherins presented by neighboring cells, which allows for adhesive recognition[70]. Differently, the cadherin cytoplasmic tails interact with proteins, such as p120-catenin and β -catenin, that link the cadherin receptor to essential intracellular processes, including cell signalling, trafficking and actin cytoskeleton[71-73].

CDH1, also known as E-Cadherin, has been considered the paradigmatic classical cadherin and is mainly expressed in epithelial tissues. Other classical cadherins include CDH2, or N-Cadherin, often present in neural tissues; CDH3, or P-Cadherin, whose expression in human tissues partially overlaps with E-Cadherin; and CDH4, or R-Cadherin, expressed in the retina[69].

E-Cadherin and P-Cadherin and cancer

E-Cadherin plays a pivotal role in important morphogenetic and differentiation processes during development, and in maintaining integrity and homeostasis in

adult tissues[46]. Studies on the role of cadherins in cancer have been focused on E-Cadherin and its relationship with tumor progression and metastasis.

It has been recognized that E-Cadherin likely functions as an invasion suppressor gene/protein, since its loss of expression, abnormal function, or both, lead to an increased ability of cells to invade neighboring tissues[19]. In addition, E-Cadherin downregulation is considered a main indicator of epithelial/mesenchymal phenotype switch, which occurs during EMT[20].

Metastasis is a complex and multi-step process in the progression of cancer[74]. Cell adhesion molecules, including cadherins, play an essential role in the development of recurrent, invasive and distant metastasis[75]. EMT is an event in which the epithelial cells lose polarity and cell adhesion molecules, transforming into migrating mesenchymal cells[76]. The loss of epithelial cell differentiation and acquisition of a migratory and invasive phenotype is necessary for the dissemination of cancer cells, and is considered to be an early step in the metastatic cascade[24]. Therefore, EMT allows for the detachment of tumor cells from the primary site, followed by intravasation into the blood stream, extravasation into distant target organs, and formation of secondary lesions or metastases[21-23].

E-Cadherin has been consistently demonstrated to be downregulated, silenced or aberrantly expressed in multiple cancer types[24] and its fundamental role in tumorigenesis has been recognized, particularly in breast[25], prostate[26,27] and gastric carcinomas [28].

Deregulation of E-Cadherin may be explained by diverse mechanisms: E-Cadherin gene loss or abnormal expression, leading to the invasion of neighboring tissues/organs by cancer cells, as it has already been shown in some cancer types, such as breast and gastric cancer; transcriptional deregulation, with hypermethylation of its gene promoter – described both in epithelial, with greater emphasis on gastric cancer, and non-epithelial tumors, such as acute myelogenous leukemia -, overexpression of E-Cadherin repressors, histone deacetylation and/or microRNAs regulation of E-Cadherin gene expression; post-translational deregulation, which might include trafficking deregulation and/or aberrant glycosylation; and aberrant activation of signaling pathways and interaction with other molecules, such as cytoskeletal components, integrins, and growth-factor receptors[46].

E-Cadherin is just one of more than 110 cadherin *superfamily* members, many of which are still poorly studied[29,77]. Consequently, other cadherins may also have tumor-suppressing activity or otherwise function synergistically or antagonistically with respect to E-Cadherin, or have entirely separated tumor-related roles[69].

P-Cadherin is another classical cadherin, which has also a crucial role in the conservation of epithelial tissues' structural integrity. P-Cadherin regulates several cellular homeostatic processes that contribute to embryonic development and maintain adult tissue architecture, similarly to other members of the cadherins *superfamily*[78-80]. It shares about 67 % of homology with the E-Cadherin protein, differing mainly in their extracellular portion[29,30]. P-Cadherin is co-expressed with E-Cadherin in a number of adult tissues, such as the basal layer of the epidermis, breast and prostate glands[53,81]. Despite its role in the maintenance of tissue architecture[82,83], P-Cadherin is markedly involved in disease states, namely in specific hereditary genetic disorders and in cancer[31].

The cancer-related function of P-Cadherin is still an object of debate, as it is considered to be context dependent. In some tumors, the induction of this cadherin is associated with E-Cadherin downregulation and possibly N-Cadherin upregulation - also known as the cadherin switch, which occurs during EMT and correlates with tumor progression[54,55]. P-Cadherin overexpression has been found in more invasive rather than *in situ* lesions, showing that its aberrant expression could be a useful marker of invasion capacity of tumor cells - for example, in breast cancer, P-Cadherin expression is strongly related with a decreased patient survival and is considered a valuable prognostic factor[84]. In other tumors, studies suggest that P-Cadherin functions as an adhesion-promoting and anti-invasive protein, behaving similarly or identically to E-Cadherin: for example, in colo-rectal cancer, P-Cadherin has been found to be strongly expressed in well-differentiated tumors, but downregulated in poorly differentiated ones[85].

The mechanisms responsible for P-Cadherin altered expression are also diverse, but by far less characterized than those of E-Cadherin. One of those mechanisms is P-Cadherin transcriptional deregulation, with hypomethylation of a specific region of P-Cadherin gene promoter[86]. Aberrant activation of signaling pathways might also play an important role in P-Cadherin altered expression, but knowledge in this area is still insufficient, as a consequence of P-Cadherin dual role as an invasion promoter or invasion suppressor molecule, depending on the cell model under study. Still, it is expected that P-Cadherin, when acting as an invasion suppressor molecule, shares common signaling pathways with E-Cadherin, even though these might not be triggered in the same way[46].

E-Cadherin and P-Cadherin and prostate cancer

To date, several published studies have examined the association between E-Cadherin expression and PCa clinicopathological features (Supplementary Table I). Using a variety of sources of patient tissues and employing either formalin-fixed and paraffin-embedded or fresh frozen tissues, several of these studies have correlated the reduced expression of E-Cadherin with loss of differentiation, increased invasiveness, advanced clinical stage, and poor survival in PCa.

Bussemakers *et al.*, using the Dunning rat model of PCa, first described a strong correlation between the reduction of E-Cadherin expression and metastatic potential, as E-Cadherin-positive tumors were found to be non-invasive, whereas E-Cadherin-negative tumors were highly metastatic[47]. Shortly afterwards, Umbas *et al.* conducted some of the first studies that investigated the aberrant expression of E-Cadherin in human PCa specimens and its relationship with tumor grade and prognosis, demonstrating that reduced expression of E-Cadherin correlated with higher tumor grade and poor prognosis[48,87]. Meng *et al.* developed a study that aimed to investigate the expression of 5-lipoxygenase (5-LOX) in metastatic PCa and whether *zileuton*, the inhibitor of 5-LOX, played a role in the metastasis of PCa. In this study, Meng *et al.* reported that PCa metastasis was inhibited in mice that received a high dose of *zileuton*, which they found to restore normal expression of E-Cadherin[88].

However, the question of whether the evaluation of the expression levels of E-Cadherin in PCa specimens provides prognostic value, independent of well-recognized markers of prognosis, is still open to debate.

In fact, a recent prospective study conducted by Ipekci *et al.* failed to demonstrate an association between E-Cadherin expression in initial prostate

tumor samples and tumor stage, grade, initial PSA level and disease-free survival, with a 36-month follow-up. The explanation given was that EMT is an early event in tumor progression and, as such, assessment of primary tumor does not give any insight into the metastatic potential of cells, that require having other alterations to survive in distant tissues. The recurrent assessment with PSA rather than with clinical and imaging results, and the short follow-up period were pointed out by the authors as possible limitations to the study[66].

A number of studies also focused on studying E-Cadherin expression in PCa metastases, and the results have been controversial. There is increasing evidence of high expression or re-expression of E-Cadherin in advanced metastatic tumors, which may also serve as a marker for tumor recurrence[89,90]. Specifically in PCa, compared to the molecular profiles of the primary lesions, E-Cadherin has been shown to be re-expressed in advanced disease[91] and in metastases[92]. In their study, De Marzo *et al.* found that most metastatic PCa cells in pelvic lymph nodes expressed E-Cadherin[93]. These findings support the hypothesis that, though E-Cadherin downregulation plays an important role in the early steps of metastasis, the establishment of a distant colonization and growth of the subsequent metastasis may be facilitated by E-Cadherin expression. Additionally, these findings also suggest that E-Cadherin downregulation is not mediated by irreversible genetic inactivation - through mutation or allelic loss -, but rather by reversible epigenetic mechanisms, such as promoter methylation and transcriptional and/or post-transcriptional regulation. Therefore, it has been suggested that the ability to modulate E-Cadherin expression - E-Cadherin plasticity-, rather than its absolute expression levels, may be more reliable when indicating cancer cell stemness and invasive capacity and a potential target for novel therapies[24].

Comparing to E-Cadherin, P-Cadherin's role in PCa has been scarcely studied. Aberrant expression of P-Cadherin associated with aggressive tumor behavior has been observed in breast[86], gastric[94], endometrial[95,96], ovarian[97], pancreatic[98], bladder[99] and colo-rectal carcinomas[53]. In most of these cases, P-Cadherin appears upregulated in the earlier stages of the malignant progress[100,101]. However, opposite effects have been found for P-Cadherin in PCa[31] and the prognostic implications of these findings are still unknown.

A study conducted by Arenas *et al.* showed that P-Cadherin expression in PCa specimens was increased compared to normal prostate tissue, suggesting an up-regulation of P-Cadherin expression in PCa neoplastic tissue/cells and, therefore, a tumor promoting behavior[49]. Moreover, Gravdal *et al.* demonstrated that positive membranous expression was significantly related to higher local Gleason score and to poorly differentiated cancer, and also to shorter time to bone metastases[50].

Differently, Jarrard *et al.* concluded in their study that P-Cadherin protein expression was absent in all of the 25 well to poorly differentiated primary prostatic cancer specimens evaluated. In addition, a decrease in P-Cadherin staining was noted during the transition from a normal acinar gland to prostatic intraepithelial neoplasia, implying that that P-Cadherin is down-regulated in PCa and, therefore, possibly acts like a tumor suppressive molecule[51].

That being said, it is still unclear whether the evaluation of E-Cadherin and P-Cadherin expression levels in PCa specimens provides prognostic information, independently of well-recognized prognostic parameters.

Supplementary Table I - Summary of published studies on E-Cadherin expression in PCa (adapted from Rubin et al[102]).

Study Design						Aberrant E-Cadherin Expression (%)	Statistically Significant Association With Decreased E-Cadherin Expression						Year
Study	N	Source	Clinical Presentation	Tissue	Staining Interpretation		PSA Level	Grade	Stage	Metastasis	Volume	Survival	
Umbas et al[87]	92	RP TURP	Loc (47)/ Adv/Met (45)	FZ	Pos/Neg/Het/Alt	45-75	-	Sig	Sig	Sig	-	-	1992
Umbas et al[48]	89	RP TURP	Loc (59)/ Adv/Met (30)	FZ	Pos/Neg/Het/Alt	33-76	-	Sig	Sig	Sig	-	-	1994
Cheng et al[103]	53	RP	Loc	FF	0-3*	50-78	-	Sig	-	Sig	-	-	1996
Richmond et al[40]	99	TURP	Loc(35)/ Adv/Met(64)	FF	Pos/Neg/Het/Alt	56	-	Sig	-	Sig	Sig	Sig	1997
Umbas et al[104]	52	RP TURP	Loc(20)/ Adv/Met(32)	FZ	Pos/Neg/Het/Alt	44	-	-	Sig	-	-	Sig	1997
Kuczyk et al[105]	67	RP	Loc	FZ/FF	40% cut off	19	Neg	Neg	-	-	-	Neg	1998
Pan et al[106]	51	NP	Loc(31) Adv/Met(22)	FZ	Pos/Neg/Het/Alt	72	-	-	-	Sig	-	-	1998
De Marzo et al[93]	76	RP	Loc	FF	70% cut off	NP	-	Sig	Sig	Neg	-	-	1999
Morita et al[107]	45	RP	NP	FF	Pos/Neg/Het	47	-	Sig	-	-	-	-	1999
Dunsmuir et al[108]	156	TURP	Loc(76)/ Adv/Met(80)	FF	Nor/Aber	NP	-	Sig	Neg	-	-	Neg	2000
Kallakury et al[109]	118	RP	Loc(50)/ Adv/Met(68)	FF	Weak/Mod/Int Dif/Int reg/Int dif	25	Neg	Sig	Sig	-	-	-	2001

Loric et al[41]	44	RP	Loc	FF	Pos/Neg/Alt	52	-	Neg	Neg	-	-	Sig	2001
Rubin et al[102]	138	RP RA	Loc (128)/ Adv/Met (12)	FF	70% cut off	20	-	Neg/Trend	Neg	Neg	Sig	Neg/Trend	2001
Köskal et al[110]	58	TURP	Loc	FF	Pos/Neg/Het	74	-	Neg	Neg	-	-	-	2002
Wu et al[111]	70	RP	Loc	FF	Pos/Neg/Het	56	-	-	-	-	-	Sig	2003
Hsu et al[112]	122	RP	Loc(72)/ Adv/Met(50)	FF	Pos/Neg/Het/Alt	65	Neg	Neg	Neg	-	-	-	2004
Jaggi et al[113]	16	RP	Loc(14) Adv/Met(2)	FF	0-3*	NP	-	Sig	Neg	-	-	-	2005
Rao et al[114]	483	RP	NP	FF	0-3*	58	-	-	Sig	-	-	Neg	2006
van Oort et al[42]	65	RP TURP	Loc(20)/ Adv/Met(45)	FZ	Nor/Aber	45	-	Sig	Neg	-	-	Sig	2007
Yuen et al[115]	107	RP NP TURP	Loc(56)/ Adv/Met(51)	FF	70% cut off	47	-	Sig	-	Sig	-	-	2007
Behnsawy et al[43]	197	RP	Loc	FF	0-3*	76	Sig	Sig	Neg	Sig	Sig	Sig	2013
Lazari et al[26]	135	RP	Loc(120)/ Adv/Met(15)	FF	1-3**	27	-	-	Sig	-	-	-	2013
Whiteland et al[44]	215	TRUSB RP TRUP	NP	FF	0-3*	NP	Neg	Sig	Sig	-	-	Sig	2013
Liu et al[116]	59	NB RP	Loc(40) Adv/Met(19)	FF	90% cut off	68	Sig	Neg	Neg***	Neg	-	-	2014
Ipekci et al[66]	103	RP	Loc(57) Adv/Met(46)	FF	0-3*	20-41	Neg	Neg	Neg	-	Neg	-	2015

Ma et al[45]	128	RP	Loc(107) Adv/Met(21)	FF	10% cut off	53	-	-	-	-	-	Sig	2015
Wang et al[117]	46	NP	Loc(18)/ Adv/Met(28)	NP	0-3*	NP	-	Sig	-	Sig	-	Neg	2015
Bengallol et al[118]	50	RP	Loc	FF	0-3*	NP	Sig	Sig	Neg	-	-	-	2016
Drivalos et al[13]	157	RP	Loc	FF	0-3*	58	Sig	Sig	Sig	Sig****	-	-	2016
Missaoui et al[119]	50	RP TURP NB TA	Loc(39)/ Adv/Met(11)	FF	Pos/Neg	NP	Neg	Neg	-	-	-	Neg	2016
Abdelrahman et al[34]	50	OP RP TRUSB	Loc(20)/ Adv/Met(30)	FF	70% cut off	62	Sig	Sig	Sig	Sig	-	Sig	2017
Figiel et al[120]	242	RP	Loc(152) Adv/Met(90)	FF	70% cut off	44	-	Sig	Neg	-	-	-	2017

N - number of patients

NP - information not provided in text

RP - radical prostatectomy; TURP - transurethral resection of prostate; TRUSB - trans-rectal ultra-sound guided biopsy; NB - needle biopsy; OP - open prostatectomy; TA - transvesical adenomectomy

Loc - localized; Adv - locally advanced disease; Met - metastatic disease

FF - formalin fixed; FZ - frozen prostate tissue

Pos - positive; Neg - negative; Alt - altered; Het - heterogeneous; Nor - normal; Aber - aberrant; Mod - moderate; Int - intense; Reg - regional; Dif - diffuse; Sig - significant

*0 (no staining at all), 1 (weak), 2 (medium) or 3 (strong)

**1 (weak positive staining), 2 (moderate staining) or 3 (strong positive staining)

*** Whitmore-Jewett staging system

**** Lymphatic metastasis

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Differential expression of E-Cadherin and P-Cadherin in pT3 prostate cancer: correlation with clinical and pathological features
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