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Abnormal E-cadherin expression and prostate cell blood dissemination as markers of biological recurrence in cancer

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Abstract

Until now, no molecular parameter has been available for predicting the metastatic potential of prostate tumours, which leaves their outcome uncertain despite an apparent benign histology or early stage. Abnormal expression of adhesion molecules, such as E-cadherin, can be contributing factors for increased invasiveness and metastatic potential. Histological analysis for E-cadherin expression was carried out on paraffin-embedded tumour tissues. Tumour metastatic potential was indirectly evaluated by detecting circulating prostate cells (CPC), using reverse transciptase-polymerase chain reaction (RT-PCR) and prostate-specific membrane antigen (PSMA) as a target. Patients were followed-up for a median of 14 months (range 10–19 months) after surgery with serum prostate-specific antigen (PSA) level measurement. Interestingly, 23 of 44 localised tumours exhibited aberrant E-cadherin expression. Prior to primary surgery, *PSMA* RT-PCR detected the spread of prostate cells to the blood in 24 patients. Statistical analysis showed that abnormal E-cadherin expression in the tumours was the only variable that was independently correlated with prostate cell dissemination in the blood ($P < 0.0001$). In logistic regression analysis, abnormal E-cadherin expression was a significant independent predictor for a later biological relapse. This impaired adhesion status was clearly correlated with a haematogenous spread of the primary tumour cells. It could therefore be an objective way to restrict the indications for radical surgery to patients not presenting with this feature.

Keywords: Adhesion; Metastasis; Cadherin; Cancer; Circulating cells

1. Introduction

Prostate cancer is the malignancy with the highest incidence in the West [1]. At clinical presentation of prostate cancer, many patients have disease extending beyond the prostate (loco-regional or distant metastases). The clinical consequences of established metastatic disease are important, since no curative therapy is available. Patients with localised tumours can be cured with radical prostatectomy (RP), but a large percentage will undergo clinical progression to metastatic disease [2]. At present, no screening method for males over 50 years old (digital rectal examination, serum prostate-specific antigen (PSA) levels, etc.) is available for making a distinction between (i) patients with small tumours that do not need any treatment, (ii) those who should undergo RP, and with a low risk of progression, and (iii) those who should undergo surgery, but who are likely to progress to a metastatic status [3].

The natural history of and prognostic factors involved in prostate cancer are not clearly defined. Hence, molecular parameters able to accurately assess the aggressiveness and the metastatic potential of the cancer are urgently needed [4]. Since vascular invasion and the spread of prostate tumour cells to the blood represent preliminary steps in the metastatic process, blood-borne detection of circulating prostate epithelial cells (CPC) could be an early marker of invasiveness. The recent development of sensitive molecular techniques evidenced such cells in the blood of patients with localised prostate cancer, and has been proposed as a new staging modality (for review, see Refs. [5,6]). Nevertheless, the

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frequency with which epithelial cells are found in the blood of patients with prostate cancer has raised doubts as to whether the discovery of such disseminated cells really matters.

The metastatic process consists of a complex pattern of sequential steps whose primary event requires the detachment of cells from the primary tumour. *In vitro* experiments have evidenced that one of the critical components is the transmembrane adhesion catenin/cadherin complex [7]. E-cadherin (CDH-1), a calcium-dependent cell adhesion molecule which is part of that complex and plays a key role in maintaining the epithelial phenotype, has been proposed as a potential biochemical marker for tumour progression [8]. This protein is commonly downregulated in various carcinomas (for review, see Ref. [9]) and is now proposed as a potent invasion-suppressor gene. Moreover, in different human cancers [8], aberrant CDH-1 expression in the tumour correlates with a poorly differentiated and invasive phenotype. This suggests the possible importance of this adhesion molecule in facilitating the spread of already invasive tumours.

Since modification of CDH-1 expression is likely to be involved in prostate cancer invasion, we analysed in this study CDH-1 expression within the prostate of patients with clinically localised prostate cancer, and also evaluated prostate cell spread prior to RP.

2. Patients and methods

2.1. Patient samples

After giving informed consent, 44 consecutive patients with localised prostate cancer in the TNM stage N0 M0 (i.e. no diagnostic signs of distant metastasis) were admitted to the study. The preoperative diagnostic staging included serum PSA levels, digital rectal examination (DRE) and transrectal ultrasonogram. No patients received neoadjuvant anti-androgen therapy prior to surgery. The patient characteristics are listed in Table 1. The staging of prostate cancer was made with TNM and pTNM/International Union Against Cancer (UICC) 92 2nd edition classification. Pathological staging was performed on serially sampled RP tissue after formaldehyde fixation and paraffin embedding. The histological grade according to Gleason's score, capsule invasion and surgical margin status were assessed on routinely stained sections. Histologically, all tumours were adenocarcinomas. All patients had survived following surgery and were followed for 10–19 months after RP.

2.2. Immunohistochemistry

Immunohistochemical procedures were performed on paraffin-embedded sections from three to five blocks per case using the streptavidin-biotin peroxidase method and the recently described E-cadherin labelling method for paraffin-embedded tissues [10] was applied using an automated immunostainer (Techmate 500, DAKO, Trappes, France). Before detection of the E-cadherin (CDH-1) epitope, slides were microwave preheated. The primary antibody (anti-CDH-1 monoclonal mouse antibody, Transduction Laboratories, Lexington, USA) was used at a 1:1000 dilution. As negative controls, we replaced primary antibody by non-immune mouse immunoglobin, phosphate-buffered saline or irrelevant antibodies. Localisation and immunostaining intensity were assessed by two independent pathologists in a simultaneous reading, who were blinded to the results of grading and staging. To assess staining, intensity was given as normal or decreased (uniformly decreased to negative staining). Besides positive (Fig. 1a and d) or negative (Fig. 1b) staining, some tumours showed cytoplasmic staining which was considered as abnormal (Fig. 1d). Uniformly positive staining patterns with strictly membranous expression were regarded as normal, while uniformly decreased or negative and cytoplasmic staining was included in the criteria for aberrant expression. This evaluation related to the mean result of staining of all the slides for a given patient.

2.3. RT-PCR procedures

Blood sample specimens were collected 1 day before RP for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, 2 weeks after RP and 1 year after for serum PSA measurements. For all patients, blood samples were collected at least 2 weeks after digital rectal examination or prostate needle biopsy. Venous blood (4×5 ml) was collected in ethylene diamine tetra acetic acid (EDTA)-treated tubes, placed at +4°C and processed within 3 h. Blood samples were diluted in two volumes of ammonium chloride 9 g/l, then shaken overnight at +4°C. After centrifugation at 1000*g* for 20 min, the supernatant was removed before RNA extraction. The RNA pellet obtained after ethanol/sodium acetate precipitation was dried under a vacuum and dissolved in 50 μl of RNAse-free water. Quantification of RNA was performed by RiboGreen™ spectrofluorometric analysis (Molecular Probes, Leyden, The Netherlands) according to the manufacturer's instructions. Precautions were taken to avoid contamination in all PCR procedures, including aliquoting all reagents, use of positive-displacement pipettes, filtered pipette tips and positive, negative, internal (*β-globin*) and reagent controls. To assess the integrity of the different RNAs extracted from blood samples, a region of the *β-globin* gene was amplified. The 283 bp-sized *β-globin* fragments could be detected in all of these samples, indicating the absence of possible inhibitors of PCR (Fig. 2b). *PSMA* RT-PCR was performed as previously described in Ref.

Table 1
Circulating prostatic cells, tumour E-cadherin expression analysis and biological outcome in localized prostate cancer patients

Patient	Age (years)	Preoperative status		Tumour analysis				One-year survey
		Serum PSA (ng/ml)	CPC	Stage/ Gleason	Surgical margins	Capsular penetration	E-cadherin expression	Serum PSAus (ng/ml)
1	64	2.1	POS	pT2/7	NEG	NEG	ABN (CYT)	0.5
2	61	6.9	POS	pT2/6	NEG	NEG	ABN (DEC)	<0.1
3	70	1.1	POS	pT2/7	NEG	NEG	ABN (CYT)	0.3
4	72	15.1	NEG	pT2/7	NEG	NEG	NOR	0.1
5	55	19.1	POS	pT2/7	NEG	NEG	ABN (CYT)	1.2
6	58	18.0	POS	pT3/7	NEG	NEG	NOR	0.1
7	55	29.7	POS	pT2/7	POS	POS	ABN (DEC)	0.7
8	56	18.5	POS	pT3/7	NEG	NEG	ABN (CYT)	0.4
9	54	6.7	NEG	pT2/7	POS	NEG	NOR	0.1
10	63	2.6	POS	pT2/6	NEG	NEG	NOR	0.2
11	67	5.5	NEG	pT3/7	NEG	NEG	NOR	<0.1
12	68	9.2	NEG	pT2/7	NEG	NEG	NOR	<0.1
13	67	21.3	POS	pT2/8	NEG	NEG	ABN (DEC)	0.5
14	69	8.4	POS	pT3/8	POS	POS	ABN (DEC)	0.1
15	69	16.0	POS	pT2/7	POS	NEG	ABN (DEC)	0.3
16	73	8.1	NEG	pT3/7	NEG	NEG	ABN (DEC)	0.2
17	44	28.0	NEG	pT3/7	POS	NEG	NOR	0.1
18	65	11.8	POS	pT3/7	POS	POS	ABN (DEC)	0.5
19	75	7.8	NEG	pT2/6	NEG	NEG	NOR	<0.1
20	66	4.6	POS	pT3/7	POS	POS	ABN (DEC)	0.4
21	69	13.4	POS	pT3/7	NEG	NEG	ABN (DEC)	0.3
22	66	1.3	NEG	pT2/6	NEG	NEG	NOR	<0.1
23	65	7.3	NEG	pT2/8	NEG	NEG	ABN (DEC)	<0.1
24	71	18.2	POS	pT3/6	POS	POS	ABN (DEC)	0.4
25	59	12.4	NEG	pT2/7	NEG	NEG	ABN (CYT)	0.3
26	54	23.0	POS	pT3/7	POS	NEG	NOR	0.3
27	70	24.1	POS	pT2/6	NEG	NEG	ABN (DEC)	0.1
28	72	9.3	NEG	pT3/7	NEG	NEG	NOR	0.1
29	70	20.0	NEG	pT3/7	POS	NEG	NOR	<0.1
30	60	7.3	POS	pT2/7	NEG	NEG	ABN (DEC)	0.3
31	66	7.1	NEG	pT2/7	NEG	NEG	NOR	<0.1
32	71	29.6	POS	pT3/8	POS	POS	ABN (DEC)	2.8
33	60	12.2	POS	pT3/7	NEG	NEG	NOR	<0.1
34	70	2.5	NEG	pT2/6	POS	NEG	NOR	<0.1
35	86	11.7	NEG	pT2/7	NEG	NEG	NOR	<0.1
36	68	12.3	NEG	pT3/6	POS	NEG	NOR	<0.1
37	67	8.9	NEG	pT2/6	NEG	NEG	NOR	<0.1
38	71	10.0	POS	pT2/6	NEG	NEG	ABN (CYT)	0.3
39	62	5.9	POS	pT3/8	POS	POS	ABN (CYT)	0.5
40	69	9.1	POS	pT2/7	POS	NEG	ABN (DEC)	0.6
41	54	5.3	NEG	pT2/6	NEG	NEG	NOR	<0.1
42	57	15.7	POS	pT3/9	POS	POS	ABN (DEC)	0.5
43	67	3.5	NEG	pT2/6	NEG	NEG	NOR	<0.1
44	72	9.7	NEG	pT2/7	NEG	NEG	NOR	<0.1

NEG, negative, POS, positive, NOR, normal; ABN, abnormal; DEC, decreased; CYT, cytoplasmic; PSAus, ultrasensitive prostate-specific antigen (PSA); CPC, circulating prostate cells.

[11]. Briefly, RNA samples were reverse-transcribed in cDNA with downstream *PSMA* oligonucleotide as primers. Then, 1 μl of cDNA was amplified by PCR with outer *PSMA* primers in an MJResearch DNA thermal cycler (TechGen, Les Ulis, France) according to the following programme parameters: 94°C for 1 min (2 min for the first cycle); 62°C for 1 min; and 72°C for 1 min (10 min for the last cycle) for 25 cycles. A 3-μl aliquot of amplimers was amplified in a nested fashion through 25 new cycles with the *PSMA* inner couple of primers (Fig. 2a). Until now, in our own experience on more than 150 negative controls, every patient without prostate disease was found to be negative for this assay. *β-globin* PCR was performed under the same conditions, except for the hybridisation temperature and the number of PCR cycles (respectively 58°C and 35 cycles). Fragment fractionation of the PCR mixtures was carried out on 2% agarose gel (FMC, Rockland, USA) stained with ethidium bromide and visualised with ultraviolet (UV).

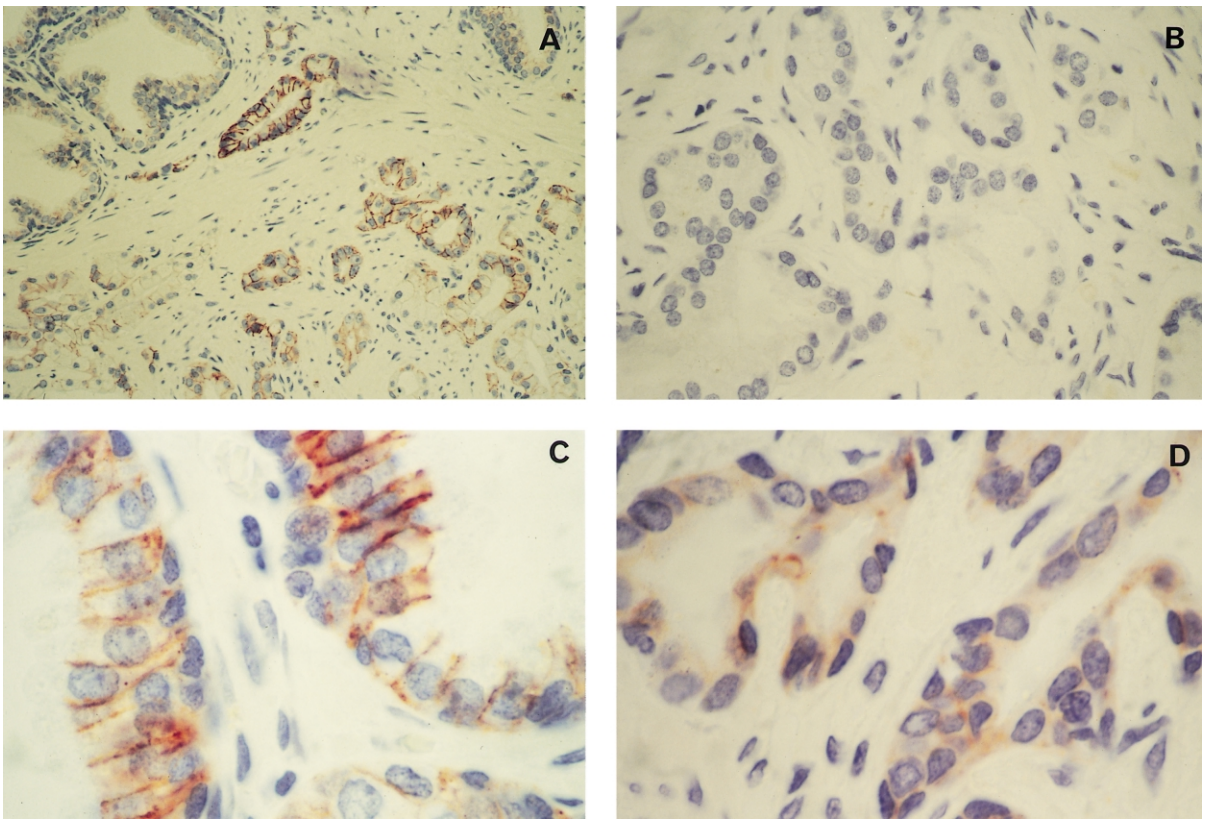

Fig. 1. Anti-CDH-1 antibody immunohistochemical analysis of normal (a, c) and neoplasic (b, d) prostate. The E-cadherin expression is normal at the cell–cell border (c: ×400 magnitude) of epithelial cells surrounding ducts (a: whole tissue ×100 magnitude). A decreased or absent expression of E-cadherin is observed within the tumour tissue (b: ×100 magnitude). In a subset of patients, a cytoplasmic expression of E-cadherin is observed (d: ×400 magnitude).

2.4. PSA measurements

Serum PSA and ultrasensitive PSA (PSAus) measurements were performed using a kit from Hybritech (Palo Alto, USA). Biological recurrence was defined as PSAus $\geqslant 0.3$ ng/ml but was considered as clinically relevant after two successive concentration measurements at or above that level.

2.5. Statistical analysis

Differences in the frequency of *PSMA*-positive circulating cells and CDH-1 tumour abnormal expression were analysed by the Fisher's exact test. The value of significance was taken as $P<0.05$. To determine the independent predictive value of the different parameters, a logistic regression model was used (NCSS 9.0 software).

3. Results

3.1. Patients

All prostate cancers were acinar adenocarcinomas and the mean ± standard deviation (S.D.) Gleason score was 6.9 ± 0.7. Twelve of them were low grade (Gleason <7) and 32 were considered as high grade (Gleason $\geqslant 7$). 24 patients were diagnosed at pathological stage pT2, and 20 were at pT3. All clinical data are summarised in Table 1.

3.2. Immunostaining of E-cadherin

In normal appearing prostate tissue, E-cadherin expression was always observed (Fig. 1a). In all prostate glands with a normal expression of CDH-1 (21 out of 44), CDH-1 immunolabelling was restricted to the basolateral membrane of the prostate epithelial cells (Fig. 1c). In contrast, 52% (23 out of 44) of the excised tumours displayed abnormal CDH-1 expression. The presence of a decreased CDH-1 expression was observed in 16 out of 23 (70%) with only 2 patients (nos. 20 and 42) with a total lack of E-cadherin staining (Fig. 1b). In the remaining 7 patients, a cytoplasmic diffuse expression of CDH-1 was evidenced (Fig. 1d).

3.3. Correlation with pathobiological data

Prior to surgery, we studied the blood of patients for prostate epithelial cell dissemination. Among the 44

patients, 24 (55%) were found pre-operatively to be positive for *PSMA* RT-PCR. 20 out of 24 patients (83%) with circulating prostate cells had abnormal expression of CDH-1 within their tumours, whereas 17 out of 20 patients (85%) without CPC had a normal plasma membrane CDH-1 expression.

The interdependence of variables (pre-operative PSA levels, tumour stage, histological grading, positive surgical margins, capsular penetration, CDH-1 expression, CPC) was examined next and results showed that aberrant CDH-1 expression ($P<0.0005$, Fisher's Exact test) and capsular invasion ($P<0.005$, Fisher's Exact test) were the only two variables significantly associated with the presence of CPC.

In multivariate analysis, aberrant CDH-1 expression was the only factor associated with the presence of CPC ($\chi^2=7.86$, $P<0.005$). This occurrence remained significant, even though only patients with decreased CDH-1, and not those with cytoplasmic CDH-1 expression were considered.

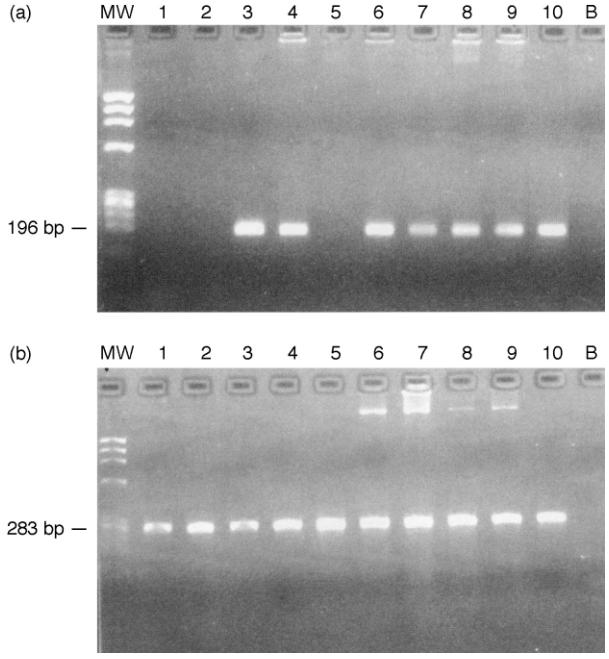

Fig. 2. Ethidium bromide stained gel depicting *PSMA* transcripts reverse transcriptase-polymerase chain reaction (RT-PCR) detection (a) in fresh blood of preoperative prostate cancer patients. Corresponding *β-globin* amplification (b) was tested prior to *PSMA* amplification to rule out degraded RNA purity and test subsequent reverse transcription efficiency. For all samples, except lane B, which illustrates a negative control without cDNA, a unique 283 bp band has been amplified for β-globin. Concerning clinical samples, patients in lanes 3, 4, 6–10 (respectively patient nos. 10, 13, 18, 26, 27, 32 and 38) were found to be positive for *PSMA* RT-PCR, whereas no *PSMA* transcripts have been amplified for the others (lanes 1, 2, 5; patient nos. 4, 9, 16). The specificity of the *PSMA* amplification was ascertained by the visualisation of a unique 196 bp band (which has been sequenced for some samples and found to correspond at every nucleotide to the GeneBank *PSMA* data (accession number m99487). MW, molecular weight.

Furthermore, during follow-up, the occurrence of a biological recurrence (serum PSAus $\geqslant 0.3$ ng/ml on two repeated experiments) was observed in 19 out of 44 patients (43%). It was most striking to see that rising PSAus occurred in 78% (18 out of 23 patients) with abnormal CDH-1 expression, whereas it was observed in only 1 out of 21 (5%) patients in the CDH-1 normal group (Fig. 3). This difference was statistically significant ($P<0.0001$, Fisher's Exact test).

Blood CPC detection paralleled this result: A prostatic blood spread was evidenced in 75% (18 out of 24) of patients. Only 1 patient out of 20 (5%) who was found negative for haematogenous dissemination underwent a 1-year biological recurrence.

In multivariate statistical analysis, CDH-1 aberrant tumour expression ($\chi^2=4.09$, $P<0.05$) proved to be an independent predictor of biological recurrence, as was pre-operative CPC ($\chi^2=6.77$, $P<0.001$), but not tumour stage ($P=0.72$), Gleason score ($P=0.30$), positive surgical margins ($P=0.23$) or capsular involvement ($P=0.38$).

4. Discussion

Slow progression and local confinement, as well as the ability of radical surgery or radiation to result in long-term disease-free survival, could account for the clinical indolence of most prostate cancers. However, a proportion of these localised cancers can metastatise and

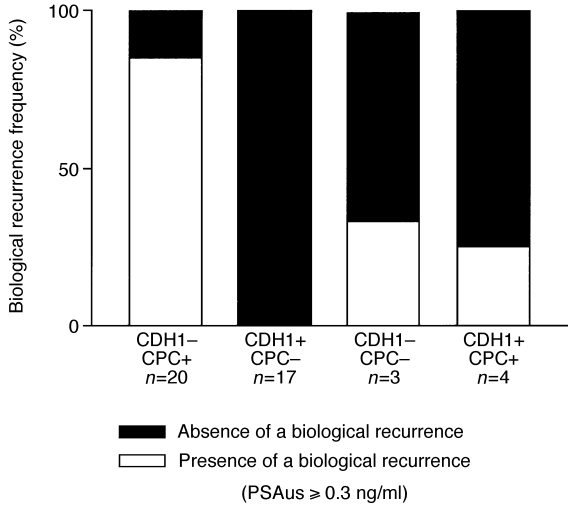

Fig. 3. Analysis of the frequency of biological recurrences at 1 year in prostate cancer patients. Four groups of patients were represented: group 1 (CDH-1-/CPC+): 20 patients found with aberrant E-cadherin tumour expression and blood circulating prostate cells; group 2 (CDH-1+/CPC-): 17 patients found with normal E-cadherin prostate expression and negative for *PSMA* RT-PCR; group 3 (CDH-1-/CPC-): 3 patients found with aberrant E-cadherin tumour expression, but without detectable circulating prostate cells; group 4 (CDH-1+/CPC+): 4 patients found with normal E-cadherin tumour expression, but positive for *PSMA* RT-PCR.

lead to significant morbidity and mortality [12]. In the earliest stages, metastatic disease is often clinically indistinguishable from organ-confined disease, although 40–50% of patients who are thought to have clinically localised carcinomas already have disseminated disease and are clinically understaged [4]. While organ-confined cancers can be cured by radical surgery, the prognosis is poorer once the disease has spread to distant sites, and operative therapy is not appropriate for these patients. As the natural history of prostate cancer is not yet clearly defined, it is crucial to develop new biological or molecular markers to assess early on the aggressiveness of the cancer [13].

The mechanisms by which a tumour cell invades the surrounding structure is poorly understood. Among numerous factors, cadherins and catenins are thought to be key molecules involved in the maintenance of the integrity of the epithelium and are likely to be involved in the earlier steps of the metastatic process [14]. Indeed, a disruption in cadherin/catenin expression could account for both haematogenous and/or lymphatic spread of cancer cells. Since vascular migration of tumour cells represents one of the first steps in the initiation of metastasis, early detection of micrometastases, which may have clinical and therapeutic implications, has recently been improved by the use of very sensitive molecular tools. In the peripheral blood of prostate cancer patients, RT-PCR-derived protocols have demonstrated the presence of CPC in many patients with overt metastases and in a subset with clinically localised disease [5]. Nevertheless, as these cells have yet to be identified as malignant, no reports have clearly established a clinical link between a defect in the primary tumour and the presence of these cells in the bloodstream. Our preliminary results from immunohistochemical studies confirm previous results on CDH-1 in prostate cancer patients and demonstrate that abnormal CDH-1 expression correlates with a haematogenous spread of prostate cells. As aberrant CDH-1 expression not only includes CDH-1 staining loss, but also cytoplasmic immunostaining generally associated with α-catenin/β-, γ-catenin and APC/CDH-1 complex dysfunction, it could be postulated that one or several members of the catenin family may also be involved in tumour cell spillage [15]. Nevertheless, a strict relationship between CDH-1 aberrant expression and CPC was not observed in every patient. These discrepant results may be explained by other metastasis-related mechanisms (such as metalloproteinase overexpression, increased angiogenesis, etc.) [16], which, in turn, may be important steps in the progression of cancer. In this respect, we have recently demonstrated that *de novo* expression of the major hyaluronan receptor CD44 occurs in a subset of prostate cancers in correlation with the systemic spreading of prostate cells and might play a role in hyaluronan-mediated facilitation of invasion [17]. Hence, the implication of these early potential cofactors of spread remain to be carefully analysed in localised prostate cancers [18].

To date, more than ten published studies have examined the relationship between CDH-1 expression and either clinical and pathological variables in prostate cancers [19–28]. Most of them documented that normal CDH-1 expression was frequently found in low Gleason score tumours, whereas high grade prostate carcinomas often showed abnormal or decreased CDH-1 expression, generally related to either loss of heterozygosity on 16q [29] or epigenetic transcriptional/traductional regulations [30]. Only three reports, with 36-, 80- and 46-months follow-up studies of RP patients, respectively [22,25,26], have shown that the loss of CDH-1 expression in prostate cancer specimens was independently associated with disease progression. Recently, De Marzo and colleagues have suggested that CDH-1 expression should be routinely assessed on RP specimens by immunohistochemistry to predict the prognosis in patients at risk of disease recurrence [28]. Such standardised evaluation should help to select men at risk of early recurrence that justify the use of experimental adjuvant therapies. Concerning CPC, only limited data have suggested that their detection may play a role in the staging of prostate tumours and in the serial follow-up of patients after treatment [6]. Our study clearly demonstrates a significant correlation between the presence of CPC prior to RP and a defect in CDH-1 within the primary tumour. Moreover, we found a significant correlation between the level of CDH-1 expression with one-year post-surgical biological recurrence that was independent of other clinical and biological prostate cancer data.

As the abnormal expression of CDH-1 may represent an early event in the natural history of prostate cancers, and seems to support the occurrence of spread of prostate epithelial cells [21], and also may be informative regarding the likelihood of biological recurrences [22,26], this strongly suggests that CDH-1 analysis may be a useful method to be undertaken prior to any invasive treatments. Since RP is not the appropriate treatment for patients with already disseminated tumours, evaluation of this new and independent determinant of risk may be used to select early stage surgical candidates and may eliminate those for whom surgery will not be curative. To achieve such a goal, the benefit of evaluating CDH-1 expression on prostate biopsies was previously suggested by Ross and colleagues [31] but, according to Ruijter and coworkers, an extensive sampling and labelling of at least 6 biopsies should be taken for each patient to avoid underestimating the aggressiveness of the cancer [32]. Therefore, it remains to be clearly established on a large cohort of prostate cancer patients whether abnormal CDH-1 expression on prostate biopsies may be an informative means to assess

tumour aggressiveness. Nevertheless, because of the slow indolent course of prostate cancer, only a longer-term follow-up should clarify the role of these procedures in the non-invasive screening of prostate cancers.

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