

Potential usefulness of CTC detection in follow up of prostate cancer patients. A preliminary report obtained by using Adnagene platform

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Summary

Objective: Prostate cancer (PCa) represents one of the most important medical problems for males, being the second major cause of cancer death. Routinely, PCa patients are followed up with both periodic evaluation of serum PSA levels and imaging. Recently, alternative laboratory methods were proposed for PCa patients' monitoring, with contrasting results.

Aim of the present study was to evaluate the usefulness of a new commercially CE-IVD kit for detection of prostate circulating tumour cells. Our intention was to verify the Adnagene platform usefulness to identify patients with disease progression, whatever treatment ongoing, in order to modify the therapeutic process even before treatment failure is evident with imaging methods.

Materials and Methods: Twenty-one patients were enrolled and subdivided into three groups: n = 10 high risk tumor PCa patients; n = 6 low risk PCa patients; n = 5 subjects without any signs of PCa. AdnaTest Prostate Cancer kit was used for enrichment and molecular characterization of prostate circulating tumour cells.

Results: Healthy subjects (with BPH) and patients without metastases resulted as negative, while 3 out of 10 high risk PCa patients were positive at least for one molecular marker like PSA, while only two showed positivity for PSMA mRNA. Our results indicate that the test specificity is 100% and the sensitivity is 100%; of course the sample is too small to give it statistical validity. In detail we verified that only the "not responder" patients resulted positive for AdnaTest.

Conclusions: The present preliminary report provides evidence that isolation and detection of circulating tumour cells (CTCs) is feasible and it may be useful in the follow-up of patients with advanced prostate cancer. If the results of this preliminary study would be confirmed by a large prospective cohort study, it could be demonstrated that this test is a rapid diagnostic method, based on the analysis of a blood sample and useful to the clinician to decide when to change therapy for patients resistant to castration or able to confirm that, at that time, the therapy is effective.

KEY WORDS: Prostate cancer; Circulating prostate tumor cells; AdnaTest.

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INTRODUCTION

Prostate cancer (PCa) represents the second major cause of cancer death in men (1). Radical prostatectomy (RP) and radiotherapy (RT) are well-established primary therapeutic options for management of localized PCa, although cancer recurrence still remains a significant concern for patients. In fact, about 27-53% of patients

undergoing RT or RP will develop local or at distance metastases within 10 years from initial treatment and 16-35% of PCa patients will receive second-line treatment within 5 years of initial therapy (2, 3). Routinely, PCa patients are followed up with periodic evaluation of serum prostate-specific antigen (PSA) levels and imaging

(TC, RMN, Bone Scintigraphy, 18-F Choline PET) (4), although undetectable serum PSA does not necessarily indicate the absence of any potential metastatic risk.

In this way, the release of tumor cells into bloodstream could represent a peculiar sign of cancer aggressiveness both before and after therapy (5, 6): therefore, circulating tumour cells (CTCs) detection could provide precious informations to the clinician regarding disease status. In this context, due to the lack of additional markers able to early detect PCa relapse, use of reliable test for prostate CTCs detection could provide novel opportunities for alternative therapeutic approaches (7, 8).

Recent studies employed RT-PCR methods for detection of prostate-specific mRNAs in whole blood without any pre-selection of CTCs (9, 10). Prostate-specific mRNAs are considered as surrogate markers of CTC presence, although with conflicting results (11, 12). However, recent studies on individuals with prostate cancer at advanced stages, performed using the CellSearch platform (13-15), found that high CTC counts correlated with poor prognosis (13-18). CTC detection rates are really variable among studies, ranging from 54% to 62%, mainly depending on the characteristics of the patients studied (19-20). Some methods for CTC characterization in PCa patients are in developing or under clinical laboratory evaluation (20, 21).

A recent CE-IVD method, namely *AdnaTest Prostate Cancer Select plus AdnaTest Prostate Cancer Detect* (AdnaGen AG, Langenhagen, Germany), has been commercialized for laboratory CTCs detection through multiple amplification for PCa-associated transcripts (reviewed as possible useful markers for monitoring of PCa patients) (22-24).

In the present work, we evaluated AdnaGene method for discriminating PCa patients at risk for metastasis from those with remission or stable disease, in order to establish if this method may be predictive or not of disease recurrence, with any ongoing therapy.

We describe preliminary data that might suggest the use of this method for management of PCa patients and support the clinical utility of the assay here described.

MATERIALS AND METHODS

Patients selection and blood sampling (more details reported in supplemental files)

We selected 21 patients who were subdivided into three groups (Table 1), according to D'Amico (25) classification: n = 10 PCa patients belonging to Group I (conventionally defined as prognostic grouping stage IV), because of the high risk tumor class (Gleason Score (GS) = 8-10 or TNM > T2b N0 M0 or PSA > 20); n = 6 PCa patients included into Group II (conventionally defined as prognostic grouping stage I), who were defined as at low risk tumor class (GS = 2-6 and TNM = T1-T2a and PSA < 10); n = 5 patients with benign prostate hypertrophy, without any clinical and biochemical signs of PCa, used as negative controls (defined as Group III; (PSA < 2 and negative Digital Rectal Exploration or TRUS). Group III cases were well known patients, with PSA < 2 for at least 5 years, taken from the database of our "stone center". Group II and

Group III were assayed as negative control groups. PCa patients belonging to Intermediate risk class (GS = 7 and TNM = 2b-T2c) were excluded. The intermediate-risk patients were excluded due to their intermediate characteristics, concerning the risk of progression and recurrence of the disease. The design of the study aims to verify, without any doubt, that patients with localized disease or without prostatic cancer may never have circulating tumor cells detectable by this method (group II and group III). For logical reasoning, the only patients who might have CTCs are those belonging at high risk group (group I). In the design of the "case-control" study, the group to be studied is the "group I". Groups II and III are control groups. We felt it was a waste of resources the introduction of a further control group consisted of intermediate risk patients. Overall PCa patients (Groups I and II) were treated with single or combined therapy: radical prostatectomy, external beam radiotherapy, total androgen blockade; two patients of Group I started chemotherapy, because of their castration resistance.

In order to reduce the biases due to the selection during the enrolment, we choose consecutively only patients who received a diagnosis of PCa within previous 36 ± 4 months, while the period of observation was 12 month from first blood sample collection for testing AdnaTest Prostate Cancer kit. In fact, for each patient enrolled we collected both 7 ml of whole blood using specific preservative tubes for CTCs capture (*AdnaGen, Langenhagen, Germany*) and 3.5 ml of vacutainer tubes for PSA measurement at diagnosis (PSA time = 0). Additional PSA assays were done during the follow up period study (2 per year). Therapeutic information and clinical characteristics of patients were collected by a trained Urologist (Table I). All subjects enrolled in this preliminary investigation gave their informed consent. All procedures were made following the Helsinki criteria for research studies.

AdnaTest Prostate Cancer select/detect

Blood (5 ml) samples were taken using AdnaCollect blood collection tubes (*AdnaGen, Langenhagen, Germany*) and immediately processed not later than 24 hours after blood withdrawal, since it has been reported the possible lack of stability of cancer cell transcripts (26). The immunomagnetic enrichment of CTCs was performed by using AdnaTest Prostate Cancer Select and total mRNA/bead mixture was retro-transcribed by Sensiscript Reverse Transcriptase (*Qiagen, Valencia, CA, USA*) according to the manufacturer's instructions. Analysis of tumor-associated mRNAs, a multiplex PCR was carried out using AdnaTest Prostate Cancer Detect according to the manufacturer's instructions. The primer mixture consisted of amplified three tumour markers (*PSMA, PSA, EGFR*) and one house-keeping gene (*Actin*). Evaluations of cDNA run were carried out using Experion 1K analysis DNA Chip (*Bio-Rad Hercules, CA*) and 4 % agarose gel.

RESULTS

For the correct interpretation of AdnaGene test results, Actin amplicons must be present in all patient's runs (internal PCR control): actin signal represents, in fact, the positive control for cell separation, reverse transcrip-

Table 1.

Therapeutic informations and pathological characteristics of PCa and control patients, following the TNM classification (UICC, 2009).

	Patients	PSA t0	Prognostic Grouping (stage)	GS	TNM	Therapy	Current PSA	ADNA MARKERS
Group I^a								
	1	332	IV	5+5	N+M+	enantone + casodex	1,68	NEG
	2	12	IV	5+4	N+M+	eligard + casodex + RT + CHT	0,98	NEG/PSA*
	3	136	IV	4+4	T4 N+ M0	casodex 50 + RT	0,49	NEG
	4	15	IV	3+5	N+	gonapeptyl + casodex RT	52	PSA/PSMA
	5	5,73	IV	4+4	pT4	RT	0,01	NEG
	6	7,05	IV	5+3	pT3b M+	eligard + RT	15	PSA
	7	128	IV	4+5	N+ M+	eligard + casodex + taxotere	68,5	PSA/PSMA
	12	9,8	IV	4+4	M+	casodex + enantone + zometa	9,29	NEG
	13	12	IV	4+4	T4	RT - Casodex suspension	0,206	NEG
	14	8,9	IV	3+5	pT3b N+	RRP R1 (margins +)	0,03	NEG
Group II^b								
	8	6,95	I	3+3	NO M0	RRP	0,04	NEG
	9	9,24	I	3+2	NO M0	eligard	0,04	NEG
	10	5,64	I	3+3	NO M0	decapeptyl + casodex + RT	0,00	NEG
	11	9,83	I	3+3	NO M0	RT	4,01	NEG
	15	4,32	I	3+3	NO M0	RRP	4,32	NEG
	21	7,13	I	3+3	NO M0	casodex	0,04	NEG
Group III								
	16			BPH	-	-	0,174	NEG
	17			BPH	-	-	1,52	NEG
	18			BPH	-	-	0,267	NEG
	19			BPH	-	-	0,94	NEG
	20			BPH	-	-	1,94	NEG

TNM = Cancer Staging System; GS = Gleason score (all GS are "biopsy GS" because they were not submitted to RRP; only pts 8-14-15 have "RP-GS");

ADNA MARKERS: PSA, PSMA, EGFR; BPH = benign prostate hypertrophy. All 15 healthy normal controls resulted as negative for the three ADNA markers.

a) Group I patients belong to stage IV of PCa disease, as reported in TNM classification of malignant tumors. UICC International Union Against Cancer. 7th edn, 2009 (ref. 39).

b) As reported in the text, Group II patients includes low-risk progression PCa individuals, as indicated in the reference (25).

*Patient n. 2 was firstly negative at the AdnaGene test, but five month later (following the disease progression) resulted as positive.

tion and multiplex PCR. AdnaTest was considered positive for CTC presence if a PCR fragment of at least one tumor-associated transcript was clearly detectable and visible (peak concentration ≥ 10 ng/ μ l was fixed as the cut-off). Peaks outside the above described criteria were defined as inconclusive. All assays run on our samples passed the quality control criteria.

Results are briefly summarized in Table 1. As expected, all healthy individuals, control group III BPH patients, as well as those belonging to group II (who presented with disease properly controlled by therapy) resulted as negative for AdnaTest. Contrastingly, among patients belonging to Group I (staging class IV), AdnaTest identified as positive only those who were no more responsive to first- or second-line therapy, while as negative those responsive to first- or second-line chemotherapy, although being escaped by hormone therapy. Three out of ten PCa Group I patients resulted as positive for at least one tumor-associated marker like PSA, while only two showed positivity also for PSMA mRNA. It is important to underline that the Group I patient (listed as number 2 in Table 1), who was negative for AdnaTest during the first-line of chemotherapy, became positive for PSA marker five months later (as shown in the comparison between Figures 1A and 1B). Surprisingly, EGFR mRNA resulted always negative in our patients: in order to establish if the negative results were

dependent on limits of Adna-kit, we spiked mRNA extracted from prostate cancer slices positive at immunohistochemical analysis, and we found a perfect amplification of EGFR target (data not shown). Our results indicate that specificity of test is 100% and sensitivity was 100% (Table 2). In detail, individuals with BPH (group III) and those defined as "Stage I" (Group II) resulted as negative, while all patients belonging to Group I (defined as Stage IV) and responsive to therapy did not show any positivity for AdnaTest markers, while all "not responder" patients resulted positive for AdnaTest. The number of patients is too small to apply the tests of analysis of variance. Furthermore, Figure 1A is representative of a group of patients' amplicons analyzed with Experion instrumentation. Clinical characteristics and main results of AdnaTest are synthetically reported below for each patient.

Details of clinical features of 15 PCa patients studied

Patient n. 1: showed a PSA relapse perhaps only for the infiltration of the bladder neck, but the bone and lymph node metastases were not metabolically active at scintigraphy examination: this test did not indicate the need of radiotherapy on the primitive mass;

Patient n. 2: was resistant to hormonal treatment. He initially responded to the first level chemotherapy, but subsequently his clinical conditions worsened. The AdnaTest

Table 2.

Patient stratification based on response to single or combined therapies.

Group I	Patients	PSA t0	GS	TNM	Therapy	Current PSA	ADNA MARKERS
Responders							
	1	332	5+5	N+M+	enantone + casodex	1,68	NEG
	2*	12	5+4	N+M+	eligard + casodex + RT + CHT	0,98	NEG
	3	136	4+4	T4 N+ M0	casodex 50 + RT	0,49	NEG
	5	5,73	4+4	pT4	RT	0,01	NEG
	12	9.8	4+4	M+	casodex + enantone + zometa	9,29	NEG
	13	12	4+4	T3a	RT - Casodex suspension	0,206	NEG
	14	8.9	3+5	pT3b N+	RRP R1 (margins +)	0,03	NEG
Not responders							
	4	15	3+5	N+	gonapeptyl + casodex RT	52	PSA/PSMA
	6	7,05	5+3	pT3b M+	eligard + RT	15	PSA
	7	128	4+5	N+ M+	eligard + casodex + taxotere	68,5	PSA/PSMA
Responder patient became not-responder							
	2*	12	5+4	N+M+	eligard + casodex + RT + CHT	6,81	POS*

has confirmed the disease status; under strict radiological follow up, at distance metastases became evident three months later the molecular test resulted positive.

Patient n. 3: The test confirmed the efficacy of enlarged radiotherapy;

Patient n. 4: after lymphadenectomy, positive obturator lymph nodes were found at the extemporary frozen histological section: therefore radical prostatectomy was not performed but enlarged radiotherapy and total androgen blockade were administered. Since patient has become refractory to total androgen blockade, an "anti-androgen withdrawal" was started;

Patient n. 5: AdnaTest confirmed the efficacy of adjuvant postoperative RT;

Patient n. 6: he is currently under chemotherapy treatment because of the resistance also to second-line hormonal therapy

Patient n. 7: he is no longer responsive to chemotherapy since he showing constant disease progression;

Patient n. 12: despite a PSA increase was registered, disease progression is not yet evident. Since AdnaTest negative, a strict monitoring of PSA and CTCs overtime has been planned.

Patient n. 13: AdnaTest confirmed the good compliance associated to anti-androgen therapy suspension after radiotherapy;

Patient n. 14: AdnaTest supported the efficacy of adjuvant Radiotherapy after surgery;

Patient n. 16: (group II). AdnaTest, performed before surgery, confirmed the preoperative staging (definitive histological examination: pT2b R0 N0).

We underline that patients who responded to the therapies listed in Table 2 were always negative for Adna markers, while the non-responder resulted as positive for both serum PSA increases and prostate CTC markers.

DISCUSSION

Several reports showed that circulating tumor cell counts correlate with prognosis in patients with advanced breast, prostate and colorectal cancers, treated by con-

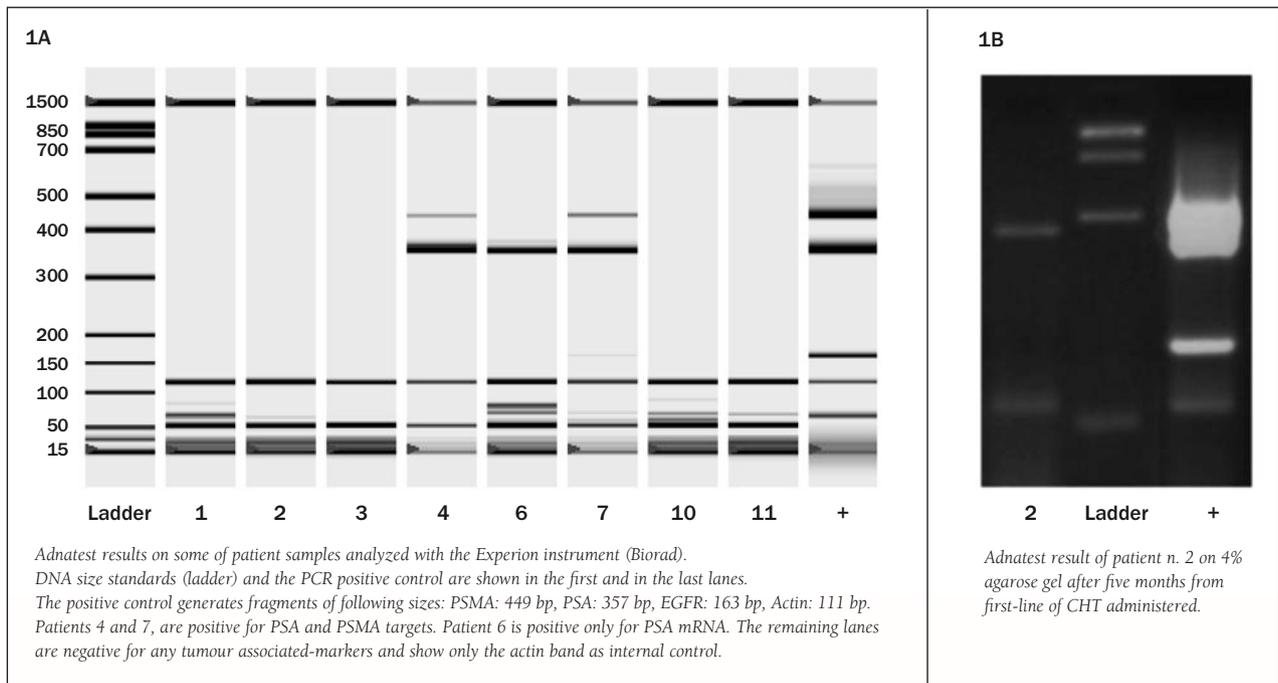
ventional and/or hormonal therapy, suggesting the use of CTCs in the clinical management of cancer patients (18, 27, 28). Furthermore, for prognostic and predictive purposes, CTCs detection, when coupled to molecular characterisation of specific cell transcripts or biomarkers, could provide important clinical information in terms of monitoring of efficacy or resistance to targeted therapy: in this way, CTCs represent an accurate laboratory clinical tool for predicting patient's outcome also in prostate cancer and providing significant advantages in the view of personalised medicine (29). Over the past few years, different approaches for enrichment of CTCs in blood have been developed (5, 30-33), associated or not to molecular approaches and/or to immunological characterization, respectively (34-35).

The present preliminary study was aimed to assess the usefulness of AdnaTest Prostate Cancer for detection of CTCs in two groups of PCa patients (with low and high risk) as compared to non PCa individuals, in order to establish if the molecular result given by this assay could be helpful for the clinical and drug management of PCa patients. This test is, in fact, able to detect some prostate or cancer-specific markers such as PSA, PSMA and EGFR (23, 24).

In our study we found that AdnaTest: a) identified patients with disease progression or with biochemical relapse; b) confirmed the clinical staging of patient n.1, who showed biologically aggressive disease (GS 10) and biochemical tumor progression in spite of total androgen blockade or combined androgen blockade; c) likewise, in patient n. 2, molecular test agreed firstly with the efficacy of chemotherapy, due to the blood negativity for AdnaTest during the first line of CHT, while only five months later AdnaTest resulted as positive, anticipating the results of radiological examination (the latter indicating at distance metastases only after three months from the evidence of a positive test for CTCs); d) AdnaTest was an useful predictive indicator for patient n.12, who was no more responsive to chemical castration so that anti-androgen therapy was currently discontinued. Patients enrolled in the present study are now under stringent follow-up, since they

Figure 1A-B.

Amplification results obtained on a group of PCa patients.



are followed by PSA assays overtime (every four months), and CT scan every six months, in order to plan other CTC tests for monitoring of disease progression. Regarding the molecular typing performed after enrichment, the use of multiplex AdnaGen PCR may improve the detection of at least one marker potentially associated to circulating prostate tumor cells and possibly associated to poor prognosis. Although mRNA-PSA is not unanimously considered as a better independent prognostic factor as compared to serum PSA detection (36), a limit of this test is that tumor-associated proteins, like PSA, are also expressed in normal cells. Nevertheless, in our test, when the suggested cut-off of 10 ng/ml was used, no false-positive results, particularly in BPH patients, were found. Furthermore, several studies suggested the use of PSMA, alone or combined to PSA-mRNA, to increase assay specificity in PCa patients (36), PSMA being over-expressed in advanced or in castration resistant PCa (37) and preferentially expressed in anaplastic cells, hormone-refractory cells and bone metastases (38).

CONCLUSIONS

In our opinion, molecular analysis of CTCs through PSMA mRNA detection could provide important information for clinicians in terms of prediction of disease recurrence, before imaging findings. This finding demonstrates the potential role of CTCs in the follow up of the Prostatic Cancer; it can be obtained in a routine practice setting, by a simple blood sampling. We underline that although the present study did not compare the efficiency of AdnaTest with that of other platforms (in particular the CellSearch System), we can assume that our test may be considered as reliable: in fact,

in this regard, Andreopoulou E et al have recently reported the concordance between these two methods (40). Finally, our results should be confirmed on larger cohorts of patients also considering that this type of test should be performed in laboratories with high expertise personnel in strict relationships with clinical departments.

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