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ORIGINAL ARTICLE

Molecular and clinico-histological data in aggressive prostate cancer patients from Bulgaria

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Summary

Purpose: *Metastatic prostate cancer (PCa) is one of the leading causes of death in men worldwide. We report Bulgarian patients with strongly aggressive, castration-resistant PCa.*

Methods: *PCA3 overexpression, GSTP1 promoter hypermethylation, TMPRSS2-ERG gene fusions, IVS1-27G>A in the KLF6 gene and mutations in androgen receptor (AR) gene, for diagnostic purposes were assessed. PCR, real-time PCR (RT-PCR), sequencing, and bisulfite conversion of DNA were applied. We correlated the molecular data to the histological and clinical findings.*

Results: *The obtained molecular profile in 11 PCa Bulgarian patients coincided with the clinico-histological data of strongly aggressive PCa. Association was detected between the tumor stage (assessed by TNM as T3 and T4) and the detected molecular profile of aggressive cancer behavior with one exception, assessed as T2. None of our patients had positive family history of prostate cancer and no somatic mutations were detected in the AR gene. All patients showed normal genotype with respect to the KLF6 IVS1-27G>A polymorphism. The rest of the markers were positive in fresh prostatic tissues and biopsies from all patients, whereas only one blood sample showed triple positive result.*

Conclusions: *The appearance of PCa-specific markers in blood was considered as a predictor for a PCa (micro) dissemination into the circulation. The GSTP1 promoter hypermethylation is the earliest epigenetic alteration, which indicates cancerous changes and the first and long-lasting marker that is detectable in blood circulation. The molecular profile needs to be strictly monitored during treatment, which is of great help in determining the patient's individual response to therapy.*

Key words: *CRPC, molecular subtyping, PCA3, prognosis, prostate cancer, TMPRSS2:ERG gene fusions*

Introduction

PCa is the most commonly diagnosed malignancy and a leading cause of cancer-related death among men in Western countries. PCa is a clinically highly heterogeneous disease with features ranging from typical indolent disease, which remains relatively insignificant to a patient's health, to rapid and often fatal progression. Clinicopathological parameters including serum PSA, Gleason score and tumor stage are not sufficient to distinguish PCa-affected men who require immediate and aggressive therapy from those who need clinical follow-up [1]. For this reason, there is an urgent need to find and to introduce into the clinical practice new markers with improved PCa specificity, both being able to identify patients with an early onset and aggressive disease progression, to determine disease prognosis and individual treatment.

Most promising, recently described, genetic markers with benefits in PCa molecular subtyping

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are gene fusions, mRNA alterations and epigenetic fluctuations. The clinical heterogeneity and marked variability of disease progression among the affected individuals makes the experimental data from studies of patient's collections significant.

Herein we report 11 Bulgarian patients with strongly aggressive PCa behavior and our aim was to develop better cancer diagnostics and management of such patients. Therefore, we correlated the molecular data from *PCA3* expression, *TMPRSS2:ERG* gene fusions, *GSTP1* promoter hypermethylation, IVS1-27G>A polymorphism in *KLF6* gene, mutations in *AR* gene to the clinico-histological findings.

Methods

Patients and samples

Eleven patients were selected on the base of their extremely aggressive PCa behavior, serum PSA levels >3 ng/mL and abnormal digital rectal examination (DRE). Prostate fresh tissues, "tru-cut" biopsies, urine and blood samples were collected in the urological outpatient clinic. The patients signed informed consent for genetic testing.

RNA and DNA extraction

Total RNA was extracted using the TRIzol reagent (Ambion, US). Total DNA was extracted using the AmpliSens DNA isolation kit (Ecoli s.r.o, Slovak Republic).

PCA3 expression

PCA3 expression levels were measured by reverse transcription (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit, Lithuania) and real time (RT) PCR analysis with Universal Master Mix (Applied Biosystems, Foster City, CA), using the primers, previously published [2].

TMPRSS2-ERG gene fusions

T2-F1/ERG-R4; T2-F1/ERG-R6; T2-F/ERG-R and T2-F2/ERG-R2 were studied by reverse transcription PCR analysis [3] and direct sequencing of the obtained PCR products (BigDye Terminator Cycle Sequencing kit v.3.1, Applied Biosystems, Foster city, CA).

GSTP1 promoter hypermethylation

This was assessed by bisulfite conversion of DNA (Zymo research, EZ DNA Methylation TM Kit, USA), followed by PCR [4].

AR gene

The *AR* gene was PCR-amplified, and PCR products were analyzed by direct sequencing.

IVS1-27G>A polymorphism in KLF6 gene

This polymorphism was sequenced (see above).

Pathological examination

Pathological examination was performed on hematoxylin-eosin-stained sections from formalin-fixed paraffin embedded prostate tissues. Sections were graded by an experienced uropathologist (I.D.), according to the Gleason scoring system.

Results

Disease onset

The molecular and histological characteristics of the 11 patients are shown in Table 1. The family history was negative in all 11 cases. In all cases PSA levels were elevated. In one of the patients (patient no.3, Table 1) dramatically high PSA levels (over 100 ng/mL) were detected by chance during the routine urological examination. The patient was referred for urological exams (DRE and TRUS). This patient was primarily negative for the classical PCa symptoms, which were well represented in the remaining 10 cases: difficulties in passing urine, splitting of urine's stream, urinary incontinence, fatigue and drowsiness and sexual dysfunction. For this reason the patient was considered as asymptomatic case of PCa. After specific urological exams all 11 patients showed abnormal DRE and TRUS findings, specific for PCa.

Histological data

Some histological data are provided in Table 1. The presented 11 PCa patients could be divided into 3 main groups according to their histological characteristics:

I. Group of moderately differentiated adenocarcinoma (patients no.1,3,4,5): The histological examination showed moderately-differentiated acinar adenocarcinoma, Gleason score 7 or 5, with perivascular and perineural tumor infiltration. The percentage of cancerous infiltration in patients was estimated to be 60-90%.

The tumor stage in the patient no.1 was estimated as pT2a NxMxG2. In this case additional pathological alterations were found: adenomyomatous prostatic hyperplasia and cystic changes in the prostate gland, presence of prostatic cancer cells in lymph clefts, PIN III, PIA including chronic prostatitis (Figure 1).

Table 1. Molecular and histological results in prostate cancer patients with strongly aggressive PCa behavior

Patient, No.	*Age (years)*	*Serum PSA levels (ng/mL)*	*Biological sample*[a]	*Histological Gleason score*	*PCA3 expression*	*GSTP1 promoter hypermethylation*	*TMPRSS2/ERG fusion*[b]	*AR*	*Treatment*	*TNM*
1	70	126	Fresh prostatic tissue	4+3=7	Overexpression	Positive	Positive	No somatic mutation	ADT Taxotere (Docetaxel)	pT2a NxMxG2
			Blood sample	----	Overexpression	Positive	Positive	ND		
2	66	67	Fresh prostatic tissue	2+4=6	Overexpression	Positive	Positive	No somatic mutation	ADT Zytiga (Abiraterone Acetate) + Deltasone (prednisone)	G1pT2bN0(0/6) MxVn.
			Blood sample	----	Negative	Positive	Negative	ND		
3	63	111	"Tru-cut" biopsy	2+3=5 left lobe 4+3=7 right lobe	Overexpression	Positive	Positive	No somatic mutation	ADT +Zometa (Zoledronic acid)	multiple bone metastases
			Blood sample	----	Overexpression	Positive	Negative	ND		
4	72	99	"Tru-cut" biopsy	4+3=7 left lobe 2+3=5 right lobe	Overexpression	Positive	Positive	No somatic mutation	ADT +Xgeva (Denosumab)	multiple bone metastases
			Blood sample	----	Overexpression	Positive	Negative	ND		
5	73	115	"Tru-cut" biopsy	2+3=5 left lobe 4+3=7 right lobe	Overexpression	Positive	Positive	No somatic mutation	ADT	----
			Blood sample	----	Negative	Positive	Negative	ND		
6	65	130	Fresh prostatic tissue	4+5=9	Overexpression	Positive	Positive	No somatic mutation	ADT	pT3bNxMx
			Blood sample	----	Negative	Positive	Negative	ND		
7	62	80	Fresh prostatic tissue	4+4=8	Overexpression	Positive	Positive	No somatic mutation	ADT	pT4 NxMx
			Blood sample	----	Negative	Positive	Negative	ND		
8	61	106	"Tru-cut" biopsy	4+5=9	Overexpression	Positive	Positive	No somatic mutation	ADT Zytiga(Abiraterone Acetate) + Deltasone (prednisone)	----
			Blood sample	----	Negative	Positive	Negative	ND		
9	55	110	Urine	4+4=8	Negative	Positive	Negative	No somatic mutation	ADT Taxotere (Docetaxel), Xgeva (Denosumab)+ Performed Orchiectomy	multiple bone metastases
			Blood sample	----	Negative	Positive	Negative	ND		

Continued on next page

10	63	57	Urine	4+5=9	Negative	Positive	Negative	No somatic mutation	ADT Suprefact depot, Cyproterone (Andocur), Eligard (Leuprolide acetate)	----
			Blood sample	----	Negative	Positive	Negative	ND		
11	87	73	"Tru-cut" biopsy	4+4=8	Overexpression	Positive	Positive	No somatic mutation	ADT	----
			Blood sample	----	Negative	Positive	Positive	ND		

[a] The fresh prostatic tissue was obtained after prostatectomy.
[b] Only the presence of one type of TMPRSS2/ERG gene fusion was determined in our group: TMPRSS2-exon1 /ERG-exon4. ND: not determined

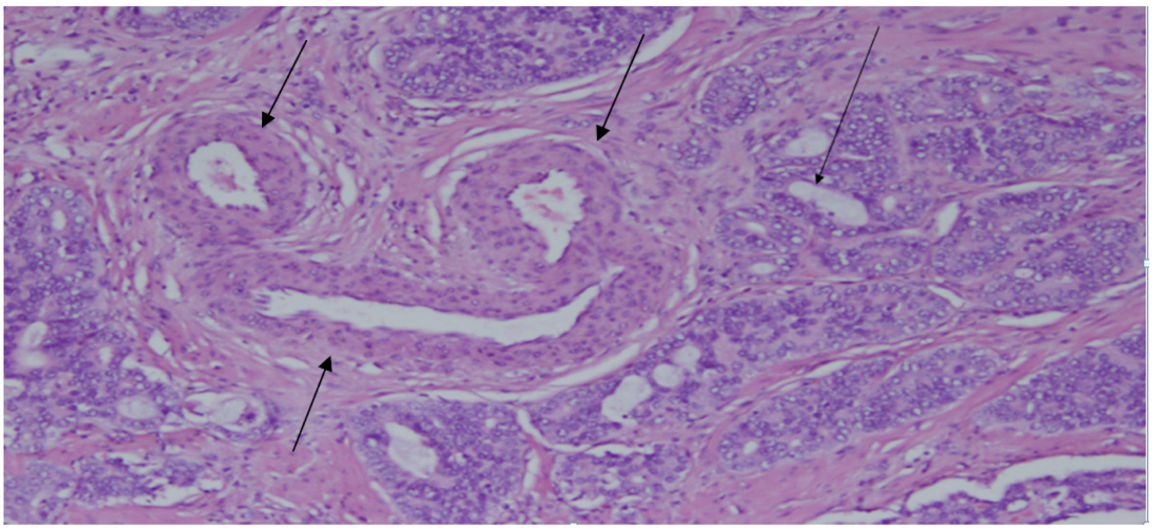

Figure 1. Histological image of prostate cancer (H&E staining x 40). Presence of perivascular tumor infiltrates (arrows); tumor nests and arterial vessels. Gleason score 4+3=7, moderately differentiated tumor.

II. Group of poorly differentiated adenocarcinoma (patients no.6,7,8,9,10,11): The histological examination showed a pattern of poorly-differentiated adenocarcinoma (Gleason score 8 or 9) with perineural and/or perivascular infiltration.

The tumor stage in the patient no.6 was pT3b-NxMx and pT4 NxMx in the patient no.7. In this patient focal infiltration and penetration in the capsule, extracapsular cancerous extension and perineural invasion inside and outside of prostate gland in the presence of BPH and acute and chronic prostatitis were observed. The seminal vesicles were not affected. The histological investigations of the patient no.9 showed ductal prostate adenocarcinoma, characterized with diffuse invasion in the periprostatic soft tissues and the seminal vesicles. The patient no.10 showed perineural invasion and cancerous extension to the periprostatic adipose tissue.

III. Group of highly differentiated adenocarcinoma (patient no.2): The histological examination showed highly-differentiated adenocarcinoma, which affected the entire left prostatic lobe (Gleason score 6). In this patient no tumor infiltration was detected in the surrounding soft tissues, seminal vesicles and lymph nodes, but a single focus of perineural infiltration in the surrounding periprostatic tissues was detected. The TNM stage was pT2bN0(0/6)MxVn.

Molecular data

In order to better clarify the molecular subtype of the PCa, the patients were subjected to molecular genetic tests. The following genetic markers were simultaneously examined: *PCA3* expression, expression of a *TMPRSS2-ERG* fusion transcript, the hypermethylation of the *GSTP1* gene promoter, *KLF6* IVS1-27G>A polymorphism

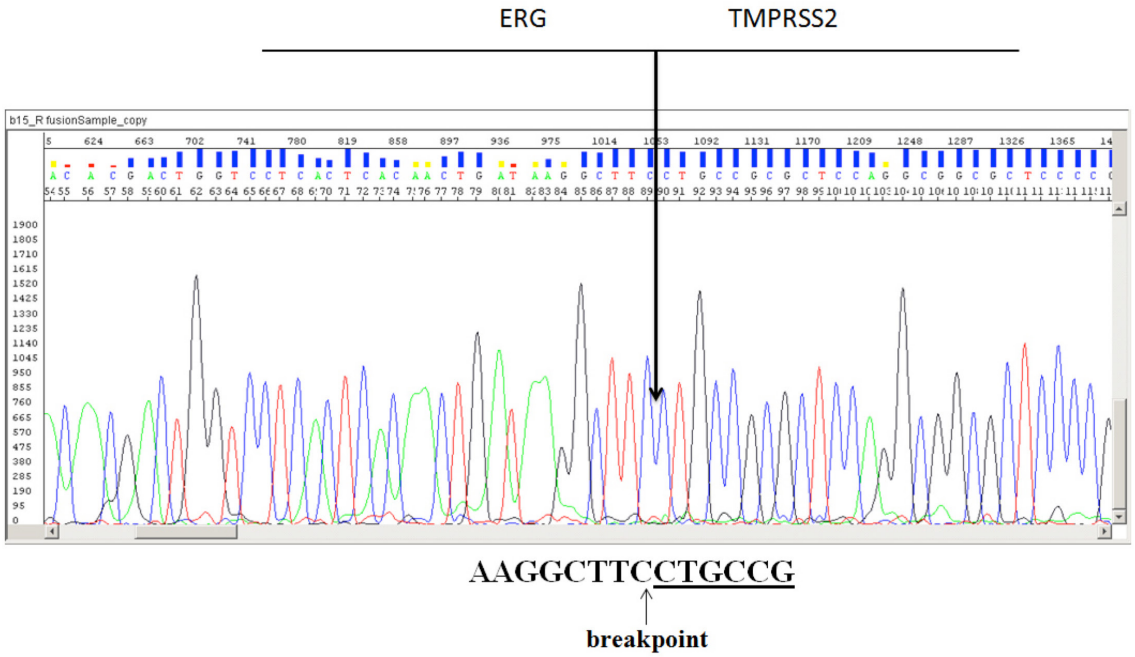

Figure 2. Sequencing profile of *TMPRSS2ex1-ERGex4* fusion detected in fresh tissue of a Gleason score 7 prostate tumor.

and AR mutation profile. The obtained molecular data are provided in Table 1.

All but two patients (no.9 and 10) with aggressive PCa were found to be positive for *PCA3*, as the levels were found to be drastically overexpressed by means of RT-PCR. For two patients (no.9 and 10), no tissue was available for analysis, and *PCA3* testing was performed on urine, which showed negative *PCA3* expression profiles. It is interesting to mention that in three cases (patients no.1,3,4) the *PCA3* marker was overexpressed also in blood samples, which indicates prostatic tumor (micro) dissemination.

GSTP1 gene promoter hypermethylation was positive in all patients and in all samples tested (data not shown).

The studied *TMPRSS2:ERG* gene fusions revealed only one type of fusion transcript: *TMPRSS2* exon 1 fused to *ERG* exon 4 (Figure 2). Again, positive results were obtained in patients tested before androgen deprivation therapy (ADT) and the last patients no.9 and 10 tested after treatment showed negative fusion profile in the urine. Positive fusion profile in blood was obtained only in two patients (no.1 and 11). The blood sample of patient no.1 was also positive for *PCA3* expression. The molecular data showed more advanced disease stage in these patients.

In the patients with aggressive PCa no somatic mutations were detected in the *AR* gene, regardless of the supplied ADT (Docetaxel, Zytiga/Abiraterone acetate + prednisone, Cyproterone, etc).

All our cases also showed normal genotype with respect to the IVS1-27G>A polymorphic variant in the *KLF6* gene.

Based on the molecular examinations, all 11 patients were classified as having PCa with pronounced migration of prostatic tumor cells (micrometastases) by means of observing specific PCa biomarkers in blood and the hormone-refractory cancer behavior, which suggests ADT and strict molecular and clinical follow-up.

Discussion

Predisposition to PCa is a result of the combined effect of polymorphic variants and mutations in genes with low penetrance. The findings from genome-wide association studies (GWAS) confirm that genetic predisposition to PCa is complex and covers multiple susceptibility loci. The most significant associations are discovered in regulatory gene regions or in non-coding gene regions and rarely in coding gene regions. Experimental data suggests that the mechanism contributing to PCa development would be rather regulatory than coding [5,6]. None of our cases was with positive family history of PCa. Altered gene expression, epigenetic modifications (such as promoter hypermethylation), gene rearrangements, and mRNA alterations are believed to play

an important role in deregulation of apoptosis and genetic misbalance in PCa. The need for new biomarkers is determined by the fact that serum PSA is a poor marker, and markers to discriminate between indolent and aggressive tumors are lacking [7-13].

The obtained molecular profile of the presented patients correlates with the clinical and histological data of aggressive, hormone-independent behavior during the period of treatment. Most of the patients were determined as having a castration-resistant prostate cancer (CRPC) based on relapse after ADT. Three of the patients were diagnosed with advanced CRPC, because of development of multiple bone metastases (patients no.3,4,9). Altogether, histological and clinical results in our patients showed an aggressive subtype of PCa, as perivascular and perineural infiltration and bone metastases were detected.

We demonstrated that an association exists between tumor stage (in our patients assessed by TNM as T3 and T4) and the detected molecular profile of aggressive cancer behavior, whereas in T2 tumor stage no such an association was found. The combined positive molecular profile (*PCA3* overexpression, *GSTP1* gene promoter hypermethylation and *TMPRSS2-ERG* fusion) was detected in patients with higher Gleason score and poor prognosis with one exception. The patient no.2, Gleason score 6, typically associated with better prognosis did not correlate to the obtained molecular profile corresponding to more aggressive PCa. For this reason Gleason scoring should not be considered as the most important prognostic parameter, and it is reasonable to be interpreted simultaneously with additional histological and molecular findings.

In regard to the *TMPRSS2-ERG* genes fusion, we studied the following types: T2-F1/ERG-R4; T2-F1/ERG-R6; T2-F/ERG-R and T2-F2/ERG-R2 [3,14], but only one variant (T2-F1/ERG-R4) was detected. This is the most prevalent fusion discussed and reported so far [7,8]. The positive fusion transcript was associated with the clinical pattern of aggressive, androgen-independent and life-threatening PCa in our patients. In patients no.3,4 and 9 multiple metastases in the skeleton and cranium were found, which led to PCa-associated death in the patient no.9. The remaining clinical cases were diagnosed as having a more intensive mineral metabolism by bone scan without bone metastases and the soft tissues. In our group, the *TMPRSS2-ERG* fusion was present predominantly in patients with higher Gleason score (no.7,8, and 9), as reported by other authors [15]. The positive fusion profile was detected also in patient no.2 with Gleason score 6, which points again to the importance of molecular findings for the final disease prognosis and PCa subtyping.

The appearance of the *TMPRSS2-ERG* fusion marker in blood is a prerequisite for micrometastatic PCa behavior. The combined testing of a number of molecular markers in blood and the appearance of a positive profile, as demonstrated in our investigation, predicts micrometastases formation. Micrometastases could develop into metastases with subsequent dissemination into adjacent tissues and then toward non-accidental locations, like bones and the CNS [16], as demonstrated in 3 of our clinical cases (patients no.3,4,9).

In one of our patients (no.1), the blood sample was positive for *PCA3* overexpression, *GSTP1* hypermethylation and *TMPRSS2-ERG (T2-F1/ERG-R4)*. It should be interpreted undoubtedly as a pronounced migration and dissemination of PCa cells in the circulation. The same patient was subjected to ADT with docetaxel and after 1 year follow-up the blood sample was found to be positive only for the *GSTP1* promoter hypermethylation. The *GSTP1* promoter hypermethylation is considered to be the earliest epigenetic alteration indicating cancerous prostatic changes and the long-time persisting marker in the blood circulation. Based on the molecular findings and the clinical observations it was concluded that the patient no.1 demonstrates good individual response to treatment.

The blood samples of the patients no.3 and 4 (Table 1) were also found to be positive for *PCA3* overexpression and for *GSTP1* promoter hypermethylation, but negative for the fusion profile. The rest of the cases were found to be positive only for the *GSTP1* promoter hypermethylation, which indicates the beginning of the micrometastases formation and their gradual dissemination.

Normal genotypes in respect to the IVS1-27G>A polymorphism in the *KLF6* gene were obtained for all 11 studied patients with aggressive PCa. This PCa-associated polymorphism generates alternative splice variants (KLF6-SV1, SV2, SV3), which directly influence and stimulate the tumor growth and metastatic formation [17,18]. This polymorphic variant has not been studied before in Bulgaria and its prevalence in Bulgarian population is unknown.

No somatic *AR* mutations were detected in our patients with CRPC, and in the rest of the aggressive subgroup. Our molecular results support once again the opinion of the urological commu-

nity, that AR mutations are comparatively rare in PCa and CRPC [19], regardless of awaiting inducible effect from the therapies.

Conclusions

In conclusion, we chose to characterize 11 patients because they had relatively similar molecular profile and clinicopathological characteristics, which determine the aggressiveness of Pca behavior. Molecular profiles in different tissues were correlated to the clinico-histological data. The molecular testing might help clarify invasive, fast progressive, metastatic and life-threatening subtypes of PCa. Our data suggests that positive molecular status in patients with aggressive PCa might be helpful for strict monitoring and for choosing better treatment. The molecular profile needs to be strictly monitored during treatment, which helps determine the patient's individual response.

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