

Investigation of Noninvasive Urine Specimens with Molecular Fluctuations for a Presence of High-Risk Human Papilloma Viruses as an Inflammatory Cofactor in the Prostate Cancer

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Abstract

Background: The aim of the study is to investigate noninvasive urine specimens in suspected prostate cancer (PCa) patients by a panel of PCA3, TMPRSS2-ERG fusions and GSTP1 promoter hypermethylation. Furthermore, we tested urine specimens for the presence of high-risk Human Papilloma Viruses (HPV) as an inflammatory cofactor in the complicated origin of prostate cancer (PCa).

Methods: A total of 50 patients with elevated PSA and/or PCa physiological symptoms were analyzed. RNA and DNA isolation; Reverse transcription; Real-time PCR; DNA sequencing; Bisulfite conversion of DNA; Methylation-specific PCR; Cytological preparations and staining were applied.

Results: Molecular fluctuations were registered in most of the patients: neoplastic GSTP1 allele, PCA3 strongly elevated expression or hyperexpression. Only in 4 cases a positive TMPRSS2-ERG status was detected. High-risk HPV types were detected in ~ 35% of our urine specimens, obtained from patients at high risk of PCa based on their molecular profiles. Approximately 96% of detected high-risk HPVs are: 16, 33, 35, 31, distributed in the subgroup with highest oncogenic potential. The estimated frequency of high-risk HPV types in control male samples with urothelial infection is significantly lower (11%). The pathological examination on cytological slides from high-risk HPV positive urine specimens showed inflammation; variable adaptations of cellular growth and differentiation and partially viral cytopathic effect. In a proportion of patients with molecular PCa disturbed profile precancerous conditions (increased primitive cells with disturbed maturation; enlarged hyperchromatic nucleus and condensed chromatin) were found.

Conclusion: Our molecular PCa findings, were confirmed on the cellular level with cytological findings of high grade alterations: coarse distributed chromatin texture with nuclear membrane irregularity and thickening; high N:C Ratio; prominence of nucleoli and irregularity in shape thereof; identical monotonous nucleoli present in all cells in a group (i.e., "Clonal" pattern); Tumor diathesis. The present study concerns novel data for Bulgarian PCa patients.

Keywords: Prostate cancer; Molecular cancer profiling; High-risk Human Papillomaviruses PCA3; TMPRSS2-ERG fusions; GSTP1 promoter hypermethylation

Introduction

Nowadays, Prostate cancer (PCa) is the most prevalent cause of cancer death among men, after lung cancer. PCa represents a heterogeneous disease with polyclonal character which can be indolent decades in some patients or life-threatening with rapid and often lethal progression in others. The predisposition to PCa may be provoked by multiple mutant variants or polymorphic alleles in genes with low phenotypic penetrance. The experimental data confirms that the mechanism contributing to prostate cancer invasion could be rather regulatory than coding. Current diagnostic approach to PCa, including clinicopathological parameters, as: serum PSA, Gleason score and tumor stage is not sufficient to distinguish PCa-affected men who

require immediate and aggressive therapy from those who need noninvasive follow-up [1].

There is an urgent necessity for molecular markers with high PCa diagnostic value to be introduced in the clinical practice for molecular subtyping of patients with aggressive, androgen-independent behavior and monitoring of the metastatic tumors.

The most promising genetic markers with important impact on PCa molecular profiling are epigenetic fluctuations (e.g. GSTP1 promoter hypermethylation), gene fusions (e.g. TMPRSS2-ERG gene fusion), and mRNA alterations (e.g. PCA3 overexpression).

In principle about 20% of human tumors in adults result from chronic inflammatory conditions [2,3], provoked by infection agents or exposure of other dangerous and harmful environmental factors. There are molecular, histopathological and epidemiological evidences that the inflammation is a crucial factor in the etiology of PCa.

The oncogenic potential of High-risk Human Papillomaviruses (HPV) and their strong association with anogenital, epithelial tumors, particularly prostate cancer (PCa) is well clarified and confirmed [4-9]. Many experimental data from PCa cellular tumor lines and animal models suggests the association between High-risk Human Papillomaviruses and the malignant transformation of the prostatic epithelium [10-12]. The HPV-mediated oncogenic potential, which has been reported by leading experts worldwide, confirms the hypothesis for the possible role of HPV as an inflammatory cofactor in the origin and progression of the prostate cancer.

Recent data underlines the increasing number of PCa cases, infected by high-risk HPV (~41%-63.5%) in the contrast to significantly lower percentage of HPV positive results in the group of healthy controls or Benign Prostatic Hyperplasia (BPH) [13-17]. These findings determined our interest to investigate this aspect of PCa in Bulgarian patients.

Here we report our novel data on 50 Bulgarian PCa suspected patients with pathogenic molecular profile in regard to the following PCa specific markers: PCA3, TMPRSS2-ERG fusions and GSTP1 promoter hypermethylation. Furthermore, we report data on HPV infection in association with prostate malignancy and results from cytological examinations of viral and cancer alterations.

Materials and Methods

Patients and samples

All patients were selected on the base of their elevated serum PSA levels >3 ng/mL and/or PCa physiological symptoms. Written informed consent was obtained from all patients prior to genetic testing.

Urine specimens were collected in the urological outpatient clinic after digital rectal examination (DRE) or before taking transrectal biopsies and immediately transferred to the laboratory for analysis. If the biopsies already have been taken, at least 2 weeks should be reassured between biopsies and urine collection. For RNA preservation 2 ml 0.5 M EDTA with pH 8.0 was used as a transport medium. The sample was centrifuged for 10 min at 3000 rpm at 4°C. The supernatant was discarded carefully without disturbing the sediment. The pellet was resuspended in 1 ml ice cold buffered NaCl. The resuspended sediment was transferred to a sterile 1.5 ml Centrifuge-tube. The sample was centrifuged for 1 minute at 3200 rpm at 4°C and it was ready to use.

RNA and DNA extraction

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

The GSTP1 promoter hypermethylation was assessed by bisulfite conversion of DNA (Zymo research, EZ DNA Methylation TM Kit, USA), followed by methylation sensitive PCR [18], using the primers: for unmethylated GSTP1 F 5'GAT GTT TGG GGT GTA GTG GTT GTT-3' and R 5'CCA CCC CAA TAC TAA ATC ACA ACA-3' and for methylated GSTP1 F 5'TTC GGG GTG TAG CGG TCG T-3' and R 5'GCC CCA ATA CTA AAT CAC GAC G-3'. The reaction master mix includes 2 µl cDNA input; 0.4 µl of specific forward and reverse primers with a concentration 20 pmol/µl; 1 µl *ddNTP* (5 mM) and 1 µl 10X Buffer; 0,05 µl Prime Taq DNA Polymerase 250 U 5 U/µl (GeNet

Bio, Korea) and 5.15 µl PCR water to final volume 10 µl and the PCR was performed under the following conditions: initial denaturation 95°C/10 min, 94°C/15s; 62°C/30s; 72°C/30s × 45 cycles, 72°C/1min.

PCA3 expression levels were measured by reverse transcription (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit, Lithuania) and real time PCR analysis with Universal Master Mix (Applied Biosystems, Foster City, CA), using the previously published primers PCA3 forward, 5'FAM6-GGTGGGAAGGACCTGATGATAC-3' in exon 1 and PCA3 reverse, 5'-GGGCGAGGCTCATCGAT-3' in exon 4 [19]. Different PSA-specific primers were used for PCR: forward, 5'-AGCATTGAACCAGAGGAGTTCT-3' (nucleotides 4024–4042 of exon 3 and 4186–4188 of exon 4 of the PSA gene, GenBank #M27274) and reverse, 5'-CCCAGCAG GTGCTTTTG-3' (nucleotides 4307–4322 of exon 4 and 5699–5700 of exon 5 of the PSA gene), as described by Hessels et al. [20]. For the amplification of the targeted PCA3 and PSA amplicons and the measurements of PCA3/PSA ratio the following PCR conditions were applied: master mix with final volume 10 µl; 2 µl cDNA input; 0.2 µl of specific PCA3 and PSA primers with a concentration 20 pmol/µl; 1 µl *ddNTP* (5 mM) and 1 µl 10X Buffer; 0.05 µl Taq DNA Polymerase 250 U 5U/µl (GeNet Bio, Korea) and 5.15 µl PCR water. The PCR cycling conditions were: initial denaturation 95°C/10min, followed by 95°C/30s; 60°C/75s; 72°C/60s × 42 cycles.

TMPRSS2-ERG gene fusions T2-F1/ERG-R4; T2-F1/ERG-R6; T2-F/ERG-R and T2-F2/ERG-R2 were studied by RT-PCR analysis by using of the next primers: T2ex1F5' CGCGAGCTAAGCAGGAG-3' and ERG ex4R 5'GTCCATAGTCGCTGGAGGAG-3'; T2ex1F5'CGCGAGCTAAGCAGGAG-3' and ERGex6R 5'CCATATTTCTTTCACCGCCACTCC-3';

T2-F 5'TAGGCGGAGCTAAGCAGGAG-3' and ERG-R 5'GTAGGCACACTCAAACAACGACTGG-3'; T2-F2 5'CAGGAGGCGGAGGCGGA-3' and ERG-R2 5'GGCGTTGTAGCTGGGGGTGAG-3'. The reaction master mix was prepared up to a final volume of 10 µl: 2 µl cDNA input; 0.4 µl of specific forward and reverse primers with a concentration 20pmol/µl; 1 µl *ddNTP* (5 mM) and 1 µl 10X Buffer; 0.05 µl Taq DNA Polymerase 250U 5U/µl (GeNet Bio, Korea) and 5.15 µl PCR water. The following PCR cycling conditions were applied: initial denaturation 95°C/2min, 94°C/45s; 64°C/45s; 72°C/45s × 40 cycles, 72°C/10 min. The fusion points between the targeted genes were determined by direct sequencing (BigDye Terminator Cycle Sequencing kit v.3.1, Applied Biosystems, Foster city, CA) as described by Tomlins et al. [21].

The specific DNA of 12 High-risk Human Papillomaviruses was registered by HPV HCR genotype-EpH PCR kit AmpliSens (Ecoli s.r.o, Slovak Republic) under the following PCR conditions: initial denaturation 95°C/15 min, 95°C/30s; 63°C/30s; 72°C/40s × 45 cycles, 72°C/1 min.

Morphological examination was performed on hematoxylin-eosin-stained preparations from urine sediments, obtained after DRE. ThinPrep® liquid-based preparations were utilized by the application of an automatic system platform resulting in more representative microscopic image [22].

The morphology of each case was estimated with specific attention to the cellular size, nuclear features, nuclear to cytoplasmic ratio, and cytoplasmic characteristics. Key findings for prostatic adenocarcinoma in urine are clustering, increased nuclear to cytoplasmic ratio (N:C), hyperchromatic nucleus and prominent nucleoli. Despite the fact that

locally advanced prostatic adenocarcinoma cells are often detected in urine, tumours in lower stage may be available in urine as well.

Results

Our experimental results cover various problematic aspects of the disease Prostate cancer and could be divided into 3 subgroups: molecular, viral and morphological results. We attempted to correlate the pathological molecular alterations in PCa suspected patients with the observed morphological findings and the presence of high-risk HPV as an inflammatory cofactor for PCa development in the same specimens. Our motive for application of this particular molecular panel as a tool for noninvasive PCa screening was based on our previous studies on larger patients' sample [23], where the powerful potential of GSTP1, PCA3 and T2-ERG markers for earlier and better primary diagnostics and monitoring of invasive, fast progressing and metastatic PCa subtypes was experimentally proven. About 2/3 of our molecular PCa-specific alterations, detected in urine of PCa suspected Bulgarian patients were confirmed by consequent biopsies. Unfortunately, not all patients were histologically tested, because some of them are under strong vigilant clinical follow up.

Molecular results

A neoplastic GSTP1 allele was found in all PCa suspected patients (Figure 1).

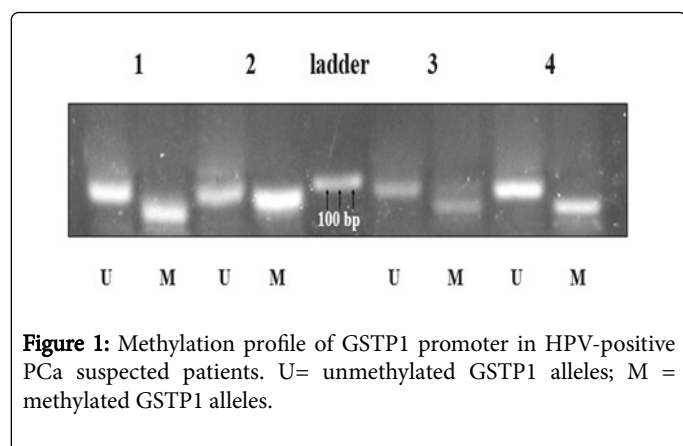


Figure 1: Methylation profile of GSTP1 promoter in HPV-positive PCa suspected patients. U= unmethylated GSTP1 alleles; M = methylated GSTP1 alleles.

The molecular profile showed in most of our patients strongly elevated PCA3 expression or hyperexpression (Figure 2).

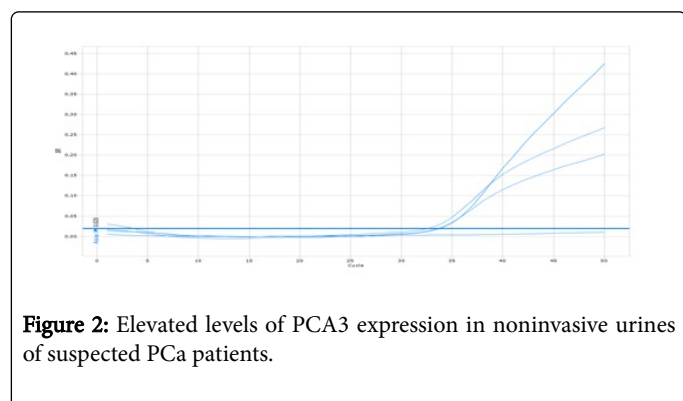


Figure 2: Elevated levels of PCA3 expression in noninvasive urines of suspected PCa patients.

Only in 4 cases a positive TMPRSS2-ERG fusion status was detected, as T2 exon 1 is fused with ERG exon 4 resulting in the most common splice variant (Figure 3).

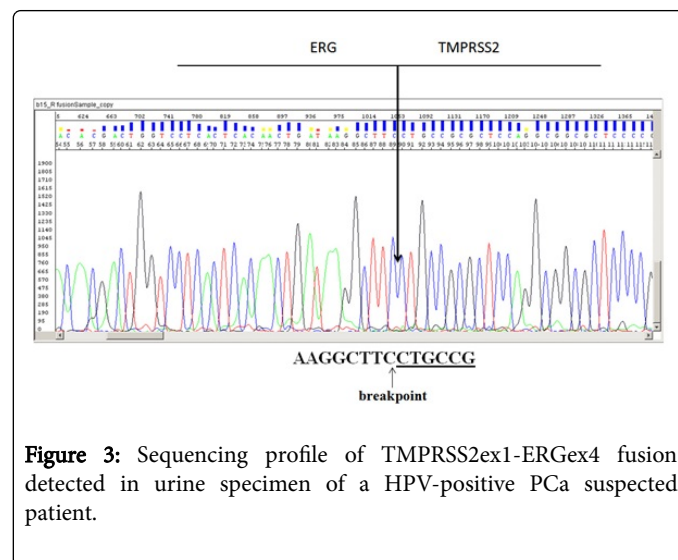


Figure 3: Sequencing profile of TMPRSS2ex1-ERGex4 fusion detected in urine specimen of a HPV-positive PCa suspected patient.

Viral results

As a result of HPV testing, positive high-risk HPV types were detected in 35% of PCa suspected patients, based on their molecular profile which corresponds to the published data for other European populations [13-17,24].

In the contrast, high-risk HPVs were detected in significantly lower percentage (only 11%) among the control group with urothelial infection.

Surprisingly, 96% of the detected high-risk HPVs are: 16, 33, 35, and 31 types, distributed in the subgroup with highest oncogenic potential and experimentally proven association with malignant transformation of the prostatic epithelium (Figure 4).

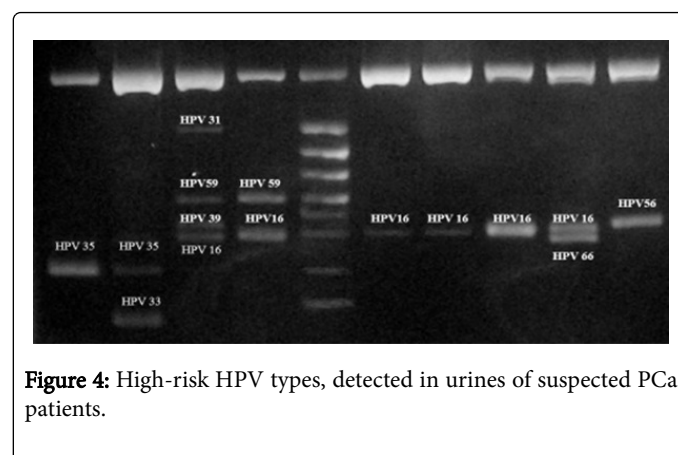


Figure 4: High-risk HPV types, detected in urines of suspected PCa patients.

Morphological results

The morphological analysis was performed on cytological urine specimens obtained from PCa suspected patients, positive for high-risk HPVs. The obtained results were divided in several subgroups: adaptations of cellular growth and differentiation; inflammation and

repair; precancerous changes; cancer changes; high-grade alterations, including cancer (Figures 5-8).

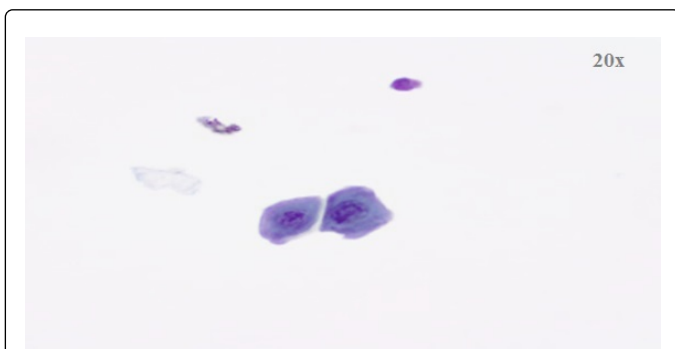


Figure 5: Adaptations of cellular growth and differentiation, metaplastic cells.

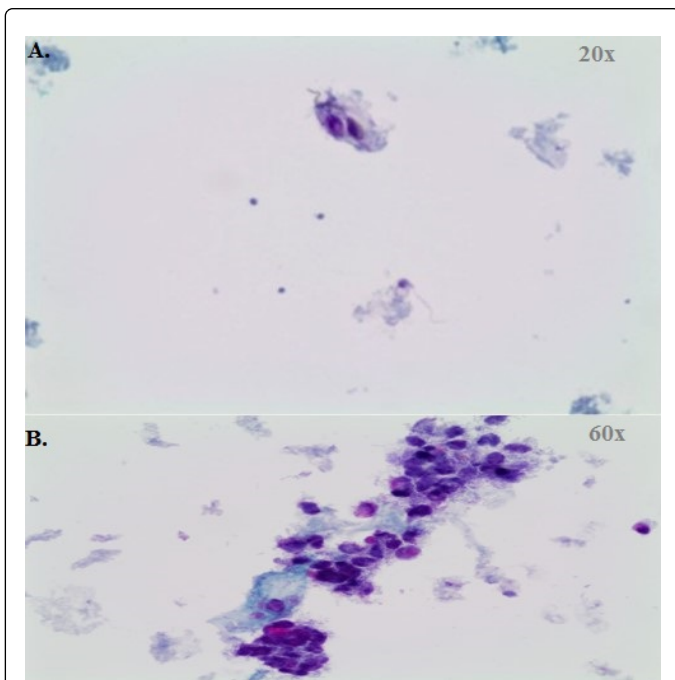


Figure 6: Inflammation and repair. A. *Trichomonas vaginalis*/Spermatozoon; B. cocci/leukocyte.

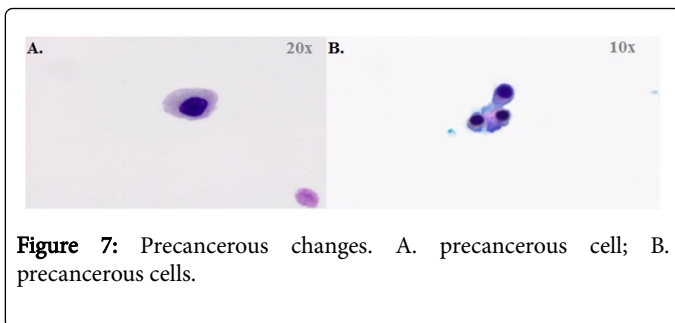


Figure 7: Precancerous changes. A. precancerous cell; B. precancerous cells.

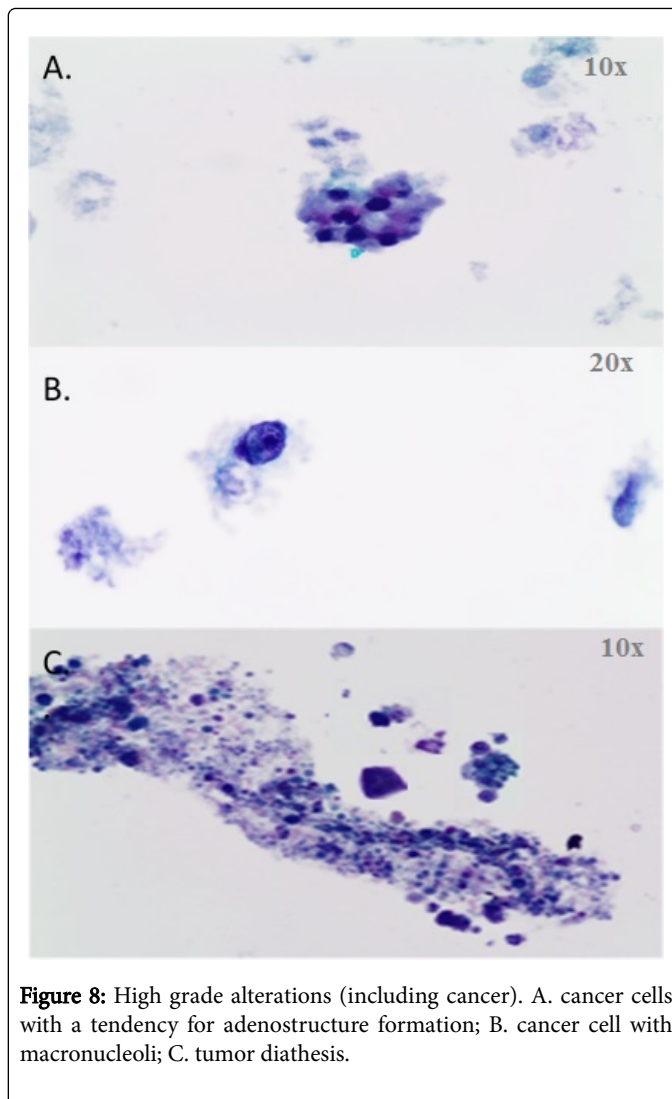


Figure 8: High grade alterations (including cancer). A. cancer cells with a tendency for adenostructure formation; B. cancer cell with macronucleoli; C. tumor diathesis.

In some cytological slides normal findings and alterations, associated with inflammation and recovery were registered. Such alterations are out of the scope of the present study.

An interesting finding is the presence of squamous epithelial cells with well determined perinuclear halo, indicating viral cytopathic effect on the cytological preparations, which confirms indirectly the presence of HPV, proven after that by DNA testing.

In a proportion of patients with molecular profile compatible with PCa, the following morphological findings were found: precancerous condition (increased primitive cells with disturbed maturation; enlarged hyperchromatic nucleus and condensed chromatin) [22].

Morphological findings of high grade alterations, including cancer, were registered in big proportion of urine sediments (~65%), such as: coarse distributed chromatin texture with nuclear membrane irregularity and thickening; high N:C Ratio; prominence of nucleoli and irregularity in shape thereof; identical monotonous nucleoli present in all cells in a group (i.e., "Clonal" pattern); Tumor diathesis [22].

Discussion

The role of the epigenetic phenomenon of DNA “de novo” methylation in the modulation of the gene expression becomes an important focus of research in the field of prostate carcinogenesis. The GSTP1 gene represents a subject of epigenetic fluctuation with implication to the pathogenesis of PCa and plays a role in the genome defense against the oxidative stress [25].

Our results for the strongly expressed hypermethylated GSTP1 alleles in the promoter area of the gene were considered as a sign for the presence of at least PIA, PIN II or PIN III lesions (precancerous findings) and a condition of primary PCa, which coincides with the published data from other authors [26,27]. It is not surprising that the hypermethylated GSTP1 alleles are registered in all PCa suspected specimens, because this epigenetic mutation is considered as the earliest event (in ~ 96% of cases), indicating fluctuations in the PCa specific molecular profile. At the same time, no hypermethylation of the GSTP1 promoter is detected in normal prostate tissues, BPH samples or other normal tissues [28]. The promoter hypermethylation of GSTP1 is reported in urine samples from PCa patients, but not in urine samples from BPH or normal patients [29,30], which is in accordance with our results.

Strongly elevated expression or overexpression of PCA3 prostatic tumor-specific marker and the presence of GSTP1 neoplastic alleles, registered in almost all specimens were considered as an indisputable evidence for prostatic tumor-specific alterations at molecular level. The elevated levels of PCA3 expression have been considered, as one of the earliest events in the PCa pathology, because the same picture has been observed in: poorly-, moderately- and highly-differentiated tumors in the prostate gland [31]. By the application of this biomarker in the clinical practice it became possible to detect PCa in very early stage, even in the absence of clinical symptoms [31]. Furthermore, these molecular fluctuations should be confirmed on cellular level in the contemporary diagnostic approach for PCa (biopsies, histological grading, etc.).

The overexpression of PCA3 in prostate tumor cells is in favour with the correct diagnosis in the clinically ambiguous cases with low levels of tumor cells (<10%) in prostate biopsies and body fluids, which coincides with the situation in our target of noninvasive urine specimens. This molecular marker allows successful detection of prostate tumor cells in tissues and fluids with low tumor content [32-36].

It is not surprising that TMRSS2-ERG positive fusion status was detected in only 4 of the PCa suspected patients. Generally, TMRSS2-ERG positive fusion status is typical for ~50-60% of the PCa probands [37,38]. A significantly lower frequency of TMRSS2-ERG positive fusions (~18%) was reported in our previous study [23]. Gene fusions typically result from primary translocations followed by alternative splicing. The established mechanism of generation of these gene fusions in PCa is androgens-induced.

The occurrence of such mutation events requires severe disturbances in the androgen homeostasis. Only 4 cases in the present sample demonstrated higher metastatic potential of PCa and more aggressive clinical behavior and, as expected only they were positive for T2/ERG fusion profile.

At present, the association between a positive T2-ERG fusion status and the risk for PCa recurrence in patients with clinically localized tumors is proven. Nowadays, TMRSS2-ERG fusion transcripts are

routinely used in the laboratory practice as a powerful prognostic tool for tumor progression [39]. TMRSS2-ERG fusion transcripts represent a potential marker for detection of circulating tumor cells (CTCs) in blood specimens of PCa probands. TMRSS2-ERG findings in the bloodstream give an opportunity to screen and diagnose primary PCa, as well as to monitor the metastatic phases.

The detected high frequency of high-risk HPVs in the investigated Bulgarian prostatic tumor-specific specimens with molecular alterations is in accordance with the reported data in Europe and confirms the hypothesis for the participation of HPV as an inflammatory cofactor in the complex etiology of PCa [40]. The prevalence of HPVs from the subgroup with highest oncogenic potential provides another evidence for the role of the viral component in the initiation and even progression of PCa. Functional analysis on PCa-specific cell cultures and animal models has demonstrated a promotion of HPV-mediated oncogenicity in the initial stage of malignant prostatic transformation and subsequent invasion and proliferation [11-14].

The morphological data showing prostatic tumor-specific alterations and partially cytopathic effect has essential impact on our current research. The assumption for the possible role of high-risk HPVs in the complex nature and dynamic behavior of PCa was proved on cellular level.

Conclusion

In conclusion, the selected molecular panel definitely contributes to earlier and better PCa diagnostics. Our results dethroned the myth that males are asymptomatic carriers of high-risk HPV. In Bulgaria, as in many other countries, chronic or acute prostatitis is treated predominantly with antibacterial drugs without taking into account the potential viral infection, for example HPV. For that reason, the present data is a take-home message for the clinicians that may benefit their medical practice.

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