Molecular analysis of multifocal prostate cancer cases

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Background. Prostate cancer (PCa) is usually a multifocal disease with different foci displaying histological and molecular heterogeneity. Biopsy-based pathology diagnosis of PCa may not be representative of the entire tumour; thus, markers more precisely characterizing every focus of multifocal PCa are highly instrumental for a better testing of PCa.

Materials and Methods. Two distant foci of prostate adenocarcinoma were obtained from prostatectomy specimens of ten patients with pT2–pT3 stage PCa. The expression of TMPRSS2 : ERG, TMPRSS2 : ETV1, and TERT was assessed by means of reverse transcription PCR and quantified using the real-time-based approach. Ki-67, ERG, EMA, AMACR, p16, and p53 expression was evaluated by means of immunohistochemistry (IHC) on tissue microarrays (TMA).

Results. Two out of ten cases were identified with different Gleason scores in paired foci of PCa. The expression of most of the IHC markers was quite even between TMA cores of each PCa case, with p16 showing the highest level of heterogeneity (44%; 4/9); 80% (8/10) of multifocal PCa expressed different variants of the TMPRSS2 : ERG transcript or showed a varying status of fusion positivity in paired foci, and the expression levels of the transcript were also heterogeneous. Similarly, 90% (9/10) of PCa showed a different positivity for TERT expression in paired foci.

Conclusions. Measurement of TMPRSS2 : ERG and TERT expression offers a valuable tool for identifying most aggressive tumour foci and selecting a relevant treatment of PCa.

Key words: prostate cancer, multifocality, TMPRSS2 : ERG, telomerase

INTRODUCTION

Prostate cancer (PCa), the most common cancer in men, usually is a multifocal disease with different foci displaying a marked histological and molecular heterogeneity. More than 80% of prostates have been shown to possess two or more separate tumours at the time of clinical diagnosis of PCa (1). Decision on PCa treatment is usually based on the analysis of a small biopsy section. Due to multifocality, a biopsy specimen may not be representative of the entire tumour, and a set of cases can be under- or over-treated. Radical prostatectomy, usually used for the treatment of PCa, although highly effective, is associated with significant morbidity. Over-treatment of localized PCa may be avoided by using minimally invasive means, such as focal therapy (2). Multiple studies (reviewed in (3)) indicate that the current diagnostic modalities, including PSA test, biopsy or imaging, are not efficient enough to distinguish unifocal cases from a multifocal disease. Molecular biomarkers, such as genetic aberra-
tions and epigenetic changes, are extensively studied for an improved prognosis of PCa. Comparison of molecular profiles of multifocal PCa cases is also an important tool for a better understanding of the molecular basis of multiclonal cancer.

In PCa, a frequent occurrence of gene fusion between the 5′ untranscribed region of TMPRSS2 (transmembrane protease, serine 2) and the 3′ region of genes of the ETS family, including ERG (v-ets erythroblastosis virus E26 oncogene homolog (avian)) or ETV1 (ets variant 1), has recently been identified (4). Fusion of transcription factors of the ETS family to androgen-inducible TMPRSS2 promoter causes an over-expression of these oncogenic proteins. The TMPRSS2 : ERG fusion is the most prevalent form of fusions in PCa and is detectable in almost half of all PCa, independently of the stage or grade (4). Multifocal PCa may harbour different foci that may be TMPRSS2 : ERG positive or TMPRSS2 : ERG negative, and for identification of the fusion positivity information from all significant foci is indispensable. Several recent studies (5–9) showed significant associations between TMPRSS2 : ERG positivity and the aggressive course of PCa; thus, identification of TMPRSS2 : ERG status in prostate tumours may assist in the stratification of PCa cases for a relevant treatment.

Many other molecular alterations, in addition to TMPRSS2 : ETS fusions, have been discovered in PCa in the recent years. Inactivation of tumour suppressor genes, including PTEN, GSTP1, CDKN2A and TP53, as well as activation of oncogenic proteins ERG, MYC and telomerase, are among the most predominant molecular alterations identified in prostate tumours (10). For a better understanding of the molecular basis of PCa, paired foci were selected from 10 cases with multifocal PCa. One of the foci from these pairs of tumours was included in our previous study (11) on a wide range of PCa molecular biomarkers; however, PCa multifocality was not addressed in that assay. In the present study, we analyse the most informative genetic biomarkers selected during our previous investigations and immunohistochemical biomarkers selected from recent publications (12–18) based on their diagnostic or prognostic value in PCa. The expression of fusion transcripts TMPRSS2 : ERG and TMPRSS2 : ETV1, reactivation of the catalytic subunit of telomerase (TERT), expression of the tumour suppressor proteins p16 (encoded by CDKN2A) and p53 (encoded by TP53), expression of the oncogenic transcription factor ERG, epithelial membrane antigen (EMA), and alpha-methylacyl coenzyme A racemase (AMACR) are analysed in the present study in paired foci of PCa cases. In addition, the cell proliferation marker Ki-67 and clinical variables (pT, Gleason score, preoperative PSA value) are assessed for their potential to characterise multifocal prostate tumours.

MATERIALS AND METHODS

Sample collection. Ten cases with multifocal tumours were selected from the large group of PSA-screened and biopsy-proven PCa cases collected during January 2008 – August 2009 at the Urology Department of the Vilnius University Hospital Santariskiu Clinics and treated by radical prostatectomy. The selection of cases was based on the availability of a sufficient amount of tumour tissue for the assessment of multiple biomarkers in two foci. Approval from the local Bioethics Committee had been obtained before initiating the study, and all patients gave informed consent for participation. After radical prostatectomy, unfixed prostate tissue was immediately transported to the Pathology Department. Adenocarcinoma was located by palpation, and cores of 0.8 cm in diameter were punched out by a large core instrument with a circular blade. One of the two punches was taken from the right and the other from the left side of the prostate, or in some cases from the basal and apical regions of the same side. The distance between multifocal tumours was 3 mm or more. The taken core was immediately frozen in liquid nitrogen. Histological sections and the staining of the same core were performed by standard procedures and evaluated by a pathologist.

The selected cases were diagnosed with pT2–pT3 tumours and were graded mainly as having the Gleason score 6 or 7 (Table 1). The mean age of the group was 63.2 ± 1.6 years and the mean preoperative PSA level 6.0 ± 0.8 ng/ml (for one case this information was missing); one case (1/10) experienced PSA-based (biochemical) progression during two months after surgery.

Tissue-arrays and immunohistochemistry. Tissue microarrays (TMA) were constructed from each patient’s archival formalin-fixed, paraffin-embedded donor block (DB) and were assembled in a recipient block (19, 20). The paraffin DB containing the largest focus of tumour was identified in a diagnostic haematoxylin/eosin stained slide. This slide was scanned with the Aperio ScanScope XT System (Aperio Technologies, Vista). The benign and cancer areas were marked with annotations by the pathologist on a virtual slide. From this section, the corresponding regions on the paraffin DB were identified, enabling TMA cores to be taken using an automated tissue arrayer (3DHISTECH, TMA Master, Budapest, Hungary). The core diameter was 1.0 mm, with a depth of 3 mm.

Cut TMA sections (4 μm) were dried, deparaffinised, and rehydrated through descending concentrations of ethanol and prepared for antigen retrieval and immunohistochemistry (IHC) staining. Heat-mediated antigen retrieval was done using microwave treatment for 2 × 5 min in a citrate buffer before being processed either in the Ventana Benchmark system (Ventana Medical Systems, Inc.)
### Table 1. Comparison of TMPRSS2:ERG (status, transcript variants and expression levels) and TERT expression in multifocal prostate tumours

<table>
<thead>
<tr>
<th>Case No.</th>
<th>pT stage</th>
<th>Gleason score</th>
<th>Expression of TMPRSS2:ERG</th>
<th>Expression of TERT</th>
<th>Gleason score</th>
<th>Expression of TMPRSS2:ERG</th>
<th>Expression of TERT</th>
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<td></td>
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QPCR – quantitative polymerase chain reaction; RT-PCR – reverse transcription polymerase chain reaction.

Differences between paired foci are marked in grey. For TMPRSS2:ERG, heterogeneity of fusion status but not the variant transcripts are marked. TMPRSS2:ERG expression levels: 1 – low level (>5.71); 2 – high level (<5.7); relative quantitation of the product was done using GAPDH as endogenous control.

Table 2. Immunohistochemistry values and heterogeneity status

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Ki-67 Mean, %</th>
<th>Range, %</th>
<th>ERG Value</th>
<th>Heterogeneity</th>
<th>EMA Value</th>
<th>Heterogeneity</th>
<th>p16 Value</th>
<th>Heterogeneity</th>
<th>p53 Value</th>
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1 – no staining, 2 – positive for staining. Differences between paired foci are marked in grey. The range was evaluated as the largest difference between Ki-67 proliferation indexes of two cores.
Transcript identification by reverse transcription-PCR. Total RNA was extracted from the frozen tumour tissues by the phenol/chloroform method. Genomic DNA was removed by treatment with DNase I (Fermentas Thermo Scientific). RNA was dissolved in RNase-free water, and RNA quality was checked using electrophoresis on a 1% agarose gel; RNA was quantified on NanoDrop 1000 (Thermo Scientific).

For the reverse transcription-PCR (RT-PCR) analysis, the TMPRSS2:ERG fusion transcript was amplified with the primers described by Tomlins et al. (21), covering the most frequently fused exons (exons 1–2 of TMPRSS2 and exon 4 of ERG): TMPRSS2 5’-CAGGAGCGGAGGCGGA-3’, ERG 5’-GTAGGCACACGACTGG-3’. TMPRSS2:ERG-negative cases were additionally analysed for expression of the TMPRSS2:ETV1 transcript. The primer pair (21) included the same sense TMPRSS2 gene primer as used in the TMPRSS2:ERG assay and the antisense primer covering the exon 4 of ETV1 gene (5’-CAGGCCATGAAAACCTT-3’). The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as endogenous control for fused gene expression, and the product was amplified with the following primer set: univdirGAPDH 5’-CAAGGTCCATC-CATGACAACCTTG-3’ and univrevGAPDH 5’-GTCCAC-CACCTGTGCTGAG-3’.

Fig. 1. Examples of different immunohistochemical staining in tissue microarray cores: a – EMA-negative prostate cancer (PCa), b – EMA-positive PCa epithelium, c – p16-negative PCa glands, d – p16-positive PCa cells, e – AMACR-negative cytoplasm of PCa cells, f – AMACR-positive cytoplasm of PCa cells, g – some Ki67-positive PCa cell nuclei, h – some p53-positive PCa cell nuclei
For detection of the TERT transcript, primers were selected from Boltze et al. (22): TERT-dir 5'-CGGAAGGTGTCTGGAGCAA-3', TERT-rev 5'-GGATGAAGCGGAGTCTGGA-3'. As an additional control, TR was amplified along with TERT, using the following set of primers: TR-dir 5'-CCTAACTGAGAAGGGCGTAGGC-3', TR-rev 5'-CTAGAATGAACGGTGGAAGGCG-3'.

In RT-PCR, 1 µg of total RNA from tissue was reverse-transcribed into cDNA using the Maxima® First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas Thermo Scientific) in the final volume of 20 µl. The reverse transcription product was amplified on Mastercycler ep gradient S (Eppendorf) in the following conditions: 95 °C for 4 min, followed by 94 °C for 1 min, annealing at 58 °C for 45 s and 72 °C for 45 s repeated 35 times, and the final elongation at 72 °C for 10 min. A negative control without reverse transcription was included for each sample, and multiple water controls were included in each run. RT-PCR products were resolved by electrophoresis on 2% agarose gels (Fig. 2). The presence of the RT-PCR product (TMPRSS2 : ERG, GAPDH, TERT or TR) was scored as positivity for the corresponding transcript (Table 1).

To identify the exact point of fusion, a set of TMPRSS2 : ERG transcripts of different length were sequenced on a 3130XL Genetic Analyzer (Applied Biosystems) using a cloned product (InsTAclone™ PCR Cloning Kit; Fermentas Thermo Scientific) and the v2.0 kit (Applied Biosystems) for sequencing reaction. In sequence analysis, the nucleotide numbering of the TMPRSS2 gene is based on cDNA sequence with the GeneBank number NM_005656 and for the ERG gene NM_004449.

**TMPRSS2 : ERG quantification.** The fused gene expression was quantified by using SYBR Green labelling and the pairs of primers described previously (21). The QPCR reaction was performed using Maxima™ SYBR Green QPCR Master Mix (Fermentas Thermo Scientific), with 1 U/µl of Uracil-DNA Glycosylase (Fermentas Thermo Scientific), 300 mol/l forward and reverse primers in the total volume of 25 µl. The PCR cycles on the ABI Prism 7500 Fast Real-time PCR System (Applied Biosystems) consisted of 50 °C for 2 min, 95 °C for 10 min, followed by 95 °C for 15 s and 60 °C for 1 min, repeated in 40 cycles, and the melting step. Threshold cycle (ct) values for each sample were generated during the exponential phase of QPCR, and ∆ct values were calculated using GAPDH as an internal control. According to the median ∆ct value, the expression level of the TMPRSS2 : ERG transcript was dichotomized into high (<5.7) and low (>5.71; Table 1).

**Statistical analysis.** Statistical analysis was carried out using GaggraphPad InStat software. Correlations were evaluated by Spearman’s test. Two-sided Fisher’s exact test was used for comparison of categorical values and the Mann–Whitney test for continuous values. The p value <0.05 was considered significant.

**RESULTS**

**Clinical and IHC characteristics of paired foci.** A total of 20 tumour foci from 10 radical prostatectomy specimens were analysed in our study. Eight pairs of multifocal tumours had an identical Gleason score, while two cases had tumours with different Gleason scores (Table 1). The expression of most of the IHC markers was quite even between TMA cores of each PCa case. EMA and ERG were homogeneously expressed in all pairs of cores (9/9 and 7/7, respectively; Table 2). TMA pairs showed a less uniform expression of p53 and AMACR proteins: 2 out of 9 (22%) and 1 out of 9 (11%) TMA pairs showed expression heterogeneity, respectively. Four out of 9 (44%) TMA pairs were heterogeneous according to positivity for the...
p16 protein. The Ki-67 PI range variation was from 1% to 7% among cores when the Ki-67 PI mean value was 1–9% among different cases. No statistically significant correlations were identified between the status of heterogeneity of a particular IHC marker and TMPRSS2 : ERG, TERT expression or PCa clinical variables. However, an association was observed between the occurrence of TMPRSS2 : ERG fusion and immunohistohemically detectable expression of ERG: two out of seven PCa cases were negative for ERG expression in IHC (Table 2), and both cases showed a low production of the TMPRSS2 : ERG transcript in the QPCR assay or were negative for TMPRSS2 : ERG in RT-PGR (Table 1).

Genetic characteristics of paired foci. Overall, 18 out of 20 tumour foci expressed the TMPRSS2 : ERG fusion product. TMPRSS2 : ERG expression was detected in 8 PCa in both foci, while two cases showed the fusion transcript only in one focus (2 / 10; 20% heterogeneity for the fusion status). No TMPRSS2 : ETV1 transcript was detected in these two foci. According to the type of TMPRSS2 : ERG transcripts, the fusion between the first exon of TMPRSS2 and the fourth exon of ERG prevailed (85%) in our study; 11 (55%) foci expressed T1 / E4 as a single transcript and 6 foci together with the T2 / E4 transcript variant (30%). One case was identified with the T1 / E1 transcript in the first focus and with the T1 / E4 in the second focus. An identical set of TMPRSS2 : ERG transcripts was detected in paired foci from two prostate carcinomas; thus, the heterogeneity of the fusion isoform was 75% (6/8). Assessment of the TMPRSS2 : ERG transcript expression levels revealed an additional level of heterogeneity. Notable differences (p < 0.0001) were identified between paired foci, with only 4 out of 8 pairs showing a similar intensity of fusion expression (Table 1). Thus, the heterogeneity of the transcription level was 50%.

Measurement of TERT expression was also helpful in verifying the molecular heterogeneity of multifocal tumours: only one case of PCa had an identical status for TERT expression in both foci (90% heterogeneity; Table 1). Expression of the TERT transcript was predominant in TMPRSS2 : ERG-positive foci with 100% (11 / 11) of TERT-positive cases concurrently having the fusion transcript (Table 1); however, the association was not statistically significant. Similarly, foci with low TMPRSS2 : ERG expression levels (50%; 7 / 14) were negative for TERT, while the cases showing a high TMPRSS2 : ERG expression (100%; 4 / 4) were also TERT-positive.

One case (No. 10) of biochemical disease progression was identified in this pilot study. A high TMPRSS2 : ERG expression level as well as positivity for TERT transcript were detected in one tumour focus, while another focus showed a low production of TMPRSS2 : ERG transcript and was TERT-negative.

DISCUSSION

Multifocal PCa has been reported in up to 91% of all cases of radical prostatectomy (1, 2). Some investigators (reviewed in 1) have reported that increased numbers of tumour foci and larger volumes of index and nonindex tumours are correlated with a more aggressive outcome of PCa. However, the currently approved PCa biomarkers, including the preoperative PSA value, are not potent predictors of multifocality, and sensitive markers for the identification of multifocal PCa are highly instrumental. In our study, 8 out of 10 pairs of foci from multifocal PCa expressed different variants of the TMPRSS2 : ERG transcript or showed a variable status of fusion positivity. Similarly, 90% of multifocal tumours showed a distinct status of TERT expression (positive or negative) in both foci. Different Gleason scores were identified in two PCa cases, but most of the IHC biomarkers showed low levels of heterogeneity between the foci.

The morphological difference of tumours is mainly characterized by the Gleason score. Arora et al. (23) showed that only 9 of 100 prostatectomy specimens had the same Gleason score in all the foci studied. In another study (24), more than half of prostates contained at least three different grades of cancer, and the number of different grades increased with increasing tumour volume. In our study, different Gleason scores were identified in two pairs of tumours with multifocal PCa. The cell proliferation index, determined by Ki-67 expression, was 1–9% among different cases of PCa, and a certain variation (up to 7%) was observed in paired PCa tissues. Other IHC biomarkers, except p16, showed quite a homogeneous expression in separate foci; 44% of paired tumour foci had a varying p16 expression. There are limited data in the literature on the heterogeneity of IHC markers in PCa. Borre et al. (25) reported some level of heterogeneity of the p53 protein expression in prostate tissue. Our study on the ERG protein expression, conducted in a larger group of PCa cases (Petroska et al., unpublished data) supports the findings of the present assay about a substantial level of ERG homogeneity in multifocal PCa.

The occurrence of TMPRSS2 : ERG fusion in prostate carcinomas has been analysed in numerous studies (reviewed in 4). However, data on the heterogeneity of TMPRSS2 : ERG fusion in multifocal PCa are really limited. Barry et al. (26) analysed 32 multifocal PCa cases and found fusion heterogeneity in 41% of them. In the study of Mehra et al. (27), 43 cases were analysed, and 70% of them showed heterogeneity. Both studies were done by means of FISH, and the heterogeneity of the fusion status was understood as either the presence or absence of fusion or the occurrence of TMPRSS2 rearrangements through different genetic events (translocation or deletion). None of these
studies evaluated the TMPRSS2 : ERG expression level or assessed fusion variants. In our study, using RT-PCR and QPCR, a high heterogeneity of TMPRSS2 : ERG transcripts was detected, and the expression levels also differed among the foci. In our study, 6 out of 8 pairs of tumours from multifocal PCa expressed different variants of TMPRSS2 : ERG, and two additional pairs showed a varying status of fusion positivity. Notably, several recent studies (5–9) have shown significant associations between TMPRSS2 : ERG positivity and the aggressive course of PCa. Expression of specific splice variants of TMPRSS2 : ERG or occurrence of fusion duplication (6, 7, 9) were shown as indicators of aggressive PCa behaviour; thus, identification of TMPRSS2 : ERG-positive foci in prostate tumours may assist in predicting the clinical outcome of the disease.

In our study, some association was identified between the expression of TERT, encoding the main subunit of telomerase, and tumour positivity for TMPRSS2 : ERG which suggests a possible association between these two oncogenic transcripts involved in prostate pathogenesis. Telomerase is a reverse transcriptase responsible for the elongation of chromosomal ends or telomeres. Telomeres are important components of molecular pathways involved in cell senescence and the control of the cell cycle. In PCa, telomerase activity has been detected in 47% to 100% of PCa cases (28). We used a RT-PCR-based approach for detecting the transcript and identified TERT in 11 of 20 (55%) tumour foci of PCa patients. Our results are in line with the data of other studies (29, 30) and for the first time show a considerable heterogeneity of multifocal prostate tumours according to TERT expression. A recent study (31) has revealed that human prostate tumours contain a cancer progenitor cell subpopulation which can be indicated by an elevated telomerase activity. The aggressiveness of tumour foci is significantly influenced by the amount of progenitor cells which can be evaluated by measuring TERT expression.

One case of biochemical progression was identified among multifocal tumours involved in our study. Results of molecular tests suggested an aggressive outcome based on the assessment of one focus, while another focus showed a non-aggressive molecular pattern – a low production of TMPRSS2 : ERG transcript and negativity for TERT. In this particular case, molecular analysis of two foci from multifocal PCa provided more accurate data on the possible biological behaviour of the tumour than those expected from the analysis of a single focus.

CONCLUSIONS

Our study revealed pronounced levels of heterogeneity in the expression of oncogenic transcripts related to prostate carcinogenesis in separate tumour foci from PCa patients. IHC biomarkers were less heterogeneous in the PCa of these cases. The application of molecular tests, particularly measurement of TMPRSS2 : ERG and TERT expression, offers a valuable tool for the identification of most aggressive tumour foci and selection of a relevant PCa treatment.

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References


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DAUGIAŽIDININIŲ PROSTATOS NAVIKŲ
MOLEKULINIAI TYRIMAI

Santrauka

Įvadas. Prostatos navikai (PN) dažniausiai yra daugiažidiniai, nes histologiškai ir molekuliniu profiliu besiskiriantys naviko žiadiniai formuoja iš karto kelio prostatos srityse. Prostatos vėžio diagnozė grindžiama patologiniu biopsijos mėginio tyrimu, tačiau šis mėginys neretai neatspindi visų piktybėjimo židinių charakteristikų. Tiksliai ligos eigos prognozavimui būtini žymenys, apibūdinantys individualius daugiažidininius PN ir įvertinantys jų agresyvumą.


Rezultatai. Skirtingos diferenciacijos pagal Gleason skale naviko židiniai nustatyti dviejųose iš dešimties daugiažidininių PN. Daugumos imunohistocheminių (IHC) žymenų raiška buvo homogeniška skirtinguose to paties naviko židiniuose, tačiau 44 % (4 iš 9) PN nustatyta skirtinga p16 baltymo raiška. 80 % (8 iš 10) daugiažidininių PN nustatyti TMPRSS2 : ERG raiškos skirtumai – sulietinio transkripto izoformų įvairės, skirtingas raiškos intensyvumas arba transkriptas buvo nustatomas tik viename židinyje. 90 % (9 iš 10) PN pasižymėjo skirtinga TERT raiška tirtuose poriniuose židiniuose.

Išvados. TMPRSS2 : ERG ir TERT transkriptų nustatymas gali būti naudojamas aptinkant agresyvius PN židinius, taip pat siekiant parinkti tinkamiausią gydymo strategiją.

Raktažodžiai: prostatos vėžys, daugiažidininiai navikai, TMPRSS2 : ERG, telomerazė