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Rasa Sabaliauskaitė

INVESTIGATION OF MOLECULAR MARKERS OF HUMAN PROSTATE CANCER

Summary of the Doctoral Dissertation

Biomedical Sciences, Biology (01 B)

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The Doctoral Dissertation was prepared at the Vilnius University in 2007–2012.

Scientific Supervisor:

Prof. Habil. Dr. Juozas Rimantas Lazutka (Vilnius University, Biomedical Sciences, Biology – 01 B)

Scientific Consultants:

Prof. Sonata Jarmalaitė (Vilnius University, Biomedical Sciences, Biology – 01 B)

The defence of doctoral dissertation will be held at the Vilnius University Scientific Council on Biology:

Chairman:

Prof. Dr. Donaldas Čitavičius (Vilnius University, Biomedical Sciences, Biology – 01 B)

Members:

Prof. Dr. Donatas Žvingila (Vilnius University, Biomedical Sciences, Biology – 01 B)

Prof. Dr. Ilona Teodora Miceikienė (Lithuanian University of Health Science, Biomedical Sciences, Biology – 01 B)

Prof. Dr. Janina Didžiapetrienė (Institute of Oncology, Vilnius University, Biomedical Sciences, Medicine – 06 B)

Prof. Habil. Dr. Izolda Pašakinskienė (Vilnius University, Biomedical Sciences, Biology – 01 B)

Opponents:

Prof. Dr. Kęstutis Sužiedėlis (Institute of Oncology, Vilnius University, Biomedical Sciences, Biology – 01 B)

Dr. Rūta Navakauskienė (Vilnius University, Physical Sciences, Biochemistry – 04 P)

The Doctoral Dissertation will be defended at the public session of the Council of Biological Science at 2 p. m. on 27th of June 2012 in the Great auditorium of the Faculty of Natural Sciences,Vilnius University.

Adress: M. K. Čiurlionio str. 21, LT–03101, Vilnius, Lithuania.

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The Doctoral Dissertation is available at the Library of Vilnius University.

VILNIAUS UNIVERSITETAS

Rasa Sabaliauskaitė

ŽMOGAUS PRIEŠINĖS LIAUKOS VĖŽIO MOLEKULINIŲ ŽYMENŲ TYRIMAI

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Mokslinis vadovas:

Prof. habil. dr. Juozas Rimantas Lazutka (Vilniaus universitetas, biomedicinos mokslai, biologija – 01 B)

Mokslinė konsultantė:

Prof. dr. Sonata Jarmalaitė (Vilniaus universitetas, biomedicinos mokslai, biologija – 01 B)

Disertacija ginama Vilniaus universiteto Biologijos mokslo krypties taryboje:

Pirmininkas:

Prof. dr. Donaldas Čitavičius (Vilniaus universitetas, biomedicinos mokslai, biologija – 01 B)

Nariai:

Prof. dr. Donatas Žvingila (Vilniaus universitetas, biomedicinos mokslai, biologija – 01 B)

Prof. dr. Ilona Teodora Miceikienė (Lietuvos sveikatos mokslų universitetas, biomedicinos mokslai, biologija – 01 B)

Prof. dr. Janina Didžiapetrienė (Vilniaus universiteto Onkologijos institutas, biomedicinos mokslai, medicina – 06 B)

Prof. habil. dr. Izolda Pašakinskienė (Vilniaus universitetas, biomedicinos mokslai, biologija – 01 B)

Oponentai:

Prof. dr. Kęstutis Sužiedėlis (Vilniaus universiteto Onkologijos institutas, biomedicinos mokslai, biologija – 01 B)

Dr. Rūta Navakauskienė (Vilniaus universitetas, fiziniai mokslai, biochemija – 04 P)

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Adresas: M. K. Čiurlionio g. 21, LT–03101, Vilnius, Lietuva.

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INTRODUCTION

Relevance of the study. Prostate cancer (PCa) is the second most common male oncologic disease, after skin cancer, and the second leading cause of cancer death in men after lung cancer (Ahmed, 2010). Prostate cancer is a complex disease caused by alterations of multiple genes involved in various molecular pathways. After surgery, some patients live a long time without symptoms, while others suffer from disease progress right afterwards. The leading cause of disease severity and high mortality is failure to respond to sex hormones signals and developed insensitivity to hormone therapy. This leads to frequent metastasis into the bone system and the limited sensitivity of the systemic chemotherapy. PCa usually is a multifocal disease with different foci displaying marked histological and molecular heterogeneity. Researchers have evidenced that more than 80% of prostates possess two or more separate tumours at the time of clinical diagnosis of PCa. Decision on the treatment of PCa is usually based on analysis of small biopsy section and widely applied marker – prostate specific antigen (PSA). Due to multifocality, biopsy specimen may not represent the entire tumour, and a set of cases can be under-treated or over-treated. High PSA levels could be found not only in PCa, but also in patients with inflammatory prostate diseases. In order to increase the survival of PCa patients, scientists are looking for new molecular biomarkers that could help to predict the course of the disease and to find the most appropriate treatment. Molecular cancer biomarkers – genetic mutation and epimutation, genes and miRNA expression changes, functionally or structurally altered proteins and other mutations that are found in tumours or pre-neoplasic tissues. Prognostic value has been shown for molecular markers, the expression of which correlates with clinical course and longer survival time. Recent studies (reviewed in Tomlins et al., 2009) revealed frequent occurrence of genetic fusion between 5' untranslated region of the androgen-regulated gene *TMPRSS2* (transmembrane protease, serine 2) and 3' region of the genes encoding transcription regulators of the ETS family, including *ERG* (v-ets erythroblastosis virus E26 oncogene homolog (avian)) or *ETV1* (ets variant 1). The fusion of transcription factors of the ETS family to androgen-inducible *TMPRSS2* promoter causes over-expression of these oncogenic proteins. The *TMPRSS2:ERG* fusion has been observed in about half of prostate tumours, and some of the studies (Demichelis et al., 2007; Nam et al., 2007; Attard et al., 2008; Hu et al., 2008; Wang et al., 2008) associated this chromosomal alteration with an increased risk of recurrence in PCa. ETS factors are especially significant for development of epithelial and endothelial tissue, blood, nervous and endocrine systems, involved in cell growth and migration. ETS proteins in mammalian cells, which activate the expression of growth factors, also are involved in *Ras* oncogene signaling. *TMPRSS2:ERG* gene fusion has been identified in prostate adenocarcinoma and intraepithelial neoplasia (PIN) tissue (approximately 20%), but they are not detectable in atrophy or normal prostate tissue. Such gene fusion is found not only in hormone-sensitive prostate metastases, but also in non-hormonal metastases.

In 1977, the existence of free circulating nucleic acids (DNA and RNA) was proved by assessing body fluids (blood serum, urine and saliva) from cancer patients. Extracellular tumour DNA, RNA or miRNA get into body fluids through the death of tumour cells. K. Porkka et al., (2007) revealed that the prostate tumours have specific

miRNA profile and showed a different miRNA expression, which is often correlated with the clinical characteristics and disease prognosis.

Epigenetic changes usually occur in the early stages of carcinogenesis. Increased DNA methylation (hypermethylation) of regulatory genes promoters inactivates the gene expression and blocks their functions. Inactivation of various tumour suppressor genes (TSGs), including *PTEN*, *GSTP1*, *CDKN2A* and *TP53*, through induction of genetic mutation or hypermethylation of promoter region, as well as activation of oncogenic proteins ERG, MYC and telomerase, are among the most predominant molecular alterations identified in prostate tumours. More than 90% of PCa are found with the hypermethylated promoter of glutathione S-transferase-P1 (*GSTP1*) gene. Inactivation of *GSTP1* through aberrant methylation of its promoter region is a major feature of most prostate tumours (Hopkins et al., 2007; Ahmed, 2010). This enzyme is involved in detoxification of carcinogenic compounds and cellular responses to oxidative stress. GSTP is abundantly detectable in prostate tissues, while expression of the enzyme is markedly suppressed in most of prostate carcinomas and high-grade prostatic intraepithelial neoplasias (Bostwick et al., 2007; Ahmed, 2010). Ras association (RalGDS/AF-6) domain family member 1 (RASSF1) is the tumour suppressor protein implicated in various cellular mechanisms: apoptosis, growth, adhesion, migration, differentiation, cell cycle control and microtubule stabilization. Frequent loss of *RASSF1* expression through promoter hypermethylation was shown in prostate carcinoma cell lines and prostate tumours (Ahmed, 2010). *RARB* gene encodes retinoic acid receptor involved in regulation of cell growth, differentiation, apoptosis and suppression of carcinogenesis. Reduced expression of *RARB* is frequent in various tumours, and one recent study (Mao et al., 2011) identified *RARB* as a target of major genetic rearrangements in PCa. Abnormal gene promoter methylation and miRNA expression could help to identify the type of cancer, predict progression of disease and to choose optimal treatment strategies for patients.

Objectives. Investigate and assess the potential prostate tumour molecular markers: by molecular methods determine oncogenes and tumour suppressing gene and miRNA expression profile also XMRV virus (*Xenotropic Murine Leukemia Virus-related virus*) sequences frequency study in prostate samples, assessing to investigate molecular markers of gene expression between the interferences relationship with clinical parameters and patient age.

Statements to be defended:

1. Presence of the *TMPRSS2:ERG* transcripts in the prostate cancer samples influences the expression profile of other genes and miRNAs.

2. Combined assessment of *TMPRSS2:ERG* and *TERT* markers in PCa has an improved prognostic value: can predict disease progression and shorter recurrence-free period.

3. Multifocal prostate cancer is characterized by variant *TMPRSS2:ERG* transcripts and diverse gene expression.

4. Detection rate of the XMRV virus sequence in samples of Lithuanian male prostate cancer population correspond the global trend.

Scientific novelty. It is one of the first attempts to analyze Lithuanian PCa samples using modern molecular techniques (a quantitative PCR (QPCR) and/or microarrays) and multi-marker approach. The study provided novel information about heterogeneity of PCa, identified prognostic biomarkers for prediction of disease progression and identified novel interactions between occurrence of *TMPRSS2:ERG* and expression of other PCa-related genes and miRNAs.

Scientific practical significance. Conventional clinical markers and PSA is currently used for prediction of clinical course of PCa. In this study, we attempted to analyze molecular changes in PCa in order to identify the novel prognostic biomarkers. Identification of molecular changes in the PCa increase knowledge on the disease development and helps to predict the likelihood of disease progression; it also could help to discover potential therapeutic targets for the next generation treatment. The success of cancer treatment depends on early diagnosis and on selection of relevant treatment based on information collected by studies of molecular profile of tumour. The genetic (RNA and miRNA) analysis is highly informative, sensitive and selective. In our study, we tried to select novel molecular biomarkers for early prediction of recurrence in PCa. We also developed a minimally invasive approach for detection of these biomarkers in urine sediment. This could assist as an additional tool for disease diagnosis and prediction of recurrence, while maintaining conventional diagnostic methods.

Presentation and approbation of results. The results of this study have been published in 3 articles. 4 poster presentations have been made on the subject for the dissertation at conferences.

Structure of the dissertation. The dissertation consists of the following chapters: Abbreviations, Introduction, Literature Review, Material and Methods, Results, Discussion, Conclusions, List of the author's publications, Acknowledgments and

References. All the material is presented in 130 pages. The list of the references includes 104 sources. The dissertation is written in the Lithuanian language.

MATERIALS AND METHODS

Sample collection. Prostate tissues were obtained from the patients with PSA-screened and biopsy-proven prostate adenocarcinoma treated with radical prostatectomy at the Urology Department of Vilnius University Hospital Santariskiu Clinics from January 2008 to August 2009. Approval from the local Bioethics Committee was obtained before initiating this study, and all patients gave informed consent for participation in the study. Adenocarcinomas were detected by palpation of removed prostate and cores of 0.8 cm diameter were punched out by a large core instrument with circular blade. For the multifocal tumours analysis, one of two punches was taken from the right and other from the left side of 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 core taken was snap-frozen in liquid nitrogen. The sections from 189 cores were available for the gene expression analysis of: 158 samples contained prostate adenocarcinoma tissue, while 21 cores included no tumour tissue, and were used as control non-cancerous tissue, also, there were 10 multifocal tumours samples. In addition, 67 specimens of urine sediments from the same group of patients were available for genetic analysis. Urine was collected by catheterization during radical prostatectomy into urine collection cups. About 30 ml of urine was centrifuged at 1000 rpm for 15 min at 4°C. Urine sediments were stored in liquid nitrogen. Follow-up data were available for 179 patients with a mean follow-up time of 23.5 months. Biochemical recurrence (BCR) was defined as increase in PSA level after surgery, using cut-off value of >0.2 ng/ml (**Table 1**).

Table 1. Clinical characteristics and age of investigated patients

1 lentelė. Tiriamosios grupės klinikiniai rodikliai ir amžius

	All cases n (%)	**Multifocal cases n (%)**	**Non-tumour cases n (%)**	**Cases used for miRNA analysis n (%)**
Mean age in years ± SEM	61,3 ± 0,59	63,2 ± 1,58	62,4 ± 1,25	61,87 ± 0,62
pT stage				
pT2	116 (73%)	7 (70%)	14 (66,67%)	80 (76,2%)
2a	16 (13,8%)	7 (100%)	2 (14,3%)	12 (15%)
2c	100 (86,2%)	-	12 (85,7%)	68 (85%)
pT3	42 (27%)	3 (30%)	7 (33,33%)	25 (23,8%)
3a	30 (71,4%)	2 (66,67%)	5 (71,4%)	17 (68%)
3b	12 (28,6%)	1 (33,33%)	2 (28,6%)	8 (32%)
Gleason score				
6	100 (63,3%)	14 (70%)	18 (85,7%)	73 (69,52%)
7	58 (36,7%)	6 (30%)	3 (14,3%)	32 (30,48%)
Preop PSA, ng/ml ± SEM	9,43 ± 0,86	6,0 ± 0,78	7,3 ± 0,98	8,85 ± 1,01
Prostate weight, g ± SEM	49,83 ± 1,74	47,5 ± 7,47	55,9 ± 5,05	50,26 ± 2,22
Tumour volume, mm^3 ± SEM	13,66 ± 0,76	19,8 ± 4,58	-	12,5 ± 1
Body mass index ± SEM	28,01 ±0,32	29,33 ± 2,53	28,32 ± 0,87	-
Recurrence				
Yes	20 (14,5%)	1 (10%)	5 (25%)	17 (17,53%)
No	118 (85,5%)	9 (90%)	15 (75%)	80 (82,47%)
Total	**158**	**10 + 10**	**21**	**105**

RNA extraction. Total RNA was extracted from the frozen tumour and non-tumour tissues by phenol/chloroform method. Genomic DNA was removed by treatment with DNase I (Fermentas Thermo Scientific). The RNA was dissolved in RNase free water and RNA quality was checked using electrophoresis on a 1% agarose gels, RNA was quantified on NanoDrop 1000 spectrophotometer (Thermo Scientific). RNeasy Plus Mini kit (Qiagen) and Total RNR Isolation NucleoSpin® RNA II kit (MACHEREY-NAGEL) were used for total RNA isolation from urine samples.

Detection of *TMPRSS2:ETS*, *TERT*, *ZAC* and XMRV. For reverse transcription-PCR (RT-PCR) analysis, the *TMPRSS2:ERG* fusion transcript was amplified with the primers described by Tomlins et al. (2005), covering the most frequently fused exons 1–2 of *TMPRSS2* and exon 4 of *ERG*. Tissue sample without *TMPRSS2:ERG* fusion transcript was amplified with the primers described by Tomlins et al. (2005), covering the most frequently fused exons 1–2 of *TMPRSS2* and exon 4 of *ETV1* using the same *TMPRSS2* primer and *ETV1*. Primers used for detection of *TERT,* described by Boltze et al. (2003), as an additional control *TR*, was amplified along with *TERT*, using the set of primers. Primers used for detection of *ZAC* described by Valleley et al. (2007). Primers used for detection of XMRV described by Urisman et al. (2006). In all genes,

the RT-PCR analysis *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as an endogenous control. All primers were from Metabion.

In RT-PCR, 1 µg of total RNA from tissue or urine sediments was reverse transcribed into cDNA using one of the kit's: RevertAid™ H Minus First Strand cDNA Synthesis Kit, RevertAid™ Premium Reverse Transcriptase or Maxima® First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas Thermo Scientific) in a final volume of 20 µl. Reverse transcription product was amplified on Mastercycler ep gradient S (Eppendorf) under the following conditions: 95˚C for 4 min, followed by 94˚C for 1 min, annealing at X˚C for 45 s (X depending from primers), and 72˚C for 45 s repeated 35 times, and the final elongation at 72˚C for 10 min. cDNA from urine sediments was analyzed under the same conditions, but the cycle number was increased up to 40. 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.

Nested PCR. We used nested PCR for XMRV virus detection for confirmation of their RT-PCR results. Expression of the housekeeping *GAPDH* gene was used as a control in nested PCR. The first-round PCR consisted of 1 µg cDNA, XMRV-1F and XMRV-1R primers under the following conditions: 95ºC for 4 min, followed by 94ºC for 1 min, 48ºC for 45 s, and 72ºC for 45 s repeated 35 times, and the final elongation at 72ºC for 10 min. The second round of PCR consisted of 1 µl of the first round PCR product, XMRV-2F and XMRV-2R primers, under the following conditions: 95ºC for 4 min, followed by 94ºC for 1 min, 54ºC for 45 s, and 72ºC for 45 s repeated 35 times, and the final elongation at 72ºC for 10 min. Both nested PCR products were amplified on Mastercycler epgradient S (Eppendorf). Nested PCR products were resolved by electrophoresis on 2% agarose gels.

Quantification of *TMPRSS2:ERG*, *TERT*, *ERG*, *SPINK1*, *GSTP1*, *RARB*, *RASSF1*, *ZAC*, *ZAC1p* and *ZAC2p*. Gene expression was assessed by quantitative RT-PCR (QPCR) with SYBR Green labeling. The pairs of primers (*TMPRSS2:ERG*, *TERT* and *ZAC*) used in the assay were as described above. Other primers were described by Tomlins et al., (2005 and 2008): *ERG* and *SPINK1*; Zambrano et al., (2005): *GSTP1, RARB, RASSF1* and Valleley et al., (2007): *ZAC1p* and *ZAC2p*. The primers for *GAPDH* analysis were the same as in the RT-PCR assay. All primers were from Metabion.

QPCR reaction was performed using Maxima™ SYBR Green QPCR Master Mix (Fermentas Thermo Scientific), with 1 U/µl of Uracil-DNA Glycosylase, 300 nmol/l forward and reverses primers, in a total volume of 20 µl. The PCR cycles on 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 by 40 cycles and the melting step. Each experiment was performed in duplicate. Threshold cycle (ct) values for analyzed genes for each sample were generated during the exponential phase of QPCR, and $2^{\Delta ct}$ values were calculated using *GAPDH* as an internal control. Δct values were divided into two groups based on median.

Genomic DNA extraction and genomic XMRV DNA amplification. Genomic DNA was extracted from XMRV-positive samples using Genomic DNA Purification Kit (Fermentas Thermo Scientific). The genomic DNA was dissolved in Nuclease free water, amount of DNA was defined with NanoDrop 1000 (Thermo Scientific). For the XMRV genome sequencing, we created 14 overlapping primers. Their used Long PCR Enzyme Mix (Fermentas Thermo Scientific) under the following conditions: 95ºC for 5 min, followed by 95ºC for 1 min, depending on the primers from 57ºC to 64ºC for 45 s, and 72ºC for 45 s repeated 42 times, and the final elongation at 72ºC for 10 min. PCR was carried out from the genomic DNA in accordance with manufacturer's instructions. The genomic DNA was amplified on Mastercycler epgradient S (Eppendorf). PCR products were resolved by electrophoresis on 1% agarose gels. PCR products were purified with GeneJET™ PCR Purification Kit (Fermentas Thermo Scientific).

Sequence analysis. For identification of the exact point of the fusion, a set of the *TMPRSS2:ERG* transcripts and XMRV RT-PCR products were sequenced. PCR products were cloned into plasmids using InsTAclone™ PCR Cloning Kit (Fermentas Thermo Scientific). Plasmids with the fragments were purified using GeneJET™ Plasmid Miniprep Kit (Fermentas Thermo Scientific) and sequenced using sequencing reaction v2.0 kit (Applied Biosystems™) on 3130XL Genetic Analyzer (Applied Biosystems™). In sequence analysis nucleotide numbering of the *TMPRSS2* gene is based on cDNA sequence with GeneBank number NM_005656, and for the *ERG* gene – NM_004449. The sequence analysis nucleotide numbering of the XMRV complete genome is based on cDNA sequence with GeneBank number EF185282.1.

miRNA Expression Profiling. miRNA expression was analyzed with Agilent human miRNA microarray v2 chips (Agilent Technologies) containing 723 human and 76 human viral miRNAs in accordance with recommended instructions. Total RNA was labeled with pCp-Cy3 using miRNA Labelling Reagent and hybridized using Hybridization Kit (Agilent Technologies) and Agilent SureHyb chambers. The microarray images were scanned with Agilent microarray scanner and analyzed using Agilent Feature Extraction Software and GeneDpring GX software (Agilent Technologies). To identify differently expressed miRNAs in prostate tissue samples, data was normalized.

miRNA validation with QPCR. For miRNA expression analyses (the same with the cell lines), first-strand cDNA synthesis was carried out from total RNA using MultiScript reverse transcriptase (Applied Biosystems™) in accordance with manufacturer's instructions. The expression of *ERG* and *TBP* (reference gene) was measured with CFX96 Real-Time System (Bio-Rad) apparatus. For the miRNA measurements, the reverse transcription was performed with MultiScript reverse transcriptase (Applied Biosystems™) in accordance with manufacturer's instructions. The RT-reactions and QPCR reactions were made separately for each miRNA with TaqMan® miRNA assays (Applied Biosystems™) in accordance with the manufacturer's instructions. QPCR were run with 2x TaqMan® Universal PCR Master Mix (Applied Biosystems™) and CFX96 Real-Time System (Bio-Rad) instrument. *RNU6B* was used as a reference miRNA. Each experiment was performed in duplicate.

Cell Culture Protocols. For the experiments, we used VCaP and LNCaP cell lines (American Type Culture Collection, ATCC) transfected with siRNA (anti *ERG*) and control siRNA. All cells were grown under the recommended conditions. After 3 days the cells were trypsinized and placed in 24-well dishes corresponding scrambled siRNA (negative control, Ambion). INTERFERin™ siRNA transfection reagent (Polyplus-Transfection) was used for transfections in accordance with manufacturer's protocol. Total RNA was extracted using Trizol (Invitrogen) reagent in accordance with recommended instructions. The level of over-expression was assessed in duplicates with QPCR as described above.

Statistical analysis. Two-sided Fisher's exact test and Mann-Whitney test were used for statistical comparisons; correlations were evaluated by Spearman's test. Mantel-Cox test was applied for analysis of follow-up data. A p value <0.05 was considered as significant.

RESULTS

Expression of *TMPRSS2:ETS* in tumour and non-tumour prostate tissue

Expression of *TMPRSS2:ETS* fused transcripts were assessed by RT-PCR in 179 prostate tissues from PCa patients. The analysed tissues consisted of 158 tumour and 21 non-tumour cases. 96 out of 179 (54%) prostate tissues from PCa patients were positive for the *TMPRSS2:ERG* fusion gene. The fusion was detected in 93 out of 158 (58%) adenocarcinomas, while only in 4 (19%) non-cancerous prostate tissues obtained from PCa patients. As identified by sequencing, T1/E4 was the most predominant fusion type, detected in 59 samples as a single transcript and in 18 samples together with the variant transcripts (**Fig. 1A**). The T1/E1 transcript was detected in 9 of the cases as a single transcript or together with the variant transcript (T1/E4 or T2/E4) and 7 cases with different transcript variant.

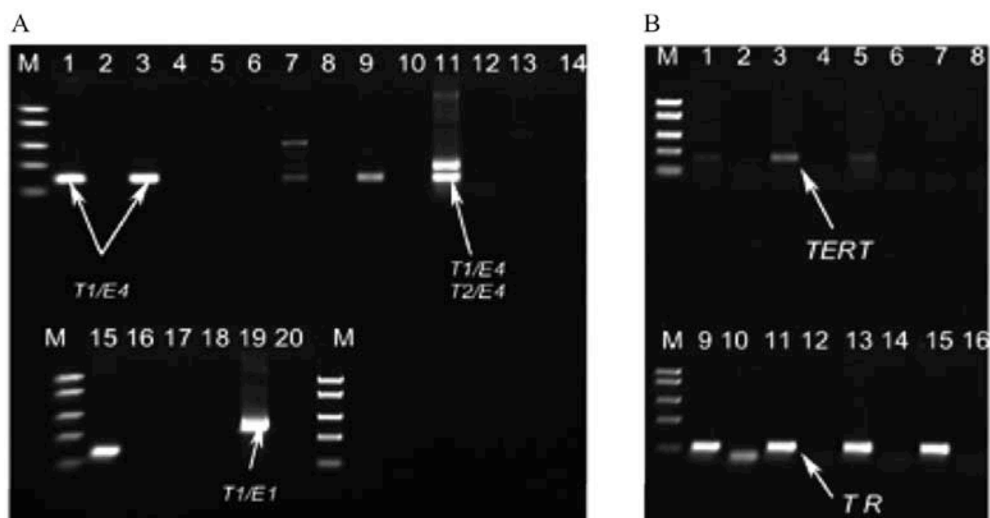

Figure 1. *TMPRSS2:ERG* and *TERT* analysis by RT-PCR. (A) An example of the *TMPRSS2:ERG* fusion products. (B) Detection of *TERT* (upper part of the gel) and *TR* (lower part of the gel) expression. Even numbers in the gels present the reaction with reverse transcriptase, odd numbers the reaction without reverse transcriptase. M – DNA size marker „FastRuler™ Low Range DNA Ladder"

1 pav. *TMPRSS2:ERG* ir *TERT* transkriptų elektrofregrama. (A) *TMPRSS2:ERG* sulietinių transkriptų raiškos pavyzdys. (B) *TERT* ir *TR* transkriptų raiškos pavyzdys. Nelyginiai skaičiai – mėginio RNR, veikta atvirkštine transkriptaze; lyginiai skaičiai – mėginio RNR, neveikta atvirkštine transkriptaze. M – DNR molekulinio svorio standartų mišinys „FastRuler™ Low Range DNA Ladder"

To confirm RT-PCR results, we examined *TMPRSS2:ERG* expression level using QPCR. Δct values ranged from 2.42 to 15.35, the Δct average was about 10 (**Fig. 2**). A low expression level of fused *TMPRSS2:ERG* transcripts could be caused by the selective assessment of PCas of early stage.

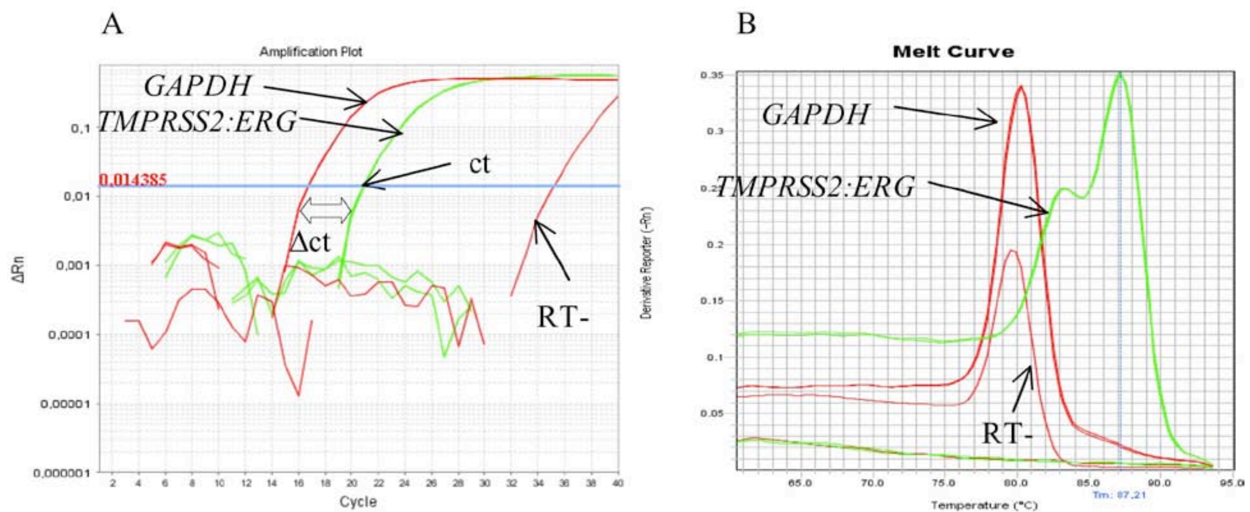

Figure 2. PCa samples analysis by QPCR. (A) QPCR logarithmic curves. (B) QPCR dissociation curves. „RT-" – cDNA sample without reverse transcriptase

2 pav. Priešinės liaukos mėginių sulietinių transkriptų analizė kiekybiniu PGR metodu. (A) logaritminės kreivės. (B) disociacinės kreivės. AT- yra kDNR mėginys, gautas sintetinant kDNR be atvirkštinės transkriptazės

We also analyzed 69 *TMPRSS2:ERG* fusion-negative samples for another type of transcript: caused by translocation between *TMPRSS2* and *ETV1* genes. In the study, we did not detect such a type of *TMPRSS2:ETV1* transcript.

Expression of *TERT* in tumour and non-tumour prostate tissue

Expression of *TERT* and *TR* was assessed by RT-PCR in 170 prostate tissues from PCa patients. In these samples, 76 cases (45%) were positive for *TERT*, while the expression of the *TR* transcript was almost ubiquitous (168/170; 98.8% (**Fig. 1B**)). *TERT* expression was identified in 75 out of 158 (47%) adenocarcinomas, and rarely occurred (1/12; 8%) in non-tumour tissues from PCa patients. To confirm RT-PCR results we examined *TERT* expression level using QPCR. Δct values were divided in to two groups: high *TERT* expression level was from 8.94 to 13.75 (41/96; 42.7%), low – from 13.76 to 19.41 (55/96; 57.3%). High *TERT* expression level was predominant in *TMPRSS2:ERG* fusion-positive cases (**Fig. 3**).

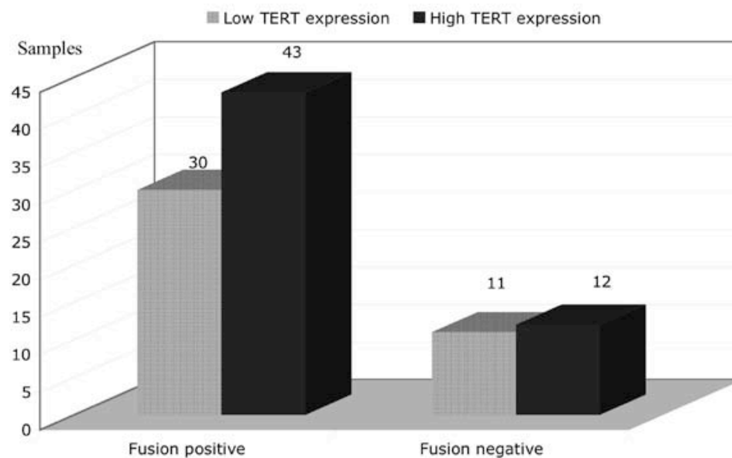

Figure 3. Distribution *TERT* gene expression between *TMPRSS2:ERG* fusion-positive and fusion-negative samples

3 pav. *TERT* geno raiškos palyginimas *TMPRSS2:ERG* teigiamuose ir neigiamuose mėginiuose

Overall, 170 cases were assessed for the presence of both transcripts, *TMPRSS2:ERG* and *TERT* and 72% (122/170) of the cases were positive for at least one of these transcripts. Among prostate adenocarcinomas the number of positive cases reached 74% (117/158).

Detection of *TMPRSS2:ERG* and *TERT* in urine sediments

Total RNA was extracted from 179 urine samples, RNA was derived from 139 urine samples, and only 67 urine samples from 139 indicated positivity with housekeeping gene *GAPDH*. These samples were assessed for the presence of the *TMPRSS2:ERG* and *TERT* transcripts in this minimally invasive material. RT-PCR revealed the presence of the *TMPRSS2:ERG* transcript in 10 out of 61 (16.4%) of urine sediments. T1/E4 variant was predominant (9/10) transcript identified in this material. In addition, *TERT* transcript was detected in 11 out of 65 (16.9%) urine sediments, and 31% (19/61) of specimens were positive for *TMPRSS2:ERG* or *TERT*. The presence of the transcripts in urine sediments was associated with status of the same transcript in tumour tissues (p=0.006 for *TMPRSS2:ERG* and p=0.051 for *TERT*).

Genetic characteristics of multifocal cases

20 multifocal PCa cases were analyzed by RT-PCR and QPCR. 18 out of 20 (90%) tumour foci expressed the *TMPRSS2:ERG* fusion product. *TMPRSS2:ERG* expression was detected in 8 PCa in both foci, while two cases expressed the fusion transcript only in one focus (2/10; 20% heterogeneity for the fusion status). *TMPRSS2:ETV1*

transcript was not detected in these two foci. 11 (55%) foci expressed T1/E4 as a single transcript and 6 foci – together with the T2/E4 transcript variant (30% (**Table 2**)). Identical set of *TMPRSS2:ERG* transcripts were detected in paired foci from two prostate carcinomas, thus heterogeneity of the fusion isoform was 75% (6/8). Only 4 out of 8 pairs showed similar intensity of the fusion expression. Thus the heterogeneity of the transcription level was 50%.

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

Table 2. Comparison of *TMPRSS2:ERG* (status, transcript variants and expression levels) and *TERT* expression in multifocal prostate tumours

2 lentelė. *TMPRSS2:ERG* ir *TERT* genų raiškos palyginimas daugiaždidininių PL navikų mėginiuose

Case No.	pT stage	Sample 1				Sample 2			
		Gleason score	Expression of *TMPRSS2:ERG*		Expression of *TERT* (RT-PCR)	Gleason score	Expression of *TMPRSS2:ERG*		Expression of *TERT* (RT-PCR)
			Type of transcript	Expression level (QPCR)			Type of transcript	Expression level (QPCR)	
1	pT3a	3+4	T1/E4 and T2/E4	1	yes	3+3	T1/E4	1	yes
2	pT2c	3+3	T1/E4 and T2/E4	1	no	3+3	T1/E4	2	yes
3	pT2b	3+3	T1/E4 and T2/E4	1	no	3+4	T1/E4	1	yes
4	pT3b	3+4	T1/E4	1	no	3+4	T1/E4	2	yes
5	pT2b	3+3	T1/E4	1	no	3+3	T1/E4	1	yes
6	pT2b	3+3	T1/E4 and T2/E4	1	yes	3+3	wt	-	no
7	pT2b	3+3	T1/E1	1	no	3+3	T1/E4	2	yes
8	pT2b	3+3	T1/E4	1	no	3+3	T1/E4 and T2/E4	1	yes
9	pT2b	3+3	wt	-	no	3+3	T1/E4	1	yes
10	pT3a	3+4	T1/E4	1	no	3+4	T1/E4 and T2/E4	2	yes

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

Quantitative analysis of *ERG* and *SPINK1* expression

The *ERG* and *SPINK1*gene expression level in PCa samples were measured by QPCR. The analysed tissues consisted of 146 tumour and 18 non-tumour cases for the *ERG* gene expression, and 151 tumour and 18 non-tumour cases for *SPINK1*. *ERG* gene QPCR Δct values were divided in to two groups: high *ERG* expression level was from -3.53 to 5.30 (88/164; 54.3%), low – from 5.31 to 10.01 (75/164; 45.7%). The same principle was applied to *SPINK1* expression results: high *SPINK1* expression level was considered from 1.88 to 12.50 (84/169; 49.7%), low from 12.51 to 19.5 (85/169; 50.3%). *ERG* and *SPINK1* expression levels were not different in tumour *versus* non-tumour prostate tissues from PCa patients (respectively: p=0.117 and p=0.393; **Figs. 4A** and **4B**). Significantly higher expression of *ERG* was detected in *TMPRSS2:ERG* fusion-positive tissues (p<0.0001), while more intense expression of *SPINK1* was found in *TMPRSS2:ERG* fusion-negative tissues (p=0.003). In addition, *TERT*-positive cases also showed higher expression of *ERG* (p=0.018).

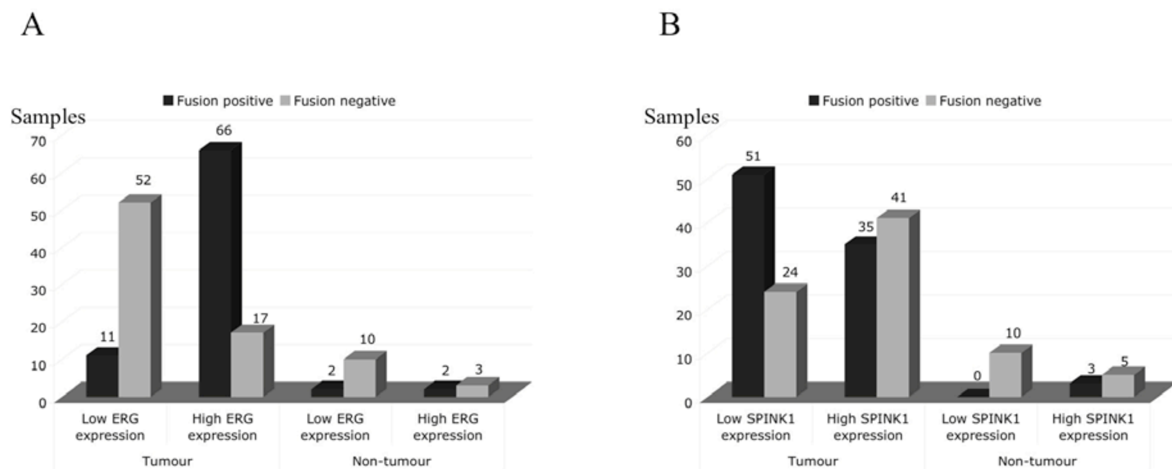

Figure 4. Expression of *ERG* (A) and *SPINK1* (B) in prostate tumours and non-tumour tissue from cancer patients

4 pav. *ERG* (A) ir *SPINK1* (B) genų raiškos palyginimas navikiniuose ir nenavikiniuose mėginiuose

Quantitative analysis of *GSTP1*, *RARB* and *RASSF1* expression

The *GSTP1, RARB* and *RASSF1* gene expression level in PCa samples was measured by QPCR. The analysed tissues consisted of 61 tumour and 17 non-tumour prostate tissues obtained from PCa patients. *GSTP1* Δct values were from -1.48 to 6.72, *RARB* Δct values were from 0.59 to 10.54, and *RASSF1* Δct values were from 1.28 to 13.01. *GSTP1*, *RARB* and *RASSF1* genes were divided in to two groups based on median. Significantly lower expression of the genes *GSTP1, RARB* and *RASSF1* was detected in tumour than non-tumour tissues (**Fig. 5**). The difference was statistically significant for *GSTP1* (p=0.042) and *RASSF1* (p=0.018) and of borderline significance for *RARB* (p=0.066).

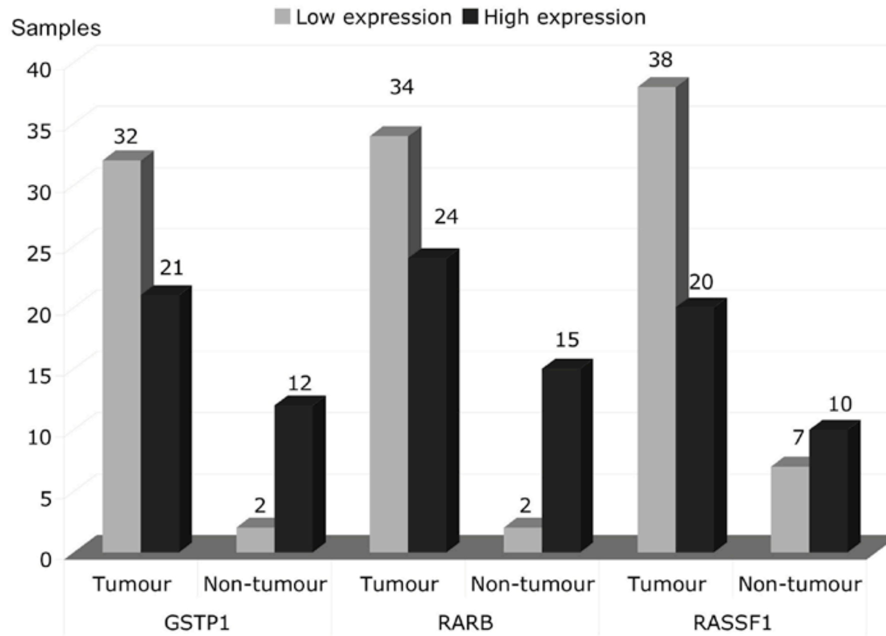

Figure 5. Expression of *GSTP1*, *RARB* and *RASSF1* in prostate tumour and non-tumour tissue from cancer patients

5 pav. *GSTP1*, *RARB* ir *RASSF1* genų raiškos palyginimas navikiniuose ir nenavikiniuose mėginiuose

TMPRSS2:ERG fusion-positive PCa showed reduced expression of analysed tumour suppressor genes (TSGs), especially *GSTP1*, but the difference was not statistically significant for *GSTP1* (p=0.201), *RARB* (p=0.409) and *RASSF1* (p=0.789). Decreased levels of TSGs production also characterized *TERT*-positive cases, but the differences were not significant.

Expression of *ZAC* in tumour and non-tumour prostate tissue

Expression of *ZAC* was assessed by RT-PCR in 168 prostate tissues from PCa patients. The analysed tissues consisted of 158 tumour and 10 non-tumour prostate tissues obtained from PCa patients. 86 out of 168 (51%) prostate tissues from PCa patients expressed *ZAC* (**Fig. 6**).

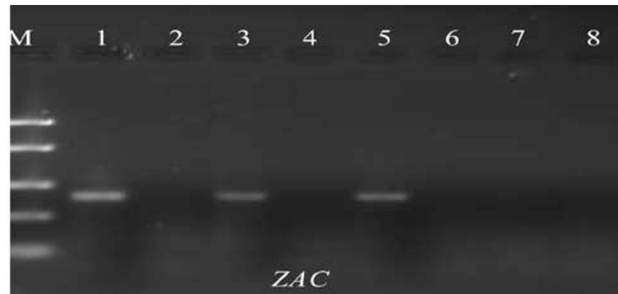

Figure 6. *ZAC* analysis by RT-PCR. Odd numbers in the gels presents the reaction with reverse transcriptase, even numbers the reaction without reverse transcriptase. M – DNA size marker „FastRuler™ Low Range DNA Ladder”

6 pav. *ZAC* transkriptų elektroforegrama. Nelyginiai skaičiai – mėginio RNR, veikta atvirkštine transkriptaze, lyginiai skaičiai – mėginio RNR, neveikta atvirkštine transkriptaze. M – DNR molekulinio svorio standartų mišinys „FastRuler™ Low Range DNA Ladder”

In the *TMPRSS2:ERG* fusion-positive samples (54; 32.14%), we found more *ZAC*-positive cases compared to *TMPRSS2:ERG* fusion-negative samples (41; 24.40%). To confirm RT-PCR results, we examined *ZAC* expression level using QPCR. Nevertheless *ZAC* expression level was not different in *TMPRSS2:ERG* fusion-positive *versus TMPRSS2:ERG* fusion-negative samples (p=0.1694). *ZAC* expression level was not different in tumour *versus* non-tumour prostate tissues from PCa patients (p=0.7461). Associations were found between *ZAC* positivity and *TERT* (p=0.0011) or *RASSF1* (p=0.0113) gene expression.

Expression levels from separate ZAC promoters (*ZAC1p* and *ZAC2p*) were measured using QPCR. The analysed tissues consisted of 52 tumour and 3 non-tumour prostate tissues obtained from PCa patients. Equal usage of both promoters was detected in ZAC-positive cases.

Correlations between clinical-pathological variables and genetic markers

In this study, Spearman's rank correlation analysis revealed statistically significant associations between clinical variables: PSA level at diagnosis was associated with pT category, Gleason score with tumour volume (p<0.0001, all), while pT correlated with Gleason score (p<0.0001).

There were no marked differences in clinical-pathological characteristics between the fusion-positive and the fusion-negative groups. However, fusion-positive patients tended to be of younger age (p=0.061) and had lower PSA level at diagnosis (p=0.046; Fisher's test). *TERT*-positive cases were characterized by larger tumours (p=0.035) and tended to have PCa of higher Gleason score (p=0.076). In tumour tissues, expression level of *RASSF1* was associated with preoperative PSA value (p=0.029), while *GSTP1* – with tumour stage (p=0.041). No correlations were found between *ZAC* gene expression and clinical-pathological variables.

Predictive markers of biochemical recurrence

The risk of biochemical recurrence (BCR) was significantly associated with pT and Gleason score ($p<0.0001$ and $p=0.001$, respectively; Fisher's test). There were no significant associations between BCR and expression of *TMPRSS2:ERG* or *TERT* as a single biomarker; however, the combination of both biomarkers showed significant association with biochemical recurrence. All but two cases (20/22; 91%) experiencing biochemical recurrence expressed *TMPRSS2:ERG* and/or *TERT* transcript in analyzed prostate tissue ($p=0.022$).

Kaplan-Meier analysis revealed significantly longer BCR-free survival after radical prostatectomy for patients with both *TMPRSS2:ERG* and *TERT*-negative tumours as compared to *TMPRSS2:ERG* fusion-positive ($p=0.023$) or *TERT*-positive ($p=0.050$) tumours, as well as to tumours expressing both transcripts ($p=0.040$) or at least one of these transcripts ($p=0.022$; Mantel-Cox; **Fig. 7**). Higher pT category and Gleason score were also significant predictors of shorter BCR-free period.

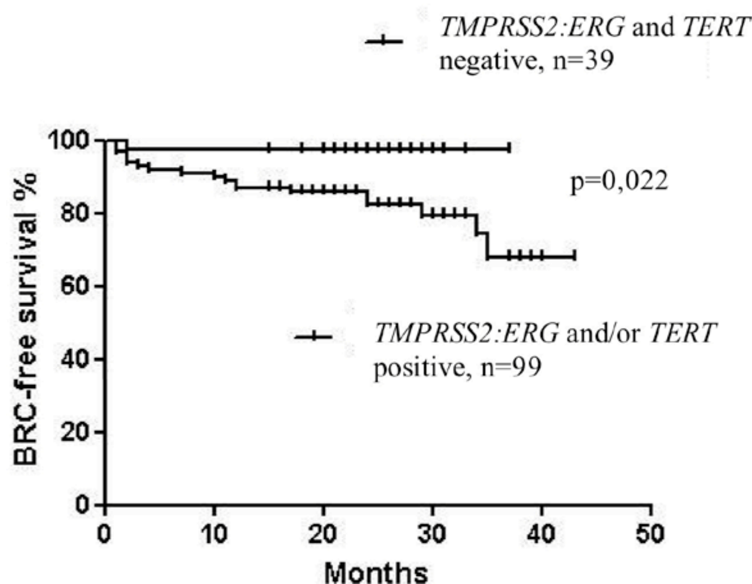

Figure 7. Kaplan-Meier curves for the biochemical recurrence (BCR)-free survival for prostate cancer patients according to combined status of *TMPRSS2:ERG* and *TERT*.

7 pav. Atkryčio priklausomybė nuo *TMPRSS2:ERG* sulietinio transkripto ir (arba) *TERT* raiškos buvimo mėginyje

In univariate analysis, pT (HR 6.80; 95% CI 2.84-16.28; $p<0.0001$), Gleason score (HR 4.92; 95% CI 2.00-12.10; $p=0.0005$) and *TMPRSS2:ERG* and/or *TERT* status (HR 4.35; 95% CI 1.01-18.73; $p=0.048$) were significant predictors of biochemical recurrence. In multivariate analysis using stepwise backward entering of covariates (pT, Gleason score, preoperative PSA value, and presence of the transcripts), *TMPRSS2:ERG* and/or *TERT* status provided prognostic information, though with the

borderline significance (HR 3.96; 95% CI 0.93-16.87; $p=0.06$), in addition to that provided by pathological stage of disease (HR 6.22; 95% CI 2.61-14.86; $p<0.0001$).

miRNAs expression profile of prostate tumours

To identify *TMPRSS2:ERG* fusion-responsive miRNA's, miRNA profile in *TMPRSS2:ERG*-positive *versus* *TMPRSS2:ERG* fusion-negative tumours was compared. Eight PCa samples were used for microarray analysis of 723 human and 76 human viral miRNAs. Based on significant difference, we selected five down-regulated miRNAs: *hsa-miR-33b*, *hsa-miR-149*, *hsa-miR-206*, *hsa-miR-370* and *hsa-miR-886-3p* (**Fig. 8**).

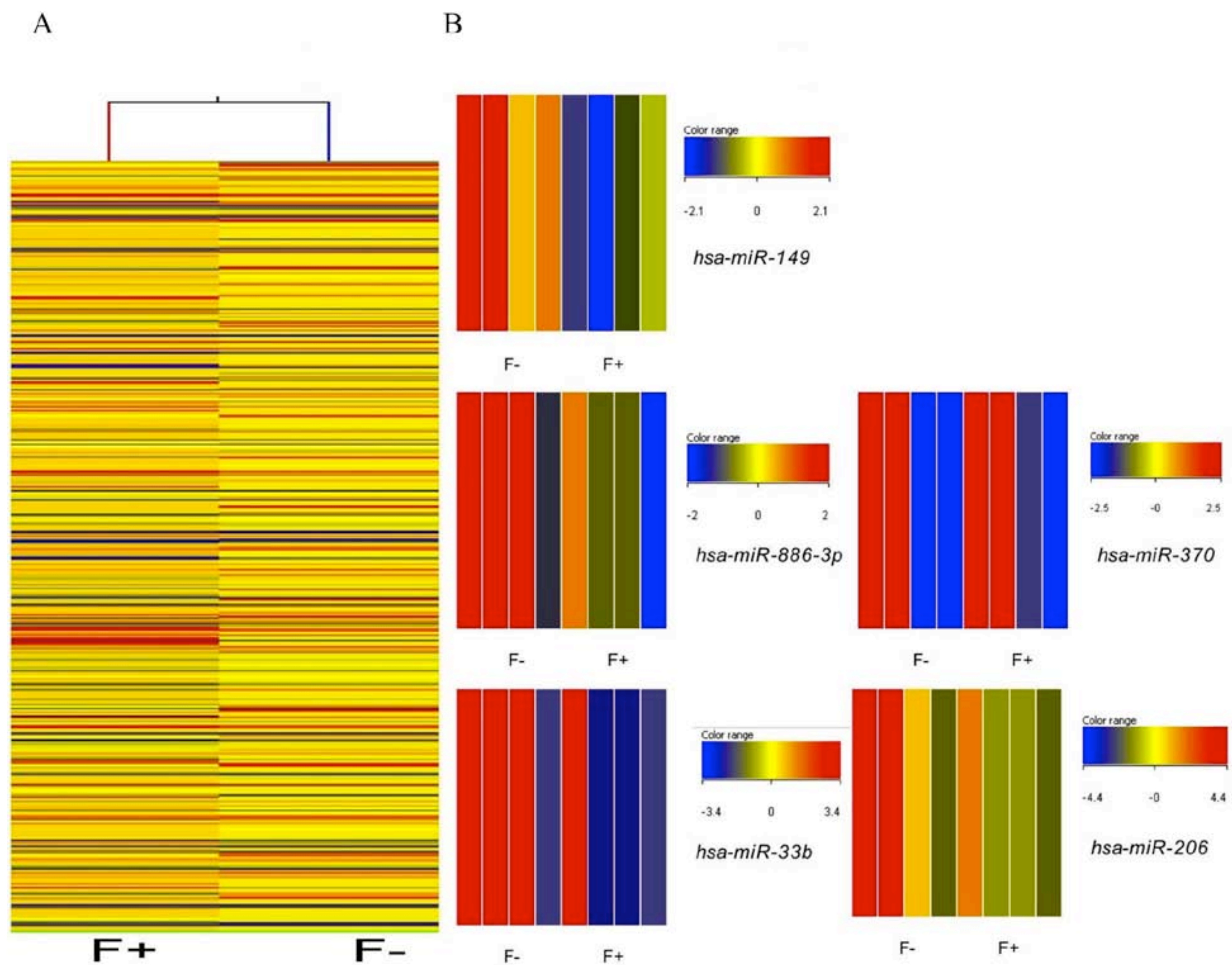

Figure 8. GeneSpring analysis results. (A) Hierarchical clustering of 8 samples based on presence of *TMPRSS2:ERG* transcript, the red color – increased miRNA expression level, the blue – a decrease and the yellow – unchanged miRNA expression level. (B) Comparison of each selected miRNA expression level, „F+" – *TMPRSS2:ERG* fusion-positive samples, „F-" – *TMPRSS2:ERG* fusion-negative samples

8 pav. Priešinės liaukos mėginių analizė GeneSpringGX kompiuterine programa: (A) aštuonių mėginių hierarchinis grupavimas, paremtas sulietinio transkripto buvimu ir jo nebuvimu; raudona spalva reiškia padidėjusią mikroRNR raišką, mėlyna – sumažėjusią, o geltona – nepakitusią raišką; (B) kiekvienos pasirinktos mikroRNR raiškos palyginimas kiekviename analizuojamame mėginyje atskirai; „F+" – *TMPRSS2:ERG* sulietinis transkriptas aptinkamas mėginyje, o „F-" – neaptinkamas

Microarray results were validated by QPCR on 105 PCa samples. The QPCR confirmed microarray data – the lower expression level of selected miRNAs in *TMPRSS2:ERG* fusion-positive samples. Significant differences were shown for *hsa-miR-149* ($p<0.0001$), *hsa-miR-33b* ($p=0.0048$), *hsa-miR-886-3p* ($p=0.0028$), *hsa-miR-370* ($p=0.0373$) and *hsa-miR-206* ($p=0.0432$) in *TMPRSS2:ERG* fusion-positive *versus* fusion-negative PCa samples.

Expression levels of miRNAs showed significant associations with clinical and molecular variables: *hsa-miR-149* expression correlated with pT category ($p=0.05$)

and *ERG* gene expression (p=0.0234); *hsa-miR-149* expression was significantly different between tumour and non-tumour PCa cases (p=0.0268); *hsa-miR-33b* expression correlated with *TERT* expression (p=0.0410); *hsa-miR-886-3p* expression correlated with Gleason score (p=0.0410), prostate mass (p=0.0228) and tumour size (p=0.0008); *hsa-miR-370* expression correlated with Gleason score (p=0.0431) and prostate mass (p=0.0361), while *hsa-miR-206* did not show significant associations with clinical variables.

hsa-miR-149 expression in VCaP and LNCaP cell lines

To confirm that *hsa-miR-149* expression depends on the presence of *TMPRSS2:ERG* fused transcripts, we examined *hsa-miR-149* expression level in the VCaP cell line, using siRNA to *ERG* for the cell line transfection. After transfection the expression of *ERG* was measured with QPCR to confirm the success of the gene silencing. *ERG* gene expression was lower in the siRNA transfected cells (**Fig. 9A**). The expression of *hsa-miR-149* was measured with QPCR, and the increase in production of *hsa-miR-149* was observed in the siRNA transfected cells (**Fig. 9B**).

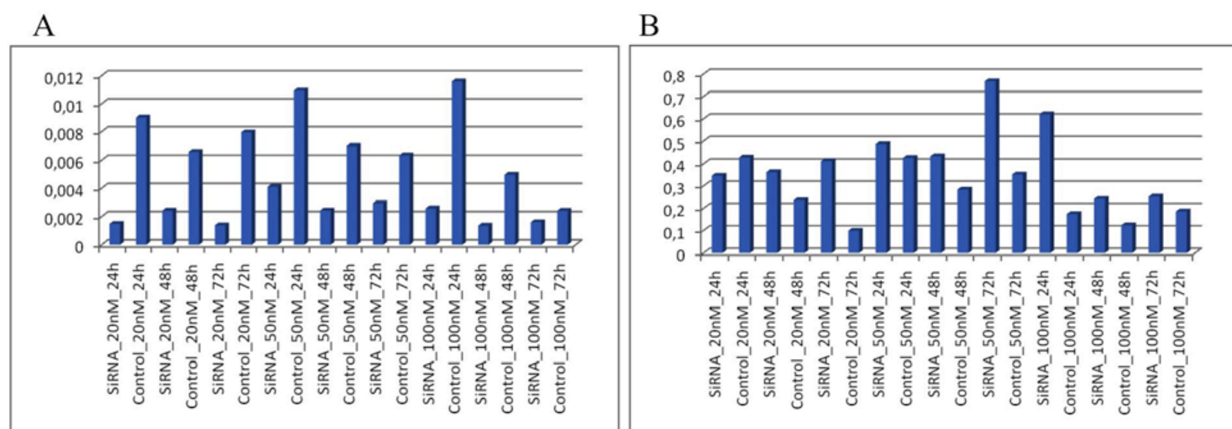

Figure 9. Expression of *ERG* (A) and *hsa-miR-149* (B) in VCaP cell line after transfection with siRNA. The Y-axis shows the normalized data

9 pav. *ERG* (A) ir *hsa-miR-149* (B) raiška VCaP ląstelių linijoje po transfekcijos siRNR nutildančiu mišiniu. Y ašyje atidėti normalizuoti duomenys

To be sure that *hsa-miR-149* expression depends on the presence of *TMPRSS2:ERG* fused transcripts, we examined *hsa-miR-149* expression level in LNCaP cell line, using siRNA for the cell line transfection. We used LNCaP cell line as a negative control, because it did not express *TMPRSS2:ERG* fused transcript. The expression of *hsa-miR-149* was measured by QPCR. As expected, detectable *hsa-miR-149* expression in LNCaP cell lines was not observed (**Fig. 10**).

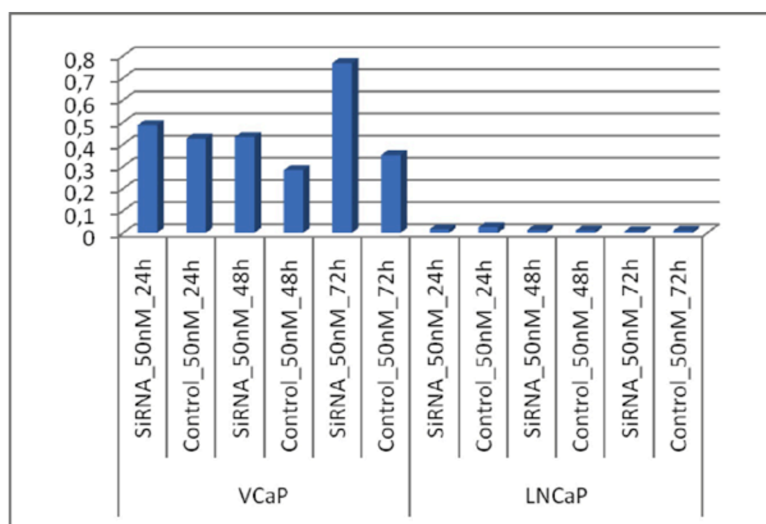

Figure 10. Comparison of *hsa-miR-149* expression level in VCaP and LNCaP cell lines. The Y-axis shows the normalized data

10 pav. *hsa-miR-149* raiškos palyginimas VCaP ir LNCaP ląstelių linijose. Y ašyje atidėti normalizuoti duomenys

Detection of XMRV virus in prostate tissues

To detect XMRV virus sequences in PCa samples, total RNA from tumour and non-tumour tissues was extracted. Total RNA was reverse transcribed into cDNA and analyzed in RT-PCR (**Fig. 11**). *GAPDH* specific primers were included as an internal control. XMRV virus sequences were found in 9 of 185 PCa samples (4.86%).

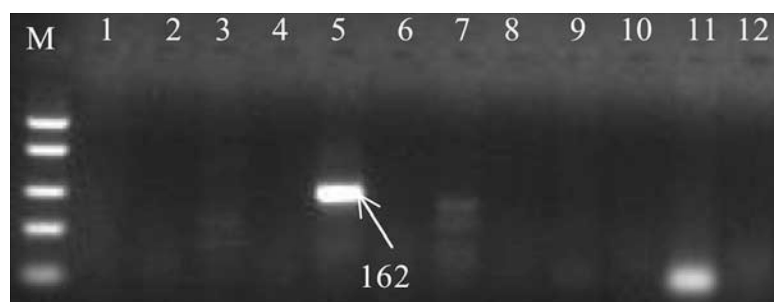

Figure 11. XMRV virus detection by RT-PCR. Even numbers in the gels present the reaction with reverse transcriptase, odd numbers the reaction without reverse transcriptase. M – DNA size marker „FastRuler™ Low Range DNA Ladder"

11 pav. XMRV viruso *gag* geno transkriptų, gautų AT-PGR metodu elektroforegrama. Nelyginiai skaičiai – mėginio RNR, veikta atvirkštine transkriptaze, lyginiai skaičiai – mėginio RNR, neveikta atvirkštine transkriptaze. M – DNR molekulinio svorio standartų mišinys „FastRuler™ Low Range DNA Ladder"

To confirm RT-PCR results, 9 XMRV-positive products were cloned, sequenced and compared with EF185282.1 sequence from the GeneBank data basis. All 9 sequences were XMRV-positive. The sequenced XMRV virus *gag* sequences are located in the GeneBank data basis: FR837936.1, FR837937.1, FR837938.1, R837939.1, FR837940.1, FR837941.1, FR837942.1, FR837943.1 and FR837944.1. Other scientists used nested PCR method for XMRV virus detection. To confirm the results, nested PCR from XMRV-positive and negative PCa samples were examined, and the same results were received (**Fig. 12**).

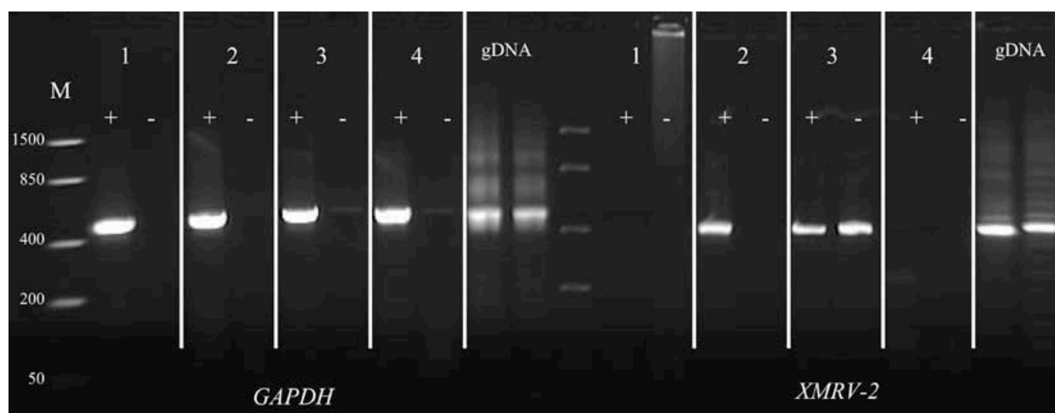

Figure 12. XMRV sequences analysis by nested PCR. Plus in the gels presents the reaction with reverse transcriptase, minus – the reaction without reverse transcriptase. 1, 4 – XMRV-negative cases; 2, 3 – XMRV-positive cases. M – DNA size marker „FastRuler™ Low Range DNA Ladder". gDNA – genomic DNA

12 pav. Lizdinės PGR reakcijos, atliktos su *GAPDH* ir XMRV *gag* pradmenimis ir priešinės liaukos mėginių kDNR, elektroforegrama. 1 ir 4 –mėginiai be XMRV viruso, 2 ir 3 – mėginiai su XMRV virusu. „+" – mėginio RNR, veikta atvirkštine transkriptaze, „-" – mėginio RNR, neveikta atvirkštine transkriptaze. M – DNR molekulinio svorio standartų mišinys „FastRuler™ Low Range DNA Ladder". gDNA – genominė DNR

There were no marked differences in clinical-pathological characteristics between the XMRV-positive and the XMRV-negative groups. One sample (case162) was used for whole virus genome amplification. Our XMRV virus genome sequence is deposited in the GeneBank data basis as FR872816.1

DISCUSSION

We used RT-PCR primers covering the most frequently fussed exons of *TMPRSS2* (exons 1–2) and *ERG* (exon 4), and were able to detect the *TMPRSS2:ERG* fusion in 58% (93/158) of PCa tumour tissues. The results are well in line with the other RT-PCR-based studies, where the fusion was identified in 50% to 80% of radical prostatectomy specimens (Nam et al., 2007; Rouzier et al., 2008; Bonaccorsi et al., 2009). In this study, occurrence of the *TMPRSS2:ERG* rearrangement was

accompanied by increased production of *ERG*, the event already detected in several microarray-based assays (Jhavar et al., 2008; Setlur et al., 2008; Barwick et al., 2010). In addition, in agreement with previous reports (Jhavar et al., 2008; Tomlins et al., 2008), this study revealed increased production of the *SPINK1* transcript in the subgroup of *TMPRSS2:ERG* fusion-negative cases. Several published studies have indicated that prostate cancer patients with the *TMPRSS2:ERG* gene fusion confer a higher risk of recurrence (Demichelis et al., 2007; Nam et al, 2007; Attard et al, 2008; Hu et al., 2008; Wang et al., 2008), while the others (Winnes et al., 2007; Saramaki et al., 2008; Hermans et al., 2009; Boormans et al., 2011) reported a significant associations of the fusion with the favorable prognosis. Expression of particular isoforms of the *TMPRSS2:ERG* transcripts or duplication of the fusion (Wang et al., 2008; Hu et al., 2008; Attard et al., 2008) were shown as significant indicators of poor prognosis in PCa, possibly due to marked activation of *ERG* in these cases.

An association between increased levels of *ERG* production and reactivation of *TERT* – the main component of telomerase – was identified in this study. Telomerase activity is tightly regulated by the availability of the *TERT* transcript. Androgen receptors (AR) are also the potent regulators of gene transcription. Recent studies (Yu et al., 2010; Wei et al., 2010) revealed a remarkable overlap between AR and ERG binding sites in promoters of multiple genes, mutation-caused over-production of ERG can disrupt normal regulation of AR-target genes. Re-activation of telomerase is a significant step toward cell malignization and de-differentiation. The data indicate a possible link between the rearrangement-induced ERG over-expression and activation of telomerase in PCa.

Non-invasive detection of molecular biomarkers in body fluids of PCa patients suggests an important tool for improved follow-up of biochemical recurrence after tumour resection. We successfully identified the *TMPRSS2:ERG* and *TERT* transcripts in catheterized urine of PCa patients. The detection rate of this minimally invasive assay reached 31% and was slightly lower than sensitivity of the tests performed on urine sediments collected after prostate massage or digital rectal examination (Laxman et al., 2006; Hessels et al., 2007; Laxman et al., 2008; Nguyen et al., 2010; Cao et al., 2011; Salami et al., 2011). However, the presence of the transcripts in urine sediments was significantly associated with the status of the same transcript in tumour tissues, showing relevant specificity of this assay.

Multifocal PCa has been reported in up to 91% of all cases of radical prostatectomy (Meiers et al., 2008; Andreoiu et al., 2010). Current approved biomarkers of PCa, including preoperative PSA value, are not potent predictors of multifocality, and sensitive markers for identification of multifocal PCa are highly instrumental. A lot of mutifocality studies were done by means of FISH, and the heterogeneity of the fusion status was understood as either the presence or absence of the fusion or the occurrence of *TMPRSS2* rearrangements through different genetic events (translocation or deletion). None of these studies evaluated the level of *TMPRSS2:ERG* expression or assessed fusion variants. In this study, 6 out of 8 pairs of tumours from multifocal PCa expressed different variants of *TMPRSS2:ERG* and 2 additional pairs showed variable status of fusion positivity. Some association was identified between expression of *TERT*, encoding the main subunit of telomerase, and tumour positivity for

TMPRSS2:ERG. *TERT* was identified in 11 of 20 (55%) tumour foci from PCa patients. The results are in line with the data of other studies (Latil et al., 2000; Kamradt et al., 2003) and for the first time show considerable heterogeneity of multifocal prostate tumours according to the expression of *TERT*. 90% of multifocal tumours showed distinct status of *TERT* expression (positive or negative) in both foci. Arora et al. (2004) showed that only 9 of 100 prostatectomy specimens had the same Gleason score in all studied foci. In this study, different Gleason score was identified in two pairs of tumours with multifocal PCa. The reason could be the early-stage of analyzed PCa.

In this study, pathological stage and Gleason score, the established prognostic factors of PCa, were significant predictors of disease recurrence, while preoperative PSA value was not predictive for biochemical recurrence (BCR). In agreement with the data of Sun et al. (2008), in this study, the *TMPRSS2:ERG* fusion-positive cases possessed a lower preoperative PSA value than the *TMPRSS2:ERG* fusion-negative cases. The latter supports the hypothesis on direct crosstalk between ERG and AR as the transcription regulators.

Our results demonstrated that the expression of *TMPRSS2:ERG* or *TERT* (catalytic subunit of telomerase) as a single biomarker was not highly informative for prediction of biochemical recurrence in PCa. However, the combination of both biomarkers showed significant association with the outcome of the disease. Up to 74% of surgically removed non-metastatic prostate adenocarcinomas were positive for *TMPRSS2:ERG* and/or *TERT*. 95% of the cases experiencing biochemical recurrence expressed *TMPRSS2:ERG* and/or *TERT* transcript in the analyzed prostate tumours. BCR-free period after radical prostatectomy was significantly longer for the *TMPRSS2:ERG*-negative and *TERT*-negative cases. The association was identified between increased levels of *ERG*, the result of the gene fusion to *TMPRSS2* promoter, and tumour positivity the *TERT* transcript. We present findings that for the first time show a possible link between the *TMPRSS2:ERG* fusion, a significant event of prostate oncogenesis, and telomerase, a key element of malignant self-renewal.

The knowledge on interaction between expression of tumour suppressor genes, like *GSTP1*, *RASSF1* and *RARB,* and the major oncogenic factors of PCa – *TMPRSS:ERG* and telomerase, are very limited. This study identified reduced expression of tumour suppressor genes in PCa as compared to non-cancerous prostate tissues, and related these changes with the status of *TMPRSS2:ERG* fusion and expression of *TERT*. Loss of *GSTP1* expression, mainly through promoter methylation, is a major hallmark of PCa (Bostwick et al., 2007). The cases experiencing recurrence showed low production of *GSTP1* in prostate carcinomas. Frequent promoter hypermethylation of *RASSF1* and *RARB* genes also have been identified in PCa by several studies (reviewed in Ahmed 2010, Jeronimo et al., 2011). In this study, reduced expression of *RARB* and *RASSF1* was identified in PCa as compared to benign prostatic tissues. In addition, *RASSF1* expression was related to preoperative PSA value. *TMPRSS2:ERG* fusion-positive tumours showed decreased production of tumour suppressor genes. Moreover, we also identified a direct link between expression levels of *ERG* and production of *RARB* and *RASSF1*.

In our analysis we included a new tumour suppressor gene – *ZAC*, which is often silenced by methylation in various solid tumours (reviewed in Theodoropoulou et al., 2010), but less studied in PCa. In *TMPRSS2:ERG* fusion-positive samples, decreased *ZAC* expression level was found, but the same results were identified in fusion-negative samples as well. From QPCR results, we can assume that in the pT2–pT3 PCa tumour *ZAC* gene is not completely methylated and expressed to some degree. As indicated in the literature, *ZAC* gene has two functional promoters. We detected almost the same expression levels from both promoters. The results are well in line with the other QPCR-based study, where the expression from the first promoter was slightly higher than from the second one (Valleley et al., 2007).

Expression profiling of 723 miRNAs in 8 PCa samples was carried out to identify miRNA expression profile of *TMPRSS2:ERG* fusion-positive PCa. Based on microarray data we selected five miRNAs (*hsa-miR-33b*, *hsa-miR-370*, *hsa-miR-149*, *hsa-miR-886-3p* and *hsa-miR-206*) that were down-regulated in *TMPRSS2:ERG* fusion-positive samples. QPCR data from PCa samples suggests that only *hsa-miR-149* showed strong statistically significant association ($p<0.0001$). For the QPCR confirmation, VCaP and LNCaP cell lines were transfected with siRNA. In the transfected VCaP cells *hsa-miR-149* was up regulated, while showed unchanged expression in LNCaP cell lines. This data shows that *hsa-miR-149* expression depends on the *TMPRSS2:ERG* transcript.

Our results of the frequency of XMRV sequences (4.86%) in PCa samples of Lithuanian are well in line with the other RT-PCR or nested PCR based XMRV studies, where XMRV sequences were identified from 1% to 22% of radical prostatectomy specimens (Urisman et al., 2006; reviewed in Sfanos et al., 2012). Recently K. S. Sfanos et al. (2012) summarized all scientific publications and came to general conclusions that XMRV virus arose in laboratory and is not related to prostate carcinogenesis.

CONCLUSIONS

1. *TMPRSS2:ERG* fusion rate in specimens (n=158) prostate tumours of Lithuanian male population is 58.9%. Most frequent *TMPRSS2:ERG* fused transcript is T1/E4 (46.6%). In urine sediment *TMPRSS2:ERG* fusion detection rate is 16.4%, and mostly predominant isoform – T1/E4. Isoforms of *TMPRSS2:ERG* transcript detected in urine sediments correspond to the isoforms detected in tumour sample.

2. The expression of *ERG*, *SPINK1* and *TERT* statistically significantly ($p<0.05$) depends on the presence of *TMPRSS2:ERG* fused transcripts in prostate tumour. The expression of *GSTP1*, *RARB*, *RASSF1* and *ZAC* genes are independent of *TMPRSS2:ERG* fused transcripts in prostate tumour samples.

3. *TMPRSS2:ERG* and *TERT*-negative prostate cancer are characterized by low risk of progression.

4. Multifocal prostate tumours are characterized by different *TMPRSS2:ERG* isoforms and different intensity of *TMPRSS2:ERG* and *TERT* expression levels.

5. Microarray, QPCR and cell line (VCaP and LNCaP) based research confirms that *hsa-miR-149* expression level depends on the presence of *TMPRSS2:ERG* transcript in prostate tissue samples ($p<0.0001$).

6. Detection rate of XMRV virus in samples of prostate tumours obtained from Lithuanian males is relatively low (4.9%; 9/185). XMRV virus, thus, could not be considered as prostate cancer inducing agent.

List of publications:

1. R. Sabaliauskaitė, S. Jarmalaitė, D. Petroška, D. Dasevičius, A. Laurinavičius, F. Jankevičius, J. R. Lazutka. Combined analysis of *TMPRSS2-ERG* and *TERT* for improved progrnosis of biochemical recurrence in prostate cancer. Genes, Chromosomes and Cancer, 2012, DOI 10.1002/gcc.21963.

2. R. Sabaliauskaitė, D. Petroška, D. Dasevičius, A. Laurinavičius, F. Jankevičius, J. R. Lazutka ir S. Jarmalaitė. Molecular analysis of multifocal prostate cancer cases. Acta Medica Lithuanica, 2012, 18, 4, pp: 147–155.

3. R. Sabaliauskaitė, S. Jarmalaitė, F. Jankevičius ir J. R. Lazutka. *TMPRSS2* ir *ERG* genų susijungimas – naujas priešinės liaukos vėžio žymuo. Medicinos teorija ir praktika. 2008; 14, 3, pp: 260–265.

Poster presentations:

1. Rasa Sabaliauskaitė, Neringa Kalinauskaitė, Sonata Jarmalaitė, Aida Laurinavičienė, Darius Dasevičius, Arvydas Laurinavičius, Juozas Lazutka and Feliksas Jankevičius. „Molecular biomarkers of early-stage prostate cancer“.

„EAU 2nd North Eastern European Meeting“ and at the „7th SCANBALT forum“ International Conferences, 2008, Vilnius, Lithuania.

2. Rasa Sabaliauskaitė, Sonata Jarmalaitė, Neringa Kalinauskaitė, Asta Sčėsnaitė, Aida Laurinavičienė, Darius Dasevičius, Arvydas Laurinavičius, Juozas Lazutka and Feliksas Jankevičius. „Telomerase gene expression is associated with clinical and molecular markers of poor prognosis in prostate cancer“. „2nd GENICA/CANGENIN Genomic Instability Workshop“ International Conference, 2009, Athens, Greece.

3. Rasa Sabaliauskaitė, Sonata Jarmalaitė, Darius Dasevičius, Donatas Petroška, Arvydas Laurinavičius, Juozas Lazutka and Feliksas Jankevičius. „*TMPRRS2-ETS* gene fusion, telomerase and *ZAC1* gene expression in early-stage prostate cancer“. Lithuanian Biochemical Society International XI Conference LBD-50, 2010, Toliejai, Lithuania.

4. Rasa Sabaliauskaitė, Sonata Jarmalaitė, Juozas Lazutka and Tapio Visakorpi. „miRNA expression analysis in early-stage prostate cancer”. LRC International PhD Conference, 2011, Vilnius, Lithuania.

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SANTRAUKA

Priešinės liaukos (PL) vėžys – dažniausia vyrų onkologinė liga, kuri pasižymi dideliu mirtingumu, ligos eigos bei molekulinių pokyčių heterogeniškumu. Po operacijos vieni ligoniai ilgai gyvena be ligos simptomų, o kitiems liga greitai progresuoja. Pagrindinė priežastis, lemianti sunkią ligos eigą ir didelį mirtingumą, yra atsako į lyties hormonų signalus sutrikimas ir išsivystęs nejautrumas hormonų terapijai. Tai lemia dažną metastazavimą į kaulų sistemą ir ribotą jautrumą sisteminei chemoterapijai. Nepaisant Lietuvoje aktyviai veikiančios PL vėžio ankstyvos diagnostikos programos, pusei ligonių liga vis dar diagnozuojama pažengusios stadijos. Šiuo metu priešinės liaukos specifinis antigenas (PSA) yra plačiausiai taikomas žymuo, kurį įdiegus pagerėjo PL vėžio diagnostika. Didelė PSA koncentracija nustatoma ne tik sergant PL vėžiu, bet ir uždegimo sukeltomis PL ligomis. Siekiant padidinti PL vėžiu sergančių ligonių išgyvenamumą, ieškoma naujų molekulinių PL vėžio žymenų, galinčių padėti prognozuoti ligos eigą ir parinkti tinkamą gydymą. Molekuliniai vėžio žymenys – tai genų mutacijos ir epimutacijos, genų ir mikroRNR raiškos pakitimai, funkciškai ar struktūriškai pakitę baltymai bei kitokios navikiniuose ar ikinavikiniuose audiniuose aptinkamos pažaidos, kurių nerandama sveikose ląstelėse. Prognozinę vertę turi tik tie molekuliniai žymenys, kurių raiška koreliuoja su ligos klinikine eiga bei ilgesne išgyvenamumo trukme, kai nėra ligos atkryčio. Perspektyviausi yra nauji PL vėžio molekuliniai žymenys – chromosomų persitvarkymai, kurie aktyvina ETS šeimos onkogenus. S. A. Tomlins ir kiti mokslininkai, 2005 metais pritaikę bioinformatinį mikrogardelių duomenų vertinimo metodą, nustatė, kad PL navikams dažnai būdinga padidėjusi *ERG* ir *ETV1* genų raiška. Šie genai priklauso ETS transkripcijos veiksnių šeimai, kurių baltymai priskiriami prie potencialių onkobaltymų, pasižyminčių didele homologija virusiniam onkobaltymui *v-ets*. ETS yra didžiausia transkripcijos veiksnių šeima, kuriai priklauso 29 žmogaus, 28 pelės, 10 *Caenorhabditis elegans* ir 9 drozofilos genai. Potencialūs onkogenai dėl chromosomų persitvarkymų yra perkeliami prie aktyviai transkribuojamų genų promotorių ir taip aktyvinama onkobaltymų raiška. ETS veiksniai ypač reikšmingi epitelio ir endotelio audinių, kraujodaros, nervų ir endokrininės sistemų vystymuisi ir dalyvauja dalijantis ir migruojant ląstelėms. Žinduolių ląstelėse ETS baltymai aktyvina augimo veiksnių raišką, taip pat dalyvauja Ras onkogeno signaliniame kelyje. *TMPRSS2:ERG* genų susijungimas nustatomas PL adenokarcinomose ir intraepitelinės neoplazijos (PIN) audiniuose (apie 20 %), bet neaptinkamas atrofavusiuose ar normaliuose PL audiniuose. Toks genų susijungimas būdingas ne tik hormonams jautrioms metastazėms, bet ir hormonams nejautriose metastazėse. Dar 1977 metais buvo nustatyta, kad onkologinėmis ligomis sergančių ligonių organizmo skysčiuose (kraujo serume, šlapime ir seilėse) gali būti aptinkama laisvai cirkuliuojančių nukleorūgščių (DNR ir RNR). Vėžinių ląstelių DNR, RNR ar mikroRNR į organizmo skysčius patenka žuvus pavienėms naviko ląstelėms. Ši DNR ar RNR yra suskaidyta į fragmentus, mažos koncentracijos, todėl genetiniai pakitimai joje gali būti tiriami tik ypač jautriais metodais. K. P. Porkka ir kiti mokslininkai 2007 metais, pritaikę bioinformatinį mikrogardelių duomenų vertinimo metodą, nustatė, kad PL navikui yra būdinga specifinių mikroRNR raiška, kuri neretai koreliuoja su klinikiniais rodikliais ir ligos prognoze. Epigenetinės pažaidos PL vėžinėse ląstelėse aptinkamos dažniau nei genų mutacijos, be to, jos pasireiškia ankstyvose stadijose.

Padidėjęs DNR metilinimas (hipermetilinimas) reguliacinių genų promotoriuose inaktyvina genų raišką ir taip blokuoja jų funkcijas. Daugelyje PL navikų (iki 90 %) nustatomas geno, koduojančio fermentą glutationo S transferazę π 1 (*GSTP1*) ir saugančio ląstelę nuo kancerogenų poveikio, promotoriaus hipermetilinimas. PL navikui charakterizuoti ir ligos eigai nustatyti svarbu žinoti pakitusių genų ir mikroRNR raišką. Šiuo metu nėra gerų žymenų, kurie padėtų efektyviai prognozuoti PL vystymąsi galbūt, mūsų pasirinkti žymenys (*TMPRSS2:ERG*, *TERT*, *ERG*, *SPINK1*, *GSTP1*, *RARB*, *RASSF1* ir *ZAC*) padės nustatyti vėžio tipą, prognozuoti ligos vystymosi eigą ir parinkti optimalias paciento gydymo strategijas.

Darbo tikslas. Ištirti ir įvertinti priešinės liaukos navikų potencialius molekulinius žymenis nustatyti onkogenų ir naviką slopinančių genų raišką, mikroRNR raiškos profilį ir XMRV viruso (*Xenotropic Murine Leukemia Virus-related virus*) sekų dažnį tirtuose priešinės liaukos mėginiuose, įvertinti tirtų molekulinių žymenų tarpusavio sąsajas bei jų ryšį su klinikiniais rodikliais ir pacientų amžiumi.

Darbo uždaviniai:

1. Priešinės liaukos mėginiuose, šlapimo nuosėdose ir daugiažidininių navikų mėginiuose nustatyti *TMPRSS2:ERG* sulietinio transkripto raiškos dažnį ir intensyvumą. Įvertinti *TMPRSS2:ERG* sulietinio transkripto raiškos sąsajas su klinikiniais rodikliais ir pacientų amžiumi.
2. Priešinės liaukos mėginiuose ištirti genų *TERT*, *ERG*, *SPINK1*, *GSTP1*, *RARB*, *RASSF1* ir *ZAC* raišką kPGR metodu, įvertinti sąsajas su klinikiniais, ir molekuliniais rodikliais bei pacientų amžiumi.
3. Mikrogardelių metodu ištirti mikroRNR raiškos profilį *TMPRSS2:ERG* sulietiniam transkriptui teigiamuose ir neigiamuose navikuose. Atrinktų mikroRNR raiškos pokyčius patvirtinti kPGR metodu. Įvertinti pasirinktų mikroRNR raiškos sąsajas su klinikiniais rodikliais ir pacientų amžiumi.
4. Priešinės liaukos mėginiuose atlikti XMRV viruso (*Xenotropic Murine Leukemia Virus-related virus*) sekų paiešką.

Ginti pristatomi teiginiai:

1. *TMPRSS2:ERG* sulietinis transkriptas dažnai aptinkamas priešinės liaukos navikuose ir įtakoja kitų genų ir mikroRNR raišką.
2. *TMPRSS2:ERG* ir *TERT* žymenys prognozuoja blogesnę ligos eigą ir trumpesnį laikotarpį be ligos atkryčio.
3. Daugiažidininiams priešinės liaukos navikams būdinga skirtinga *TMPRSS2:ERG* sulietinio transkripto genetinė įvairovė ir skirtinga geno raiška.

4. Lietuvos vyrų populiacijos priešinės liaukos mėginiuose aptinkamos XMRV viruso sekos, jų aptikimo dažnis atitinka pasaulines tendencijas. XMRV virusas nėra priešinės liaukos vėžio išsivystymo priežastis.

Mokslinio darbo naujumas. Lietuvos vyrų populiacijos PL navikų mėginiuose iki šiol nebuvo atlikta molekulinių žymenų analizė taikant naujausius molekulinius metodus: kiekybinį PGR ir (ar) mikrogardeles. Darbo metu gauti vertingi genų raiškos PL mėginiuose rezultatai. Naviko DNR ir RNR molekulinė analizė, genetinių pažaidų ir (ar) pakitimų įvairovė gali suteikti informacijos apie vėžio jautrumą gydymui bei padėti prognozuoti ligos progresavimo tikimybę.

Praktinė darbo reikšmė. PL vėžio vystymasis vis dar prognozuojamas remiantis įprastine klinikinių žymenų sistema (PSA), o gydymui tik pavieniais atvejais skiriami vaistai, kurie specifiškai veikia ligą sukėlusį genetinį pakitimą. Mes bandėme kuo išsamiau atlikti PL mėginių molekulinę analizę: ištirti *TMPRSS2:ERG*, *TERT*, *ERG*, *SPINK1*, *GSTP1*, *RARB*, *RASSF1* ir *ZAC* genų raišką, aptikti pakitusią mikroRNR raišką PL mėginiuose, kurie tarpusavyje skyrėsi *TMPRSS2:ERG* sulietinio transkripto buvimu. Mūsų gauti rezultatai patikimai įrodo, kad *TMPRSS2:ERG* sulietinis transkriptas ir *TERT* raiška mėginyje siejasi su didesne ligos atsinaujinimo tikimybę. *TMPRSS2:ERG* sulietinio transkripto buvimas PL mėginyje turi įtakos mikroRNR raiškai – nustatyta sumažėjusi *hsa-miR-149* mikroRNR raiška PL mėginiuose. Mes atlikome *TMPRSS2:ERG* sulietinio transkripto ir *TERT* geno raiškos analizę šlapimo nuosėdose. Šlapimo nuosėdų analizė gali būti papildoma priemonė ligos diagnozei, neatsisakant įprastinių diagnostikos metodų. Atliktais tyrimais metu mes siekėme prisidėti prie molekulinių žymenų sistemos, naujos kartos prevencijos ir gydymo strategijos kūrimo.

Rezultatų pristatymas ir jų aprobavimas. Disertacijos darbo rezultatai paskelbti 3 publikacijose ir pristatyti 4 stendiniuose pranešimuose.

Disertacijos struktūra. Disertacijos rankraštį sudaro: Santrumpos, Įvadas, Literatūros apžvalga, Metodai, Rezultatai, Rezultatų aptarimas, Išvados, Mokslinių darbų sąrašas, Padėka, Literatūros sąrašas ir Priedai. Darbo medžiaga išdėstyta – 130 puslapių. Literatūros sąraše pateikiami 104 šaltiniai.

Rezultatai. *TMPRSS2:ERG* sulietinių transkriptų aptikimo priešinės laiukos (PL) mėginiuose dažnis – 58,86 %, dažniausia izoforma – T1/E4 (46,62 %). PL mėginiuose aptikome padidėjusią *ERG* geno raišką, kuri siejama su *TMPRSS2:ERG* sulietinio transkripto buvimu PL mėginyje. *TMPRSS2:ERG* sulietiniam transkriptui neigiamuose PL mėginiuose aptinkome padidėjusią *SPINK1* geno raišką. Mūsų atlikti tyrimai

parodė, kad vieno *TMPRSS2:ERG* arba *TERT* biožymens aptikimas PL mėginiuose nėra informatyvus, tačiau abiejų biožymenų derinys rodo patikimas sąsajas su ligos progresavimu. Neinvazinio tyrimo metu aptikome *TMPRSS2:ERG* ir *TERT* transkriptus šlapimo nuosėdose. *TMPRSS2:ERG* sulietinio transkripto aptikimo dažnis siekė 16,39 % ir buvo šiek tiek mažesnis, nei nurodomas kitų tyrėjų. PL vėžys yra daugiažidininės kilmės, ištyrus tokius mėginius nustatėme, kad genų raiška ir *TMPRSS2:ERG* sulietinio transkripto izoformos yra skirtingos daugiažidininio PL vėžio mėginiuose. Mes nustatėme sumažėjusią naviką slopinančių genų (*GSTP1*, *RARB*, *RASSF1* ir *ZAC*) raišką nenavikiniuose ir *TMPRSS2:ERG* sulietinį transkriptą turinčiuose mėginiuose, nors patikimų sąsajų ir negavome. Mikrogardelių tyrimui atsirinkome penkias mikroRNR (*hsa-miR-149*, *hsa-miR-33b*, *hsa-miR-886-3p*, *hsa-miR-370* ir *hsa-miR-206*), kurių raiška yra sumažėjusi *TMPRSS2:ERG* sulietinį transkriptą turinčiuose mėginiuose. Atlikus tinkamumo patikrinimą kPGR metodu, o gautus rezultatus patvirtinus su VCaP ir LNCaP ląstelių linijų tyrimais, rasta stipri *hsa-miR-149* mikroRNR sąsaja su *TMPRSS2:ERG* sulietiniu transkriptu. XMRV viruso sekų tyrimo rezultatai yra palyginami su kitų tyrėjų rezultatais, gautais panaudojus AT-PGR ir lizdinės PGR metodus. XMRV virusas yra laboratorinės kilmės ir nėra susijęs su PL kancerogeneze.

Išvados:

1. Priešinės liaukos navikų mėginiuose (n = 158) aptinkamas *TMPRSS2:ERG* sulietinio transkripto dažnis yra 58,9 %. Dažniausias *TMPRSS2:ERG* sulietinis transkriptas – T1/E4 (46,6 %). Šlapimo nuosėdose *TMPRSS2:ERG* sulietinio transkripto aptikimo dažnis 16,4 %, dažniausiai aptinkama izoforma – T1/E4. Šlapimo nuosėdose randama *TMPRSS2:ERG* sulietinio transkripto izoforma atitinka jo izoformą priešinės liaukos mėginyje.

2. *ERG*, *SPINK1* ir *TERT* genų raiška statistiškai patikimai ($p < 0{,}05$) priklauso nuo *TMPRSS2:ERG* sulietinio transkripto buvimo priešinės liaukos mėginyje, o *GSTP1*, *RARB*, *RASSF1* ir *ZAC* genams tokia priklausomybė nenustatyta.

3. Priešinės liaukos navikai, kuriuose nėra *TMPRSS2:ERG* sulietinio transkripto ir *TERT* geno raiškos, pasižymi mažesne progresavimo rizika.

4. Daugiažidininiuose priešinės liaukos navikuose aptinkamos skirtingos *TMPRSS2:ERG* sulietinių transkriptų izoformos bei skirtingo intensyvumo *TMPRSS2:ERG* ir *TERT* raiška.

5. Mikrogardelių ir kPGR tyrimų rezultatai bei jų patvirtinimas su VCaP ir LNCaP ląstelėse įrodo, kad *hsa-miR-149* mikroRNR raiška statistiškai patikimai ($p < 0{,}0001$) priklauso nuo *TMPRSS2:ERG* sulietinio transkripto buvimo mėginyje.

6. XMRV viruso aptikimo dažnis priešinės liaukos mėginiuose yra mažas (4,9 %; 9/185), todėl šis virusas negali būti siejamas su priešinės liaukos navikų išsivystymu.

General information about the author

Name: Rasa Sabaliauskaitė

Date of birth: 26th of September, 1982

Contact e-mail: rasa.sabaliauskaite@gmail.com

Education:

1989-2001	Elektrėnai „Versmės” Gymnasium
2001-2005	Vilnius University, Faculty of Natural Sciences, Bachelor degree in Biology
2005-2007	Vilnius University, Faculty of Natural Sciences, Master degree in Biology
2007-2011	Vilnius University, Faculty of Natural Sciences, PhD student in Biology

Working Experience:

2005-2007	Institute of Biotechnology, Department of Eukaryote Gene Engineering, technician
2008-2010	„Fermentas Thermo Scientific” MTEPC Junior research fellow
From 2012	„Fermentas Thermo Scientific” MTEPC Junior research fellow

International Training:

„High-throughput screens in genome integrity and cancer“, 2010, Oksford, United Kingdom

„Phospho flow workshop“, 2011, Bergen, Norway

An Intership:

An intership in Finland, Tampere University of Technology Medical Institute in prostate cancer research laboratory from 18th of October 2010 until 31th of May 2011.