Landesklinikum Weinviertel
MISTELBACH GÄNSERNDORF

A. Schöller[1], C. Freibauer[1], E. Dlouhy-Schütz[2], M. Wang[3,4], Y. Hu[3,4], M. Stearns[3,4] and G. Lunglmayr[2]

[1]Institute of Clinical Pathology, [2]Department of Urology, [1,4]Karl Landsteiner Institute for Andrology and Prostate Research, LK Weinviertel, 2130 Mistelbach, Austria
[3]Department of Pathology and Laboratory Medicine, Drexel University, [4]MacroArray Technologies LLC., PA 19102-1192, Philadelphia, USA

KARL LANDSTEINER INSTITUT für Andrologie und Prostataforschung im Landesklinikum Weinviertel Mistelbach

RELATIVE QUANTIFICATION OF PROSTATE CANCER TUMOR MARKER mRNAs IN URINE CELL FILTRATES BY SYBR GREEN LIGHTCYCLER qPCR

ABSTRACT

In the last ten years hundreds of genes which are over-/under-expressed or specifically produced in prostate cancer tissue have been discovered. The growing prostate tumor exfoliates cells (molecules) into spontaneous urine via the urethra during natural shedding/apoptosis/lysis. In addition the number of released prostate cells can be increased by an attentive digital rectal examination (DRE) prior to urine collection. Diagnostic urine real time PCR assays have been developed recently (specific mRNAs: PCA3, AMACR, TMPRSS2:ERGa fusion gene). In our long term research program we address the question if quantitative information on selected prostate cancer biomarker mRNAs can be extracted from a heterogeneous urine cell fraction by real time qPCR. Furthermore, we explore if urine qPCR data can be used for a comprehensive non-invasive prostate tumor survey (differentiation latent/clinical tumor, malignancy, progression, metastasis, relapse, survival).

Methods: Urinary cells were isolated by filtration (50ml; n=42 urine from pathologically confirmed prostate cancer patients; n=36 urine samples from males aged <35 years as negative controls; additionally urine was collected from patients prior and after an attentive DRE). Total RNA was isolated from lysed cells (Zymo Research urine RNA isolation kit) and reverse transcribed into cDNA using anchored primers (Roche Transcriptor Kit). Quality control was performed by Lightcycler II real time SYBR Green I PCR with a 18s RNA primer pair. A geNORM and Normfinder analysis was performed with 15 housekeeping genes (PrimerDesign Ltd.) with RNA samples in duplicate reactions. A panel of 12 mRNAs known to be over-expressed in prostate cancer was quantified after correction for PCR efficiencies. GenEx software (MultiD) was used for calculations and statistical data analyses.

Results: A cell filtration assay allows highly efficient, inhibitor free total RNA isolation from spontaneous and post-DRE urine. Urine cell filtrates contain a mixture of kidney, bladder, prostate, urethral epithelial cells and leukocytes of unknown percentage composition. Hence, a relative mRNA quantification strategy has to be based on mRNAs of reference genes which are stable expressed in normal and cancer or pre- and post-DRE urine. A geNORM/Normfinder analysis identified GAPDH and CYC1 as the two best overall fitted genes for a prostate cancer urine mRNA analysis. Subsequently Lightcycler II SYBR Green assays were performed for 12 prostate cancer tumor marker genes (ABCA5, AMACR, DD3/PCA3, PCa-002, PCGEM1, PSA, PSCA, PSGR, PSMA, RPS2, TMPRSS2, TMPRSS2:ERGa). Results of relative Lightcycler real time qPCR assays with selected biomarker mRNAs will be presented.

Conclusions: Prostate cancer tumor marker mRNAs can be quantified in spontaneous and post-DRE urine of non-diseased and prostate cancer patients by real time qPCR. Multi-parameter qPCR assays might in the near future supplement the urological diagnosis (OENB P11491).

MATERIAL AND METHODS

Urine collection. Fresh spontaneous urine was collected from prostate cancer patients prior to radical prostatectomy (n = 46) and from young males aged below 35 years as control group according to local ethical regulations.

Urine nucleic acid isolation. To establish a working method for urine RNA isolation we compared three different protocols: I. Rneasy Mini Kit (QIAGEN), II. MagNA Pure Compact RNA Isolation Kit (Roche) and III. Urine RNA Kit (ZYMO Research). 50ml total urine was either centrifuged at 3500 rpm for 15 min (I, II) or filtered through a filter syringe to collect cells and cell fragments (III). Pellets (I,II) were resuspended in 350µl of the respective lysis buffer and centrifuged through a shredder column. All further isolation steps were according the original supplied kit instructions. Purified RNAs were treated with RNAse free DNAse to digest potentially present genomic DNA (I: RNase-Free Dnase Set; QIAGEN. II: Dnase is included in the RNA isolation kit; Roche. III: DNA-Free RNA Kit; ZYMO Research). RNA quality was assessed with an Eppendorf Biophotometer, a RNA 6000 Nano Assay (Bioanalyzer 2100, Agilent Technologies) and a 18 S RNA LightCycler II real time PCR assay. RNA was stored at -80 C° in the presence of 15 units Prime Rnase Inhibitor (Eppendorf).

Conventional RT-PCR. 10µl total RNA respectively were reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) with anchored oligo-dT primer. PCR reactions were performed with an Eppendorf Mastercycler ep using HOTMASTER *Taq* DNA Polymerase (Eppendorf): initial denaturation 94°C for 2 min; 40 cycles: denaturation at 94°C for 20 s, primer annealing at 55°C for 10 s, primer extension at 70°C for 30 s, final extension at 94°C for 2 min. Primer sequences for prostate cancer tumor markers and control genes (exon/intron junction primer either adapted from the literature or self-designed with the Primer 3 software):

tumor marker	forward primer	reverse primer
AMACR	5'-GGAATTCATGGCTGTTGGAG-3'	5'-CTTCATTTCTGGCCAATCATC-3'
DD3/PCA3	5'-TGGGAAGGACCTGATGATAC-3'	5'-AGGGCGAGGCTCATCGAT-3'
PCGEM1	5'-GGTAGGCACGTGGAGGACTA-3'	5'-TGCTTTTGTGGGTTTGTTCA-3'
PSA	5'-TTCTGGTGCACCCCCAGT-3'	5'-ATATCGTAGAGCGGGTGTGG-3'
PSCA	5'-CTGCCTGCAGGTGGAGAA-3'	5'-CTGTGAGTCATCCACGCAGT-3'
PSGR	5'-ATCCTTGCCCTTTTCTGGTT-3'	5'-ATGCCAATCTGGGCTGTTAC-3'
PSMA	5'-AGCTGAAAAGCCCTGATGAA-3'	5'-GCCGCTGAATTTGTTTGTTT-3'
RPS2	5'-GCCAAGCTCTCCATCGTC-3'	5'-GTGCAGGGATGAGGCGTA-3'
TMPRSS2	5'-GCTGCAGAAGCCTCTGACTT-3'	5'-ACAGATCATGGCTGGTGTGA-3'
ABCA5	5'-AGGCAGAGGCTGTCTGTGAT-3'	5'-GGCTTGCATTTGGGAAAATA-3'
PCa-002	5'-AGGTCAGCAGAGATGTCTGT-3'	5'-TAGTCCCCGAGAAAGAATTA-3'
control genes		
cytokeratin 5	5'-TGGAGAAGGAGTTGGACCAG-3'	5'- GCTTGCACTGAAGCCAGAG-3'
HOXB13	5'-GTTGCCAGGGAGAACAGAAC-3'	5'-GCTGTACGGAATGCGTTTCT-3'

PCR products were resolved on 2% agarose gels (containing GelStar Nucleic Acid Stain; CAMBREX) and documented with an UVP AutoBioChemi System.

LightCycler II real time PCR. 2µl undiluted or 5µl 1:10 diluted cDNAs were amplified on a LightCycler II (Roche) using Absolute QPCR SYBR Green Capillary Mixes (Abgene) or QuantiTect SYBR Green (QIAGEN). The amplification program was: 15 min enzyme activation at 95°C, 15 s denaturation at 95°C, 20 s annealing at 60°C, 20 s extension at 72°C (45 cycles), final extension at 72°C. A melting curve analysis was performed between 45°C and 95°C. Amplification runs were evaluated and documented with the LightCycler 4.05 software (Roche).

GeNORM analysis and relative quantification strategy. 13 housekeeping genes were evaluated with 11 urine sample cDNAs of prostate cancer patients. Each PCR reaction (including controls) was performed in duplicate. The averaged CPs were transformed into quantities by the ΔCt method and the average expression stabilities (M values) were calculated by the GeNORM software applet (Vandesompele et al. , 2002, Genome Biology 3, RESEARCH0034. Epub. Jun 18).

RESULTS

URINE RNA ISOLATION METHODS

PSA RT-PCR

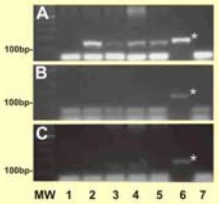

Fig.1 Comparison of three urine RNA isolation methods. **A:** urine filtration (ZYMO Research); **B:** urine centrifugation (QIAGEN); **C:** urine centrifugation (MagNA Pure Roche). RNA was reverse transcribed and 2µl undiluted cDNA used in a PCR reaction. 10µl PCR products were resolved on 2% agarose gels stained with SYBR Green (Gelstar). Lanes: molecular weight markers (MW), 1 - 5: urine RNA of tumor patients; 6: tumor RNA control; 7: negative control without cDNA. The star sign (*) indicates the height of the 151 bp fragment of prostate specific antigen (PSA).

URINE RNA QUALITY CONTROL

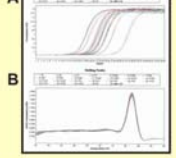

Fig.2 RNA integrity is evaluated by 18 S RNA LightCycler II real time PCR. **A:** Amplification curves. **B:** Melting temperature analysis of the 18 S RNA PCR fragments.

PROSTATE SPECIFIC ANTIGEN (PSA) mRNA IN URINE

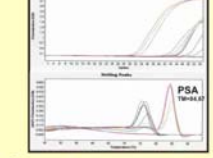

Fig.3 Prostate specific antigen (PSA) mRNA as a marker for the presence of prostate cells in spontaneous urine samples. The amplification specificity was validated by melting temperature analysis and electrophoretic size fragmentation.

RELATIVE mRNA QUANTIFICATION STRATEGY

Accession Number	Sequence Definition
NM_001101	Homo sapiens actin, beta (ACTB), mRNA
NM_002046	Homo sapiens glyceraldehyde 3-phosphate dehydrogenase (GAPDH), mRNA
NM_021009	Homo sapiens ubiquitin C (UBC), mRNA
NM_004048	Homo sapiens beta-2-microglobulin (B2M), mRNA
NM_003406	Homo sapiens phospholipase A2 (YWHAZ), mRNA
NM_001005409	Homo sapiens splicing factor 3a, subunit 1(SF3A1), mRNA
M10098	Human 18S rRNA gene
NM_001916	Homo sapiens cytochrome c-1 (CYC1), mRNA
NM_001967	Homo sapiens eukaryotic translation initiation factor 4A, isoform 2 (EIF4A2), mRNA
NM_004168	Homo sapiens succinate dehydrogenase complex, (SDHA), mRNA
NM_003286	Homo sapiens topoisomerase (DNA) I (TOP1), mRNA
NM_001686	Homo sapiens ATP synthase, (ATP5B), mRNA
NM_000194	Homo sapiens hypoxanthine phosphoribosyl transferase I, mRNA

Fig.4 Gene list for the GeNORM analysis.

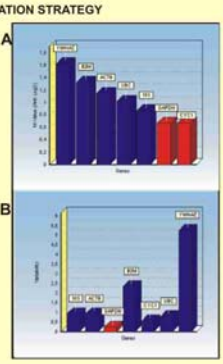

Fig.5 GenEX analysis of 13 housekeeping genes in urine of 11 normal and 11 prostate cancer patients (SF3A1, SDHA, Elf4A2, TOP1, ATP5B and HPRT1 had to be excluded). **A:** geNORM **B:** Normfinder.

GAPDH and CYC1 are identified by both methods as the optimal reference genes for an urine mRNA analysis.

mRNA EXPRESSION PROFILE OF PROSTATE TUMOR MARKERS IN URINE

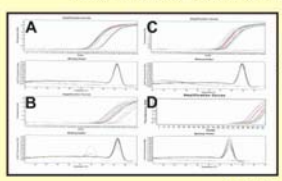

Fig.6 mRNA expression of prostate cancer tumor markers in urine. Total RNA was purified from 12 samples and LightCycler II SYBR Green real time PCR was performed as described:

- **A.** RPS2: expressed in 100%
- **B.** PSCA: expressed in 100%
- **C.** TMPRSS2: expressed in 92%
  hepsin: expressed in 82% (data not shown)
- **D.** AMACR: expressed in 75%

CONCLUSIONS

In the present work we established a quantitative LightCycler real time PCR SYBR Green assay for several potential prostate cancer tumor markers in spontaneous patient urine. We have developed an experimental tool to extend the analysis to benign prostatic hyperplasia (BPH) and "normal" (males aged between 18 to 35 years) urine after DRE (prostate massage). Such a comparative study could prove a potential application to diagnose prostatic disease for urological routine.