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Relative Gene Expression Analysis from Urine Samples of Patients with Prostate Disease

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Abstract

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Conventional screening and diagnostic procedures are leaned on PSA (Prostate specific antigene) which is not specific for prostate cancer and frequently leads to unnecessary invasive procedures for the exclusion of malignant disease. It is estimated that approximately 50% of persons underwent tissue biopsy is done upon false positive PSA value. Therefore a proper and timely differential diagnosis of malignant disease using non-invasive techniques remains one of the biggest challenges in medicine. Urine is the invaluable source of biological information contained in small molecules i.e. RNA that is easily reachable and detectable using molecular genetics techniques. We describe economical and fast method for relative expression analysis applicable to any target gene from urine samples and associated risk factors. Efficient non-invasive method for identification of malignant or high risk cases prove useful in reduction of patient distress during the diagnostic procedure and healthcare costs.

Keywords

*prostate cancer,
urine, relative
expression, non-
invasive method*

Introduction

Prostate disease, including prostatitis, benign prostatic hyperplasia and carcinoma, represent a group of the most common diseases that occur in

Caucasian men after fifty years of age (Bussemakers et al., 1999). Prostate diseases are characterized by similar symptoms, containing frequent urge to urinate, an intermittent or incomplete urination, increased frequency of nocturnal urinating and erectile dysfunction. Healthcare authorities worldwide support the annual screening strategy for prostate cancer using PSA for all men above age of 50. Because of similar symptoms, especially between benign prostatic hyperplasia and various types of cancer, precise diagnostics can be done only

after patohistological assessment of the prostate tissue biopsy. In practice, the diagnostic screening starts with PSA blood serum analysis, and the findings of digital rectal examination. According to the recommendations of the US FDA serum PSA value up to 4 ng/ml are considered normal. If serum PSA levels exceed the concentration of 4 - 10 ng/ml, the sample is considered as potentially positive for cancer or benign prostatic increasement, after a digital rectal examination, patient is directed to biopsy. Prostate tissue biopsy is performed in cancer suspect cases. The sample is collected with long, sterile needle using transrectal access.

Digital rectal examination reveals possible increase in the prostate volume and the analysis of the blood serum determines concentration of PSA which is elevated in all the above mentioned diseases (Hessels et al., 2003). According to available published data (Rigau et al., 2013) 20-60% of prostate biopsies return negative for tumor. In addition to that ASAP (Atypical Small Acinar Proliferation) and HGPIN (High - grade Prostatic Intraepithelial Neoplasia), by medical parameters, belong to the category of benign hyperplasia or prostatitis (Bretton et al., 1994; Catalona et al., 1994).

Prostate biopsy is an invasive, painful, and unpleasant diagnostic procedure. Because of relatively high proportion of negative outcomes after tumor biopsies the trend in modern urology is to develop new methods of efficient screening for malignant prostate diseases (Mengual et al., 2016; Drake et al., 2009; Bryzgunova et al., 2015). Some countries even introduced molecular-genetic profiling as a support to non-invasive diagnostic classification (Mottett et al., 2015). We find that alternative sources of such clinical information can be sought in other types of specimen i.e. blood and urine. The benefit of validation of new and non-invasive screening procedure would significantly reduce patients' psychological distress but also alleviate financial costs of public screening programs (Adeola et al., 2015; Neves et al., 2008; Bussemakers et al., 1999).

The aim of our study was to evaluate methods of isolation of total RNA from urine samples and to assess the performance of relative gene expression

analysis with such RNA as template using Real time PCR. We also aimed at identification of critical procedural factors associated with the quality and quantity of isolated genetic information.

Materials and methods

Ethical aspects of the human samples collection for purposes of this study were affirmed by institutional ethics committee of the Clinical Center University of Sarajevo (CCUS). All subjects signed informed consent prior to the sample collection. Urine samples were collected from a total of 162 participants, patients at the Urology Clinic of CCUS. The patients underwent standard clinical evaluation for prostate cancer (transrectal examination, biochemical PSA measurement from blood, needle biopsy and patohistological assessment). Immediately after rectal examination a sample of urine was collected into 50 ml volume sterile tubes in the amount not lower than 35 ml. Since mRNA is rather unstable molecule susceptible to degradation, the samples were transported to the Laboratory for human genetics of the Institute for genetic engineering and biotechnology within 12 hours of collection. When it was not possible to observe the procedure, a sample was maintained refrigerated at temperature of 4°C until processing.

The samples containing less than 50 ml of urine, were supplemented with PBS buffer (Phosphate buffered saline, Thermofisher scientific, Gibco, USA) until the total volume of 50 ml. Urine samples were centrifuged at 4000 rpm for 15 minutes to collect the tissue and cell residues as source of RNA. The debris was resuspended in 25 ml of PBS and centrifuged in additional purification step at 4000 rpm for 15 minutes. After decanting the supernatant, solid, purified fraction which contained cells originating from prostate was used as starting material for RNA extraction.

Total RNA was extracted using NucleoSpin® RNA isolation kit, according to the protocol recommended by manufacturer (Macherey - Nagel GmbH & Co., Duren, Germany). This kit uses a membrane filtration system for selective separation of nucleic acid which yields 50-60 µl of purified and solubilized RNA. Extracted RNA was stored at the

temperature of -20°C until further processing step. Extracted RNA was quantitated by fluorometry using Qubit FS RNA Assay kit and Qubit[®] 2.0 fluorometer (Invitrogen, Life Technologies, Oregon, USA). This method is based on the measurement of fluorometric extinction in samples in comparison with the values of the fluorometric extinction obtained from the set of standards of known concentrations.

Reverse transcription reaction was done using GeneAmp[®] Gold RNA PCR Core Kit (Applied Biosystems, USA) on an Eppendorf Mastercycler Gradient PCR unit. Components of the kit used in reverse transcription reactions for one sample were: 2 μl of RT buffer, 0,8 μl of dNTPs, 2 μl of random hexanucleotide primers, 1 μl of reverse transcriptase and RNA inhibitor, 3,2 μl of dd H₂O and 10 μl of RNA of equalized concentration of 1 ng/ μl for all samples. Verification of reverse transcription was performed by PCR of segment of gene coding for beta-actin (NM_001101) using ACTB primers (F – 5'-GCTATCCCTGTACGCCTCTG-3' ; and R – 5'-GTGGTGGTGAAGCTGTAGCC-3') described elsewhere (Valeri et al., 2015; Murphy et al., 2013) with the synthesized cDNA template, and the product was visualized in 2% agarose gel (Fig 1).

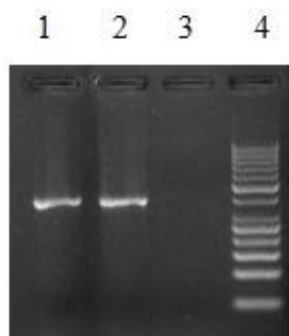


Figure 1. *β-actin* PCR product - first lane is a positive control *β-actin* product obtained by reaction with genomic DNA as a template, in second lane is *β-actin* product synthesized from the newly cDNA, and in the third lane is negative PCR control without. 50 bp DNA marker is used to confirm size of expected PCR product (353 bp)

PCR mastermix contained 2,5 μl of 10X PCR buffer (Applied Biosystems, USA), 1,5 μl of 25 mM MgCl₂ (Applied Biosystems, USA), 0,5 μl of 20 mM dNTP's mix (Fermentas, Lithuania), 0,5 μl of 20 mM of F and R primers (Biotez, Germany), 0,2 μl of

5 U/ μl of Taq polymerase (Applied Biosystems, USA) and 18,3 μl of ddH₂O with 1 μl of cDNA template. Amplification conditions were as follows: initial denaturation at 94°C for 3 min, denaturation 94°C for 30 seconds, primer binding 57°C 30 seconds, elongation 72°C for 40 seconds, and a final elongation of 72°C for 7 minutes with repeat until 30 cycles.

Results and Discussion

Out of 162 collected urine samples, RNA was isolated from 133, which makes 82% success rate for RNA extraction from urine.

In addition to the analysis of the concentration of the isolated RNA, we performed (qualitative) analysis using electrophoresis method to assess level of defragmentation of isolated nucleic acids as well as to check for residual protein from the extraction process. Defragmentation of nucleic acids is an important quality parameter and may affect later steps in terms of reduction of successful reverse transcription reaction, and therefore on the measurement of gene expression (Roberts et al., 2016). All samples showed high quality of the extracted RNA. The average concentration of total RNA was 4,60 ng/ μl with highest concentration of 16,03 ng/ μl in prostate cancer specimens subset (Table 1).

Reverse transcription reaction was done for RNA samples with minimum concentration of 1 and maximum concentration of 96 ng/ μl .

Having in mind instability of RNA molecule which is high even in the blood and tissue samples that are considered to be ideal, achieved 82% extraction can be considered high success. Urine, as a byproduct of numerous physiological reactions, is a very demanding material for this type of isolation due to the presence of a large number of various compounds affecting RNA degradation. Although RNA yield was relatively low, it was possible to obtain cDNA of sufficient quantity and quality for different downstream analysis by reverse transcription. According to our results, the critical step in extraction of total mRNA from urine is quick transport to the laboratory where extraction is to be performed. Also, equally important factor is the

Table 1. Total RNA quantity assessment and comparisons between diagnostic groups per DRE status and type of diagnosis

Patients (specimen) group	Average quantity of isolated RNA ug/ul	N	Comparison against RNA quantity isolated from prostate cancer patients (P-value)	Assessment of DRE factor (P-value)
Prostate cancer	16,03	37		
Benign hyperplasia	2,40	14	0,0294	
Non-specific precancerous cell dysplasia	2,99	41	0,0011	
Healthy controls with DRE	3,80	41	0,0020	0,0031
Healthy controls w/o DRE	2,40	102	0,0031	

stabilization of the urine sample in the laboratory for which we used PBS buffer and centrifugation step prior to the extraction. The selected kits for RNA extraction (NucleoSpin® RNA isolation kit, Macherey - Nagel GmbH & Co., Duren, Germany) and reverse transcription kit (GeneAmp® Gold RNA PCR Core Kit, Applied Biosystems, Ca., USA) proved to be good choice for obtaining high quality material for further analysis using relatively small amount of extracted starting material.

In the clinical evaluation for prostate disease most patients undergo a digital – rectal examination (DRE) procedure, and it is suggested that urine can be taken immediately after this procedure (Pellegrini et al., 2017). It was found that this procedure results in larger quantity of isolated RNA and therefore with larger amount of cDNA which is synthesized from total mRNA. It is probably related to an increase in number of epithelial cells of prostate tissues in urine after DRE.

After the prostate massage greater amount of prostate epithelial cells occur in urine because of mechanical pressure on the walls of prostate gland.

Complementary DNA obtained from total RNA isolated from urine samples was used for relative quantification of constitutive gene ACTB1 which could also be used as a reference gene in relative gene expression analysis for any target gene or gene products specific for prostate tissue or disease (Roberts et al., 2016).

A large number of potentially useful markers for diagnostic evaluation of prostate disease have no

detectable expression levels in patient's serum or the values are not reproducible. Therefore, expression of potential markers of prostate disease are located mainly in the tissue of the prostate gland and not necessarily present in the blood. Biological material for analysis includes proteins, total messenger RNA, genomic and mitochondrial DNA, microRNAs and extracellular vesicles (Sharova et al., 2016; Pellegrini et al., 2017). Following investigational assessment in various clinical populations, a number of alternative strategies based on molecular genetics markers are being proposed. In addition to that, a fact is that in many molecular-genetic laboratories other materials for the expression analysis are validated. DNA cannot be used as a material for the measurement of gene expression as DNA is only the carrier of qualitative information for amino acid sequence in protein composition. For functional diagnostic procedures, mRNA is a molecule of choice for this type of analysis because it is a direct precursor of synthesized protein and its quantity correlates with the quantity of the protein. With the method of reverse transcription it is converted to the doublestranded cDNA form, which as a transcript from the mRNA contains only gene coding segments. It is reproducible for any gene expression analysis technique (gene panels or targeted tissue or disease specific gene expression).

Conclusions

Using urine as a potential source of biological material could provide insight into the physiological condition of the prostate. Urine is a biological material which is relatively easy to collect and/or

recollect and sampling procedure is non-invasive. The following factors are important for successful isolation of total RNA and its further use in gene expression profiling: (1) method of sample collection – digital rectal exam or prostate gland massage prior to urine collection is very important for the increase of abundance of prostate epithelial cells in given urine sample and therefore for total RNA yield because, after all, those cells are the primary source of RNA which is used in following analyses, and (2) urine samples should be processed as soon as possible after the collection because of well known fast degradation of total RNA which is extremely unstable molecule because of the presence of large number of RNA degradation enzymes in urine. Alternatively, urine samples can be kept under refrigerated conditions (+4°C - 8°C) to prevent biological degradation but not longer than 24 hours. In addition, urine contains many products and by-products of physiological and pathological processes that take place in an organism and is potential source of diagnostic information for various diseases.

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