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Reference gene validation for relative quantification analysis of transcripts in urinary exfoliated cells among urothelial bladder carcinoma patients

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ABSTRACT

The specific mRNA signature of urinary exfoliated cells (UECs) can be helpful for biomarker discovery and validation studies related to urological malignancies including cancers of bladder, kidney, prostate and testicles, as well as kidney allograft rejection, systemic autoimmune diseases such as lupus, and acquired proteinuric diseases. To the best of our knowledge, no systematic validation of reference genes has been reported for UECs Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) analysis up to now.

To determine the stability of candidate housekeeping genes (HKGs) across UECs, they were isolated from first morning urine of patients diagnosed as high grade and low grade urothelial bladder cancer as cases and clinical controls including the patients suffering from bladder stone, benign prostatic hyperplasia, obstructive uropathy and healthy individuals. RT-qPCR was performed to determine 9 candidate HKGs stability.

geNorm, Normfinder, and BestKeeper statistical algorithm were applied to determine the most stable reference genes. The non-parametric Mann-Whitney *U* test applied to compare HKGs expression levels between cases and controls samples.

GAPDH and HSP90AB1 were selected as two most stable expressed HKGs by all three algorithms, while ACTB demonstrated to have the least stability.

Although, GAPDH is a suitable reference gene for quantitative analysis of UECs in urological malignancies, based on normalization factor calculated by geNorm, we recommend using GAPDH, and HSP90AB1 together as normalizer to obtain more realistic expression analysis via relative qPCR.

1. Introduction

Gene expression analysis, using Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR), broadly conducts to discover new potential diagnostic biomarkers (Ginzinger, 2002). In 2009, "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" was published as a guideline to achieve more reliable and unequivocal interpretation from qPCR experiments (Bustin et al., 2009). It introduced precise normalization, as an important requirement to ensure the accuracy of this method and obtain reliable gene expression measurement. Compensate intra- and inter-kinetic RT-qPCR, using reference genes as internal controls, is the most accepted normalization strategy (Huggett et al., 2005).

Internal references usually select among cells natural constitutive housekeeping genes (HKGs) that are essential for maintenance of basic cellular function. Therefore, they are expected to express similarly in all cells under normal and patho-physiological conditions. Actin Beta (ACTB), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), subunit A of Succinate dehydrogenase complex (SDHA),

Abbreviations: UEC, urinary exfoliated cells; RT-qPCR, Reverse Transcription-quantitative Polymerase Chain Reaction; HKGs, housekeeping genes; ACTB, Actin Beta; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; SDHA, subunit A of Succinate dehydrogenase complex; TBP, TATA box binding protein; UBC, Ubiquitin C; HPRT1, Hypoxanthine phosphoribosyltransferase 1; RPS13, Ribosomal protein S13; HSP90AB1, Heat shock protein 90 kDa alpha; PGM1, Phosphoglucomutase 1; BPH, benign prostatic hyperplasia; PBS, Phosphate Buffered Saline; cDNA, complementary DNA; qPCR, quantitative Polymerase Chain Reaction; R2, correlation coefficients square; E, efficiency; Ct, cycle threshold; Std. Dev, standard deviation; CV, coefficient of variations.

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TATA box binding protein (TBP), Ubiquitin C (UBC), Hypoxanthine phosphoribosyltransferase 1(HPRT1), Ribosomal protein S13(RPS13), Heat shock protein 90 kDa alpha (HSP90AB1), and Phosphoglucomutase 1 (PGM1) are known HKGs applied as internal controls for quantitative expression analysis of human transcriptome by qPCR.

Although pre-assumed constant, many studies demonstrated that expression levels of some common HKGs are affected by experimental conditions, and changed dramatically under pathological states such as cancer (Thellin et al., 1999; Hendriks-Balk et al., 2007; Guénin et al., 2009; Rocha-Martins et al., 2012). It has been demonstrated that using inappropriate gene as an internal control, can lead to data misinterpretation of target gene expression signature (Dupasquier et al., 2014; dos Santos et al., 2015). Therefore, HKGs must be experimentally validated in different specimens in order to define the most reliable reference gene (Livak and Schmittgen, 2001).

Many bioinformatics algorithms and statistical approaches have been developed to assess expression stability of HKGs. Among them the most frequently used are geNorm, Normfinder, sBestKeeper, qBasePlus, and comparative delta Ct. None of them seems to be optimal enough to rely on, and latest study recommended applying more than two for selection of the most stable reference gene (Jacob et al., 2013).

Bladder carcinoma considers among male's most frequent cancers in more developed countries (Torre et al., 2015). Its diagnosis and recurrence screening currently managed by using combination of urine cytology and cystoscopy (Gregg et al., 2015). Although, cystoscopy provides valuable biopsy, it is too invasive and expensive for routine screening and surveillance (Lotan et al., 2008). Voided urine cytology is almost inexpensive and absolutely noninvasive, but does not provide sufficient sensitivity(Turco et al., 2011). Developing substitute urine-based test for bladder carcinoma diagnosis and recurrence surveillance with reasonable cost, and adequate specificity-sensitivity, which can be accessible by applying high-throughput simple molecular techniques, would be promising alternative to conquer invasiveness and expensiveness of current system.

Most urinary epithelial cells are due to the normal sloughing of epithelial tissue from ureters, to urethra, including bladder. Bladder epithelial turnover is low, and epithelium detachment and desquamation more occur under abnormal malignant states such as cancer (Patriarca et al., 2008). The specific mRNA signature of epithelial cells, originated from cancerous tissue, among other urinary cell, might improve urine based molecular markers sensitivity for noninvasive diagnosis and recurrence surveillance of bladder carcinoma (Siracusano et al., 2005).

Selecting the most reliable gene that has been systematically validate as reference gene for certain specimen under expected experimental conditions via precise literature review would be sufficient. But no systematic validation of reference genes has been reported for urinary exfoliated cells (UECs) RT-qPCR analysis. This study has been designed to identify the most reliable reference genes as well as most suitable combination of them for gene expression normalization of UECs from a panel of 9 commonly used HKGs genes according to the literature.

2. Material and methods

2.1. HKGs selection

Nine potential HKGs were selected based on their frequent usage as internal control in RT-qPCR as well as their functional characteristic. Based on frequent usage, ACTB, GAPDH, HPRT1, and HSP90AB1 are classical HKGs commonly used as internal control, while SDHA, TBP, UBC, and PGM1 occasionally applied. RPS13 is nearly novel HKG (De Jonge et al., 2007). Considering their products biological function, ACTB encodes one of highly conserved actin proteins involved in cell motility, structure, and integrity. The rest encode functional proteins that are involved in maintenance of basic cellular function; from gene expression, and signaling, to metabolism. GAPDH encodes member of the glyceraldehyde-3-phosphate dehydrogenase protein family, which has many functions besides being the most well known involved in the glycolytic pathway. The protein encoded by HPRT1 gene is a transferase, and plays central role in purine generation through the salvage pathway. HSP90AB1 encodes a member of the heat shock protein 90 family; the constitutive form of the cytosolic 90 kDa heat-shock protein, which seems to be involved in gastric apoptosis and inflammation. Catalytic subunit of succinate-ubiquinone oxidoreductase encoded by SDHA is a complex of the mitochondrial respiratory chain. TBP encodes the TATA-binding protein that modulates the DNA binding activity and affects the rate of transcription complex formation. Ubiquitin C, encoded by UBC is a polyubiquitin precursor, involved in protein degradation, DNA repair, cell cycle regulation, kinase modification, endocytosis, and regulation of other cell signaling pathways. PGM product is predominant isozyme of phosphoglucomutase in most cell types, belonged to the phosphohexose mutase family. Ultimaltly, recently described HKG; RPS13 encodes a ribosomal protein, which is a component of the 40S subunit.

2.2. Patients and samples

Sampling was conducted from May-2015 to Sep-2015 in department of urology, *Imam* Khomeini general hospital, Tehran, Iran. The study was approved by research ethics committee of TUMS. A total number of 32 individuals including 16 non-bladder carcinomas (consist of patients suffering from bladder stone (n = 3), benign prostatic hyperplasia (BPH) (n = 2), obstructive uropathy (n = 3) and healthy individuals (n = 8)), along with 8 high grade and 8 low grade pathologically confirmed bladder carcinoma patients (based on the World Health Organization system) (Fig. 1) were included in this study. All individuals signed informed consent after receiving detailed description of purpose and procedures of investigation.

First morning complete urine samples were collected in 25–370 mL bulk and stored at 4 °C for maximum 4 h. Samples were centrifuged in 50 mL falcon tubes at 800*g* for 10 min at 4 °C (Chiu et al., 2002). Cell pellets were washed with 1× Phosphate Buffered Saline (PBS), treated

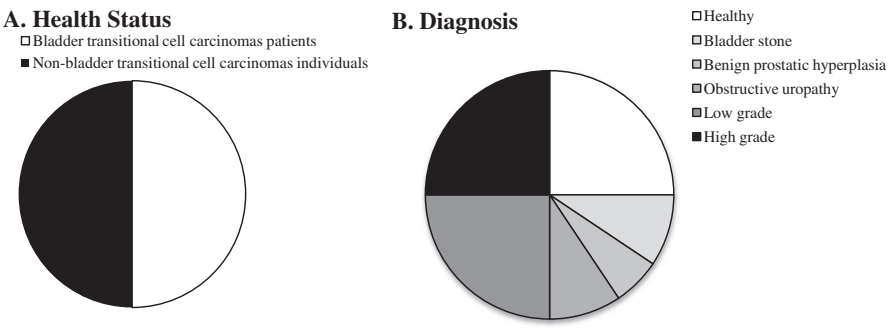

Fig. 1. Case and control groups based on A: Health status and B: Diagnosis.