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Research paper

Comprehensive selection of reference genes for expression studies in meniscus injury using quantitative real-time PCR

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ABSTRACT

The meniscus plays critical roles in the knee function. Meniscal tears can lead to knee osteoarthritis. Gene expression analysis may be a useful tool for understanding meniscus tears, and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) has become an effective method for such studies. However, this technique requires the use of suitable reference genes for data normalization. We evaluated the suitability of six reference genes (*18S*, *ACTB*, *B2M*, *GAPDH*, *HPRT1* and *TBP*) using meniscus samples of (1) 19 patients with isolated meniscal tears, (2) 20 patients with meniscal tears and combined anterior cruciate ligament injury (ACL), and (3) 11 controls without meniscal tears. The stability of the candidate reference genes was determined using the NormFinder, geNorm, BestKeeper DataAssist and RefFinder software packages and comparative ΔCt method. Overall, *HPRT1* was the best single reference gene. However, GenEx software demonstrated that two or more reference genes should be used for gene expression normalization, which was confirmed when we evaluated *TGFβR1* expression using several reference gene combinations. *HPRT1* + *TBP* was the most frequently identified pair from the analysis of samples of (1) meniscal tear samples of patients with a concomitant ACL tears, (2) all meniscal tears, and (3) all samples. *HPRT1* + *GAPDH* was the most frequently identified pair from the analysis of samples of isolated meniscal tear samples and controls. In the analysis involving only controls, *GAPDH* + *18S* was the most frequently identified pair. In the analysis of only isolated meniscal tear samples and in the analysis of meniscal tear samples of patients with concomitant ACL tears and controls, both *HPRT1* + *TBP* and *HPRT1* + *GAPDH* were identified as suitable pairs. If the gene expression study aims to compare non-injured meniscus, isolated meniscal tears and meniscal tears of patients with ACL tears as three independent groups, the trio of *HPRT1* + *TBP* + *GAPDH* is the most suitable combination of reference genes.

1. Introduction

Menisci are important components in joint biomechanics with crucial roles in the knee joint: distributing joint forces, load bearing, and enhancing joint stability (Lee et al., 2014; Kaleka et al., 2014). Lesion of this structure can cause pain, joint swelling, and osteoarthritis in the long term. Younger people are more likely to have acute lesions due to trauma, whereas older people are more likely to have lesions due to degeneration (Englund et al., 2009; Pauli et al., 2011; Rai et al., 2013). Patients with traumatic meniscal tears commonly present an associated rupture of the anterior cruciate ligament (ACL) (Poulsen and Johnson, 2011). Once present, meniscus tears are associated with an accelerated progression of cartilage degeneration in the knee compared with individuals with osteoarthritis but without tears (Biswal et al., 2002; Hunter et al., 2006).

Recent studies have been performed to understand the gene expression alterations that may have a role in human meniscal tears. In a transcriptome analysis, several genes were identified that were differentially expressed with age and chondrosis in patients with meniscus tears (Rai et al., 2013a). Moreover, Brophy et al. investigated whether the expression of osteoarthritis markers (matrix components, cytokines, chemokines, aggrecanases, metalloproteinases, and transcription factor genes) are age- and sex-related in meniscal tears with and without a concomitant ACL tear (Brophy et al., 2012). The authors demonstrated that the meniscus in younger patients reacts with an intrinsic response and is more prone to inflammatory changes. Conversely, there were no

Abbreviations: ACL, anterior cruciate ligament; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MRI, magnetic resonance imaging; TLDA, TaqMan Low-Density Array; AAV, adeno-associated virus; Crt, relative cycle threshold; Ct, cycle threshold; SD, standard deviation; CV, coefficient of variance; RQ, relative quantification; Acc.SD, accumulated standard deviation.

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differences in inflammatory cytokines or chemokines in the group of patients over forty years old (Brophy et al., 2012). If confirmed in larger studies, these markers may monitor local events at the surgical sites and detect osteoarthritis progression (Kambic, 2012).

Investigation of gene expression in human meniscal samples may help in improving the understanding of meniscal tears as well as osteoarthritis progression. Moreover, gene expression analysis will be important for guiding patient management and the development of new therapeutic options for these knee afflictions.

Although powerful techniques, including microarrays and high-throughput measurements, have been developed to detect gene expression levels, the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is commonly used in many laboratories (Wang et al., 2015). Moreover, because of its accuracy, sensitivity, and capacity for high-throughput analysis, RT-qPCR is currently considered to be the gold standard technique for evaluation of gene expression (Derveaux et al., 2010). RT-qPCR is one of the most commonly utilized approaches in functional genomics research, and its use in gene expression analysis may become more routine; furthermore, this technique is commonly used to validate data obtained by other methods (Kozera and Rapacz, 2013), including the data of transcriptomic analysis.

To obtain reliable data using RT-qPCR, a common method is to normalize the target gene expression using an endogenous reference gene. Ideally, reference genes should be stably expressed or at least vary only slightly in expression in all tissues or cells under the experiment conditions (Li et al., 2009); therefore, a validation experiment for the evaluation of reference gene expression stability for each target tissue and disease is recommended (Bustin and Mueller, 2005; Hruz et al., 2011). Normalization with unstable internal controls may result in different values, leading to erroneous results (Yuzbasioglu et al., 2010). However, many authors do not critically evaluate their RT-qPCR experiments; therefore, the experiments are improperly designed and difficult to repeat because of insufficient data quality (Bustin, 2010). Consequently, the use of suitable reference genes with stable expression in the studied tissue (normal and/or injured) is essential for effective data normalization and the acquisition of accurate and meaningful biological data.

Suitability of reference genes has been evaluated in some human musculoskeletal diseases, such as shoulder instability (Leal et al., 2014), rotator cuff tears (Leal et al., 2015a), ACL tears (Leal et al., 2015b), osteoarthritic articular cartilage (hip and knee) (Pombo-Suarez et al., 2008), human lumbar vertebral endplate with modic changes (Zhou et al., 2014), and skeletal muscle with chronic degenerative changes (Yuzbasioglu et al., 2010). To our knowledge, no previous studies have described the best individual or set of reference genes for gene expression analysis in human meniscus samples. A previous study used *GAPDH* for gene expression normalization in meniscal tear samples of patients with and without a concomitant ACL tear (Brophy et al., 2012).

In this study, we assessed the suitability of six reference genes frequently reported in the literature (*18S*, *ACTB*, *B2M*, *GAPDH*, *HPRT1* and *TBP*) using meniscus injured samples of patient with or without concomitant ACL tears as well as meniscus non-injured samples by analyzing gene stability with five software packages and comparative delta cycle threshold (ΔCt) method.

2. Materials and methods

2.1. Patients

Tissue samples were obtained from 39 patients with medial meniscal tears, including 19 samples of patients with isolated medial meniscal tears and 20 samples of patients with meniscus injury and a concomitant ACL injury. The following inclusion criteria were employed: age between 18 and 50 years old, clinical history compatible to meniscal injury (such as pain, swelling, stiffness, catching and locking), at least one specific physical examination test positive among McMurray (McMurray, 1949), Appley (Apley, 1947)and Steiman (Tria and Klein, 1992) tests that were used to diagnose meniscus injury (Speziali et al., 2015), magnetic resonance imaging (MRI) diagnosis of medial meniscus injury with abnormal signal extending to at least one articular surface involving the posterior horn and or the body of the medial meniscus (Crues et al., 1987), and arthroscopic confirmation of the medial meniscus lesion involving its posterior horn and or its body. The Lachman test (Torg et al., 1976), Anterior Drawer test (Marshall et al., 1975), and Pivot-Shift tests (Galway et al., 1972) were used to diagnose ACL injury (Astur et al., 2014a, b). Coronal and sagital MRI view were used to identify meniscal and ACL lesions. All injuries were confirmed during arthroscopic procedure and reclassified if necessary.

The following exclusion criteria were also applied: medial meniscus lesions treated by suture (outside-in, inside-out or all-inside), medial meniscus stable lesions tested by probe palpation such as some longitudinal lesion <1 cm, radial lesions <5 mm, partial thickness lesions. The stable, unstable criteria was defined intra-operatively by the surgeon.

Additionally, 11 patients without any history of meniscal tears were included in this study as a control group. These patients were arthroscopically operated for other knee injuries, such as isolated ACL injury. All control patients were physically active. Table 1 displays the main clinical outcomes of the studied cases and controls.

This study was performed with the approval of the Ethics Committee of the Universidade Federal de São Paulo (UNIFESP), Brazil (CEP #51,436). Written informed consent with approval of the ethics committee was obtained from all patients prior to specimen collection.

2.2. Tissue samples

To collect tissue samples, patients were prepared in the standard fashion for arthroscopy meniscus surgery. A standard arthroscopic joint evaluation was carried out, confirming the meniscus injury or meniscus and ACL injuries diagnosis. During surgery, about 5 mm^3 samples of the innermost part of the injured area of the posterior horn and the body of the medial meniscus were collected for gene expression analysis.

In the controls, a sample fragment of about 5 mm^3 was resected from the innermost part of the healthy medial meniscus body by arthroscopy.

All tissue specimens were immediately immersed in Allprotect Tissue Reagent (Qiagen, USA) and stored at −20 °C until RNA extraction.

2.3. RNA extraction

Total RNA was extracted from 10 to 20 mg of tissue sample using an AllPrep DNA/RNA/miRNA Mini Kit (Qiagen, USA) according to the manufacturer's protocol. The mechanical lysis step was performed using the Tissue Lyser LT equipment (Qiagen, USA). RNA concentration and quality were immediately determined using a Nanodrop ND-1000 (Thermo Scientific, USA) and the integrity of the RNA was verified by gel electrophoresis on a 1% agarose gel. Aliquots of the total RNA were stored at −80 °C until further use.

2.4. RT-qPCR

RT-qPCR gene expression quantifications were performed according to MIQE guidelines (Taylor and Mrkusich, 2014). Only RNA samples

Table 1
Distribution of the clinical outcomes of meniscal tear patients and controls.

Variable	Cases (N = 39)	Controls (N = 11)
Age at surgery, years (mean ± SD)	39.5 ± 12.6	29.4 ± 7.3
Gender (% of male)	79.5%	81.8%
Duration of condition, months (mean ± SD)	9.4 ± 10.7	4.5 ± 3.6
Mechanism (% of traumatic onset of symptoms)	84.6%	90.9%

N: number of samples; SD: standard deviation.