Research Square

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Identification of Appropriate Housekeeping Genes for Gene Expression Studies in Human Renal Cell Carcinoma Under Hypoxic Conditions

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Research Article

Keywords: Renal cell carcinoma, hypoxia, endogenous genes, normalization

Posted Date: July 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-706573/v1

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Version of Record: A version of this preprint was published at Molecular Biology Reports on March 11th, 2022. See the published version at https://doi.org/10.1007/s11033-022-07236-0.

Abstract

Background: Due to the loss of von Hippel-Lindau tumor suppressor function, clear renal cell carcinoma (ccRCC) deregulates hypoxia pathways. Quantitative PCR is a powerful tool for quantifying differential expression between normal and cancer cells. Reliable gene expression analysis requires the use of genes encoding housekeeping genes. Therefore, in this study, eight reference candidate genes were evaluated to determine their stability in 786-0 cells under normoxic and hypoxic conditions.

Methods and Results: Four different tools were used to rank the most stable genes: GeNorm, NormFinder, BestKeeper, and Comparative Ct (ΔCt), and a general ranking was performed using the RankAggreg. According to the four algorithms, the *TFRC* reference gene was identified as the most stable, and therefore, no agreement was observed for the 2nd and 3rd positions. A general classification was then established using the RankAggreg tool. Finally, the three most suitable reference genes to be used in 786-0 cells under normoxic and hypoxic conditions were *TFRC*, *RPLP0*, and *SDHA*.

Conclusions: To our knowledge, this is the first study to evaluate reliable genes that can be used in gene expression analysis in ccRcc under a hypoxic environment.

Introduction

Renal cell carcinoma (RCC) is a group of malignant histological subtypes that arise from epithelial cells, accounting for 2%–3% of all malignancies in adults [1,2]. The three major RCC histological subtypes are clear cell RCC (ccRCC), papillary RCC (pRCC), and chromophobe RCC (ccRCC). Each subtype is associated with unique genetic mutations, clinical characteristics, and sensitivity to treatment [3].

The ccRCC is the most common histologic subtype of RCC, and it accounts for approximately 75% of kidney cancer diagnoses [4]. The majority of ccRCC patients have a mutation in the von Hippel-Lindau (*VHL*) gene, which is located on the short arm of chromosome 3 and serves as an autosomal dominant tumor suppressor [5]. The *VHL* protein (pVHL) interacts with other proteins, such as hypoxia-inducible factor (HIF), to form the complex E3-ubiquitin, and this complex targets specific proteins to undergo proteasomal degradation. HIF is a heterodimeric transcription factor (HIF1α and HIF1β) that coordinates the expression of several genes responsible for cellular adaptation to hypoxia [6]. Under normoxic conditions, HIF1α protein is hydroxylated, recognized by pVHL, which drives them to degradation. On the other hand, under hypoxic conditions, HIF1α is not hydroxylated and cannot be recognized by pVHL and its intracellular concentration rises [7]. In RCC patients, pVHL is nonfunctional and consequently unable to target HIF1α protein for degradation. Thus, free HIF1α promotes the transcription of a wide variety of target genes. Therefore, molecular studies of hypoxia-responsive pathways are challenging because they require genes with stable expression to be used as reference genes [8].

Determination of gene expression profiles is an important tool in the field of molecular oncology. The analysis of differential gene expression between tumors and normal tissues is extremely important for identifying possible therapeutic targets [9]. Real-time quantitative polymerase chain reaction (qRT-PCR) has been used to measure mRNA in a given cell type. Owing to its high sensitivity and accuracy, this technique is the gold standard for gene expression measurements [10]. In qRT-PCR, target gene expression was obtained by normalization with any housekeeping genes (HKG). HKGs are a set of genes that are constitutively expressed and play a fundamental role in maintaining the existence of cells, and their expression should not be modulated by experimental conditions [11].

In this study, we investigated the performance of a panel of eight HKGs in a ccRCC cell line under normoxic and hypoxic conditions, aiming to identify suitable reference genes for the purpose of normalization in RCC gene expression studies.

Materials And Methods

Cell cultureand induction of hypoxic conditions

The RCC cell line 786-0 was obtained from American Type Culture Collection (catalog no.: CRL-2947™, Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco® Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco® Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco® Invitrogen) in a humidified incubator with 5% CO_2 at 37°C.

A day before hypoxia assay was performed, approximately 2 × 10^5 cells were seeded in 60 mm petri dishes and incubated for 6 h in a hypoxia-inducing humid chamber (StemCellTM Technologies, USA) with an atmosphere of 1% O_2, 5% CO_2, and 94% N_2, and stored in an oven at 37°C. The Altair PRO Single-Gas Detector (Code: 217597, MSA, Cranberry Township, Pennsylvania, USA) was used to measure the O_2 concentration inside the chamber.

RNA extraction

RNA was extracted from the cells of the control and hypoxia-treated groups. The cells were washed with PBS, then RNA was extracted using RNeasy® Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. Extracted RNA was diluted in RNase-free water. Subsequently, RNA concentration (ng/µL) and purity (OD260/280) were determined by spectrophotometry using Nanodrop® ND-100 (Thermo Scientific). The RNA was considered pure if the OD260:OD280 ratio falls within the range of 1.8–2.1. The integrity of the samples was confirmed through electrophoresis using 1% agarose gel for 40 min. The RNA samples were stored at -80°C.

Complementary DNA (cDNA) synthesis

The QuantiTec Reverse Transcription kit (Qiagen) was used. The cDNA was synthesized using 2 µg of total RNA, followed by a step of eliminating genomic DNA using the same buffer from the kit. Then, the resulting mixture was incubated in the thermocycler at 42 °C for 2 min and then on ice soon after. A second mix was prepared, complementing the previous mix, containing the RT primer, and amplified using a cycle at 42 °C for 15 min, and the response stop with a cycle of 95 °C for 3 min. Then, the samples were incubated in an ice bath for 2 min and stored at -20 °C until qRT-PCR.

Real-time quantitative polymerase chain reaction (qRT-PCR)

Amplification by qRT-PCR was performed using SYBR Green® (Applied Biosystems, NY, USA). StepOnePlus® (Applied Biosystems) was used for amplification, and the protocol was as follows: 2 min at 50 °C and 10 min at 95 °C; two cycles of 15 s at 95 °C and 1 h at 60 °C (completing 40 cycles), then final cycle for 15 s at 95 °C.

The human genes used were: RRN18S (lateral stem subunit P0 of ribosomal protein), ACTB (beta-actin), GAPH (Glyceraldehyde-3-Phosphate dehydrogenase), HPRT1 (hypoxanthine phosphorbosiltransferase 1), PGK1 (phosphoglycerate kinase 1), RPLP0 (Ribosomethermal Protin P0), SDHA (Subunit A of the flavoprotein of the succinate dehydrogenase complex), and TFRC (transferrin receptor). The forward and reverse primer were designed using the Applied Biosystems website (Table 1). The Primer Express 3.0 (Life Technologies, MD, USA) was used to confirm human sequences in BLAST.

Analysis of the stability of reference genes

Four algorithms were used to determine the stability of the candidate HKG: NormFinder [12], geNorm [13], BestKeeper [14], and Delta-Ct (ΔCт) method [15]. NormFinder calculates the stability of reference genes based on intra- and inter-group variability. The weighted measure of these two parameters is expressed as the S value, and the most stable reference gene has the lowest S value [12]. GeNorm calculates the average expression stability (M). The algorithm functions by first identifying two genes with the highest expression agreement and, therefore, high stability for each gene. Lower M values indicate greater stability [13]. The BestKeeper program calculates a Pearson's correlation coefficient for each gene, wherein p values closer to 1.0 indicates greater stability [14]. Comparative ΔCt uses a basic ΔCt approach to compare the relative expression of pairs of genes, creating a stability rank based on ΔCt and average standard deviations. The genes with the lowest average standard deviation (SD) and constant ΔCt values were considered to be the most stable [15]. In addition, once all the stability values for all tools were obtained, the BruteAggreg function, a weighted rank aggregation tool from the RankAggreg package was used [16]. This is an R package that uses a Monte Carlo algorithm to calculate the Spearman distance to obtain the overall ranking among the evaluated genes and tools (NormFinder, geNorm, BestKeeper, and Delta-Ct (ΔCт)).

Results

In this study, eight genes were selected and evaluated as potential gene candidates to use as reference for RT-qPCR in hypoxia-cultured human adenocarcinoma cells. All samples had high RNA yield, quality, and integrity. Mean RNA quantification was 3603.38 ± 176.50 ng/µL for the normoxia samples and 3111.76 ± 54.90 ng/µL for the hypoxia samples. Mean A260/280 ratio was 2.05 ± 0.02 in normoxia samples and 2.08 ± 0.01 in hypoxia samples. The integrity was assessed using an agarose gel, and two sharp bands (28S and 18S RNAs) were observed.

Primer's specificity and efficiency

The specificity of the primers designed for the amplification of HKGs was determined using melting curve analysis. A single fluorescence peak was detected for each primer, indicating that only one fragment was amplified during qPCR amplification (Fig 1). The efficiency of the primers (E) ranged from 1.98 to 2.02, and the correlation coefficient (R^2) ranged from 0.99–1.00.

Expression stability of reference genes normoxic and hypoxic conditions

The cycle threshold (Ct) values of eight reference genes in 786-0 cells under normoxic and hypoxic conditions were used to compare gene expression patterns. A wide range of Ct expression variances were observed. *ATCB* had the highest Ct variation, while *TFRC, RPLPO, SDHA,* and *HPRT1* had the lowest variation (Fig 2).

Determination of expression stability of candidate reference genes.

The expression stability of the eight candidate genes was assessed under hypoxic conditions and evaluated using the statistical algorithms BestKeeper, geNorm, NormFinder, and Delta-C_T (ΔCт) analyses.

The stability of the HKGs was determined by BestKeeper based on the extent of standard deviation (SD±CP), with a higher SD value corresponding to the low stability of the HKGs. According to the BestKeeper ranking, *TFRC* (0.38) was the best candidate, followed by *SDHA* (0.60) *and HPRT1* (0.63)(Table 2).

GeNorm analysis ranked the target reference gene according to their M value using the Ct values of all samples. Samples with the lowest M value were considered to be the most stable and *vice versa*. The M value of HKGs ranged from 0.65–1.55. *TFRC* and *RPLP0* showed the highest stability (0.65), followed by *SDHA* (0.75) and *PGK1* (0.89) (Table 2).

NormFinder analysis was employed in intra- and intergroup variations for the estimation of stability values. Following this approach, *TFRC* (S-value = 0.20) was identified as the most stable gene, followed by *SDHA* (S = 0.51) and *RPRP0* (S = 0.51) (Table 2).

Finally, the stability of the HKGs was determined using the comparative ΔCт methods based on (SD). A lower SD value is correlated with higher stability of the HKGs. The *TFRC* with an SD value of 1.15 was observed to be the most stable HKG, followed by *SDHA* (1.20) and *RPLP0* (1.20) (Table 2).

The rank-ordered genes calculated by the four algorithms presented in Table 2 were further analyzed by RankAggreg [16] to obtain a consensus rank list of genes. The stability of the candidate reference genes was in the order *TRFC > RPLPO > SDHA> PGK1> HPRT1 > GAPDH > ACTB >* X *18S* (Fig. 3).

Discussion

Accurate relative quantification in gene expression analysis requires the use of normalized reference genes, since the stability of target genes could vary according to the experimental design, making it essential for the reliability of the results [10,13,14]. The selection of suitable reference genes for gene expression analyses has recently been highlighted in several studies [17–21].

RCC cell line-based research has a major impact on understanding signaling pathways and discovering new therapeutic targets [22]. *In vitro* assays mimic the tumor microenvironment conditions as closely as possible. Hypoxia is present in nearly 80% of RCCs, and it modulates the gene expression profile, resulting in an aggressive phenotype of this tumor [23]. Despite the great importance of hypoxia in the pathophysiology of RCC, studies on more adequate HKGs are scarce. To the best of our knowledge, this is the first study on the appropriate HKG under hypoxic conditions in ccRCC cells. For this purpose, eight putative reference genes (*RRN18S, ACTB, GAPDH, HPRT1, PGK1, RPLP0, SDHA, and TFRC*) in ccRCC cell lines under normoxic and hypoxic conditions were evaluated according to their expression stability and consistency with four different specific tools: geNorm, NormFinder, BestKeeper, and ΔCt method. In addition, Rankagreg was used to generate a consensus ranking.

In general, it was possible to observe a partial accordance of the three best normalizer genes (*TFRC*, *SDHA,* and *RPLP0*) chosen among the four methodologies (Table 2). *X18S* and *ACTB* were considered to be the least stable genes. According to the four algorithms, the *TFRC* reference gene was identified as the most stable gene, followed by *SDHA* and *RPLP0*. *RPLP0* was the gene that showed great discrepancy among the four algorithms, ranging from 1st by geNorm to 4th by BestKeeper (Table 2, Fig. 3). This disagreement is possibly due to the different principles among the algorithms [21],To provide comprehensive rankings integrating the four different programs, the RankAggreg, a Monte Carlo cross-entropy algorithm, was employed to reach a consensus among data obtained by the others four algorithms [11,12]. High stability of *TFRC* has been demonstrated in breast cancer [23] and pancreatic cancer [24]. Furthermore, *TFRC* was observed to be the most suitable reference gene for human umbilical vein endothelial cells (HUVECs) subjected to hypoxic experimental conditions [25]. *RPLP0* was observed to be an optimal reference gene for expression analysis using formalin-fixed paraffin-embedded renal tumors [26]. *RPLP0* was also a suitable reference gene to normalize gene expression levels in qRT-PCR experiments in hypoxic and/or hyperglycemic HUVEC cultures [27]. Finally, the *SDHA* reference gene ranged 3rd in RankAgreeg. This gene was used as a reference for renal tissue sample gene expression evaluation by Hansson et al. [28].

Conclusions

TFRC, *RPLP0, and SDHA* were considered the most stable genes among the eight evaluated genes using the analysis tools, and they might be recommended for normalization of gene expression data in qPCR in studies of the impact of hypoxia on renal tumor cells.

Declarations

Funding: This study was supported by **Nuclear and Energy Research Institute** - IPEN (process number no. 2018.05.IPEN.08).

Conflicts of interest: The authors declare that they have no conflicts of interest.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Author contribution –

Luiz Felipe S. Teixeira - Performed the analysis and wrote de paper

Rodrigo Gigliotti – Performed the analysis, wrote the paper

Luana da Silva Ferreira - Performed the analysis and wrote de paper

Maria Helena Bellini - Conceived and designed the analysis, wrote the paper

Consent to Participate (Ethics)

Not applicable

Consent to Publish (Ethics)

All other Authors have read the manuscript and have agreed to submit it in its current form for consideration for publication in the Journal

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Tables

Table 1 - Primer sequences used to evaluate relative gene expression and primer's efficiency

Symbol	Gene name	Function	Accession No.	Forward Primer	Revrse primer	Amplicon size (nt)	Efficiency (E)	Correlation coefficient (R^2)
RRN18S	18S ribosomal RNA	Human 18 S ribosomal RNA sequence inferred from DNA sequence	X03205.1	CGGACCAGAG CGAAAGCAT	CCTCCGACTT TCGTTCTTGATT	61	1.99	1.00
ACTB	Actin β	Encodes protein involved in motility, structure, integrity, and intercellular signaling.	NG_007992.1	CGTGGACA TCCGCAAAGAC	GCATCCTG TCGGCAATGC	82	2.00	0.99
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Encodes a member of the glyceraldehyde-3-phosphate dehydrogenase protein family	NG_007073.2	CACATGGCCT CCAAGGAGTAA	TGAGGGTCTCTC TCTTCCTCTTGT	75	1.98	1.00
HPRT1	Hypoxanthine phosphorib osyltransferase 1	Conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate	NG_012329.2	GCTTTCCTTG GTCAGGCAGTA	GGTCCTTTTC ACCAGCAAGCT	66	2.00	1.00
PGK1	Phosphogly cerate kinase 1	Production of glycolytic enzyme that catalyzes the conversion of 1,3-diphosphogly cerate to 3-phosphoglycerate	NG_008862.1	GCTGCTGGGT CTGTCATCCT	GCATCTTTTCCC TTCCCTTCTT	70	2.02	1.00
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0	Encodes a ribosomal protein	NP_000993.1	TGCTCAACAT CTCCCCCTTCT	ATGCTGCCAT TGTCGAACAC	63	2.00	1.00
SDHA	Succinate dehydrogenase complex, subunit A	Encodes a major catalytic subunit of succinate-ubiquinone oxidoreductase	NG_012339.1	TCTCTGCGATAT GATACCAGCTATTT	GGCACTCCCC ATTCTCCAT	72	1.99	1.00
TFRC	Transferrin receptor protein 1	Encodes a cell surface receptor necessary for cellular iron uptake by the process of receptor-mediated endocytosis	NG_046395.1	GGAGGACGCG CTAGTGTTCT	TGCTGATCTAG CTTGATCCATCA	61	2.01	0.99

Table 2 T Expression stability ranking of the candidate reference genes according to BestKeeper, geNorm, NormFinder and the Delta C_T analysis

Gene	BestKeeper (Power of the gene)	geNorm (M-value)	NormFinder (S-value)	ΔCt	Final ranking
TFRC	0.38 (1)	0.65 (1)	0.20 (1)	1.15 (1)	1
RPLP0	0.70 (4)	0.65 (1)	0.51 (3)	1.24 (3)	2
SDHA	0.60 (2)	0.74 (2)	0.38 (2)	1.20 (2)	3
PGK1	0.74 (5)	0.89 (3)	0.74 (4)	1.65 (6)	4
HPRT1	0.63 (3)	0.97 (4)	0.81 (5)	1.46 (4)	5
GAPDH	1.18 (6)	1.09 (5)	1.06 (6)	1.48 (5)	6
ACTB	1.63 (8)	1.30 (6)	1.56 (7)	2.00 (7)	7
X18S	1.43 (7)	1.55 (7)	1.94 (8)	2.29 (8)	8

Figures

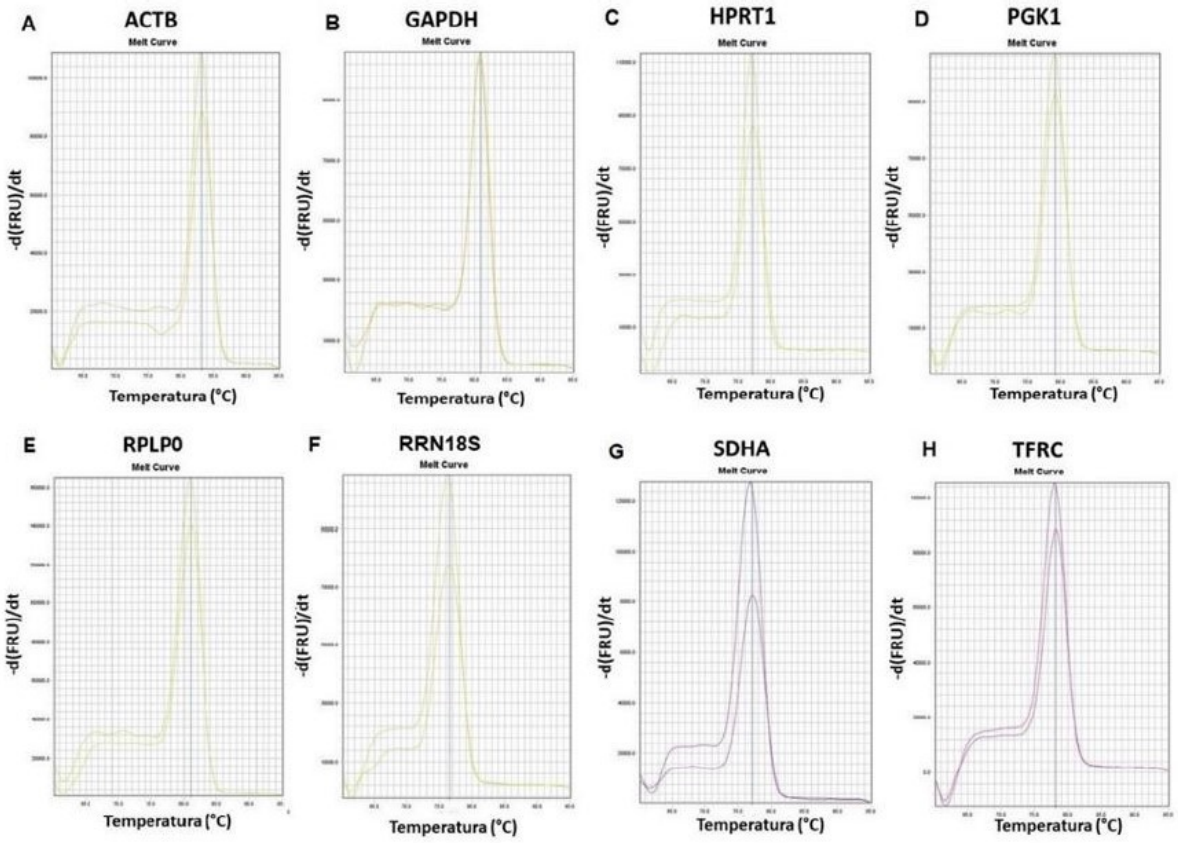

Figure 1

Melting curve analyses of the eight reference candidate genes

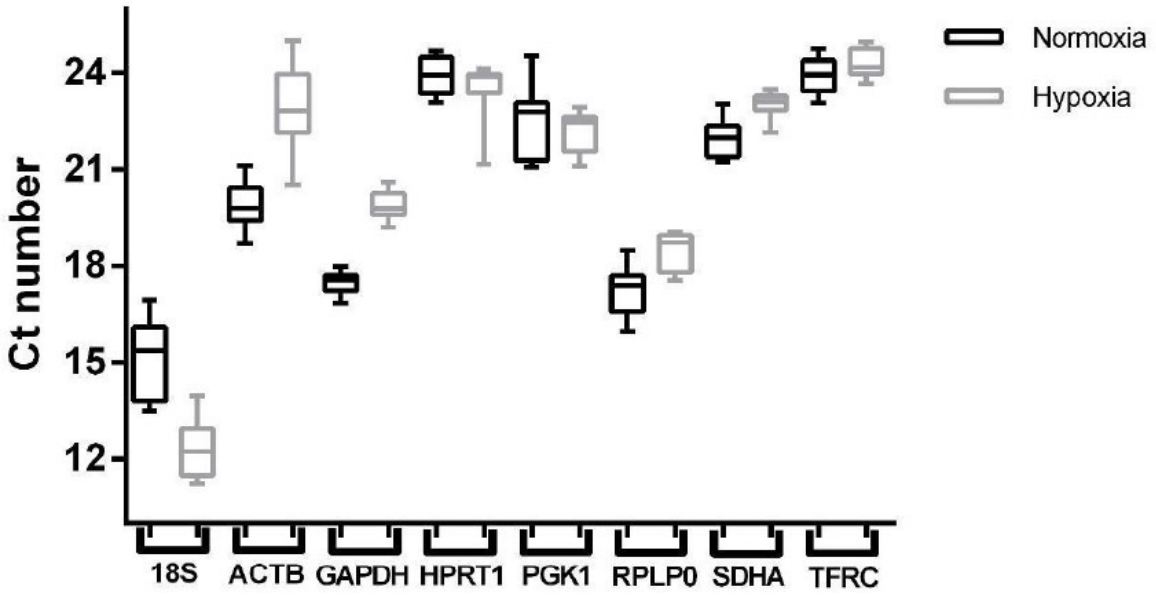

Figure 2

Comparison of expression level for the eight indicated housekeeping genes (HKGs) in 786-0 cells. Values are expressed as cycle threshold (Ct) cross-points as defined in Material and Methods

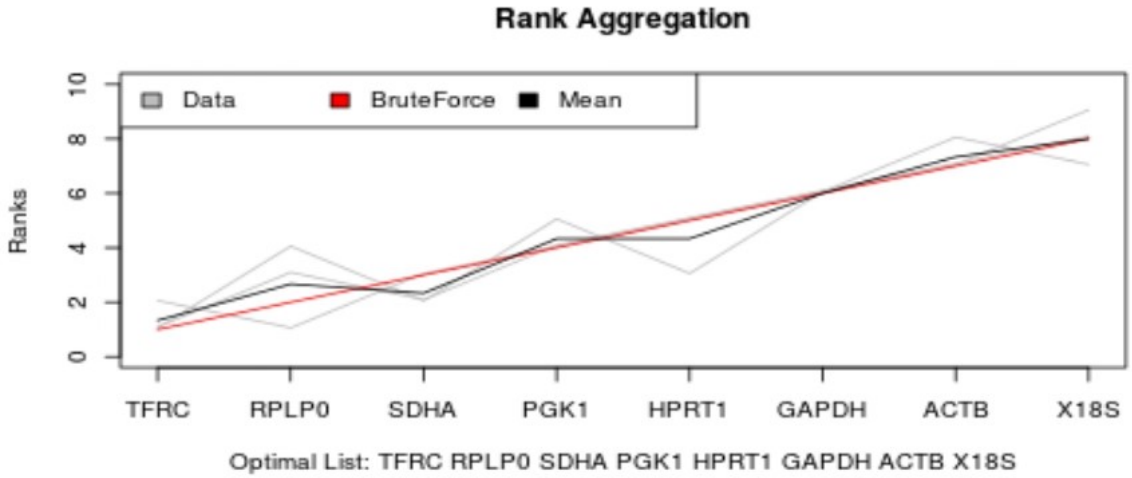

Figure 3

Rank aggregation of the eight candidate reference genes. The RankAggreg package was loaded into R software. The BestKeeper, NormFinder, and geNorm and ΔCt ranks are represented as grey lines. The black line represents the mean rank of each gene according to each method. The red line indicates the result of the Cross-Entropy algorithm