Molecular Biology Reports (2022) 49:3885–3891
https://doi.org/10.1007/s11033-022-07236-0

ORIGINAL ARTICLE

Identification of appropriate housekeeping genes for gene expression studies in human renal cell carcinoma under hypoxic conditions

Luiz Felipe S. Teixeira[1] · Rodrigo Gigliotti[2] · Luana da Silva Ferreira[1] · Maria Helena Bellini[1]

Received: 14 July 2021 / Accepted: 7 February 2022 / Published online: 11 March 2022

Abstract

Background Hypoxia pathways are deregulated in clear renal cell carcinoma (ccRCC) because of the loss of the von Hippel-Lindau tumor suppressor function. 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 RankAggreg. According to the four algorithms, the *TFRC* reference gene was identified as the most stable. There was no agreement among the results from the algorithms for the 2nd and 3rd positions. A general classification was then established using the RankAggreg tool. Finally, the three most suitable reference genes for use in 786-0 cells under normoxic and hypoxic conditions were *TFRC*, *RPLP0*, and *SDHA*.

Conclusions To the best of our knowledge, this is the first study to identify reliable genes that can be used for gene expression analysis in ccRCC in a hypoxic environment.

Keywords Renal cell carcinoma · Hypoxia · Endogenous genes · Normalization

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 most common histologic subtype of RCC, ccRCC, accounts for approximately 75% of kidney cancer diagnoses [4]. A majority of patients with ccRCC 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 protein encoded by the *VHL* gene, termed as pVHL, interacts with other proteins, such as hypoxia-inducible factor (HIF), to form an E3-ubiquitin complex, which targets proteins for 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 patients with RCC, pVHL is nonfunctional and is consequently unable to target the

✉ Maria Helena Bellini
mbmarumo@ipen.br

[1] Centro de Biotecnologia, Instituto de Pesquisas Energéticas e Nucleares, São Paulo, Brazil

[2] Centro de Pesquisa de Genética e Reprodução Animal, Instituto de Zootecnia (IZ), Nova Odessa, São Paulo, Brazil

HIF1α protein for degradation. Thus, free HIF1α promotes the transcription of various target genes. 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 essential for identifying possible therapeutic targets [9]. Real-time quantitative polymerase chain reaction (qRT-PCR) is 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 analysis, the target gene expression is determined by normalizing it with the expression of 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 is not 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, with the aim of identifying suitable reference genes for normalization in RCC gene expression studies.

Materials and methods

Cell culture and induction of hypoxic conditions

The RCC cell line 786-0 was obtained from the 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 (Gibco® Invitrogen), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco® Invitrogen) in a humidified incubator with 18.6% O_2, 5% CO_2, and 76.4% N_2 at 37 °C.

A day before the 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 placed in an incubator at 37 °C. The Altair PRO Single-Gas Detector (Code: 217,597, MSA, Cranberry Township, Pennsylvania, USA) was used to measure the O_2 concentration inside the chamber.

Protein extraction and western blot analysis

Proteins were extracted from the cells grown under normoxic and hypoxic conditions using CelLytic™ M reagent (Sigma-Aldrich, St Louis, MO, USA). After centrifugation at 20.000xg for 15 min, the supernatant was collected and added to protease inhibitor cocktail powder (Sigma-Aldrich, St Louis, MO, USA). The protein concentration was detected by using the bicinchoninic acid assay (BCA) method and stored at -80 °C until use. The protein samples were loaded at a concentration of 50 µg per lane, separated using 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis, and transferred onto a GE Hybond-P polyvinylidene difluoride membrane. The membrane was then blocked in 5% skim milk in Tris–buffered saline at room temperature for 1 h. The membranes were incubated overnight at 4 °C with the primary antibody anti-HIF-2α (rabbit polyclonal anti-mouse; Abcam, Cambridge, UK), diluted 1:300, followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Dallas,TX, USA), diluted 1:1000, for 2 h at room temperature. Signals were detected using the SuperSignal® West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Waltham, MA, USA). Photographs were taken using the Uvitec Cambridge Alliance 4.7 equipment. Protein bands were quantified using ImageJ software.

RNA extraction

RNA was extracted from the cells grown under normoxic and hypoxic conditions. The cells were washed with PBS, and RNA was extracted using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The extracted RNA was diluted with RNase-free water. Subsequently, RNA concentration (ng/µL) and purity (A260/280) were determined using a Nanodrop® ND-100 (Thermo Scientific). The RNA was considered pure if the A260/280 ratio was within the range of 1.8–2.1. The integrity of the samples was confirmed using agarose gel electrophoresis. The RNA samples were stored at -80 °C.

Complementary DNA (cDNA) synthesis

The QuantiTec reverse transcription kit (Qiagen) was used to synthesize cDNA. The cDNA was synthesized using 2 µg of total RNA, followed by elimination of genomic DNA using the buffer from the kit. The resulting mixture was then incubated in a thermocycler at 42 °C for 2 min and then immediately transferred on ice. A second mix was prepared, complementing the previous mix, containing the RT primer, and amplified at 42 °C for 15 min. The reaction was stopped 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 analysis.

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

The amplification qRT-PCR was performed using SYBR Green® (Applied Biosystems, NY, USA). StepOnePlus® (Applied Biosystems), 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 (40 cycles); followed by a final cycle of 15 s at 95 °C.

The human genes used were as follows: *RRN18S* (lateral stem subunit P0 of ribosomal protein), *ACTB* (beta-actin), *GAPDH* (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 primers were designed using the Applied Biosystems website (Table 1). Primer Express 3.0 (Life Technologies, MD, USA) was used to confirm human sequences using 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 (ΔCT) 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 first identifies 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, where p values closer to 1.0 indicate greater stability [14]. Comparative ΔCt method 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 are considered 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 ΔCT).

Results

Hypoxic response

The effectiveness of the hypoxic microenvironment was confirmed using western blot analysis. The 786-0 cells cultured under hypoxia had significantly increased HIF-2 α protein levels compared to those observed in 786-0 cells grown under normoxia (normoxia vs. hypoxia, $P < 0.05$) (Fig. 1 A and A1).

RNA quality

The RNA extracted from all samples showed high yield, quality, and integrity. The mean RNA concentration in the cells from the normoxia and hypoxia group was 3603.38 ± 176.50 ng/µL and 3111.76 ± 54.90 ng/µL, respectively. The mean A260/280 ratio in the cells from the normoxia and hypoxia group was 2.05 ± 0.02 and 2.08 ± 0.01, respectively. Integrity was assessed using agarose gel, and two sharp bands (28 S and 18 S rRNA) were observed.

Primer specificity and efficiency

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

Expression stability of reference genes under 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* showed the lowest variation (Fig. 3).

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 (ΔCT).

The stability of the HKGs was determined using BestKeeper based on the extent of standard deviation (SD ± CP), with a higher SD value corresponding to the low stability

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

Symbol	Gene name	Function	Accession No.	Forward Primer	Reverse primer	Amplicon size (nt)	Efficiency (E)	Correlation coefficient (R^2)
RRN18S	18 S ribosomal RNA	Human 18 S ribosomal RNA sequence inferred from DNA sequence	X03205.1	CGGACCAGAGCGAAAGCAT	CCTCCGACTTTCGTTCTTGATT	61	1.99	1.00
ACTB	Actin β	Encodes protein involved in motility, structure, integrity, and intercellular signaling.	NG_007992.1	CGTGGACATCCGCAAAGAC	GCATCCTGTCGGCAATGC	82	2.00	0.99
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Encodes a member of the glyceraldehyde-3-phosphate dehydrogenase protein family	NG_007073.2	CACATGGCCTCCAAGGAGTAA	TGAGGGTCTCTCTCTTCCTCTTGT	75	1.98	1.00
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate	NG_012329.2	GCTTTCCTTGGTCAGGCAGTA	GGTCCTTTTCACCAGCAAGCT	66	2.00	1.00
PGK1	Phosphoglycerate kinase 1	Production of glycolytic enzyme that catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate	NG_008862.1	GCTGCTGGGTCTGTCATCCT	GCATCTTTTCCCTTCCCTTCTT	70	2.02	1.00
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0	Encodes a ribosomal protein	NP_000993.1	TGCTCAACATCTCCCCCTTCT	ATGCTGCCATTGTCGAACAC	63	2.00	1.00
SDHA	Succinate dehydrogenase complex, subunit A	Encodes a major catalytic subunit of succinate-ubiquinone oxidoreductase	NG_012339.1	TCTCTGCGATATGATACCAGCTATTT	GGCACTCCCCATTCTCCAT	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	GGAGGACGCGCTAGTGTTCT	TGCTGATCTAGCTTGATCCATCA	61	2.01	0.99

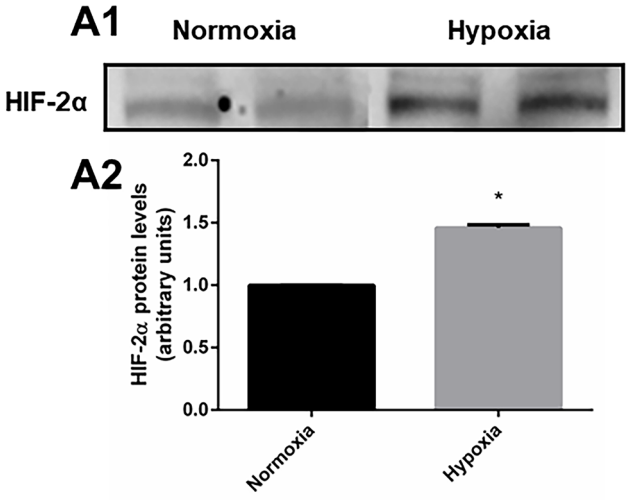

Fig. 1 Effect of hypoxia on the expression of HIF-1 α in 786-0 cells. (A) The protein levels of HIF-1 α was detected using western blot. (A1) Quantitative analysis of the protein levels using ImageJ 1.6.0_24 software. The values shown represent the mean ± SD. **P < 0.05 vs. normoxia, Student's t-test

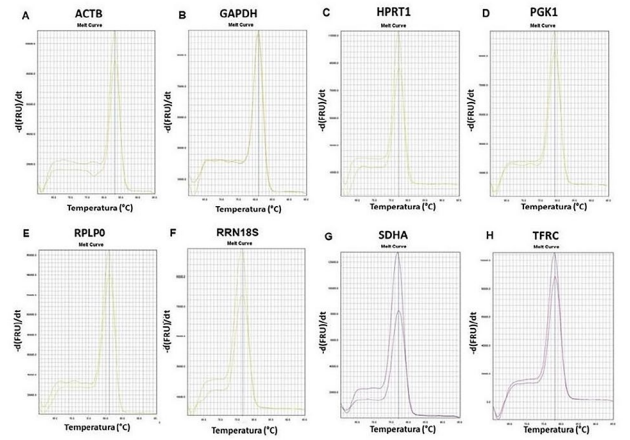

Fig. 2 Melting curve analyses of the eight reference candidate genes

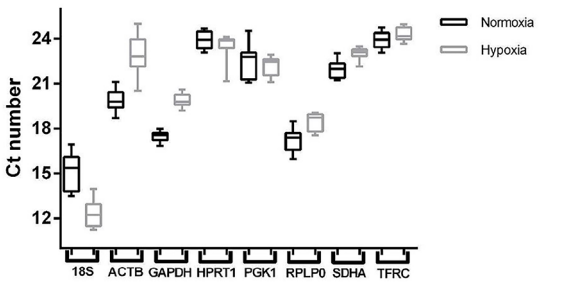

Fig. 3 Comparison of expression level for the eight indicated housekeeping genes (HKGs) in 786-0 cells. Values are expressed as cycle threshold (Ct) cross-points as defined in Material and Methods

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 genes according to their M values using the Ct values of all of the samples. Samples with the lowest M values were considered to be the most stable, and *vice versa*. The M value of HKGs ranged from 0.65 to 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 for intra- and inter-group variations to estimate 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 correlated with higher stability of the HKGs. The *TFRC* with an SD value of 1.15 was perceived to be the most stable HKG, followed by *SDHA* (1.20) and *RPLP0* (1.20) (Table 2).

The rank-ordered genes calculated using the four algorithms presented in Table 2 were further analyzed by using RankAggreg [16] to obtain a consensus rank list of genes. The stability of the candidate reference genes was in the following order: *TRFC* > *RPLPO* > *SDHA* > *PGK1* > *HPRT1* > *GAPDH* > *ACTB* > X*18S* (Fig. 4).

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 importance of selecting 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 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 elucidating 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

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

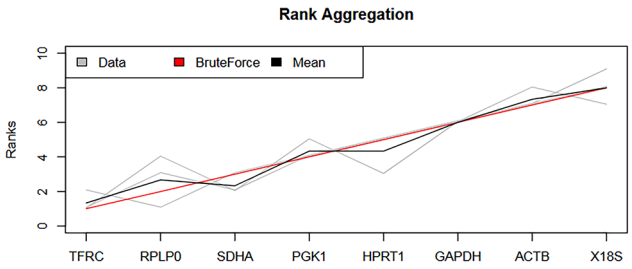

Fig. 4 Rank aggregation of the eight candidate reference genes. The RankAggreg package was loaded into R software. The BestKeeper, NormFinder, geNorm, and ΔCt ranks are represented as grey lines. The black line represents the mean rank of each gene according to each method. The red line indicates the result of the Cross-Entropy algorithm

according to their expression stability and consistency with four different specific tools: geNorm, NormFinder, BestKeeper, and the ΔCt method. In addition, Rankagreg was used to generate a consensus ranking.

A partial accordance among the four methodologies was observed for the three best normalizer genes (*TFRC*, *SDHA*, and *RPLP0*) selected (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 the greatest discrepancy among the four algorithms, ranking 1st by geNorm and 4th by BestKeeper (Table 2; Fig. 3). This disagreement is possibly due to the different principles used by 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 other four algorithms [11, 12]. High stability of *TFRC* has been demonstrated in breast [23] and pancreatic cancers [24]. Furthermore, *TFRC* was found to be the most suitable reference gene for human umbilical vein endothelial cells (HUVECs) subjected to hypoxic conditions [25]. *RPLP0* was found 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 ranked 3rd in the RankAggreg analysis. 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.

Author contributions Luiz Felipe S. Teixeira: Analysis and Writing. Rodrigo Gigliotti: Analysis and Writing. Luana da Silva Ferreira: Analysis and Writing. Maria Helena Bellini: Conceptualization, Design, Analysis, and Writing.

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

Declarations

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

Consent to participate Not applicable.

Consent to publish All authors have read the manuscript and have agreed to submit it in its current form for consideration for publication in the Journal.

References

1. Escudier B, Porta C, Schmidinger M, Rioux-Leclercq N, Bex A, Khoo V, Grünwald V, Gillessen S, Horwich A (2019) Renal cell carcinoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 30:706–720. https://doi.org/10.1093/annonc/mdz056
2. Rocha FG, Chaves KC, Chammas R, Peron JP, Rizzo LV, Schor N, Bellini MH (2010) Endostatin gene therapy enhances the efficacy of IL-2 in suppressing metastatic renal cell carcinoma in mice. Cancer Immunol Immunother 59:1357–1365. https://doi.org/10.1007/s00262-010-0865-6
3. D'Avella C, Abbosh P, Pal SK, Geynisman DM (2020) Mutations in renal cell carcinoma. Urol Oncol 38:763–773. https://doi.org/10.1016/j.urolonc.2018.10.027
4. Jonasch E, Walker CL, Rathmell WK (2021) Clear cell renal cell carcinoma ontogeny and mechanisms of lethality. Nat Rev Nephrol 17:245–261. https://doi.org/10.1038/s41581-020-00359-2

5. Büscheck F, Fraune C, Simon R, Kluth M, Hube-Magg C, Möller-Koop C, Sarper I, Ketterer K, Henke T, Eichelberg C, Dahlem R, Wilczak W, Sauter G, Fisch M, Eichenauer T, Rink M (2020) Prevalence and clinical significance of VHL mutations and 3p25 deletions in renal tumor subtypes. Oncotarget 11:237–249. https://doi.org/10.18632/oncotarget.27428
6. Schödel J, Grampp S, Maher ER, Moch H, Ratcliffe PJ, Russo P, Mole DR (2016) Hypoxia, hypoxia-inducible transcription factors, and renal cancer. Eur Urol 69:646–657. https://doi.org/10.1016/j.eururo.2015.08.007
7. Ivan M, Kaelin WG (2017) The EGLN-HIF O2-sensing system: Multiple inputs and feedbacks. Mol Cell 66:772–779. https://doi.org/10.1016/j.molcel.2017.06.002
8. Zhang J, Zhang Q (2018) VHL and hypoxia signaling: Beyond HIF in cancer. Biomedicines 6. https://doi.org/10.3390/biomedicines6010035
9. Lemma S, Avnet S, Meade MJ, Chano T, Baldini N (2018) Validation of suitable housekeeping genes for the normalization of mRNA expression for studying tumor acidosis. Int J Mol Sci 19. https://doi.org/10.3390/ijms19102930
10. Lorenzetti WR, Ibelli AMG, Peixoto JO, Mores MAZ, Savoldi IR, Carmo KBD, Oliveira HC, Ledur MC (2018) Identification of endogenous normalizinggenes for expression studies in inguinal ringtissue for scrotal hernias in pigs. PLoS ONE 13:e0204348. https://doi.org/10.1371/journal.pone.0204348
11. Marciano CMM, Ibelli AMG, Peixoto JO, Savoldi IR, do Carmo KB, Fernandes LT, Ledur MC (2020) Stable reference genes for expression studies in breast muscle of normal and white striping-affected chickens. Mol Biol Rep 47:45–53. https://doi.org/10.1007/s11033-019-05103-z
12. Andersen CL, Jensen JL, Ørntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496
13. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034. https://doi.org/10.1186/gb-2002-3-7-research0034
14. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515. https://doi.org/10.1023/B:BILE.0000019559.84305.47
15. Silver N, Best S, Jiang J, Thein SL (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol 7:33. https://doi.org/10.1186/1471-2199-7-33
16. Pihur V, Datta S, Datta S (2009) RankAggreg, an R package for weighted rank aggregation. BMC Bioinformatics 10:62. https://doi.org/10.1186/1471-2105-10-62
17. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622. https://doi.org/10.1373/clinchem.2008.112797
18. Zhang X, Ding L, Sandford AJ (2005) Selection of reference genes for gene expression studies in human neutrophils by real-time PCR. BMC Mol Biol 6:4. https://doi.org/10.1186/1471-2199-6-4
19. Brym P, Ruść A, Kamiński S (2013) Evaluation of reference genes for qRT-PCR gene expression studies in whole blood samples from healthy and leukemia-virus infected cattle. Vet Immunol Immunopathol 153:302–307. https://doi.org/10.1016/j.vetimm.2013.03.004
20. Arrowsmith S (2021) Identification and validation of suitable reference genes for quantitative real-time PCR gene expression analysis in pregnant human myometrium. Mol Biol Rep 48:413–423. https://doi.org/10.1007/s11033-020-06066-2
21. Wang Y, Zhang Y, Liu Q, Tong H, Zhang T, Gu C, Liu L, Huang S, Yuan H (2021) Selection and validation of appropriate reference genes for RT-qPCR analysis of flowering stages and different genotypes of Iris germanica L. Sci Rep 11:9901. https://doi.org/10.1038/s41598-021-89100-y
22. Brodaczewska KK, Szczylik C, Fiedorowicz M, Porta C, Czarnecka AM (2016) Choosing the right cell line for renal cell cancer research. Mol Cancer 15:83. https://doi.org/10.1186/s12943-016-0565-8
23. Che J, Yao X, Wang C, Zheng J, Guo C (2020) Hypoxia promoted renal cell carcinoma cell migration through regulating lncRNA-ENST00000574654.1. Am J Transl Res 12:3884–3894
24. Majidzadeh -AK, Esmaeili R, Abdoli N (2011) TFRC and ACTB as the best reference genes to quantify urokinase plasminogen activator in breast cancer. BMC Res Notes 4:215. https://doi.org/10.1186/1756-0500-4-215
25. Iyer G, Wang AR, Brennan SR, Bourgeois S, Armstrong E, Shah P, Harari PM (2017) Identification of stable housekeeping genes in response to ionizing radiation in cancer research. Sci Rep 7:43763. https://doi.org/10.1038/srep43763
26. Bakhashab S, Lary S, Ahmed F, Schulten HJ, Bashir A, Ahmed FW, Al-Malki AL, Jamal HS, Gari MA, Weaver JU (2014) Reference genes for expression studies in hypoxia and hyperglycemia models in human umbilical vein endothelial cells. G3 (Bethesda) 4:2159–2165. https://doi.org/10.1534/g3.114.013102
27. Glenn ST, Jones CA, Liang P, Kaushik D, Gross KW, Kim HL (2007) Expression profiling of archival renal tumors by quantitative PCR to validate prognostic markers. Biotechniques 43:639–640. https://doi.org/10.2144/000112562
28. Hansson J, Lindgren D, Nilsson H, Johansson E, Johansson M, Gustavsson L, Axelson H (2017) Overexpression of functional SLC6A3 in clear cell renal cell carcinoma. Clin Cancer Res 23:2105–2115. https://doi.org/10.1158/1078-0432.CCR-16-0496