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
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% O₂, 5% CO₂, and 76.4% N₂ at 37 °C.

A day before the hypoxia assay was performed, approximately 2 × 10⁵ 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% O₂, 5% CO₂, and 94% N₂, 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 O₂ 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 Hyper-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.
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-2α protein levels compared to those observed in 786-0 cells grown under normoxia (normoxia vs. hypoxia, \(P < 0.05\)) (Fig. 1A 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-Ct (\(\Delta\text{Ct}\)).
| 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 | CCTCCGACTTTCGTTCCTTGATT | 61 | 1.99 | 1.00 |
| ACTB   | Actin β   | Encodes protein involved in motility, structure, integrity, and intercellular signaling. | NG_007992.1 | CGTGGAACATCCGAAAGAC | GCATCTGTCGGCAAATGC | 82 | 2.00 | 0.99 |
| GAPDH  | Glyceraldehyde 3-phosphate dehydrogenase | Encodes a member of the glyceraldehyde-3-phosphate dehydrogenase protein family | NG_007073.2 | CACATGGCCTCCAAGGAGTA | TGAGGTTCTCTCTCTCTCTGT | 75 | 1.98 | 1.00 |
| HPRT1  | Hypoxanthine phosphoribosyltransferase 1 | Conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate | NG_012329.2 | GCTTTCTTTGTCAGGCAAGTA | GGTCCTTTTACCCAGCAAGCT | 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 | GCATCTTTTCCTCCCTTCTT | 70 | 2.02 | 1.00 |
| RPLP0  | Ribosomal Protein Lateral Stalk Subunit P0 | Encodes a ribosomal protein | NP_000993.1 | TGCTCAACATCTCCCCCTTCT | ATGCTGCAATTGCGAACAC | 63 | 2.00 | 1.00 |
| SDHA   | Succinate dehydrogenase complex, subunit A | Encodes a major catalytic subunit of succinate-ubiquinone oxidoreductase | NG_012339.1 | TCTCTGGATATGATCCAGTATTT | GGCACTCCCAATTTCCAT | 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 | GGAGGACGCGCTAGTGGTCT | TGCTGATCTAGTGGTACCATC | 61 | 2.01 | 0.99 |
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 intergroup 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 RRP0 (S = 0.51) (Table 2).

Finally, the stability of the HKGs was determined using the comparative ΔCt 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 > X18S (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.
The authors declare that they have no competing interests.

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

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

| Gene   | BestKeeper (Power of the M-value gene) | geNorm | NormFinderΔCt (S-value) | 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, RankAggreg 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.

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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.

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