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Published in:
Journal of Ovarian Research

DOI:
10.1186/1757-2215-6-60

2013

Citation for published version (APA):
Kolkova, Z., Arakelyan, A., Casslén, B., Hansson, S., & Kriegova, E. (2013). Normalizing to GADPH jeopardises correct quantification of gene expression in ovarian tumours-IPO8 and RPL4 are reliable reference genes. Journal of Ovarian Research, 6(60). https://doi.org/10.1186/1757-2215-6-60

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5

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Normalizing to GADPH jeopardises correct quantification of gene expression in ovarian tumours – IPO8 and RPL4 are reliable reference genes

Zuzana Kolkova1*, Arsen Arakelyan2, Bertil Casslén1, Stefan Hansson1 and Eva Kriegova3

Abstract

Background: To ensure a correct interpretation of results obtained with quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR), it is critical to normalize to a reference gene with stable mRNA expression in the tissue of interest. GADPH is widely used as a reference gene in ovarian tumour studies, although lacking tissue-specific stability. The aim of this study was to identify alternative suitable reference genes for RT-qPCR studies on benign, borderline, and malignant ovarian tumours.

Methods: We assayed mRNA levels for 13 potential reference genes – ABL1, ACTB, CDKN1A, GADPH, GUSB, HPRT1, HSP90AB, IPO8, RPL30, RPL4, RPLPO, and TBP – with RT-qPCR in 42 primary ovarian tumours, using commercially pre-designed RT-qPCR probes. Expression stability was subsequently analysed with four different statistical programs (GeNorm, NormFinder, BestKeeper, and the Equivalence test).

Results: Expression of IPO8, RPL4, TBP, RPLPO, and ACTB had the least variation in expression across the tumour samples according to GeNorm, NormFinder, and BestKeeper. The Equivalence test found variation in expression within a 3-fold expression change between tumour groups for: IPO8, RPL40, RPL30, GUSB, TBP, RPLPO, ACTB, ABL1, and CDKN1A. However, only IPO8 satisfied at a 2-fold change as a cut-off. Overall, IPO8 and RPL4 had the highest, whereas GADPH and HPRT1 the lowest expression stability. Employment of suitable reference genes (IPO8, RPL4) in comparison with unsuitable ones (GADPH, HPRT1), demonstrated divergent influence on the mRNA expression pattern of our target genes – GPER and uPAR.

Conclusions: We found IPO8 and RPL4 to be suitable reference genes for normalization of target gene expression in benign, borderline, and malignant ovarian tumours. Moreover, IPO8 can be recommended as a single reference gene. Neither GADPH nor HPRT1 should be used as reference genes in studies on ovarian tumour tissue.

Background

Most cases of ovarian cancer are diagnosed at an advanced stage, with poor prognosis for the patients. Early stages of ovarian cancer are, on the other hand, more accessible to treatment and have much better prognosis. There is an ongoing search for biomarkers with capacity to detect in particular early stages of the disease in screening programs, since this would be the single most important step towards improving the prognosis. A selective biomarker might, furthermore, be helpful in the preoperative assessment of ovarian lesions in order to employ optimal surgery.

Analysis of gene expression by quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR), a sensitive technique with broad dynamic range, is a frequent approach for the biomarker discovery in tumour tissue. However, in order to obtain reliable results by RT-qPCR in heterogeneous clinical samples, the expression of a target gene needs to be normalized to a stably expressed reference gene (RG) to minimize the influence of variations in, e.g. extraction yield, reverse-transcription yield, and amplification efficiency [1].
Stability of such reference genes has to be validated in normal ovarian tissue, and benign and malignant ovarian tumours. The obtained results, however, differ; Li et al. recommended combination of GUSB, PPIA, and TBP [4], whereas Fu et al. concluded that combination of RPL4, RPLPO, and HSP90AB1 (HSPCB) are more suitable [6]. Both studies were performed on Chinese populations, did not include borderline tumours, and used SYBR Green RT-qPCR technique.

The present study was performed on a Scandinavian population, included borderline tumours, used predesigned commercial RT-qPCR probes, and applied four different statistical software programs. In addition to the above mentioned traditionally used and earlier recommended RGs for ovarian tissue, we also selected four genes from a commercially printed array (ABL1, CDKN1A, IPO8, and RPL30). Thus, altogether 13 genes we included in the study. Finally, two target genes were chosen to demonstrate the divergent results, which may be obtained by normalizing their mRNAs to suitable vs. unsuitable RGs: G protein-coupled estrogen receptor (GPER), which has no differences in expression between benign and malignant ovarian tumours and urokinase plasminogen activator receptor (uPAR), which is up-regulated in malignant tumours.

Methods

Ovarian tumour tissue

Tissue samples (n = 42) were obtained from primary ovarian tumours during surgery at the Department of Obstetrics and Gynaecology, Lund University Hospital, during 2001–2007. None of the patients had received chemotherapy prior to the operation. The samples were cut in 5 × 5 × 5 mm cubes, quick frozen on dry ice, and stored at −80°C until used. In addition to the routine histo-pathological examination, each specimen was re-evaluated by a second pathologist. Histological differentiation was classified as benign (n = 9), borderline (n = 11), and malignant (n = 22); the histological types were serous (n = 21), mucinous (n = 13), and endometrioid (n = 8) (Table 1). The mean age of included patients was 59 years (range 22–80) in the benign group, 55 years (35–86) in the borderline group, and 62 years (43–85) in the malignant group. The Ethical Review Board at Lund University Hospital approved the study design and informed consent was obtained from each patient.

Extraction of total RNA

Total RNA was extracted from about 125 mg frozen ovarian tumour tissue. The tissue was homogenized in Trizol 50 mg/mL (Invitrogen, Carlsbad, CA) using rotating-knives (Polytron). All RNA samples were checked for concentration and purity by NanoDrop Spectrophotometer ND-1000 (Saveen Werner, Linhamn, Sweden) having A260/280 and A260/230 ~ 2. RNA quality and integrity was verified by Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA), i.e. all samples had RNA Integrity Number > 7.7.

cDNA synthesis

GeneAmp® RNA PCR kit (Applied Biosystems, Foster City, CA) was used for reverse transcription of total RNA (0.2 μg) to cDNA. The final concentration of cDNA was 1 μg/μL (+/− 7%) and A260/280 ratio ~1.8 as assessed by NanoDrop. The cDNA samples were stored at −20°C until further use.

Quantitative RT-qPCR amplification

RT-qPCR was performed using a StepOnePlus™ cycler (Applied Biosystems) under standard thermal cycling conditions (activation of contamination preventing enzyme at 50°C for 2 min, enzyme activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 1 min). PCR reactions were run in duplicates and negative controls were included in each amplification set. For each gene analysed, premanufactured real-time qPCR assays were used (Ap-
plied Biosystems or Integrated DNA technologies, Inc., Coralville, IA, USA) (Table 2), with probes spanning exon junctions and not detecting genomic DNA. Using one malignant tumour sample and a universal human reference RNA (Stratagene, La Jolla, CA, USA), quantification experiments were performed using two standard curves from 10-fold serial dilutions of the cDNA (80–0.08 ng).

**Identification of new potential reference genes**

In order to identify new candidate reference genes in ovarian tumour tissue, we employed a commercial array (TaqMan® Express Endogenous Control Plate, cat no 4396840, Applied Biosystems) consisting of 32 potential RGs (18S, GADPH, HPRT1, GUSB, ACTB, B2M, HMBS, IPO8, PGK1, RPLPO, TBP, TFRC, UBC, YWHAZ, PP1A, POLR1A, CASC3, CDKN1A, CDKN1B, GADD45A, PUM1, PSMC4, EIF2B1, PESI, ABL1, ELF1, MT-AT6, MRPL19, POP4, RPL37A, RPL30, RPS17).

We analysed one benign and one malignant sample of ovarian tumour, which were selected based on the greatest difference in expression of traditionally used RGs (ACTB, GADPH, and HPRT1), as measured by RT-qPCR. The difference between the threshold cycles (ΔCt) of the two samples was then calculated for each of the 32 genes in the array. Four genes with the lowest ΔCt were selected for inclusion in our main study.

**Statistical analysis**

Descriptive statistics, F-test for Ct variance equality and Kolmogorov-Smirnov test for normality of log-transformed relative expression values were calculated by software SPSS 19.0 (SPSS Inc, Chicago, IL). The Equivalence test [7-9] and statistical applets BestKeeper [10], geNorm [11], and NormFinder [12] were used for analysis of genes expression stability. GeNorm calculates a gene-stability measure, M-value, as the average pair-wise variation of a particular gene to all other candidate reference genes [11]. On the other hand, the stability value calculated with NormFinder combines estimated both intra-group and inter-group variations [12]. Genes with the lowest M-values have the most stable expression (least variability). Relative expression values for target genes were analysed by Kruskal-Wallis and Mann-Whitney tests, and the log-transformed values by one-way ANOVA. P < 0.05 was considered significant.

**Results**

**Selection of best RGs from the commercial gene array**

In order to select optimal candidate RGs for this study on ovarian tumours, ΔCt between one benign and one malignant sample of ovarian tumour tissue, we employed a commercial array (TaqMan® Express Endogenous Control Plate, cat no 4396840, Applied Biosystems) consisting of 32 potential RGs (18S, GADPH, HPRT1, GUSB, ACTB, B2M, HMBS, IPO8, PGK1, RPLPO, TBP, TFRC, UBC, YWHAZ, PP1A, POLR1A, CASC3, CDKN1A, CDKN1B, GADD45A, PUM1, PSMC4, EIF2B1, PESI, ABL1, ELF1, MT-AT6, MRPL19, POP4, RPL37A, RPL30, RPS17).

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### Table 2 Reference genes, target genes and assays used

| Gene symbol | Gene name (synonyms) | Function | NCBI Gene reference | Assay ID |
|-------------|---------------------|----------|---------------------|---------|
| ABL1        | C-abl oncogene 1, non-receptor tyrosine kinase | Cell differentiation, division, adhesion and stress response. | NM_005157.3, NM_007313.2 | Hs00245445_m1 |
| ACTB        | Actin, beta | Cell motility, structure, integrity | NM_001101.3 | Hs99999903_m1 |
| CDKN1A      | Cyclin-dependent kinase inhibitor 1A (p21, Cip1) | Regulation of cell cycle progression at G1. | NM_004064.3 | Hs00355782_m1 |
| GADPH       | Glyceraldehyde-3-phosphate dehydrogenase | Catalysis of an important energy-yielding step in carbohydrate metabolism. | NM_002046.3 | Hs99999905_m1 |
| GUSB        | Glucuronidase, beta | Degradation of glycosaminoglycans | NM_000181.2 | Hs99999908_m1 |
| HPRT1       | Hypoxanthine phosphoribosyltransferase 1 | Generation of purine nucleotides through the purine salvage pathway. | NM_000194.3 | Hs99999909_m1 |
| HSP90AB1    | Heat shock protein 90 | Protein folding, response to stress. | NM_007355 | Hs.PT.49a.20846338 |
| IPO8        | Importin 8 | Nuclear transport. | NM_001190995.1, NM_0063903 | Hs00183533_m1 |
| PP2A        | Peptidylprolyl isomerase A (cyclophilin A) | Protein folding, ligand for Cyclosporin A. | NM_021130.3 | Hs0012218451 |
| RPL30       | Ribosomal protein L30 | Component of 60S subunit. Catalysis of protein synthesis. | NM_000989.2 | Hs00265497_m1 |
| RPL4        | Ribosomal protein L4 | Component of 60S subunit. | NM_000968 | Hs.PT.49a.20266660 |
| RPLPO       | Ribosomal protein, large, PO | Component of 60S subunit. | NM_053275.3, NM_0010023 | Hs99999902_m1 |
| TBP         | TATA box binding protein | Initiation of transcription of RNA polymerases. | M34960.1 M53654.1 | Hs99999910_m1 |
| GPER        | G protein-coupled estrogen receptor | Rapid estrogen signalling. | NM_001505.2 | Hs00173506_m1 |
| uPAR        | Urokinase plasminogen activator receptor | Cell invasion, migration, signalling via ERK1/2. | NM_001005376.2, NM_001005377.2, NM_002659.3 | Hs00182181_m1 |
malignant ovarian tumour sample with the greatest difference in expression of the traditionally used RGs (ACTB, GADPH, and HPRT1), was measured by RT-qPCR and calculated for all 32 genes included in the arrays. The lowest \( \Delta C_t \), i.e. the least variation, was found for CDKN1A (\( \Delta C_t = 0.47 \)), ABL1 (0.76), RPL30 (0.83), RPS17 (1.09), MT-ATP6 (1.42), and IPO8 (1.71), whereas POP4 (6.11), GADPH (5.04), HPRT1 (4.91), POLR2A (4.41), CASC3 (3.48) had the highest \( \Delta C_t \). The most abundant genes were 18S (mean \( C_t \pm SD: 22.12 \pm 0.82 \)) and MT-ATP6 (21.64 \( \pm \) 1.00), the genes with lowest expression were YWHAZ (31.42 \( \pm \) 2.14) and TBP (31.37 \( \pm \) 2.06). CDKN1A, ABL1, RPL30 and IPO8 were chosen to be included in our panel of potential reference genes.

Expression of selected candidate reference and target genes in primary ovarian tumours

We analysed altogether 13 candidate reference genes (ABL1, ACTB, CDKN1A, GADPH, GUSB, HPRT1, HSP90AB1, IPO8, PPIA, RPL30, RPL4, RPLPO, and TBP) and two target genes (GPER and uPAR) by RT-qPCR. Expression levels and variability of \( C_t \) values are shown in Table 3. The genes were ranked according to expression stability as follows: the most stable-TBP > RPLPO > ACTB > RPL4 > PPIA > HSP90 > GADPH > HPRT1 > CDKN1A > RPL30 > GUSB > ABL1. Of all genes, PPIA had the highest expression stability (\( M \)-value) was calculated based on the average pair-wise variation between aM \( C_t \) values, which had 82% efficiency. The linear regression analysis of expression stability by BestKeeper and the Equivalence test for variations in expression in the whole data set and between tumours groups as described above. IPO8 had the lowest standard deviation (SD) of the \( C_t \) value across the groups (mean \( C_t \pm SD: 29.10 \pm 0.65 \)). The best-ranked genes by GeNorm and NormFinder — IPO8, ACTB, TBP, RPL4, and RPLPO — fulfilled the BestKeeper criteria for stability variation of the \( C_t \) value with SD < 1 (Table 3).

Table 3 Descriptive and correlation analysis of the candidate RGs obtained by BestKeeper

|       | ABL1 | ACTB | CDKN1A | GADPH | GUSB | HPRT1 | HSP90 | IPO8 | PPIA | RPL30 | RPL4 | RPLPO | TBP |
|-------|------|------|--------|-------|------|-------|-------|------|------|-------|------|-------|-----|
| n     | 41   | 42   | 42     | 41    | 42   | 42    | 42    | 42   | 42   | 42    | 42   | 42    | 42  |
| aM \( C_t \) | 28.05 | 23.73 | 28.54 | 25.39 | 31.20 | 29.02 | 26.81 | 29.10 | 22.12 | 28.78 | 25.88 | 24.86 | 28.70 |
| gM \( C_t \) | 28.07 | 23.75 | 28.57 | 25.42 | 31.23 | 29.04 | 26.84 | 29.11 | 22.15 | 28.78 | 25.88 | 24.86 | 28.71 |
| min \( C_t \) | 25.90 | 21.80 | 26.43 | 23.02 | 27.75 | 26.63 | 24.30 | 27.48 | 19.91 | 26.34 | 23.79 | 22.91 | 27.28 |
| max \( C_t \) | 30.39 | 25.87 | 31.23 | 27.80 | 34.06 | 31.91 | 29.55 | 30.64 | 24.53 | 31.06 | 27.98 | 26.66 | 31.55 |
| SD \( C_t \) | 0.87 | 0.73 | 1.05 | 1.05 | 0.91 | 0.91 | 0.86 | 0.65 | 0.82 | 1.09 | 0.77 | 0.81 | 0.75 |
| CV \( C_t \) | 3.10 | 3.07 | 3.69 | 4.11 | 3.17 | 3.13 | 3.19 | 2.22 | 3.71 | 3.78 | 2.98 | 3.27 | 2.62 |
| min \( x \)-fold | \(-3.62\) | \(-3.36\) | \(-4.00\) | \(-4.33\) | \(-10.12\) | \(-4.17\) | \(-5.66\) | \(-2.76\) | \(-4.17\) | \(-4.53\) | \(-3.79\) | \(-3.37\) | \(-2.62\) |
| max \( x \)-fold | 4.04 | 3.85 | 5.85 | 4.41 | 6.78 | 5.64 | 6.62 | 2.61 | 4.73 | 4.11 | 3.82 | 3.06 | 6.15 |
| SD \( x \)-fold | 1.68 | 1.55 | 1.88 | 1.87 | 1.81 | 1.72 | 1.67 | 1.63 | 1.96 | 1.61 | 1.66 | 1.39 |

Geometric mean of \( C_t \) (gM \( C_t \)), arithmetic mean (aM \( C_t \)), minimum and maximum values of \( C_t \) (min \( C_t \), max \( C_t \)), standard deviation of \( C_t \) (SD \( \pm C_t \)), coefficient of variance expressed as a percentage on the \( C_t \) level (CV \( \% C_t \)), extreme values of expression levels expressed as an absolute \( x \)-fold over- or under- regulation coefficient (min \( x \)-fold, max \( x \)-fold), and standard deviation of the absolute regulation coefficients (SD \( \pm x \)-fold).
GADPH had SD > 1 and hence did not meet the stability criteria.

Further, we applied the Equivalence test including both cut-offs of 2-fold and 3-fold expression change to identify the best candidates according equivalent expression in group-wise comparison (Figure 2) [8]. The Equivalence test criteria at 3-fold expression change were fulfilled for IPO8, RPL4, RPL30, GUSB, TBP, RPLPO, ACTB, ABL1, and CDKN1A in all subgroups (Table 6). GADPH was stably expressed only in two out of the five subgroups, followed by HPRT1, HSB90AB1, and PPIA that were equivalently expressed in three subgroups using cut-off of 3. However, IPO8 was the only gene with equivalent expression within 2-fold change in all subgroups.

Table 4 Ranking of 13 candidate RGs according to their expression stability by GeNorm and NormFinder

| Gene   | GeNorm M-value | Gene   | NormFinder M-value |
|--------|----------------|--------|--------------------|
| IPO8   | 0.55           | TBP    | 0.225              |
| RPL4   | 0.55           | RPLPO  | 0.251              |
| TBP    | 0.58           | IPO8   | 0.253              |
| RPLPO  | 0.60           | ACTB   | 0.264              |
| ACTB   | 0.62           | RPL4   | 0.272              |
| PPIA   | 0.65           | PPIA   | 0.339              |
| HSP90  | 0.67           | HSP90  | 0.357              |
| HPRT1  | 0.72           | GADPH  | 0.373              |
| GADPH  | 0.77           | HPRT1  | 0.396              |
| ABL1   | 0.86           | CDKN1A | 0.433              |
| CDKN1A | 0.93           | RPL30  | 0.441              |
| GUSB   | 1.00           | GUSB   | 0.444              |
| RPL30  | 1.10           | ABL1   | 0.515              |

Figure 1 Expression levels of 13 candidate reference genes in benign (BE), borderline (BO), and malignant (MA) primary ovarian tumours. Values are given as the cycle threshold (Ct) and are inversely proportional to the amount of template. Expression levels of the genes studied are shown as whiskers box plots.

Interpretation of target genes expression

In order to show the effect of the unstable RGs on the final expression of target genes, GPER and uPAR mRNAs were related to either IPO8 and RPL4, or GADPH and HPRT1 mRNA. The choice of target genes was based on our previous observations that GPER mRNA expression did not show any variation between benign, borderline, and malignant ovarian tumour samples [13], whereas uPAR mRNA was higher in borderline and malignant than benign ovarian tumour samples [14].

In accordance with our previously published results, the tissue content of GPER mRNA normalized to IPO8 or RPL4 mRNA showed no significant differences between benign, borderline, and malignant tumour samples. In contrast, GPER mRNA normalized to GADPH or HPRT1 mRNA was higher in benign and borderline tumours than in malignant tumours (Figure 3). uPAR mRNA normalized to IPO8 or RPL4 was significantly up-regulated in borderline and malignant tumours as compared to benign tumours, whereas when it was
We included IPO8 in our study because it showed low variation in expression between the benign and the malignant sample in the commercial array. This gene was equivalently expressed across the tumour subgroups of different malignant potential and histology. IPO8 is a Ran-binding protein mediating nuclear import [15] and has been previously reported stably expressed in lung tissues [16], gliomas [17], and colon cancer [18].

The second best RG for group-wise comparison, RPL4, encodes a protein that is a component of the 60S ribosome subunit [19]. Apart from ovarian tissue, it has previously been recommended as RG in combination with PGK1 for exfoliated cervical cells [20]. RPLPO, another gene from the ribosomal protein family, had stable expression in HPV-positive as in HPV-negative cervical samples [21] and in tamoxifen or estrogen treated breast cancer cells [22]. TBP, a key regulator of gene expression, has previously been identified as a suitable RG for expression studies on human hepatitis B virus-related hepatocellular carcinoma [23], human renal cell carcinoma [24], and glioblastomas [17]. RPLPO and TBP also belonged to one of the most stably expressed genes in breast carcinomas [25].

Two other candidates that have not previously been tested as RGs in ovarian tumour tissue, ABL1 and CDKN1A, were selected from the commercial gene array. Both genes satisfied the Equivalence test at 3-fold expression change. ABL1, originally identified as a homologue of the transforming gene of the Abelson murine leukemia virus, is a proto-oncogene, which has been implicated in mitogenesis, regulation of gene transcription, and inhibition of apoptosis [26]. Nucleotide polymorphism in the ABL1 gene has been associated with
risk of ovarian cancer [27]. CDKN1A (also known as p21) was initially described as an inhibitor of cancer cell proliferation [27]. However, recent studies suggest that it has dual functions since it also may promote tumour progression [28] and be associated with cisplatin resistance in ovarian cancer [29].

According to BestKeeper and Equivalence test criteria, we found that GADPH had the worst expression stability in our set of ovarian tumour samples. Similar unfavourable results were obtained for HPRT1. These observations are in line with previous studies on other tissue types that have discouraged use of GADPH and HPRT1 as RGs for clinical lung specimens [16] and renal cell cancer [24]. Most recently, a microarray study identified a group of genes highly correlated to GADPH upregulation in various solid tumours, which were and proportionally associated with advanced stages [30]. Previous reports on GADPH in ovarian tissue have either pointed out higher expression in malignant than in benign tumours and normal tissue [6], or not meeting the GeNorm stability criteria [4]. We further demonstrated that employment of GADPH or HPRT1 for
Table 6 Expression stability of the candidate RGs analysed by equivalence test

|               | BE × BO + MA | BE + BO × MA | BE × MA | Ser × Muc (BE + BO) | Ser × End (MA) | Total passes 2-fold/3-fold |
|---------------|--------------|--------------|---------|---------------------|----------------|--------------------------|
| ABL1         | 0 /1         | 0 /1         | 0 /1    | 1 /1                | 0 /1           | 1 /5                     |
| ACTB*        | 0 /1         | 0 /1         | 0 /1    | 1 /1                | 0 /1           | 1 /5                     |
| CDKN1A       | 0 /1         | 1 /1         | 0 /1    | 0 /1                | 0 /1           | 1 /5                     |
| GADPH        | 0 /0         | 0 /0         | 0 /0    | 0 /1                | 0 /1           | 0 /2                     |
| GUSB         | 0 /1         | 0 /1         | 1 /1    | 1 /1                | 0 /1           | 2 /5                     |
| HPRT1        | 0 /1         | 0 /0         | 0 /0    | 0 /1                | 0 /1           | 0 /3                     |
| HSP90        | 0 /1         | 0 /0         | 0 /0    | 0 /1                | 0 /1           | 0 /3                     |
| IPO8*        | 1 /1         | 1 /1         | 1 /1    | 1 /1                | 1 /1           | 5 /5                     |
| PPIA         | 0 /1         | 0 /0         | 0 /0    | 1 /1                | 0 /1           | 1 /3                     |
| RPL30        | 1 /1         | 0 /1         | 0 /1    | 0 /1                | 1 /1           | 2 /5                     |
| RPL4*        | 1 /1         | 0 /1         | 0 /1    | 0 /1                | 1 /1           | 2 /5                     |
| RPLPO*       | 0 /1         | 0 /1         | 0 /1    | 0 /1                | 1 /1           | 1 /5                     |
| TBP*         | 1 /1         | 0 /1         | 0 /1    | 0 /1                | 1 /1           | 2 /5                     |

The expression within (1) or outside (0) 2-fold/3-fold expression change cut-off and the total number of meeting the cut-off criteria in the five subgroups.

* Genes best-ranked by GeNorm, NormFinder and BestKeeper.

Figure 3 GPER mRNA assayed and normalized to IPO8, RPL4, GADPH, and HPRT1 mRNA. Ovarian tumours were sub-grouped according to the histological malignant potential as benign (BE, n = 9), borderline (BO, n = 11) and malignant (MA, n = 22). Normalization to IPO8 and RPL4 showed no significant variation of the GPER mRNA content between BE, BO and MA tumours (A, B). In contrast, GPER mRNA was higher in BE/BO compared to MA when normalized to GADPH (p = 0.002) or HPRT1 (p = 0.008) (C, D).
normalization resulted in erroneous conclusions on expression of target genes.

To our knowledge, this is the first report on RGs in ovarian tumours that include borderline tumours in addition to benign and malignant tumours. Since they are considered a non-invasive pre-stage of molecular type I ovarian cancer, it is important to include them in any study on biomarker discovery [31].

Ovarian cancer comprises tumours of different morphology and pathogenesis, which may have different gene expression profiles [32]. Therefore we wished to see whether the histology of ovarian tumours influences the stability of RGs. Thus, in contrast to the previous studies conducted exclusively on serous malignant tumours, our study also included mucinous and endometrioid tumours. However, small number of samples in some groups limited the comparisons that could be performed.

**Conclusions**

In conclusion, thorough statistical evaluation of our 13 candidate RGs identified IPO8 followed by RPL4 as the most suitable for the normalization of gene expression data in benign, borderline, and malignant ovarian tumours. For the first time, IPO8 is presented as the best normaliser for gene expression studies on ovarian tumour tissue with heterogeneous histology when used as a single RG. Neither GADPH nor HPRT1 should be used as RGs for ovarian tissue studies, because of poor expression stability. Normalizing to these genes may erroneously influence the quantification of the target gene(s) and hence reduce the reliability of the RT-qPCR results.

**Abbreviations**

RT-qPCR: Quantitative real-time reverse transcription-polymerase chain reaction; RG: Reference gene; IPO8: Importin 8; RPL4: Ribosomal protein 4; GADPH: Glyceraldehyde-3-phosphate dehydrogenase; HPRT1: Hypoxanthine phosphoribosyl transferase 1.


Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

ZK carried out the gene expression experiments and drafted the manuscript. AA performed the statistical analysis. BC drafted the manuscript. SH contributed methodological know-how. EK participated in the study design and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by the Swedish Cancer society, Skåne University Hospital and Region Skåne.

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Received: 10 May 2013 Accepted: 18 August 2013

References

1. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaff MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009, 55(4):611–622.

2. Sirower MA: New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochimica et biophysica acta 1999, 1432(2):159–184.

3. Chang TJ, Juan CC, Yin PH, Chi CW, Tsay HJ. Up-regulation of beta-actin, cytoplilhin and GAPDH in N1S1 rat hepatoma. Oncol Rep 1998, 5(2):469–471.

4. Li YL, Ye F, Hu Y, Lu WG, Xie X: Identification of suitable reference genes for gene expression studies of human serous ovarian cancer by real-time polymerase chain reaction. Anal Biochem 2009, 394(1):110–116.

5. Sun Y, Li Y, Liu D, Liao D: Pseudogenes as weakens of ACTB (Acb) and GAPDH (Gpdh) used as reference genes in reverse transcription and polymerase chain reactions. PLoS One 2012, 7(8):e41659.

6. Fu J, Bian L, Zhao L, Dong Z, Gao X, Luan H, Sun Y, Song H: Identification of genes for normalization of quantitative real-time PCR data in ovarian tissues. Acta biochimica et biophysica Sinica 2010, 42(8):568–574.

7. Stefan W: Testing Statistical Hypotheses of Equivalence and Noninferiority. 2003.

8. Haller F, Kuller B, Schwager S, Gunawan B, von Heydebreck A, Sultmann H, Kolek V, du Bois RM, Petrek M: Identification of housekeeping genes, differentially regulated target genes and sample denominators to normalize gene expression profiles in bronchoalveolar lavage fluid. BMC Mol Biol 2004, 5:9.

9. Pfaff MW, Tichopad A, Prgomet C, Neuvians TP: Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–excel-based tool using pair-wise correlations. Biotechnol Lett 2004, 26(6):509–515.

10. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002, 3(7):RESEARCH0034.11.

11. Andersen CL, Jensen JL, Orntoft TF: 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 2004, 64(15):5245–5250.

12. Kolkova Z, Caislen V, Henic E, Ahnadi S, Ehinger A, Jirström K, Caislen B: The G protein-coupled estrogen receptor 1 (GPER/GPR30) does not predict survival in patients with ovarian cancer. J Ovarian Res 2012, 5(9).

13. Borgfeldt C, Hansson SR, Gustavsson B, Måstback A, Caislen B: Dedifferentiation of serous ovarian cancer from cystic to solid tumors is associated with increased expression of mRNA for urokinase plasminogen activator (uPA), its receptor (uPAR) and its inhibitor (PAI-1). Int J Cancer 2001, 92(4):497–502.

14. Dean KA, von Ahesen Q, Gorlich D, Fried HM: Signal recognition particle protein 19 is imported into the nucleus by importin 8 (RanBP8) and transportin. J Cell Sci 2001, 114(Pt 19):3479–3485.

15. Nguyeva PA, Agorejta J, Blanco D, Lorenzo MD, Gomez-Roman J, Sanchez BA, Valles I, Pajares MJ, Pio R, Rodriguez MJ, Montuenga LM, Calvo A: Identification of importin 8 (IPOB) as the most accurate reference gene for the clinicopathological analysis of lung specimens. BMC Mol Biol 2008, 9:103.

16. Kiehl S, Heyn J, Grau S, Kretzschmar HA, Eggersperger R, Kiehl FW: Identification of valid endogenous control genes for determining gene expression in human glioma. Neuro Oncol 2010, 12(6):570–579.

17. Sorby LA, Anderssen SN, Bokhholm IR, Jacobsen MB: Evaluation of suitable reference genes for normalization of real-time reverse transcription PCR analysis in colon cancer. J Exp Clin Cancer Res 2010, 29:144.

18. Klinger S, Voigts-Hoffman F, Liebundgut M, Arpagaus S, Ban N: Crystal structure of the eukaryotic 60S ribosomal subunit in complex with initiation factor 6. Science 2011, 334(6058):944–948.

19. Steinau M, Rajeevan MS, Unger ER: DNA and RNA references for qRT-PCR assays in exfoliated cellular samples. JMD 2006, 8(1):113–118.

20. Daud I, Scott ME: Validation of reference genes in cervical cell samples from human papillomavirus-infected and -uninfected women for quantitative reverse transcription-PCR assays. Cytometry 2008, 73(9):1369–1373.

21. Shah KN, Faridi IJ: Estrogen, tamoxifen, and Aft modulate expression of putative housekeeping genes in breast cancer cells. J Steroid Biochem Mol Biol 2011, 125(3–5):219–225.

22. Fu LY, Ja HL, Dong QZ, Wu JC, Zhao Y, Zhou HJ, Ren Y, Ye QH, Qin LX: Suitable reference genes for real-time PCR in human HBV-related hepatocellular carcinoma with different clinical prognoses. BMC Cancer 2009, 9:49.

23. Jung M, Ramankulov A, Roigas J, Johannsen M, Ringsdorf M, Kristiansen G, Jung K: In search of suitable reference genes for gene expression studies of human renal cell carcinoma by real-time PCR. BMC Mol Biol 2007, 8:47.

24. Lyng MB, Laenholm AV, Pallisgaard N, Ditzel HJ: Identification of genes for normalization of real-time RT-PCR data in breast carcinomas. BMC Cancer 2008, 8:20.

25. Colicelli J: ABL tyrosine kinases: evolution of function, regulation, and specificity. Sci Signal 2010, 3(139):re6.

26. Cunningham JM, Vierkant RA, Sellers TA, Phelan C, Rider DN, Liebow M, Schildkraut J, Berchuck A, Couch FJ, Wang X, Fridley BL, Gentry-Maharaj A, Menon U, Hogdall E, Kjaer S, Whitemore A, DiCioccio R, Song H, Gaylor SA, Ramsu SJ, Pharoah PD, Goode EL: Cell cycle genes and ovarian cancer susceptibility: a tagSNP analysis. Br J Cancer 2009, 101(8):1461–1468.

27. Stivala LA, Cazzalini O, Prosperi E: The cyclin-dependent kinase inhibitor p21CDKN1A as a target of anti-cancer drugs. Curr Cancer Drug Targets 2012, 12(2):85–96.

28. Xia X, Ma Q, Li X, Ji T, Chen P, Xu G, Meng L, Zhou J, Ma D: Cytoplasmic p21 is a potential predictor for cisplatin sensitivity in ovarian cancer. BMC Cancer 2011, 11:399.

29. Wang D, Moothart DR, Lowy DR, Qian X: The expression of glyceraldehyde-3-phosphate dehydrogenase associated cell cycle (GACC) genes correlates with cancer stage and poor survival in patients with solid tumors. PloS One 2013, 8(4):e61262.

30. Romero I, Bost RC: Minireview: morphologic and molecular characterization of ovarian cancer by gene-expression profiling. Gynecol Oncol 2010, 118(1):88–92.

Cite this article as: Kolkova et al.: Normalizing to GADPH jeopardises correct quantification of gene expression in ovarian tumours – IPO8 and RPL4 are reliable reference genes. Journal of Ovarian Research 2013 6:60.