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Selection and Validation of Reliable Reference Genes for RT-qPCR Analysis in a Large Cohort of Pituitary Adenomas

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Introduction

Context:

Real-time reverse transcription quantitative PCR (RT-qPCR) has become the method of choice for quantification of gene expression changes. Inappropriate data normalization and inconsistent data analyses are some limitations of RT-qPCR. Pituitary adenomas are frequent tumors and the interpretation of increasingly published data within this field is hindered by the lack of a proper selection and validation of stable expressed reference genes[1].

Hypothesis:

Multiple reference genes increase the stability value for normalization of gene expression in RT-qPCR.

Objective:

To find an optimal combination of reference genes for RT-qPCR in pituitary adenomas.

Materials & Methods

- 30 commonly used reference genes (PCR array reference gene panel, BioRad, Hercules, CA) were quantified by RT-qPCR in 24 pituitary adenomas (12 NFPA, 7 GH and 4 ACTH).
- Data was analysed using three programs: geNorm, Normfinder and BestKeeper having different algorithms to identify the most stable reference gene/combination of reference genes.
- Three genes (SDHA,TFRC, and CDKN1A) and one sample (GH) were omitted from analysis due to missing values and poor quality.
- The top candidate genes, based on the geNorm and Normfinder algorithms, were validated in a large cohort of adenomas (141 NFPA, 63 GH and 19 ACTH).

References

1. Bustin SA et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry 2009;55(4):611-22.

2. Bujko M, Rusetska N, Mikula M. Validating candidate reference genes for qRT-PCR-based gene expression analysis in nonfunctioning pituitary adenomas. Pituitary 2016;19(1):110-2.

Results

Selection of Reference Genes

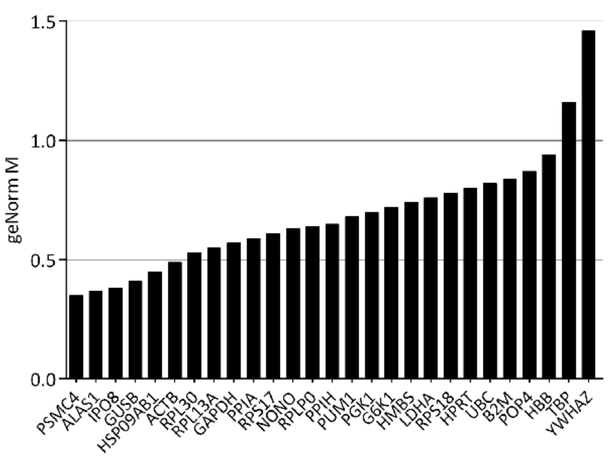

Figure 1. Reference gene stability assessed by geNorm (n=23). Lower M predicts higher stability. PSMC4 and ALAS1 are the top ranked genes and were also the best combination for gene expression normalization.

Rank	ALL (n=23)	NFPA (n=12)	GH (n=7)	ACTH (n=4)
1	PSMC4	PSMC4	ALAS1	POP4
2	ALAS1	RPL30	LDHA	RPS18
3	IPO8	ALAS1	PPIH	PPIA
4	GUSB	IPO8	PUM1	TBP
5	HP90AB1	HSP90AB1	PSMC4	IPO8

Table 1. GeNorm ranking of the top 5 stably expressed reference genes in: all adenomas, NFPA, GH and ACTH producing adenomas. PSMC4 and ALAS1 are amongst the top 5 stably expressed genes in all groups but the ACTH producing adenomas (possible due to type 2 error).

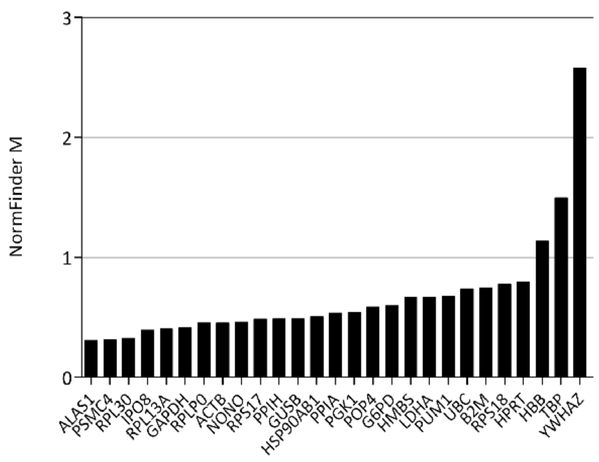

Figure 2. Reference gene stability by NormFinder (n=23). Lower M predicts higher stability. ALAS1 is the top ranked gene (M=0.312), whereas PSMC4 and GAPDH were the best gene combination (M=0.221).

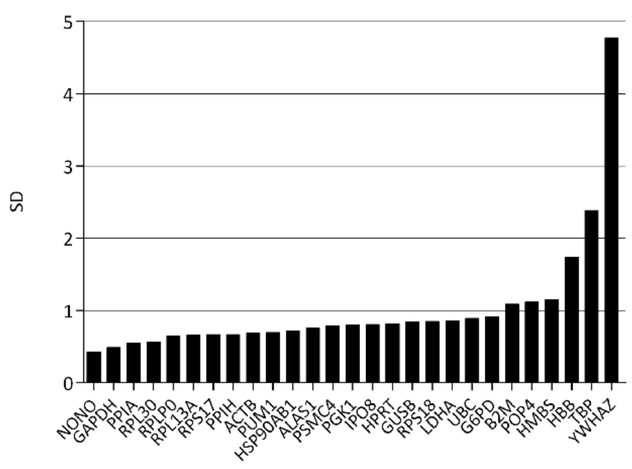

Figure 3. Reference gene stability assessed by standard deviation (SD) of the Ct-values (n=23). Of the 27 genes analysed, 22 had a SD less than 1, indicating several reference genes with good stability.

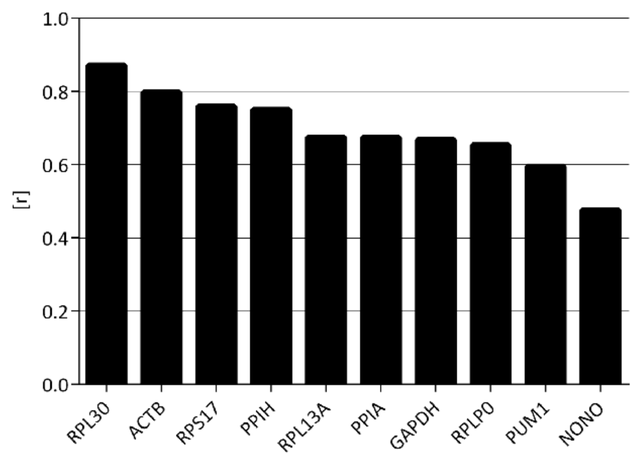

Figure 4. BestKeeper stability assessment ranged by each reference genes correlation coefficient [r]. BestKeeper analyzed the ten most stable genes based on SD, yielding RPL30 as the most stably expressed gene.

Rank	GeNorm	NormFinder	BestKeeper
1	PSMC4	ALAS1	RPL30
2	ALAS1	PSMC4	ACTB
3	IPO8	RPL30	RPS17
4	GUSB	IPO8	PPIH
5	HSP901A	RPL13A	RPL13A
6	ACTB	GAPDH	PPIA
7	RPL30	RPLP0	GAPDH
8	RPL13A	ACTB	RPLP0
9	GAPDH	NONO	PUM1
10	PPIA	RPS17	NONO

Table 2. Top 10 ranked reference genes from geNorm, NormFinder and BestKeeper (n=23). PSMC4 and ALAS1 are top ranked in both geNorm and NormFinder, whereas RPL30 was the most stable gene in BestKeeper.

Validation of Selected Reference Genes

NFPA (n=143)		GH (n=63)		ACTH (n=19)	
Gene combination	M	Gene combination	M	Gene combination	M
PSMC4/ALAS1	0.787	PSMC4/GAPDH	0.646	PSMC4/GAPDH	0.786
PSMC4/ALAS1/GAPDH	1.020	PSMC4/GAPDH/ALAS1	0.941	PSMC4/GAPDH/ALAS1	1.098

Table 2. Validation of the selected reference genes by geNorm software in a large cohort of NFPA, GH and ACTH producing adenomas. Lower M predicts higher stability. Best stability is achieved using two reference genes in combination, adding a third gene results in a lower stability.

Abbreviations:
NFPA – non functioning pituitary adenomas, RT-qPCR – Real-time reverse transcription quantitative PCR, Ct – cycle threshold, SD – standard deviation.

Conclusions

- The reference gene panel revealed several stably expressed genes in the selection study.
- GeNorm showed that two reference genes generated a valid stability value in the validation study.
- PSMC4 and ALAS1 were validated as the best combination of reference genes for NFPAs.
- PSMC4 and GAPDH were validated as the best combination of reference genes for the hormone producing adenomas.

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