Validation of Appropriate Reference Genes for Gene Expression Studies in Human Thyroid Gland Using Real-Time RT-PCR

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Background: *Gene-expression analysis is increasingly important in biological research, with real-time reverse transcription PCR (qRT-PCR) becoming the method of choice. The selection of reference genes is critical for gene expression studies because the expression of these genes may vary among tissues or cells and may change under certain circumstances. However, there has not been any study that compares the stability of these reference genes in human thyroid specimens. Therefore, the authors studied the stability values and the appropriate reference genes expressed in thyroid specimens.*
Material and Method: *25 human thyroid specimens were prospectively collected and extracted for their RNA. The candidate reference genes (hypoxanthine phosphoribosyl-transferase1 (HPRT1), ribosomal protein LI3a (RPLIA), β-2-micro-globulin (B2M), β-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPD)) were amplified from these thyroid specimens using real-time RT-PCR. The stabilities of these candidate reference genes were analyzed using Normfinder® software.*
Results: *The authors found that HPRT1 has the highest stability value (40.38×10^9) while GAPD has the lowest stability value (85.46×10^7). Therefore GAPD is the most stably expressed gene in thyroid specimens.*
Conclusion: *Of the 5 genes studied, GAPD was found to be the best reference gene for gene expression studies in the thyroid gland. The present results may facilitate the choice of reference genes for expression studies in thyroid diseases.*

Keywords: *Gene expression, Housekeeping gene, RT-PCR, Thyroid diseases, Normfinder*

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In the present day, real-time RT-PCR was used in order to measure transcript abundance in gene expression analysis study. This method has gained much popularity and is frequently used due to its less time consuming, high reliability, high sensitivity and specificity. Real time RT-PCR provides a simultaneous measurement of gene expression in many different samples for the limited number of genes and is especially suitable when only a small number of cells are available[1-3]. Nevertheless, real time RT-PCR is an indirect measurement. The result depends on quantity, quality of starting material, enzymatic efficiencies and differences between tissues or cells in overall transcriptional activity. Many strategies have been attempted to control or normalize these variations in order to acquire the most accurate result. To date, internal control genes or reference genes or Housekeeping genes are most frequently used to normalize the mRNA fraction. The concept of reference gene is that this gene should not vary in the tissues or cells under any investigation, or in response to experimental treatment. The selection of reference genes is critical for gene expression studies. If the studies use improper reference genes, their result would be mistaken. Several commonly used reference genes have been applied in real time RT-PCR studies, including hypoxanthine phosphoribosyl-transferase1 (HPRT1), ribosomal protein LI3a (RPLIA), β-2-micro-globulin (B2M), β-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPD)[4-8].

Recently, gene expression analysis is increasingly important in the fields of thyroid research. Understanding patterns of expressed genes is expected to provide an insight into complex regulatory networks and will most probably lead to an understanding of organ functions or causes of thyroid diseases. Previous studies have attempted to diagnose or predict thyroid malignancy using gene expression analysis.

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However, they make use of reference genes without proper validation of their presumed stability of expression[9-15]. In the present study, the authors carried out an extensive evaluation of commonly used 5 reference genes in thyroid tissues using real time RT-PCR to find the most stable reference gene for studying the gene expression profiles in human thyroid specimens.

Material and Method

The thyroid tissue samples were extracted from 25 patients who required thyroid surgery in Rajavithi Hospital. Rajavithi Hospital Ethic Committee approved the present study.

Each thyroid tissue was extracted and amplified from 25 different human thyroid tissues using semi-quantitative real-time PCR. The reference genes selection was made from the common reference genes used from previous studies or related literature reviews including HPRT1, RPLIA, B2M, ACTB and GAPD (Table 1)[9-15]. The sequences of primers used in the present study are demonstrated in Table 2. The authors calculated number of amplified genes using two to the power of n (2^n format); n refers to number of cycles of each gene. The most stable gene refers to less variable number of genes expression among various thyroid tissues. The stabilities of genes were analyzed using Normfinder® software. NormFinder® is an algorithm for ranking the set of candidate normalization genes according to their expression stability in a given sample set and a given experimental design[16].

Results

Thyroid specimens and the purity of extracted RNA25 thyroid specimens were obtained from 25 patients. The pathology of each specimen was reviewed by Rajavithi Hospital pathologists. 6 patients were follicular adenoma. 13 patients were adenomatous nodules. 2 patients were lymphocytic thyroiditis. 2 patients were follicular carcinoma and the remaining 2 patients were papillary carcinoma. All these thyroid specimens were extracted for their RNA. The optical density (OD) ratio A260/A280 nm was 2.0 $\pm$ 0.25 (OD A260/A280 ratio $\pm$ SD). The average RNA concentration after extraction was 62.14 µg/ml $\pm$ 22.25 (µg/ml $\pm$ SD).

Expression levels of candidate reference genes

The cycle threshold (Ct) values of the candidate reference genes obtained from these thyroid specimens are demonstrated in Fig. 1. The authors demonstrated that the average Ct value of the HRP

Table 1. Internal control (reference) genes evaluated in this study

Symbol	Accession number	Name	Function	Localization
ACTB	NM_001101	Beta actin	Cytoskeletal structural protein	7p15-p12
B2M	NM_004048	Beta-2-micro-globulin	Beta-chain of major histocompatibility complex class 1 molecules	15q21-q22
GAPD	NM_002046	Glyceraldehyde-3 -phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis	12p13
HPRTI	NM_000194	Hypoxanthine phosphoribosyl-transferase1	Purine synthesis in salvage pathway	Xq26
RPLIA	NM_012423	Ribosomal protein LI3a	Structural component of the large 60s ribosomal subunit	19q13

Table 2. Primer sequences for internal control (reference) genes

Symbol	Forward primer	Reverse primer
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
GAPD	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
HPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
RPLIA	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA

gene was significantly different from the other reference genes ($p < 0.001$).

Identification of the optimal reference genes

The NormFinder® Software was used to rank the candidate reference genes based on their stability values. The most stable gene refers to less variation among 25 thyroid specimens. The results from NormFinder® showed that the stability values of GAPD was 854689680.112, ACTB was 2510978994.428, B2M was 2693895816.910, RPLIA was 2986940313.904 and HPRT1 was 40383536363.800 (Fig. 2). GAPD was the least variable gene. This means GAPD is the most stable gene in thyroid expression studies.

Discussion

Gene-expression analysis using real-time RT-PCR is increasingly important in biological research, and becoming the method of choice. The selection of reference genes is critical for gene expression studies. In gene expression studies of the human thyroid gland, various reference genes have been selected as internal control genes to normalize mRNA fraction. In this study, we compared the 5 most common reference genes in 25 thyroid specimens using real-time RT-PCR. The authors found that the most stable genes were GAPD, ACTB, B2M, RPLIA and HPRT1, respectively.

GAPD (glyceraldehyde-3-phosphate dehydrogenate) gene located on chromosome 12p13 was found to be the single best reference gene for gene expression studies in the human thyroid gland. This finding may facilitate the choice of reference genes for expression studies in thyroid gland diseases. However, there are some limitations of the present study. This is a pilot study. There is no other paper that can be compared these reference genes in thyroid specimens before. Therefore, the accuracy of the result of the present study would be more reliable if more samples and more variations among samples were tested. The number of samples of the present study was only 25 and there was less variation among thyroid samples, 4 samples were carcinoma, 2 samples were thyroiditis and 18 samples were thyroid adenoma. Further study with more calculated samples and more variation may be needed to confirm the result of the present study.

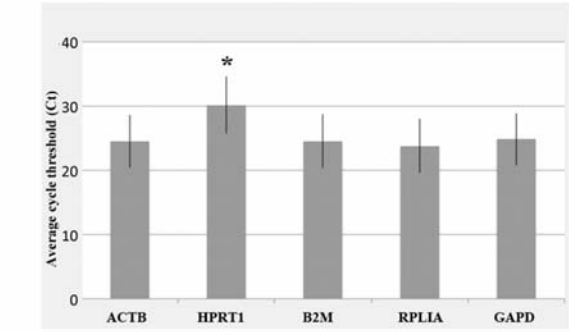

Fig. 1 The average cycle threshold (Ct) values of the candidate reference genes obtained from 25 thyroid specimens are demonstrated

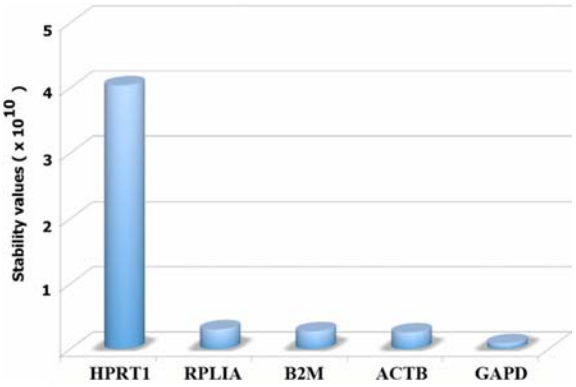

Fig. 2 The stability values of the candidate reference genes are calculated by NormFinder®. HPRT1 has the highest stability valuewhile GAPD has the lowest stability value

Potential conflicts of interest

None.

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การตรวจสอบความเหมาะสมของจีนจะนำมาใช้เป็นจีนอ้างอิงสำหรับการศึกษาการแสดงออกของจีนจากต่อมไทรอยด์

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วัตถุประสงค์: การศึกษาการแสดงออกของจีนมีความสำคัญมากขึ้นในกระบวนการวิจัยทางด้านชีวภาพปัจจุบันเราใช้ PCR เป็นวิธีการหลัก ด้วยวิธีนี้จำเป็นต้องใช้จีนอ้างอิง (Reference gene) เพื่อเป็นหลักในการคำนวณการเปลี่ยนแปลงของจีนที่ศึกษา ดังนั้นจีนอ้างอิงจำเป็นต้องมีการแสดงออกที่คงที่ในแต่ละตัวอย่างที่นำมาศึกษา อย่างไรก็ตาม ยังไม่มีการศึกษาเปรียบเทียบความคงตัวของ reference genes เหล่านี้ในต่อมไทรอยด์มนุษย์ ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อหาความคงที่ของการแสดงออกของจีนอ้างอิงแต่ละตัวเพื่อหาจีนที่เหมาะสมที่สุดเพื่อใช้เป็นจีนอ้างอิงในการศึกษาเรื่องจีนในต่อมไทรอยด์

วัสดุและวิธีการ: การศึกษานี้ได้ตรวจสอบความคงที่ของการแสดงออกของจีนอ้างอิงที่ใช้บ่อย (hypoxanthine (B2M), -actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPD)) ในเนื้อเยื่อจากต่อมไทรอยด์ในผู้ป่วยจำนวน 25 ราย โดยการสกัด RNA และเพิ่มจำนวนด้วยวิธี RT-PCR ค่าความคงตัวของจีนทุกตัวถูกวิเคราะห์โดยใช้โปรแกรม Normfinder

ผลการศึกษา: พบว่า HPRT1 มีค่าสูงสุดคือ 40.38×10^{9} ในขณะที่ GAPD มีค่าต่ำสุดคือ 85.46×10^{7} ดังนั้น GAPD จึงเป็นจีนที่มีการแสดงออกคงที่มากที่สุด

สรุป: จากการศึกษาพบว่า GAPD เป็นจีนอ้างอิงที่มีการแสดงออกคงที่มากที่สุด จึงแนะนำให้ใช้ GAPD เป็นจีนอ้างอิงสำหรับการศึกษาการแสดงออกของจีนในต่อมไทรอยด์