RESEARCH ARTICLE

Optimization of Reference Genes for Normalization of the Quantitative Polymerase Chain Reaction in Tissue Samples of Gastric Cancer

Lian-Mei Zhao[1,2], Zhao-Xu Zheng[1], Xiwa Zhao[2], Juan Shi[3], Jian-Jun Bi[1], Wei Pei[1], Qiang Feng[1]*

Abstract

For an exact comparison of mRNA transcription in different samples or tissues with real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR), it is crucial to select a suitable internal reference gene. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTB) have been frequently considered as house-keeping genes to normalize for changes in specific gene expression. However, it has been reported that these genes are unsuitable references in some cases, because their transcription is significantly variable under particular experimental conditions and among tissues. The present study was aimed to investigate which reference genes are most suitable for the study of gastric cancer tissues using qRT-PCR. 50 pairs of gastric cancer and corresponding peritumoral tissues were obtained from patients with gastric cancer. Absolute qRT-PCR was employed to detect the expression of GAPDH, ACTB, RPII and 18sRNA in the gastric cancer samples. Comparing gastric cancer with corresponding peritumoral tissues, GAPDH, ACTB and RPII were obviously up-regulated 6.49, 5.0 and 3.68 fold, respectively. Yet 18sRNA had no obvious expression change in gastric cancer tissues and the corresponding peritumoral tissues. The expression of GAPDH, β-actin, RPII and 18sRNA showed no obvious changes in normal gastric epithelial cells compared with gastric cancer cell lines. The carcinoembryonic antigen (CEA), a widely used clinical tumor marker, was used as a validation gene. Only when 18sRNA was used as the normalizing gene was CEA obviously elevated in gastric cancer tissues compared with peritumoral tissues. Our data show that 18sRNA is stably expressed in gastric cancer samples and corresponding peritumoral tissues. These observations confirm that there is no universal reference gene and underline the importance of specific optimization of potential reference genes for any experimental condition.

Keywords: Gastric cancer tissue - qRT-PCR - internal reference gene - variability

Asian Pac J Cancer Prev, **15 (14)**, 5815-5818

Introduction

With the unique features of accurate quantification, high sensitivity and throughput (Huggett et al., 2005; Radonić et al., 2004), the technique of real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) has been applied extensively for detection of specific gene expression. Especially, in recent years, genomic studies have revealed that the number of noncoding RNA is far greater than that of coding RNA (the ENCODE Project Consortium, 2007; Mattick et al., 2009). And, incapable of effective protein encoding, it is impossible to detect its protein expression. As a result, the technique of qRT-PCR has become one of implified and feasible detection methods of gene expression variation.

While performing qRT-PCR experiments, a reference gene, also called internal standard gene or normalized gene is required to calibrate the expression level of target gene to obtain objective and trustworthy results. This method is based on the assumption that the expression level of the normalizing gene does not change from sample to sample. The commonly used housekeeping genes include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), 18SRNA, 28SRNA, RNA polymerase II (RPII) and tublin, etc (Vandesompele et al., 2002; Koon et al., 2004). An ideal internal reference gene should be stably expressed in all types of tissues and cells or various experimental settings. However, as demonstrated by the results of many experiments, a stable expression of these internal reference genes was only detectable in some particular tissues and cells or under some special experimental conditions. And it varied with clinical

*[1]Department of Abdominal Surgical Oncology, Cancer Hospital, Peking Union Medical College & Chinese Academy of Medical Sciences, [3]National Laboratory of Medical Molecular Biology, Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing, [2]Tumor Research Institute, the Fourth Hospital of Hebei, Medical University, Shijiazhuang, China *For correspondence: fengqiang2008@vip.sina.com*

status of the tissue studies and experimental conditions (Goidin et al., 2001; Hamalainen et al., 2001 Glare et al., 2002; Steele et al., 2002;). Therefore, the more promising strategy is to identify the best normalizing gene for each tissue type, pathology, or experimental design setting.

Gastric cancer ranks the second in cancer-related mortality (Jemal et al., 2010). Thus it is regarded as one of the greatest health hazards to human beings. Thus it is of vital importance to elucidate its occurring and progressive mechanisms and discover new molecular markers for an early diagnosis. The most common method of screening gastric cancer tumor markers is the technique of high-throughput array or qRT-PCR, necessitating the use of stable housekeep gene as internal reference. The present study is intended to discover the best internal reference gene stably expressing in gastric cancer, peritumoral tissues, gastric epithelial cell line and gastric cancer cell lines.

Materials and Methods

Surgical specimen

A total of 50 specimens of gastric cancer and paired peritumoral tissues were all collected from surgical resections of hospitalized gastric cancer patients at Hebei Provincial Cancer Hospital in 2012. All cases were confirmed as gastric cancer after preoperative endoscopy or postoperative pathological examination and without preoperative radiotherapy, chemotherapy or endocrine therapy. All tissues were cut into pieces of approximately 100-200 mg, snap frozen in liquid nitrogen immediately after surgery.

Cell lines

Normal human gastric mucous epithelial cell line GSE-1 was kindly provided by Division of Genetics, Beijing Tumor Control & Research Center. Gastric cancer cell lines of MGC-803, SGC-7901, MKN-28, MKN-45, SNU-1 and HGC-27 were all purchased from Cell Center, Chinese Academy of Medical Sciences.

RNA isolation and qRT-PCR amplification

Tumor tissues or cultured cells were lysed in Trizol reagent (Life Technologies, CA). Total RNAs were extracted using the methods as recommended by the manufacturer. RNA quantity was assayed using NanoDrop 2000 (Thermo Scientific, PA). Reverse transcription polymerase chain reaction (RT-PCR) was performed using Superscript first-strand synthesis system (Life Technologies, CA). RNA concentration. 1% agrose gel electrophoresis was used for detecting the RNA integrity. The instructions of reverse transcription reagent kit were followed for reverse transcription of tissue RNA. Quantitative real-time PCR (q-PCR) was performed for 40 cycles using SYBR Green (Takara, Japan) measured by 9700HT Step one (Life Technologies, CA)with cDNA at 1 μl of which belongs to the total 10μl RNA reversed reaction system and primers at 10 nM concentrations. Primer designed with primer5 were summarized in (Table 1). Reaction conditions: 95°C5 min; 95°C 30s, 60°C 30s, 72°C 30s (40 cycles). For each degree of 70°C to 99°C, 8 fluorescent spots were collected to plot the dissolution curve. And the curve was observed for whether or not conforming to a single peak to evaluate the specificity of PCR product.

Statistical analysis

Statistical analysis was performed with geNorm program. In Excel spreadsheet, the expression level for the lowest value of Ct of a housekeeping gene was 1 for different samples. For other samples, the lowest expression level of this housekeeping gene was 1. For other samples, the relative expression level of this housekeeping gene was $2^{-\triangle Ct}$ ($\triangle Ct$= Ct value of each sample - lowest Ct value). These data were inducted into the geNorm program to calculate the mean (M) of gene expression stability. And the expression stabilities of housekeeping genes were ordered. The smaller M, the more expression stability.

And T test were processed with software SPSS17.0. Firstly the experimental data were tested for a normal distribution. If conforming to a normal distribution, paired T test was used; if not, rank-sum test was applied. All values were expressed as mean + standard deviation. $p<0.05$ denoted statistical significance.

Results

Markedly up-regulated Expressions of GAPDH, Beta-actin, RPII except 18sRNA in Gastric Cancer

From 50 pairs of gastric cancer and peritumoral tissues, total RNA was extracted for accurate quantification. After qRT-PCR, in gastric cancer tissues, GAPDH was up-regulated by 2.7±0.41 cycles (6.49±1.32folds), Beta-actin up-regulated by 2.33±0.32cycles (5.0±1.24folds), RPII up-regulated by 1.88±0.21cycles (3.68±1.15 folds). All the results had statistical differences ($p<0.05$). However, there is no significant difference of 18sRNA between gastric cancer and peritumoral tissues (Figure 1A, B). It indicated that GAPDH and ACTB, the most commonly used internal genes, were up-regulation greatly in gastric cancer tissue. And the expression of 18sRNA gene was most stable in the genes we detected in gastric cancer and peritumoral tissues.

No obviously Altered Expression of Four Internal Reference Genes in Gastric Cancer Cell Lines and Normal Gastric Epithelial Cells

As shown in (Figure 2), the Ct values of GAPDH were 16.7±1.13, 16.1±0.85, 15.8±1.32, 15.9±1.40 and 17.2±2.10 respectively in human cancer cell lines, SGC-7901, MGC-803, HGC-27, MKN-28 and human normal gastric epithelial cell GSE-1 demonstrated by

Table 1. Primer Sequences and Amplification Conditions

Gene name	Primer sequence	Product length
18Srna	F:5'-CAGCCACCCGAGATTGAGCA-3' R: 5'-TAGTAGCGACGGGCGGTGT -3'	244bp
GAPDH	F: 5'- CGGATTTGGTCGTATTGGG -3' R:5'-TGCTGGAAGATGGTGATGGGATT -3'	283bp
ACTB	R: 5'- GAGGCGTACAGGGATAGCAC -3' F: 5'-GTCACCAACTGGGACGACAT -3'	279bp
RPII	R: 5'-GTGCGGCTGCTTCCATAA -3'	362bp

DOI:http://dx.doi.org/10.7314/APJCP.2014.15.14.5815

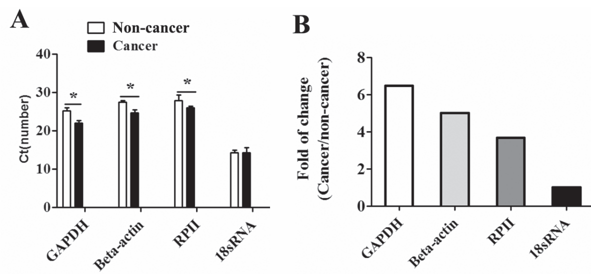

Figure 1. Comparative Expression as Determined by Q-RT-PCR for Four Housekeeping Genes. The standard deviations for each gene were calculated based on Q-RT-PCR analysis of 50 pairs tissue samples (A) total cycle of Q-RT-PCR for each gene in cancer tissue samples and non-cancer tissue samples, Ct = mean±SD, *P <0.05, compared between the two groups. (B) Fold increases above 1 indicate gene overexpression.

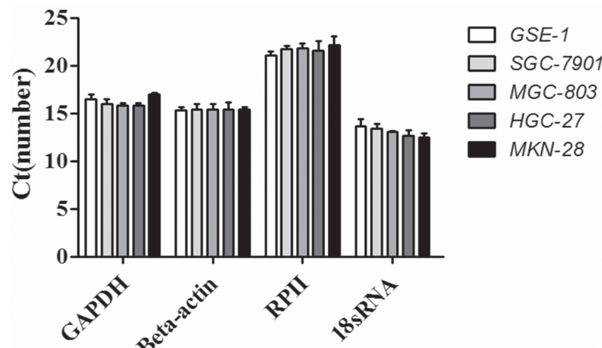

Figure 2. Comparative Expression as Determined by Q-RT-PCR for Four Housekeeping Genes in Normal Stomach and Stomach Cancer Cells

the experimental results of qRT-PCR. Compared with GSE-1, none of four gastric cancer cell lines showed statistical difference. In 5 cell lines, the Ct values of beta-actin were 15.2±0.54, 15.4±1.41, 15.4±1.62, 15.5±1.70 and 15.7±0.73; the Ct values of RPII were 22.3±2.01, 23.1±1.84, 23.7±2.03, 22.6±2.65 and 22.4±2.21; the Ct values of 18sRNA were 14.7±2.61, 14.1±1.78, 13.1±0.57, 12.6±1.31 and 11.5±1.20, respectively. For each internal gene, a comparison of Ct value for gastric cancer versus GSE-1 revealed no statistical difference. However, RPII had a low basal expression in gastric cancer cells and gastric immortalized epithelial tissues so that RPII was not suitable as an internal reference gene. Therefore GAPDH, beta-actin and 18sRNA might be used as normalized genes in the research based on gastric cancer cell lines.

Expression of Carcinoembryonic Antigen (CEA) in Gastric Cancer Tissues with Different genes as Internal Reference Genes

The carcinoembryonic antigen (CEA), one of widely used clinical tumor marker (Duffy et al., 2001; Malati et al., 2007; Cidón et al., 2011) was used as validation gene to further analyze the reliability of the internal reference genes. Using GAPDH, beta-actin, RPII and 18sRNA as internal reference genes, the expression of CEA was detected with qRT-PCR in 50 specimens of gastric cancer and peritumoral tissues. The expression of CEA was decreased by 1.71±0.38 folds in gastric cancer tissues when normalized by GAPDH. And when normalized

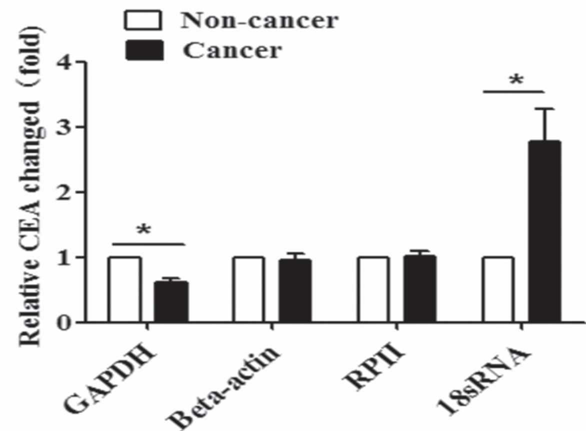

Figure 3. Comparative Expression as Determined by Q-RT-PCR for Four Housekeeping Genes. The standard deviations for each gene were calculated based on Q-RT-PCR analysis of 50 pairs tissue samples Fold increases above 1 indicate gene overexpression. *P <0.05, compared between the two groups.

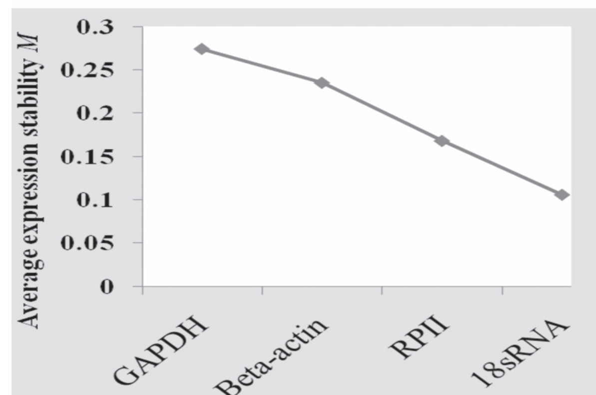

Figure 4. Average Expression Stability of Four Housekeeping Genes Tested in this Study. The average expression stability value (M) was determined using the geNorm program. Housekeeping genes in order of increasing stability are ranked from left toright, which are indicated by lower M values

with beta-actin and RPII, CEA expression had no obvious changes in gastric cancer and peritumoral tissues (p>0.05). However, CEA increased markedly in gastric cancer tissues (p<0.05)and it rose 2.67 folds compared with it rose by 2.67±0.51 folds when using 18sRNA as internal reference gene (Figure 3). Based on these results, 18sRNA was considered as an optimal internal reference gene.

Statistical Analysis with genNorm Program

For validating the above experimental results, genNorm was employed to select the optimal references according to the expression levels of internal reference genes of 50 specimens of gastric cancers and para-gastric cancer tissues. Vandesompele et al developed the program of genNorm in 2002 for selecting the internal reference genes. Within the present study system, the mean (M) values of expression stability for GAPDH, beta-actin, RPII and 18sRNA were 0.274, 0.235, 0.168 and 0.106 (Figure 4). According to the principle of smaller M value denoting more stability, 18sRNA had the greatest expression stability in gastric cancer and peritumoral tissues while GAPDH was the least stable gene.

Discussion

In this study, we have examined the expression of four commonly used reference genes (Vandesompele et al., 2002; Koon et al., 2004) by qRT-PCR. And we also confirmed that normalization with suitable reference genes is important for reliable qRT-PCR results of a target gene. Comparing gastric cancer with paired peritumoral tissues, the lower expression of the reference gene GAPDH, ACTB and RPII in gastric cancer led to the misinterpretation of the expression of the target gene CEA, whereas there was no significant differential expression of CEA between gastric cancer with paired peritumoral tissues after normalization to the reference gene GAPDH, ACTB and RPII. That was to say, comparing gastric cancer with paired peritumoral tissues, a tendency of the target gene CEA for a higher expression in gastric cancer was masked by a lowerr expression of the unconfirmed reference gene GAPDH, ACTB and RPII in gastric cancer itself. The results demonstrated how use of unconfirmed reference genes results in misinterpretation of the expression of the target genes.

Moreover, among these four housekeeping genes, GAPDH and beta-actin were moderately basal expressed in gastric cancer and peritumoral tissues, RPII had a lowest basal expression and 18sRNA was expressed at a highest basal level. These results indicated 18sRNA was a suitable reference gene for the comparison of gastric cancer with paired peritumoral tissues.

With rapid advances in high-throughput gene technologies, researchers have uncovered a large number of non-coding RNAs besides mRNA (the ENCODE Project Consortium, 2007; Mattick et al., 2009; Zhang et al., 2013; Zhao et al., 2013). It is doubtless that these non-coding RNAs play important roles in the occurrence and evolution of biological organisms. During their functional studies, an initial step is always judging the differential expression of a target gene in a particular system. qRT-PCR is a widely and commonly used method to quantify noncoding RNA because of its high sensitivity, good reproducibility and wide dynamic quantification range. The significance of the obtained results strongly depends on the normalization of the data to compensate for differences between the samples (Mattick et al., 2009; Gao et al., 2013; Zhang et al., 2013; Zhao et al., 2013). Thus it is necessary to employ an internal reference gene to calibrate the expression level of this target gene. And selecting a suitable housekeeping gene as internal reference gene is of vital importance. As a typical internal reference gene, such a gene has to maintain a stable expression in all cells and tissues independently of the effects of any influencing factor. However, in fact, the so-called immutable gene is non-existent. While implementing a specific experiment, it is necessary to select a least altering and most stably expressed gene within the system as a candidate internal reference gene. Our study clearly shows that there is no universal reference gene and underline the importance of specific validation of potential reference genes for any experimental condition. We recommend the usage of 18sRNA as normalized gene in analyzing the target gene expression in gastric cancer tissues.

Acknowledgements

This work was partially supported by open project of State Key Laboratory of molecular oncology (Grant No. SKL-KF-2010-06) and Research Foundation of Chinese Medical Association (Y-L2010-002). No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References

Cidón EU1, Bustamante R. (2011). Gastric cancer: tumor markers as predictive factors for preoperative staging. *J Gastrointest Cancer*, **42**, 127-30.

Duffy MJ. (2001). Carcinoembryonic antigen as a marker for colorectal cancer: is it clinically useful? *Clin Chem*, **47**, 624-30.

Gao Y, Li BD, Liu YG. (2013). Effect of miR27a on proliferation and invasion in colonic cancer cells. *Asian Pac J Cancer Prev*, **14**, 4675-8.

Glare EM, Divjak M, Bailey MJ, Walters EH. (2002). Beta-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. *Thorax*, **57**, 765-70.

Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O. (2001). Ribosomal 18S RNA prevails over glyceraldehyde-3- phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. *Anal Biochem*, **295**, 17-21.

Hamalainen HK, Tubman JC, Vikman S, et al (2001). Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. *Anal Biochem*, **299**, 63-70.

Huggett J, Dheda K, Bustin S, Zumla A. (2005). Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun*, **6**, 279-84.

Jemal A, Siegel R, Xu J, Ward E. (2010). Cancer statistics, 2010. *CA Cancer J Clin*, **60**, 277-300.

Koon N, Schneider-Stock R, Sarlomo-Rikala M, et al (2004). Molecular targets for tumour progression in gastrointestinal stromal tumours. *Gut*, **53**, 235-40.

Malati T. (2007). Tumour markers: An overview. *Indian J Clin Biochem*, **22**, 17-31.

Mattick JS (2009). The genetic signatures of noncoding RNAs. *PLoS Genet*, **5**, 1000459.

Radonić A1, Thulke S, Mackay IM, et al (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun*, **313**, 856-62.

Steele BK, Meyers C, Ozbun MA. Variable expression of some ''housekeeping'' genes during human keratinocyte differentiation. *Anal Biochem*, **307**, 341-7.

The ENCODE Project Consortium (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature*, **447**, 799-816.

Vandesompele J, De Preter K, Pattyn F, et al (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*, **3**, 34.

Zhang Q, Geng PL, Yin P, et al (2013). Down-regulation of long non-coding RNA TUG1 inhibits osteosarcoma cell proliferation and promotes apoptosis. *Asian Pac J Cancer Prev*, **14**, 2311-5.

Zhao BS, Liu SG, Wang TY, et al (2013). Screening of microRNA in patients with esophageal cancer at same tumor node metastasis stage with different prognoses. *Asian Pac J Cancer Prev*, **14**, 139-43.