Optimization of internal reference genes for qPCR in human pancreatic cancer research

Wan-Li Ge[1,2#], Guo-Dong Shi[1,2#], Xu-Min Huang[1,2#], Qing-Qing Zong[1,3], Qun Chen[1,2], Ling-Dong Meng[1,2], Yi Miao[1,2], Jing-Jing Zhang[1,2], Kui-Rong Jiang[1,2]

[1]Pancreas Center, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China; [2]Pancreas Institute, Nanjing Medical University, Nanjing 210029, China; [3]Ultrasonography, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China
[#]These authors contributed equally to this work.
Correspondence to: Kui-Rong Jiang; Jing-Jing Zhang. Pancreas Center, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China. Email: jiangkuirong@njmu.edu.cn; zhangjingjing@njmu.edu.cn.

Background: Pancreatic cancer (PC) has been becoming a common cancer with high mortality and quantitative real-time polymerase chain reaction (qPCR) is one of the best choices for researching gene expression. Internal reference genes, such as actin beta (ACTB) and glyceraldehyde-3-phosphatide hydrogenase (GAPDH) have long been used in relative quantification analysis. But evidence shows that some internal reference genes expression may vary in different tissues, cell lines and different conditions. The present study aimed to find more stable internal reference gene for qPCR experiment in PC.
Methods: Total RNA of human PC tissues were prepared using TRIZOL reagent. qPCR was performed using FastStart Universal SYBR Green Master to reflects the expression of target genes. Normfinder and geNorm were used to analyze the stability of chosen internal reference genes
Results: According to the results of NormFinder and geNorm, eukaryotic translation initiation factor 2B subunit alpha (EIF2B1) and importin 8 (IPO8) were the same most stable internal reference genes in PCs and non-neoplastic tissues. In addition, EIF2B1 and IPO8 remained the most stable internal reference genes only in PCs. Using a normalization factor NF2 by geNorm as reference, the normalized GAPDH and ACTB expression levels were obviously up-regulated by 3.29- and 2.23-fold change, meanwhile ribosomal protein S17 (RPS17) were down-regulated by 0.77-fold change in PCs comparing with corresponding adjacent tissues.
Conclusions: The use of the combination of EIF2B1 and IPO8 would provide more stable results in differential expression analysis and prognostic analysis of PC.

Keywords: Pancreatic cancer (PC); quantitative real-time polymerase chain reaction (qPCR); internal reference gene; normalization

Introduction

Pancreatic cancer (PC) is the third leading cause of cancer mortality followed by lung cancer and colorectal cancer in the United States (1). The incidence and mortality have also been increasing in China over the years (2). PC shows no symptom in the early stage and surgery is the only way to cure it. However, majority of the patients were diagnosed at a distant stage and could not be surgically resected (3). The 5-year overall survival rate remains low at about 8% (4).

Quantitative real-time polymerase chain reaction (qPCR) is a relatively easy and common technique that is widely used to detect gene expression in molecular biology studies. The advantages of qPCR are its high accuracy, enhanced sensitivity, and rapid speed (5). The qPCR experiment is depended on the bond of fluorescent dyes into amplified

products exponentially (6). The ideal internal reference gene should be expressed in all kinds of tissues and cells under various experimental conditions and its expression is similar without significant difference (7). There is also a requirement that there be no coamplification of pseudogenes, overlapping genes or other genes to avoid unexpected products of the genome which may result in inaccuracy (8,9). A reliable internal reference gene is of great importance for qPCR because the expression of one gene of interest (GOI) is normalized by selected reference gene (10). In general, some housekeeping genes, such as 18S ribosomal RNA (*RS18*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and actin beta (*ACTB*) are often used as internal reference genes for relative quantification analysis currently. However, evidences show that the expression of these reference genes may vary in different tissues, cell lines and different conditions (11-13). Our previous microarray study also found that *ACTB* and *GAPDH* were up-regulated in PCs comparing with adjacent non-neoplastic tissues (*Figure S1*) (14). Meanwhile, the effect of qPCR may be hindered by some technical challenges such as sample preparation, storage and use of unspecific primers.

By reviewing the literature on the selection of internal reference genes in qPCR, and combining with the result of our previous microarray analysis, we included 11 most frequently used internal reference genes with non-difference or small difference between PCs and adjacent non-neoplastic tissues, which were *RS18*, *ACTB*, beta-2-microglobulin (*B2M*), *GAPDH*, ribosomal protein L13a (*RPL13A*), eukaryotic translation initiation factor 2B subunit alpha (*EIF2B1*), hydroxymethylbilane synthase (*HMBS*), importin 8 (*IPO8*), RNA polymerase II subunit A (*POLR2A*), ribosomal protein S17 (*RPS17*) and TATA-box binding protein (*TBP*). They were supposed to optional internal reference gene for relative quantification analysis. The aim of our study was to analyze the stabilities and look for the optimal internal reference gene of these 11 genes for relative quantification analysis by qPCR in 16 pairs of human PCs and non-neoplastic tissues.

Methods

Human tissue samples

At first 20 pairs of PCs and adjacent non-neoplastic tissues were collected from patients undergoing surgery in the Pancreas Center of the First Affiliated Hospital of Nanjing Medical University between November 2013 and January 2014. No chemotherapy or radiotherapy was performed before tumor removal. The pathology of all tissue specimens was diagnosed by pathologists. All resected specimens were taken immediately during operation and frozen quickly by liquid nitrogen until RNA extraction. Only histologically verified PCs and non-tumor tissues were used in this study. All patients signed informed consent for the use of clinical specimens for research and the Ethics Committees of the First Affiliated Hospital of Nanjing Medical University approved the study.

Internal reference gene selection and primer design

The primers of *RS18* and *ACTB* were supplied by Sangon Biotech (Shanghai, China). The primers of *GAPDH*, *B2M* and *RPL13A* were referenced in the literature (15). The primers of remaining 6 pairs of internal reference genes were designed by using Primer Premier 5 and following principles: (I) specific primers were designed across intron as far as possible. In this way, the size of the fragments amplified from cDNA and genomic DNA was different, and the influence of genomic DNA was eliminated by limiting the amplification of large fragments in genome by appropriate extension time; (II) the amplification products were as short as possible in order not to affect the amplification efficiency; (III) the amplification region was designed at the 3'-end as far as possible for reducing the effect of sample degradation; (IV) other requirements: the length of primers was 18–25 bp; the Tm value was 58–62 ℃; the Tm value of forward and reverse primers was no more than 2 ℃; the content of GC% was 40–65%; there was no dimer and hairpin as far as possible; the length of products was limited to 50–350 bp. To meet above mentioned requirements, base modification could be performed at 5'-end. After the primers were qualified, they were synthesized by Nanjing Realgene Biotechnology.

RNA extraction and reverse transcription

Total RNA samples were prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and then stored at –80 ℃. The concentration and purity of the isolated RNA were determined by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). Samples under the A260/A280 ratio of 1.8 (n=4) and corresponding cancer tissues or non-neoplastic tissues were excluded from

the 20 pairs of PCs and adjacent non-neoplastic tissues for further analysis. After spectrophotometric quantification, 2 µg of total RNA was used for reverse transcription in a final volume of 40 µL with PrimeScript™ reverse transcription Master Mix (TAKARA, Tokyo, Japan) according to the manufacturer's instructions in PTC-200 PCR instrument (Bio-Rad, Hercules, CA, USA). In order to make the input of RNA among different samples as close as possible for reducing experimental error, the concentration of aliquot approximate 3.5 µg RNA of each sample was diluted to 62.5–100 ng/µL (the least concentration of RNA was 62.5 ng/µL because there is a requirement to add 8 µL 5× reverse transcription reagent and the maximum volume of 2 µg RNA is 32 µL). Next, cDNA product from 2 ng RNA was diluted to 100 µL for 100 reactions.

Real-time qPCR and quantification

qPCR was performed using FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) in a StepOne Plus Real-time qPCR System (Thermo Fisher Scientific). Reactions were performed in a volume of 10 µL containing 1 µL diluted cDNA (equivalent to 20 ng RNA), 2× SYBR Green Master. Thermal cycling conditions consisted of an initial denaturation step at 95 ℃ for 10 min, 40 cycles at 95 ℃ for 15 s, and 60 ℃ for 1 min, a melting curve analysis step at 95 ℃ for 15 s, 60 ℃ for 1 min, and 95 ℃ for 15 s. SYBR Green Master was used to detect 11 candidate internal reference genes. All samples for each reference gene were run on the same plate, and different reference genes were run on separate plates. Each qPCR was performed in triplicate. The genes are quantified on the same batch of cDNA to minimize experimental variation according to the manufacturer's instructions.

Evaluation of stabilities of internal reference genes

Two software programs, including NormFinder (16) and geNorm (15) were used to analyze the stabilities of the 11 candidate internal reference genes. In both programs, ΔCt was calculated [ΔCt = sampleCt – minCt (the lowest average Ct from all tested samples of each internal reference gene)]. Then, $2^{-\Delta Ct}$ was calculated to input into the Microsoft Excel to perform programs according to requirement. NormFinder program ranks the candidate genes by calculating the intra-group variance and inter-group variance of each gene in two groups of PCs and adjacent non-neoplastic samples. GeNorm program also

ranks the genes according to the gene expression stability measure M-value which is the average pairwise variation V of a particular gene with all other tested reference genes. The gene with the highest M-value was stepwise excluded. GeNorm expects to find two best genes with the same expression ratio in all samples. If the variation of expression ratios increases, the expression stability of the gene decreases.

Results

The specificity of qPCR amplification

A total of 11 candidate internal reference genes were evaluated in 16 pairs (4 of the 20 pairs were excluded) of PCs and adjacent non-neoplastic tissues in this study. The full gene name, ID number, primer sequences, location of probe indicating whether primers span an intron, amplicon length and melting temperature were shown in *Table 1*. As expected, the expression of *RS18* was abundantly expressed in samples. *RPL13A*, *ACTB*, *B2M* and *GAPDH* were highly expressed with median Ct between 19 and 23, and the expression levels of the rest genes were low with Ct between 25 and 28 (*Figure 1A*). The melting curve analysis of each gene all revealed a single peak, which suggested the specificity of qPCR amplification (*Figure 1B,C,D,E,F,G,H,I,J,K,L*).

The results of NormFinder algorithm

NormFinder evaluated the stabilities of these 11 genes and calculated the intra-group variance and inter-group variance of each gene in two groups (*Figure 2A*). To investigate the expression of GOI in PCs and adjacent non-neoplastic tissues, we hoped that the expression of internal reference gene in two groups were consistent as far as possible. So the ideal internal reference gene should meet the conditions that the inter-group variance was as close to zero as possible, and at the same time the intra-group variation was as small as possible. Observed from *Figure 2A*, *TBP*, *POLR2A*, *HMBS*, *IPO8* and *EIF2B1* were the top ranked candidate genes.

The results of geNorm algorithm

The stability M value of each internal reference gene is equal to the arithmetical mean of the standard deviation of the log2 transformed expression ratio between each gene

Table 1 Eleven candidate internal reference genes evaluated in this study

Gene symbol	Gene name	ID	Forward primer	Reverse primer	Location of probe	Amplicon length (bp)	Tm (°C)
RS18	18S ribosomal RNA	No	CAGCCACCCGAGATTGAGCA	TAGTAGCGACGGGCGGTGTG	Not applicable	253	85
RPL13A	Ribosomal protein L13a	23521	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA	Exon 7–8 boundary	126	77.38
ACTB	Actin beta	60	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG	Exon 5–6 boundary	205	84.25
B2M	Beta-2-microglobulin	567	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT	Exon 4	86	79.04
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	2597	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	Exon 7–8 boundary	87	79.17
RPS17	Ribosomal protein S17	6218	CCCCCGAGGTATCTCCATCA	CCCCCCAGCATTTCCTTAGT	Exon 3–4 boundary	129	77.41
POLR2A	RNA polymerase II subunit A	5430	TTGACTTGCGTTTCCACCC	AGAATGCGGACCCGATGC	Exon 8–9 boundary	151	81.14
EIF2B1	Eukaryotic translation initiation factor 2B subunit alpha	1967	GGAAGGAGTTGTTGAAAACGGAG	GGAAGGGGCAGTGTAGTCG	Exon 7–9 boundary	247	80.09
IPO8	Importin 8	10526	GCAGTAGATGCTGTGGTGGG	GGGTTGTCAAGGTCAAGTGGAG	Exon 23–24 boundary	305	78.01
TBP	TATA-box binding protein	6908	CGTGCTCACCCACCAACAA	CGCAGGAAATAACTCTGGCTCATA	Exon 6–7 boundary	52	72.63
HMBS	Hydroxymethylbilane synthase	3145	CCCTGGAGAAGAATGAAGTGG	ATTTTGGGTGAAAGACAACAGC	Exon 6–7 boundary	135	80.38

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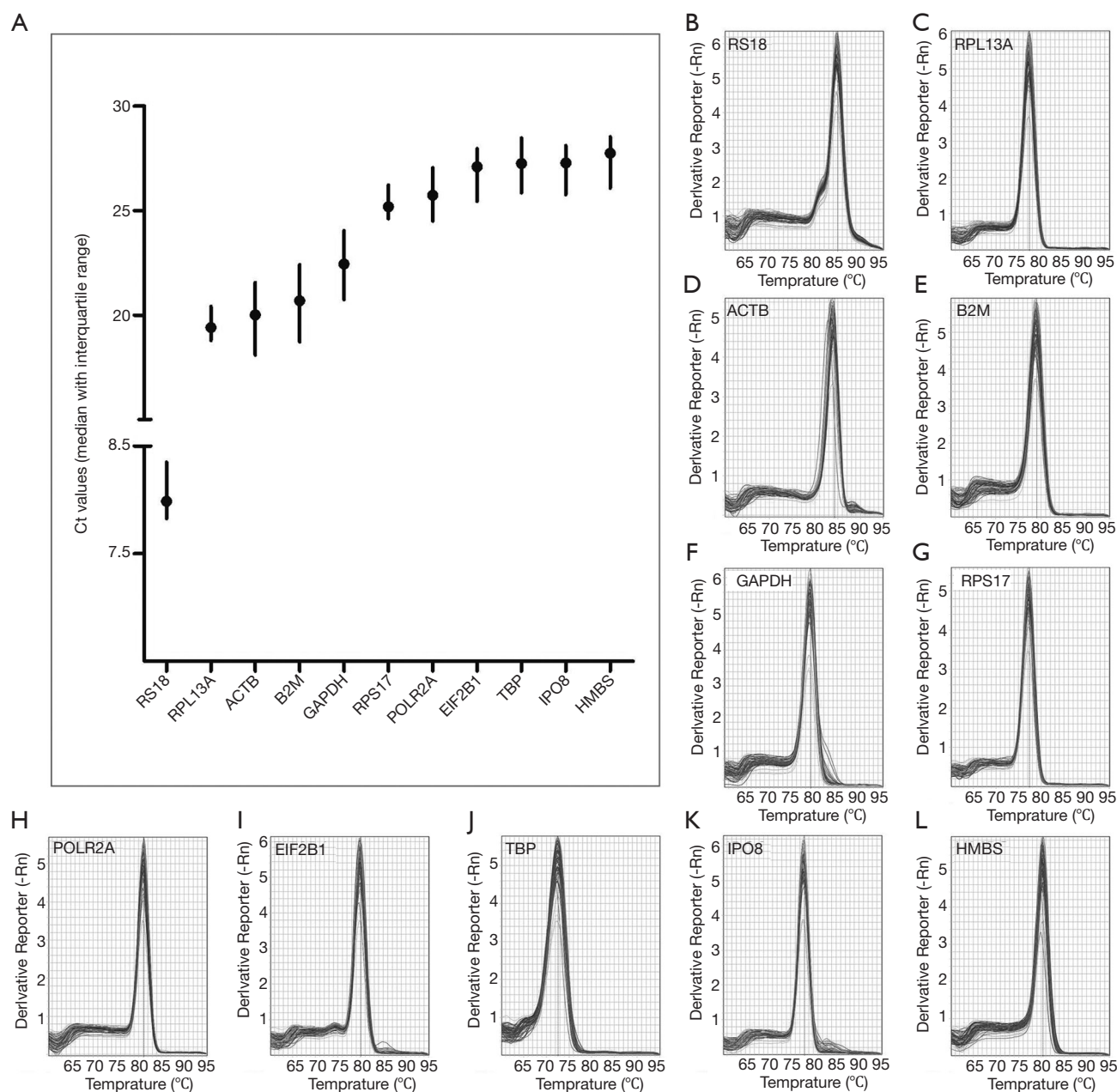Ge et al. Internal reference optimization for qPCR in PC

Figure 1 Median Ct values and melt curve analysis of candidate internal reference genes expressed in 16 pairs of histologically verified pancreatic cancers and adjacent non-neoplastic tissues. (A) Variation in 11 internal reference gene expression. Y-axis represents median Ct with interquartile range and X represents genes. (B,C,D,E,F,G,H,I,J,K,L) The specificity of qPCR amplification observed from the plots for 11 internal reference genes. Y-axis represents delta Rn and X represents temperature (℃). Delta Rn values represent the magnitude of the signal generated by the given set of PCR conditions. Sixteen pairs of histologically verified PCs and adjacent non-neoplastic tissues were used.
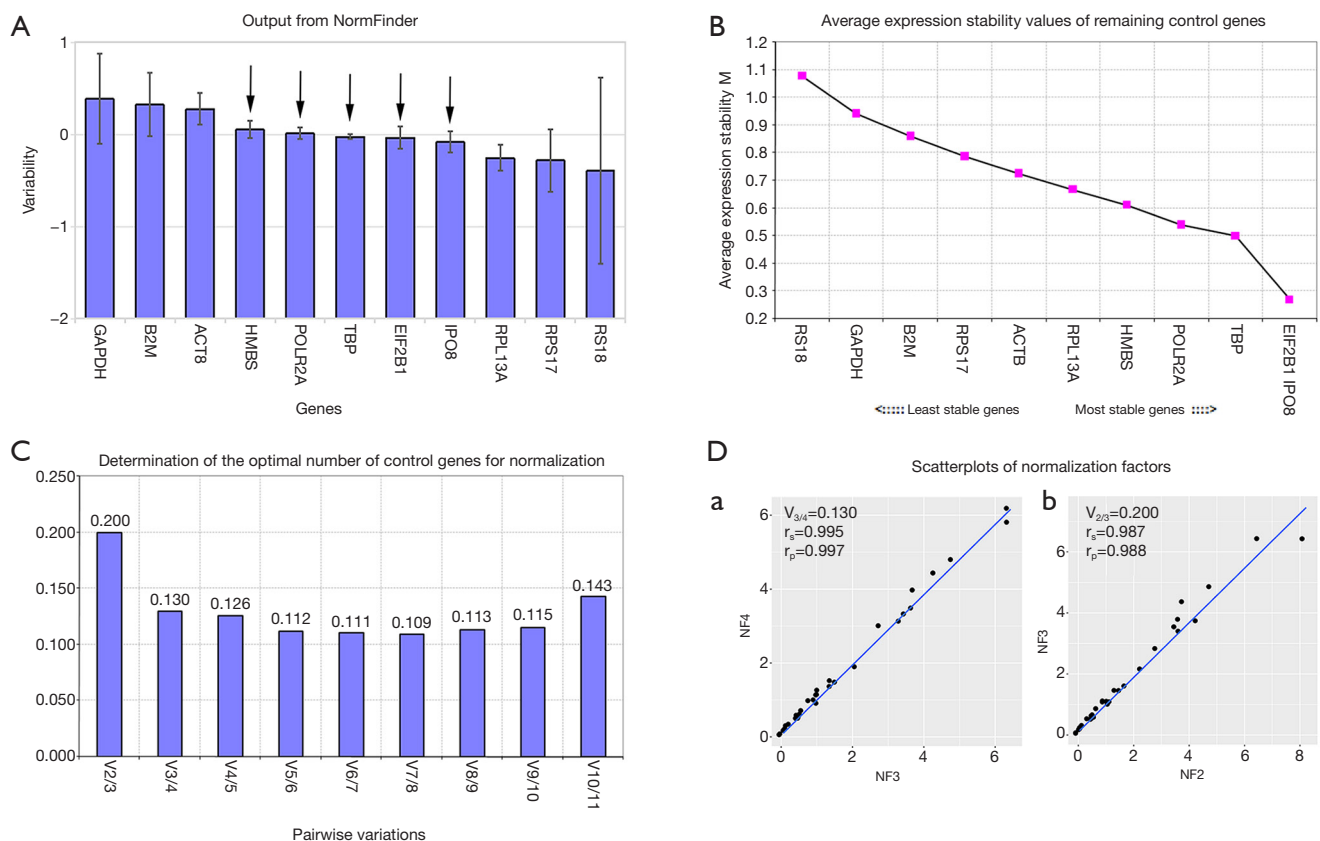
Figure 2 The evaluation of stabilities of 11 candidate internal reference genes expressed in 16 pairs of histologically verified pancreatic cancers and adjacent non-neoplastic tissues. (A) Output of NormFinder. Y-axis represents the inter-group variance of each gene in two groups of pancreatic cancers and adjacent non-neoplastic samples. Error bars represent the average of the inter-group variances of each gene in two groups. The candidate gene with an inter-group variance as close to zero as possible, and having as small errors bars as possible will be picked by NormFinder. The arrows indicate several internal reference genes with low variability. (B) Average expression stability values (M) of remaining control genes. The gene with the highest M-value was stepwise excluded. (C) Determination of the optimal number of control genes for normalization. Y-axis represents pairwise variation between the normalization factor NFn+1 and NFn. When it first appears that Vn/n+1 >0.15 and at the same time Vn+1/n+2 <0.15, n+1 is the optimal number of reference genes for normalization. (D) Scatterplots of normalization factors between NFn (x-axis) and NFn+1 (y-axis) when it includes an (n+1)th most stable control gene. Vn/n+1 represents pairwise variation (n=2 or 3, r_s = spearman correlation coefficient, r_p = person correlation coefficient). The spearman correlation coefficient (r_s) between NF2 and NF3 was up to 0.987. So we chose the NF2 to replace the NF3 to test the expression of one gene of interest.

and all other genes in the entire samples. The lower M value of the gene is, the higher stability is. The result of geNorm showed that the expression of EIF2B1 and IPO8 was the most stable (*Figure 2B*).

In addition, geNorm also had the function on how to select the optimal combination of internal reference genes. Because the V2/3 value was greater than 0.15 and the V3/4 value was less than 0.15, the normalization factor should contain at least the 3 best reference genes including EIF2B1, IPO8 and the TBP (*Figure 2C*). Moreover, according to manufacturer's instructions, 0.15 is not a

non-adjustable cut-off value. Through the calculation of correlation analysis, we found that the consistency between NF2 and NF3 was so high and the spearman correlation coefficient (r_s) was up to 0.987 (*Figure 2D*). So we chose a NF2 by geNorm or calculated the geometric mean of two internal reference genes (i.e., EIF2B1 and IPO8) to test the expression of GOI.

We also considered whether the most stable genes—EIF2B1 and IPO8 expressed in two groups were stable in individual PC samples for GOI's prognostic analysis (*Figure 3*). The result was that nothing changed.

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A

Average expression stability values of remaining control genes

B

Determination of the optimal number of control genes for normalization
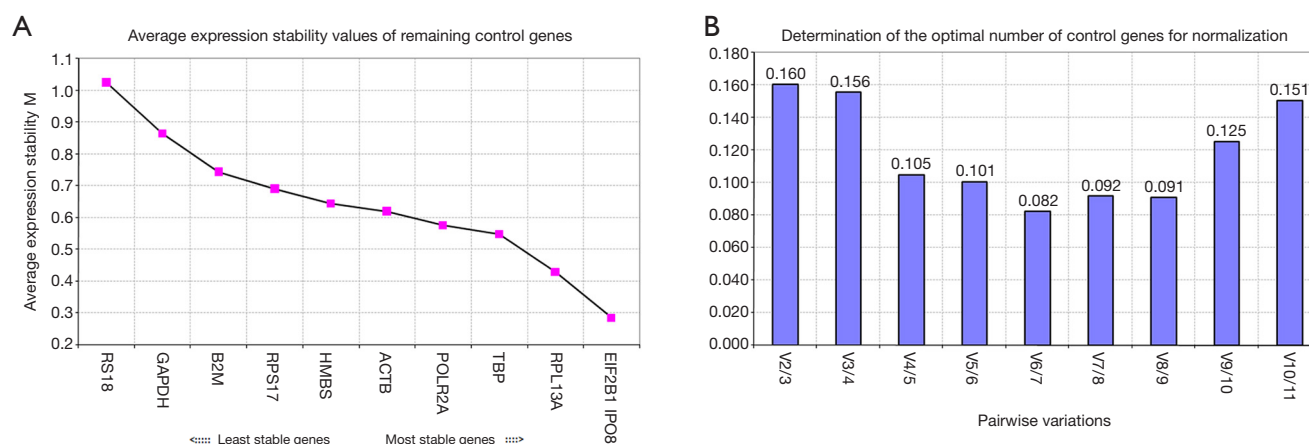

Figure 3 The evaluation of stabilities of 11 candidate internal reference genes expressed in 16 pancreatic cancers by geNorm. (A) Average expression stability values (M) of remaining control genes. The gene with the highest M-value was stepwise excluded. (B) Determination of the optimal number of control genes for normalization. Y-axis represents pairwise variation between the normalization factor NF_{n+1} and NF_n. When it first appears that $V_{n/n+1} > 0.15$ and at the same time $V_{n+1/n+2} < 0.15$, n+1 is the optimal number of reference genes for normalization.
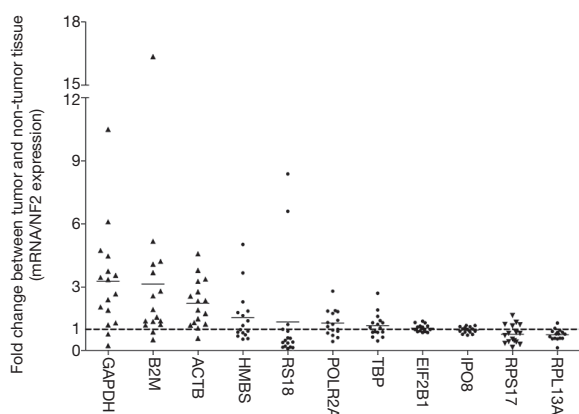

Figure 4 Re-quantification of internal reference genes by the normalization factor NF2. The normalization factor NF2 was calculated by geNorm. The horizontal line segments represent mean fold change. Sixteen pairs of histologically verified PCs and adjacent non-neoplastic tissues were used. Genes with significant P value in paired *t*-test were labeled with triangular spots.

Re-quantification of internal reference genes

Hence, after calculation of a NF2 from the expression levels of EIF2B1 and IPO8 by geNorm, we computed the normalized levels of these 11 candidate internal reference genes again (*Figure 4*). The normalized *GAPDH* and *ACTB* expression levels were obviously up-regulated by 3.29- and 2.23-fold change, meanwhile *RPS1*7 were down-regulated

by 0.77-fold change in PCs comparing with corresponding adjacent tissues.

Discussion

The stability of internal reference gene is of great importance for relative quantitative analysis of gene expression. However, many researchers have found that the internal reference gene expression can vary considerably in distinct tissues, cell lines and conditions. Therefore, to get accurate results, it is necessary to evaluate whether the selected reference gene is expressed stably and find more stable internal reference gene for qPCR experiment.

In this study, 11 common internal reference genes on the histological verified 16 pairs of PCs and non-neoplastic tissues were used for qPCR in order to find reliable internal reference gene for relative quantification analysis. In consideration of many factors, such as sample storage method, temperature and laboratory reagent may affect the results, samples preparation, RNA extraction, reverse transcription and real time qPCR were all performed under the same standard in our study. Total RNA was extracted according to the manufacturer's protocol strictly. The concentration and purity of the isolated RNA were determined by measuring the absorbance values. Only extracted RNA with satisfying concentration and high purity was used in next steps. After the design of primers, all amplification products of these primers were compared with the reference sequence of the

human genome by using the Ensembl tool BLAST. Although both the Ensembl database and the NCBI website could do this blast comparison work, the annotation information of the genome was updated more comprehensively in the Ensembl database. So we used it and knew which transcripts were amplified in advance and whether the amplification products included other pseudogene, overlapping gene and so on. The single peak observed from melt curve analysis suggested the specificity of these primers.

NormFinder and geNorm were two of the most commonly used softwares for exploring the stabilities of the candidate internal reference genes. The NormFinder algorithm focus more on inter-group and intra-group variances, making it possible to confirm reference genes in different sample groups, but the program is less sensitive to co-regulation. The geNorm algorithm calculates pairwise variation of each selected gene with all other internal reference genes included in the study and then uses the average of the pairwise variation as the stability M-value for each internal reference gene. For geNorm analysis, 10 samples are the minimum recommended. When estimating expression stability in PCs and adjacent non-neoplastic tissues, we used 32 samples that exceeded minimum sample size, and made sure that the number of samples from each group was same in order to avoid bias. The stabilities of 11 common internal reference genes, including *ACTB*, *B2M*, *EIF2B1*, *GAPDH*, *HMBS*, *IPO8*, *POLR2A*, *RPL13A*, *RPS17*, *RS18* and *TBP* were investigated in this study. According to the results of NormFinder, *TBP*, *POLR2A*, *HMBS*, *IPO8* and *EIF2B1* were suitable reference genes with high stability. But it didn't offer the number of genes and quantitative method in the best combination. geNorm could cover the shortage, and it recommended *EIF2B1* and *IPO8* as the best internal reference genes with high stabilities. There are only limited studies to assess the stabilities of internal reference genes in PCs. Kosinová *et al.* [2016] found that unstable expression of commonly used reference genes in rat pancreatic islets early after isolation affected results of gene expression studies (17). *RPL13A* was the most suitable reference gene in mouse acute pancreatitis among 10 reference genes including *ACTB* and *GAPDH* (18). Mohelnikova-Duchonova *et al.* investigated stabilities of 24 reference genes in human pancreas tissue (19). They found both *ACTB* and *GAPDH* were considered the least stable genes, and *EIF2B1* exhibited the most stable gene while *IPO8* showed medium expression stability. Only 10 pairs of samples in their study were tested and fewer than ours. Our single center operated several hundred cases of pancreatic

surgery every year, so the source of samples was sufficient. And the collection, preservation and extraction of samples were basically the same in order to reduce bias. *EIF2B1* and *IPO8* performed excellent stabilities in other cancers, such as non-small cell lung cancer (20) and meningiomas (21). We also considered whether most stable genes expressed in both PCs and adjacent non-neoplastic groups were stable in individual PC samples for GOI's prognostic analysis. The result showed that the combination of *EIF2B1* and *IPO8* were also suitable. We also used a NF2 by geNorm as a composite variable to normalized the expression of 11 reference genes. The *GAPDH* and *ACTB* expression levels were actually up-regulated within experimental validation. It reflected that some common reference genes were not stable in PCs and should not be utilized without validation.

In conclusion, the use of the combination of *EIF2B1* and *IPO8* would provide more stable results in differential expression analysis and prognostic analysis of PCs.

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2020.02.48). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee of the First Affiliated Hospital with Nanjing Medical University (2017-SR-171).

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All patients signed informed consent for the use of clinical specimens for research.

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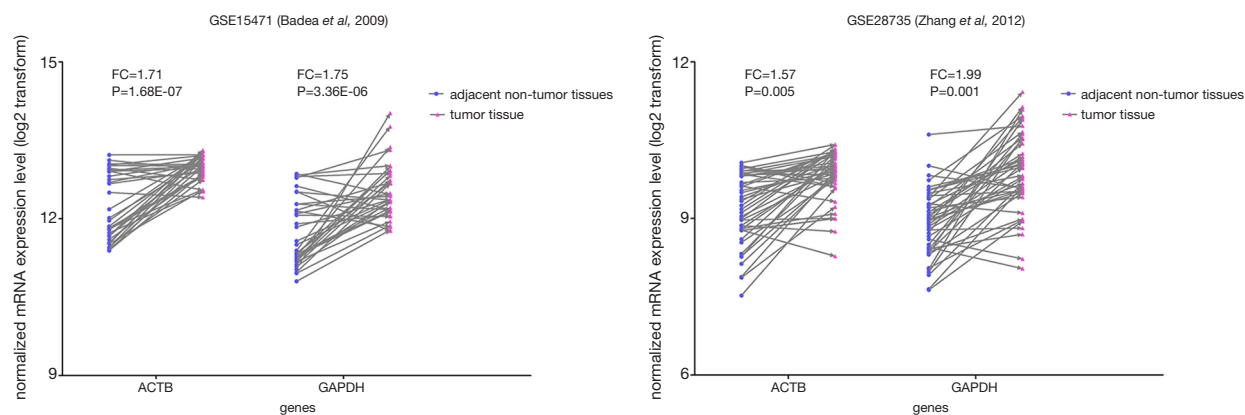
Figure S1 The expression of *ACTB* and *GAPDH* in pancreatic cancers (PCs) and matched adjacent non-neoplastic tissues observed from transcriptional microarrays. The post-normalized matrix data was used. If multiple probes were annotated with the same gene, their mean value was computed to represent expression level of this common gene. Gene with P-value of paired *t*-test <0.05 was considered as differentially expressed gene. According to the result, *ACTB* and *GAPDH* were up-regulated in PCs comparing with non-neoplastic tissues.