bjh research paper

Selection of reference genes for quantitative polymerase chain reaction studies in purified B cells from B cell chronic lymphocytic leukaemia patients

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Summary

The clinical heterogeneity of B-cell chronic lymphocytic leukaemia (B-CLL) makes it necessary to identify potent prognostic indicators to predict individual clinical course and select risk-adapted therapy. In recent years, numerous gene expression models have been suggested as prognostic factors of B-CLL. Today, quantitative polymerase chain reaction (qPCR) is a preferred method for rapid quantification of gene expression and validation of microarray data. The reliability of qPCR data is highly dependent on the use of appropriate reference genes for normalization. To date, no validated reference genes have been reported for the normalization of gene expression in B-CLL. Therefore, the present study was conducted to identify suitable reference genes for gene expression studies in $CD19^+$ B cells isolated from B-CLL patients' peripheral blood. The stability of *ACTB*, *B2M*, *GAPDH*, *GUSB*, *HMBS*, *HPRT1*, *MRPL19*, *TBP* and *UBC* genes was determined by three different descriptive statistics, geNorm, NormFinder and BestKeeper-1, which produced highly comparable results. Based on our results, *B2M*, *HPRT1*, and *GUSB* were found to be the most suitable reference genes for qPCR studies in B-CLL patients' peripheral blood B cells.

Keywords: B-CLL, B cells, qPCR, reference genes, normalization.

B-cell chronic lymphocytic leukaemia (B-CLL) is the most common form of adult leukaemia in the Western world. It follows a highly heterogeneous clinical course; some patients survive for many years without any treatment, whereas others progress very quickly and die within a few years (Zenz *et al*, 2010). To predict individual clinical course and select optimal therapy, powerful prognostic factors are needed. The clinical heterogeneity of B-CLL was shown to correlate with the pattern of genetic changes (Stilgenbauer *et al*, 2002). Gene expression profiling of B-CLL samples using microarray platforms identified a number of genes that appeared to be under- or overexpressed in B-CLL. (Klein *et al*, 2001; Rosenwald *et al*, 2001; Jelinek *et al*, 2003; Falt *et al*, 2005). Based on these findings, the role of gene expression levels in B-CLL prognosis assessment has been intensively studied (Oppezzo *et al*, 2005; Joshi *et al*, 2007; Stamatopoulos *et al*, 2007; Van Bockstaele *et al*, 2007; Nuckel *et al*, 2009). The high expression of *ZAP70* (coding for tyrosine-protein kinase ZAP-70) (Catherwood *et al*, 2006; Stamatopoulos *et al*, 2007) and *AICDA* (coding for activation-induced cytidine deaminase) (Palacios *et al*, 2010) was found to be associated with unfavourable prognosis of B-CLL. Similar observations were made for lipoproteinlipase (encoded by *LPL*) and disintegrin and metalloproteinase domain-containing protein 29 (encoded by *ADAM29)* (Oppezzo *et al*, 2005; Van Bockstaele *et al*, 2007). All these data indicate a significant role of gene expression parameters in the evaluation of biological mechanisms of B-CLL and identification of potential markers for prognostic assessment.

Today quantitative polymerase chain reaction (qPCR) has become a method of choice for gene expression studies. In addition, it is also used to validate microarray data. Most frequently, gene expression quantification involves the analysis of target gene expression relative to a reference gene. This allows control for possible non-biological variations, as the reference genes are exposed to the same preparation steps as

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the gene of interest. An ideal reference gene has to be expressed constitutively and uniformly in all test samples. Significant fluctuations or changes in the expression of chosen reference genes between samples can lead to the loss of detection of small differences between expression of the genes of interest or to other erroneous results. Therefore, it is extremely important to find appropriate reference genes with minimal variability between the test samples. However, this task is often omitted and many studies have used previously published reference genes without appropriate analysis of their expressional stability in the test tissue.

The accurate selection of the reference control for gene expression studies in B-CLL is crucial as sometimes even small changes in the gene expression level may differentiate patient groups with different prognosis (Catherwood *et al*, 2006; Stamatopoulos *et al*, 2007; Van Bockstaele *et al*, 2007). To the best of our knowledge, there have been no reports on the suitability of reference genes for qPCR studies in B-CLL samples.

The aim of this research was to select the reference genes best suited for gene expression studies in B cells purified from B-CLL patients' blood. The expression stability of nine potential reference genes was compared using BestKeeper-1, NormFinder and geNorm applications.

Materials and methods

Samples

Peripheral blood samples were obtained from 30 B-CLL patients. The diagnosis of B-CLL required a persistent lymphocytosis of more than $5{\cdot}0 \times 10^9$/l and a typical $CD5^+$, $CD19^+$, $CD20^+$ (low), $CD23^+$, sIg low, $CD79b^+$ (low) immunophenotype as revealed by flow cytometry of peripheral blood cells. $CD19^+$ cells from blood samples were isolated by positive immunomagnetic selection with Dynal® magnetic beads against CD19 (Invitrogen, Carlsbad, CA, USA) according to the vendor's recommendations. CD19 selection typically resulted in >97% purity as assessed by flow cytometry. The study was approved by the Lithuanian Bioethics Committee and all patients gave informed consent to use their blood samples.

Primers

Primer pairs for *B2M*, *HPRT1*, *GUSB*, *MRPL19*, *HMBS* and *UBC* were designed using Primer3Plus software (Untergasser *et al*, 2007) taking into account primer dimer, self-priming formation, and primer melting temperature. Primers for *ACTB*, *GAPDH* and *TBP* were published previously (Lossos *et al*, 2003; Zhang *et al*, 2004). All primer pairs were chosen to span an exon-intron boundary to exclude amplification of genomic DNA. The non-specific amplification was tested on genomic DNA (gDNA) samples. Primers for *ACTB*, *GAPDH*, *GUSB* and *TBP* were synthesized by Integrated DNA Technologies (Coralville, IA, USA) whereas primer pairs for *B2M*, *HMBS*, *HPRT1*, *MRPL19* and *UBC* were synthesized by Metabion (Martinsried, Germany). Primer sequences and GenBank accession numbers of selected reference genes are listed in Table I.

qPCR analysis

Total RNA was extracted from purified $CD19^+$ cells using a QIAmp RNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of each RNA sample was evaluated spectrophotometrically by Smart-Spec Plus (Bio-Rad Laboratories, Hercules, CA, USA). First strand cDNA synthesis was carried out in triplicates using 1 μg of RNA, random hexamers and First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's recommendations. After the reaction, cDNA samples were diluted up to 60 μl with nuclease-free water, triplicate samples were pooled, aliquoted and stored at −20°C. To minimize experimental variation all reference genes were quantified on the same batch of cDNA. For each reference gene all cDNA samples were amplified in the same qPCR run. All samples were amplified in duplicates, as well as non-template control (NTC) samples without cDNA to check for reagent contamination.

qPCR reactions were performed in a 20 μl reaction volume containing Maxima™ SYBR Green I qPCR Master Mix (Fermentas), 0·2 μmol/l final concentration of each primer, and 5 μl cDNA solution. Uracil-DNA glycosylase (Fermentas) was added into each reaction mix (0·4 units) to prevent qPCR cross-contamination.

qPCR was performed on RotorGene 6000 (Corbett Life Science, Australia) using the following cycling conditions: 2 min at 50°C followed by 5 min at 95°C and 45 amplification cycles at 95°C for 20 s, 60°C for 15 s and 72°C for 20 s. The melting curve analysis involved a rise of temperature to 94°C for 5 s, cooling to 65°C for 90 s, and then heating back to 95°C while continuously monitoring fluorescence.

qPCR amplification efficiencies for all primer pairs were evaluated using the serial 10-fold dilutions of the same cDNA sample (a pool of cDNAs prepared from $CD19^+$ cells purified from five B-CLL patients' blood samples). Amplification curves were plotted using RotorGene 6000 software and amplification efficiencies were calculated automatically from raw fluorescence data taken from the RotorGene 6000 detection system.

Data analysis

Individual quantification cycle (C_q) values were obtained by setting a threshold manually and obtained mean C_q values were used for further analysis. Data from RotorGene 6000 were imported into Microsoft Excel and transformed to relative quantities Q using $Q = E^{(\min C_q - \text{sample} C_q)}$ equation, where amplification efficiency (E) was specific for each

Table I. Characteristics of qPCR primer pairs.

Gene symbol	Gene name	GenBank number	Primer sequence (5′-3′)	Amplicon size (bp)	qPCR amplification efficiency (%)
ACTB	Actin, beta	NM_001101	F- CCCCGCGAGCACAGA R- CCACGATGGAGGGGAAGAC	171	88·9
B2M	Beta-2-microglobulin	NM_004048	F- CACCCCCACTGAAAAAGATGAG R- CCTCCATGATGCTGCTTACATG	106	102
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	F- CCCGCGAGCACAGA R- CCACGATGGAGGGGAAGAC	122	99·6
GUSB	Glucuronidase, beta	NM_000181	F- GAAAATACGTGGTTGGAGAGCTCATT R- CCGAGTGAAGATCCCCTTTTTA	101	99·5
HMBS	Hydroxymethyl-bilane synthase	NM_000190	F- TGCCAGAGAAGAGTGTGGTG R- CTGAACTCCTGCTGCTCGTC	142	99·6
HPRT1	Hypoxanthine guanine phosphoribosyl transferase I	NM_000194	F- ATGACCAGTCAACAGGGGAC R- TGCCTGACCAAGGAAAGCAA	136	99·9
MRPL19	39S ribosomal protein L19, mitochondrial	NM_014763	F- GGGATTTGCATTCAGAGATCAGG R- CTCCTGGACCCGAGGATTATAA	117	99·7
TBP	TATA box binding protein	NM_003194	F- GCACAGGAGCCAAGAGTGAA R- TCACAGCTCCCCACCATATT	127	98·1
UBC	Ubiquitin C	NM_021009	F- TGGGTCGCAGTTCTTGTTTG R- CCTTCCTTATCTTGGATCTTTGCC	150	99·8

Table II. Descriptive statistics of the tested reference genes.

	GUSB	*ACTB*	*GAPDH*	*HMBS*	*TBP*	*HPRT1*	*B2M*	*UBC*	*MRPL19*
Amplification efficiency (*E*)*	1·996	1·889	1·996	1·997	1·982	2·000	2·022	2·044	1·999
Geometric mean (C_q)†	25·29	19·71	18·60	23·18	24·71	23·75	16·40	19·40	25·74
Arithmetic mean (C_q)†	25·31	19·81	18·62	23·23	24·97	23·77	16·42	19·50	25·75
Minimum (C_q)†	23·42	16·29	17·08	19·49	21·15	22·00	14·13	13·63	23·96
Maximum (C_q)†	27·54	25·02	20·92	26·95	31·83	25·06	17·78	22·64	27·43
M value‡	1·303	1·812	1·276	1·454	3·643	1·274	1·304	1·947	1·353
Stability value§	0·113	0·150	0·144	0·151	0·233	0·111	0·099	0·172	0·159
SD_{Cq}†	0·686	1·547	0·800	1·086	3·206	0·715	0·723	1·532	0·678
SD threshold	1·003	1·090	1·003	1·002	1·013	1·000	0·984	0·969	1·001
CV (%C_q)†	2·71	7·81	4·30	4·67	12·84	3·01	4·40	7·86	2·63

C_q, quantification cycle; SD_{Cq}, C_q standard deviation; SD threshold, ln2/lnE; CV, C_q coefficient of variation.
Calculated by RotorGene 6000*, BestKeeper-1†, geNorm‡ and NormFinder § softwares.

reference gene. The data obtained were converted into correct input files, according to the requirements of the particular software, and analysed using three different Visual Basic for Applications (VBA) applets, geNorm (version 3.5) (Vandesompele *et al*, 2002), NormFinder (Pfaffl *et al*, 2004) and BestKeeper-1 (Andersen *et al*, 2004).

Results

Expression profile of reference genes

In order to identify the best reference genes for gene expression studies in B cells from B-CLL patients, we designed a qPCR assay based on SYBR Green I detection for the expression analysis of nine selected genes (*ACTB*, *B2M*, *GAPDH*, *GUSB*, *HMBS*, *HPRT1*, *MRPL19*, *TBP* and *UBC*). The amplification specificity for each primer pair was confirmed by a single band of expected size in agarose gel electrophoresis and by a single-peak melting curve of PCR products (data not shown). No signals were detected in NTC samples. Amplification efficiency ranged from 88·9 % for *ACTB* to 102% for *B2M* (Table I).

The RNA transcription levels were analysed by directly comparing C_q values. The C_q is defined as the number of cycles needed for the fluorescence to reach a specific threshold level of detection and is inversely correlated with the amount of template nucleic acid present in the reaction (Bustin *et al*, 2009). The lowest C_q value recorded was 13·63 cycles for *UBC* and the highest was 31·83 for *TBP*. The lowest geometric mean of C_q values was 16·40 for *B2M*, and the highest was 25·74 for *MRPL19* (Table II and Fig 1). *MRPL19*, *B2M*, *HPRT1* and

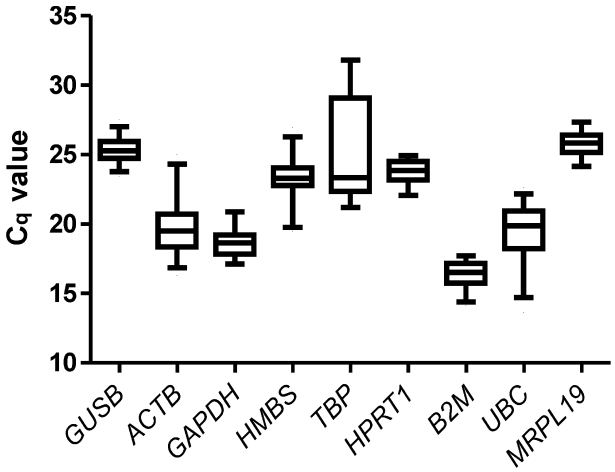

Fig 1. C_q values for reference genes. Expression data displayed as C_q values for each reference genes in all B cell samples. The median is depicted as a line across the box. The box indicates the 25th and 75th percentiles. Whiskers represent the maximum and minimum values.

GAPDH exhibited the smallest gene expression variation (<4 cycles) among the studied reference genes, while *UBC* and *TBP* had much higher expression variation (more than nine cycles).

geNorm analysis

The geNorm calculates the gene expression stability measure (*M*) for the reference genes. The *M* value is defined as an average pair-wise variation (*V*) of a particular gene with all other tested reference genes, whereas the variation of this gene to another is determined as the standard deviation of the log2-transformed expression level ratios (Vandesompele *et al*, 2002). The gene with the lowest *M* value was considered to have the most stable expression, while the gene with the highest *M* value had the least stable expression. Table II indicates the *M* values for all tested reference genes. *GUSB*, *GAPDH*, *HPRT1*, *B2M* and *MRPL19* had *M* values less than the geNorm default threshold of 1·5, while *ACTB*, *HMBS*, *TBP* and *UBC M* values exceeded the threshold. In a stepwise progression, geNorm excludes the least stable gene, and recalculates *M* values for the remaining genes, which results in the characterization of the stability of each gene on a ranked scale and the identification of the two most stably expressed genes. The average expression *M* values of the nine tested reference genes were plotted in Fig 2A. *TBP* and *UBC* were respectively the first and the second genes excluded from the analysis on the basis of instability whereas *HPRT1* and *B2M* were identified as the most stable reference gene pair in purified $CD19^+$ cells (Fig 2A).

To evaluate the optimal number of genes required for accurate normalization, geNorm calculates whether the stepwise addition of a less stable gene to the normalization factor (NF_n) affects the variance ($V_{n/n+1}$) compared to (NF_{n+1}). A large pair-wise variation implies that the added gene has a significant effect on normalization and should be included in calculation of a reliable normalization factor (Vandesompele *et al*, 2002). To meet the default cut-off *V* value of 0·15 (the point at which it is unnecessary to include additional genes in a normalization strategy) the geNorm indicated the use of four of the five most stable genes (Fig 2B).

BestKeeper-1 analysis

BestKeeper-1 estimates inter-gene relationships of possible reference gene pairs by performing numerous pair-wise correlation analysis using raw C_q values of each gene (Pfaffl *et al*, 2004). Unstable genes show the standard deviation (SD_{Cq}) of >1·0, indicating variation in the starting template by the factor 2. However, the qPCR reaction efficiency in biological samples is rarely 100%, and default setting of BestKeeper-1 might be too strict. Therefore it was suggested to adjust the SD threshold (ln2/lnE) for each gene to its specific efficiency (Axtner & Sommer, 2009). As indicated in Table II, *ACTB*, *HMBS*, *TBP* and *UBC* had SD_{Cq} values of >1·0 and were ranked by BestKeeper-1 as unstable, whereas *GAPDH*, *HPRT1*, *B2M*, *GUSB* and *MRPL19* showed SD_{Cq} values of <1·0 and were considered to be stably expressed. The calculated SD threshold did not change the tested gene stability: *GAPDH*, *HPRT1*, *B2M*, *GUSB* and *MRPL19* were considered as stable reference genes as the SD_{Cq} was lower than their individual SD threshold value whereas the remaining genes were ranked as unstable (Table II). The range of expression stability calculated by BestKeeper-1 was (from the most stable to the least stable): *MRPL19*, *GUSB*, *HPRT*, *B2M*, *GAPDH*, *HMBS*, *UBC*, *ACTB* and *TBP* (Table III).

NormFinder analysis

NormFinder calculates gene expression stability value (not comparable to the *M* value of genNorm) for each single gene independently and ranks the set of potential reference genes according to their expression stability (Andersen *et al*, 2004). The more stable gene expression is indicated by a lower expression stability value. The NormFinder calculated stability values for tested reference genes and their ranking order are indicated in Tables II and III, accordingly. The expression of *B2M* was defined to be the most stable, whereas *TBP* was indicated as the most unstable.

Evaluation of reference gene stability

In order to select the most stable reference gene for accurate normalization of gene expression in purified B cells from B-CLL patients' blood, we compared data obtained by three different statistical approaches: geNorm, NormFinder and BestKeeper-1. All three applications gave comparable results (Table III). The five stable reference genes identified by BestKeeper-1 (*GAPDH*, *HPRT1*, *B2M*, *GUSB* and *MRPL19*) did not differ from those identified by geNorm application,

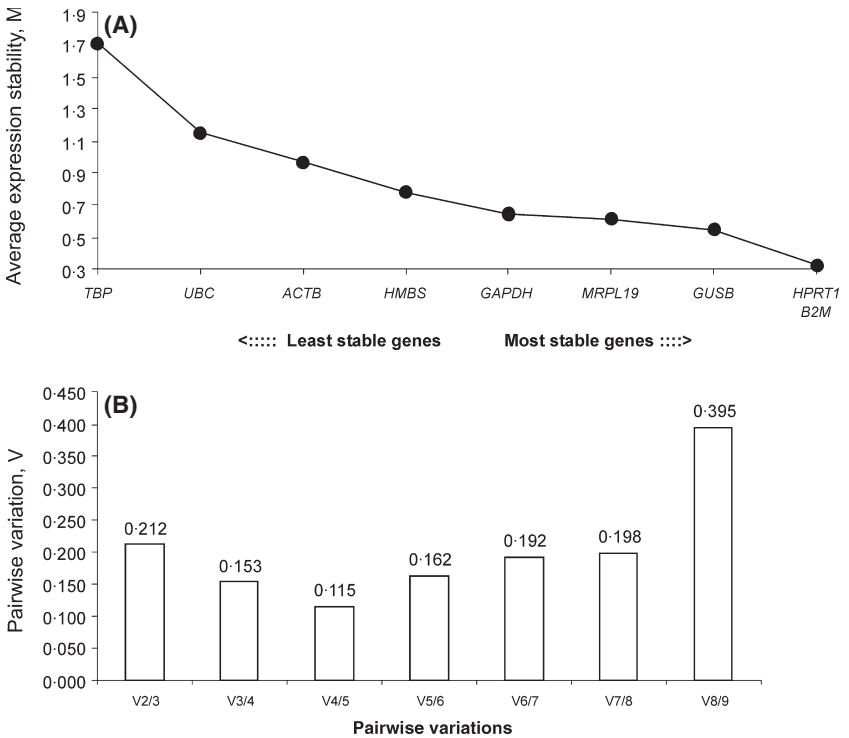

Fig 2. geNorm analysis of reference genes. Results are presented as an output file of the geNorm program. (A) Average expression stability (*M*) of reference genes during stepwise exclusion of the least stable gene. (B) Determination of the optimal number of reference genes for normalization. Every bar represents change in normalization accuracy when more reference genes are added stepwise according to the ranking. The geNorm proposed cut-off value below which the inclusion of an additional reference gene is not required is 0·15.

Table III. Ranking order of the reference genes calculated by NormFinder, BestKeeper-1 and geNorm algorithms.

	B2M	*HPRT1*	*GUSB*	*MRPL19*	*GAPDH*	*HMBS*	*ACTB*	*UBC*	*TBP*
NormFinder	1	2	3	7	4	6	5	8	9
BestKeeper-1	4	3	2	1	5	6	8	7	9
geNorm	1·5	1·5	3	4	5	6	7	8	9
Mean rank	2·17	2·17	2·67	4·00	4·67	6·00	6·67	7·67	9·00

though they were arranged in a different order. NormFinder ranking order differed from geNorm and BestKeeper-1 ranking, but *B2M*, *HPRT1* and *GUSB*, the most stable genes in NormFinder analysis, were among the top four most stable genes identified by geNorm and BestKeeper-1. *TBP* and *UBC* were the most unstable genes in all three applications. The calculated mean ranking indicated *B2M*, *GUSB* and *HPRT1* as the most stable and these genes could be considered as suitable reference genes for gene expression studies in B-CLL patients' B cells (Table III).

Discussion

Gene expression studies by qPCR play an important role in understanding the pathogenesis of B-CLL, and in identification of novel prognostic markers as well as potential targets for therapy. In order to obtain reliable qPCR results, appropriate normalization of relative qPCR data is required to control for non-biological variation (Bustin, 2000; Nolan *et al*, 2006; Bustin *et al*, 2009).

Nine potential reference genes were analysed for their expression stability in the purified B cells obtained from B-CLL patients' blood samples. These genes were *ACTB*, *B2M*, *GAPDH*, *GUSB*, *HMBS*, *HPRT1*, *MRPL19*, *TBP* and *UBC*. To select the most stably expressed genes, three publicly available programs: BestKeeper-1 (Pfaffl *et al*, 2004), geNorm (Vandesompele *et al*, 2002) and NormFinder (Andersen *et al*, 2004) were used.

Although geNorm, BestKeeper-1 and NormFinder are based on different algorithms and analytical programs, all three programs showed consistent results with only slight differences in the ranking order except for *MRPL19,* which was the most stable gene in BestKeeper-1 analysis but was seventh in the NormFinder gene stability ranking. In general, analysis

indicated *B2M*, *HPRT1* and *GUSB* as the most stable reference genes, as these three genes were always classified among the four best performing reference genes. On the other hand, *UBC* and *TBP* were ranked poorly based on all three software programs, indicating that these two genes should not be used as reference controls for gene expression studies in B-CLL patients. Based on the summarized data (Table III), the use of *ACTB*, *GAPDH* and *HMBS* as reference controls also could not be recommended.

Several studies have scrutinized the stability of commonly known reference genes *GAPDH* and *ACTB* and have demonstrated that these genes should be used with caution as their expression varied considerably and they were consequently unsuitable as reference genes in some cases (Deindl *et al*, 2002; Glare *et al*, 2002; Tong *et al*, 2009). The present study also determined *ACTB* and *GAPDH* as unstable reference genes. However, a literature search revealed that *GAPDH* and *ACTB* were among the most often used reference genes in B-CLL gene expression studies (Jelinek *et al*, 2003; Joshi *et al*, 2007; Nuckel *et al*, 2009; Kienle *et al*, 2010). This underscores the importance of careful selection of reference genes.

The geNorm model makes an assumption that the candidate reference genes are not co-regulated as this may lead to the erroneous choice of the best gene pair. Whereas geNorm is susceptible to identification of co-regulated genes as an optimal reference gene pair, NormFinder and BestKeeper-1 do not have this problem. As all three programs gave consistent results, one can assume that the studied reference genes were not co-regulated in the analysed samples.

Although most studies used only a single reference gene for normalization, it has been suggested that the use of two or more reference genes for qPCR studies might generate more reliable results (Tricarico *et al*, 2002; Vandesompele *et al*, 2002). geNorm analysis indicated that four genes would be required for accurate normalization of qPCR data in B-CLL patients' blood cells (Fig 2B). However, the number of reference genes needs to be balanced between accuracy and practical consideration, thus the cut-off V value of 0·15 suggested by geNorm should not be taken too strictly, as suggested by the geNorm manual itself. The calculated $V_{3/4}$ value was 0·153, which was only slightly higher than the geNorm cut-off value and we would suggest using three reference genes from the four most stable ones. The optimal set of reference genes should be selected and defined for each study, depending on the gene of interest. For example, *B2M* is a highly expressed gene in most tissues (Vandesompele *et al*, 2002; Rulcova *et al*, 2007) and also in peripheral blood B cells from B-CLL patients (Fig 1). Highly expressed reference genes are not recommended for the normalization of target genes with low expression levels because the copy number of reference and target gene should fall in the same linear measurement scale.

In conclusion, *B2M*, *HPRT1*, and *GUSB* genes were identified as the most stably expressed genes in $CD19^+$ B cells purified from B-CLL patients' peripheral blood and they are recommended for the normalization of gene expression results in these cells.

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Disclosures

The authors have no financial conflict of interest.

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