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Optimized Internal Control and Gene Expression Analysis in Epstein-Barr Virus-Transformed Lymphoblastoid Cell Lines

Hye-Young Nam, Hye-Ryun Kim, Sung-Mi Shim, Jae-Eun Lee, Jun-Woo Kim, Hye-Kyung Park, Bok-Ghee Han* and Jae-Pil Jeon*

National Biobank of Korea, Center for Genome Science, Korea National Institute of Health, Korea Centers for Disease Control & Prevention, Osong Health Technology Administration Complex (OHTAC), Chungbuk 363-951, Korea

Abstract

The Epstein-Barr virus-transformed lymphoblastoid cell line (LCL) is one of the major genomic resources for human genetics and immunological studies. Use of LCLs is currently extended to pharmacogenetic studies to investigate variations in human gene expression as well as drug responses between individuals. We evaluated four common internal controls for gene expression analysis of selected hematopoietic transcriptional regulatory genes between B cells and LCLs. In this study, the expression pattern analyses showed that *TBP* (TATA box-binding protein) is a suitable internal control for normalization, whereas *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) is not a good internal control for gene expression analyses of hematopoiesis-related genes between B cells and LCLs at different subculture passages. Using the *TBP* normalizer, we found significant gene expression changes in selected hematopoietic transcriptional regulatory genes (downregulation of *RUNX1*, *RUNX3*, *CBFB*, *TLE1*, and *NOTCH2*; upregulation of *MSC* and *PLAGL2*) between B cells and LCLs at different passage numbers. These results suggest that these hematopoietic transcriptional regulatory genes are potential cellular targets of EBV infection, contributing to EBV-mediated B-cell transformation and LCL immortalization.

Keywords: lymphoblastoid cell line, internal control, quantitative real-time polymerase chain reaction

*Corresponding authors: E-mail jpjeon@cdc.go.kr
Tel +82-43-719-8911, Fax +82-43-719-8949
E-mail bokghee@nih.go.kr
Tel +82-43-719-8801, Fax +82-43-719-8802
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Introduction

EBV-infected B cells are transformed into continuously proliferating lymphoblastoid cell lines (LCLs), which provide genomic resources for human genetics and immunological studies. However, there is a limitation of LCL utilization, due to possible genetic changes in the process of EBV transformation (Shukla and Dolan, 2005; Haas *et al.*, 2006). It is controversial that LCLs are suitable as an experimental material of genomewide association studies (Simon-Sanchez *et al.*, 2007). Nonetheless, the use of LCLs is currently extended to pharmacogenetic studies to investigate variations in human gene expression as well as drug responses between individuals. For example, gene expression phenotypes were investigated in LCLs for the relative impact of nucleotide and copy number variation (Stranger *et al.*, 2007).

EBV infection induces gene expression changes in B-cells, contributing to immortalization. A number of cellular genes have been identified as targets of EBV infection in various systems, including inducible and stable lymphoma cell lines that have been transfected with particular viral gene expression vectors or infected with EBV (Carter *et al.*, 2002; Kelly *et al.*, 2002; Cahir-McFarland *et al.*, 2004; Kang *et al.*, 2005). For gene expression analysis, appropriate internal controls should be carefully selected to normalize the expression of target genes in LCLs. Generally, housekeeping genes are used as internal controls for gene expression studies, assuming that such genes exhibit relatively constant basal levels of gene expression under all conditions. However, not all housekeeping genes are always applied to the normalization of gene expression data under all conditions (Thellin *et al.*, 1999). It has been reported that the comparison of target gene expression needs particular internal controls that are suitable in different tissues or different physiological conditions in human and other organisms (Selvey *et al.*, 2001; Vandesompele *et al.*, 2002; Lossos *et al.*, 2003; Filby and Tyler, 2007; Theis *et al.*, 2007; Rho *et al.*, 2010). Among housekeeping genes, *GAPDH* has been demonstrated to be a good internal control in tumor cell lines (Janssens *et al.*, 2004), while *HPRT* has been identified as a good reference gene for cancer research when comparing solid tumor tissue samples with normal tissue samples (de Kok *et al.*, 2005; Ohl *et al.*, 2005). Thus, it is recommended that suitable internal controls should be determined for accu-

rate normalization of gene expression data.

For peripheral blood cells or leukemia/lymphoma cell lines, *GAPDH* is frequently used to normalize gene expression data (Natkunam *et al*., 2005; Saunders *et al*., 2005). According to our previous microarray data, the choice of *GAPDH* may be problematic as an internal control of gene expression analysis before and after EBV infection. In addition, as the use of LCLs is expanded to various areas of genomics, including pharamcogenomics, gene expression analysis of LCLs needs optimized internal controls. Here, we evaluated four common internal controls for gene expression analysis between B cells and LCLs, applying them to an expression analysis of selected hematopoietic transcriptional regulatory genes. *TBP* was finally selected as an optimal internal control for gene expression analysis between B cells and LCLs.

Methods

Cell culture and sample preparation

Peripheral blood was obtained from the Jungang Blood Center of the Korean Red Cross. Ficoll-Hypaque gradient centrifugation was performed to isolate peripheral blood mononuclear cells according to the manufacturer's instructions (Amersham). Primary CD19(+) B cells were then purified by negative selection using a B cell isolation kit (Miltenyi Biotech). EBV viral stock was collected from the culture of an EBV-transformed B95-8 marmoset cell line, which was maintained at 37°C and 5% CO_2 for 4～7 days and then stored at −80°C until needed. EBV infection of primary B cells and LCL generation were performed as described elsewhere (Hur *et al*., 2005). Briefly, EBV-infected B-cells were incubated in RPMI-1640 supplemented with 10% FBS and penicillin-streptomycin (100 μg/100 unit) for 10～20 days until clumps of EBV-infected B cells were visible. The culture medium was then changed with fresh complete medium for subsequent subculture. When cell numbers of EBV-infected B-cells reached approximately 10 million cells per culture flask, the cells were used for the next passage at a seeding density of $2 \sim 5 \times 10^5$ cells/ml. At this point, the LCL was referred as passage number 1. Next, the LCL culture was divided for continuous subculture with fresh medium approximately twice per week and maintained to passage number 100.

Expression analysis

Total RNA was isolated from B-cells and counterpart LCLs from three donors using Trizol (Invitrogen) and treated with DNase I prior to the synthesis of first-strand cDNA using oligo (dT) primer and the Superscript II reverse transcription kit (Invitrogen). Semi-quantitative RT-PCR was performed for some selected genes. Gene expression levels of some selected genes were estimated by semiquantitative RT-PCR with the following conditions: 25 cycles of 40 sec at 94°C, 40 sec at 60°C, and 100 sec at 72°C for *RUNX3*, *MSC*, and *GAPDH* and 32 cycles for *NOTCH2* and *TLE1*.

For quantitative real-time PCR, amplification mixtures (50 ul) contained 10～100 ng of first-strand cDNA template, 25 ul of 2× SYBR Green Master Mix buffer (Invitrogen), and 600 nM of forward and reverse primers (Table 1) for: control genes β-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β2-microglobulin (*B2M*), and TATA box-binding protein (*TBP*); and target genes pleiomorphic adenoma gene-like 2 (*PLAGL2*), musculin (*MSC*)/activated B-cell factor (*ABF1*), transducin-like enhancer of split 1 (*TLE1*), Notch homolog 2 (*NOTCH2*), Runt-related transcription factor 1 (*RUNX1*), Runt-related transcription factor 3 (*RUNX3*), and core binding factor beta subunit (*CBFB*). PCR cycles were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Reactions were run on an ABI HT 7900 (Applied Biosystems). Relative expression levels, $\Delta(CT_{gene}-CT_{control})$, were displayed as the difference in C_T (threshold cycle) between genes of interest and internal control genes.

Table 1. Sequence information of PCR primers

Gene		Primer sequence	Size (bp)
ACTB	Forward	5'-GTCTTCCCCTCCATCGTG-3'	280
	Reverse	5'-TGATCTGGGTCATCTTCTCG-3'	
GAPDH	Forward	5'-CAGGGCTGCTTTTAACTCTGGTAA-3'	99
	Reverse	5'-GTGGAATCATATTGGAACATGTAAACC-3'	
B2M	Forward	5'-TTTCATCCATCCGACATTGA-3'	228
	Reverse	5'-CCTCCATGATGCTGCTTACA-3'	
TBP	Forward	5'-TTCGGAGAGTTCTGGGATTGTA-3'	227
	Reverse	5'-TGGACTGTTCTTCACTCTTGGC-3'	
PLAGL2	Forward	5'-AGGCAGCTGAGCTTTCAGAG-3'	191
	Reverse	5'-CTCCCGATTCAGGTCAAAAA-3'	
MSC	Forward	5'-CAACCTGTGTGGGGTCTTCT-3'	215
	Reverse	5'-TCGCAGGATCACTTGCTATG-3'	
NOTCH2	Forward	5'-AAGCAGAGTCCCAGTGCCTA-3'	171
	Reverse	5'-CAGGGGGCACTGACAGTAAT-3'	
TLE1	Forward	5'-CGACAAGTCCATCAGCAGAA-3'	150
	Reverse	5'-CCCAGATCACCCAGAAAGAA-3'	
RUNX1	Forward	5'-TCTAGCTCAGCACTGCTCCA-3'	232
	Reverse	5'-TCATGCAAAACTGGCTTCAG-3'	
RUNX3	Forward	5'-TAGGAAGCACGAGGAAAGGA-3'	189
	Reverse	5'-GCAAACGATAGTGCAAAGCA-3'	
CBFB	Forward	5'-GCAGGCAAGGTATATTTGAAGG-3'	218
	Reverse	5'-TTGGAACCAGGACTAGGGTCT-3	

For the correlation coefficient analysis of gene expression patterns, expression levels of particular genes of interest were normalized to four different reference genes. Then, correlation coefficients were calculated from the expression levels (C_T values), normalized to pairs of reference genes (e.g., *ACTB* vs *TBP*, *ACTB* vs *B2M*, *ACTB* vs *GAPDH*, *TBP* vs *B2M*, *TBP* vs *GAPDH*, *B2M* vs *GAPDH*).

Results

To address the question of whether *GAPDH* is a suit-

Table 2. Expression levels extracted from our previous microarray data

Genes	Accession	Description	Fold[a]
GAPDH	AA419281	Glyceraldehyde-3-phosphate dehydrogenase	18.90±5.59
B2M	AA670408	Beta-2-microglobulin	1.2±0.14
TBP	N50549	TATA box binding protein	0.7±0.09
ACTB	AW081870	Beta, actin	4.13±0.90
PLAGL1	AI346457	Pleiomorphic adenoma gene-like 2	4.95±1.93
MSC	AA470081	Musculin (activated B-cell factor-1)	3.99±1.65
TLE1	AI268473	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	0.06±0.03
RUNX1	AI521317	Runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	0.27±0.09
RUNX3	N67778	Runt-related transcription factor 3	1.5±0.05
CBFB	AA187148	Core-binding factor, beta subunit	0.68±0.03
NOTCH2	AA827551	Notch homolog 2 (Drosophila)	0.05±0.01

[a]Fold-changes were shown in reference to B-cells

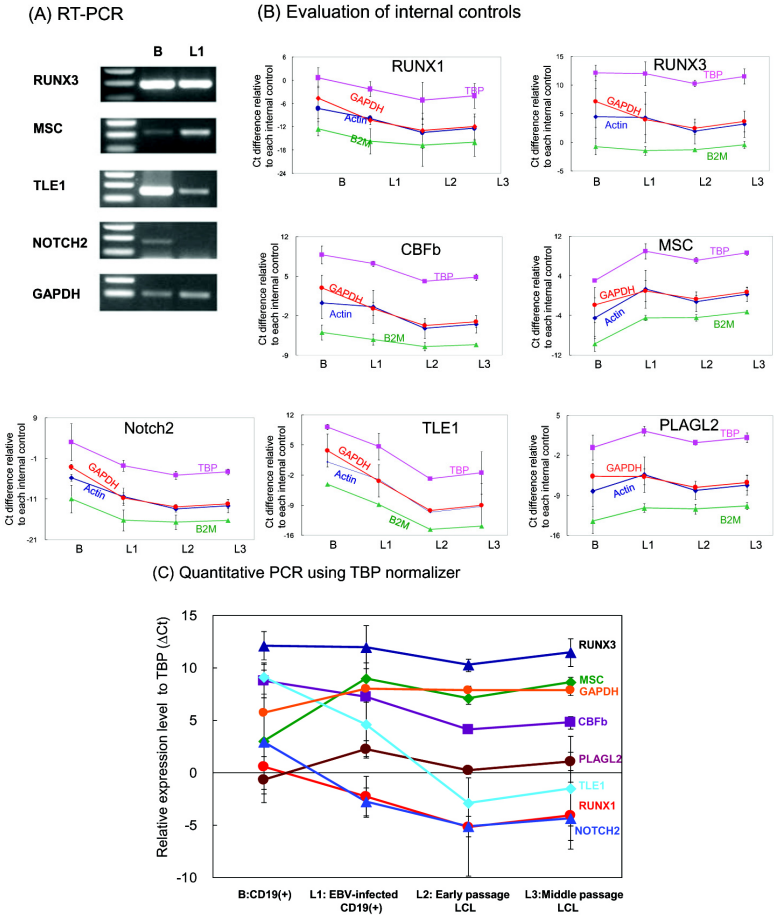

Fig. 1. Expression analysis of selected hematopoietic transcriptional regulatory genes using four common internal controls between B-cells and LCLs. Semi-quantitative RT-PCR was performed for some of target genes (A), followed by quantitative real-time PCR of selected hematopoietic transcriptional regulatory genes using four common internal controls (B). Next, the relative expression levels were obtained using the *TBP* internal control (C). B indicates B-cells, L1: LCLs at 5-8 weeks after EBV infection, L2 and L3: long-term subculture of LCLs at 50 and 100 passages, respectively. Relative expression levels, $\Delta(CT_{gene}-CT_{control})$, were displayed as C_T (threshold cycle) difference between genes of interest and internal control genes. Error bars indicate standard deviations of three experiments in duplicate.

able internal control for gene expression analysis of various types of LCLs, we evaluated four common housekeeping genes as internal control genes. First, we extracted expression data on *ACTB*, *TBP*, *B2M*, and *GAPDH* as well as some genes of interest from our previous microarray data (Baik *et al.*, 2007), indicating upregulation of *GAPDH* and *ACTB* in LCLs compared to B cells. However, *TBP* and *B2M* exhibited less than a 2-fold change between B-cells and LCLs (Table 2). In addition, with regard to B cell transformation, some genes of interest were randomly selected for expression studies of hematopoiesis and lymphomagenesis-related genes. Our microarray data showed that *PLAGL2*, *MSC*, *TLE1*, *RUNX1*, and *NOTCH2* exhibited significant changes in gene expression levels between B-cells and LCLs (Table 2), whereas the other genes (i.e., *RUNX3*, *CBFB*) exhibited less than a 2-fold change. For these selected genes, RT-PCR had similar gene expression patterns for *RUNX3*, *MSC*, *TLE1*, and *NOTCH2* as the microarray data (Fig. 1).

Four common housekeeping genes were included in the quantitative real-time PCR of hematopoietic transcriptional regulatory genes so that relative gene expression levels could be obtained by normalization. In a comparison of gene expression only between B cells and early passages of LCLs, similar patterns in hematopoietic gene expression were observed when normalized by *TBP*, *ACTB*, and *B2M* but not by *GAPDH* (Fig. 1B), indicating that the expression pattern of *GAPDH* deviated from those of the other housekeeping genes. This result suggested that *GAPDH* expression might be upregulated in EBV-infected B cells during LCL generation. Indeed, *GAPDH* upregulation was seen in LCLs of different passage numbers compared with B cells when normalized by *TBP* (Fig. 1C).

Next, expression levels of hematopoietic genes were plotted in reference to each internal control (Fig. 1C). In a comparison of expression patterns between B cells and three different time points of long-term subcultures of LCLs (i.e., L1: EBV-infected B cells, L2: early passage LCLs, L3: late passage LCLs), correlation coefficients of individual expression patterns were calculated by pairwise comparison of normalized expression levels using four different internal controls (Table 3). For seven genes that were tested, *TBP*-normalized expression levels of each gene were correlated with *ACTB*-normalized expression levels, whereas the worst correlation coefficients of normalization between *GAPDH* and *B2M* were seen. This result suggested that *TBP* or *ACTB* might be a suitable internal control for gene expression analyses of hematopoiesis-related genes in various subculture passages of LCLs. However, our previous microarray data showed that *ACTB* was upregulated by about 4-fold in LCLs compared with B cells (Baik *et al*, 2007). Therefore, *TBP* was finally selected as an internal control for further gene expression analysis of hematopoietic transcriptional regulatory genes of interest.

Next, the optimized internal control, *TBP*, was applied to the gene expression analysis of seven randomly selected hematopoietic transcriptional regulatory genes in LCLs. Real-time PCR results showed that gene expression levels of *MSC*, *TLE1*, *RUNX1*, *PLAGL2*, and *GAPDH* changed dramatically between B cells and LCLs, whereas those of *RUNX3* and *CBFB* changed slightly after EBV infection (Fig. 1C). Interestingly, the upregulation or downregulation patterns that were detected in EBV-infected cells were sustained until the middle passage number (100) of LCLs. Thus, these results suggest that dysregulation of these selected hematopoietic genes may contribute to EBV-mediated B cell transformation and LCL immortalization.

Discussion

Gene expression studies of EBV-transformed LCLs have commonly used *GAPDH* or *ACTB* as internal controls for normalization (Baran-Marszak *et al.*, 2002). In our previous study, global gene expression patterns between B

Table 3. Pairwise comparison of correlation coefficients of gene expression patterns.

	Correlation coefficients[a]					
Gene name	ACTB vs TBP	ACTB vs B2M	ACTB vs GAPDH	TBP vs B2M	TBP vs GAPDH	B2M vs GAPDH
RUNX1	0.995	0.928	0.963	0.959	0.983	0.992
RUNX3	0.979	0.102	0.788	0.281	0.778	0.379
CBFB	0.985	0.938	0.920	0.984	0.974	0.998
PLAGL2	0.952	0.529	0.406	0.763	0.130	-0.514
MSC	0.988	0.903	0.975	0.958	0.956	0.856
NOTCH2	0.993	0.948	0.983	0.979	0.998	0.990
TLE1	1.000	0.995	0.987	0.997	0.991	0.998
AVE[b]	0.985	0.763	0.860	0.846	0.830	0.671

[a]Correlation coefficients of the expression patterns of individual genes using normalization controls; [b]average correlation coefficients.

cells and LCLs were obtained from our microarray analysis, in which *GAPDH* was used as an internal control for validation of gene expression (Baik *et al*., 2007). In general, internal control genes need relatively constant basal levels of gene expression in experimental conditions. However, our microarray data suggested that some housekeeping genes might be dysregulated between B cells and LCLs. Therefore, we evaluated four common housekeeping genes as suitable internal controls for the normalization of mRNA expression and applied them to a gene expression study of potential cellular target genes of EBV infection. Our results showed that the transcriptional level of *GAPDH* increased in the process of EBV-mediated B-cell transformation. The upregulation of *GAPDH* might be involved in B-cell reprogramming during LCL generation. Alternatively, *GAPDH* might be a direct cellular target gene of EBV infection. Generally, the expression of B cell-specific genes is lost, and B lineage-inappropriate genes are upregulated during malignant transformation of lymphoid cells, suggesting B-cell reprogramming during EBV-mediated B-cell transformation (Janz *et al*., 2006). Therefore, *GAPDH* may not be suitable for internal controls in gene expression analyses between B-cells and LCLs. Furthermore, a correlation coefficient analysis for gene expression profiles, normalized by four housekeeping genes, showed that *TBP* was found to be a suitable internal control in the gene expression analysis between B cells and LCLs. Moreover, *TBP* expression level was found to be moderately abundant in B cells and LCLs, as determined by the C_T difference between genes of interest and *TBP* (Fig. 1B). Although many other housekeeping genes were not evaluated in this study, at least *TBP* can be used as an optimum internal control for gene expression analysis, regardless of the status of B-cells (e.g., primary, EBV-infected B-cells) or long-term subculture of LCLs. Thus, we finally selected *TBP* as a normalizer for further gene expression studies of hematopoietic transcriptional regulatory genes.

It is known that the hematopoietic genes that were selected for this study are involved in hematopoiesis and lymphoid malignancies. For example, *PLAGL2*, a developmentally regulated C2H2 zinc finger on chromosome 8q12, is the main target for pleomorphic adenomas of the salivary gland. Recently, it was reported that *PLAGL2* induced acute myeloid leukemia in cooperation with *CBFB-MYH11* in mice (Landrette *et al*., 2005). Our results showed that *PLAGL2* was upregulated in EBV-infected B-cells and LCLs, suggesting that *PLAGL2* may be a potential target of EBV-mediated B-cell transformation. On the other hand, RUNX family members are heterodimeric transcription factors composed of an α subunit, RUNX, and a β subunit, CBFβ. Of the three members of the RUNX family, *RUNX1* is known as a master regulatory gene of hematopoiesis. According to the literature, *RUNX3* was identified as a cellular target of *EBNA-2* in the inducible cell line (Spender *et al*., 2002). The distal P1 promoter of *RUNX1* controls expression of the *RUNX1*c isoform, while the proximal P2 promoter expresses *RUNX1a* (*AML-1a*) and *RUNX1b* (*AML-1b*) (Miyoshi *et al*., 1991). *RUNX3* represses transcription from the *RUNX1* P1 promoter, indicating transcriptional crossregulation of *RUNX1* by *RUNX3* in human B cells (Spender *et al*., 2005). On the contrary, our PCR results showed that *RUNX1* expression was downregulated in parallel with slight downregulation of *RUNX3* between B cells and LCLs, which is inconsistent with a previous report of crossregulation by *RUNX3*. This discrepancy might be due to a different PCR target of *RUNX1* isoforms, because the *RUNX* isoform that we examined in our study was the *RUNX1* P2 transcript rather than *RUNX1* P1 transcripts. Musculin (activated B-cell factor-1, *MSC*) is a basic helix-loop-helix transcription factor, and its expression is restricted to a subset of lymphoid tissues, lymphoblastoid cell lines, and activated human B cells. *MSC* (*ABF-1*) expression is restricted to a subset of lymphoid tissues, lymphoblastoid cell lines, and activated human B cells (Massari *et al*., 1998), suggesting that *MSC* is a downstream target of the B-cell receptor signal transduction pathway. With respect to cell growth, EBV infection mimics B-cell activation signals or factors, such as CD40-CD40L, IgM, and LPS, so that EBV-infected B-cells exhibit greater proliferation than resting B-cells (Kilger *et al*., 1998). Thus, the upregulation of *MSC* in LCLs might be responsible for greater proliferative activity in EBV-infected B-cells compared with B-cells. *TLE* (transducin-like enhancer of split) genes are human homologs of the *Drosophila* groucho gene. *TLE1* binds to the Runt domain and the C terminus of *RUNX1* to inhibit *RUNX1*-induced transactivation of the *CSF1* (colony stimulating factor 1) receptor. Our PCR analysis showed that *TLE1* and *NOTCH2* were downregulated in LCLs compared with B-cells, suggesting that Notch signaling may be involved in EBV-mediated B-cell transformation. Taken together, our results suggest that the hematopoietic transcriptional regulatory genes that were tested in this study may be potential cellular targets of EBV infection during LCL generation.

In conclusion, we evaluated four common internal controls for a gene expression analysis between B cells and LCLs and found *TBP* as a suitable internal control. Expression analysis using a *TBP* normalizer allowed us to identify dramatic changes in selected hematopoietic transcriptional regulatory genes (downregulation of *RUNX1*, *RUNX3*, *CBFB*, *TLE1*, and *NOTCH2*; upregula-

tion of *MSC* and *PLAGL2*) during EBV-mediated B-cell transformation and LCLs at different passages. Therefore, these genes may be potential cellular targets of EBV infection and potential reference biomarkers of LCL generation.

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