# β-2 microglobulin is unsuitable as an internal reference gene for the analysis of gene expression in human colorectal cancer

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**Abstract.** It is well-known that gene expression levels should be normalized to a carefully selected and appropriately stable internal control gene. However, numerous studies have demonstrated that the expression of housekeeping (HK) genes, typically used as internal control genes varies considerably. A number of studies have shown that β-2 microglobulin (B2M), an HK gene, frequently used as an internal reference gene, is expressed at low levels in colorectal cancer tissue, when assessed using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Due to the fact that the expression levels of various HK genes vary depending on the tissue type or experimental conditions, it has been suggested that several control genes should be analyzed in parallel for certain tissues. In the present study, mRNA expression levels of toll-like receptors 2 (TLR2) and 4 (TLR4) in sporadic human colorectal cancerous and non-cancerous tissues were analyzed relative to three HK genes, β-glucuronidase (GUS), β-actin (BA) and B2M, using a commercially available tool. Relative expression levels were quantified using the three genes individually and together, and TLR2 as well as TLR4 expression was compared in cancerous and non-cancerous colorectal tissue specimens. Consistent data were obtained in most cases when GUS and BA were used as internal control genes. When B2M was used as the internal control gene, TLR2 and TLR4 expression was demonstrated to be higher in cancerous compared to non-cancerous colorectal tissues. These results were consistent with previous observations of low-level B2M expression in cancerous colorectal tissue and suggest that B2M may be inappropriate as an internal control gene for gene expression studies of colorectal cancer.

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## Introduction

Internal control genes are necessary for the accurate determination of gene expression using techniques such as quantitative real-time polymerase chain reaction (PCR). High-throughput real-time reverse transcriptase (RT)-PCR, with its outstanding sensitivity and accuracy, has rendered the selection of a housekeeping (HK) gene used as the internal standard for the estimation and comparison of mRNA levels even more important (1-3). Previous studies showed that the expression levels of commonly used HK genes are different in various tissues or between normal and diseased tissues (1,4-9).

It has been argued that since the expression levels of various HK genes differ depending on the tissue type or experimental conditions, more than one or even several various control genes should be assessed in parallel for certain tissues (1,3,10-12). In colorectal cancer (CRC), downregulation of the HK gene β-2 microglobulin (B2M) has been confirmed using real-time RT-PCR (13,14).

In a previous study, we examined the expression of toll-like receptors 2 (TLR2) and 4 (TLR4) in sporadic human CRC tissue (15). TLRs are known to be involved in innate immunity and to play an important role in immune surveillance (16-20). In this study, we used a commercially available kit for analysis of the preferred internal control genes and selected three reference genes: β-glucuronidase (GUS), β-actin (BA) and B2M. Using these three internal control genes, the mRNA expression levels of TLR2 and TLR4 in cancerous and non-cancerous colorectal tissue specimens were quantified using TaqMan real-time PCR and compared. Based on the results, we discuss the validity of each of the internal control genes in terms of accurate analysis of gene expression in CRC tissue under the present experimental conditions.

## Materials and methods

*Tissue specimens and internal control genes.* Surgical specimens of colorectal tissue were obtained from 50 CRC patients

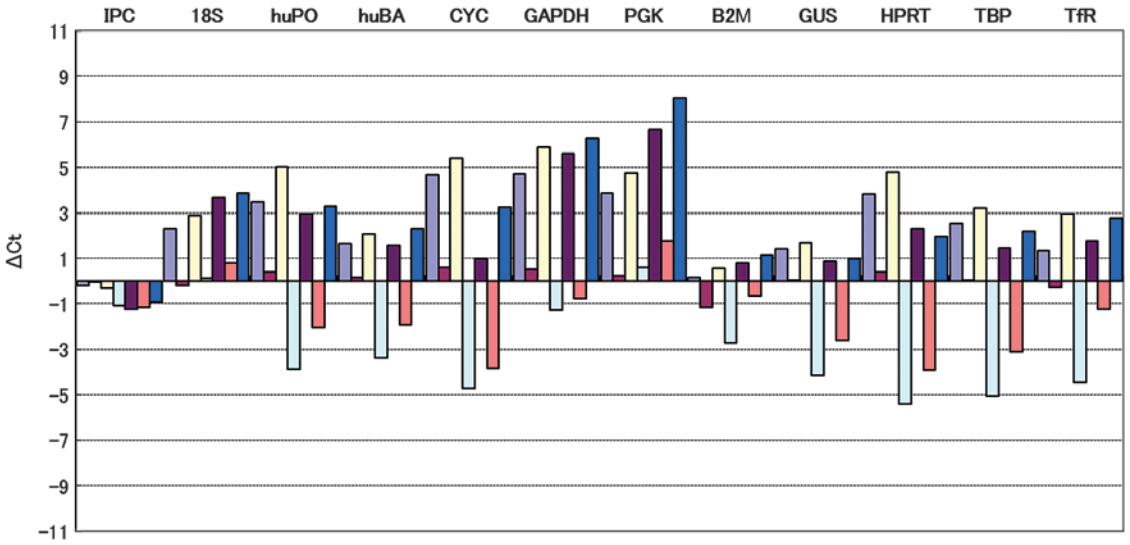


Figure 1. Columns represent individual samples analyzed using TaqMan primers and probes for the detection of the indicated target gene in order to compare gene expression between samples. Less fluctuation in amplification indicated less variable gene expression. The three HK genes used as internal reference genes (GUS, BA and B2M) were selected based on these results. IPC, internal positive control; 18S, 18S rRNA; huPO, acidic ribosomal protein; huBA, β-actin; CYC, cyclophilin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerokinase; B2M, β-2 microglobulin; GUS, β-glucronidase; HPRT, hypoxanthine ribosyl transferase; TBP, transcription factor IID, TATA binding protein; TfR, transferrin receptor.

at the Toho University Sakura Medical Center (Sakura, Japan) as previously described (15). Written informed consent was obtained from the subjects according to the terms of the Declaration of Helsinki. Non-cancerous tissue, located proximal to the tumor and macroscopically free of disease, was obtained immediately after surgery. The characteristics of the 50 subjects were previously described (15) and there was no bias in the sample population.

The 50 cancerous specimens were grouped according to the histopathological stage (pStage I, II, III and IV), based on the tumor-node-metastasis (TMN) classification of the International Union against Cancer (UICC). Differences in TLR2 and TLR4 expression in each of the pStages and non-cancerous tissues were examined.

To select the optimal internal control gene, the TaqMan® Human Endogenous Control Plate (Applied Biosystems, Inc., Foster City, CA, USA) was used, which contained TaqMan primers and probes for 11 commonly used HK genes and one internal positive control sequence. Non-cancerous and cancerous tissue specimens obtained from CRC patients were used as the test specimens (8 samples from 4 patients) to determine the most suitable internal reference genes. One of the non-cancerous tissue specimens was used to calibrate the plate.

This study was approved by the Ethics Committee of the Toho University Sakura Medical Center.

*Real-time PCR*. RNA was extracted using an RNeasy® Plus Mini kit (Qiagen, Hilden, Germany) and cDNA was synthesized using an AffinityScript® QPCR cDNA synthesis kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions, as previously described (15).

Quantitative real-time PCR was performed using a Stratagene Mx3000P® QPCR System with TaqMan® Gene Expression Master mix. The thermal profile consisted of precycle heat activation at 95˚C for 10 min, followed by 95˚C for 15 sec and then 60˚C for 60 sec for a total of 40 cycles. Data were expressed as fold-induction relative to the RNA amplified at the lowest level. Relative quantification of total gene product in each sample was performed using the comparative CT (ΔΔCT) method.

The primers and probes used in this study can be found using the assay IDs: Hs00610101_ml for TLR2, Hs00152939_ml for TLR4, and Hs99999903_ml for BA (http://products.appliedbiosystems.com/ab/en/US/adirect/ab).

*Statistical analysis*. Data were expressed as the means ± standard deviation (SD). Mean values were compared in groups using the unpaired Student's t-test with two-tailed P-values using StatMate III (ATMS Co., Ltd., Tokyo, Japan). $P<0.05$ was considered to indicate statistically significant difference.

## Results

*Detection of the indicated target genes to compare gene expression between sample*. The effect of several potential internal control genes on relative expression levels was assessed using the TaqMan® Human Endogenous Control Plate, according to the manufacturer's instructions. The three HK genes selected as internal control genes, GUS, BA and B2M, exhibited minimal variability at expression levels, based on the total threshold cycle, in samples under the experimental conditions used (Fig. 1). Relative quantification of gene expression was performed using each gene individually and together.

*TLR2 and TLR4 expression in cancerous and non-cancerous tissues*. TLR4 expression in non-cancerous and cancerous tissues was confirmed in all the 50 subjects using real-time PCR. TLR4 mRNA expression was significantly higher in cancerous compared to non-cancerous tissues when B2M

Table I. TLR2 and TLR4 expression in cancerous and non-cancerous tissues (mean ± SD).

| | BA | | GUS | | B2M | |
|---|---|---|---|---|---|---|
| Receptor | NCT | CT | NCT | CT | NCT | CT |
| TLR2 | 2.61±1.82 | 6.60±4.15[b] | 5.08±4.44 | 13.61±12.26[a] | 5.14±3.01 | 30.84±22.61[b] |
| TLR4 | 1.61±1.04 | 2.17±2.22 | 3.24±3.40 | 2.72±2.37 | 2.92±21.13 | 5.22±3.23[b] |

[a]P<0.01; [b]P<0.001. BA, β-actin; GUS, β-glucuronidase; B2M, β-2 microglobulin; NCT, non-cancerous; CT, cancerous.

Table II. TLR2 and TLR4 expression at various histological stages (mean ± SD).

| | BA | | GUS | | B2M | |
|---|---|---|---|---|---|---|
| Receptor (pStage) | NCT | CT | NCT | CT | NCT | CT |
| TLR2 (I) | 1.76±0.96 | 7.02±4.55[b] | 2.70±1.20 | 8.84±6.13[a] | 4.02±0.15 | 11.21±5.64[a] |
| TLR4 (I) | 1.14±0.43 | 2.10±1.41 | 1.15±0.11 | 1.31±0.13 | 1.30±0.64 | 5.43±3.17[a] |
| TLR2 (II) | 2.89±2.05 | 10.90±8.61[b] | 5.10±4.35 | 18.02±14.85[a] | 5.32±3.40 | 52.84±58.88[b] |
| TLR4 (II) | 1.11±0.55 | 1.82±1.74 | 2.47±2.63 | 2.03±1.37 | 4.16±4.37 | 4.69±3.37 |
| TLR2 (III) | 4.02±3.19 | 7.73±5.55[b] | 5.07±5.40 | 12.44±9.71[a] | 6.11±2.83 | 34.17±19.96[c] |
| TLR4 (III) | 1.72±0.78 | 1.33±1.09 | 4.09±3.08 | 2.86±2.50 | 3.79±2.00 | 6.80±4.00[a] |
| TLR2 (IV) | 1.77±1.06 | 5.59±1.94[b] | 1.50±0.72 | 5.94±2.18[b] | 3.94±1.83 | 16.64±10.22 |
| TLR4 (IV) | 1.67±1.04 | 3.50±3.33 | 2.65±1.63 | 3.92±3.17 | 2.86±1.81 | 4.63±0.03 |

[a]P<0.05; [b]P<0.01; [c]P<0.001. BA, β-actin; GUS, β-glucuronidase; B2M, β-2 microglobulin; NCT, non-cancerous; CT, cancerous.

was used as the internal control gene (P<0.001). No significant difference was observed in TLR4 expression between non-cancerous and cancerous tissues when GUS and BA were used as the control genes (Table I). TLR2 mRNA expression was also confirmed in the 50 cancerous and in 49 of the non-cancerous tissue specimens using real-time PCR. TLR2 expression was significantly higher in cancerous compared to non-cancerous tissues when analyzed using each of the three internal control genes (P<0.001) (Table I).

*TLR2 and TLR4 expression according to histological stage*. TLR2 and TLR4 expression was compared between cancerous and non-cancerous tissues at various pStages. TLR4 expression was significantly higher in pStage I and II cancerous compared to non-cancerous tissues when B2M was used as an internal control gene, while no statistically significant differences were detected in pStage II and IV cancerous tissues (Table II). TLR2 expression was significantly higher in pStage I, II and III cancerous compared to non-cancerous tissues with the three control genes (pStage I, P<0.05 for the control genes; pStage II, P<0.05 with GUS and P<0.01 with B2M and BA; pStage III, P<0.05 with GUS and BA and P<0.001 with B2M). In pStage IV tissues, significant differences in TLR2 expression were observed only with GUS and BA (P<0.01) (Table II). TLR2 expression did not differ significantly in non-cancerous and cancerous tissues in pStage IV with B2M as the control gene.

## Discussion

Common HK genes, such as GAPDH and BA are traditionally used as internal controls for the assessment of gene expression using techniques such as quantitative real-time PCR (12). However, previous studies have indicated that the expression levels of HK genes may be different in various tissues or between normal and diseased tissues (2,4). Shrout *et al* (21) and Bianchini *et al* (13) reported that low levels of B2M in CRC tissue might serve as a prognostic indicator in CRC patients with lymph node metastasis. Vandesompele *et al* (1) have recommended the use of at least three appropriate control genes for the calculation of a normalization factor in view of the inherent variation in the expression of HK genes.

Bustin (22) proposed the use of the TaqMan® Human Endogenous Control Plate for normalizing mRNA levels in tissue culture cells using HK genes. Other groups have similarly emphasized the convenience of this system for initial screening to select an appropriate internal reference gene (11,23). In the present study, three HK genes (GUS, B2M and BA) were selected as the internal control genes. These genes exhibited a relatively stable expression under the present conditions.

In the majority of cases, consistent results were obtained when GUS and BA were used as the internal control genes. However, TLR4 expression was higher in cancerous compared to non-cancerous tissues, when B2M was used as the internal

control gene. These results may be associated with the low level of B2M expression in cancerous tissues and are consistent with the findings of Shrout *et al* (21) and Bianchini *et al* (13).

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