Comparative Biochemistry and Physiology, Part C 153 (2011) 259–268

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Validation of housekeeping genes as internal controls for studying biomarkers of endocrine-disrupting chemicals in disk abalone by real-time PCR

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ARTICLE INFO

Article history:
Received 25 March 2010
Received in revised form 13 November 2010
Accepted 15 November 2010
Available online 17 December 2010

Keywords:
Reference genes
Internal control
Real-time PCR
EDCs
Biomarker

ABSTRACT

Our experiments were designed to identify suitable housekeeping genes (HKGs) in disk abalone as internal controls to quantify biomarker expression following endocrine disrupting chemicals (EDCs). Relative expression levels of twelve candidate HKGs were examined by real-time reverse transcription PCR (qRT-PCR) in gill and hepatopancreas of abalone following a 7-day challenge with either tributyltin chloride (TBT) or 17β-estradiol (E2). The expression levels of several conventional HKGs, such as 18s rRNA, glyceraldehyde-3-phosphate dehydrogenase and β-actin, were significantly altered by the challenges, indicating that they might not be suitable internal controls. Instead, the geNorm analysis pinpointed ribosomal protein L-5/ elongation factor 1 and ribosomal protein L-5/ succinate dehydrogenase as the most stable HKGs under TBT and E2 challenges, respectively. Moreover, these three HKGs also showed the highest stabilities overall amongst different tissues, genders and EDC challenges. The expression of a biomarker gene, cytochrome P450 4B (CYP4), was also investigated and exhibited a significant increase after the challenges. Importantly, when unsuitable HKGs were used for normalization, the influence of two EDCs on CYP4 expression was imprecisely overestimated or underestimated, which strongly emphasized the importance of selecting appropriately validated HKGs as internal controls in biomarker studies.

1. Introduction

Endocrine disrupting chemicals (EDCs) are a class of chemicals that can interfere with natural hormone actions in the body (Kavlock et al., 1996). As a result of exposure to EDCs, humans and wildlife may exhibit several signs of ill-health including abnormal plasma hormone levels, abnormal reproductive organs, reduced germ cell count, dysfunctional immune system, and increased cancer risk (Mills and Chichester, 2005; Sonnenschein and Soto, 1998). Every year, however, great quantities of EDCs from various sources are discharged into the ambient environment. As the ultimate recipient of all kinds of waste effluents, the marine environment is suffering the most serious contamination with EDCs. The instances of endocrine disruption in marine organisms are also continuously increasing worldwide (Ford et al., 2004; Matthiessen et al., 2002; Porte et al., 2006). As a consequence, monitoring, risk assessment and management of marine EDC pollution have given rise to significant concerns from the scientific community, government agencies and the general public. To date, several biomarker genes, such as vitellogenin (VTG), cytochrome p450 1A (CYP1A), glutathione S-transferase (GST) and metallothionein (MT), have been well established in marine organisms to monitor contamination with EDCs (Fossi et al., 2002; Rhee et al., 2009; Wan et al., 2008).

In recent ecotoxicological studies, qRT-PCR is one of the most widely used methods to evaluate the expression of biomarker genes as affected by environmental contaminants. However, researchers have shown little concern about the selection and validation of suitable HKGs for biomarker quantification. To the best of our knowledge, there are only a few published works in this regard, which were respectively conducted in marine mammals (Spinsanti et al., 2008), fish (Filby and Tyler, 2007), crustacean (Heckmann et al., 2006) and insects (de Boer et al., 2009). There is a substantial lack of information in the mollusc phylum, although molluscs have been frequently used as sentinel species in pollution monitoring programs (Hall et al., 2009; Valdez Domingos et al., 2007). Most of the studies that quantify biomarker expression in mollusc species have used HKGs randomly, without any validation of their expression stabilities (Banni et al., 2007; Brooks et al., 2009; Puinean and Rotchell, 2006; Zapata et al., 2009). Recently, a large body of evidence has

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doi:10.1016/j.cbpc.2010.11.009

demonstrated that the expression of several commonly used HKGs such as 18s rRNA, β-actin and GAPDH us subject to considerable variable in certain tissues or under certain experimental conditions (Goidin et al., 2001; Ruan and Lai, 2007; Selvey et al., 2001). It is well-known that selection of an inappropriate HKG for normalization can increase experimental noise and thus strongly influence the reliability of qRT-PCR analyses. In the present study, therefore, we attempted to identify suitable HKGs in abalone (a marine gastropod mollusc) for studying biomarkers of contamination with EDCs. We selected twelve HKGs and evaluated their expression stabilities in disk abalone (*Haliotis discus discus*) gill and hepatopancreas after 7 days water-borne exposure to either TBT or E2. Furthermore, the influence of using different HKGs on the quantification of biomarker expression was also investigated.

2. Materials and Methods

2.1. Animals

Two-year-old disk abalones (*Haliotis discus discus*) weighing 50-60 g and with well-developed gonads were obtained from an abalone farm on Jeju Island, South Korea. The abalone were acclimated in laboratory aquaria for 1 week prior to the challenge experiment. The seawater was filtered and aerated continuously, with the salinity and temperature maintained at 32 ± 1‰, and 20 ± 1 °C, respectively. After acclimation, the abalone were divided into one control group and two challenge groups, having three males and three females in each group. The stock solutions of TBT (200 μg/mL) and E2 (20 mg/mL) were prepared in dimethyl sulfoxide (DMSO). The abalone in the two challenge groups were exposed to seawater containing 1 μg/L of TBT and 100 μg/L of E2, respectively. In the control group, only DMSO vehicle was added into the seawater. The final concentration of DMSO in the seawater of all the groups is 0.0005%. Toxicant treated seawater was renewed every 24 h. After 7 days exposure, gill and hepatopancreas tissues were dissected and frozen in liquid nitrogen immediately for further experiments.

2.2. Total RNA extraction and cDNA synthesis

Total RNA of gill and hepatopancreas was extracted from the tissue pools containing the same weight of tissue from three abalones using TRI Reagent (Sigma). A secondary purification using the S.N.A.P. total RNA isolation kit (Invitrogen) was subsequently carried out for hepatopancreas samples due to the abundance of polysaccharides and pigments, which have interfered with subsequent processes. Total RNA concentration was obtained by measuring absorbance at 260 nm. The quality of RNA was also verified by formaldehyde agarose gel electrophoresis. First-strand cDNA synthesis was carried out using the SuperScript III First-Strand Synthesis System (Invitrogen). Briefly, 2.5 μg RNA, 1 μL of 50 μM oligo $(dT)_{20}$, 1 μL of 50 ng/μL random hexamers and 10 mM dNTP mix were combined to incubate at 65 °C for 5 min and then placed on ice for at least 1 min. Subsequently, 2 μL 10 × RT buffer, 2 μL 25 mM $MgCl_2$, 2 μL 0.1 M DTT, 40 U RNaseOUT and 200 U SuperScript III were added to a total volume of 20 μL and then incubated at 25 °C for 10 min followed by 50 °C for 50 min. The reaction was terminated by incubating at 85 °C for 5 min and the residual RNA was removed by incubating at 37 °C for 20 min with the addition of 1 μL RNaseH. Finally, the cDNA was diluted 1:20 for use in qRT-PCR.

2.3. Primer design and qRT-PCR analysis

Sequences of twelve HKGs and one biomarker gene (CYP4) obtained from a disk abalone cDNA library constructed by our earlier work (Munasinghe et al., 2006) were used to design primers using Primer 3.0. The primer pairs were selected using the following criteria: melting temperature (T_m) of approximately 60 °C, primer length ranging from 18 base pairs (bp) to 24 bp with 22 bp as the optimum, and an amplicon size of approximately 150 bp (Table 1). qRT-PCR was run in triplicate for each samle in 0.2 mL 8-strip PCR tubes with a Takara™ real time PCR detector TP800 (Takara Inc., Japan). Each reaction consisted of 12.5 μL of SYBR Premix Extaq (Takara), 4 μL cDNA from 25 ng total RNA, and 1 μmol of a pair of specific primers in a final volume of 25 μL. The thermal profile was programmed as follows: 3 min at 94 °C, and 40 cycles of 20s at 95 °C for, 20 s at 60 °C, and 30 s at 72 °C. In order to ensure amplification specificity, the dissociation curve of the PCR product was investigated by heating from 60 °C to 95 °C at the end of each reaction.

2.4. Data processing and statistics

The PCR results were analyzed by the software platform of the Takara TP800 thermocyler. Baseline and threshold values were automatically set by the program. The number of PCR cycles required to reach the fluorescence threshold in each sample was defined as the C_t value. The C_t values for each of the twelve housekeeping genes were transformed into relative quantities using the $2^{-\Delta Ct}$ method where $\Delta C_t = C_{t\ treated} - C_{t\ control}$ (Livak and Schmittgen, 2001). Expression stability values for the 12 HKGs under either E2 or TBT challenge were then calculated by the geNorm VBA applet for Microsoft Excel (Vandesompele et al., 2002). To determine the effect of using the different internal controls on biomarker gene quantification, the expression of CYP4 in gill and hepatopancreas after TBT and E2 exposure was normalized to the 12 HKGs. Statistical comparisons between the control and experimental groups were carried out using Student's *t*-test in SPSS 16.0 for Windows (SPSS, Chicago, IL, USA). Statistical significance was assigned at $p < 0.05$. Experimental data are shown as the mean ± S.E.M.

3. Results

3.1. Absolute expression of the twelve HKGs

To reduce possible errors due to co-regulation of different genes, we purposely selected twelve HKGs that are involved in different cellular functions: 18s rRNA, ACTB, BGLU, CY, ELF, GAPDH, HH2A, HPRT, RPL5, SDHA, TUBB and UBC (Table 1). All twelve HKGs exhibited high similarities (>50% in protein sequence) to their respective gene homologues in the NCBI database. The expression of the twelve HKGs was investigated by qRT-PCR using gene-specific primers. Each different primer pair used in the qRT-PCR analysis had an expected efficiency of 1.9-2.0 and displayed a single dissociation peak at the expected temperature (data not shown), indicating a highly efficient and specific PCR amplification. The C_t values of the twelve HKGs are shown in Fig. 1. C_t values are proportional to the negative logarithm of the specific transcript copy number in the input cDNA. In both gill and hepatopancreas, 18s rRNA registered the highest abundance ($C_t < 10$). It was followed by ACTB, ELF and RPL5 ($C_t \approx 15$). At the end of the range, BGLU and HPRT1 were expressed at the lowest levels ($C_t \approx 25$). It is noteworthy that most HKGs exhibited remarkably similar expression levels between two different tissues. In addition, there was very little effect of either gender or EDC treatment on absolute expression of any of the HKGs.

3.2. Relative expression levels of twelve abalone HKGs following EDCs exposure

As shown in Fig. 2, significant expression change ($P < 0.05$) in relative expression were found for all twelve HKGs, and these changes were highly associated with tissue type, gender and/or treatment condition. In gill, the expression patterns of the twelve HKGs were remarkably similar. Almost all twelve HKGs were induced by TBT in male gill and slightly repressed in female gill, except for CY, which was

Table 1
Candidate reference genes, biomarker gene of interest and their specific primers used in the study.

Symbol	Gene name	Function	Forward primer	Reverse primer	Size (bp)
18rRNA	18s ribosomal RNA	Structural constituent of ribosome	AGACTGTCGATGGTAAGTGCTATGC	ACTACCTCCTCGTATCGAGATTGG	153
ACTB	Beta-actin	Cytoskeletal structural protein	GAATCTGCTGGTATCCATGAAACC	GGGATGTGATCTCCTTCTGCAT	151
BGLU	Beta-glucuronidase	Carbohydrate metabolic process	ATAGTCCAGGCCTTTCCCTCGAAT	CCTCTTCAGCTGGAATGGATGTGT	155
CY	Cyclophilin	Protein folding	GATCCAAGGTGGAGACTTCACTAAG	AACTGGGAACCATTGGTGTTG	153
ELF	Elongation factor 1-alpha	Essential component of the eukaryotic translational apparatus	CTGCCACACAGCCCATATTG	CCACACACATGGGCTTGCT	152
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme	TGGGCGTGAACCACACTAAGTAC	GCGGTGTAAGCATGAACTGTTG	151
HH2A	Histone H2A	Chief structural protein of the nucleosomes	GCAATGCCAGTAAGGATTTGAAAG	TTTCCGATCAGAGACTGTGGATA	151
HPRT	Hypoxanthine phosphoribosyltransferase 1	Metabolic salvage of purines	ACGACATCTCAACAGGGAACATC	GACTTGGGCTTCACTTCCTTCA	151
RPL5	Ribosomal protein L5	Ribosomal protein in ribosomal large subunit	TCACCAACAAGGACATCATTTGTC	CAGGAGGAGTCCAGTGCAGTATG	152
SDHA	Succinate dehydrogenase	Electron transporter in the TCA cycle and respiratory chain	CTAGCTCTGCCTGTCACATACCA	TACCAAACACAAACCCTGTGGATA	151
TUBB	Beta-tubulin	Member of the tubulin family of structural proteins	AGTTCTGGGAGGTGATTTCAGATG	TCCAAATCGACGAGGATAGCA	151
UBC	Ubiquitin-conjuction enzyme	Protein degradation	CACTGGCAAGCAACAATAATGG	CCATTGCTGTTGATGTTTGGA	152
CYP4	Cytochromep450 family 4	Phase I detoxification enzyme	AGAAGAAGGACACCTACGCATACA	CGTCCGACTTTGTGGCTTATATC	151

induced in both genders. In contrast, ten HKGs were induced by E2 in gill for both genders. There was no statistically significant change in the expression of either 18s rRNA or RPL5 for two challenges in gill tissue.

In contrast, the responses of twelve HKGs in hepatopancreas were fairly variable. In male hepatopancreas, TBT significantly induced the expression of nine of the HKGs by up to 2-fold; while in female, five HKGs were significantly repressed, three HKGs were significantly induced and four HKGs showed no statistically significant change. Similarly, E2 challenge also caused significant induction of nine HKGs in male hepatopancreas; while in female, five HKGs were significantly repressed, three HKGs were significantly induced and four HKGs showed no statistically significant change. Amongst the twelve HKGs, RPL5 was comparatively stable in hepatopancreas, showing a significant expression change only in TBT treated male abalone.

3.3. Expression stability analysis and selection of the most suitable HKGs for normalization

As shown in Fig. 3, the twelve HKGs were ranked by average expression stability (M) values from the geNorm analysis, starting from the least stable gene at the left (highest M value), and ending with the two most stable genes at the right (lowest M value). All twelve HKGs exhibited generally high stability, with the M values lower than 0.35. For TBT challenge, ELF and RPL5 were the two most stable genes (Fig. 3-A); while for E2 challenge, RPL5 and SDHA showed the lowest M values (Fig. 3-B). Finally, RPL5 and SDHA (followed by ELF) were determined as the most stable HKGs in the overall in the 12 samples from the two EDC challenges (Fig. 3-C). In contrast, the 18s rRNA and BGLU were shown to be the worst HKG candidates by the geNorm classification, due to their high M values.

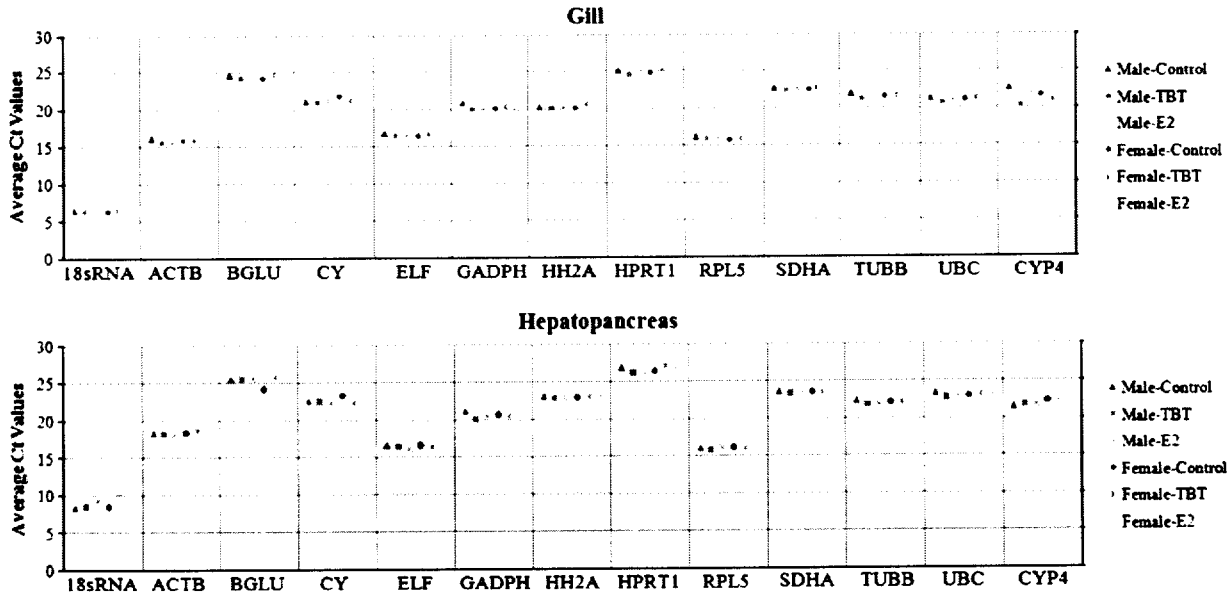

Fig. 1. Raw C_t values of twelve abalone HKGs and one biomarker gene CYP4 for different tissues (gill and hepatopancreas), genders (male and female), and experimental conditions (control, 7-day exposure of 1.0 μg/L TBT and 100 μg/L E2). The results are represented as the arithmetical mean of Ct values obtained from three replicates in the qRT-PCR assay.

3.4. Effect of different HKGs on biomarker gene quantification

The expression of CYP4 in abalone gill or hepatopancreas after TBT or E2 challenges was examined by qRT-PCR (Fig. 4). Without normalization by HKGs, assuming that the same amount of input cDNA has been used for each sample, the expression of CYP4 was significantly induced by TBT challenge ($P<0.001$) in both gill and hepatopancreas and for both genders. Similarly, CYP4 expression following E2 challenge also demonstrated significant induction ($P<0.001$) in male abalone. However, the E2 effect in female abalone was generally not significant, indicating a highly male-specific effect. When the expression data for CYP4 were normalized against different HKGs, the expression patterns in gill were generally similar, although the fold-changes and/or significances were remarkably variable (Fig. 5-A). In contrast, the normalized patterns of CYP4 expression in hepatopancreas against the different HKGs were quite distinct as the result of the stronger influence of EDCs on individual HKGs in this tissue (Fig. 4-B). Finally, we normalized CYP4 expression data with an optimal normalization factor (NF), which was obtained by the geometric mean of the two most stably expressed HKGs in the geNorm analysis. Abalone CYP4 displayed significant induction by TBT and E2 challenges in male abalone ($P<0.001$). While in female abalone, a significant change in CYP4 expression was observed only in TBT treated gill ($P<0.001$).

4. Discussion

Validation of HKGs for accurate normalization of real-time PCR data in specific biological samples or experimental conditions has been extensively carried out in many cell lines and model organisms for research in different fields. In contrast, the relevant work in non-model organisms has received much less attention (Siah et al., 2008). Our study represents the first effort aimed toward the systematic comparison of HKGs for environmental biomarker studies in mollusc species. We selected abalone (a marine gastropod) as an experimental animal, not only because of its high economic value in fisheries, but also due to its important roles in marine ecosystems and biological diversity. Even though no risk of extinction has been perceived yet, the wild populations of abalone in Korea, *H. sieboldii* in particular, have shown a rapid decline over the past few years, as a result of overfishing, climate change, environmental pollution and disease outbreak. Like most mollusc species, abalone appears particularly sensitive to EDCs due to a lack of an efficient system to metabolize and eliminate exogenous organic chemicals. In abalone, TBT and

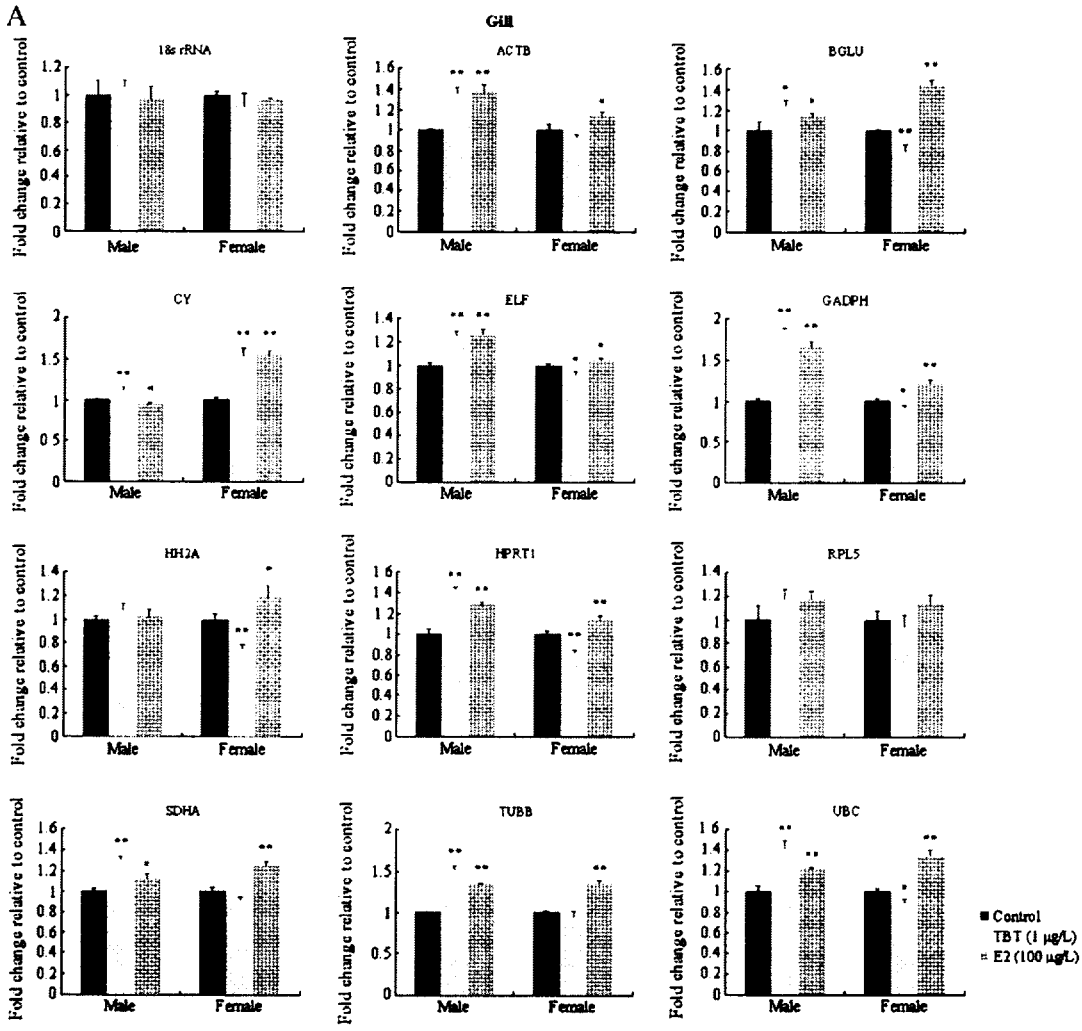

Fig. 2. Relative gene expression of twelve abalone HKGs following 7-day TBT and E2 challenges. Relative expression fold of each HKG in gill (A) and hepatopancreas (B) was calculated based on the Ct values from qRT-PCR in the method of $2^{-\Delta Ct}$. The results are represented as means ($n=3$) ± S.E.M. Statistically significant differences in gene expression between control and TBT/E2 treated abalone are denoted as follows: * $P<0.05$, ** $P<0.01$ (Student's *t*-test).

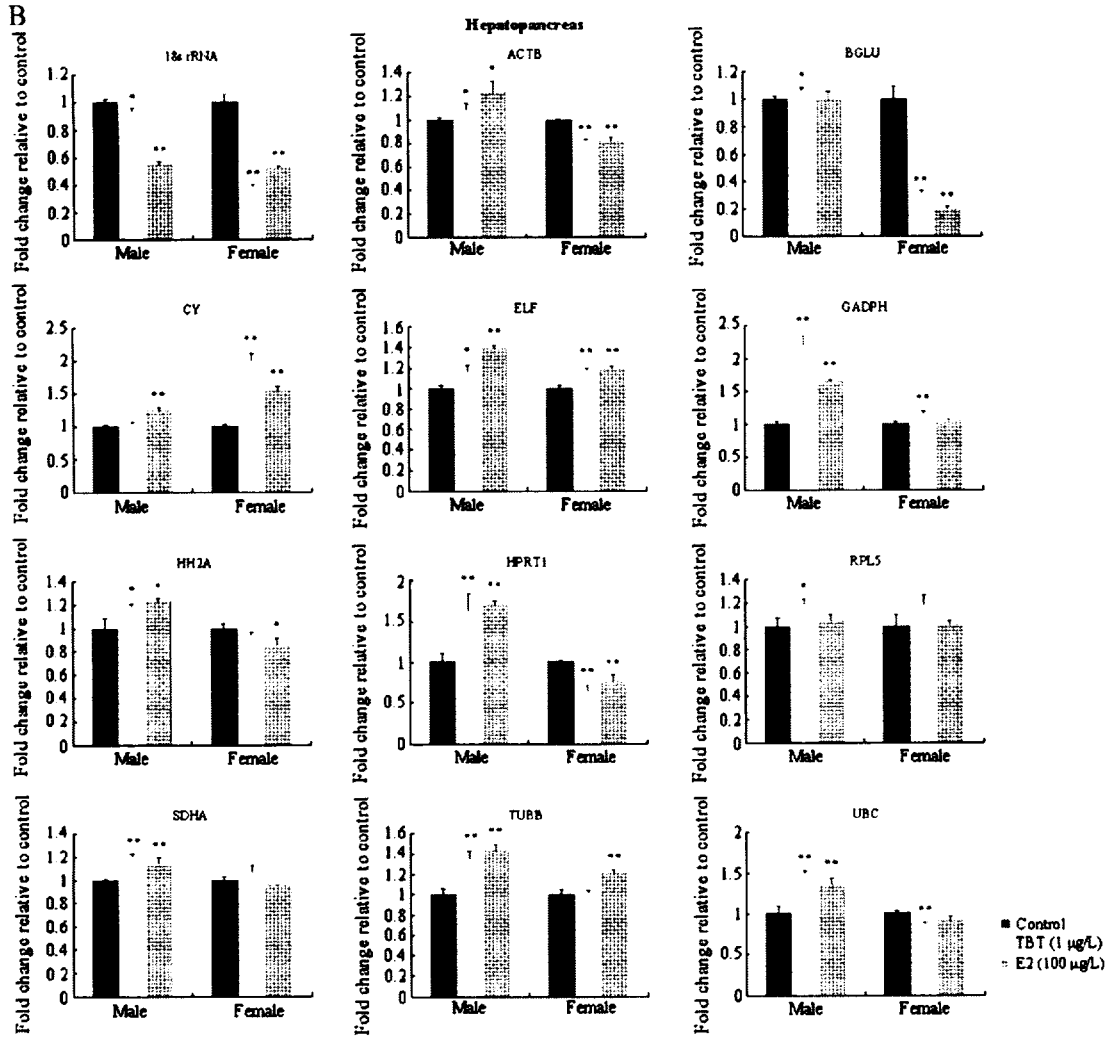

Fig. 2 (*continued*).

derivatives were reported to disturb the reproductive cycle and cause ovarian spermatogenesis in a female ovary, which contributed to abalone population decline in the examined site (Horiguchi et al., 2000, 2002; Sloan and Gagnon, 2004). On the other hand, excessive E2 exposure could evoke a "superfemales" syndrome in female molluscs and massive egg production and elevated mortality (Duft et al., 2006). We purposely selected these two model EDCs for the challenge experiment because we wish our findings to be representative for other EDCs with similar androgenic or estrogenic properties.

The study of biomarkers places strong emphasis on quantitative analysis of gene expression, and hence the qRT-PCR assay undoubtedly is one of the most important tools. In our earlier studies, however, we had met serious problems with the use of a randomly selected β-actin gene to normalize qRT-PCR data in abalone until we realized the importance of examining other appropriate HKGs. Although genes of actin family have been largely used as internal controls in quantitative analysis of biomarker expression in mollusc species (Park et al., 2009; Rhee et al., 2008), the expression of our gene was tightly regulated by many challenge conditions, and also showed huge variations among different tissues of abalone (data was not shown). This prevented the accurate analysis of biomarker genes of interest. Similar findings regarding varying expression of α- and β-actin genes have been widely reported in model organisms and cell lines when they were subjected to various stimuli such as temperature, hypoxia, microbial infection, oxidative stress and diseases (Araya et al., 2008; Chen and Ruan, 2009; Ruan and Lai, 2007). These results strongly challenged the role of actins as the conventional reference gene. Moreover, an association between hormones and regulation of actin expression has been revealed by several studies (Schroder et al., 2009; Verma and Shapiro, 2006). Thus, the use of actins as an internal control in a study about the effects of EDCs, which could potentially alter normal hormone levels, especially requires careful examination. In the present study, we have tested another actin gene (ACTB) of disk abalone, which showed highest similarity to cytoplasmic β-actin from other organisms. Its expression was much more stable than the previously utilized one, and it showed moderate expression stability among the twelve HKG candidates by geNorm analysis, especially following exposure to E2 (Fig. 3). However, we noted that this β-actin gene had a significant tissue-specificity with approximately 10-time higher expression in gill than in hepatopancreas (Fig. 1), which could potentially lead to a false tissue distribution pattern of the target gene if used as an internal control. In addition to β-actin, GADPH and 18s rRNA are the two most used HKGs as internal controls in many studies, but they were grouped into the least stably expressed HKGs since their expression was dramatically altered after exposure to the two EDCs (>0.5-fold change). In addition, there is still a debate over whether rRNA is suitable as an internal control in qRT-PCR mainly due to its high abundances (80–90% of total RNA) compared with target mRNA

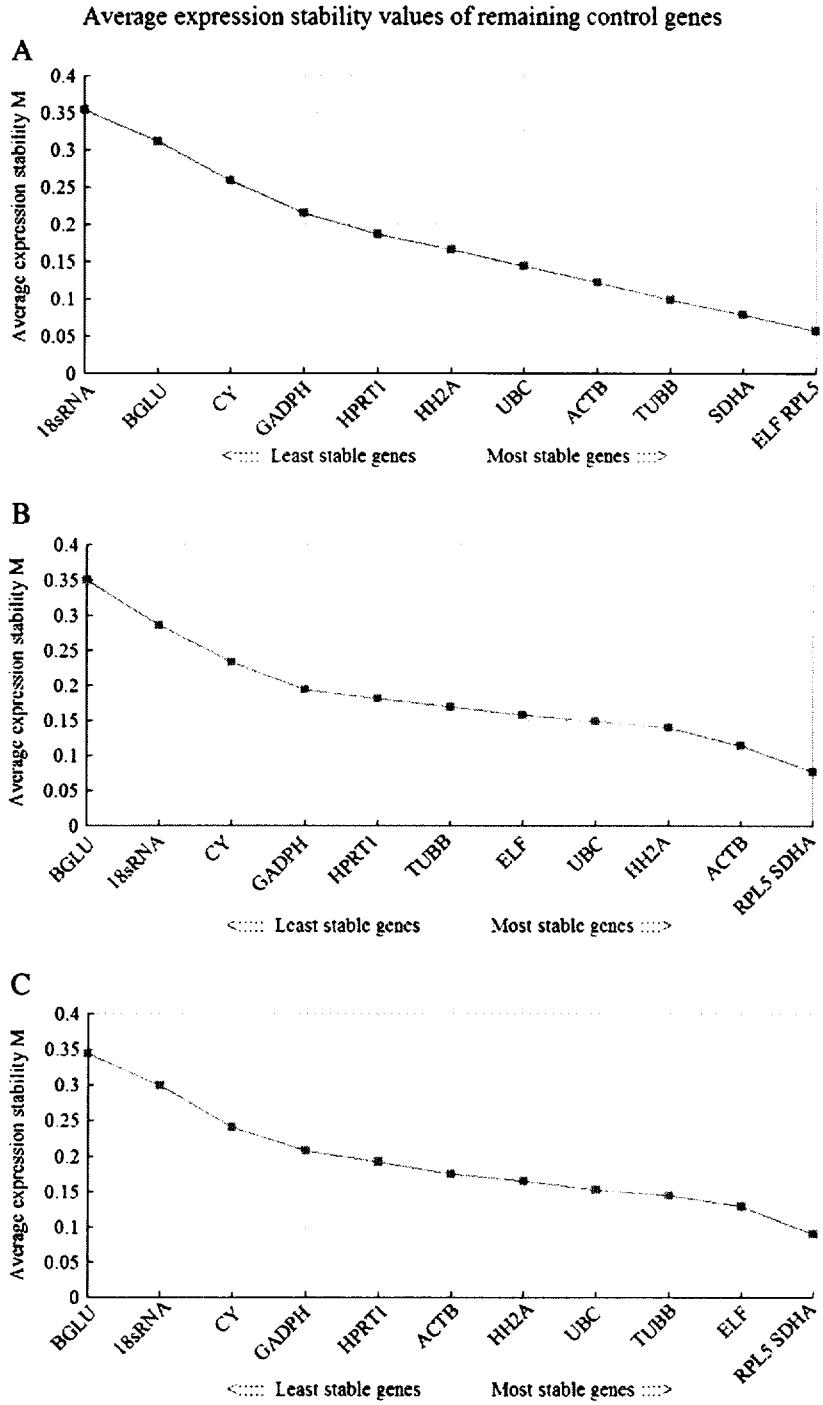

Fig. 3. Average expression stability measure (M) of twelve abalone HKGs following exposure to 1.0 μg/L TBT (A), 100 μg/L E2 (B) and overall (C), respectively. In overall analysis, 12 relative expression values from two tissues, two genders and two challenges were together input into the geNorm program.

transcripts within cells, which makes it difficult for accurate baseline subtraction in qRT-PCR analysis (Vandesompele et al., 2002). In contrast, some unconventional HKGs exhibited better expression-stability in our study and seemed to be more useful for the experiments involving challenge by EDCs.

Nevertheless, we noted that the simple comparison of expression patterns (up- or down-regulation) is not adequate to identify the most appropriate HKGs as there are several gene candidates showing similar expression changes. To address this issue, many computer programs such as geNorm, NormFinder and BestKeeper are available. Despite different statistical algorithms used to measure the expression stability, scientists found that the results produced by these programs usually showed only minor deviations (Radonic et al., 2005). Therefore, it is recommended to use only one of these tools to pinpoint stable HKGs under most experimental conditions. In our study, we chose geNorm to evaluate expression stability of the twelve HKGs because this program ranks the genes according to the average pairwise variation between a particular gene and all other control

genes, and also provides a measure of the minimum optimal number of reference genes (Vandesompele et al., 2002). Although the geNorm algorithm is highly dependent on the proposition that the expression of HKG candidates should not be co-regulated in experimental conditions, the 12 HKGs that we selected are involved in different biological processes, and the effect of such co-regulation thus theoretically should be minor. Based on the results of the geNorm analysis, ELF, SDHA and especially RPL5 were identified as the most stable genes under TBT and E2 exposure. The high stability of three HKGs in our study is highly consistent with the findings for E2 exposure on cetacean fibroblast culture (Spinsanti et al., 2008) and 17α-ethinylestradiol (EE2) exposure on fathead minnow (Filby and Tyler, 2007), which were the only works regarding validation of HKGs in the challenge of EDCs other than ours. RPL5 is one of the constitutive proteins in the large ribosomal subunit that catalyzes mRNA-directed protein synthesis. It binds specifically to the 5 S rRNA and forms a L5/5 S RNA protein complex that is a precursor to ribosome assembly. Moreover, the expression of RPL5 is not coordinated with the synthesis of other ribosomal proteins. As the most stable gene in our study, the expression of RPL5 was altered by

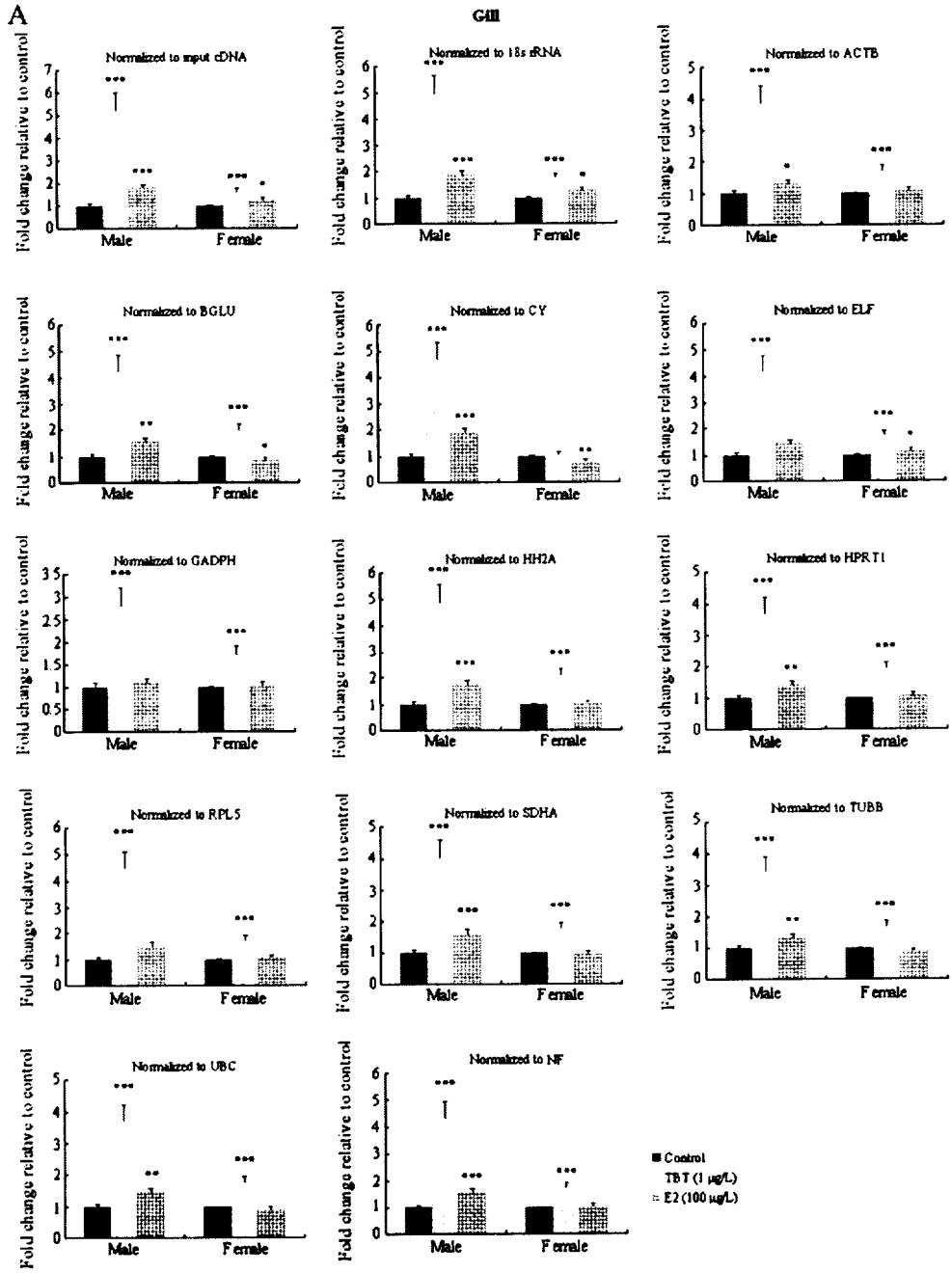

Fig. 4. Relative gene expression of biomarker gene CYP4 in abalone gill (A), and hepatopancreas (B), following 7-day TBT and E2 challenges, with or without normalization to twelve reference genes and a normalization factor (NF). The expression of CYP4 without normalization (normalized to input cDNA) was obtained by using the $2^{-\Delta Ct}$ method same as the calculation of HKGs, while the normalized result was obtained by the standard $2^{-\Delta\Delta Ct}$ method. The results are represented as means ($n=3$) ± S.E.M. Statistically significant differences in gene expression between control and TBT/E2 treated abalone are denoted as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (Student's t-test).

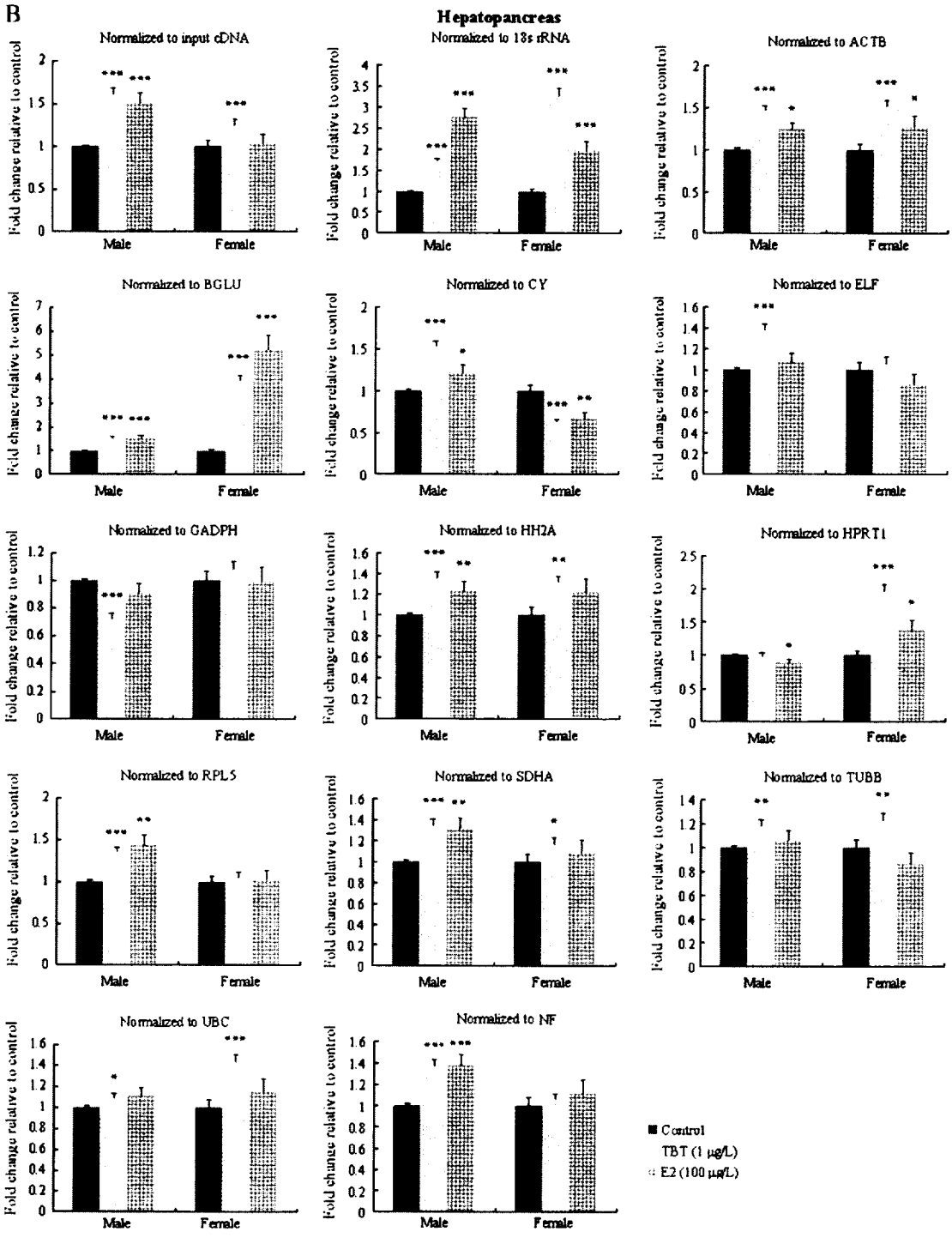

Fig. 4 (*continued*).

less than 0.2-fold in all of the samples, in contrast to over 0.5-fold for the other HKGs. Although ribosomal proteins were previously recommended for the use in only less sensitive detection methods like Northern blot (Thellin et al., 1999), many recent HKG validation studies have reported that ribosomal proteins showed remarkable stabilities in different cell lines and tissues of mammals (Brinkhof et al., 2006; Janovick-Guretzky et al., 2007; Spinsanti et al., 2006), fish (Infante et al., 2008), shellfish (Siah et al., 2008), and plants (Barsalobres-Cavallari et al., 2009). Overall, we would suggest ribosomal proteins can be a good candidate to substitute for the traditional HKGs as an internal controls in real time PCR assays.

In addition to the 12 HKGs, we also investigated the expression of one biomarker gene in abalone not only to ensure the impact of our experimental conditions on abalone, but also to assess the influences of using inappropriate HKGs as internal controls. In vertebrates like mammals and fish, vitellogenin (Vtg), the serum phospholipoglycoprotein precursor to egg yolk, has been proposed as an ideal biomarker for estrogenic contaminations due to its remarkaby

elevated expression both at the level of transcription and translation in response to the exposure (Matozzo et al., 2008). Unfortunately, however, the Vtgs of molluscs have been isolated and characterized in only a few bivalve species, but not in abalone. A similar predicament also occurred for selection of a biomarker for TBT, i.e., that there is no definitive biomarker gene available other than some general stress proteins like CYP1A and GSTs. Consequently, we chose the CYP4 gene of abalone, which is highly identical to mammalian cytochrome P450 4B enzymes, as the biomarker gene of interest. As the major enzymes in phase I biotransformation, many cytochrome P450s, such as CYP1B1, CYP17 and CYP19, are involved in the biosynthetic and metabolic pathways of estradiol (Tsuchiya et al., 2005). Although the enzymes in cytochrome P450 family 4 are typically related to cholesterol metabolism as fatty acid omega hydroxylases, they are also known to play a prominent role in xenobiotic metabolism and thereby serve as biomarkers for xenobiotic exposure (Jorgensen et al., 2005; Okita and Okita, 2001). The expression of abalone CYP4 was highly induced after 7 days waterborne exposure to TBT in gill but only slightly up-regulated in hepatopancreas, indicating that our challenge possibly caused acute oxidative stress in gill tissue that comes into directly contact with chemicals. On the other hand, if our challenge could be long term (>30 days), TBT might be accumulated in hepatopancreas and alter the levels of circulating androgens that would induce the expression of CYP4 as well (Imaoka et al., 1992). Compared to TBT, the exposure to E2 produced a weak effect on CYP4 expression in both tissues ($\leq$0.5 fold-change). This observation is consistent with the reports in humans that mRNA level and omega-hydroxylase activity of CYP4 genes were unaffected by E2 treatment (McCabe et al., 2001; Savas et al., 2005). It is noteworthy that the normalization procedure for CYP4 should be paid more attention since its low induction levels are easily altered by variations in the internal control. As shown in Fig. 4, through normalization against the unstable HKGs including 18s rRNA, BGLU and CY, we could possibly obtain erroneous results for biomarker quantification and thus mislead the monitoring and environment risk assessment of EDCs.

5. Conclusions

We analyzed the expression-stability of 12 housekeeping genes in abalone under the conditions of TBT and E2 challenges. Our validation results indicated that ELF, SDHA and especially RPL5 might be suitable internal controls to normalize expression data of EDC biomarkers in abalone. Our findings also provide insights into the application of orthologs of these genes as internal controls in other mollusc or invertebrate species exposed with EDCs. Nevertheless, our data were obtained through only analyzing the pooled samples of abalone where biological variation between abalone individuals could be masked, further examinations thereby may be necessary.

Acknowledgments

This work was supported by the Marine and Extreme Genome Research Center Program of the Ministry of Land, Transportation and Maritime Affairs, Republic of Korea; the MKE (The Ministry of Knowledge Economy), Korea, under the ITRC (Information Technology Research Center) support program supervised by the NIPA (National IT Industry Promotion Agency (NIPA-2009-C1090-0903-0007).

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