Expression profiling of choline and ethanolamine kinases in MCF7, HCT116 and HepG2 cells, and the transcriptional regulation by epigenetic modification

CHUA SIANG LING¹, KHOO BOON YIN², SEE TOO WEI CUN¹ and FEW LING LING¹

¹School of Health Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan 16150;
²Institute for Research in Molecular Medicine, Universiti Sains Malaysia,
Penang 11800, Malaysia

Received December 10, 2013; Accepted September 4, 2014

DOI: 10.3892/mmr.2014.2707

Abstract. The function of choline kinase (CK) and ethanolamine kinase (EK) is to catalyse the phosphorylation of choline and ethanolamine, respectively, in order to yield phosphocholine (PCho) and phosphoethanolamine (PEtn). A high expression level of PCho, due to elevated CK activity, has previously been associated with malignant transformation. In the present study, a quantitative polymerase chain reaction was performed to determine the mRNA expression profiles of ck and ek mRNA variants in MCF7 breast, HCT116 colon and HepG2 liver cancer cells. The ck and ek mRNA expression profiles showed that total cka was expressed most abundantly in the HepG2 cells. The HCT116 cells exhibited the highest ckβ and ek1 mRNA expression levels, whereas the highest ek2α mRNA expression levels were detected in the MCF7 cells. The ckβ variant had higher mRNA expression levels, as compared with total cka, in both the MCF7 and HCT116 cells. Relatively low ek1 mRNA expression levels were detected, as compared with ek2α in the MCF7 cells; however, this was not observed in the HCT116 and HepG2 cells. Notably, the mRNA expression levels of cka2 were markedly low, as compared with cka1, in all three cancer cell lines. The effects of epigenetic modification on ck and ek mRNA expression, by treatment of the cells with the histone deacetylase inhibitor trichostatin A (TSA), were also investigated. The results of the present study showed that the mRNA expression levels of cka1, ckβ and ek2α were affected by TSA. An increase >8-fold was observed in ek2α mRNA expression upon treatment with TSA, in a concentration- and time-dependent manner. In conclusion, the levels of ck and ek transcript variants in the three cancer cell lines were varied. The effects of TSA treatment on the mRNA expression levels of ck and ek imply that ck and ek mRNA expression may be regulated by epigenetic modification.

Introduction

Choline kinase (CK) and ethanolamine kinase (EK) are enzymes that initiate the first step in the Kennedy pathway, resulting in the biosynthesis of phosphatidylcholine and phosphatidylethanolamine (1). In the presence of Mg²⁺, CK and EK catalyse the ATP-dependent phosphorylation of choline and ethanolamine into phosphocholine (PCho) and phoshoethanolamine (PEtn), respectively. In humans, CK is encoded by two separate genes, cka and ckβ, which are located on chromosomes 11q13.2 and 23q13.33 (Ensembl Genome Browser v48, Gene view: http://www.ensembl.org/), respectively. The cka transcript has been shown to undergo alternative splicing, resulting in the generation of two mRNA splice variants: cka1 (NCBI reference: NM_212469.1) and cka2 (NCBI reference: NM_001277.2). These splice variants are later translated into two functional protein isoforms, CKα1 and CKα2, consisting of 439 and 457 amino acids, respectively (2). The cka2 variant contains an additional stretch of 54 nucleotides, as compared with the cka1 variant. CKβ is translated from the ckb (NCBI reference: NM_005198.4) mRNA transcript, and consists of 395 amino acids and the protein sequence has 60% homology with the CKα1 and CKα2 isoforms (2). Mammalian CKβ was initially characterised in rat liver (3). In humans and mice, CKβ, alongside muscular carnitine-palmitoyl transferase 1b (M-CPT1b), are encoded by a bicistronic gene (4). Due to the overlapping of the ckb and m-cpt1b genes on the chromosome; therefore, Yamazaki et al (5) previously suggested that these two genes may share the same regulators.

Human EK exists as three isoforms: EK1, EK2α and EK2β, encoded by two separate genes; ek1, which produces the ek1 transcript variant 1 (NCBI reference: NM_018638.4); and ek2, which undergoes alternative splicing to produce ek2α (NCBI reference: NM_018208.2) and ek2β (GenBank reference: AK001623.1) transcript variants. ek2α and ek2β are respectively translated into EK2α and EK2β (6).
CK actively participates in cell proliferation, which is modulated by various kinases and metabolites derived from phospholipids. PCho is a crucial lipid metabolite associated with cell proliferation and tumor development. Previously, a significant increase in CK activity and elevation of PCho levels was shown to be associated with cancer progression (7). Malignant transformation and progression have previously been reported to alter phospholipid metabolism, by increasing the production of PCho (8,9). A high concentration of PCho is a common characteristic of tumor-derived cells, and may be used as a novel biomarker for cancer diagnosis and assessment of cancer progression (10,11). CKα has also been proposed as a potential prognostic marker, to predict the outcome in patients with non-small-cell lung cancer (12).

The aim of the present study was to determine and analyze the expression profiles of ck and ek mRNA variants in MCF7 breast, HCT116 colon and HepG2 liver cancer cells, using quantitative polymerase chain reaction (qPCR). The cell lines were chosen due to the previously reported differential expression of CKα in breast, colon and liver tissues (13). Although cka overexpression is usually associated with carcinogenesis (12,14), it remains unclear whether this is always the case, as CKα protein expression levels have shown significant variation in numerous tumour tissues (13). The expression levels of ckb, relative to cka, have also been shown to affect the regulation of the cell cycle (15). Similarly, EK, which belongs to the same family of enzymes as CK, has been suggested to have a role in carcinogenesis, by promoting the growth of transformed cells (16). However, the expression levels of the ek gene transcripts in various cancer cells have not yet been compared. Quantification of ck and ek expression levels in numerous cancer cells may provide a new insight into their roles in promoting cancer cell growth. In addition, the regulation of ck and ek transcriptional activities in cancer cells by epigenetic modification remains to be elucidated. Epigenetic modification, such as histone deacetylation, modulates the expression of numerous genes at the transcriptional level, including genes that encode tumor protein p53 (17,18), E2F (19) and nuclear factor-xB (19,20). Numerous histone deacetylase inhibitors (HDACi) have been synthesised, including trichostatin A (TSA), which belongs to the hydroxamic acids group. HDACi have been shown to regulate gene transcription by inducing histone acetylation, and modulating the recruitment of transcription factors and other proteins to the promoter region of target genes (21). Notably, HDACi has also been reported to display significant demethylating activity by indirectly altering DNA and histone methylation (22). TSA has been demonstrated to indirectly induce gene promoter demethylation in fungi (23). To investigate whether the expression levels of ck and ek transcripts are affected by DNA methylation, a qPCR analysis was performed to determine the effects of TSA treatment in HepG2 cells.

Materials and methods

Cell culture. The MCF7 breast adenocarcinoma cell line [American Type Culture Collection (ATCC) no. HTB-22], HCT116 colorectal carcinoma cell line (ATCC no. CCL-247) and HepG2 liver hepatocellular carcinoma cell line (ATCC no. HB-8065) were obtained from ATCC (Manassas, VA, USA). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies). The cell lines were maintained at 37°C in 95% humidity and 5% CO₂.

TSA treatment of HepG2 cells. The HepG2 cells were seeded at 1 x 10⁶ cells/well, in a 24-well plate, and cultured in DMEM, supplemented with 10% FBS, overnight. The following day, the cells were treated with a final concentration of 670 nM TSA (Sigma-Aldrich, St Louis, MO, USA) for 24 h (24). The TSA was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and diluted in fresh medium. The control well was seeded with the same cellular density and treated with DMSO diluted with phosphate-buffered saline. To determine the effects of TSA concentration and treatment duration on ek2α expression levels, the HepG2 cells were treated with the indicated doses of TSA for 24 h, and at the indicated treatment durations with 750 nM TSA. The cells were observed using an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Total cellular RNA extraction and cDNA synthesis. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNase-Free DNase I (Qiagen) was used to eliminate any contaminating genomic DNA. The yield and purity of the total RNA was assessed by measuring the absorbance at 260 nm using a BioPhotometer Plus (Eppendorf, Hamburg, Germany). The integrity and size distribution of the total RNA were examined by agarose gel electrophoresis. cDNA was reverse transcribed from 1 µg of total RNA, using the RevertAid™ H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA).

Preparation of plasmids as homologous standards for qPCR. Plasmids containing the coding sequences of the various CK and EK isoforms (pET14b-cka, pGEXRB-cka2, pET14b-ckb, pET14b-ek1, pMALK4-ek2α, and pGEXRB-ek2β) were used as standards for the qPCR. The plasmid copy number (PCN) was calculated using the following equation, as described by previous methods (25):

\[
\text{PCN} = \left(\frac{[6.02x10^{23} \text{copy/mol}] \times \text{DNA amount (g)}}{[\text{DNA length (bp)}] \times 660 \text{ (g/mol/bp)}}\right)
\]

A 10-fold serial dilution of each plasmid DNA, ranging from 1 x 10⁴ to 1 x 10⁵ copies/µl, was used to construct a standard curve. The PCR amplification efficiency of each gene was calculated from the corresponding standard curve using the following equation, as described previously (24):

\[
E(\%) = (10^{-\text{dexp}-1}) \times 100\%
\]

qPCR. A qPCR was performed using the ABI PRISM 7000 Sequence Detection system (Applied Biosystems Life Technologies, Foster City, CA, USA). Each reaction was performed in a 25 µl volume containing 12.5 µl Power SYBR® Green I Master Mix (Applied Biosystems), specific primer (300 nM of target primer or 1 µl of reference primer) and 1 µl template DNA (1:2 diluted cDNA or plasmid DNA).
Thermal cycling was performed by 2-PCR and Primer were purchased from TATAA Biocenter. The Tukey Honestly Significant Difference using a >50% a defined as a region of >200 bp nucleotides, with a GC content statistically significant difference. Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Statistical analyses were performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression profiling of ck and ek mRNA variants in MCF7, HCT116 and HepG2 cells. In the present study, both absolute and relative quantification strategies were used to quantify and compare the mRNA expression levels of ck and ek variants in MCF7, HCT116 and HepG2 cells. The specificity of the PCR amplification of ck and ek variants was confirmed using a melt curve analysis and agarose gel electrophoresis. The slope of the generated standard curves for all of the amplicons was between -3.2 and -3.6, confirming that the amplifications were highly efficient.

The comparative cycle threshold (Ct) method was used to determine the expression levels of total-cka, cka2, ckβ, ek1, eka2 and ek2β variants, relative to the MCF7 cells and normalised to the geometric means of UBC and YWHAZ (Fig. 1A). A previous study identified UBC and YWHAZ as the most suitable reference genes for normalisation in gene expression studies using MCF7, HCT116 and HepG2 cells (27). In addition, an absolute mRNA copy number of each ck and ek variant was calculated from respective standard curves (Fig. 1B). The cka gene, which has previously been reported to be associated with carcinogenesis (7), had the highest mRNA expression levels detected in the HepG2 cells; total-cka had a value of 2031±415.78 mRNA copies/ng total RNA. The highest mRNA expression levels of ckβ and ek1, which has previously been reported to be associated with carcinogenesis (7), had the highest mRNA expression levels detected in the HepG2 cells; total-cka had a value of 2031±415.78 mRNA copies/ng total RNA. The highest mRNA expression levels of ckβ and ek1 were found in the HCT116 cells. The expression levels, relative to the MCF7 cells, were ~2- and 4-fold higher, respectively (Fig. 1A). The absolute copy numbers of the ckβ and ek1 mRNA variants in the HCT116 cells, however, as determined by absolute quantification, were markedly lower. The eka2 variant displayed the highest mRNA expression levels detected in the HepG2 cells, however, as determined by absolute quantification, were markedly lower.

Identification of CpG islands in the gene promoter region. The location of CpG islands at the cka, ckβ, ek1 and ek2 promoter regions were determined using CpG Plot, which is available from the European Molecular Biology Open Software Suite (http://www.EMBL-EBI.org). A CpG island is defined as a region of >200 bp nucleotides, with a GC content >50% and an observed/expected ratio >0.6.

Statistical analysis. Statistical evaluations were performed using a paired t-test and a one-way analysis of variance with the Tukey Honestly Significant Difference post-hoc test. All analyses were performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Table I. ck and ek primer sequences.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>PCR product size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>total-cka</td>
<td>F 5’-TCAGAGCAAACATCCGGAAGT-3’</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>R 5’-GGCGTAGTAGCCATGTACCACAAAAT-3’</td>
<td></td>
</tr>
<tr>
<td>cka2</td>
<td>F 5’-GGCCCTAGACAGTGCTGC-3’</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>R 5’-AGCTGGTATGAGCCCTCTCCTTT-3’</td>
<td></td>
</tr>
<tr>
<td>ckβ</td>
<td>F 5’-ATGTTCCAGCTACCTTGGGA-3’</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>R 5’-AATGCGGCTCATTCCGTGG-3’</td>
<td></td>
</tr>
<tr>
<td>ek1</td>
<td>F 5’-AAAGGTTCATTAGTGATACCC-3’</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>R 5’-GCCAGGTAGTTGTATCCAGA-3’</td>
<td></td>
</tr>
<tr>
<td>eka2</td>
<td>F 5’-TTCAATGAGTTTGCAGGGTG-3’</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>R 5’-CAGAAGAAGTGAGAGGCGCCAG-3’</td>
<td></td>
</tr>
<tr>
<td>ek2β</td>
<td>F 5’-TGTGTCTTCCCAATAGACTGC-3’</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>R 5’-TGATGGTGTGAGTACTGTTC-3’</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse; bp, base pairs; PCR, polymerase chain reaction.
and 18±4.24 mRNA copies/ng total RNA, as determined by absolute quantification; whereas ek2β mRNA expression was 10±6.36, 18±4.24 and 1±0.71 mRNA copies/ng total RNA in the MCF7, HCT116 and HepG2 cells, respectively.

In the present study, due to overlapping cDNA sequences, a general cka primer pair, known as total-cka and a specific cka2 primer pair were designed to quantify the mRNA expression levels of the cka variants in the MCF7, HCT116 and HepG2 cells. The cka1 mRNA expression levels were quantified only by the absolute quantification strategy. The absolute mRNA copy numbers of the cka1 variants were determined by calculating the difference between the copy number of the total-cka and cka2 variants. Due to the markedly low expression levels of the cka2 variant detected in all of the cancer cell lines tested, the mRNA copy number of the cka1 variant was shown as equal to the copy number of total-cka (Fig. 1B).

Besides determining the quantification of each ck and ek mRNA variant in various cancer cells, the qPCR approach permits the comparison of mRNA expression levels of all of the ck and ek variants in the same cancer cell line. For the relative quantification strategy, the ckβ gene was chosen as the calibrator and its relative mRNA expression was arbitrarily set at 1.0. The mRNA expression levels of total-cka, cka2, ek1, ek2α and ek2β were therefore compared, relative to ckβ (Fig. 2A). The ek2α variant had the highest mRNA expression levels among ck and ek variants, in MCF7 cells, as determined by absolute quantification, with a difference of 2762 mRNA copies/ng total RNA, as compared with ckβ (Fig. 2B). This finding is concordant with the 2.9-fold higher expression level of ek2α, as compared with ckβ, as determined by the relative quantification strategy (Fig. 2A).

The mRNA expression levels of cka1 were slightly lower, as compared with ckβ, in the MCF7 cells with ~248 copies difference between total cka1 and ckβ mRNA expression levels, as determined by the absolute quantification strategy. In the HCT116 cells, the mRNA expression levels of ckβ were the highest, with 1975±695.93 mRNA copies/ng total RNA. The relative mRNA expression levels of total-cka were 0.59-fold lower, as compared with ckβ. Conversely, the expression levels of total-cka were the highest, as compared with the other ck and ek variants, in the HepG2 cells. The expression of total-cka was 1.26-fold higher, as compared with ckβ; whereas the relative ek1 mRNA expression level was ~50% that of ckβ. The ek2α mRNA expression level was 0.08-fold, as compared with ckβ, which was equal to 111±39.85 mRNA copies/ng total RNA, in the HepG2 cells. The mRNA expression levels of the cka2 and ek2β variants in the MCF7, HCT116 and HepG2 cells were negligible as compared with ckβ.

**Prediction of CpG islands at ck and ek gene promoters.** The presence of CpG islands at the gene promoter region potentially increases the DNA methylation activities, which may affect the expression of ck and ek genes. To examine this possibility, the cka, ckβ, ek1 and ek2 promoter regions were analysed for the presence of CpG islands. The promoter regions of cka, ckβ and ek2 were all predicted to contain putative CpG islands (Fig. 3). This hypothesis was supported by the relatively high GC content (>70%) and an observed/expected ratio of >0.70. An analysis of the cka gene promoter region predicted two CpG islands located at the 5' upstream regions between -695 and -907 (213 bp) and between -55 and -566 (512 bp). The former CpG island contained 138 CpGs, a GC content of 64.79% and

---

**Figure 1.** Expression profiling of ck and ek mRNA variants in MCF7 breast, HCT116 colon and HepG2 liver cancer cells. (A) Relative quantification of mRNA expression levels of ck and ek variants in the three cancer cell lines, relative to MCF7 cells, post-normalisation to the geometric means of UBC and YWHAZ reference genes. (B) Absolute quantification of mRNA expression levels of ck and ek variants in copy number/ng total RNA in MCF7, HCT116 and HepG2 cells. Each bar represents the means ± standard deviation of three independent experiments.

**Figure 2.** Comparison of ck and ek mRNA expression levels in MCF7 breast, HCT116 colon and HepG2 liver cancer cells. (A) Relative and (B) absolute quantifications of total-cka, cka2, ek1, ek2α and ek2β mRNA variants in the three cell lines. Each bar represents the means ± standard deviation of three independent experiments.
an observed/expected ratio of 0.76, whereas the latter CpG island consisted of 405 CpGs, a GC content of 79.10% and an observed/expected ratio of 0.96. Similarly, two CpG islands were predicted at the gene promoter region of \(\text{ck} \beta\). The first CpG island region was located between -712 and -988 (277 bp), consisted of 164 CpGs, had a 59.21% GC content and an observed/expected ratio of 0.73. The other CpG island covered a larger region, between -55 and -665 (611 bp), consisted of 437 CpGs with a 71.52% GC content and an observed/expected ratio of 0.94. A 512 bp CpG island between -59 and -570 containing 398 CpGs, with a 77.73% GC content and an observed/expected ratio of 0.80 was predicted in the \(\text{ek}2\) promoter region. There were no predicted CpG islands at the 5' upstream region of the \(\text{ek}1\) gene. These results indicate that the regulation of \(\text{ck}\) and \(\text{ek}\) expression may be affected by DNA methylation at the promoter region. This prompted the exploration into the effects of DNA demethylation on the expression levels of \(\text{ck}\) and \(\text{ek}\) mRNA variants.

**Effects of TSA treatment on \(\text{ck}\) and \(\text{ek}\) mRNA expression profiles.** The HDAi TSA was used to investigate the effects of DNA methylation on \(\text{ck}\) and \(\text{ek}\) mRNA expression. The expression levels were determined using the absolute quantification strategy, with YWHAZ reference gene used as the internal control. TSA, which indirectly causes DNA demethylation, altered total-\(\text{ck} \alpha\), \(\text{ck} \beta\) and \(\text{ek}2\alpha\) mRNA expression levels in the HepG2 cells (Fig.4). The mRNA expression levels of total-\(\text{ck} \alpha\), which were high in the HepG2 cells, exhibited a significant upregulation in response to TSA treatment, as compared with the control (\(P<0.05\)). Conversely, TSA treatment resulted in a substantial downregulation in the mRNA expression levels of \(\text{ck} \beta\), with a \(>2\)-fold decrease to 438±198.01 mRNA copies/ng total RNA, as compared with the control (\(P<0.05\)). There were no significant changes to the \(\text{ek}1\) mRNA expression levels in the HepG2 cells, in response to the TSA treatment. This observation correlates with the absence of a predicted CpG island in the \(\text{ek}1\) promoter region. Notably, the mRNA expression levels of \(\text{ek}2\alpha\), which were repressed in the HepG2 cells, exhibited a substantial increase of \(>8\) fold to 744 mRNA copies/ng total RNA, in response to TSA treatment.

**Effects of TSA treatment dosage and duration on \(\text{ek}2\alpha\) mRNA expression.** The substantial induction of \(\text{ek}2\alpha\) mRNA expression in response to TSA treatment, led to further investigations regarding the effects of various TSA concentrations and treatment durations on \(\text{ek}2\alpha\) expression levels. A TSA concentration as low as 250 nM was able to significantly induce \(\text{ek}2\alpha\) mRNA expression in the HepG2 cells (Fig. 5). A dose-dependent increase in the mRNA expression levels of \(\text{ek}2\alpha\) was observed in response to TSA treatment up to 1000 nM. At 1000 nM TSA, the expression of \(\text{ek}2\alpha\) was 666±46.73 mRNA copies/ng total RNA, as compared with the control of 101±11.27 mRNA copies/ng total RNA. There were no significant differences in the mRNA expression
levels of ek2α between 750 and 1000 nM TSA (P>0.05). Therefore, a TSA concentration of 750 nM was used for the subsequent experiment.

TSA treatment also induced ek2α mRNA expression, in the HepG2 cells, in a time-dependent manner (Fig. 6). A significant increase in the mRNA expression levels of ek2α, in response to TSA treatment, was observed throughout the duration tested, starting from 6 h (P<0.05). The highest ek2α mRNA expression levels were detected 18 h post-TSA treatment with 2061 mRNA copies/ng total RNA, as compared with 184 mRNA copies/ng total RNA in the control. The longer treatment durations, >18 h, resulted in a gradual decrease in the mRNA expression levels of ek2α.

Discussion

Two qPCR quantification approaches, absolute and relative, are usually applied to determine the mRNA expression levels of ck and ek variants. Different experimental protocols are required for these approaches and each of them has advantages over the other. Relative quantification takes into account the expression levels of target genes, as well as the reference gene, therefore excluding the need to use a calibration curve. However, the use of a stably expressed reference gene throughout the experiment is required and must be identified prior to quantification. To perform a reliable comparison, the amplification efficiencies of both the target and reference genes must be similar (28). Normalisation with a suitable reference gene greatly minimises the variation in total RNA input, qPCR efficiencies and sample to sample variation; therefore, producing accurate and reliable results.

Conversely, normalization to a reference gene is not required in the absolute quantification strategy. The absolute quantification approach uses a highly reproducible calibration curve, which is generated using a serial dilution of a standard DNA molecule, such as recombinant plasmid DNA, which was used in the present study. The absolute quantification strategy is dependent on the accuracy of the standard; therefore, similar qPCR efficiencies for both the calibration curve and target gene, as well as the accurate determination of the concentration of the standard, are of critical importance. Absolute quantification allows the absolute copy number of the mRNA transcript to be determined, in contrast to relative quantification which only measures the relative change to the mRNA expression level. A wide dynamic range of the constructed calibration curve, up to a few orders of magnitude, also allows genes that are very lowly expressed to be quantified.

In the present study, quantification of the mRNA expression levels of the ck and ek variants in MCF7, HCT116 and HepG2 cells was determined using both the absolute and relative qPCR approaches, which generated similar results. In relative quantification, application of a mathematical model with a correction for the PCR kinetic efficiency (26) has previously been shown to reflect the actual cell situation, therefore allowing the generation of reliable results similar to absolute quantification (29). The relative quantification strategy provides a ratio of the expression of the gene of interest, rather than the absolute copy number in a defined concentration of total RNA. In the present study, each ratio was generated based on the expression of total-cka, cka2, ckb, ek1, ek2α and ek2β, normalised to the geometric means of UBC and YWHAZ expression levels. However, a high ratio produced in relative quantification does not necessarily reflect a high expression level of the target gene. Pfaffl (30) stated that these expression ratios are sensitive and dependent on the expression levels of the genes used for normalization. Therefore, the outcomes derived from relative and absolute qPCR strategies are not completely comparable. This may explain the differences observed in the mRNA expression levels of ckb, ek1, ek2α and ek2β determined using both approaches. For example, the mRNA expression levels of ek2β in the HCT116 cells were shown to be >2-fold higher, as compared with the expression levels detected in the MCF7
cells, as determined by the relative quantification strategy. However, the mRNA expression levels of ek2β were only different by a small number of copies in the HCT116 and MCF7 cells, when the absolute quantification approach was used.

The results obtained from both quantification strategies supported the earlier hypothesis that the mRNA expression levels of each ck and ek variants in MCF7, HCT116 and HepG2 cells varies. Total-cfα mRNA expression was detected in all three cancer cell lines tested. Notably, cfα, which has previously been shown to be overexpressed in breast cancer-derived cell lines (12,14), was expressed at lower expression levels in the MCF7 breast cancer cells, as compared with the HepG2 liver cancer cells in the present study. Currently, there have been few comparisons made between cfα and cfβ mRNA expression levels in cancer cells. The present study demonstrated that cfβ transcript expression levels exceed that of total cfα in breast and colon cancer cell lines. This observation is in agreement with a previous qPCR analysis of cfα and cfβ mRNA expression ratios in T47D human breast cancer cells, that showed higher mRNA expression levels of cfβ (10). The balance of cfα and cfβ mRNA expression levels has also been shown to have a vital role in cancer cell survival (15).

EK1 protein was previously demonstrated as being uniformly distributed in various human tissues, whereas EK2 was selectively expressed in the kidney, liver, ovary, testis and prostate (6). Tian et al (31) detected significantly higher ek2 mRNA expression levels in the mouse liver, as compared with the kidney and testis. In the present study, ek2β, the splice variant of ek2, exhibited a markedly low mRNA transcript level in all three of the cancer cell lines examined, thus suggesting that transcription of the ek2β gene may be negligible and this isoform may not have a significant role in phosphatidylethanolamine synthesis in these three cancer cell lines.

The present study demonstrated that TSA treatment did not have the same effects on the gene expression of all of the ck and ek variants. This is concordant with previous studies, which showed that inhibition of HDAC activity by HDACi affected only 2-5% of expressed genes in numerous cancer cell lines (32-34). The presence of CpG islands was predicted at the promoter regions of cfα, cfβ and ek2α, which may explain the effects of TSA treatment on the expression of these genes. It has previously been stated that 94% of TSA-induced genes have CpG islands at the gene promoter region (35). It may be hypothesized that TSA influences the gene expressions of cfα, cfβ and ek by DNA demethylation at the methylated CpG islands in the promoter region, as is the case for p16, SALL3 and GATA4 genes (36).

Treatment with various HDACi, such as vorinostat, in a human myeloma cell line was shown to increase the degree of acetylation and methylation of lysines in histones H3 and H4 at the proximal promoter region of the target gene (37). Chromatin immunoprecipitation analysis previously detected an accumulation of acetylated histones H3 and H4 at the promoter regions of the upregulated genes post-TSA treatment (38). Therefore, it may be possible that the elevated ek2α mRNA expression was due to the accumulation of acetylated histones H3 and H4, located around the promoter region, which subsequently induced the expression of ek2α. Furthermore, TSA may also induce the recruitment of euchromatic markers and RNA polymerase II to the transcription factor complex that binds to the promoter, thus facilitating gene transcription (39).

Previously, magnetic resonance spectroscopy demonstrated that the HDACis LAQ824 and SAHA, increased the cellular levels of PCho in human colon cancer cells and tumor xenografts (40). The rise in PCho de novo synthesis was shown to be closely associated with an induction of cfα expression. The present study showed that the mRNA expression levels of cfα in HepG2 cells were upregulated in response to TSA treatment, and supported the use of PCho as a potential biomarker to monitor the activities of HDACi.

The downregulation of cfβ expression upon TSA treatment, as observed in the present study, may be the result of either a direct or indirect inhibitory effect. The indirect effect of TSA on HDAC regulated gene transcription may be through specific recruitment of non-histone HDAC targets to various gene regulatory regions (33). The attenuating effects on cfβ expression may result in a reduction in cellular CK activity and phosphatidylcholine biosynthesis in liver cancer cells. However, the enhanced expression of total cfα by TSA would compensate for phosphatidylcholine biosynthesis which is required for cell proliferation, especially during tumor progression.

The dose- and time-dependent induction of ek2α by TSA is similar to previous reports that showed the variations in TSA dose and treatment duration that altered the expression profile of genes involved in apoptosis and the cell cycle (38,41). The mRNA expression levels of genes affected by HDAC inhibition have been shown to increase proportionally with the concentration and treatment duration of HDACi (42).

The mRNA expression levels of ck and ek variants in MCF7, HCT116 and HepG2 cells were determined and quantified in the present study. Both relative and absolute quantification strategies generated similar mRNA expression patterns. In response to epigenetic drug treatment, TSA upregulated the expression levels of total-cfα and ek2α, but downregulated the expression levels of cfβ. The induction of ek2α by TSA was both concentration- and time-dependent. The effects of TSA may be mediated by the presence of CpG islands in the promoter regions of the affected genes. However, further experiments are required to confirm this assumption. A future aim may be to identify the CpG sites that are responsible for the pronounced effects on cfα, cfβ and ek2α gene expressions in HepG2 cells during TSA treatment.

Acknowledgements

The present study was funded by the Research University Grant (no. 1001/PPSK/813034, 1001/PPSK/815101) from the Universiti Sains Malaysia. Chua Siang Ling was supported by a fellowship from the Institute of Postgraduate Studies, Universiti Sains Malaysia. The authors of the present study would also like to acknowledge the technical support provided by the UPM, Cell Culture and Biomedicine Laboratories, School of Health Sciences, Universiti Sains Malaysia.
REFERENCES


