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PCFT/SLC46A1 promoter methylation and restoration of gene expression in human leukemia cells

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ABSTRACT

The proton-coupled folate transporter (PCFT/SLC46A1) displays optimal and prominent folate and antifolate transport activity at acidic pH in human carcinoma cells but poor activity in leukemia cells. Consistently herein, human leukemia cell lines expressed poor PCFT transcript levels, whereas various carcinoma cell lines showed substantial PCFT gene expression. We identified a CpG island with high density at nucleotides −200 through +100 and explored its role in PCFT promoter silencing. Leukemia cells with barely detectable PCFT transcripts consistently harbored 85–100% methylation of this CpG island, whereas no methylation was found in carcinoma cells. Treatment with 5-Aza-2′-deoxycytidine which induced demethylation but not with the histone deacetylase inhibitor trichostatin A, restored 50-fold PCFT expression only in leukemia cells. These findings constitute the first demonstration of the dominant epigenetic silencing of the PCFT gene in leukemia cells. The potential translational implications of the restoration of PCFT expression in chemotherapy of leukemia are discussed.

DNA methylation of promoters at position 5 of cytosine in CpG dinucleotides has evolved as an epigenetic mechanism in higher eukaryotes, which proved essential for development, genomic imprinting and inactivation of the X chromosome [1,2]. The major outcome of promoter methylation is long-term silencing of the associated genes [3,4]. Interest in elucidating the molecular mechanisms of DNA methylation has gained a major momentum in recent years for two main reasons. First, silencing of multiple tumor suppressor genes in many different primary malignancies is frequently correlated with methylation of their promoters [5]. Second, mutations in two key protein factors involved in methylation-mediated silencing including DNA methyltransferase 3b and methyl-CpG binding protein 2 (MeCP2), were found to be responsible for the human diseases ICF (immunodeficiency, centromeric instability and facial anomalies) and Rett syndromes, respectively [6]. In principle, DNA methylation can repress gene transcription either by inhibiting the binding of positive factors to the promoter and/or by recruiting transcriptional co-repressors. However, in general, methylation of CpG islands does not usually impair binding of transcription factors to their cognate elements. The silencing of methylated promoters requires methyl-CpG binding proteins (MBDs) that specifically recognize symmetrically methylated CpG. MBD1 and MBD2 as well as MeCP2 recruit histone deacetylases (HDACs) thereby resulting in repression of gene expression via of methylation of their promoters.

The recently cloned proton-coupled folate transporter (PCFT/SLC46A1) is the primary transporter mediating intestinal folate absorption [7]. PCFT displays optimal folate influx activity at acidic pH and recognizes folic acid, reduced folates (e.g. 6S-5-CH_3-THF) and the antifolate methotrexate (MTX) as transport substrates with comparable high affinities (K_m = 0.5–2 μM) [8–11]. Moreover, PCFT exhibits an extremely high transport affinity for the antifolate anticancer drug pemetrexed (Alimta) with a transport K_m of 90 nM [12]. Belonging to the solute carrier superfamily (i.e. SLC) of transporters [13], PCFT is a representative of various proton-coupled low pH transport systems that mediate intestinal absorption of various essential nutrients including vitamins, amino acids, peptides, metal-ions and various organic anions [14–17]. Consistent with the important role that PCFT plays in intestinal folate absorption, it was recently found that hereditary (congenital) folate malabsorption (HFM) [7,18,19] is due to loss-of-function mutations in the PCFT gene [7,19]. HFM (OMIM 229050) is a rare autosomal recessive disorder caused by impaired intestinal folate absorption and defective folate uptake from the blood into the CNS with an early onset of a few months after birth. The disease manifests itself with very low levels of folate in the blood as well as in the CSF. HFM patients suffer from megaloblastic anemia, immune deficiency and recurrent infections (e.g. upper respiratory infections), recurrent or chronic diarrhea, failure to thrive, seizures, neurological deficits and moderate to severe mental retardation. Despite the central role that this folate transporter plays in cellular physiology

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and pathology states associated with folate deficiency after birth, nothing is known about the mechanisms that regulate PCFT gene expression including the epigenetic control of gene expression. As a step toward this end, we here show that the human PCFT gene is silenced via promoter hypermethylation in leukemia cells but not in carcinoma cells. We further demonstrate that high level restoration of PCFT gene expression can be achieved by demethylation but not by HDAC inhibition. These results constitute the first demonstration of the epigenetic regulation of PCFT gene expression. Potential translational implications for the chemotherapeutic treatment of leukemia after restoration of PCFT expression are discussed.

Materials and methods

Cell culture. All cell lines were maintained in RPMI-1640 medium (GIBCO®) containing 10–20% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Biological Industries) in a humidified atmosphere of 5% CO_2.

Drug treatment with 5-Aza-2′-deoxycytidine (5-Aza-dC) or trichostatin A (TSA). CCRF-CEM or Jurkat cells were grown in medium containing 2 μM or 5 μM 5-Aza-dC, respectively (Sigma) for 72 h and the 5-Aza-dC-containing medium was replaced every 24 h. TSA (Sigma) was added to the appropriate cultures at a concentration of 75 ng/ml and incubated for 20 h. Following treatment, RNA was isolated and RT-PCR was performed as described below.

RNA extraction and RT-PCR. Total RNA was extracted using the TriReagent protocol (Sigma). Residual genomic DNA was eliminated by treatment with RQ1 RNase-Free DNase (Promega). cDNA synthesis was carried out as previously described [18,20].

Quantification of PCFT gene expression by real-time PCR analysis. PCFT mRNA levels were determined by Absolute™ QPCR SYBR Green quantitative real-time PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems). PCFT expression levels were normalized using the β-2 microglobulin (β2M) gene as an internal control.

Isolation of genomic DNA and bisulfite conversion of DNA. Genomic DNA was isolated using The DNeasy® Blood & Tissue Kit, followed by bisulfite conversion using the EpiTect® Bisulfite Kit according to the instructions of the manufacturer (Qiagen).

DNA methylation analysis. CpG island was identified as described in Supplementary data. The methylation status of the PCFT promoter was examined using combined bisulfite restriction analysis (COBRA) as well as bisulfite DNA sequencing. Sodium bisulfite-treated DNA was amplified using primers (Table 1, Supplementary data); specifically designed with the Methyl Primer Express software v1.0 (Applied biosystems). β2M primers were obtained from Harvard Primer bank (http://pga.mgh.harvard.edu/primerbank/index.html). PCR was performed in the presence of 200 ng bisulfite-modified DNA, 0.4 μM of primers and 1× ReddyMix PCR Master Mix (Thermo Fisher Scientific). For detailed PCR program see Supplementary data. COBRA assay required a second round of PCR. Purification of PCR products was performed using the Wizard® SV Gel and PCR Clean-Up System (Promega).

Combined bisulfite restriction analysis (COBRA). For semi-quantitative restriction analysis, 1 μg of purified bisulfite-modified PCR products were digested by 20 U of BstUI (NEB) overnight at 60 °C and resolved on 2% agarose gels containing ethidium bromide. The DNA methylation level was assessed using a 2-fold serial dilution of the undigested PCR fragment.

Bisulfite DNA sequencing analysis. Bisulfite-modified PCR products were purified, sub-cloned into pGEM®-T Easy vector system (Promega) and transformed into DH5α competent bacterial cells (RBC). DNA sequencing was carried out using an ABI Prism 310 DNA sequencer (AME Bioscience).

Results

Quantification of PCFT gene expression in various tumor cell lines

While folate transport activity at low pH was documented in multiple tumor cell lines [12,21], PCFT gene expression was reported solely for Caco-2 and HeLa cells [7]. Hence, we herein undertook a survey of PCFT gene expression in various malignant cell lines of the following lineages: colon (Caco-2, SW620), cervical (HeLa, HeLa-R5), lung (A549) and breast cancer (MCF-7) as well as T-cell leukemia (CCRF-CEM and Jurkat) using real-time RT-PCR (Fig. 1). While Caco-2 showed the highest level of PCFT gene expression, HeLa-R5 (a derivative of HeLa cells with a genomic deletion of the RFC locus), SW620 and A549 cells also displayed high levels of PCFT transcripts. HeLa and MCF-7 cells displayed moderate levels of PCFT expression. In contrast, PCFT mRNA levels in the two T-cell leukemia lines CCRF-CEM and Jurkat were barely detectable.

Identification of CpG island in the putative PCFT promoter region

The very poor expression of PCFT mRNA in these T-cell leukemia lines prompted us to determine whether the mechanism underlying gene silencing was promoter methylation. Toward this end, the putative promoter region of PCFT was examined for the presence of CpG island using two different programs including "CpG island searcher" and "Methyl Primer" (the search parameters are described in Supplementary data). These programs which revealed identical results identified a 1085-bp long CpG island spanning from nucleotide position −600 in the 5′ region to nucleotide +485 in the PCFT open reading frame (Fig. 2). Specifically, this CpG island comprised 504 bp from the putative PCFT promoter, the entire 5′-UTR (96 bp), the first exon (228 bp) as well as part (i.e. 257 bp) of the first intron. The entire CpG island contained 79 CpG dinucleotides with the highest density found at nucleotide positions −200 through +100.

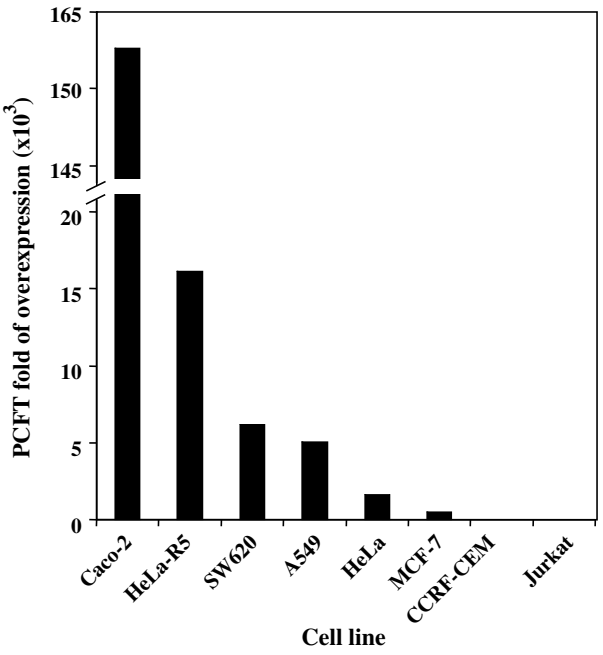

Fig. 1. Real-time PCR analysis of PCFT expression in human tumor cell lines. PCFT mRNA levels in different tumor cell line were determined using real-time PCR analysis. β2M mRNA was used to normalize PCFT expression. Results are presented relative to the expression of PCFT mRNA levels in CCRF-CEM cells assigned as a value of 1.

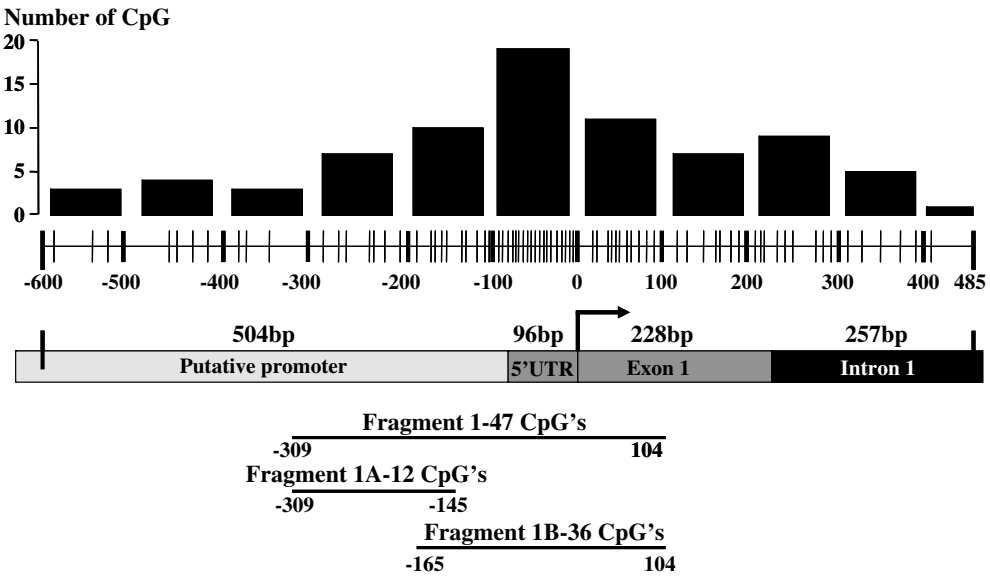

Fig. 2. Identification of CpG island in the putative PCFT promoter. We identified a 1085bp CpG island located between −600 and +485 of the PCFT genomic region (+1-translation start site, represented by an *arrow*). Vertical lines on the scale represent CpG sites, whereas the CpG dinucleotide densities are denoted above the scale by wide columns. Fragment-1 was used for COBRA, and subdivided into fragment-1A and fragment-1B for bisulfite-based sequencing.

Actual methylation of the PCFT promoter

After identifying the high density CpG island in the putative PCFT promoter, the actual status of DNA methylation was explored using two complementary techniques: COBRA and bisulfite-dependent DNA sequencing. A 414-bp long fragment (termed fragment-1) spanning nucleotide position −309 to +104 with as many as 47 CpG dinucleotides was hence amplified (Fig. 2). The COBRA technique was performed using the restriction enzyme BstUI which cuts at eight different CG-CG sites within fragment-1. Thus, following bisulfite treatment, each CpG dinucleotide in a methylated fragment remains unchanged and will be digested with BstUI. In contrast, in an un-methylated fragment, each CpG dinucleotide becomes TpG after bisulfite treatment, and will be no longer digested with BstUI. As such, it is possible to quantify the level of promoter methylation by comparing the residual levels of fragment-1 upon serial dilutions of DNA following digestion with BstUI, when compared to undigested DNA. The results obtained by COBRA (Fig. 3A) tightly correlate with the PCFT gene expression data presented in Fig. 1; CCRF-CEM and Jurkat cells which exhibited very low levels of PCFT mRNA were consistently found to be highly methylated in their PCFT promoter with DNA methylation status of ~100% in CCRF-CEM and ~85% in Jurkat cells. Consistently, Caco-2 cells with the highest PCFT mRNA levels (Fig. 1) showed no promoter methylation. Furthermore, HeLa cells which showed moderate levels of PCFT expression (Fig. 1) were also devoid of PCFT promoter methylation (Fig. 3A). These DNA methylation results were further corroborated by direct DNA sequencing after bisulfite treatment (Fig. 3B); this analysis revealed high methylation levels in CCRF-CEM and Jurkat, whereas Caco-2 cells showed background methylation levels that are likely to be due to the incomplete conversion of all CpG to TpG dinucleotides by bisulfite.

Demethylation with 5-Aza-dC restores PCFT gene expression

The tight correlation between promoter methylation and silencing of the PCFT gene led us to explore whether or not DNA methylation is indeed the mechanism underlying transcriptional silencing of the PCFT gene in leukemia cells. Toward this end, we focused our analysis on the leukemia CCRF-CEM cell line displaying the highest level of PCFT promoter methylation. We therefore determined whether exposure either to the demethylation agent 5-Aza-dC and/or the HDAC inhibitor TSA can restore PCFT gene expression. Expectingly, RT-PCR analysis revealed no PCFT expression in CCRF-CEM cells; however, treatment with the demethylation agent 5-Aza-dC restored PCFT expression (Fig. 4A). In contrast, treatment with TSA showed no restoration of PCFT expression. Moreover, a combined treatment with the two agents showed no significant increase in restoration of PCFT expression beyond the level achieved with 5-Aza-dC treatment alone (Fig. 4A). These results were further confirmed by real-time PCR analysis demonstrating that 5-Aza-dC dramatically increased PCFT gene expression by 50-fold (Fig. 4B); a combination of 5-Aza-dC and TSA increased PCFT expression to a similar extent. Finally, experiments were undertaken to provide direct evidence that restoration of gene expression by 5-Aza-dC treatment was associated with demethylation of the PCFT promoter. Thus, both the COBRA technique and the method of direct DNA sequencing of multiple genomic clones after bisulfite treatment were used (Fig. 1, Supplementary data). Expectingly, treatment with 5-Aza-dC induced a significant level of PCFT promoter demethylation as judged by serial dilutions of genomic DNA upon the COBRA technique (Fig. 1A, Supplementary data) as well as using direct DNA sequencing of multiple genomic clones after bisulfite treatment (Fig. 1B, Supplementary data).

Discussion

The current paper provides the first evidence that the PCFT gene of human T-ALL cells but not carcinoma cell lines is silenced by dense promoter methylation. First, a search for CpG island revealed a cluster of 79 CpG dinucleotides with the highest density at nucleotide positions −200 through +100 of the PCFT gene, proximal to the ATG start site. Using two complementary and established techniques including the semi-quantitative COBRA analysis as well as high resolution bisulfite-dependent DNA sequencing, the putative PCFT promoter region of leukemia cells but not of carcinoma cell lines was found to be densely methylated. It is noteworthy that the dense block of methylated CpG dinucleotides in the PCFT promoter mapped to the epi-center of various consensus transcription

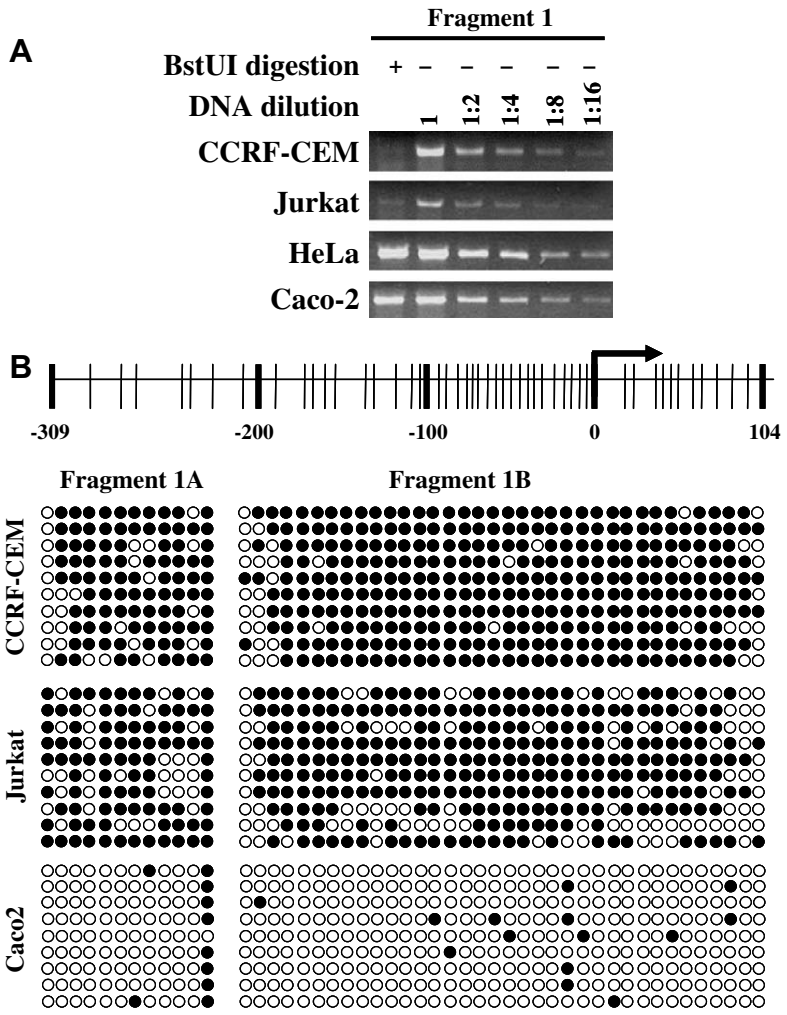

Fig. 3. DNA methylation status of the PCFT promoter. (A) COBRA analysis of fragment-1 using the restriction enzyme BstUI on four different human tumor cell lines. The left lane represents digestion with BstUI and the following lanes denote serial dilutions of uncut fragment-1. (B) Bisulfite sequence-based methylation status of fragment-1A and 1B (described in Fig. 2) in different tumor cell lines. Each line represents a different clone, whereas each row represents a different CpG site. Nine to ten independent clones were used for each cell line. *Solid circles* represent CpG dinucleotides whereas *open circles* represent TpG dinucleotides.

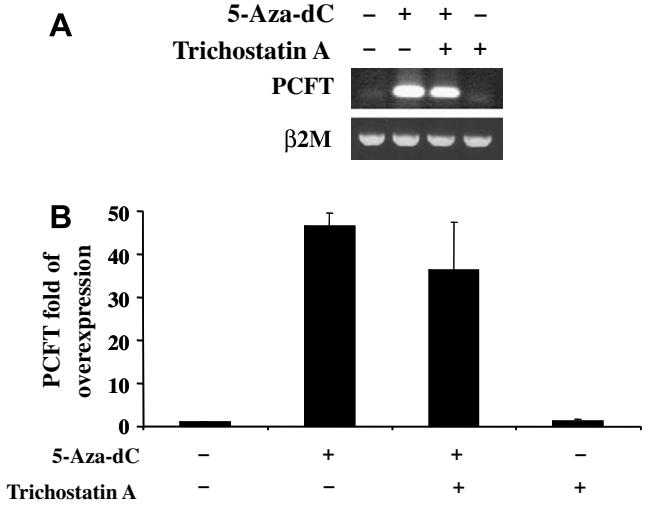

Fig. 4. Restoration of PCFT expression with the demethylation agent 5-Aza-dC. CCRF-CEM cells were treated either with the demethylation agent 5-Aza-dC or the HDAC inhibitor TSA or both, and PCFT mRNA levels were determined using RT-PCR (A) and real-time PCR (B). β2M levels were used to normalize PCFT expression. (B) The results are presented as fold of PCFT expression relative to CCRF-CEM mRNA expression levels (set as a value of 1), and represent means ± SD of three independent experiments.

factor binding sites that may play an important role in transcriptional up-regulation of PCFT gene expression. Hence, this methylated CpG island may facilitate the pinpointing of key *cis*- and *trans*-acting elements that may contribute to regulation of PCFT gene expression. Studies are underway in our laboratory to evaluate the role of this cluster of transcription factor binding sites in regulation of PCFT gene expression. In support of this presumption is a recent paper reporting that a 1.4 kb fragment of the 5′-regulatory region of the PCFT gene proximal to the ATG start site displayed marked promoter activity when transfected into confluent Caco-2 cells [22]. Second, treatment with 5-Aza-dC which resulted in promoter demethylation induced a marked restoration of PCFT gene expression only in human leukemia cells, whereas TSA failed to do so. Hence, on the one hand, these results establish that the promoter of the PCFT gene in leukemia cells is silenced via DNA methylation. On the other hand, these findings illustrate the key role that demethylation of the PCFT promoter but not histone reacetylation plays in restoration of PCFT gene expression in leukemia but not in carcinoma cells. The epigenetic silencing of the PCFT gene and restoration of gene expression by DNA demethylating agents is in good agreement with the methylation-dependent silencing of various tumor suppressor genes in pediatric tumors including acute leukemia [23].

It should be noted that although Caco-2 and HeLa cells presented comparable, very low (i.e. background) levels of promoter

methylation, colon cancer Caco-2 cells expressed ~80-fold more PCFT mRNA than cervical carcinoma HeLa cells. This intriguing finding suggests that while promoter methylation plays a key role in PCFT gene silencing, it is of no relevance in accomplishing a gradient of PCFT gene expression. Rather, this major differential in PCFT gene expression among carcinoma cells of distinct tissue origin may possibly be due an intricate expression of tissue-specific transcription factors that may play an important role in transcriptional up-regulation of the PCFT gene. Moreover, *trans*-acting factors that promote histone acetylation and chromatin remodeling may also contribute to promotion of transcriptional activation of PCFT gene expression. These results warrant further studies to determine the identity and role of these putative factors that up-regulate PCFT gene expression.

In a recent study, it was found that a spectrum of human carcinoma cell lines displays a prominent folate transport activity at acidic pH (5.5) that exceeded the influx obtained at pH 7.4 [21]. In contrast, folate transport activity at pH 7.4 in CCRF-CEM leukemia cells exceeded the influx obtained at pH 5.5. These results suggested that whereas PCFT is expressed at substantial levels in various carcinoma cell lines [7], it is poorly expressed in leukemia cells which readily express the RFC, another folate transporter [24,25]. Our present findings are in accord with these results; hence, whereas tumor cell lines of epithelial origin including colon, lung and cervical carcinoma cells exhibit prominent PCFT mRNA levels, both of the human T-cell leukemia lines CCRF-CEM and Jurkat had barely detectable levels of PCFT transcripts. The current paper provides the first mechanistic basis for the differential silencing of PCFT gene in human T-leukemia cells as opposed to the marked expression in carcinoma cells. Hence, the PCFT gene in leukemia cells was silenced via promoter methylation, whereas the PCFT promoter in carcinoma cells (e.g. Caco-2), with marked expression of PCFT transcripts was devoid of substantial DNA methylation. It is therefore possible that these findings reflect the physiological need of various mucosal epithelia, some of which may be readily exposed to an acidic microclimate including the upper intestinal folate absorptive epithelium [7], to express prominent PCFT levels in order to take up sufficient folates necessary to sustain DNA replication and cellular proliferation. In contrast, white blood cells of a lymphocytic lineage that are largely confined to the circulation at the physiological pH (pH 7.4) may fail to efficiently take up folates via PCFT that bears optimal activity at acidic pH, thereby resulting in permanent PCFT promoter silencing. Hence, it is possible that leukemia cells and normal lymphocytes that express prominent levels of the ubiquitously expressed RFC, rely on the latter for reduced folate uptake from the blood and not on the PCFT [24,25].

It was recently shown that PCFT and RFC produced comparable increases in pemetrexed (Alimta) cytotoxicity in HeLa cell transfectants in growth medium containing 5-formyltetrahydrofolate, thereby illustrating the unique role that PCFT plays in the transport and pharmacological activity of pemetrexed [26]. Moreover, using a MTX displacement assay of cellular F-MTX labeling, we recently reported that substantial levels of MTX were taken up into stable PCFT transfectants at physiological pH [18]. Hence, one plausible modality to overcome antifolate-resistance in leukemia cells that are devoid of PCFT activity due to promoter methylation may be to pulse-treat cells with the relatively well-tolerated 5-Aza-dC [27–29]. As such, exposure to pemetrexed of leukemia cells with restored PCFT expression may result in efficient eradication of antifolate-resistant cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.074.

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