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A novel mitochondrial C_1-tetrahydrofolate synthetase is upregulated in human colon adenocarcinoma

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Abstract

To seek the genes involved in the development of colorectal cancer, we analyzed the microarray gene expression profiles of human normal and cancerous colon tissues using the BioExpress database platform. Through the analysis we found one gene named DKFZp586G1517 that was upregulated in colon adenocarcinomas. The full-length cDNA of the DKFZp586G1517 cloned by polymerase chain reaction (PCR) encodes a protein with 978 amino acids, which is homologous to the human cytosolic C_1-tetrahydrofolate synthetase and contains a mitochondrial target signal at N-terminus. The gene product expressed in 293 cells was localized in mitochondria and processed at the predicted signal cleavage site, supporting the idea that DKFZp586G1517 is a novel mitochondrial C_1-tetrahydrofolate synthetase (mtC_1-THFS). The overexpression of mtC_1-THFS in 293 cells stimulated the colony formation. These results suggest that mtC_1-THFS may participate in the progression of colorectal cancer by conferring growth advantage and could be a new molecular target for cancer therapy.

Keywords: Microarray; C_1-tetrahydrofolate synthetase; Colon cancer; One-carbon unit pathway; Mitochondria

It is well accepted that colorectal cancer develops in multiple steps with accumulation of genetic aberrations, such as activation of oncogene or inactivation of tumor suppressor gene [1]. However, the detailed delineation of the carcinogenesis process is not yet accomplished and, naturally, a complete cure for patients with advanced colorectal cancer must await new targets and strategies despite a fair number of therapeutic approaches so far exploited. As a consequence, colorectal carcinoma is one of the most common causes of cancer-related deaths in the Western world. More desirable therapies need diagnosing colorectal cancer at an early stage and treating the patients with more efficient drugs. One of the ways to achieve these goals is to identify and characterize key molecules participating in colon tumorigenesis [2]. To this end, we have resorted to the BioExpress database developed by Gene Logic Inc., which provides the gene expression profile of normal and diseased human tissue samples based on Affymetrix GeneChip technology [3,4]. Thus, the database enables exploration of the genes that are differentially expressed between normal and cancer tissues of various origins.

During the genome-wide new target search we identified a novel C_1-THFS as a candidate target molecule for colon cancer. The trifunctional enzyme, C_1-THFS, consists of an N-terminal domain containing 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) and 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) activities linked to a 10-formyltetrahydrofolate synthetase (EC 6.3.4.3) activity domain. It participates in the one-carbon transfer system, contributing to amino acid (glycine, methionine, and serine) and nucleic acid (purines and thymidine) synthesis [5]. In yeast, the cytoplasm and mitochondria each contain a trifunctional enzyme, encoded by different nuclear genes [6]. In mammals only the cytosol type enzyme has thus far been reported and no trifunctional enzyme was purified from mitochondria [7]. Here we found one gene named DKFZp586G1517 upregulated in colon tumors and identified it as a missing C_1-THFS of mitochondria. Our findings demonstrated that mitochondrial C_1-THFS is a potential molecular target for colon cancer.

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Materials and methods

Identification of the differentially expressed genes in human colorectal cancers. We collected 117 normal and 77 adenocarcinoma colon samples on the BioExpress database (Gene Logic Inc., Gaithersburg, MA) containing Affymetrix U133 GeneChip expression profiles [3,4]. The Fold Change analysis tool equipped in the database was utilized to identify genes expressed at least 2-fold greater in colon cancers than in normal colon tissues. We also collected normal tissue samples with the sample number >30 for tissue expression pattern analysis. The BioExpress Web Export tool allowed us to retrieve the expression values of each sample for the gene of interest. The extracted values were used to visualize the expression pattern of each tissue as in Figs. 1A and B.

Cloning of the full-length human DKFZp586G1517 cDNA. Among the genes overexpressed in the colon cancers, we selected one gene termed DKFZp586G1517 (GenBank Accession No. AL117452) as a candidate for the target gene but its sequence deposited in GenBank lacked the first ATG, meaning that it was truncated at N-terminus. We, therefore, made an attempt to predict the sequence of the deleted part. The BLAST program analysis (http://www.ncbi.nih.gov/BLAST) with the AL117452 sequence as a query gave us the human genomic DNA sequence (Accession No. AL035086). The translation of the upstream sequence of DKFZp586G1517 in AL035086 gave rise to the putative 61 amino acids up to the possible first ATG. The sequence around the ATG (GCCATGG) is consistent with the Kozak sequence (A/GCCATGG) [8]. On the other hand, in the search for another sequence similar to DKFZp586G1517 by the BLAST analysis, we acquired the full-length mouse ortholog sequence (Accession No. AK038579). Comparison of the tentative N-terminal 61 amino acid sequence of DKFZp586G1517 obtained as above with the mouse counterpart indicated a high identity (56.1%). These analyses suggested that the predicted sequence is, indeed, the N-terminus of the human DKFZp586G1517 gene sequence. The N-terminal 61 amino acids and the deposited AL117452 sequence were assembled to yield a hypothetical full-length sequence of DKFZp586G1517.

We designed the following primers on the basis of the predicted full-length DKFZp586G1517 sequence; 5′-CGGGATCCGCCATGGGCACGCGTCTGCCGCTCGTCCTG-3′ (forward) and 5′-CCGCTCGAGGAACAAGCCTTTAACTTGTTCTGTTTCGG-3′ (reverse) (underlined are added *Bam*HI and *Xho*I restriction sequences, respectively). With these primers and KOD-Plus-DNA polymerase (Toyobo, Osaka), we performed PCR using human testis QUICK-Clone cDNA (Clontech, Palo Alto, CA) as a template in the presence of 5% DMSO under the following conditions: 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 68 °C for 4 min, and finally at 68 °C for 3 min. PCR products were cloned into pCR4Blunt-TOPO Vector (Toyobo). Two independent colonies were picked up and sequenced with Thermosequenase Cy5 or Cy5.5 dye TerminatorSequencing kit (Amersham–Biosciences, Uppsala, Sweden) using Long-Read Tower (Amersham–Biosciences). The determined sequences were identical with the hypothesized full-length sequence except for one common change (C to G of 2315 in AL117452), without any amino acid alteration.

RT-PCR analysis of the differential expression of mtC_1-THFS between normal and tumor colon cell lines. Human colon cancer cell lines SW620 and HCT116 were purchased from Dainippon Pharmaceuticals (Osaka). They were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS, Iwaki glass, Tokyo). Normal colon epithelial cell line CCD841CoN was obtained from American Tissue Culture Collection (Manassas, VA) and grown in ACL-4 serum-free medium [9]. Total RNA was isolated from these cells by using ISOGEN (Nippon Gene, Tokyo). Complementary DNA was synthesized from 1 μg total RNA with RNA PCR Kit (AMV) Ver.2.1 (Takara Shuzo, Kyoto) under the conditions of 30 °C for 10 min, 99 °C for 5 min, and 5 °C for 5 min. One-tenth of the produced cDNA was subsequently employed as a template for PCR with 5′-GCTTTGGTGCTGACATCGGAATG-3′ (forward) and 5′-CCC GGACGTCACTGATAGGTAAG-3′ (reverse) as primers. To avoid the influence of genomic DNA contamination, the primers were designed so that intron exists between the two sequences. PCR with Advantage2 polymerase mix (Clontech) consisted of 94 °C for 1.5 min and 30 cycles of 94 °C for 30 s, 60 °C for 30 min, and 72 °C for 1 min, followed by 72 °C for 3 min. The product was electrophoresed on 1% agarose gel stained with ethidium bromide. As a control, PCR for glycerol 3-aldehyde phosphate dehydrogenase (GAPDH) was conducted, in parallel, with 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′- TCCACCACCCTGTTGCTGTA-3′ (reverse) as primers.

Expression of mtC_1-THFS in mammalian cells. Human embryonic kidney 293 cells were obtained from RIKEN cell bank (Wako, Saitama) and cultured in DMEM containing 10% FCS (complete DMEM). To express mtC_1-THFS tagged with the FLAG sequence at C-terminus, the *Bam*HI–*Xho*I fragment of the PCR-cloned DKFZp586G1517 was inserted into pCMV-Tag4 (Stratagene, La Jolla, CA) cut with the same restriction enzymes. Four micrograms of the resulting vector (mtC_1-THFS/pCMV-Tag4) or the empty vector (pCMV-Tag4) was transfected into subconfluent 293 cells in 10 cm plates with LipofectAMINE (Invitrogen), following the supplier's instruction. Forty-eight hours later the cultures were lysed in 0.3 ml lysis buffer (50 mM Tris–HCl, pH 7.4, with 300 mM NaCl, 5 mM EDTA, 1% Triton X, and a protease inhibitor cocktail (Roche Diagnosis, Basel, Switzerland)) on ice for 30 min. After the lysates were centrifuged, the FLAG-tagged protein was purified with anti-FLAG M2 agarose beads (Sigma, St. Louis, MO) according to the manufacturer's protocol. The proteins adsorbed onto the beads were recovered in the SDS sample buffer with 10% of 2-mercaptoethanol. At the same time the lysates were directly treated with the sample buffer under the reducing conditions. After heating for 5 min, the samples were run on 4–20% SDS gel (Daiichi Chemicals, Tokyo) and transferred onto a nitrocellulose membrane (Schleicher and Schuel, Kassel, Germany). The membrane was treated with 800-fold diluted anti-FLAG M2 (Sigma), developed with anti-mouse Ig linked to peroxidase (Amersham–Biosciences), and visualized with 4-chloronaphthol. The N-terminal end of the purified protein was determined at Toray Research Center (Kamakura, Kanagawa).

Subcellular fractionation. Subconfluent 293 cells grown in 10 cm plates were transfected with the mtC_1-THFS/pCMV-Tag4 vector as described above. At 48 h posttransfection cells were harvested in 0.5 ml mitochondrial isolation buffer (MIB) (200 mM mannitol, 70 mM sucrose, 35 mM of 2-mercaptoethanol, 5 mM EDTA, and 50 mM potassium phosphate, pH 7.3, containing a protease inhibitor cocktail). The cells were homogenized with a Dounce homogenizer and centrifuged at 600*g* for 5 min at 4 °C twice. The supernatant was further subjected to centrifugation at 15000 rpm for 20 min at 4 °C. The supernatant (cytosol) and precipitate (mitochondria) fractions equivalent to one-fifth of the original lysate amount were processed for immunoblotting with anti-FLAG M2 or anti-mitochondrial HSP70 (1:500 dilution: Affinity Bioreagent, Golden, CO) antibody.

Effect of mtC_1-THFS overexpression on the colony formation. The subconfluent 293 cells cultured in 12- or 24-well dishes were transfected with the mtC_1-THFS/pCMV-Tag4 or pCMV-Tag4 vector as above. The next day, cells were replated in 10 cm plates with the complete DMEM supplemented with the antibiotic G418 (Promega, Madison, WI) at 1 mg/ml. After 10–14 days, the surviving colonies were fixed with 3% formaldehyde in PBS, followed by staining with 0.2% crystal violet.

Miscellaneous. Oligonucleotides were available from Sigma Genosis (Ishikari, Hokkaido). All other reagents were of the highest grade. Sequence comparison was done with Genetyx software (Software Development, Tokyo).

Results

Increased expression of the DKFZp586G1517 gene in human colon cancer tissues

To seek the genes differentially expressed in the human cancer tissues, we relied on the BioExpress database developed by Gene Logic Inc. [3,4]. The platform contains Affymetrix U133 oligonucleotide microarray expression profiles of numerous human samples from >60 normal and diseased tissues types, making it possible to compare the expression level of the genes globally between normal and tumor tissues. After completing the analysis with the BioExpress, we paid our attention to probeset 225520_at whose expression intensity was increased more than 2-fold in human colon adenocarcinomas compared with normal colon tissues ($P < 0.0000001$). As the 225520_at probe sequence was derived from the gene named DKFZp586G1517 deposited in GenBank (http://www.affymetrix.com/index.affix), the expression pattern of the 225520_at probe can be viewed as that of DKFZp586G1517. The expression level of DKFZp586G1517 analyzed by the BioExpress platform was increased to some degree (1.45-fold) in benign adenoma tissues but the increase was more prominent (2.38-fold) in colon adenocarcinomas (Fig. 1A). The BioExpress was further examined to determine the expression level of DKFZp586G1517 in different normal human tissues (Fig. 1B). The gene was expressed ubiquitously in normal tissues with the average expression intensity between 100 and 150. The highest levels of the expression were noted in ovary (average intensity, 349), lung (194), and thymus (189), whereas the expression level was the lowest in white blood cells (59), muscle (76), and lymphocytes (87). These expression results were obtained using the BioExpress database specified by the oligonucleotide microarray technology with human tissue samples. Although probesets on the Affymetrix microarray are designed carefully, there still remain concerns about the cross-reactivity [10]. Furthermore, we cannot eliminate the possibility of the contamination of the human tissue specimens with unrelated cell populations. Given these issues, we tested the human cell lines for the expression of the DKFZp586G1517 gene to assure the BioExpress results. As can be seen from Fig. 1C, RT-PCR analysis clearly demonstrated upregulation of the gene in two human colon tumor cell lines (HCT116 and SW620) compared to the normal colon cell line (CCD841CoN).

Full-length cDNA cloning of DKFZp586G1517 and sequence analysis

Because the sequence deposited in the GenBank (AL117452) was lacking in N-terminus, we extended the missing part from the genomic DNA sequence as

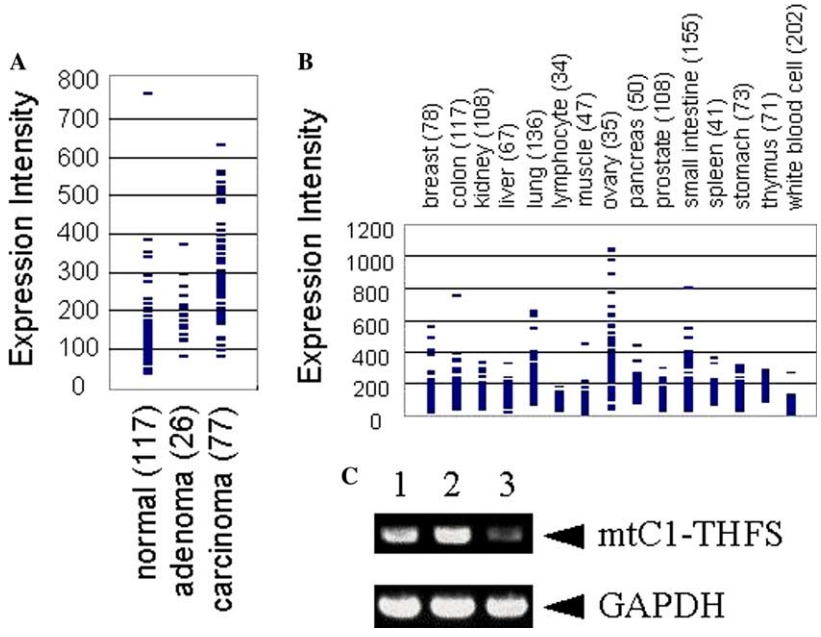

Fig. 1. Expression patterns of DKFZp586G1517 (mtC$_1$-THFS). (A) Expression profile of DKFZp586G1517 (probeset 225520_at) in human colon tissues. The expression intensity value of the 225520_at probe for each sample was retrieved from the BioExpress database and used for drawing the graph. The bar represents the intensity value of each sample. The number in parentheses depicts the sample number. (B) Expression profile of DKFZp586G1517 in various human normal tissues. The graph is drawn as for (A). (C) Differential expression of DKFZp586G1517 between human cancerous and normal colon cell lines. RT-PCR was performed for DKFZp586G1517 (upper) and GAPDH (lower) as control. Lane 1, HCT116; lane 2, SW620; and lane 3, CCD841CoN. The expected size of the DKFZp586G1517 band was 699 bp and that of GAPDH, 452 bp.

A

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Human  mtC1-THFS    1 MGTRLPLVLRQLRRPPQPPGPPRRLRVPCRASSGGGGGGGGGREGLLGQRRPQDGQARSSCSPGGRTPAARDSIVREVIQNSKEVLSLLQ
Mouse  mtC1-THFS    1 MSVRLPLLLRQLGRQQLPSGPACRLRELCRSGSRSSSSGGGDPEGLRG-RRLQDGQTFSSHGPGNPEAPGMDSIVRDVIHNSKEVLSLLQ
                      *..****.**** *.. *.**. ***  **..* ....***..*** * ** ****. ** .**.......*****.**.**********

Human  mtC1-THFS   91 EKNPAFKPVLAIIQAGDDNLMQEINQNLAEEAGLNITHICLPPDSSEAEIIDEILKINEDTRVHGLALQISENLFSNKVLNALKPEKDVD
Mouse  mtC1-THFS   90 EKNPAFKPVLVVIQAGDDNLMKDMNQNLAKEAGLDITHICLPPDSGEDEIIDEILKINEDPRVHGLTLQISEDSLSNKVLNALKPEKDVD
                      **********..**********...*****.****.**********.*.***********.*****.*****. .***************

Human  mtC1-THFS  181 GVTDINLGKLVRGDAHECFVSPVAKAVIELLEKSGVNLDGKKILVVGAHGSLEAALQCLFQRKGSMTMSIQWKTRQLQSKLHEADIVVLG
Mouse  mtC1-THFS  180 GVTDINLGKLVRGDAPECFVSPLAKAAVELVEKSGITLDGKKVLVVGAHGPLEAALQWLFQRKGSMTMSCPWATPQLPDKLREADIVVLG
                      ***************.******.***..**.****..*****.*******.****** ********** .* *.**..**.********

Human  mtC1-THFS  271 SPKPEEIPLTWIQPGTTVLNCSHDFLSGKVGCGSPRIHFGGLIEEDDVILLAAALRIQNMVSSGRRWLREQQHRRWRLHCLKLQPLSPVP
Mouse  mtC1-THFS  270 SPKPEEIPAVWIPSGTTILNCFHDFLSGKLSGGSPGVPVDKLIAEESVSLLAAALRIQNMVSSGRRWLREQQHRRWRLHCLKLQPLSPVP
                      ******** .**..***.*** *******. .*** .. . **.*..* ****************************************

Human  mtC1-THFS  361 SDIEISRGQTPKAVDVLAKEIGLLADEIEIYGKSKAKVRLSVLERLKDQADGKYVLVAGITPTPLGEGKSTVTIGLVQALTAHLNVNSFA
Mouse  mtC1-THFS  360 SDIEISRGQTPKAVDVLAKEIGLLADEIEIYGKSKAKVQLSLLERLKDQTDGKYVLVAGVTPTPLGEGKSTVTIGLVQALTAHLKVNSFA
                      ************************************.**.*******.*********.**********************.*****

Human  mtC1-THFS  451 CLRQPSQGPTFGVKGGAAGGGYAQVIPMEEFNLHLTGDIHAITAANNLLAAAIDTRILHENTQTDKALYNRLVPLVNGVREFSEIQLARL
Mouse  mtC1-THFS  450 CLRQPSQGPTFGVKGGAAGGGYAQVIPMEEFNLHLTGDIHAITAANNLLAAAIDTRILHESTQTDKALYNRLVPLVNGVREFSEIQLSRL
                      ************************************************************.**************************.**

Human  mtC1-THFS  541 KKLGINKTDPSTLTEEEVSKFARLDIDPSTITWQRVLDTNDRFLRKITIGQGNTEKGHYRQAQFDIAVASEIMAVLALTDSLADMKARLG
Mouse  mtC1-THFS  540 KKLGIHKTDPSTLTEEEVRKFARLNIDPATITWQRVLDTNDRFLRKITIGQGSTEKGYSRQAQFDIAVASEIMAVLALTDSLTDMKERLG
                      *****.************.*****.***.**********************.****. **********************.***.***

Human  mtC1-THFS  631 RMVVASDKSGQPVTADDLGVTGALTVLMKDAIKPNLMQTLEGTPVFVHAGPFANIAHGNSSVLADKIALKLVGEEGFVVTEAGFGADIGM
Mouse  mtC1-THFS  630 RMVVASDKDGQPVTAEDLGVTGALTVLMKDAIKPNLMQTLEGTPVFVHAGPFANIAHGNSSVLADKIALKLVGEEGFVVTEAGFGADIGM
                      ********.******.*********************************************************************

Human  mtC1-THFS  721 EKFFNIKCRASGLVPNVVVLVATVRALKMHGGGPSVTAGVPLKKEYTEENIQLVADGCCNLQKQIQITQLFGVPVVVALNVFKTDTRAEI
Mouse  mtC1-THFS  720 EKFFNIKCRASGLVPNVVVLVATVRALKMHGGGPSVTAGVPLKKEYTEENIQLVADGCCNLQKQIQIAQLFGVPVVVALNVFKTDTRAEI
                      ************************************************************.**********************

Human  mtC1-THFS  781 DLVCELAKRAGAFDAVPCYHWSVGGKGSVDLARAVREAASKRSRFQFLYDVQVPIVDKIRTIAQAVYGAKDIELSPEAQAKIDRYTQQGF
Mouse  mtC1-THFS  780 DLVCELAKRAGAFDAVPCYHWSAGGKGSVDLARAVREAANKRSRFQFLYDVQLPIVEKIRVIAQTVYGAKDIELSPEAQSKIDRYTQQGF
                      **********************.***************.************.***.***.***.**************.**********

Human  mtC1-THFS  901 GNLPICMAKTHLSLSHQPDKKGVPRDFILPISDVRASIGAGFIYPLVGTMSTMPGLPTRPCFYDIDLDTETEQVKGLF   978
Mouse  mtC1-THFS  900 GNLPICMAKTHLSLSHEPDKKGVPRDFTLPISDVRASIGAGFIYPLVGTMSTMPGLPTRPCFYDIDLDTETEQVKGLF   977
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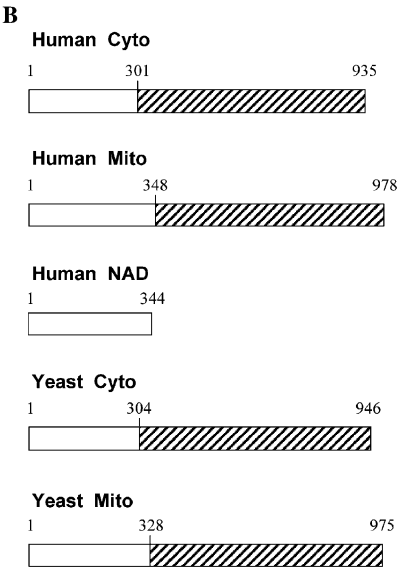
B



D/C domain	Human Cyto	Human Mito	Human NAD	Yeast Cyto	Yeast Mito
Human Cyto	–	33.2	48.0	47.8	38.7
Human Mito	–	–	32.3	24.8	23.7
Human NAD	–	–	–	53.3	36.1
Yeast Cyto	–	–	–	–	42.3
Yeast Mito	–	–	–	–	–

S domain	Human Cyto	Human Mito	Yeast Cyto	Yeast Mito
Human Cyto	–	75.7	62.8	60.4
Human Mito	–	–	61.2	60.7
Yeast Cyto	–	–	–	80.7
Yeast Mito	–	–	–	–

Fig. 2. Analysis of the deduced amino acid sequence of DKFZp586G1517 (mtC_1-THFS). (A) Sequence alignment of the cloned human DKFZp586G1517 (upper) and the mouse ortholog (lower). The newly isolated 61 amino acids of the human sequence are in italic. The dash symbol (-) is introduced to optimize alignment. The symbols below the alignment refer to residues that are similar (.) or identical (*). The arrowhead denotes the cleavage site determined by N-terminal sequence analysis. (B) Comparison of the structures between the C_1-THFS family members. Left, schematic diagram of the domain structures. Human Cyto, human cytosol C_1-THFS; Human Mito, human mitochondrial C_1-THFS; Human NAD, human NAD-dependent mitochondrial dehydrogenase/cyclohydrolase; Yeast Cyto, yeast cytosol C_1-THFS; and Yeast Mito, yeast mitochondrial C_1-THFS. C_1-THFS is composed of N-terminal D/C domain (open box) and C-terminal S domain (hatched box) except for human NAD (D/C domain only). Right, homology analysis of separate C_1-THFS domains. Dehydrogenase/cyclohydrolase (upper) and synthetase (lower) domains were analyzed individually.

described in Materials and methods. With the primers designed from the predicted sequence, the full-length DKFZp586G1517 cDNA could be cloned by PCR with human testis cDNA library as a template. The BLAST analysis revealed that the deduced amino acid sequence of DKFZp586G1517 (Fig. 2A) has a high similarity to those of the human cytosolic C_1-THFS (57.4% identity throughout the entire protein) and its cognates. Besides, MITOP analysis (http://mips.gsf.de/proj/medgen/mitop) indicated the existence of a mitochondrial target motif at the N-terminal portion that is rich in basic and hydrophobic amino acid residues [11]. Taken together, we considered the DKFZp586G1517 gene as a novel human mitochondrial C_1-THFS and hereafter we refer to the protein encoded by the DKFZp586G1517 gene as mtC_1-THFS. BLAST search program additionally yielded the complete murine ortholog sequence, which is highly homologous to the sequence of the human counterpart (88.2% identity) (Fig. 2A). As C_1-THFSs are composed of two different domains (dehydrogenase/cyclohydrolase (D/C) domain and synthetase (S) domains) [5], homology analysis between related genes was conducted separately for individual domains (Fig. 2B). It is noteworthy that in general homology in S domain is fairly high (>60% identity) while homology in D/C domain is much lower (around 30% identity).

Localization of recombinant mtC_1-THFS

To express a putative mtC_1-THFS we transiently transfected the C-terminally FLAG-tagged gene into 293 cells. The recombinant protein was immunoprecipitated with anti-FLAG antibody beads and subjected to Western blot analysis. Only in the sample of the cells transfected with the plasmid harboring the mtC_1-THFS gene, we detected a band at around 110 kDa, which is in good accordance with the theoretical molecular mass of the gene product (Fig. 3A, lane 6). The band was not detected in the lysate of the same sample without purification presumably due to the low productivity (Fig. 3A, lane 3).

We next examined the subcellular localization of mtC_1-THFS. Cytosolic and mitochondrial fractions were isolated from the mtC_1-THFS transfected 293 cells by differential centrifuge. Fig. 3B shows that recombinant mtC_1-THFS colocalized with mitochondrial HSP70, a typical marker of the mitochondrial protein, demonstrating that mtC_1-THFS was targeted to the mitochondria, as expected from the sequence analysis. During the mitochondrial transport the target sequence is cleaved by mitochondrial import peptidase [12]. To further ascertain the processing of mtC_1-THFS, we determined the N-terminal sequence of the purified protein. The determined sequence, SSGGG, implicated that the cleavage occurred between amino acids 31 and 32, which was pointed out accurately by the MITO algorithm as a cleavage site.

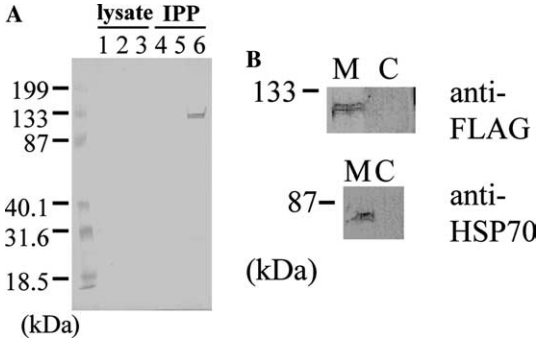


Fig. 3. Localization of mtC_1-THFS. (A) Transient expression of recombinant mtC_1-THFS in 293 cells. Recombinant mtC_1-THFS tagged with FLAG was transiently expressed in 293 cells and purified with the anti-FLAG antibody agarose. The recombinant protein was detected with immunoblot. Lanes 1–3 contain the samples from the lysate whereas lanes 4–6, the immunopurified samples. Lanes 1 and 4, non-treated 293 cells; lanes 2 and 5, 293 cells transfected with the empty vector; and lanes 3 and 6, 293 cells transfected with the mtC_1-THFS vector. (B) Mitochondrial (M) and cytosolic (C) fractions were isolated from the FLAG-tagged mtC_1-THFS expressing 293 cells. The fractions were processed for Western blot with anti-FLAG (top) or anti-mitochondrial HSP70 (bottom) antibody.

Overexpression of mtC_1-THFS stimulated cell growth

Since C_1-THFS is deemed to supply amino acids and nucleic acids, components required for cell growth, we examined the effects of the mtC_1-THFS overexpression on clonal cell growth. After transfection of the mtC_1-THFS gene, 293 cells were selected in the medium with the antibiotic G418, allowing for the colony formation 10–14 days later. As evidenced from Fig. 4, colony formation of 293 cells was stimulated when transfected

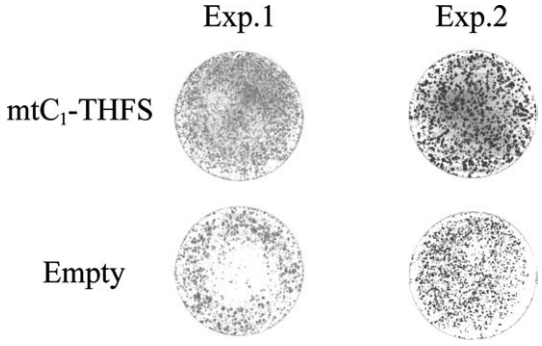


Fig. 4. Effect of overexpression of mtC_1-THFS on colony formation of 293 cells. The subconfluent 293 cells cultured in 12- (Exp. 1) or 24 (Exp. 2)-well dishes were transfected with the empty or mtC_1-THFS expression vector. The next day, cells were replated in 10 cm plates and selected with G418. After 10–14 days, the surviving colonies were fixed with 3% formaldehyde in PBS, followed by staining with 0.2% crystal violet. The experiments were done twice in duplicate and the representative views are indicated.

with the mtC_1-THFS gene, implying that mtC_1-THFS is capable of accelerating cell proliferation.

Discussion

The one-carbon derivatives of tetrahydrofolate serve as donors of one-carbon units in a variety of biosynthetic reactions involving purine bases, pyrimidines, amino acids, and vitamins [6]. In eukaryotic cells the interconversion of one-carbon substituted folates is accomplished by a number of activities in a complex pathway, some of which are present as a part of multienzyme [6]. The trifunctional C_1-THFS is central to this metabolic pathway and performs sequential reactions [5]. Although yeast cells possess both cytosolic and mitochondrial types of C_1-THFS, in mammals, only the cytosolic type of the enzyme has so far been documented. Instead, bifunctional NAD-dependent methylenetetrahydrofolate dehydrogenase–cyclohydrolase exists in mitochondria of mammalian cells [13]. This bifunctional enzyme specifically evolved in mammals may replace the role of the trifunctional enzyme in mitochondria [7]. Yet, it has also been postulated that mammals would contain the mitochondrial type C_1-THFS as well [6,14]. In this study, we have identified a gene overexpressed in human colon cancer as the missing mammalian mitochondrial C_1-THFS. The BLAT program analysis [15] showed that human mtC_1-THFS is mapped to 6q25.1 while its relatives, cytosolic C_1-THFS and bifunctional mitochondrial NAD-dependent THF dehydrogenase/cyclohydrolase, mapped to 14q24 and 2p12, respectively. Apparently, these enzymes are encoded by different genes and should play distinct roles in the one-carbon metabolic pathway in different compartments.

It is recognized that cancers are caused as a result of the accumulation of the genetic mutations such as oncogene activation and tumor suppressor gene inactivation [1]. In the case of the colorectal cancer the malignant transformation is believed to occur from colorectal adenomatous polyps with the deregulated gene expression by β-catenin [16]. Mutations mimicking Wnt stimulation, that is, inactivating APC mutations or activating β-catenin mutations resulted in nuclear accumulation of β-catenin which subsequently complexes with T cell factor/lymphoid enhancing transcription factors to activate gene transcription. In approximately 90% of colorectal cancer, mutations stimulating the Wnt signal pathway are noted [17]. One of the target genes of the β-catenin signaling pathway is c-myc oncogene [18]. Myc is a crucial transcription factor in the control of cell proliferation, differentiation, and apoptosis [19], however, its detailed mechanism of actions is still unclear. It has recently been revealed that one of the myc-responsive genes is mitochondrial serine hydroxymethyltransferase (mtSHMT), another enzyme involved in the one-carbon unit metabolism [20,21]. SHMT catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and 5,10-methylenetetrahydrofolate, which is offered by a 5,10-methylenetetrahydrofolate dehydrogenase activity of C_1-THFS. In mammalian cells two isozymes of SHMT reside in cytosol and mitochondria, respectively. Meanwhile, with the murine cell lines NIH3T3 and Balb/c3T3 conditionally expressing c-myc gene, one cDNA clone (I-8-29) was isolated as a gene responsive to c-myc [22]. When the paper was published, the identity of the clone was not clarified. We ourselves analyzed the deposited sequence of the clone (Accession No. U06665) and concluded that the sequence of this clone is a part of mouse mtC_1-THFS. Thus, both mtC_1-THFS and mtSHMT, working closely in the one-carbon unit metabolism, appear to be targets of c-myc. A myc consensus sequence (CACGTG) found in the first intron of human mtSHMT gene [21] also exists in the tentative first intron of the human mtC_1-THFS (data not shown). The BioExpress data, too, showed a significant increase in the mtSHMT expression in colon cancer tissues (2.16-fold), as in the case of mtC_1-THFS. It is reasonable that the genes coding for the enzymes acting together in the same compartment are upregulated simultaneously to facilitate the metabolic pathway. Hence, we propose that the mitochondrial one-carbon unit metabolism is activated during colon carcinogenesis through the Wnt pathway (Fig. 5). In addition to colon cancer, the increased expression of mtC_1-THFS was denoted by the BioExpress analysis in other tumors such as stomach (1.97-fold) or pancreatic cancer (1.44-fold). The degree of fold induction was, however, not so great compared with that in colon cancer (2.38-fold). This can be explained by the most frequent occurrence of the mutations activating the Wnt signaling pathway in colon cancer [17].

In view of the function of C_1-THFS, catalysis of reactions in the one-carbon metabolism, it is conceivable

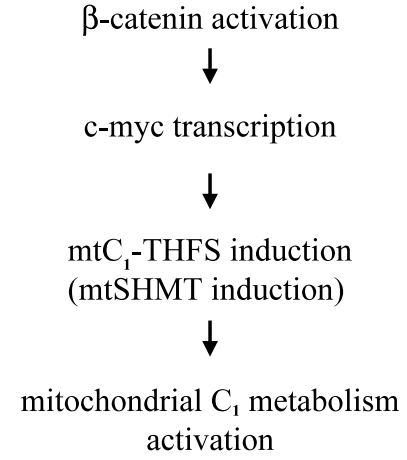


Fig. 5. Proposed cascade for the mitochondrial one-carbon unit metabolism activation.

that the mtC_1-THFS expression plays a pivotal role in cell proliferation. In fact, we demonstrated that the overexpression of mtC_1-THFS stimulated the colony formation of 293 cells (Fig. 4). This observation supports the notion that the augmented expression of mtC_1-THFS in colon adenoma and adenocarcinoma (Fig. 1A) could impart clonal cell growth advantage during initiation or progression of colon tumors. Ubiquitous expression of mtC_1-THFS in normal tissues underscores its importance in maintaining basic metabolism. Normal tissues comprising rapidly dividing cells such as ovary and thymus displayed higher expression levels of mtC_1-THFS (Fig. 1B). In these tissues the role of mtC_1-THFS would be primarily to aid in active cell proliferation rather than to meet the needs of the fundamental metabolism. The function of mtC_1-THFS might be diversified depending on the temporal and spatial conditions.

D/C domain of the C_1-THFS family members is more variable (about 30% identity) than S domain (>60% identity) (Fig. 2B). D/C domain contains two activities (5,10-methylenetetrahydrofolate dehydrogenase and 5,10-methenyltetrahydrofolate cyclohydrolase) which are difficult to separate on the sequence. Interestingly, during evolution there has been flexibility instead of constraint in the D/C domain sequence while it retained the enzyme activities. We tried to express C-terminally tagged D/C domain in the *Escherichia coli* and baculovirus expression systems. The purified product of either system exhibited no or, if any, little activity (data not shown). C-terminal S domain could lend itself to stabilize the D/C domain activity [23]. Another possibility is that a very weak activity of the D/C domain in vitro is sufficient for exhibiting the function in vivo. Alternatively, prevalent low homology in D/C domain may mask the loss of the some residues(s) in mtC_1-THFS essential for the activity. In the last situation, the role of mtC_1-THFS should be presenting scaffold to multienzyme complex in mitochondria, as has been proposed for other C_1-THFSs [24,25].

The enzymes involved in the one-carbon unit pathway, dihydrofolate reductase (DHFR) or thymidilate synthase (TS), are the targets of anti-cancer drugs such as methotrexate (MTX) or 5-fluoruracil (5-FU), respectively [26]. These drugs are widely used for the cancer patients, which reflects the significance of the whole one-carbon unit process in the cancer therapy. Nevertheless, those agents still suffer from obvious limitations. The adverse effects of the agents may be at least partially attributed to the expression profiles of their target molecules in normal tissues. In fact, the BioExpress database revealed that the average expression intensities of DHFR and TS in thymus exceed those in varied neoplastic tissues extremely (>3-fold). In this respect, the mtC_1-THFS expression level in colon cancer is much higher than those in most normal tissues. The exceptional organ is ovary, in which the expression level of mtC_1-THFS is the highest among normal tissues, nevertheless, comparable with that in colon cancer. In conclusion, mtC_1-THFS could be an ideal target for the therapeutic intervention of colorectal cancer.

Note. While this manuscript was in preparation, the cloning of mtC_1-THFS gene was reported in [27].

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