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13. ABSTRACT *(Maximum 200 Words)*

Methylthioadenosine phosphorylase (MTAP) is a key enzyme in the conversion of methylthioadenosine (MTA) into methionine and adenine. The MTAP gene is frequently deleted in a variety of different cancers. Our lab has found a link between loss of MTAP and the phenomena of methionine dependent growth, defined as the inability to grow on media containing methionine's metabolic precursor homocysteine. Cells lacking MTAP have increased sensitivity to purine biosynthetic inhibitors such as methotrexate and 5,10-dideazatetrahydrofolate. These observations suggest that an effective two-pronged strategy could be used to eliminate MTAP negative breast cancer cells in vivo. We have created two isogenic breast cancer derived cell lines, one that is MTAP+ and the other that is MTAP-. The MTAP expressing line can use MTA to make methionine, but is still unable to grow on media lacking homocysteine. This result suggests that MTAP deletion is not the primary cause of methionine dependent growth. Our MTAP deleted cells have increased sensitivity to growth in low levels of methionine and are more sensitive to drugs that inhibit purine biosynthesis. These results suggest that these treatments may be useful in treating MTAP deficient cancers in vivo.

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Introduction

Ideally, one would like to take advantage of genetic alterations in malignant tumor cells that could be used to target these cells for destruction. One such alteration found in metastatic breast cancer cells, as well as a variety of other tumor types, is loss of expression of the methylthioadenosine phosphorylase gene (MTAP). MTAP is involved in the methionine salvage pathway that converts the methylthioadenosine into methionine and adenosine (see appendix for metabolic pathway diagram). The *MTAP* gene is located adjacent to the *p16* tumor suppressor gene and is frequently found homozygously deleted in a variety of different cancers (1-4). Our lab has found a link between loss of MTAP expression and the phenomenon of methionine dependent growth. Methionine dependence refers to the inability of certain tumor derived cell lines to grow on media containing homocysteine, a metabolic precursor to methionine (5). Thus, cells lacking MTAP seem to require excess methionine for growth. Other labs have shown that cells lacking MTAP have increased sensitivity to purine biosynthetic inhibitors such as methotrexate and 5,10-dideazatetrahydrofolate (6). These observations suggest that an effective two pronged strategy could be used to eliminate MTAP negative breast cancer cells *in vivo*. In this study we will determine the relationship between MTAP deletion and methionine dependent growth, attempt to identify conditions which maximally differentiates the growth effects of $MTAP^-$ and $MTAP^+$ cells, and finally we will determine the frequency of MTAP deletion in primary breast tumors.

Body

Technical objective 1: Determine if MTAP is responsible for methionine dependent growth observed in MCF-7 breast carcinoma cell lines.

Task 1: Months 1-3. Construct MTAP expression vector and make stable isogenic MCF-7 cell lines with and without MTAP expression.

Progress: A construct was made by cloning the MTAP cDNA into pcDNA3.1, such that MTAP is now under the control of the CMV enhancer-promoter. This vector also contains the neomycin resistance gene for selection of stable transfectants. The construct, and a control construct lacking MTAP were used to make stable MCF-7 transfectomas. Twelve neomycin resistant clones were analyzed by Western blot analysis and one was selected for further study (See Figure 1). Thus this task has been completed.

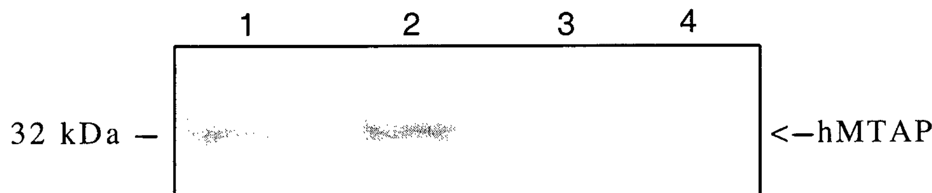


Figure 1. Western Analysis of MTAP. Western blot probed with anti-MTAP antiserum. Lane 1-Raji cell extract. Lane 2-MCF-7 cells transfected with pCR3.1 with MTAP in the sense orientation. Lane3-MCF-7 cells transfected with pCR3.1 with MTAP in the anti-sense orientation. Lane4-MCF-7 cells transfected with pCR3.1 with no insert.

Task 2: Months 4-6. Examine growth characteristics of MTAP-expressing and control cells on media containing various methionine metabolites.

Progress: MCF-7 cells expressing MTAP in the sense, anti-sense, and vector alone were examined for growth rates in media containing either 100uM methionine, homocysteine (Hcy), methylthioadenosine (MTA), or 4-methylthio-2-oxobutanoate (MTOB) (see appendix). Cells lacking MTAP were unable to grow in media containing either MTA or Hcy, but grew well in Met or MTOB media. Cells with MTAP reintroduced grew well in Mer, MTOB, and MTA containing media, but still could not grow in media containing Hcy (see Table below). These results, in combination with our survey of methionine dependent cell lines, suggest that although lack of MTAP expression is common in methionine dependent cell lines, it is not the cause of the methionine dependent behavior.

Table 1 Growth behavior of MTAP transfected MCF-7 cells[a]

A)

Media (after 7 days)	pCR3.1/sense hMTAP MCF-7	pCR3.1/antisense hMTAP MCF-7	pCR3.1/CAT MCF-7
Met	100	100	100
Hcy	3	0	5
MTA	60	0	3
MTOB	88	80	98

B)

Time/Media	Raji	pCR3.1/sense hMTAP MCF-7	pCR3.1/antisense hMTAP MCF-7	pCR3.1/CAT MCF-7
3 days Met	100	100	100	100
3 days Hcy	60	4	3	5
5 days Met	100	100	100	100
5 days Hcy	56	2	1	5
7 days Met	100	100	100	100
7 days Hcy	37	2	1	0

[a]MCF-7 cells were transfected with MTAP in the sense, anti-sense, or with the vector alone. Cells were assayed for growth as described in the text. The growth values for each line are relative to the growth observed in the same cell line in the presence of methionine. Cell growth was assessed by measuring total cellular protein. A value of 0 implies there was no additional after plating. Table A shows relative growth after seven-days in media containing the indicated methionine precursor. Table B shows growth after 3,5, and 7 days compared to Raji cells, a methionine-independent cell line.

Technical objective 2: Determine if MTAP deficient cell lines are at increased sensitivity for purine biosynthetic inhibitors in combination with methionine starvation.

Task 1: Months 7-9: Determine the IC-50 of the purine biosynthetic inhibitors methotrexate (MTX), L-alanosine and 5,10-dideazatetrahydrofolate.

Progress: The IC-50 for the MTAP+ MCF-7 cells is approximately 40uM for alanosine, 1uM for methotrexate, and 100uM for azaserine. For the isogenic MTAP- cells the IC-50 for alanosine is approximately 10uM, for methotrexate 0.1uM, and 25uM for azaserine. We used azaserine instead of 5,10-dideasztetrahydrofolate because of difficulties in obtaining this compound.

Task 2: Months 7-9: Determine the rates of growth of MTAP expressing and non-expressing cell lines in media containing various methionine concentrations.

Progress: We have determined that MTAP deficient cells grow more slowly in low concentrations of methionine relative to MTAP positive cells (see Figure 2).

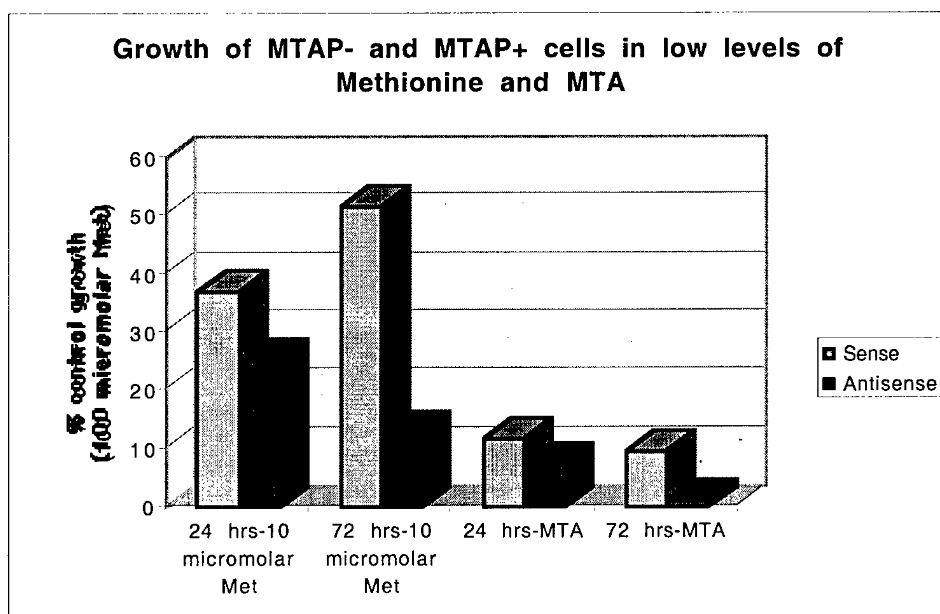


Figure 2. Effect of MTAP expression on growth in 10mM methionine or 100mM MTA. Identical numbers of cells were plated out on media containing either 10mM methionine or 100uM MTA. Growth was measured after 24 and 72 hours. Results are expressed as percentage of growth compared to the same cells grown on 100mM methionine

Task 3: Months 10-12: Determine the optimum combination of methionine restriction and purine biosynthetic inhibition which gives the largest difference in IC-50 between MTAP expressing and non-expression cell lines.

Progress: We have not been able to generate convincing data that there is any synergy between these two treatments in mammalian cells. Part of the problem is that there is too much variation between experiments due to the effects of initial plating density, and "evolution" of the cell line in culture. For these reasons we have decided to explore this question using isogenic MTAP+ and MTAP- strains of the yeast *Saccharomyces cerevisiae*. These experiments are currently underway.

Technical objective 3: Determine the frequency of MTAP expression defects in primary breast carcinoma cell lines.

Task 1: Months 6-12: Obtain primary breast carcinoma material from FCCC tumor bank and from FCCC patients.

Progress: We have analyzed MTAP expression in 20 samples from primary tumor material. We have decided to use Western analysis as opposed to RT-PCR as it is more directly related to MTAP function. As shown in Figure 3, at least four of the twenty samples show greatly reduced levels of MTAP activity. However, one problem with these experiments is that interpretation can be difficult because primary breast tumor material is generally contaminated with a large amount of stromal material. For definitive results it will be necessary to develop reagents that can be used to do immunohistochemistry for MTAP levels. Unfortunately, our current anti-serum (obtained for Dr. Carson's group) has too much cross reactivity to be effective. In addition we do not have enough material to affinity purify.

To solve this problem we have developed *an E. coli* strain that produces large quantities of a TRPE::hMTAP fusion protein. We will use this to immunize rabbits in order to produce high quality MTAP antiserum. This serum will then be affinity purified and used for immunohistochemistry.

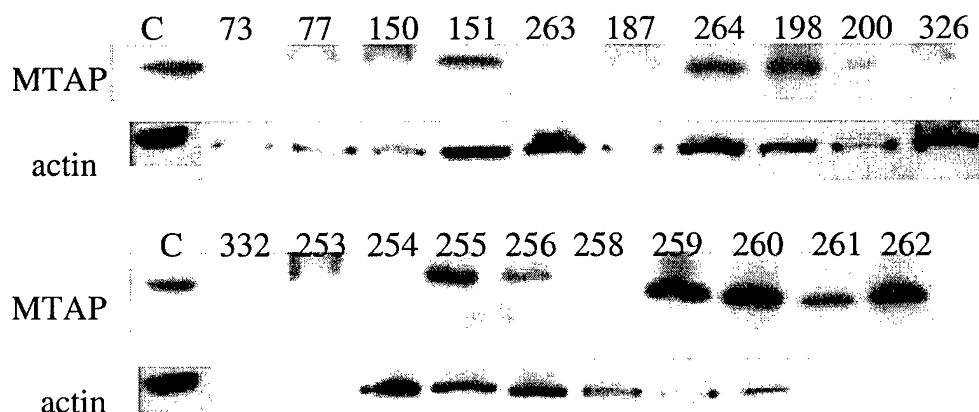


Figure 3. MTAP protein in primary breast tumors. Total protein extracts were prepared from breast tumor material from twenty patients undergoing surgical tumor resection. Proteins were separated by electrophoresis on 12% SDS-PAGE gels. The gels were then immunoblotted and probed with MTAP antiserum. Extracts were also tested for beta-actin expression as a control. The numbers above the lanes indicate the different sample numbers as coded by the Fox Chase Tumor Bank Facility. The lane labeled C contains extract from MCF-7 cells transfected with MTAP.

Task 2: Months 12-18: Isolate RNA from material and assay for MTAP expression by quantitative RT-PCR.
This task was determined to be overly redundant with Task 1 and was therefore abandoned.

Key Accomplishments

- Created isogenic MTAP expressing and non-expressing MCF-7 cell lines.
- Showed that MTAP deficient cells are more sensitive to low levels of methionine and alanosine.
- Showed that at least 4/20 primary breast tumor samples have absent or greatly reduced levels of MTAP.

Reportable outcomes

We have published part of this work in *Cancer Research* **60:5543-5547 (2000).** This work was also presented at the FASEB Research Conference, Folic Acid, Vitamin B_{12} and One-Carbon Metabolism, Snowmass Village, CO, August 5-10, 2000, "Disruption of Methionine Metabolism in Tumor Cells."

Conclusions

Based on our work we can conclude that methionine dependent growth is associated with, but not caused by defects in MTAP. However, the fact that they are commonly associated together suggests that a combination of purine inhibition and methionine starvation may be a way to selectively kill breast tumor cells. We have also shown that reduced MTAP expression is a relatively common occurrence in primary breast tumors.

List of Personnel

The following have received pay from the research effort: Warren D. Kruger, Liqun Wang

Bibliography of Publications

Tang, B., Li, Y.N., Kruger, W.D. Defects in methylthioadenosine phosphorylase are associated with but not responsible for methionine-dependent tumor cell growth. Cancer Res. 60:5543-5547, 2000.

References

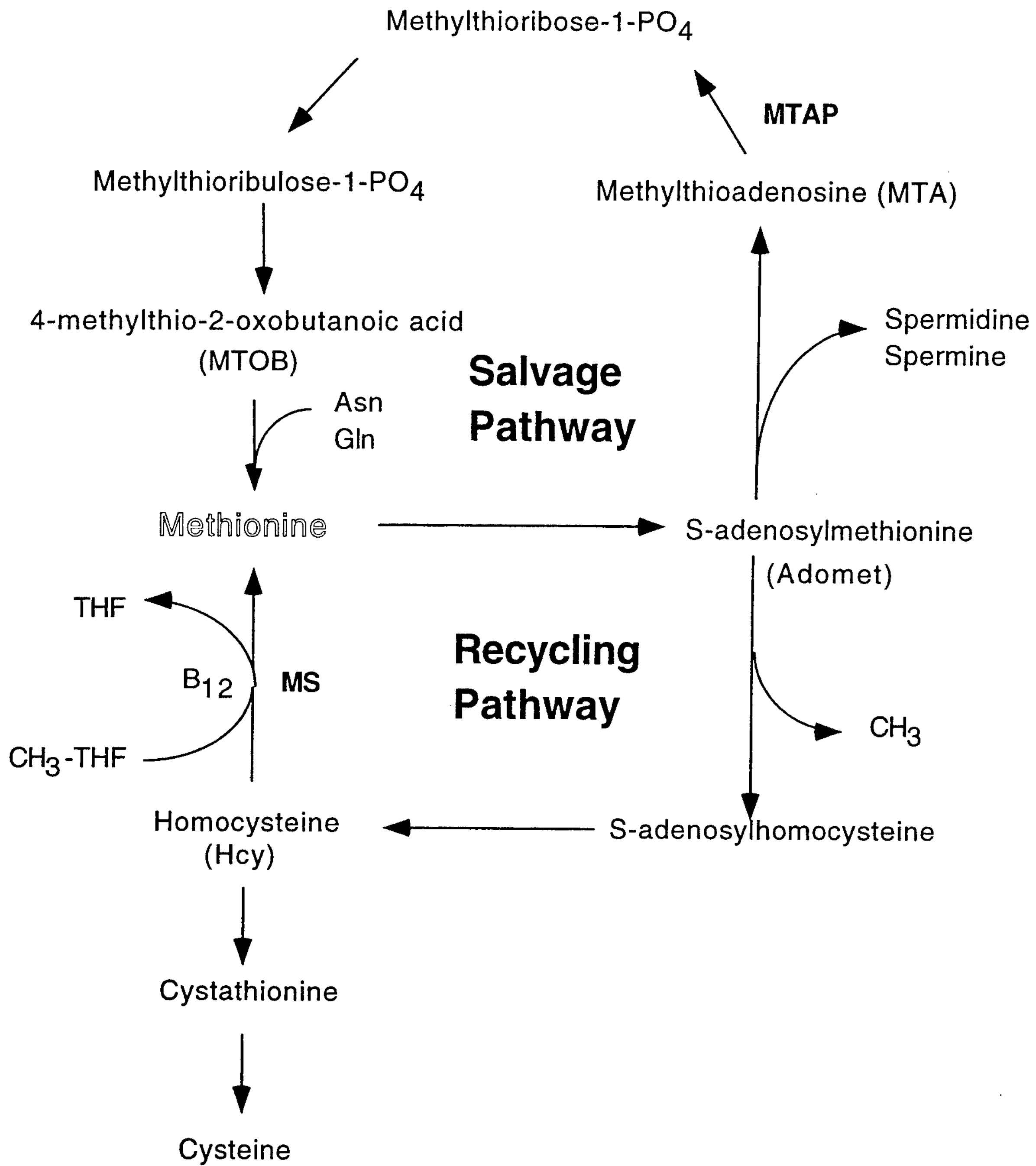
1. Zhang, H., Chen, Z. H., and Savarese, T. M. Codeletion of the genes for p16INK4, methylthioadenosine phosphorylase, interferon-alpha1, interferon-beta1, and other 9p21 markers in human malignant cell lines, Cancer Genet. Cytogenet. *86:* 22-8, 1996.
2. Stadler, W. M., Sherman, J., Bohlander, S. K., Roulston, D., Dreyling, M., Rukstalis, D., and Olopade, O. I. Homozygous deletions within chromosomal bands 9p21-22 in bladder cancer, Cancer Res. *54:* 2060-3, 1994.
3. Schmid, M., Malicki, D., Nobori, T., Rosenbach, M. D., Campbell, K., Carson, D. A., and Carrera, C. J. Homozygous deletions of methylthioadenosine phosphorylase (MTAP) are more frequent than p16INK4A (CDKN2) homozygous deletions in primary non- small cell lung cancers (NSCLC), Oncogene. *17:* 2669-75, 1998.
4. Hori, Y., Hori, H., Yamada, Y., Carrera, C. J., Tomonaga, M., Kamihira, S., Carson, D. A., and Nobori, T. The methylthioadenosine phosphorylase gene is frequently co-deleted with the p16INK4a gene in acute type adult T-cell leukemia, Int. J. Cancer. *75:* 51-6, 1998.
5. Hoffman, R. M. Altered methionine Metabolism and Transmethylation in Cancer, Anticancer Res. *5:* 1-30, 1985.
6. Hori, H., Tran, P., Carrera, C. J., Hori, Y., Rosenbach, M. D., Carson, D. A., and Nobori, T. Methylthioadenosine phosphorylase cDNA transfection alters sensitivity to depletion of purine and methionine in A549 lung cancer cells, Cancer Res. *56:* 5653-8, 1996.

APPENDIX

1. FIGURE
   Metabolic Pathway

2. Tang, B., Li, Y.N., Kruger, W.D. Defects in methylthioadenosine phosphorylase are associated with but not responsible for methionine-dependent tumor cell growth. Cancer Res. 60:5543-5547, 2000.

APPENDIX



[CANCER RESEARCH 60, 5543–5547, October 1, 2000]

Defects in Methylthioadenosine Phosphorylase Are Associated with but not Responsible for Methionine-dependent Tumor Cell Growth[1]

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ABSTRACT

A large proportion of human tumor-derived cell lines and primary tumor cells show methionine-dependent growth. This phenomenon refers to the ability of cells to grow in media containing methionine and the inability of cells to grow in media supplemented with methionine's precursor, homocysteine (Hcy). Methionine can be formed by two different pathways, the recycling pathway and the salvage pathway. To discover the basis for methionine-dependent growth, we have analyzed 12 tumor cell lines and 2 non-tumor-derived cell lines for defects in two key genes in different methionine synthetic pathways. We found little evidence that defects in methionine synthase expression or mutations in the *MS* gene are correlated with methionine-dependent growth. However, we did find a correlation between methionine-dependent growth and defects in expression of methylthioadenosine phosphorylase (MTAP), a key enzyme in the salvage pathway. Three of the four cell lines lacking detectable MTAP protein were unable to grow in Hcy-containing media, whereas all six of the MTAP-positive cell lines tested showed strong growth. However, when we introduced MTAP cDNA into MTAP-deficient MCF-7 cells, the resulting cell line was still defective in growth on Hcy, although it could now grow on the salvage pathway precursor methylthioadenosine. These findings indicate that salvage pathway defects are not causally related to methionine-dependent growth.

INTRODUCTION

Methionine dependence denotes the inability of cells to grow *in vitro* when methionine is replaced with Hcy[3] in the culture medium (1, 2). The phenomenon has been demonstrated in a large number of malignant cell lines and in primary cultures of human malignant tumors (3, 4). With few exceptions, nontransformed cells or tissue is Met independent (1, 5, 6). These observations have led to an interest in using recombinant methioninase as an anticancer agent (7).

The enzyme that converts Hcy to methionine, MS, is a conceivable site of a defect in Met-dependent tumor cells. This enzyme catalyzes a cobalamin-dependent reaction that transfers a methyl group from 5′-methyltetrahydrofolate onto Hcy to form methionine (see Fig. 1). Although some studies of methionine-dependent cells have shown decreases in MS activity measured in cell extracts (8, 9), other studies have not (10, 11). Additional studies have presented evidence that some methionine-dependent cells have defects in cobalamin (B_{12}) metabolism (9, 12). Thus, the role of MS in methionine dependence is still unclear.

Other literature has emphasized the importance of the methionine salvage pathway in explaining methionine-dependent growth (13). This pathway salvages the MTA that is released after *S*-adenosylmethionine donates an aminopropyl group for polyamine biosynthesis. The MTA is then converted to adenine and methionine in a multistep enzymatic procedure (Fig. 1). Ogier *et al.* (14) found that four different methionine-dependent cell lines could be rescued by addition of the penultimate compound in the salvage pathway, MTOB. It has been claimed that Met-dependent tumor cells do not lack the ability to form methionine from Hcy but rather have a metabolic defect in the salvage pathway (13). Indeed, one of the key enzymes in this pathway, MTAP, is frequently absent in many kinds of cancer cells and primary tumors including leukemias, brain tumors, non-small cell lung cancers, and bladder cancers (15–19).

To understand the mechanisms of methionine-dependent growth in tumor cells, we surveyed 12 human tumor cell lines for alterations in the *MS* and *MTAP* gene and reexamined the growth behavior of a subset of these cell lines in media containing various methionine precursors. Our results indicate that although methionine-dependent growth and MTAP deletion frequently occur together in the same cells, MTAP deletion is not responsible for the methionine-dependent growth phenotype.

MATERIALS AND METHODS

Reagents. L-Met, Hcy, and MTA were purchased from Sigma Chemical Co. (St. Louis, MO). MTOB was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Cell Lines and Media. All of the cell lines used in this study are listed in Table 1. FC1010 cells were isolated in the FCCC tissue culture facility. All other tumor cell lines were purchased from ATCC. Cell lines from ATCC were grown in the media recommended by ATCC. FC1010 was grown in RPM 1640 supplemented with dialyzed 10% FBS. The corresponding type of Met-free media supplemented with 10 μM B_{12} were used to test Met dependence. Eagle's Met-free MEM was ordered from Sigma, and Met-free DMEM was obtained from Life Technologies, Inc. (Grand Island, NY).

Cell Growth Assays. Cells ($1–5 \times 10^5$) were plated depending on the type of cell line and the size of the cells. Cells were seeded into 3-ml of Met-free medium plus FBS in 6-well plates or in 10 ml of medium in T25 flasks. Four h later, 100 μM of either Hcy, MTA, MTOB, or Met was added. Each treatment was carried out in duplicate or triplicate at the same time. After 4 days, the cells were either trypsinized or scraped off the dishes using cell scrapers. Cell growth was assessed by cell counting and/or measuring the total proteins using the Bio-Rad protein assay dye kit. Total protein was found to correlate very closely with cell counting (data not shown). Each value is the average of two or three repeats.

Isolation of Total RNA, RT-PCR, and Sequencing. Total RNA was isolated from cultured cells ($1–5 \times 10^6$) using Perfect RNA Total RNA Isolation Kit (5′-3′, Inc., Boulder, CO). First-strand cDNA synthesis was performed on 1 μg of total RNA from an individual cell line using oligo$(dT)_{16}$ as a primer with the Advantage RT-for-PCR kit (Clonetech, Palo Alto, CA). PCR was carried out using Advantage KlenTaq Polymerase Mix (Clonetech). PCR-DNAs were either sequenced directly or cloned into pCR2.1 vector (Invitrogen, San Diego, CA), and several individual clones were pooled for sequencing.

SDS-PAGE and Western Blotting. Cells were lysed and sonicated in 1× PBS containing 1 mM phenylmethylsulfonyl fluoride (Sigma). The lysate was suspended in 1× SDS sample buffer, and proteins were separated by SDS-PAGE. Proteins were then transferred onto an Immobilon P membrane (Millipore Corp., Bedford, MA). The subsequent steps of Western blotting were performed using the Immun-Lite Assay Kit from Bio-Rad according to the

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[3] The abbreviations used are: Hcy, homocysteine; MS, methionine synthase; MTA, methylthioadenosine; MTOB, 4-methylthio-2-oxobutanoate; MTAP, methylthioadenosine phosphorylase; ATCC, American Type Culture Collection; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; MTHFR, methylenetetrahydrofolate reductase.

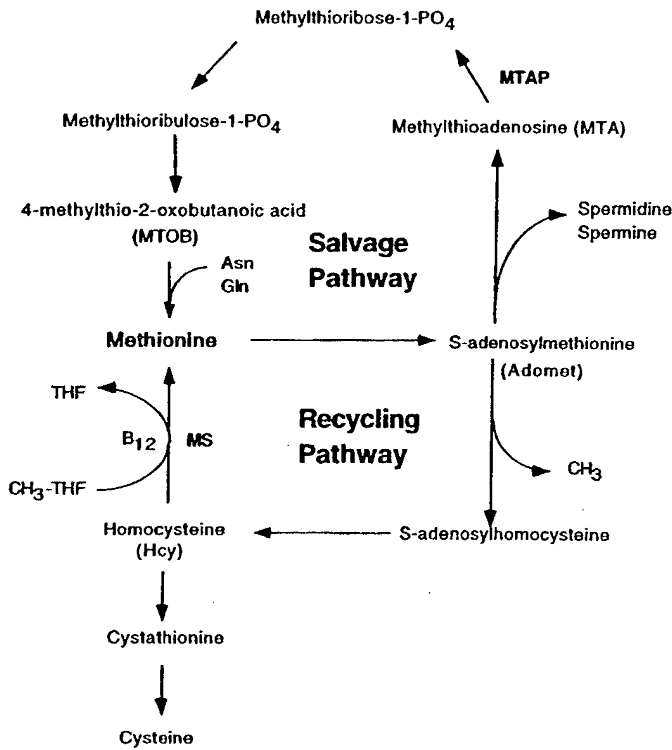

Fig. 1. Methionine biosynthetic pathways. The recycling and salvage pathways are presented. Relevant enzymes are shown in *bold*.

Table 1 *Cell lines used in this study*

Cell line	Source	Reported Met growth status	Ref. no. for Met status
MCF-7	Breast carcinoma	AD[a]	5
SW480	Colon carcinoma	AD	3
SK-N-SH	Neuroblastoma	AD	5
HOS	Osteogenic sarcoma	AD	5
A498	Kidney carcinoma	AD	5
J82	Bladder carcinoma	AD	5
HT1080	Fibrosarcoma	AD	5
SK-CO-1(HTB-39)	Colon carcinoma	MD	5
A172	Glioblastoma	MD	5
HeLa	Cervical carcinoma	MD	5
MRC-5	Lung fibroblast	Independent	14
Raji	Burkitt's lymphoma	Independent	35
LCL-DABU	Human EBV-transformed lymphocyte	Unknown	None
NIH-3T3	Mouse fibroblast	Unknown	None
FC1010	Human skin fibroblast	Unknown	None

[a] AD, absolutely Met dependent; MD, mostly Met dependent.

manufacturer's instructions. The primary antibodies used in this study were MS anti-sera [from Dr. Ruma Banerjee's laboratory (University of Nebraska, Omaha, NE; Ref. 20)] and MTAP antibody produced from chicken yolk (from Dr. Dennis Carson's laboratory, University of San Diego, CA).

Cloning and Transfection of MTAP. MTAP cDNAs were obtained by PCR using Raji cell cDNA as template with primers 5′-AATTCCGCTC-CGCACTGCTCACTCCCG and 5′-CTCCTGGGCAGCCATGCTACTTTA-ATG. MTAP cDNA was cloned into the eukaryotic expression vector pCR3.1 (Invitrogen) and then transformed into TOP10F competent cells according to the manufacturer's instructions. The orientation of the insert was determined by digesting plasmid DNA with *Hin*cII, and the clones were sequenced to screen for PCR-induced errors. The sense construct was designated pCR:sMTAP, whereas the antisense construct was designated pCR:asMTAP. Five μg of pCR:sMTAP and pCR:asMTAP were used to transfect MCF-7 cells with pCR3.1/Cat expression vector as a positive control by using the Qiagen Superfect Transfection Reagent Kit (Qiagen, Inc., Chatsworth, CA). Twenty-four h after transfection, medium was replaced by MEM with 2 mM L-glutamine, 1.5 mg/ml sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 0.01 mg/ml bovine insulin, 10% FBS, and 600 mg/ml G418 (Life Technologies, Inc.). After culture for 5 weeks in the presence of G418, colonies were picked and subcloned. Six clones were tested for the expression of MTAP protein by Western blot analysis.

RESULTS

MS Protein Expression and Mutation Analysis. We examined 12 human tumor and 2 nontransformed cell lines for alterations in MS protein levels (Table 1). Ten of the cell lines had previously been characterized as having either absolute or partial methionine-dependent growth. We examined MS protein levels by Western analysis. The antiserum was generated against purified porcine MS, but it cross-reacts with human, mouse, and *Escherichia coli* MS due to the high degree of evolutionary conservation of the MS protein (21). Among the 14 cell lines, we saw no significant difference in steady-state MS levels, with one exception (Fig. 2). We observed a slight decrease in MS levels in SK-CO-1, a colon carcinoma-derived cell line that has previously been reported to exhibit partial methionine-dependent growth behavior.

We next examined whether any of the cell lines contained mutations in the MS coding region. RNA from each of the human cell lines was isolated, and RT-PCR was used to amplify the entire MS coding region. The resulting PCR product was then sequenced directly. We did not observe any nonsense or missense mutations in any of the cell lines. However, we did identify several silent changes (Table 2) present in some cell lines, indicating that our methods were sensitive enough to detect heterozygous alterations. These results show that mutations that inactivate the MS protein are not found frequently in methionine-dependent cells.

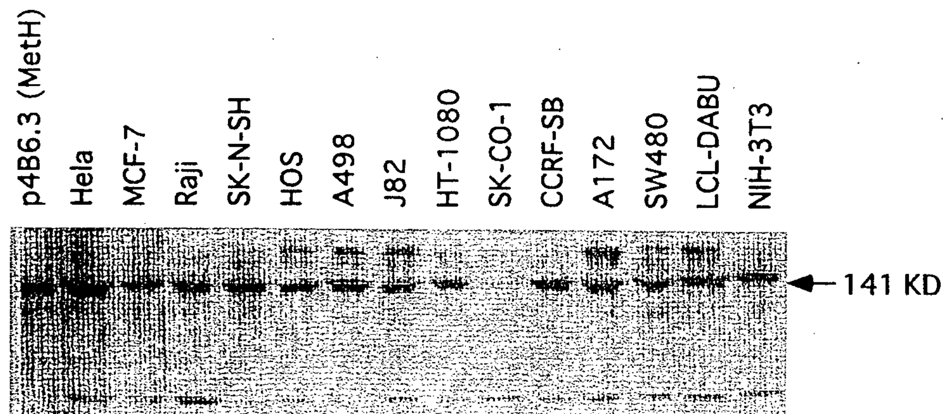

Fig. 2. Western analysis of MS protein. Total cellular protein was separated on 10% SDS-polyacrylamide gel and transferred to nylon membranes as described in "Materials and Methods." p4B6.3 is an *E. coli* strain overexpressing the METH protein, which is the bacterial homologue of MS.

Table 2 *Polymorphisms present in the MS gene*

Polymorphism (wt/mut)	Hetro- or homozygote	Tumor cell lines
A/G 3144, Ala/Ala 1048	Hetrozygote	HT1080
	Hetrozygote	MCF-7
	Hetrozygote	A172
C/A 3492, Arg/Arg 1164	Hetrozygote	HOS
	Hetrozygote	J82
	Hetrozygote	HT1080
	Hetrozygote	MCF-7
	Homozygote	A172
	Homozygote	TC13
C/T 3576, Leu/Leu 1192	Hetrozygote	J82
	Hetrozygote	SK-CO-1
	Hetrozygote	HeLa
	Hetrozygote	MCF-7
	Hetrozygote	Raji
	Hetrozygote	SK-N-SH
	Homozygote	HOS
G/A 3332, Ser/Ser 1111	Hetrozygote	SK-CO-1
	Hetrozygote	CCRF-SB

MTAP Expression in Tumor Cell Lines. Next we examined the MTAP status in these 12 tumor cell lines by RT-PCR and Western blotting. The primers used for RT-PCR of MTAP cDNA were derived from the immediate flanking sequences of MTAP open reading frame (22). We were unable to detect MTAP RNA in 4 of the 12 tumor-derived cell lines (see Fig. 3*A*). As a control for the quality of the RNA and the cDNA synthesis, the glyceraldehyde-3-phosphate dehydrogenase gene was amplified successfully in all samples (Fig. 3*C*).

To confirm the RT-PCR data, we also examined MTAP protein by Western blot. All of the cell lines lacking MTAP RNA as judged by RT-PCR also lacked detectable MTAP protein by Western analysis (Fig. 3*B*).

Cell Growth. We wanted to assess whether failure to express MTAP correlates with a distinguishing growth pattern in media containing methionine and various precursors. We examined the growth of 10 cell lines: (*a*) 4 MTAP+ tumor cell lines; (*b*) 4 MTAP− lines; and (*c*) two nontransformed cell lines (Table 3). Cell growth was evaluated in the presence of 100 μM methionine, MTA, MTOB, or Hcy (see Fig. 1). The two nontransformed control cell lines (MRC-5 and FC1010) both showed excellent growth in media containing methionine or MTOB and significant growth in media containing Hcy or MTA. All four of the MTAP+ cell lines tested (HeLa, SK-N-SH, J82, and Raji) exhibited growth behavior that was essentially identical to that of the nontransformed cell lines. This was somewhat surprising because three of these four cell lines (HeLa, SK-N-SH, and J82) have previously been characterized as either absolutely or partially methionine dependent (5). However, the four MTAP− cell lines (MCF-7, HOS, HT1080, and A172) exhibited very different growth characteristics. As expected, all four MTAP− cell lines were unable to grow in media containing MTA as the sole source of methionine. Interestingly, three of the four MTAP− cell lines were also unable to grow in Hcy-containing media. The single exception was the A172 glioblastoma cell line, which showed no growth in MTA but showed clear growth in Hcy-containing media. Thus, all three of the cell lines that showed absolutely no growth in Hcy-containing media were MTAP−.

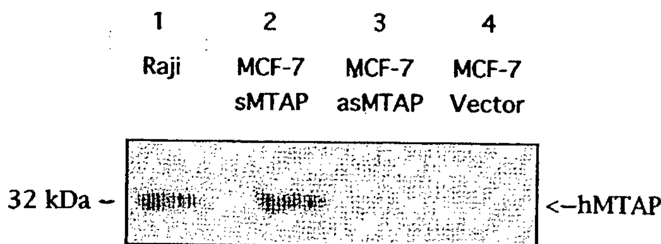

Fig. 3. MTAP analysis of tumor-derived cell lines. *A*, RT-PCR analysis of MTAP expression in the indicated cell lines; *B*, Western analysis of MTAP in the same cells; *C*, RT-PCR of glyceraldehyde-3-phosphate dehydrogenase.

Reintroduction of MTAP into MCF-7 Cells. We next tested directly whether deletion of MTAP was responsible for the methionine-dependent growth. Wild-type MTAP cDNA was cloned into expression vector pCR3.1 into the sense (pCR:sMTAP) and antisense (pCR:asMTAP) orientations. These constructs, along with pCR3.1 itself, were used to transfect the MTAP− MCF-7 cells to create stable transfected lines. Western analysis indicated that one of the cell lines transfected with the sense orientation construct, pCR3.1:sMTAP, expressed MTAP levels comparable to Raji cells, a methionine-independent cell line (see Fig. 4). Control transfectants containing either pCR3.1:asMTAP or pCR3.1 had no detectable MTAP protein.

We next examined the ability of these cell lines to grow on media containing various sources of sulfur. Cells expressing MTAP were able to grow on media with MTA as the sole source of methionine precursor (see Table 4), but the control cells were not able to do so. However, no significant improvement in growth on Hcy-containing media was observed. Examination of growth in comparison to Raji cells at 3, 5, and 7 days indicated that no significant growth took place on Hcy-containing media (Table 5). Thus, expression of MTAP in MCF-7 cells corrected the MTAP− phenotype, but not the methionine-dependent growth phenotype.

DISCUSSION

The results reported here support the view that defects in the *MS* gene are unlikely to be a major cause of methionine-dependent growth. We failed to identify a single missense or nonsense mutation in the *MS* gene in 12 tumor cell lines, 10 of which have been previously characterized as being at least partially methionine dependent for growth. Only one cell line, a colon carcinoma-derived line (SK-CO-1), had lower levels of MS protein compared with all of the other cell lines. Interestingly, this cell line was previously classified as only partially methionine dependent.

Although defects in MTAP expression are often observed in methionine-dependent cell lines, these do not appear to be the cause of methionine-dependent growth. Four of the 12 cell lines examined lacked MTAP RNA and MTAP protein. All four of these cell lines could not use MTA as a sulfur source, and three of these cell lines were also unable to use Hcy. These results suggested that MTAP deficiency could be related to methionine-dependent growth. However, when we reintroduced MTAP into one of these cell lines (MCF-7), we corrected the MTA growth defect but not the methi-

Table 3 *Cell growth studies*[a]

Media	MRC-5	FC1010	HeLa	J82	Raji	SK-N-SH	MCF-7	HOS	HT1080	A172
Hcy+	46	50	53	30	64	30	0	0	0	41
MTA+	46	55	47	49	81	49	0	0	0	0
MTOB+	100	100	86	100	100	80	100	100	86	100
Met+	100	100	100	100	100	100	100	100	100	100
MTAP status	+	+	+	+	+	+	−	−	−	−

[a] Cells were assayed for growth as described in the text. The growth values for each line are relative to the growth observed in the same cell line in the presence of methionine (100 μM). A value of 0 implies there was no additional after plating. The MTAP status (see Fig. 3) is shown for each cell line.

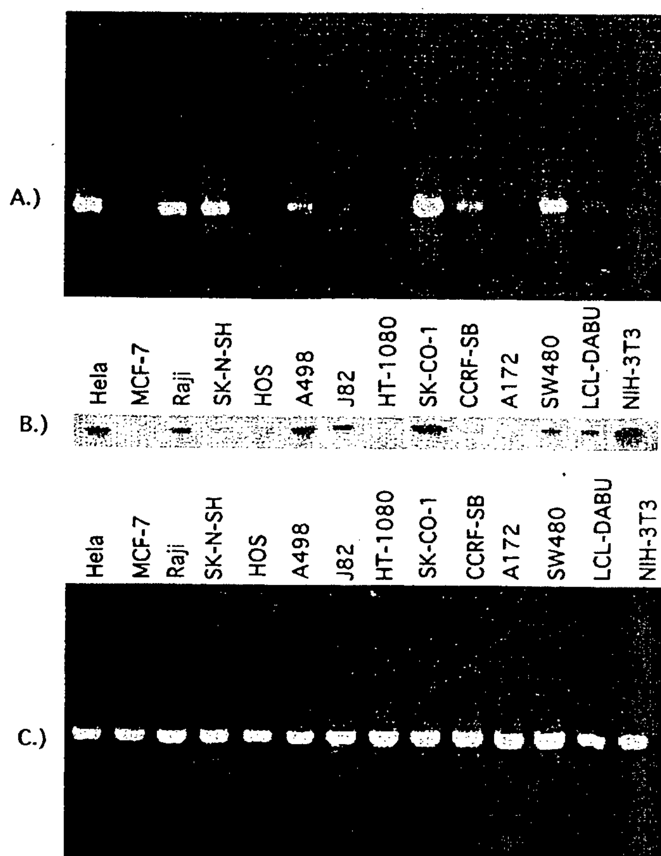

Fig. 4. MTAP protein in MCF transfectants. Western blots were probed with anti-MTAP antiserum. *Lane 1*, Raji cell extract. *Lane 2*, MCF-7 cells transfected with pCR3.1 with MTAP in the sense orientation. *Lane 3*, MCF-7 cells transfected with pCR3.1 with MTAP in the antisense orientation. *Lane 4*, MCF-7 cells transfected with pCR3.1 with no insert.

onine-dependent growth. Thus, the defect in MTAP expression is separate from methionine-dependent growth.

The gene for MTAP resides on chromosome 9p21 in close proximity to the tumor suppressor gene p16 and is frequently found to be deleted in a variety of cancers (15–19). It is generally assumed that MTAP is deleted "by accident" simply because of its proximity to p16. The p16 gene is unusual among tumor suppressor genes in that the mechanism of inactivation generally involves homozygous deletion as opposed to loss of heterozygosity and point mutation (23). Whether deletion of MTAP is coincidental or gives the tumor cell a selective advantage is unknown. Interestingly, it has recently been described that in a set of non-small cell lung carcinomas, MTAP deletion occurs frequently in the absence of p16 deletion, suggesting that MTAP dysfunction may give the tumor cell some advantage (24).

One unexpected finding of this study is that many of the cell lines that had previously been characterized as either fully or partially methionine dependent exhibited methionine-independent growth in our hands. For example, two of the cell lines characterized in other studies (5) as showing no growth on Hcy (SK-N-SH and J82) exhibited only a slightly retarded growth on Hcy compared with the two control cell lines. In addition, two other cell lines previously characterized as only slightly independent (HeLa and A172) exhibited growth in Hcy that was essentially undistinguishable from that of our control cells. A possible explanation for these discrepancies is that the cell lines might not be identical to the ones used in the earlier studies. Fiskerstrand *et al.* (12) have observed that a human glioma cell line that was initially methionine independent became methionine dependent when the passage number increased. Perhaps our cells (most of which were obtained from ATCC) are at earlier passages than the cells used in the study of Mecham *et al.* (5).

We observed that although MTAP deletion did not explain methionine-dependent growth, MTAP deletion and methionine-dependent growth frequently occurred together. Three of the four MTAP-deficient cell lines showed no growth on Hcy. The observation that these two phenotypes frequently occur together may be potentially useful in cancer treatment. Cells deleted for MTAP are known to be more sensitive to the effects of purine biosynthetic inhibitors such as methotrexate, L-alonosine, 5,10-dideazatetrahyhydrofolate, and azaserine *in vitro* (25–27). Methionine-dependent tumor cells have been shown to be sensitive to depletion of plasma methionine *in vivo*. Methionine-dependent human cancer xenografts in nude mice grow much more slowly when the mice are put on a methionine-free diet (28). In addition, methionine-dependent cancer xenografts can have their growth reduced by the use of recombinant methioninase to lower plasma methionine levels (29). Thus, by combining purine inhibitors with plasma methionine-lowering procedures, it may be possible to get increased selective killing of MTAP− methionine-dependent tumors.

Our observations still leave the question of what is the molecular basis of methionine dependency unanswered. One possibility is that methionine-dependent cells could have defects in MTHFR. This enzyme makes methyltetrahydrofolate, which is the methyl donor for the conversion of Hcy to methionine. It has been reported that in some ovarian carcinomas, there is loss of heterozygosity at the MTHFR locus that is associated with decreased activity (30). However, RT-PCR analysis indicates that none of the cell lines used in this study have defects in MTHFR expression (data not shown). Another possible mechanism for methionine-dependent growth involves impairment in the uptake/metabolism of cobalamin. Cobalamin is a vitamin cofactor required for MS function. Studies of the methionine dependence of P60 human glioma cells indicate that these cells have reduced levels of both methylcobalamin and adenosylcobalamin (12). Methionine-dependent MeWoLC1 melanoma-derived cells also seem to have defects in cobalamin metabolism (9). Complementation analysis suggests that the defect is in the same gene as that observed in cells from individuals suffering from the *cblC* inborn error of metabolism (31). However, this same group also showed that inactivation of the *cblC* gene is unlikely to be involved in the methionine dependence of

Table 4 *Growth behavior of MTAP transfected MCF-7 cells*[a]

Media (after 7 days)	pCR3.1/sense hMTAP MCF-7	pCR3.1/antisense hMTAP MCF-7	pCR3.1/CAT MCF-7
Met	100	100	100
Hcy	3	0	5
MTA	60	0	3
MTOB	88	80	98

[a] MCF-7 cells were transfected with MTAP with sense, antisense, or vector alone. Cells were assayed for growth as described in the text. The growth values for each line are relative to the growth observed in the same cell line in the presence of methionine. A value of 0 implies there was no additional after plating. The table shows relative growth after 7 days in media containing the indicated methionine precursor.

Table 5 *Growth behavior of MTAP-transfected MCF-7 cells compared with Raji cells*[a]

Time/media	Raji	pCR3.1/sense hMTAP MCF-7	pCR3.1/antisense hMTAP MCF-7	pCR3.1/CAT MCF-7
3 days Met	100	100	100	100
3 days Hcy	60	4	3	5
5 days Met	100	100	100	100
5 days Hcy	56	2	1	5
7 days Met	100	100	100	100
7 days Hcy	37	2	1	0

[a] The table shows growth after 3, 5, and 7 days compared to Raji cells, a methionine-independent cell line.

most other methionine-dependent tumors, including the MCF-7 cells studied here.

A final possibility is that methionine dependence results not from a defect in the production of methionine but rather from overutilization of methionine. Several studies have demonstrated that methionine-dependent cells tend to have reduced ratios of *S*-adenosylmethionine: *S*-adenosylhomocysteine, suggesting that these cells have increased rates of transmethylation (32–34). Thus, it may be that the production of methionine simply cannot keep up with its utilization.

In summary, the studies described here suggest that mutation in *MS* or *MTAP* is not the cause of the methionine-dependent growth observed in most tumor cells. However, *MTAP* deletion and methionine dependence often occur in the same cells, suggesting that a combination of purine inhibitors and methionine starvation may be a reasonable approach to kill these cells. Future efforts toward understanding the genetic basis for methionine dependence could help development of a genetic test to identify methionine-dependent tumors, and this information could be incorporated into studies examining the efficacy of methionine-lowering treatments.

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REFERENCES

1. Halpern, B. C., Clark, B. R., Hardy, D. N., Halpern, R. M., and Smith, R. A. The effect of replacement of methionine by homocysteine on survival of malignant and normal adult mammalian cells in culture. Proc. Natl. Acad. Sci. USA, *71:* 1133–1136, 1974.
2. Hoffman, R. M. Methionine dependence in cancer cells: a review. In Vitro, *18:* 421–428, 1982.
3. Hoffman, R. M. Altered methionine metabolism and transmethylation in cancer. Anticancer Res., *5:* 1–30, 1985.
4. Guo, H. Y., Herrera, H., Groce, A., and Hoffman, R. M. Expression of the biochemical defect of methionine dependence in fresh patient tumors in primary histoculture. Cancer Res., *53:* 2479–2483, 1993.
5. Mecham, J. O., Rowitch, D., Wallace, C. D., Stern, P. H., and Hoffman, R. M. The metabolic defect of methionine dependence occurs frequently in human tumor cell lines. Biochem. Biophys. Res. Commun., *117:* 429–424, 1983.
6. Kreis, W., and Goodenow, M. Methionine requirement and replacement by homocysteine in tissue cultures of selected rodent and human malignant and normal cells. Cancer Res., *38:* 2259–2262, 1978.
7. Hoffman, R. M. Methioninase: a therapeutic for diseases related to altered methionine metabolism and transmethylation: cancer, heart disease, obesity, aging, and Parkinson's disease. Hum. Cell, *10:* 69–80, 1997.
8. Ashe, H., Clark, B. R., Chu, F., Hardy, D. N., Halpern, B. C., Halpern, R. M., and Smith, R. A. N5-methyltetrahydrofolate: homocysteine methyltransferase activity in extracts from normal, malignant and embryonic tissue culture cells. Biochem. Biophys. Res. Commun., *57:* 417–425, 1974.
9. Liteplo, R. G., Hipwell, S. E., Rosenblatt, D. S., Sillaots, S., and Lue-Shing, H. Changes in cobalamin metabolism are associated with the altered methionine auxotrophy of highly growth autonomous human melanoma cells. J. Cell. Physiol., *149:* 332–338, 1991.
10. Hoffman, R. M., and Erbe, R. W. High *in vivo* rates of methionine biosynthesis in transformed human and malignant rat cells auxotrophic for methionine. Proc. Natl. Acad. Sci. USA, *73:* 1523–1527, 1976.
11. Tautt, J. W., Anuszewska, E. L., and Koziorowska, J. H. Methionine regulation of N-5-methyltetrahydrofolate: homocysteine methyltransferase and its influence on the growth and protein synthesis in normal, neoplastic, and transformed cells in culture. J. Natl. Cancer Inst., *69:* 9–14, 1982.
12. Fiskerstrand, T., Christensen, B., Tysnes, O. B., Ueland, P. M., and Refsum, H. Development and reversion of methionine dependence in a human glioma cell line: relation to homocysteine remethylation and cobalamin status. Cancer Res., *54:* 4899–4906, 1994.
13. Dumontet, C., Roch, A. M., and Quash, G. Methionine dependence of tumor cells: programmed cell survival? Oncol. Res., *8:* 469–471, 1996.
14. Ogier, G., Chantepie, J., Deshayes, C., Chantegrel, B., Charlot, C., Doutheau, A., and Quash, G. Contribution of 4-methylthio-2-oxobutanoate and its transaminase to the growth of methionine-dependent cells in culture. Effect of transaminase inhibitors. Biochem. Pharmacol., *45:* 1631–1644, 1993.
15. Della Ragione, F., Russo, G., Oliva, A., Mastropietro, S., Mancini, A., Borrelli, A., Casero, R. A., Iolascon, A., and Zappia, V. 5′-Deoxy-5′-methylthioadenosine phosphorylase and p16^{INK4} deficiency in multiple tumor cell lines. Oncogene, *10:* 827–833, 1995.
16. Zhang, H., Chen, Z. H., and Savarese, T. M. Codeletion of the genes for p16^{INK4}, methylthioadenosine phosphorylase, interferon-$\alpha 1$, interferon-$\beta 1$, and other 9p21 markers in human malignant cell lines. Cancer Genet. Cytogenet., *86:* 22–28, 1996.
17. Nobori, T., Karras, J. G., Della Ragione, F., Waltz, T. A., Chen, P. P., and Carson, D. A. Absence of methylthioadenosine phosphorylase in human gliomas. Cancer Res., *51:* 3193–3197, 1991.
18. Nobori, T., Szinai, I., Amox, D., Parker, B., Olopade, O. I., Buchhagen, D. L., and Carson, D. A. Methylthioadenosine phosphorylase deficiency in human non-small cell lung cancers. Cancer Res., *53:* 1098–1101, 1993.
19. Stadler, W. M., Sherman, J., Bohlander, S. K., Roulston, D., Dreyling, M., Rukstalis, D., and Olopade, O. I. Homozygous deletions within chromosomal bands 9p21–22 in bladder cancer. Cancer Res., *54:* 2060–2063, 1994.
20. Chen, Z., Crippen, K., Gulati, S., and Banerjee, R. Purification and kinetic mechanism of a mammalian methionine synthase from pig liver. J. Biol. Chem., *269:* 27193–27197, 1994.
21. Li, Y. N., Gulati, S., Baker, P. J., Brody, L. C., Banerjee, R., and Kruger, W. D. Cloning, mapping, and RNA analysis of the human methionine synthase gene. Hum. Mol. Genet., *5:* 1851–1858, 1995.
22. Olopade, O. I., Pomykala, H. M., Hagos, F., Sveen, L. W., Espinosa, R., III, Dreyling, M. H., Gursky, S., Stadler, W. M., Le Beau, M. M., and Bohlander, S. K. Construction of a 2.8-megabase yeast artificial chromosome contig and cloning of the human methylthioadenosine phosphorylase gene from the tumor suppressor region on 9p21. Proc. Natl. Acad. Sci. USA, *92:* 6489–6493, 1995.
23. Liggett, W. H., Jr., and Sidransky, D. Role of the p16 tumor suppressor gene in cancer. J. Clin. Oncol., *16:* 1197–1206, 1998.
24. Schmid, M., Malicki, D., Nobori, T., Rosenbach, M. D., Campbell, K., Carson, D. A., and Carrera, C. J. Homozygous deletions of methylthioadenosine phosphorylase (MTAP) are more frequent than p16^{INK4A} (CDKN2) homozygous deletions in primary non-small cell lung cancers (NSCLC). Oncogene, *17:* 2669–2675, 1998.
25. Hori, H., Tran, P., Carrera, C. J., Hori, Y., Rosenbach, M. D., Carson, D. A., and Nobori, T. Methylthioadenosine phosphorylase cDNA transfection alters sensitivity to depletion of purine and methionine in A549 lung cancer cells. Cancer Res., *56:* 5653–5658, 1996.
26. Chen, Z. H., Olopade, O. I., and Savarese, T. M. Expression of methylthioadenosine phosphorylase cDNA in p16-, MTAP- malignant cells: restoration of methylthioadenosine phosphorylase-dependent salvage pathways and alterations of sensitivity to inhibitors of purine *de novo* synthesis. Mol. Pharmacol., *52:* 903–911, 1997.
27. Kamatani, N., Nelson-Rees, W. A., and Carson, D. A. Selective killing of human malignant cell lines deficient in methylthioadenosine phosphorylase, a purine metabolic enzyme. Proc. Natl. Acad. Sci. USA, *78:* 1219–1223, 1981.
28. Hoshiya, Y., Guo, H., Kubota, T., Inada, T., Asanuma, F., Yamada, Y., Koh, J., Kitajima, M., and Hoffman, R. M. Human tumors are methionine dependent *in vivo*. Anticancer Res., *15:* 717–718, 1995.
29. Tan, Y., Xu, M., Guo, H., Sun, X., Kubota, T., and Hoffman, R. M. Anticancer efficacy of methioninase *in vivo*. Anticancer Res., *16:* 3931–3936, 1996.
30. Chen, J., Giovannucci, E., Kelsey, K., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Spiegelman, D., Willett, W. C., and Hunter, D. J. A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. Cancer Res., *56:* 4862–4864, 1996.
31. Watkins, D. Cobalamin metabolism in methionine-dependent human tumour and leukemia cell lines. Clin. Invest. Med., *21:* 151–158, 1998.
32. Tisdale, M. J. Utilization of preformed and endogenously synthesized methionine by cells in tissue culture. Br. J. Cancer, *49:* 315–320, 1984.
33. Stern, P. H., Wallace, C. D., and Hoffman, R. M. Altered methionine metabolism occurs in all members of a set of diverse human tumor cell lines. J. Cell. Physiol., *119:* 29–34, 1984.
34. Judde, J. G., Ellis, M., and Frost, P. Biochemical analysis of the role of transmethylation in the methionine dependence of tumor cells. Cancer Res., *49:* 4859–4865, 1989.
35. Christa, L., Kersual, J., Auge, J., and Perignon, J-L. Salvage of 5′-deoxy-5′-methylthioadenosine and L-homocysteine into methionine in cells cultured in a methionine-free medium: a study of "methionine-dependence." Biochem. Biophys. Res. Commun., *135:* 131–138, 1986.